UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA, TOXICOLOGÍA Y MEDICINA LEGAL

"RIESGOS TÓXICOS POR CIANOTOXINAS EN PECES DE CONSUMO: OPTIMIZACIÓN DE MÉTODOS DE ANÁLISIS, POTENCIALES MECANISMOS DE TOXICIDAD Y TRATAMIENTO"

Memoria que presenta la Licenciada REMEDIOS GUZMÁN GUILLÉN

para optar al título de Doctor por la Universidad de Sevilla con la Mención Internacional

Sevilla, 2015

UNIVERSIDAD DE SEVILLA

AREA DE TOXICOLOGÍA
C/ Profesor García González, nº 2
41012 Sevilla (España)
Teléfono: 954 55 67 62
Fax: 954 55 64 22

Dª ANA MARÍA CAMEÁN FERNÁNDEZ, Catedrática de Universidad y Directora del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla,

CERTIFICA: Que la Tesis Doctoral titulada "**RIESGOS TÓXICOS POR CIANOTOXINAS EN PECES DE CONSUMO: OPTIMIZACIÓN DE MÉTODOS DE ANÁLISIS, POTENCIALES MECANISMOS DE TOXICIDAD Y TRATAMIENTO**", presentada por la Lda. Dª REMEDIOS GUZMÁN GUILLÉN para optar al grado de Doctor por la Universidad de Sevilla con la Mención Internacional, ha sido realizada en el Área de Toxicología de este Departamento bajo la dirección de la Dra. Ana Mª Cameán Fernández, la Dra. Isabel Mª Moreno Navarro y la Dra. Ana Isabel Prieto Ortega. Así mismo, se incluyen las investigaciones llevadas a cabo en el Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) de la Universidad de Porto, bajo la dirección del Dr. Vitor Vasconcelos.

Y para que así conste, firmo el presente en Sevilla,
Enero de 2015.

Dra. Ana María Cameán Fernández

UNIVERSIDAD DE SEVILLA

AREA DE TOXICOLOGÍA
C/ Profesor García González, nº 2
41012 Sevilla (España)
Teléfono: 954 55 67 62
Fax: 954 55 64 22

Dª ANA MARÍA CAMEÁN FERNÁNDEZ, Catedrática del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal, Dª ISABEL MARÍA MORENO NAVARRO y Dª ANA ISABEL PRIETO ORTEGA, Profesoras Titulares del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla,

CERTIFICAN: Que siendo directoras de la Tesis Doctoral de la Licenciada Dª REMEDIOS GUZMÁN GUILLÉN, con título "**RIESGOS TÓXICOS POR CIANOTOXINAS EN PECES DE CONSUMO: OPTIMIZACIÓN DE MÉTODOS DE ANÁLISIS, POTENCIALES MECANISMOS DE TOXICIDAD Y TRATAMIENTO**", AVALAN la idoneidad de la presentación de la misma en la modalidad de COMPENDIO POR PUBLICACIONES.

Y para que así conste, firmamos el presente en Sevilla,
Enero de 2015.

Dra. Ana María Cameán Fernández

Dra. Isabel M. Moreno Navarro

Dra. Ana Isabel Prieto Ortega

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Dª ANA MARÍA CAMEÁN FERNÁNDEZ, Catedrática del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal, Dª ISABEL MARÍA MORENO NAVARRO y Dª ANA ISABEL PRIETO ORTEGA, Profesoras Titulares del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla,

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41012 Sevilla (España)
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Fax: 954 55 64 22

Este trabajo ha sido realizado en el Área de Toxicología del Departamento de Nutrición y Bromatología, Toxicología y Medicinal Legal de la Facultad de Farmacia de la Universidad de Sevilla y financiado por los Proyectos de Investigación:

- AGL2009-10026, de la CICYT (MINECO): Transferencia y bioacumulacion de cilindrospermopsina en pescados de consumo público a escala de laboratorio, y evaluación de su toxicidad (Investigadora Principal: Ana María Cameán Fernández).
- P09-AGR-4672 de la Junta de Andalucía: Transferencia y Bioacumulación de Cilindrospermopsina en Pescados de Consumo Público a Escala de Laboratorio, y Evaluación de su Toxicidad (Investigadora Principal: Ana María Cameán Fernández).

La Doctoranda Dª Remedios Guzmán Guillén ha disfrutado de una ayuda Predoctoral de Personal Investigador en Formación (PIF), del IV Plan Propio de la Universidad de Sevilla, del 16 de Abril al 30 de Noviembre del 2011, así como de una ayuda Predoctoral de Formación del Profesorado Universitario (FPU), del Ministerio de Educación, Cultura y Deporte, desde Diciembre del 2011.

Asimismo, para la realización de las estancias en el extranjero, la Doctoranda Dª Remedios Guzmán Guillén ha disfrutado de una Ayuda de Movilidad derivada del IV Plan Propio de Investigación de la Universidad de Sevilla (2012), y de una ayuda para Estancias Breves del Programa de Formación del Profesorado Universitario (FPU) (2014).

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Adda: ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienoico

AChE: acetilcolinesterasa

ADN: ácido desoxirribonucleico

AOAC: Association of Official Analytical Chemists

ARN: ácido ribonucleico

ARNm: ácido ribonucleico mensajero

C: carbono

Ca: calcio

CAT: catalasa

CoA: coenzima A

Cu: cobre

CYN: cilindrospermopsina

CYP3A4: citocromo P450 3A4

CYP450: citocromo P450

Da: daltons

D-Ala: D-alanina

DDT: Dicloro Difenil Tricloroetano

D-Glu: ácido D-glutámico

D-MeAsp: ácido D-eritro- β-metil-aspártico

ELISA: ensayo de inmunoafinidad

EPA: Agencia de Protección Ambiental de los Estados Unidos

ERO: especies reactivas de oxígeno

Fe: hierro

g: gramos

γ-GCS: γ-glutamilcisteina sintetasa

G.I.: gastrointestinal

GPx: glutatión peroxidasa

GR: glutatión reductasa

GSH: glutatión reducido

GSSH: glutatión oxidado

GST: glutatión-S-transferasa

HAPs: Hidrocarburos Aromáticos Policíclicos

HCB: hexaclorobenceno

HOCl: ácido hipocloroso

HPBLs: linfocitos de sangre periférica humana

H_2O_2: peróxido de hidrógeno

IARC: Agencia Internacional para la Investigación sobre el Cáncer

IDT: Ingesta Diaria Tolerable

IHC: inmunohistoquimica

i.p.: intraperitoneal

K: potasio

kg: kilogramos

L: litro(s)

LC: cromatografia líquida / L carnitina

LC-DAD: cromatografia líquida con detector diodo-array

LC-MS: cromatografia líquida-espectrometría de masas

LC-MS/MS: cromatografia líquida-espectrometría de masas en tándem

LC-UV: cromatografia líquida-espectroscopía ultravioleta visible

LPO: peroxidación lipídica

LPS: lipopolisacáridos

MCs: microcistinas

MC-LA: microcistina-LA

MC-LR: microcistina-LR

MC-RR: microcistina-RR

MC-YR: microcistina-YR

Mdha: N-metildehidro-alanina

Mg: magnesio

mg: miligramo(s)

mL: mililitro(s)

Mn: manganeso

MNBNC: micronúcleos de células binucleadas

Mo: molibdeno

Na: sodio

NAC: N-acetilcisteína

NADPH: β-nicotinamida adenina dinucleótido fosfato reducido

NF-κB: factor nuclear kappa B

$OH^{\bullet}$: radical hidroxilo

OMS: Organización Mundial de la Salud

$OONO^-$: anión peroxinitrito

$^1O_2$: oxígeno singulete

$O2^{-\bullet}$: radical superóxido

P: fósforo

PCBs: Bifenilos Policlorados

PCR: Reacción en Cadena de la Polimerasa

PP1: fosfatasas de proteínas 1

PP2A: fosfatasas de proteínas 2A

p.c.: peso corporal

p.s.: peso seco

7-desoxi-CYN: 7-desoxi-cilindropsermopsina

7-epi-CYN: 7-epi-cilindrospermopsina

SE: selenio

SOD: superóxido dismutasa

SPE: Extracción en Fase Sólida

STX: saxitoxinas

UPLC-MS2: cromatografia líquida de ultra rendimiento acoplada a espectrometría de masas en tándem

VIH: Virus Inmunodeficiencia Humana

Zn: zinc

µg: microgramo(s) / **µmol:** micromol(es) / **µM**: micromolar

I. RESUMEN / SUMMARY

RESUMEN

Las cianobacterias han ido adquiriendo gran importancia a lo largo de los años debido a su capacidad de formar floraciones o "blooms", las cuales pueden llegar a ser tóxicas debido a la producción de cianotoxinas, por lo que se han convertido en una preocupación a nivel mundial en materia de contaminación ambiental, toxicológica, sanitaria y económica, ya que pueden afectar tanto a animales y plantas como a seres humanos. De todas las cianotoxinas, y dado que son las únicas que han causado fenómenos masivos de intoxicación en poblaciones humanas, merecen especial atención las Microcistinas (MCs) y la Cilindrospermopsina (CYN), constituyendo esta última el principal objeto de estudio de la presente Tesis Doctoral. Por todo ello, decidimos realizar una serie de estudios para investigar, en primer lugar, los efectos que pueden tener diferentes técnicas usuales de cocinado en las concentraciones de MCs en el pescado contaminado destinado al consumo humano. En el caso de la CYN, en primer lugar, nos pareció importante la puesta a punto y validación de métodos analíticos para cuantificar la toxina en diferentes matrices, y posteriormente decidimos profundizar en su mecanismo de toxicidad y sus efectos tóxicos, así como estudiar alternativas que nos permitieran contrarrestar los daños producidos por dicha toxina. Asimismo, también estudiamos su distribución en tejidos de peces contaminados. Todo ello en una de las especies acuáticas de consumo humano que pueden verse afectadas por esta toxina en su hábitat natural, como es el pez tilapia (*Oreochromis niloticus*). Por otra parte, también se ha abordado, mediante proteómica, el estudio del repertorio de Glutatión-S-transferasas (GSTs) en branquias de bivalvos, y los efectos de la CYN sobre plantas de consumo humano, como son las zanahorias (*Daucus carota*), expuestas a la toxina mediante el agua de riego.

Una de las vías de exposición a MCs más importantes es la vía oral. Además, las MCs pueden bioacumularse en organismos acuáticos, pudiendo ser transferidas a lo largo de la cadena trófica hasta los escalones más altos, y llegando así a los seres humanos, por lo que el consumo de estos animales acuáticos contaminados representa un riesgo potencial para la salud humana. Así, una correcta evaluación de riesgos de la exposición humana a MCs a través de los alimentos requiere de un detallado conocimiento acerca de la influencia del procesado y cocinado del alimento sobre estas toxinas. Es por ello que nos propusimos investigar los posibles cambios en la concentración de MCs libres en músculo de tilapias (*Oreochromis niloticus*) (porción comestible) a las que se les añadió una mezcla de MCs puras (MC-LR, MC-RR y MC-YR), conteniendo 1,5 µg/mL de cada toxina, y sometidas

posteriormente a dos técnicas de cocinado habituales, como son el horno microondas y el hervido en dos modalidades. Se ha comprobado que ambas técnicas de cocinado son capaces de reducir los niveles de MCs libres en el pescado (*Oreochromis niloticus*) en un rango entre 25-50%, siendo mayor con el hervido continuo en el caso de la MC-RR. Concretamente, el hervido continuo ha demostrado ser el más eficaz, debido tal vez al mayor tiempo de permanencia de la muestra en el agua. Asimismo, tras detectar MCs en el agua de hervido, mostramos que existe un riesgo adicional inherente a la utilización de esta agua para hacer sopas de pescado. En general, estas técnicas podrían ser consideradas como medidas de control de estas toxinas en la dieta tras el consumo humano de los alimentos cocinados. Los resultados de este experimento han dado lugar a la siguiente publicación:

1. EFFECTS OF THERMAL TREATMENTS DURING COOKING, MICROWAVE OVEN AND BOILING, ON THE UNCONJUGATED MICROCYSTIN CONCENTRATION IN MUSCLE OF FISH (OREOCHROMIS NILOTICUS). (*Guzmán-Guillén y col., 2011. Food and Chemical Toxicology 49, 2060-2067).*

La monitorización de CYN en medios contaminados por cianobacterias es de gran importancia para evaluar los riesgos que supone su exposición para la salud y el medioambiente. Las técnicas analíticas empleadas para detectar y cuantificar esta toxina, tanto en aguas como en otras matrices, son de diverso fundamento y complejidad (ELISA, LC-DAD, LC-MS y LC-MS/MS). Sin embargo, no todos estos métodos ofrecen la misma sensibilidad. La LC-MS/MS ha sido elegida como el método ideal y de referencia para detectar y cuantificar pequeñas cantidades de toxina en muestras de agua (Eaglesham y col., 1999; Bogialli y col., 2006; Oehrle y col., 2010) y en muestras más complejas, como tejidos de peces (Gallo y col., 2009) o muestras de suero y orina humanas (Foss y Aubel, 2013). Por ello nos propusimos poner a punto y validar diferentes métodos cuantitativos, incluyendo ensayos de robustez, para la determinación de CYN en distintas matrices (aguas, cultivos de cianobacterias y tejidos de peces contaminados con CYN) por LC-MS/MS. Con el empleo de cartuchos de carbón grafitizado se obtuvieron resultados fiables en la extracción de CYN a partir de aguas y de cultivos de cianobacterias, usando una mezcla acidificada de diclorometano:metanol como disolvente. En el caso de los tejidos de peces (hígado y músculo), mediante un sistema SPE de doble cartucho (uno de C18 y uno de carbón grafitizado) conseguimos extractos limpios que permitieron la recuperación de la toxina en un alto porcentaje (80-110%). Por tanto, los métodos que proponemos han sido adecuadamente validados: con amplios rangos de concentraciones de CYN, límites de detección y

cuantificación aceptables (que en el caso del agua, permiten la determinación de la toxina por debajo del valor guía propuesto por la OMS de 1 μg/L), recuperaciones en el intervalo 80-110%, y valores adecuados de precisión según la guía AOAC (Association of Official Analytical Chemists). Asimismo, la combinación de factores variables en la metodología demostró la robustez de los métodos propuestos para poder ser reproducidos bajo diferentes condiciones sin sufrir variaciones significativas en los resultados. Los métodos desarrollados en esta Tesis Doctoral pudieron ser aplicados con éxito y rapidez posteriormente para el análisis de CYN en las diferentes matrices estudiadas (aguas y matrices biológicas), permitiendo un control de la exposición, degradación, transferencia y bioacumulación de la CYN. Los resultados de estos experimentos han dado lugar a las siguientes publicaciones:

2. CYLINDROSPERMOPSIN DETERMINATION IN WATER BY LC-MS/MS: OPTIMIZATION AND VALIDATION OF THE METHOD AND APPLICATION TO REAL SAMPLES. *(Guzmán-Guillén y col., 2012. Environmental Toxicology and Chemistry 31 (10), 2233-2238).*

3. DEVELOPMENT AND OPTIMIZATION OF A METHOD FOR THE DETERMINATION OF CYLINDROSPERMOPSIN FROM STRAINS OF APHANIZOMENON CULTURES: INTRA-LABORATORY ASSESSMENT OF ITS ACCURACY BY USING VALIDATION STANDARDS. *(Guzmán-Guillén y col., 2012. Talanta 100, 356-363).*

4. CYN DETERMINATION IN TISSUES FROM FRESHWATER FISH BY LC-MS/MS: VALIDATION AND APPLICATION IN TISSUES FROM SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2015. Talanta 131, 452-459).*

Debido a que la CYN es una toxina emergente, su mecanismo de acción tóxica en las distintas especies acuáticas es menos conocido que el de las MCs. Nos propusimos entonces profundizar en el conocimiento del papel que juega el estrés oxidativo en el mecanismo de toxicidad de la CYN, investigando el efecto de diversas variables experimentales como son las dosis administradas (10 y 100 μg CYN/L) y el tiempo de exposición (7 y 14 días) sobre la alteración de biomarcadores de estrés oxidativo en hígado y riñón de peces de consumo humano (*O. niloticus*) expuestos a CYN por inmersión en aguas con un liofilizado de células de *A. ovalisporum* con 10 y 100 μg CYN/L, y 0,46 y 4,6 μg 7-desoxi-CYN/L, respectivamente. Nuestros resultados demuestran la implicación del estrés oxidativo como un mecanismo de acción tóxica de la CYN, manifestado por alteraciones en los niveles de LPO,

oxidación de proteínas y del ADN, la relación GSH/GSSG, y cambios en las actividades de GST, GPx, SOD, CAT, y γ-GCS. Además, se observaron en general cambios más significativos con la concentración más alta y durante el mayor período de exposición, lo que muestra una toxicidad dependiente de la concentración y del tiempo de exposición. Además, el riñón se mostró como el principal órgano afectado por la exposición a CYN. Demostrada la alteración de parámetros bioquímicos de estrés oxidativo, nos propusimos estudiar las alteraciones histopatológicas producidas en tilapias bajo las mismas condiciones experimentales. Esta relación dosis y tiempo-dependiente mostrada anteriormente se confirma con las alteraciones histopatológicas observadas en el hígado, riñón, corazón, tracto gastrointestinal (G.I.) y branquias de tilapias expuestas durante 7 ó 14 días a 10 ó 100 μg CYN/L. Los resultados de estas investigaciones se reflejan en las siguientes publicaciones:

5. CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES OXIDATIVE STRESS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUB-CHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2013. Chemosphere 90, 1184-1194).*

6. CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES HISTOPATHOLOGICAL CHANGES AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2013. Environmental Toxicology (in press). DOI: 10.1002/tox.21904).*

Una vez demostrado el papel del estrés oxidativo como mecanismo de acción tóxica de la CYN, así como las alteraciones histopatológicas producidas en varios órganos de tilapia, nos preguntamos si esta toxina también sería capaz de inducir alteraciones neurológicas en esta especie, basándonos en algunos estudios previos realizados en mamíferos y animales acuáticos expuestos a CYN (Kiss y col., 2002; Schoeb y col., 2002; Zagatto y col., 2012). Nuestros resultados muestran por primera vez los efectos neurotóxicos de la CYN en peces, con disminución del 35% en la actividad de la acetilcolinesterasa (AChE), aumento del 71% en los niveles de LPO y alteraciones histopatológicas en el cerebro de tilapias expuestas a dosis repetidas de CYN por inmersión en un cultivo de *A. ovalisporum* durante 14 días. Además, la toxina fue detectada en el 100% de los cerebros de peces expuestos a la toxina. Los resultados obtenidos se reflejan en la siguiente publicación:

7. CYLINDROSPERMOPSIN INDUCES NEUROTOXICITY IN TILAPIA FISH (OREOCHROMIS NILOTICUS) EXPOSED TO APHANIZOMENON OVALISPORUM. *(Guzmán-Guillén y col., 2015. Enviado a Aquatic Toxicology. Aceptado).*

Tras comprobar los efectos tóxicos producidos por la CYN en nuestro modelo experimental bajo las condiciones de ensayo expuestas, nos preguntamos sobre la efectividad del uso de algunos antioxidantes como suplementos en la dieta, como la L-carnitina (LC) y la vitamina E, para prevenir la aparición de estos efectos. En primer lugar, se comprobó que la LC, administrada como pretratamiento de 21 días, ejerce un efecto protector a partir de la menor dosis utilizada (400 mg LC/kg p.c./día) frente al daño oxidativo producido por la exposición aguda a 400 µg CYN/kg p.c., tanto pura como liofilizada, al prevenir la alteración de los niveles de biomarcadores de estrés oxidativo ya mencionados. Asimismo, también demostramos la utilidad de la LC para prevenir las alteraciones histopatológicas en hígado, riñón, corazón, tracto G.I. y branquias de peces intoxicados con CYN. Al mismo tiempo, nos planteamos investigar la efectividad de este antioxidante para aliviar o mejorar las variaciones en la expresión génica de las enzimas GPx y GST, así como la abundancia relativa de GST en hígado y riñón bajo las mismas condiciones, ya que estos marcadores pueden proporcionar una información temprana sobre el estado del pez. Los resultados preliminares mostraron que la LC consiguió restaurar a valores control los niveles de actividad GPx en hígado y GST en riñón de los peces expuestos a CYN. Asimismo, se observó un aumento de la expresión génica de GPx y GST en hígado de los peces suplementados con la dosis menor de LC (400 mg LC/kg) e intoxicados con CYN.

Asimismo, demostramos el uso de la vitamina E (pretratamiento de 7 días con 700 mg vitamina E/kg pez/día) como un quimioprotector seguro para la profilaxis de intoxicaciones de tilapias por CYN, mediante la restauración de los valores de biomarcadores oxidativos, así como la prevención de lesiones histopatológicas inducidas por CYN pura en el hígado, riñón, corazón, tracto G.I., branquias y cerebro de los peces expuestos. Todo ello ha tenido como resultado las publicaciones siguientes, y seguimos preparando otras publicaciones:

8. THE PROTECTIVE ROLE OF L-CARNITINE AGAINST CYLINDROSPERMOPSIN-INDUCED OXIDATIVE STRESS IN TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2013. Aquatic Toxicology 132-133, 141-150).*

9. L-CARNITINE (LC) PRETREATMENT PREVENTS HISTOPATHOLOGICAL CHANGES IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén y col., 2015. Pendiente de envío).*

10. PROTECTIVE ROLE OF DIETARY L-CARNITINE ON ACTIVITY AND TRANSCRIPTION OF ANTIOXIDANT ENZYMES IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén y col., 2015. Pendiente de envío).*

11. BENEFICIAL EFFECTS OF VITAMIN E SUPPLEMENTATION AGAINST THE OXIDATIVE STRESS ON CYLINDROSPERMOPSIN-EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2015. Pendiente de envío).*

12. VITAMIN E PRETREATMENT PREVENTS HISTOPATHOLOGICAL EFFECTS IN TILAPIA (OREOCHROMIS NILOTICUS) ACUTELY EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén y col., 2015. Pendiente de envío).*

Y ha dado lugar a las siguientes solicitudes de patentes:

USO DE L-CARNITINA PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA. PATENTE. *(Cameán y col., 2012).*

USO DE VITAMINA E PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA. PATENTE. *(Cameán y col., 2014).*

Posteriormente nos pareció interesante estudiar la posible reversión de los efectos tóxicos producidos por CYN tras transferir a los peces a un medio no contaminado, para su depuración. Los resultados mostraron que cortos periodos de depuración (3 ó 7 días) podrían ser efectivos para restaurar hasta los niveles basales algunos marcadores de estrés oxidativo (LPO, oxidación de proteínas y del ADN, actividades SOD, CAT y γ-GCS, y la relación GSH/GSSG), en hígado y riñón de tilapias expuestas a células de *A. ovalisporum* que contienen CYN y 7-desoxi-CYN durante 7 y 14 días. Asimismo, la depuración demostró la mejora de las alteraciones a nivel neurológico (actividad AChE, niveles de LPO, efectos histopatológicos y presencia de CYN en cerebro) en las mismas condiciones, sugiriendo que este puede ser un proceso de destoxicación efectiva en peces potencialmente expuestos a CYN. Los resultados han quedado patentes en la siguiente publicación:

13. EFFECTS OF DEPURATION ON OXIDATIVE BIOMARKERS IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER SUBCHRONIC EXPOSURE TO

CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN. *(Guzmán-Guillén y col., 2014. Aquatic Toxicology 149, 40-49).*

Por otra parte, consideramos de interés evaluar la distribución de la CYN en tejidos de peces contaminados, mediante inmunohistoquímica (IHC). Los resultados demostraron que el órgano que presentó mayor tinción fue el hígado, seguido por el riñón, el intestino y las branquias de tilapias expuestas a CYN. Además, la señal se intensificó con el aumento del tiempo tanto en el ensayo agudo como en el subcrónico, confirmando la toxicidad retardada de CYN, y también con el aumento de la dosis, como se muestra en el ensayo subcrónico. Las señales en el intestino y las branquias fueron más intensas en el ensayo subcrónico, debido a que los peces están continuamente sumergidos en el agua que contiene la toxina. Por lo tanto, se demuestra la utilidad de la IHC para estudiar la distribución de la CYN en estos organismos. Los resultados de este experimento han dado lugar a la siguiente publicación:

14. IMMUNOHISTOCHEMICAL APPROACH TO STUDY CYLINDROSPERMOPSIN DISTRIBUTION IN TILAPIA (OREOCHROMIS NILOTICUS) UNDER DIFFERENT EXPOSURE CONDITIONS. *(Guzmán-Guillén y col., 2014. Toxins 6, 283-303).*

Para la realización de esta Tesis Doctoral, la doctoranda realizó tres estancias de investigación en el Centro Interdisciplinar de Investigación Marina y Ambiental (CIIMAR) de la Universidad de Oporto, bajo la dirección del Dr. Vitor Vasconcelos (de Septiembre a Diciembre del 2012, de Agosto a Octubre del 2013, y de Junio a Agosto del 2014), en la que se abordaron varios objetivos.

Profundizamos en la diversidad proteica de las Glutatión-S-transferasas (GSTs), caracterizando el conjunto de sus isoformas presentes en las branquias del mejillón marino *Mytilus galloprovincialis*, mediante técnicas proteómicas. Los resultados preliminares muestran una eficiente extracción, purificación y separación de varias isoformas de GST mediante electroforesis bidimensional (2D), y su identificación por MALDI-TOF/TOF, constituyendo la primera caracterización de la familia de GSTs en este órgano.

Por último, por ser escasos los estudios que relacionan a la CYN con plantas, se consideró interesante el estudio del efecto de la CYN sobre organismos vegetales destinados al consumo humano, ya que ésta puede encontrarse en los reservorios de aguas destinadas al riego, y por tanto pasar a las plantas, constituyendo con ello un importante riesgo para la salud

humana. Así, nos propusimos conocer los efectos de la CYN sobre las condiciones fisiológicas, capacidad fotosintética y valor nutritivo (contenido en minerales) de zanahorias (*Daucus carota*) expuestas a diferentes dosis de la toxina durante 30 días. Los resultados preliminares sugieren que son capaces de hacer frente a concentraciones ambientales de CYN (10 y 50 μg/L) contenida en extractos de *A. ovalisporum*, con un mantenimiento y aumento del peso fresco de las raíces, sin afectar negativamente la fotosíntesis, aunque sí se afectan procesos metabólicos tales como la acumulación de minerales.

SUMMARY

Cyanobacteria have acquired a big importance along the years due to their ability to produce "blooms", which can become toxic due to the production of cyanotoxins. For this reason, cyanobacteria constitute a worldwide concern regarding environmental pollution, toxicology, health and economy, because they can affect animals, plants and humans. Among all the cyanotoxins, and since they are the only which have caused massive phenomena of intoxication in human populations, one of the most important are Microcystins (MCs) and Cylindrospermopsin (CYN), constituting the latter the main object of study of this Doctoral Thesis. Therefore, we decided to conduct a series of studies that allow us to understand, in the first place, the influence of different usual cooking techniques on MCs concentrations in contaminated fish for human consumption. In the case of CYN, firstly, we found it essential developing and validating diverse analytical methods to quantify the toxin in different matrices. Later, we decided to go into detail about CYN mechanism of toxicity and toxic effects and to consider alternatives that enable to counteract the damage caused by the toxin. Moreover, we also study the tissue distribution of contaminated fish. All in an aquatic species for human consumption that may be affected by this toxin in its natural habitat, such as tilapia fish (*Oreochromis niloticus*). Moreover, the characterization of Glutathione-S-transferases (GSTs) in gills of bivalves has also been studied by proteomics, as well as the effects of CYN on plants intended for human consumption, such as carrots (*Daucus carota*), exposed to the toxin by water irrigation.

One of the most important routes of exposure to MCs is the oral route. Furthermore, MCs can bioaccumulate in aquatic organisms, and they may be transferred along the food web, reaching humans; so eating these contaminated aquatic animals represents a potential risk for human health. Therefore, a proper risk assessment of human exposure to MCs through food requires a detailed knowledge about the influence of food processing and cooking on

these toxins. That is the reason why we decided to investigate possible changes in the concentration of free MCs in tilapia muscle (*Oreochromis niloticus*) (edible portion) spiked with a mixture of pure MCs (MC-LR, MC-RR and MC-YR), containing 1.5 µg/mL of each toxin, and then subjected to two usual cooking techniques, such as microwave and two types of boiling. It was found that both techniques are capable of cause a reduction of free MCs in fish (*Oreochromis niloticus*) in a range between 25-50%, obtaining higher reductions with the continuous boiling and in the case of MC-RR. Specifically, the continuous boiling has proved to be the most effective, perhaps due to the increased contact time between the sample and the water. Also, after detecting MCs in the waters in which fish boiled, it is deduced that there is an additional risk inherent to the use of this water for fish soups. In general, these techniques could be considered as control measures of these toxins in the diet after human consumption of cooked food. The results of this experiment have led to the following publication:

1. EFFECTS OF THERMAL TREATMENTS DURING COOKING, MICROWAVE OVEN AND BOILING, ON THE UNCONJUGATED MICROCYSTIN CONCENTRATION IN MUSCLE OF FISH (OREOCHROMIS NILOTICUS). (*Guzmán-Guillén et al., 2011. Food and Chemical Toxicology 49, 2060-2067).*

CYN monitoring in environments contaminated by cyanobacteria is of great importance in order to assess the risks of exposure to health and the environment. Analytical studies used to detect and quantify this toxin both in water and in other matrices are of diverse technical basis and complexity (ELISA, LC-DAD, LC-MS and LC-MS/MS); however, not all these methods offer the same sensitivity. LC-MS/MS has been chosen as the ideal method to detect and quantify small amounts of toxin in water samples (Eaglesham et al., 1999; Bogialli et al., 2006; Oehrle et al., 2010) and in more complex samples such as fish tissues (Gallo et al., 2009) or human serum and urine (Foss and Aubel, 2013). Therefore we decided to develop and validate different quantitative methods, including robustness tests for the determination of CYN in different matrices (water, cultures of cyanobacteria and CYN-contaminated fish tissues) by LC-MS/MS. By the use of graphitized carbon cartridges reliable results were obtained on CYN extraction from waters and cyanobacterial cultures using an acidified mixture of dichloromethane:methanol as solvent. In the case of fish tissues (liver and muscle), through a double SPE cartridge (C18+graphitized carbon) we obtained clean extracts with the recovery of the toxin in a high percentage (80-110%). Therefore, the methods we propose have been adequately validated: wide ranges of CYN concentrations, acceptable limits of detection and quantitation (in the case of water allowing the determination of toxin

below the proposed guideline value of 1 µg CYN/L by WHO), recoveries in the range 80-110%, and adequate precision values according to AOAC (Association of Official Analytical Chemists). Moreover, the combination of diverse factors in the methodology demonstrated the robustness of the proposed methods to be assayed under different conditions without significant variations in the results. The methods developed in this Thesis could be successfully and rapidly applied for analysis of CYN in water and biological matrices, allowing exposure control, degradation, transference and bioaccumulation of CYN. The results of these experiments have led to the following publications:

2. CYLINDROSPERMOPSIN DETERMINATION IN WATER BY LC-MS/MS: OPTIMIZATION AND VALIDATION OF THE METHOD AND APPLICATION TO REAL SAMPLES. *(Guzmán-Guillén et al., 2012. Environmental Toxicology and Chemistry 31 (10), 2233-2238).*

3. DEVELOPMENT AND OPTIMIZATION OF A METHOD FOR THE DETERMINATION OF CYLINDROSPERMOPSIN FROM STRAINS OF APHANIZOMENON CULTURES: INTRA-LABORATORY ASSESSMENT OF ITS ACCURACY BY USING VALIDATION STANDARDS. *(Guzmán-Guillén et al., 2012. Talanta 100, 356-363).*

4. CYN DETERMINATION IN TISSUES FROM FRESHWATER FISH BY LC-MS/MS: VALIDATION AND APPLICATION IN TISSUES FROM SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén et al., 2015. Talanta 131, 452-459).*

Because CYN is an emerging toxin, its mechanism of toxic action in different aquatic species is less known than in the case of MCs. We decided to go into detail in the understanding of the role of oxidative stress in the mechanism of toxicity of CYN, investigating the effects of various experimental variables such as the assayed dose (10 and 100 µg CYN/L) and the exposure time (7 and 14 days) on the alteration of biomarkers of oxidative stress in the liver and kidney of fish for human consumption (*Oreochromis niloticus*) exposed by immersion to lyophilized *A. ovalisporum* cells containing 10 and 100 µg CYN/L, and 4.6 and 0.46 µg 7-deoxy-CYN/L, respectively. Our results demonstrate the involvement of oxidative stress as a mechanism of action of CYN shown by alterations in LPO levels, proteins and DNA oxidation, GSH/GSSG ratio, and changes in the activities of GST, GPx, SOD, CAT, and γ-GCS. Generally, more important significant changes were observed at the highest concentration and the longest period of exposure, which shows

toxicity depends on concentration and exposure time. In addition, the kidney was shown as the main affected organ by exposure to CYN. Once demonstrated the alteration of biochemical parameters of oxidative stress, we decided to study the histopathological changes induced by CYN in tilapia under the same experimental conditions. This dose- and time-dependent toxicity shown above is confirmed by histopathological alterations observed in the liver, kidney, heart, gastrointestinal (G.I.) tract and gills of tilapia exposed for 7 or 14 days to 10 or 100 μg CYN/L. The results of these investigations are reflected in the following publications:

5. CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES OXIDATIVE STRESS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUB-CHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2013. Chemosphere 90, 1184-1194).*

6. CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES HISTOPATHOLOGICAL CHANGES AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén y col., 2013. Environmental Toxicology (in press). DOI: 10.1002/tox.21904).*

Once demonstrated the role of oxidative stress as a mechanism of toxic action of CYN and histopathological changes in various organs of tilapia, we wondered if this toxin was also able to induce neurological changes in this species, based on previous studies in mammals and aquatic animals exposed to CYN (Kiss et al., 2002; Schoeb et al., 2002; Zagatto et al., 2012). Our results show for the first time the neurotoxic effects of CYN in fish, with a decrease of 35% in the activity of acetylcholinesterase (AChE), increased levels of LPO (71%) and histopathological changes in the brains of tilapia exposed to repeated doses of CYN by immersion if an *A. ovalisporum* culture for 14 days. Furthermore, the toxin was detected in 100% brains of fish exposed to the toxin. The results obtained are shown in the following publication:

7. CYLINDROSPERMOPSIN INDUCES NEUROTOXICITY IN TILAPIA FISH (OREOCHROMIS NILOTICUS) EXPOSED TO APHANIZOMENON OVALISPORUM. *(Guzmán-Guillén et al., 2015. Aquatic Toxicology. Accepted).*

After investigating the effects of CYN in our experimental model under the assayed conditions, we wondered about the effectiveness of the use of some antioxidants as dietary

supplements, such as L-carnitine (LC) and vitamin E, to prevent the appearance of these effects. First, it was found that LC, administered as a pretreatment for 21 days, has a protective effect from the lowest dose used (400 mg LC/kg bw/day) against oxidative damage caused by acute exposure to 400 µg CYN/kg bw, both pure and lyophilized cells, to prevent alterations in the levels of oxidative stress biomarkers already mentioned. Furthermore, we also demonstrate the utility of LC to prevent the histopathological changes in liver, kidney, heart, G.I. tract and gills of fish intoxicated with CYN. At the same time, we proposed to investigate the effectiveness of the antioxidant to alleviate or improve the variations induced by CYN in gene expression of GPx and GST enzymes, and the relative abundance of GST in liver and kidney under the same conditions, as these markers can provide early information about the status of the fish. Preliminary results showed that LC managed to restore to control levels the activity of GPx in the liver and GST in the kidney of fish exposed to CYN. Also, an increase in gene expression of GST and GPx in liver of fish supplemented with the lowest dose of LC and intoxicated with CYN was observed.

Furthermore, we demonstrated the use of vitamin E (7 days pretreatment with 700 mg vitamin E/kg fish/day) as a chemoprotectant for the prophylaxis of CYN intoxications in tilapia, by restoring the values of oxidative biomarkers and prevention of histopathological lesions induced by pure CYN in liver, kidney, heart, G.I. tract, gills and brain of exposed fish. All of this had resulted in the following publications, and we continue working on the future ones pending submission:

8. THE PROTECTIVE ROLE OF L-CARNITINE AGAINST CYLINDROSPERMOPSIN-INDUCED OXIDATIVE STRESS IN TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén et al., 2013. Aquatic Toxicology 132-133, 141-150).*

9. L-CARNITINE (LC) PRETREATMENT PREVENTS HISTOPATHOLOGICAL CHANGES IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén et al., 2015. Pending submission).*

10. PROTECTIVE ROLE OF DIETARY L-CARNITINE ON ACTIVITY AND TRANSCRIPTION OF ANTIOXIDANT ENZYMES IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén et al., 2015. Pending submission).*

11. BENEFICIAL EFFECTS OF VITAMIN E SUPPLEMENTATION AGAINST THE OXIDATIVE STRESS ON CYLINDROSPERMOPSIN-EXPOSED TILAPIA (OREOCHROMIS NILOTICUS). *(Guzmán-Guillén et al., 2015. Pending submission).*

12. VITAMIN E PRETREATMENT PREVENTS HISTOPATHOLOGICAL EFFECTS IN TILAPIA (OREOCHROMIS NILOTICUS) ACUTELY EXPOSED TO CYLINDROSPERMOPSIN. *(Guzmán-Guillén et al., 2015. Pending submission).*

And it had led to the following patent applications:

USO DE L-CARNITINA PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA. PATENT. *(Cameán et al., 2012).*

USO DE VITAMINA E PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA. PATENT. *(Cameán et al., 2014).*

Later we found it interesting to study the possible reversion of the toxic effects of CYN after transference of fish to a clean environment for depuration. The results showed that short periods of depuration (3 or 7 days) may be effective in restoring to basal levels some oxidative stress biomarkers (LPO, protein and DNA oxidation, activities of SOD, CAT and γ-GCS, and the relationship GSH/GSSG), in liver and kidney of tilapia exposed to *A. ovalisporum* cells containing CYN and deoxy-CYN for 7 and 14 days. Moreover, depuration demonstrated the improvement of the neurological alterations (AChE activity, LPO levels, and presence of histopathological effects in brain CYN) under the same conditions, suggesting that this process can be effective in detoxification of fish potentially exposed to CYN. The results are shown in the following publication:

13. EFFECTS OF DEPURATION ON OXIDATIVE BIOMARKERS IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER SUBCHRONIC EXPOSURE TO CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN. *(Guzmán-Guillén et al., 2014. Aquatic Toxicology 149, 40-49).*

Furthermore, we decided to study the distribution of CYN in contaminated fish tissues by immunohistochemistry (IHC). As the results show, the organ with more staining was the liver, followed by the kidney, intestine and gills of tilapia exposed to CYN. Furthermore, the signal intensified with increasing time in both the acute and the subchronic assay, confirming the CYN delayed toxicity, and also with increasing of the dose, as shown in the subchronic assay. Signals in the intestine and the gills were more intense in the subchronic

experiment, because fish are continuously immersed in the water containing the toxin. Therefore, the usefulness of the IHC study for CYN distribution in these organisms is demonstrated. The results of this experiment have led to the following publication:

14. IMMUNOHISTOCHEMICAL APPROACH TO STUDY CYLINDROSPERMOPSIN DISTRIBUTION IN TILAPIA (OREOCHROMIS NILOTICUS) UNDER DIFFERENT EXPOSURE CONDITIONS. *(Guzmán-Guillén et al., 2014. Toxins 6, 283-303).*

For the fulfillment of this thesis, the PhD student performed three internships abroad, in the Centre of Marine and Environmental Research (CIIMAR) from the University of Porto, under the direction of Dr. Vitor Vasconcelos (September-December 2012, August-October 2013, and June-August 2014), where different objectives were addressed.

We decided to study the protein diversity of Glutathione-S-transferases (GSTs), characterizing the isoforms present in the gills of the marine mussel *Mytilus galloprovincialis*, by proteomic techniques. Preliminary results show an efficient extraction, purification and separation of various isoforms of GST by two-dimensional electrophoresis (2D), and their identification by MALDI-TOF/TOF, constituting the first characterization of the family of GSTs in this organ.

Finally, taking into account the scarce studies relating CYN and plants, we considered interesting the study of the effect of CYN on plant organisms intended for human consumption, since this can be found in water reservoirs for irrigation, affecting plants and constituting a significant risk for human health. Thus, we decide to study the effects of CYN on the physiological condition, photosynthetic capacity and nutritional value (mineral content) of carrots (*Daucus carota*) exposed to different doses of the toxin for 30 days. Preliminary results suggest that they are able to cope with environmental CYN concentrations (10 and 50 μg/L) contained in extracts of *A. ovalisporum*, with no changes or increased fresh weight of roots, without adverse effects on photosynthesis, although CYN appears to affect metabolic processes such as mineral reservoirs.

II. INTRODUCCIÓN / INTRODUCTION

1. CIANOBACTERIAS Y CIANOTOXINAS

Las cianobacterias son organismos procarióticos similares a las bacterias Gram negativas con capacidad de realizar la fotosíntesis oxigénica y fijar nitrógeno (Quesada y col. 2006a). Estas protofitas pigmentadas han jugado un papel clave en la oxigenación de la atmósfera de la tierra por su capacidad de captar la luz solar a longitudes de onda en las que la mayoría de los organismos fotosintéticos no pueden (Aráoz y col., 2010). Desde el punto de vista morfológico y de sus capacidades metabólicas son un grupo muy diverso, lo que refleja billones de años de evolución (Herrero y Flores, 2008).

El cambio climático global, más concretamente el incremento apreciable de la temperatura, junto con los flujos de ciertos nutrientes (nitratos, fosfatos, etc.) y la eutrofización de las aguas procedentes de cultivos agrícolas, de plantas de tratamiento de aguas residuales y otras fuentes antropogénicas, está afectando al crecimiento y distribución de las cianobacterias (Paerl y Huisman, 2009). Los agrupamientos masivos de cianobacterias se denominan "floraciones" o "blooms" y presentan un crecimiento en biomasa de una o varias especies. Ocurren generalmente a finales de verano, en períodos de horas a días y se disponen en la superficie de las aguas formando una lámina verde más o menos viscosa que puede tener aspecto de espuma (Pizzolon, 1996; de León, 2002). Esto es cada vez más frecuente en algunas regiones donde el crecimiento de la actividad agrícola y la industrialización no han ido acompañadas de un desarrollo adecuado de los tratamientos de aguas residuales (Azevedo y col., 2002) (**Figura 1**).

Algunas especies de cianobacterias son altamente tolerantes a condiciones extremas y pueden encontrarse en aguas termales a más de 60°C (Ward y col., 1994), o en lagos de regiones polares (Vincent 2000; Bonilla y col., 2009). Algunas cianobacterias habitan ecosistemas semiacuáticos como plataformas de hielo en latitudes extremas (Vincent y col., 2004), desiertos (Whitton y Potts, 2000) o colonizan superficies de cualquier tipo, incluso pinturas artísticas como frescos (Cappitelli y col., 2009).

Por lo tanto, debido a la amplia distribución de las cianobacterias por todo el mundo, por el cambio climático y el aumento de la eutrofización de las aguas, se ha propuesto que las reservas de agua y la fauna acuática deben estar sujetas regularmente a una monitorización y control de calidad (Chorus y Bartman, 1999).

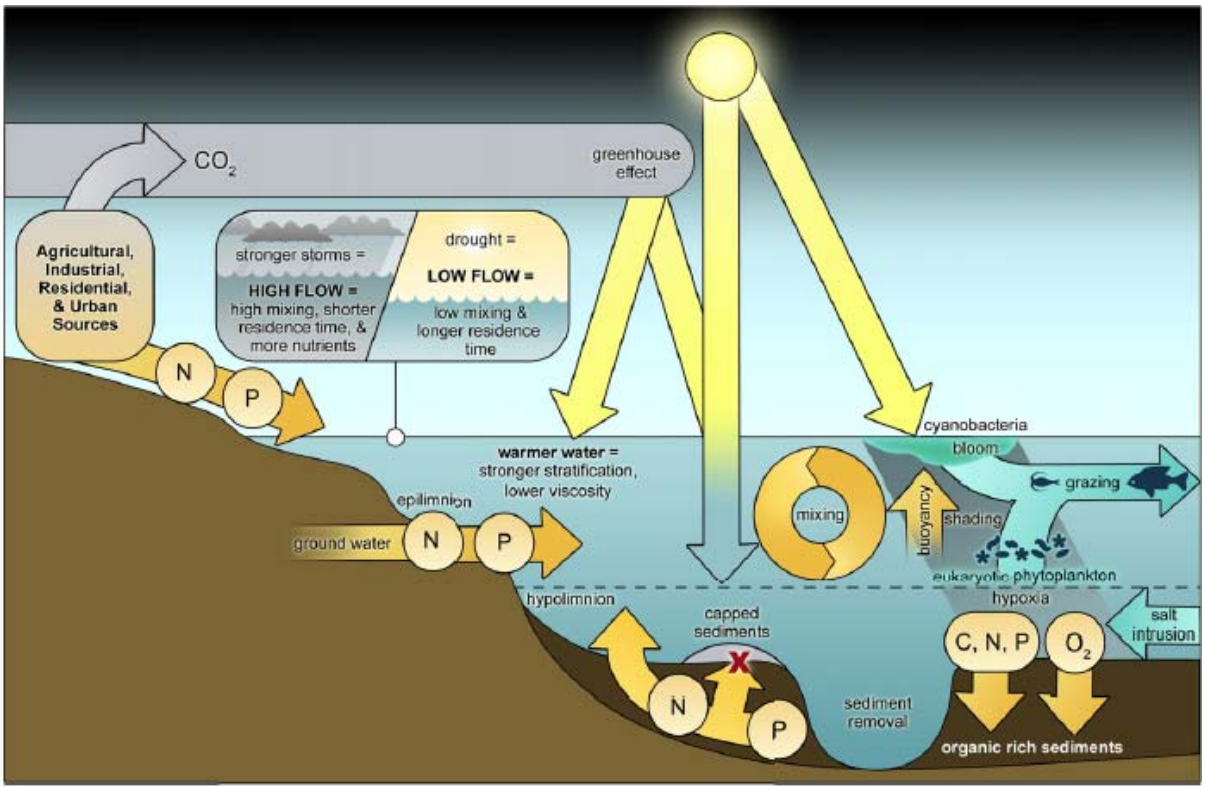

Figura 1. Procesos ambientales que controlan las floraciones de cianobacterias, incluidas las acciones de manejo llevadas a cabo por el hombre y los impactos del cambio climático (tomada de Paerl y col., 2011).

Existe una gran variedad de especies y cepas de cianobacterias que producen toxinas y otros compuestos biológicamente activos, representando un riesgo tanto para la salud de los seres humanos y los animales que las consumen o están en contacto con aguas contaminadas (Codd y col., 2005; Quesada y col., 2006a), como para el medio ambiente (O´Neil y col., 2012). Su presencia afecta a la calidad de las aguas mediante cambios de pH, sabor, transparencia y olor (Bláhová y col., 2009). Se ha estimado que aproximadamente el 25-75% de las floraciones de cianobacterias son tóxicas (Lawton y Codd, 1991; Chorus, 2001). Los principales géneros de cianobacterias por su repercusión en la salud humana, animal y en el medio ambiente por su capacidad de producir toxinas son *Microcystis, Cylindrospermopsis*, *Aphanizomenon*, *Nodularia*, *Anabaena, Nostoc*, *Oscillatoria* y *Lyngbya* (Corbel y col., 2014).

Por todo esto, algunos autores advierten que, en un momento dado, sea posible que todas las reservas de agua del mundo puedan contener cianobacterias (Sivonen, 1996; Fastner y col., 1999; Mankiewicz y col., 2001), convirtiéndose así en un problema de la calidad del agua en muchos países del mundo (Park y col., 1996; Zhang y col., 2009) y con importantes consecuencias económicas (Carmichael, 2001, 2008; Paerl, 2008; Paerl y Huisman, 2008), y sobre la salud (Falconer y col., 1994; Pouria y col., 1998; Chorus y col., 2000; Codd y col., 2005).

Las cianotoxinas son muy diversas tanto en su estructura química como en su toxicidad (Briand y col., 2003), y se pueden clasificar en función de los efectos tóxicos que producen en cinco grupos diferentes (Gutiérrez-Praena y col., 2013a):

- **Hepatotoxinas**: **microcistinas** y nodularia.
- **Neurotoxinas**: anatoxina-a, anatoxina-a (s), saxitoxinas.
- **Citotoxinas**: **cilindrospermopsina**.
- **Dermatotoxinas**: aplisiatoxinas, lyngbyatoxinas.
- **Toxinas irritantes**: lipopolisacáridos.

Figura 2. Ejemplo de grandes masas de agua que han experimentado aumentos en la frecuencia, magnitud y duración de floraciones de cianobacterias. Fotografía de una floración en la orilla del Lago Erie (Ohio, EEUU) (izquierda) e imagen por satélite de la cuenca occidental del lago (derecha), durante el verano de 2009 (tomada de Paerl y col., 2011).

De todas las cianotoxinas, y dado que son las únicas que han causado fenómenos masivos de intoxicación en poblaciones humanas, merecen especial atención las Microcistinas (MCs) y la Cilindrospermopsina (CYN), constituyendo esta última el principal objeto de estudio de la presente Tesis Doctoral.

2. MICROCISTINAS

2.1. Estructura química

Las microcistinas (MCs) deben su nombre al organismo del cual se aislaron por primera vez, *Microcystis aeruginosa* (Carmichael y col., 1988). Estas comprenden el grupo de cianotoxinas más ampliamente distribuido y estructuralmente más diverso. Las MCs son heptapéptidos cíclicos que comparten una estructura común (Carmichael y col., 1988) (**Figura 3**):

D-Ala (1)-**X** (2)-D-MeAsp (3)-**Z** (4)-Adda (5)-D-Glu (6) -MdhA (7)

Donde:

(1) D-Ala: D-alanina

(3) D-MeAsp: Ácido D-eritro-β-metil-aspártico

(5) Adda: Ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienoico

(6) D-Glu: Ácido D-glutámico

(7) Mdha: N-metildehidro-alanina

(2) **X** y (4) **Z** son los L-aminoácidos variables

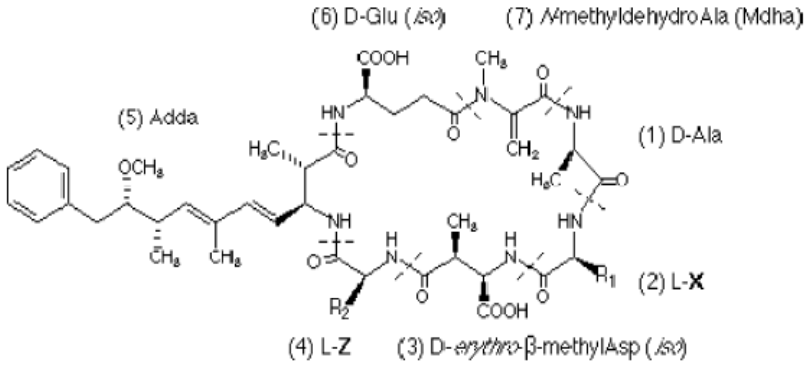

Figura 3. Estructura general de las microcistinas (tomada de Msagatti y col., 2006)

Hoy día se conocen alrededor de 100 variantes de MCs en función del grado de metilación, hidroxilación, epimerización, secuencia del péptido y toxicidad (Wu y col., 2014). Estas variaciones se producen en todos los aminoácidos, pero se encuentran con más frecuencia en los L-aminoácidos de las posiciones 2 (X) y 4 (Z) y en la metilación o desmetilación de los aminoácidos en las posiciones 3 (D-MeAsp) y/o 7 (Mdha) (Namikoshi y col., 1998; Sekadende y col., 2005).

Las MCs más frecuentemente encontradas en la naturaleza son: la microcistina-LR (MC-LR) que contiene leucina (L) en la posición 2 y arginina (R) en la posición 4, siendo la más abundante y suponiendo el 46,0-99,8% de la concentración total de MCs en floraciones naturales (Vasconcelos y col., 1996; Pereira y col., 2011); la microcistina-RR (MC-RR) que contiene arginina en ambas posiciones; la microcistina-YR (MC-YR) que contiene tirosina (Y) y arginina (R), y la MC-LA que contiene leucina (L) y alanina (A) (Gurbuz y col., 2009;

Martins y Vasconcelos, 2009; Vasas y col., 2010). El aminoácido Adda en posición 5 es característico y común en todas ellas, considerándose el principal responsable de su toxicidad (Dawson, 1998).

2.2. Mecanismo de acción tóxica de las MCs

Los principales mecanismos de acción tóxica de las MCs son la inhibición de las fosfatasas de proteínas, formación de especies reactivas de oxígeno (ERO) y daño del ADN (Filipič y col., 2011). También pueden actuar como promotores tumorales y la MC-LR ha sido clasificada como "posiblemente cancerígena para los seres humanos" (grupo 2B) por la Agencia Internacional para la Investigación sobre el Cáncer (IARC) (http://www.iarc.fr/).

2.3. Vías de exposición y efectos tóxicos de las MCs

La mayoría de las MCs son intracelulares (Kaebernick y Neilan, 2001; Juttner y Luthi, 2008), siendo sus niveles generalmente altos (Wiedner y col., 2003) y pudiendo así dañar a los organismos que se alimentan de cianobacterias tóxicas (Rohrlack y col., 2001; Lürling, 2003; Juhel y col., 2006; Smith y Haney, 2006; Trubetskova y Haney, 2006).

Uno de los brotes de intoxicación por MCs más importantes en seres humanos fue el ocurrido en Caruaru (Brasil) en 1996, donde 100 pacientes de hemodiálisis desarrollaron un cuadro tóxico de diversa gravedad. Presentaron neurotoxicidad aguda y hepatotoxicidad subaguda, y 76 de ellos murieron (Pouria y col., 1998). Las MCs también tienen capacidad de producir irritación en ojos, oídos, y piel, descamación de la piel, erupción y ampollas en la piel y en la boca, debido a exposiciones por vía dérmica y/o inhalatoria. Por vía oral, otros síntomas son el daño renal con pérdida de electrolitos, sangrado en la orina, deshidratación, gastroenteritis, diarrea, náuseas, vómitos, tos perruna, dolor de garganta, alergias, dolor de cabeza, y letargo (Bell y Codd, 1994; Falconer, 1998; Jochimsen y col., 1998).

En lo que se refiere a legislación, España es uno de los pocos países que tienen legislación concreta respecto a las MCs, donde se ha elegido el valor guía recomendado por la Organización Mundial de la Salud (OMS) de 1 µg/L para aguas de consumo humano.

2.4. Estabilidad y degradación de las MCs

Las MCs son compuestos químicamente estables, resistentes a diferentes condiciones de luz, pH y temperatura, y a la hidrólisis química a pH casi neutro (Harada y col., 1996). Estos autores demostraron que a altas temperaturas (40°C) se producía una hidrólisis lenta,

requiriéndose, para lograr más del 90% de su disminución, aproximadamente 10 semanas a pH 1 y más de 12 semanas a pH 9. Además, los cambios de pH (1-12) demostraron tener poco impacto en la degradación de la MC-LR (Yu y col., 2009). Estos mismos autores observaron tras una incubación de 6 h, que la MC-LR no se degradaba de 10°C a 150°C, mientras que su vida media a 200°C era inferior a media hora. Según otro estudio, las MCs son químicamente estables incluso a temperaturas superiores a 300°C en condiciones de laboratorio (Wannemacher, 1989), y pueden soportar varias horas de ebullición (Van Apeldoorn y col., 2007). Por otra parte, un estudio en el que MCs disueltas en agua fueron sometidas a una prueba de disolución con fluidos gástrico e intestinal según las condiciones de la Farmacopea de EEUU, demostró que las MC-RR, MC-YR y MC-LR sufrieron hidrólisis bajo condiciones gástricas (pepsina y pH 1,2), siendo la MC-RR la toxina más afectada. Sin embargo, ninguna de ellas se degradó por la digestión intestinal (pancreatina y pH 7,5) (Moreno y col., 2004).

2.5. Efectos del procesado de alimentos sobre la concentración de MCs

Como ya se ha comentado, una de las vías de exposición a MCs más importantes es la vía oral. Además, las MCs pueden bioacumularse en organismos acuáticos, pudiendo ser transferidas a lo largo de la cadena trófica hasta los escalones más altos, y llegando así a los seres humanos, por lo que el consumo de estos animales acuáticos contaminados representa un riesgo potencial para la salud humana (Zhang y col., 2010).

Una vez conocido el peligro y los posibles riesgos derivados del consumo de MCs, deben tomarse las medidas oportunas para su control. En pescados y mariscos, dichas medidas a menudo incluyen restricciones en su recolección cuando los niveles de alerta se superan. Otra medida de control potencialmente eficaz consiste en eliminar los partes de estos animales en las que las cianotoxinas se suelen acumular, como las vísceras (principalmente el hígado) de los peces o las agallas y hepatopáncreas de los moluscos, antes de su procesamiento, cocinado y consumo.

Ibelings y Chorus (2007) sugirieron que las técnicas domésticas de cocinado no son capaces de eliminar o alterar las MCs presentes en estos alimentos. Esta afirmación no es sorprendente teniendo en cuenta la estabilidad expuesta anteriormente de estas toxinas.

Por otra parte, la estimación de la exposición a MCs se ha llevado a cabo siempre comparando directamente su concentración en el alimento crudo con el valor de la Ingesta Diaria Tolerable (IDT) de 0,04 µg/kg de peso corporal/día, establecida para la MC-LR por la

OMS, asumiendo que la concentración de MCs en el alimento crudo y en el que está listo para consumir es similar. Sin embargo, los alimentos suelen ser consumidos tras someterlos a diferentes procesos de almacenamiento (refrigeración, congelación) y/o cocinado (hervido, microondas, fritura, etc.). Estos tratamientos pueden hacer variar de diferente forma la concentración de MCs libre en dicho alimento. Además, una vez ingeridos, los alimentos están sujetos a las condiciones fisicoquímicas del estómago y del intestino delgado, que pueden alterar la bioaccesibilidad de las MCs (Freitas y col., 2014). Por lo tanto, una correcta evaluación de riesgos de exposición humana a MCs a través de los alimentos requiere un detallado conocimiento cuali- y cuantitativo acerca de la influencia de estos procesos sobre estas toxinas.

En concreto, los efectos de las prácticas de cocinado pueden depender tanto del tipo de método aplicado (hervido, microondas, horno de asado, etc.), del tiempo de calentamiento y temperatura empleada, como también de las características fisicoquímicas del nutriente/contaminante, ya que influyen en su estabilidad. Así, por ejemplo, la influencia del cocinado sobre los niveles de metales tóxicos y diversos contaminantes ambientales orgánicos no se basa sólo en el tipo de práctica empleada, sino también en el tipo de alimento en cuestión (Domingo, 2011).

A pesar de esto, el número de estudios con respecto a la influencia del procesado de alimentos sobre los xenobióticos no es tan amplio, centrándose principalmente en un reducido número de contaminantes, tales como metales (Devesa y col., 2008; Perelló y col., 2008), bifenilos policlorados (PCBs) (Hori y col., 2005), difeniléteres polibromados (PBDEs), hexaclorobenceno (HCB) e hidrocarburos aromáticos policíclicos (HAPs) (Perelló y col., 2009), radionúclidos (Burger y col., 2004), y algunos pesticidas, siendo los estudios muy escasos en el caso de las MCs (Morais y col., 2008; Zhang y col., 2010; Freitas y col., 2014).

El primer estudio sobre el uso del horno microondas y el hervido para extraer MCs a partir de células de cianobacterias fue el realizado por Metcalf y Codd (2000). En comparación con la liofilización y posterior extracción de las MCs de los extractos con metanol, el tratamiento mediante hervido y microondas permitió la máxima detección de la toxina tras 9 y 1 min, respectivamente. Aunque su objetivo no era precisamente investigar el efecto de estas técnicas en los tejidos de organismos acuáticos de consumo humano, establecieron la base para futuros estudios sobre la eliminación de MCs de los alimentos. En este sentido, Morais y col. (2008) demostraron que el hervido durante 5 y 30 min de

mejillones (*Mytilus galloprovincialis*) conteniendo MCs no disminuyó significativamente los niveles de éstas. Sin embargo, tras 1 y 5 min al microondas, el contenido en MCs disminuyó significativamente, incluso tras 1 min, sugiriendo que esta era una nueva forma de evitar intoxicaciones por consumo de mejillones contaminados con MCs.

Posteriormente, Zhang y col. (2010) estudiaron por primera vez la estabilidad de las MCs acumuladas en músculo de pescado tras el cocinado, utilizando la carpa cabezona (*Aristichthys nobilis*) como modelo experimental. Estos autores postulan que el hervido permite una mejor liberación de las MCs a partir del músculo del pescado, quizás al acelerar la liberación de MCs de las fosfatasas de proteínas PP1 y PP2A, al encontrar mayores niveles de MCs en el músculo hervido que en el músculo sin cocinar. También detectaron MCs en el agua de hervido, sugiriendo que existe un riesgo adicional inherente a la utilización de esta agua para hacer sopas de pescado.

Recientemente, Freitas y col. (2014) mostraron cómo la temperatura ambiente (25°C) y la refrigeración (4°C) hacían disminuir con el tiempo la concentración de MC-LR libre en mejillones (*Corbicula fluminea*) expuestos a la toxina, mientras que la congelación (-20°C) la aumentaba con el tiempo. En general, las técnicas de cocinado empleadas (microondas por 30 segundos y 1 min, y hervido por 5, 15 y 30 min, principalmente) resultaron en una mayor concentración de MC-LR libre en los mejillones. Además, estos autores simularon una digestión gastrointestinal (G.I.) completa y más realista del alimento al someterlo a una digestión ácida seguida de la digestión intestinal, lo que redujo la concentración de MC-LR libre hasta niveles inferiores al tejido sin cocinar.

Todos estos estudios indican que los valores detectados en alimentos crudos no son representativos de la exposición humana real, pudiéndose subestimar o sobrevalorar la exposición real a estas toxinas a través del consumo de alimentos contaminados que serán cocinados previos a su consumo.

3. CILINDROSPERMOPSINA

3.1. Estructura y propiedades fisicoquímicas

La cilindrospermopsina (CYN) es un alcaloide tricíclico derivado de la guanidina unido a un grupo hidroximetiluracilo en el C7 (Ohtani y col., 1992), con un peso molecular relativamente bajo (415 Da) (Sivonen y Jones, 1999).

Hasta hace poco se habían identificado dos variantes de la cilindrospermopsina (CYN) con estructuras análogas (**Figura 4**):

- 7-epicilindrospermopsina (7-epi-CYN), es un epímero de la CYN con toxicidad parecida a ésta (Looper y col., 2005). Se ha comprobado su producción como metabolito secundario por *Aphanizomenon ovalisporum* (Banker y col., 1997, 2000), y como metabolito principal por *Oscillatoria sp*. (Mazmouz y col., 2010; de la Cruz y col., 2013). Está presente en pequeñas cantidades, representando menos del 5% del total de la CYN producida por estas especies (Davis y col., 2014).
- 7-desoxicilindrospermopsina (7-desoxi-CYN), producida como metabolito secundario por *Cylindrospermopsis raciborskii* (Norris y col., 1999; Li y col., 2001a) y como su principal metabolito por *Raphidiopsis curvata* y *Lyngbya wollei* (Li y col., 2001b; Seifert y col., 2007). Está presente en grandes cantidades, representando junto con la CYN más del 95% del total producido por estas especies (Davis y col., 2014).

El descubrimiento de estos metabolitos llevó al estudio de la relación entre la estructura química de los compuestos y su toxicidad. Banker y col. (2001) sugirieron que la molécula de uracilo es crucial para la toxicidad de la CYN. Por otro lado, la orientación del grupo hidroxilo en el centro epimérico del C7 no está relacionada con la toxicidad de la CYN (Reisner y col., 2004; Seifert y col., 2007), lo que explica la toxicidad similar entre la CYN y la 7-epi-CYN. Sin embargo, la toxicidad de la 7-desoxi-CYN aún está debatiéndose (Sotton y col., 2014). Aunque en un principio se pensara que no era tóxica (Runnegar y col., 2002), posteriores estudios demostraron que es capaz de inhibir la síntesis de proteínas con potencia similar a la de CYN y que probablemente tenga su correspondiente toxicidad *in vivo* (Looper y col., 2005, 2006).

Recientemente, Wimmer y col. (2014) detectaron la presencia no sólo de CYN, sino también de dos nuevos análogos en una cepa de *C. raciborskii* aislada en la costa de Nueva Zelanda: 7-desoxi-desulfo-cilindrospermopsina y 7-desoxi-desulfo-12-

acetilcilindrospermopsina (**Figura 4d,e**). Previamente, Runnegar y col. (2002) habían demostrado que el grupo sulfato de la estructura de CYN no desempeña ningún papel ni en la actividad tóxica, ni en el transporte de la misma hacia el interior de las células de los órganos diana. Teniendo esto en cuenta, es probable que estos nuevos análogos también posean las actividades biológicas nocivas mostradas por el resto de la familia de cilindrospermopsinas.

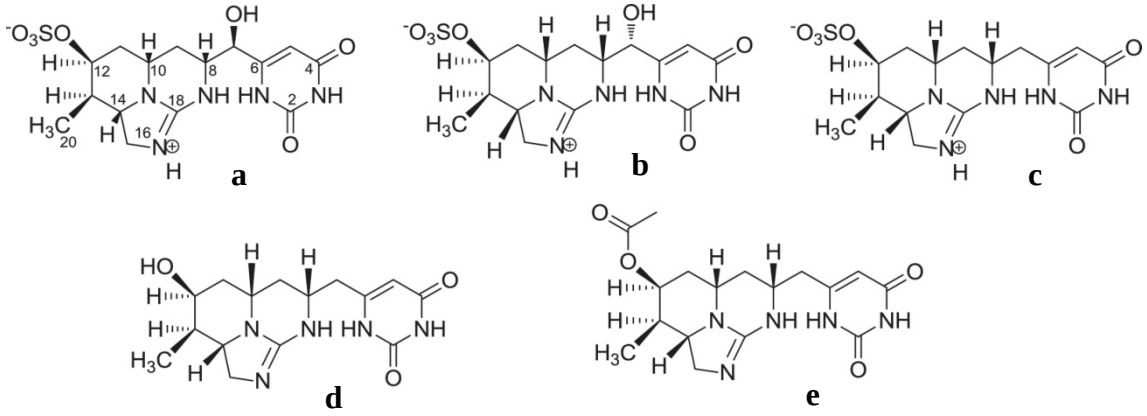

Figura 4. Estructura de las tres cilindrospermopsinas conocidas (**a-c**) y de los dos nuevos análogos (**d** y **e**): (**a**) cilindrospermopsina; (**b**) 7-epicilindrospermopsina; (**c**) 7-desoxicilindrospermopsina; (**d**) 7-desoxi-desulfo-cilindrospermopsina; (**e**) 7-desoxi-desulfo-12-acetilcilindrospermopsina (Wimmer y col., 2014).

La CYN es altamente hidrosoluble debido a su forma zwitterión (Shaw y col., 2000), encontrándose de hecho disuelta en el agua en un 70-98%, lo que la hace una toxina principalmente extracelular (Rücker y col., 2007; van Apeldoorn y col., 2007). Hasta el momento, los principales estudios de estabilidad molecular han sido realizados con CYN, debido a la presencia minoritaria de 7-epi-CYN y a la dudosa toxicidad de 7-desoxi-CYN. En este sentido, la CYN se muestra muy estable a altas temperaturas de hervido (100°C durante 5 min). Además, cuando se expone a cambios de pH entre 4 y 10 durante 8 semanas, sólo se degrada un 25%, y no muestra ninguna degradación en estado sólido o en soluciones acuosas puras. Sin embargo, cuando está en cultivo se descompone rápidamente por exposición a la luz solar (vida media de 1-5 h) (Chiswell y col., 1999). El tratamiento del agua por cloración a pH 6 produce la degradación de CYN inmediatamente; no obstante, la molécula es más estable a valores más bajos de pH (Senogles y col., 2000).

3.2. Especies productoras y distribución geográfica

Actualmente, se conocen trece especies de cianobacterias capaces de producir CYN en cuatro de los cinco continentes (Oceanía, América, Asia y Europa), siendo *Cylindrospermopsis raciborskii* (Ohtani y col., 1992; Li y col., 2001a) y *Aphanizomenon ovalisporum* (Banker y col., 1997; Shaw y col., 1999) las principales productoras, estando distribuidas de forma diferente por todo el mundo (**Tabla 1**). También se ha comprobado la producción de esta toxina por *Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Aphanizomenon flos-aquae, Aphanizomenon flos-aquae* var. *klebahnii, Aphanizomenon gracile, Lyngbya wollei, Oscillatoria* sp. PCC 6506*, Raphidiopsis curvata, Raphidiopsis mediterranea y Umezakia natans* (Moreira y col., 2012).

Se ha detectado la presencia de CYN predominantemente en aguas tropicales y subtropicales, donde se presenta en diferentes entornos, como lagos, embalses, ríos, estanques y presas (Pearson y col., 2010). Algunos estudios de campo han encontrado altas concentraciones de CYN en aguas de diferentes países, alcanzando valores de 1,1 y 800 μg/L en Australia (Hoeger y col., 2004; Shaw y col., 2000), 97,1 μg/L en Florida, EEUU (Burns y col. 2002), 12,1 μg/L en Alemania (Rucker y col., 2007) y 18,4 μg/L en Italia (Bogialli y col., 2006). En España, Quesada y col. (2006b) detectaron concentraciones de 9,4 μg/L. Todas estas concentraciones superan el valor de referencia propuesto para CYN en agua potable por Humpage y Falconer (2003) de 1 μg/L.

Especie productora	País	[CYN] µg/L o µg/g de peso seco*	Referencia
C. raciborskii	Alemania	0,2 (49-741*)	Fastner y col., 2003
	Arabia Saudi	0,03-23,3	Mohamed y Al-Shehri, 2013
	Australia	5500* 3,4 1,1 38,2	Hawkins y col., 1997 McGregor y Fabbro, 2000 Hoeger y col., 2004 Everson y col., 2009
	EEUU (Florida)	50-202	Falconer y Humpage, 2006; Williams y col., 2006
	Egipto	n.r.	Mohamed, 2007
	Grecia	0,3-2,8	Gkelis y Zaoutsos, 2014
	Italia	15 2,6-126	Manti y col., 2005 Messineo y col., 2010
	Polonia	0,2–1,8	Kokocinski y col., 2009
	Tailandia	1020*	Li y col., 2001 a
A. ovalisporum	Australia	4-120	Shaw y col, 1999
	España	1,5-9,4	Quesada y col., 2006b
	EEUU	7390-9330*	Yilmaz y col, 2008
	Italia	2,6-126	Messineo y col., 2010
Otras	Alemania	12,1	Fastner y col., 2007; Rücker y col., 2007
	Australia	101,4	Everson y col., 2011
	EEUU	8,1-97,7	Burns, 2008
	Francia	1,6-1,9	Brient y col., 2009
	Italia	0,4- 126	Messineo y col., 2010
	República Checa	0,4-4,4	Bláhová y col., 2009

(*) Las concentraciones se presentan en µg/g de peso

Tabla 1. Estudios que muestran concentraciones de CYN detectadas en diferentes países de cuatro continentes (Oceanía, América, Asia y Europa). Las concentraciones se presentan en µg/L o bien en µg/g de peso seco. Modificado de Corbel y col., 2014.

3.3. Mecanismos de acción tóxica de la CYN

Aunque el mecanismo de acción tóxica de la CYN aún no ha sido totalmente esclarecido y las dianas moleculares específicas de la CYN no están caracterizadas (Campos y col., 2013), se considera que su toxicidad está mediada por citocromo P450 (CYP450) (Humpage y col., 2005). Los principales mecanismos de toxicidad conocidos de la CYN son la inhibición irreversible de la síntesis de proteínas (Runnegar y col., 2002; Froscio y col., 2003) y de glutatión (GSH) (Runnegar y col., 1994, 1995; Humpage y col., 2005), la fragmentación del ADN resultado de la genotoxicidad mediada por la toxina (Žegura y col., 2011), así como el estrés oxidativo demostrado por estudios *in vitro* e *in vivo* realizados en peces más recientemente (Gutiérrez-Praena y col., 2011a, b; Puerto y col., 2011).

3.3.1. Inhibición de la síntesis de proteínas

La inhibición de la síntesis de proteínas fue demostrada por Terao y col. (1994) tanto *in vivo* como *in vitro*. Estos autores investigaron los efectos morfológicos y bioquímicos producidos en ratones expuestos por vía i.p. a una dosis de 200 µg CYN/kg, aislada de un cultivo de *Umezakia natans* y sacrificados tras 72 h. Observaron que el órgano más afectado fue el hígado y establecieron cuatro fases de cambios patológicos, consistiendo la fase inicial en la inhibición de la síntesis de proteínas. En el estudio *in vitro*, se observó la inhibición de la síntesis de globina en lisados de reticulocitos de conejo (Terao y col., 1994). Otro estudio realizado en hepatocitos primarios de ratón mostró que la inhibición de la síntesis de proteínas fue un indicador temprano de la respuesta celular a la exposición a CYN, presentándose a concentraciones subtóxicas y mucho antes de la aparición de toxicidad a concentraciones más altas (Froscio y col., 2003). Tras comparar los efectos de la CYN con otras sustancias inhibidoras de la síntesis de proteínas, se observó que la inhibición era irreversible, incluso tras la eliminación de la toxina. Estos efectos irreversibles pueden ser generados por la unión covalente de la toxina con los ribosomas y/o sus componentes, o por la modificación intracelular de la toxina que evita su salida de la célula (Froscio y col., 2003).

Por otro lado, algunos estudios indican que la CYN interfiere en el paso de elongación de las proteínas, ya que se comprobó que la adición de la misma a un lisado de reticulocitos dio lugar al cese inmediato de la síntesis de proteínas (Froscio y col., 2002). Además, Runnegar y col. (2002) demostraron que las alteraciones en el anillo de guanidina

presentes en los análogos de CYN, también potencian la acción inhibidora de ésta en lisados de reticulocitos de conejo.

Estudios posteriores sugirieron que la CYN más que unirse al ribosoma, se une a una de las proteínas solubles asociadas al sistema de traducción eucariótica a través de un enlace no covalente y de esta forma ejerce su efecto inhibidor sobre la síntesis de proteínas (Froscio y col., 2008).

3.3.2. Inhibición de la síntesis de glutatión

La molécula de GSH forma parte del sistema antioxidante no enzimático, por lo que juega un papel fundamental en la defensa celular frente a las especies reactivas de oxígeno (ERO) y participa en las vías de señalización celular. Además, es necesario en el mantenimiento de la homeostasis celular y en las muchas reacciones de conjugación (Fase II), interviniendo así en procesos de destoxicación de una gran variedad de xenobióticos (Boyland y Chasseaud, 1969).

La inhibición de la síntesis de GSH ha sido descrita como uno de los mecanismos de toxicidad de CYN (Runnegar y col., 1995; Humpage y col., 2005), lo que puede generar un aumento del estrés oxidativo. El papel que juegan tanto el GSH como el CYP450 en el mecanismo de toxicidad de la CYN se ha investigado *in vitro* e *in vivo*. Así, Runnegar y col. (1994) comprobaron que la exposición a CYN condujo a una disminución del contenido de GSH en cultivos de hepatocitos de rata. Este mismo equipo realizó un seguimiento de la inhibición de la síntesis de GSH que precedía al efecto tóxico, observando daño celular a altas dosis de CYN, probablemente producido por una reducción de la capacidad de destoxicación de la célula, atribuyendo así la reducción de GSH inducida por CYN a la inhibición de su síntesis, más que a un aumento de su consumo (Runnegar y col., 1995). Además, observaron la implicación del CYP450 en el mecanismo de toxicidad de CYN por activación metabólica tras ensayar con bloqueantes de esta enzima y hallar una protección parcial de los mismos frente a la toxicidad de la CYN. De forma similar, Gutiérrez-Praena y col. (2011a) observaron una reducción significativa en el contenido de GSH y de la actividad de la enzima γ-glutamilcisteina sintetasa (γ-GCS), implicada en su síntesis, en líneas celulares de peces (PLCH-1) expuestas a distintas concentraciones de CYN pura (2, 4 y 8 μg/mL durante 24 y 48 h), llegando así a establecer la misma causa para el agotamiento del GSH.

Posteriormente, Shaw y col. (2000) observaron efectos tóxicos en la región periacinar del hígado, donde el CYP450 participa activamente en la metabolización de los xenobióticos, sugiriendo que su activación estaba implicada en la toxicidad de la CYN. A diferencia de otras cianotoxinas, como las MCs, esto se ha comprobado ya que sus metabolitos obtenidos tras la actuación del CYP450 se unen covalentemente al ADN, provocando roturas en la doble hélice y dando lugar a posibles efectos carcinogénicos (Humpage y col., 2005).

En cuanto a los estudios *in vivo*, Norris y col. (2002) también encontraron reducciones de GSH en hígado de ratones expuestos a CYN. Mediante cálculos teóricos demostraron que la cantidad de CYN administrada no sería suficiente para producir el agotamiento del GSH por conjugación, estando en principio de acuerdo con los resultados *in vitro* respecto a la inhibición de su síntesis (Runnegar y col., 1994, 1995). Sin embargo, al emplear inhibidores de la síntesis del GSH, Norris y col. (2002) observaron disminuciones aún mayores en sus contenidos, concluyendo que la inhibición de la síntesis de GSH no sería el principal mecanismo tóxico de la CYN, mientras que la activación de la toxina por CYP450 tendría un papel más importante en su toxicidad. Una reducción de los niveles de GSH junto con una disminución de la actividad de la γ-GCS, también fue observada por Gutiérrez-Praena y col. (2011b) en peces expuestos tanto por vía i.p. como por sonda nasogástrica a 200µg CYN/kg, confirmando los resultados obtenidos previamente en estudios *in vitro*.

3.3.3. Inducción de estrés oxidativo

El estrés oxidativo se puede definir como el daño celular causado como consecuencia del desequilibrio entre las sustancias prooxidantes y antioxidantes del organismo, a favor de las primeras. Este hecho puede deberse bien a una excesiva producción de especies reactivas de oxígeno (ERO), a una deficiencia en los mecanismos antioxidantes, o bien a una combinación de ambas situaciones (Halliwell, 2007). Las principales especies reactivas de oxígeno son: radical superóxido ($O_2^{-\bullet}$), peróxido de hidrógeno (H_2O_2), radical hidroxilo (OH•), oxígeno singulete ($^1O_2$), ácido hipocloroso (HOCl) y anión peroxinitrito ($OONO^-$) (**Figura 5**).

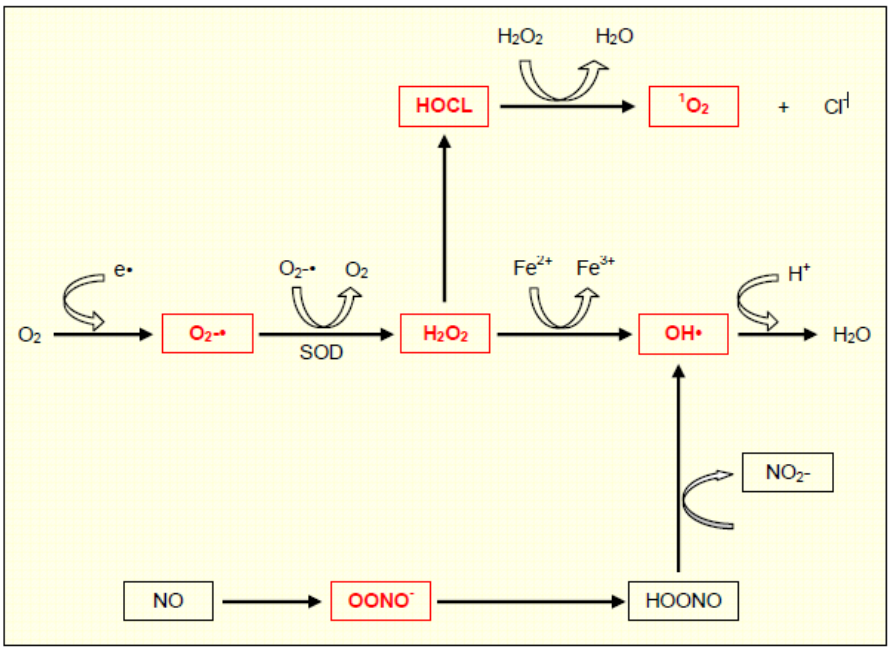

Figura 5. Principales especies reactivas de oxígeno (ERO).

Los primeros estudios *in vitro* sobre el estrés oxidativo inducido por CYN como posible mecanismo de toxicidad fueron realizados en hepatocitos primarios de ratones expuestos a 5 µM de CYN durante 12 h, sugiriendo que el estrés oxidativo y las ERO no tenían un papel en la toxicidad de la CYN. No obstante, estos estudios sólo se basaban en la determinación de los niveles de LPO sin considerar otros biomarcadores como son la oxidación de proteínas y la actividad de enzimas antioxidantes (Humpage y col., 2005). También se ha observado un aumento de LPO en hepatocitos primarios de *Hoplias malabaricus* al ser expuestos a 100 µg/L CYN (Silva y col., 2010) y en hepatocitos primarios de *Prochilodus lineatus* expuestos a 10 µg/L CYN pura (Liebel y col., 2011). En la línea celular de peces PLHC-1, se demostró una relación entre los efectos tóxicos inducidos por CYN pura y la producción de ERO (Gutiérrez-Praena y col., 2011a). Posteriormente, se observó por primera vez un aumento de la formación de ERO en líneas celulares humanas CaCo-2 y HUVEC (Gutiérrez-Praena y col., 2012a, b).

Además, estudios *in vivo* han demostrado que la CYN induce estrés oxidativo mediado por las ERO, produciendo aumento de LPO, así como afectación de la expresión génica y la abundancia relativa de los marcadores enzimáticos GST y GPx en hígado y riñón de tilapias (*Oreochromis niloticus*) expuestas a CYN (200 y 400 µg CYN pura/kg pez) por sonda nasogástrica (Puerto y col., 2011). Resultados similares obtuvieron Gutiérrez-Praena y col. (2011b) cuando los peces fueron expuestos a 200 µg CYN pura/kg pez, por sonda y por vía i.p., durante 24 h ó 5 días, además de un aumento de la oxidación de proteínas, alteración

de las γ-GCS y descensos del GSH. Con estas mismas condiciones experimentales, dichos autores comprobaron la alteración de las actividades enzimáticas, la expresión génica y la abundancia relativa de GST y GPx en hígado y riñón de las tilapias expuestas (Gutiérrez-Praena y col., 2013b). Por otro lado, Ríos y col. (2013) observaron la inducción de estrés oxidativo en hígado y riñón de tilapias expuestas cada dos días a dosis repetidas de CYN (10 µg/L) mediante inmersión en biomasa de *A. ovalisporum* o por vía oral con sus células liofilizadas durante 7, 14 y 21 días, observando alteraciones en los niveles de LPO, oxidación de proteínas y de ADN, así como en las actividades de las enzimas antioxidantes estudiadas (GST, GPx, SOD, CAT, y γ- GCS), y en los niveles de GSH a lo largo de los tres períodos de exposición. Estos autores demostraron que estos efectos se ven influenciados por la vía y el tiempo de exposición. Posteriormente, estos autores observaron distintas respuestas en la actividad, expresión génica y abundancia relativa de GST y GPx en hígado y riñón de tilapias expuestas cada 2 días a dosis repetidas de 10 µg CYN/L mediante inmersión en cultivo de *A. ovalisporum* durante 14 días, y posteriormente depuradas durante 3 ó 7 días (Ríos y col., 2014).

A pesar de estos trabajos que señalan al estrés oxidativo como otro de los posibles mecanismos de acción tóxica de CYN, sigue siendo necesario profundizar y llevar a cabo más estudios al respecto, así como sobre la diferente toxicidad entre la CYN pura y las células liofilizadas de cultivos productores de CYN, y distintos tiempos y vías de exposición.

3.3.4. Genotoxicidad

Varios estudios *in vitro* e *in vivo* han demostrado la genotoxicidad de CYN y la necesidad de una activación metabólica para que produzca este efecto, aunque los mecanismos implicados no son bien conocidos (Fessard y Bernard, 2003; Bazin y col., 2010). En este sentido se han propuesto dos mecanismos responsables del daño citogenético: uno es la inducción de la rotura de las cadenas de ADN y el otro es la pérdida de cromosomas completos en el nivel de la función del cinetocoro/uso mitótico (Shen y col., 2002).

En estudios de micronúcleos *in vitro*, se ha observado daño cromosómico en la línea celular linfoblastoide humana WIL2-NS expuesta a 1, 3, 6 y 10 µg CYN/mL (Humpage y col., 2000a). Además, se ha detectado fragmentación del ADN en hepatocitos primarios de ratones tratados con CYN, mediante ensayo del cometa (Humpage y col., 2005). No obstante, este mismo efecto no pudo ser confirmado en la línea celular CHO K1 de ovario de hámster

(Fessard y Bernard, 2003). En las líneas celulares humanas de enterocitos Caco-2 y hepatocitos HepaRG tratadas con varias dosis de CYN (0,5- 2µg/mL), se observó un aumento de la frecuencia de micronúcleos en células binucleadas (MNBNC). La comparación de la citotoxicidad entre estas reveló: (a) una mayor sensibilidad de las células HepaRG frente a células Caco-2, y (b) una mayor sensibilidad de las etapas diferenciadas con respecto a las no diferenciadas, afirmando que el estado de diferenciación es un factor clave en la determinación de la respuesta celular a CYN. Más recientemente, Štraser y col. (2011) evaluaron la genotoxicidad de CYN en la línea celular humana HepG2, donde la toxina indujo rotura del ADN, desequilibrio de la estructura nuclear y formación de micronúcleos y puentes nucleoplásmicos. Por otro lado, Žegura y col. (2011) fueron los primeros en evidenciar la genotoxicidad de CYN en linfocitos de sangre periférica humana (HPBLs), observando también un desequilibrio de la estructura nuclear, inducción de micronúcleos y, en menor medida, fragmentación del ADN y puentes nucleoplásmicos.

Para entender el papel que juega el CYP450 en la genotoxicidad de CYN mediada por su activación metabólica, Humpage y col. (2005) demostraron que el omeprazol, un inhibidor de CYP3A4, fue eficaz en la protección de los hepatocitos primarios de ratón frente a la genotoxicidad inducida por CYN. Resultados similares obtuvieron Bazin y col. (2010) utilizando el ketoconazol, otro inhibidor de varias isoformas del CYP450, en la línea celular Caco-2 inducida por CYN. Estos resultados confirman por un lado que la genotoxicidad de CYN es dependiente de los metabolitos generados por CYP450, y por otro la implicación de la activación metabólica de CYN en la mediación de su toxicidad, sugiriéndose que CYN es progenotóxica. Otros autores mostraron, a nivel molecular, que la CYN indujo una sobrerregulación de la expresión de los genes CYP1A1 y CYP1A2 de las isoenzimas metabólicas del CYP450, proporcionando una prueba más de que estas enzimas están involucradas en la activación metabólica de la CYN (Štraser y col., 2011; Žegura y col., 2011).

En relación a los estudios *in vivo* que se han llevado a cabo sobre la genotoxicidad de CYN, estos son más escasos. Se han detectado aductos de ADN en hígado de ratones expuestos a dosis únicas de CYN por inyección i.p. (Shaw y col., 2000) y fragmentación del ADN en el hígado de los ratones tratados con 200 µg CYN/kg tras inyección i.p. (Shen y col., 2002). Por último, Bazin y col. (2012) evaluaron el daño del ADN en hígado, sangre, médula ósea, riñón, intestino y colon de ratones suizos albinos tratados por vía i.p. con 50, 100 y 200 µg CYN/kg o por sonda nasogástrica con 1, 2, y 4 mg CYN/kg. Observaron fragmentación

del ADN y daño cromosómico en todos los órganos evaluados y el examen histológico mostró focos de muerte celular en hígado y riñón de los ratones que recibieron las dos dosis más altas de CYN por ambas vías de administración.

3.4. Vías de exposición a CYN

Existen diferentes vías de exposición a la CYN, como son la vía oral, dérmica, inhalatoria e intravenosa.

La vía más importante en cuanto a la frecuencia de la exposición humana es la vía oral, mediante ingesta de agua contaminada, o por ingesta accidental durante la realización de actividades recreativas (Funari y Testai, 2008). La exposición también es posible a través de la cadena alimentaria, aunque la información actual es más limitada. Los seres humanos pueden estar expuestos a CYN a través del consumo de alimentos contaminados, especialmente pescado, crustáceos o bivalvos, que han estado en contacto con cianobacterias y sus toxinas, o mediante la ingesta de verduras regadas con aguas contaminadas con cianotoxinas y que han acumulado concentraciones tóxicas para los seres humanos y animales (Berry y Lind, 2010; Gutiérrez- Praena y col., 2013a).

La exposición oral, dérmica e inhalatoria pueden producirse al mismo tiempo durante la realización de deportes acuáticos en aguas recreativas contaminadas con cianotoxinas (Merel y col., 2013).

Además, puede ocurrir excepcionalmente que la exposición a cianotoxinas se produzca por vía intravenosa, lo que supondría una entrada directa de las toxinas a la sangre, hecho a tener en cuenta en la evaluación del riesgo para la salud humana (Funari y Testai, 2008). Este caso pudo darse en el incidente del centro de hemodiálisis en Caruaru (Brasil) en 1996. Los análisis de las resinas del sistema de tratamiento del agua de la clínica llevaron a la identificación de MCs y CYN en estas muestras, aunque se piensa que las MCs fueron el principal factor contribuyente a la muerte de los pacientes (Azevedo y col., 2002).

Por otro lado, las cianobacterias también se utilizan a menudo como complementos alimenticios, debido a la amplia variedad de nutrientes que poseen, tales como aminoácidos, minerales, vitaminas, ácidos grasos, β-carotenos y clorofila (Dittmann y Wiegand, 2006). Sus diversas propiedades (anticancerígenas, anti-inflamatorias, hipolipidémicas, antioxidantes, antibacterianas, antivirales, etc.) también las hacen útiles para aplicaciones médicas (Deng y

Chow, 2010; Dittmann y Wiegand, 2006; Gantar y Svircev, 2008; Sharma y col., 2011; Yang y col., 2011). El problema de su empleo radica en la contaminación de estas sustancias nutritivas con especies de cianobacterias tóxicas, al coexistir en una misma floración (Schaeffer y col., 1999; Saker y col., 2005). Estudios realizados para la detección de CYN no han podido confirmar la presencia de esta toxina en diferentes suplementos alimenticios (Liu y Scott, 2011). El valor guía de cianobacterias en suplementos alimenticios es de 10 µg/g (Schaeffer y col., 1999) y concretamente en Oregón (EEUU), se ha establecido un máximo de 1 µg/g en los alimentos (USEPA, 2001). A pesar de que los suplementos alimenticios a base de cianobacterias son clasificados en algunos países como alimento (Canadá) o como "otros suplementos dietéticos" (EEUU), la Comisión del Codex Alimentarius rechazó la propuesta de que estos productos se pudieran considerar como "alimento" debido a la falta de evidencias sobre su uso seguro (Comisión del Codex Alimentarius, 2003).

3.5. Efectos tóxicos de la CYN

La forma en que la CYN entra en los organismos no es del todo conocida. Por un lado, se piensa que su bajo peso molecular hace factible su absorción por difusión pasiva mediante un proceso lento y progresivo, ya que es dependiente del gradiente de concentración (Chong y col., 2002; Runnegar y col., 2002). No obstante, la naturaleza hidrofílica de la CYN sugiere que es poco probable que pueda atravesar la pared celular (Froscio y col., 2009). Otro posible mecanismo de transporte de la CYN al hígado es mediante el sistema de transporte de las sales biliares, a las cuales se une la CYN y de esta forma llega al hígado (Chong y col., 2002).

3.5.1. Efectos tóxicos de la CYN en mamíferos y organismos acuáticos

La CYN que ingresa en el organismo por vía oral se transfiere desde el tracto G.I. a la circulación sistemática, y ejerce su efecto tóxico principalmente en el hígado y riñón. Sin embargo, también es capaz de causar daños en otros órganos como pulmones, timo, bazo, glándulas suprarrenales y corazón, lo que hace que sea clasificada como una citotoxina (Hawkins y col., 1985; Terao y col., 1994; Falconer y col., 1999; Humpage y col., 2000a,b; Norris y col., 2001a; Oliveira y col., 2012), generalmente con una toxicidad retardada (Kinnear, 2010).

Los efectos de CYN se han estudiado más ampliamente en mamíferos, y más recientemente se han ampliado los modelos de toxicidad para mostrar los efectos de esta

toxina en peces e invertebrados como langostas, anfibios o camarones, así como en poblaciones de fitoplancton y zooplancton, demostrándose la variabilidad de sus efectos tóxicos entre los diferentes modelos animales, e incluso entre los diferentes individuos de una misma especie (Kinnear, 2010).

A pesar de las evidencias descritas, la toxina no ha sido evaluada por la OMS, pero la Agencia de Protección Ambiental de EEUU (EPA) la ha clasificado en la lista de compuestos con alta prioridad para su caracterización (USEPA, 2001).

Tras la revisión bibliográfica realizada, los efectos tóxicos producidos por la CYN pueden clasificarse en:

a) Hepatotoxicidad

Hasta la fecha, sólo se conoce un incidente de intoxicación del ganado por CYN, en Australia (Thomas y col., 1998), con la muerte de 21 vacas tras consumir agua contaminada con *C. raciborskii*. En las necropsias se detectaron derrame abdominal, mesenterios hiperémicos e inflamación y palidez hepática (Briend y col., 2013).

No obstante, el primer estudio que demostró en ratones que el órgano más afectado por la exposición a CYN es el hígado fue el de Hawkins y col. (1985), tras administrarles por inyección i.p. la cepa de *C. raciborskii* aislada del incidente ocurrido en Palm Island (1979). Los ratones aparecieron acinados, con anorexia y diarrea, y la autopsia reveló hígados pálidos con focos blancos y necrosis coagulativa a nivel histopatológico. Estos resultados fueron confirmados y ampliados más tarde, diferenciándose cuatro etapas de cambios morfológicos en los hepatocitos de ratones expuestos intraperitonealmente (i.p.) a CYN (Terao y col., 1994):

- Desprendimiento de los ribosomas del retículo endoplasmático y acumulación de éstos en el citoplasma, acompañado de condensación y reducción del nucleolo.
- Proliferación de la membrana a las 24 h de la administración de CYN con reducción de la cantidad de CYP450 en los microsomas hepáticos.
- Acumulación de grasa en la parte central de los lóbulos hepáticos, probablemente inducido por los radicales libres generados en la lesión.
- Muerte celular con necrosis hepática grave.

Posteriormente, otros estudios en ratones han seguido presentando al hígado como uno de los principales órganos diana para la acumulación y la toxicidad de CYN (Falconer y col., 1999; Carmichael y col., 2001; Norris y col., 2001a,b).

En animales acuáticos, los efectos tóxicos de CYN también han sido estudiados, como en renacuajos del anfibio *Bufo marinus* expuestos a cultivos de *C. raciborskii* y a extractos celulares del mismo, a concentraciones subletales de CYN (Kinnear y col., 2007a; White y col., 2007). Se mostraron lesiones principalmente en el hígado, aunque también en intestino, conductos renales y epitelio de las branquias. Además, más de un 66% de los *B. marinus* adultos murieron, mientras que los renacuajos sobrevivieron, sugiriendo que las floraciones tóxicas de *C. raciborskii* pueden presentar riesgos importantes para la salud de las poblaciones de anfibios.

Recientemente, nuestro equipo de investigación ha demostrado en peces (*O. niloticus*) expuestos de forma aguda a CYN pura (200 y 400 µg/kg pez) por sonda nasogástrica e inyección i.p., la aparición de efectos histopatológicos dependientes de la dosis (Gutiérrez-Praena y col., 2012c; Puerto y col., 2014). Los resultados obtenidos confirmaron que el hígado y riñón son los principales órganos afectados por esta toxina, pero también se observaron cambios histopatológicos en corazón, intestino y branquias. Las principales alteraciones histológicas en el hígado fueron la pérdida de la arquitectura del parénquima, degeneración glucogénica general y esteatosis. También se observó una degeneración generalizada en los acinos pancreáticos con células necróticas, y en el riñón, glomerulopatía con atrofia glomerular capilar, cápsula de Bowman dilatada, hiperemia de los capilares y disminución en la anchura de los túbulos contorneados proximal y distal.

b) Neurotoxicidad

La generación de efectos tóxicos por la CYN en el sistema nervioso es un tema controvertido aún.

Saker y col. (2003) observaron diferentes síntomas neurológicos en ratones expuestos por inyección i.p. a suspensiones celulares de *C. raciborskii* (rango de dosis 1337-1572 mg CYN/kg), tales como piloerección, letargo y dificultad en la respiración que llevó a la muerte en un máximo de 24 h después de la exposición a CYN.

Zagatto y col. (2012) estudiaron el efecto neurotóxico de la CYN tras inyección i.p. de un extracto de cepas tóxicas de *C. raciborskii* en ratones suizos. La administración de 50

mg/kg peso corporal (p.c.) reveló síntomas típicos de neurotoxicidad tales como temblor, ataxia, convulsiones y muerte por fracaso respiratorio en 1-2 min. También se observaron los mismos efectos en ratones tratados con el extracto hervido o filtrado en membrana AP20. Cambios en el pH del extracto (ajuste a 1,2 y a 12) tampoco conseguían disminuir su toxicidad.

Se encontró que un extracto de *C. raciborskii* tanto aplicado por microperfusión como localmente sobre la superficie celular causa un efecto directo despolarizante o hiperpolarizante sobre las neuronas de dos especies de caracoles, *Helix pomatia* y *Lymnaea stagnalis* (Kiss y col., 2002). Por otro lado, se demostró que los caimanes que habitaban en el Lago Griffin (Florida) durante una floración de *C. raciborskii* mostraban respuestas clínicas deprimidas, reducida velocidad en la conducción nerviosa, degeneración axonal y necrosis de los loci específicos en el mesencéfalo (Schoeb y col., 2002).

c) Inmunotoxicidad

El efecto de la CYN sobre la respuesta inmune no ha sido bien estudiado hasta el momento. Terao y col. (1994) encontraron necrosis masiva de linfocitos en la capa cortical del timo en ratones expuestos a 0,2 mg CYN/kg p.c. por inyección i.p. También se ha observado degeneración y necrosis de linfocitos corticales, así como linfofagocitosis en el tejido linfoide del bazo de ratones después de la exposición a un rango de 4,4-8,3 mg CYN/kg p.c. (Seawright y col., 1999). En otros estudios, también aparecieron lesiones en el bazo de ratones intoxicados durante 14 días con 50 μg CYN/kg p.c. procedente de un extracto celular, mientras que la administración de CYN pura no causó el mismo efecto (Shaw y col., 2000, 2001). Estos resultados indican que se requieren más estudios para determinar si la CYN puede ser clasificada como un inmunotóxico.

d) Dermatotoxicidad

Se ha observado la aparición de eritema y edema en conejos californianos albinos a las 24 h de la exposición a un extracto liofilizado de *A. ovalisporum* por vía intradérmica (Torokne y col., 2001). Otros efectos cutáneos tales como piel seca, costras, descamación y supuración de sangre o de líquido seroso en el área expuesta, fueron detectados también tras la aplicación tópica (en piel abdominal) de 100 μg CYN/mL en ratones Balb/c (Stewart y col., 2006a). Además, las orejas de los ratones tratados con esta dosis de CYN presentaron edema, engrosamiento e infiltración de células inflamatorias (principalmente células mononucleares) tras 24 h y 48 h de exposición, observándose tumefacción a la dosis de 73 μg CYN/mL.

e) Carcinogenicidad

Hasta el momento, son escasas las evidencias científicas sobre la potencial carcinogenicidad de CYN. Sólo hay un estudio en el que se han observado tumores iniciados por CYN (Falconer y Humpage, 2001), encontrando cinco tumores en 53 ratones suizos tratados durante 30 semanas con un extracto de *C. raciborskii*, en comparación con los ratones control. Aunque el número de animales era demasiado bajo para proporcionar evidencia estadística significativa de la carcinogénesis, el estudio sugiere que sería imprudente rechazar esta posibilidad. Otro estudio más reciente demostró que la CYN tiene capacidad de transformar la morfología de células embrionarias de hámster sirio (Maire y col., 2010).

La IARC aún no ha clasificado la CYN por su carcinogenicidad, por lo que se necesitan nuevos estudios para dictaminar si esta toxina está involucrada en procesos de carcinogénesis (Grosse y col., 2006; de la Cruz y col., 2013).

f) Toxicidad fetal

La toxicidad fetal de CYN ha sido demostrada por Rogers y col. (2007) en ratones intoxicados con 50 μg CYN kg^{-1} por inyección i.p., observando un número significativo de muertes fetales o nacimientos prematuros y reducción del crecimiento postnatal de las crías macho.

En peces, el único trabajo al respecto hasta la fecha ha sido el realizado en embriones del pez cebra (*Danio rerio*) (Berry y col., 2009). Estos autores demostraron que la CYN sólo era tóxica al inyectar la toxina purificada directamente al embrión, pero no por su inmersión en extractos de *C. raciborskii* y *A. ovalisporum,* a dosis de hasta 50 μg/mL. Estos resultados sugirieren que la CYN pura resulta letal a este nivel sin producir disfunción ni malformaciones, mientras que otros metabolitos presentes en los extractos parecen inhibir el desarrollo.

En invertebrados, Metcalf y col. (2002) estudiaron los efectos de la CYN en el crustáceo *Artemia salina* tras exposición a CYN pura, observando una respuesta dependiente de la dosis en la mortalidad de este organismo y disminuyendo los valores de la DL50 desde las 24h a las 72h. Nogueira y col. (2004a) expusieron poblaciones naturales de *Daphnia magna* a floraciones de *C. raciborskii*, observando una alta mortalidad.

3.5.2. Efectos tóxicos de la CYN en plantas

A pesar de que los estudios de los efectos de CYN en plantas de consumo humano son de interés, ya que esta toxina puede encontrarse en las aguas de riego, y transferirse a través de las plantas a la cadena alimentaria, con el consiguiente peligro para la salud humana, la bibliografía existente al respecto es escasa. Vasas y col. (2002) demostraron por primera vez la toxicidad de la CYN en plantas al observar la inhibición del crecimiento de brotes de *Sinapis alba* expuestos a la toxina aislada a partir de *A. ovalisporum.* También se ha demostrado que la CYN inhibe la germinación del polen en plantas de tabaco (*Nicotiana tabacum*) cuando son expuestas a concentraciones superiores a 5 μg/mL (Metcalf y col., 2004). Asimismo, el crecimiento de las raíces y brotes del macrófito acuático *Phragmites australis* se vio disminuido tras la exposición a concentraciones de entre 0,5 y 40 μg CYN/mL, aunque el número de raíces experimentó un aumento (Beyer y col., 2009). En *Hidrilla verticillata* expuesta a extractos extracelulares de *C. raciborskii* se observaron efectos dependientes de la concentración de CYN (25-400 μg/mL) y la duración de la exposición (Kinnear y col., 2008). En ocasiones, la producción de raíces aumentaba y la elongación del tallo disminuía; la disminución del contenido en clorofila y los cambios en el cociente clorofila *a:b* apuntaban a que los extractos pueden tener efectos complejos en la fotosíntesis (Kinnear y col., 2008). Prieto y col. (2011) encontraron alteraciones de diferentes marcadores de estrés oxidativo en hojas y raíces de plantas de arroz (*Oryza sativa*) expuestas a un extracto de *A. ovalisporum*. Estos autores observaron un efecto sinérgico entre CYN y MCs cuando las plantas se exponían a una mezcla de *A. ovalisporum* y *M. aeruginosa* productoras de las toxinas.

3.5.3. Efectos tóxicos de la CYN en humanos

Hasta la fecha, es escaso el conocimiento que se tiene sobre la potencial cito-, neuro-, dermatotoxicidad y toxicidad fetal de CYN en humanos, como se explica a continuación. La información al respecto proviene de estudios llevados a cabo en líneas celulares humanas que revelan algunos efectos citotóxicos inducidos por CYN, y algunos casos de exposición accidental humana a la toxina.

La CYN tiene la capacidad de entrar en diferentes tipos de células y causar efectos tóxicos (Froscio y col., 2009b). Es el caso de las células humanas CKO-K1, que mostraron necrosis y disminución de la proliferación y de los índices mitóticos de forma dependiente de

la concentración (0,05-2 µg CYN/mL) y del tiempo (3-21 h) (Lankoff y col., 2007). En un ensayo realizado con células humanas HepG2 tratadas con 2,5 µg CYN/mL se observó un aumento en el número de células en suspensión (Neumann y col., 2007). Asimismo, los efectos patológicos de CYN se han estudiado en las líneas celulares humanas Caco-2 y HUVEC, reduciéndose la viabilidad de Caco-2 en un 90%, así como las de las HUVEC tras 48 h de exposición a la concentración más alta ensayada (40 µg CYN/mL). Las alteraciones morfológicas observadas en células HUVEC tras 24 de exposición a 1,5 µg CYN/mL fueron segregación nucleolar con núcleos alterados, degeneración del Aparato de Golgi, alta presencia de gránulos y apoptosis, siendo este daño más severo tras 48 h de exposición (Gutiérrez-Praena y col., 2012a,b).

Por otra parte, los efectos tóxicos ejercidos por la CYN sobre la reproducción han sido también estudiados en células de la granulosa humana obtenidas a partir de ovarios de mujeres sometidas a fertilización *in vitro*, mostrando resultados contradictorios. En un primer lugar, Young y col. (2008) observaron una inhibición de la progesterona basal, hormona femenina principal en promover la gestación, tras 24 h de exposición a 0.0625 µg CYN/mL, y después de 6 h de exposición a 1 µg CYN/mL. Sin embargo, estudios posteriores revelaron que concentraciones similares de CYN ensayadas (0,04-1,25 µg CYN/mL) no afectaban a la producción de progesterona y estrógeno tras 24 h de exposición (Young y col., 2012). Estos resultados indican que son necesarios más estudios para investigar los efectos de CYN sobre el embarazo.

El único caso documentado hasta la fecha de intoxicación humana por ingesta de agua contaminada con CYN es el episodio ocurrido en Palm Island (Australia, 1979), donde se registraron 148 casos de hepatoenteritis, acompañado de vómitos, diarreas, deshidratación y de daño tubular renal, en una población aborigen que había consumido el agua local de una presa contaminada con una floración de *C. raciborskii* (Byth, 1980; Bourke y col., 1983; Hawkins y col., 1985). El seguimiento de las historias clínicas de los niños intoxicados desde ese episodio en 1979, determinó una mayor tasa de cáncer G.I. en el período 1982-1999, en comparación con una población similar no expuesta, aunque el hecho carece de significación estadística dado el bajo número de individuos expuestos (Falconer y Humpage, 2006).

Aunque este es el único incidente confirmado, también se llegó a estudiar la posible implicación de la CYN en el episodio ocurrido en el centro de hemodiálisis en Caruaru (Brasil) en 1996, tras identificarse tanto MCs como CYN en muestras del carbono y otras

resinas del sistema de tratamiento del agua de la clínica. Aún así, la muerte de los pacientes se sigue atribuyendo principalmente a las MCs. De cualquier manera, no hay estudios que demuestren signos de neurotoxicidad en ninguno de estos dos casos conocidos de intoxicación humana (Palm Island, Australia, y Caruaru, Brasil).

Como se ha indicado previamente, otra de las vías de exposición a CYN es a través de baños en agua contaminada con CYN. En Australia, se encontró una correlación entre el tiempo transcurrido en el agua contaminada, el número de cianobacterias presentes en ella y la aparición de síntomas como diarrea, vómitos e irritación ocular, aunque no se observaron efectos alergénicos (Pilotto y col., 1997). Igualmente, la dermatotoxicidad de CYN tampoco pudo confirmarse en otro estudio posterior de toxicidad cutánea con voluntarios humanos (Stewart y col., 2006b). No obstante, debe tenerse en cuenta la existencia de otros metabolitos secundarios producidos por cianobacterias que sí pueden tener un efecto dérmico evidente en los seres humanos tras una exposición directa (Rzymski y Poniedziałek, 2012). A este respecto, la dermatotoxicidad de especies productoras de CYN podría también deberse a la presencia de lipopolisacáridos (LPS) en la pared de las células cianobacterianas (Stewart y col., 2006c).

En realidad, el número de intoxicaciones humanas en las que podría estar involucrada la CYN puede ser mayor del que se conoce, ya que muchos casos no han sido identificados debido a complicados procedimientos de detección de la toxina o a la falta de conocimiento sobre las toxinas de cianobacterias hace años.

Debido a la toxicidad y ubicuidad de la CYN, la OMS está estudiando establecer como valor guía en aguas de consumo el propuesto por Humpage y Falconer (2003) de 1 μg CYN/L. Este valor de seguridad también ha sido adoptado por el gobierno de Nueva Zelanda, aunque Brasil ha establecido uno muy distinto (15 μg/L) (Burch, 2006). El resto de los países, entre ellos España, carece de legislación al respecto. Del mismo modo, no se ha adoptado todavía ningún valor guía para la CYN en aguas recreativas y de baño.

Por otra parte, otro aspecto a tener en cuenta es la potencial diferente toxicidad de los distintos análogos de CYN, aún en estudio; de ahí la recomendación de incluir tanto CYN como 7-desoxi-CYN en las evaluaciones de riesgos (Neumann y col., 2005; Orr y col., 2010). Se ha llegado a sugerir que, mientras exista ambigüedad sobre la toxicidad de la 7-desoxi-CYN, debería considerarse la toxicidad total como la suma de la toxicidad individual de cada molécula (Seifert y col., 2007).

3.6. Acumulación de CYN en organismos acuáticos y plantas

La bioacumulación de cianotoxinas en organismos acuáticos puede suponer un riesgo potencial para las especies superiores de la cadena trófica. Gutiérrez-Praena y col. (2013a) y Kinnear (2010) han publicado revisiones sobre la presencia y bioacumulación de MCs y/o CYN en bivalvos, crustáceos, gasterópodos, peces y plantas, así como sobre la eficacia de algunas técnicas para la disminución de sus concentraciones. Además, el orden general propuesto para la capacidad de bioacumulación sería gasterópodos > bivalvos > crustáceos > anfibios > peces, cumpliéndose la relación inversa para la susceptibilidad de estos organismos a la toxicidad de CYN (Kinnear, 2010).

La capacidad que tienen los invertebarados acuáticos de acumular cianotoxinas puede dar lugar a una transferencia de las mismas a través de la cadena alimentaria en los organismos más sensibles (Liras y col., 1998). Los bivalvos son los organismos acuáticos que pueden estar más amenazados por la presencia de floraciones de cianobacterias tóxicas, ya que son organismos filtradores de agua. Por otro lado, la acumulación de cianotoxinas en los gasterópodos aumenta el riesgo para sus consumidores mayores, ya que se encuentran en la cadena trófica acuática (Habdija y col., 1995). De la misma manera, la acumulación en los anfibios aumenta el riesgo de exposición a estas toxinas para los animales terrestres (Papadimitriou y col., 2012).

Desde que hace más de una década se realizara el primer estudio de bioacumulación de CYN por Saker y Eaglesham (1999), las investigaciones sobre la acumulación de esta toxina han demostrado su bioacumulación en organismos invertebrados y vertebrados, aunque estos estudios siguen siendo más escasos en comparación con las MCs.

Saker y Eaglesham (1999) estudiaron la bioacumulación de CYN en el cangrejo de río *Cherax quadricarinatus* y el pez arcoiris *Melanotaenia eachamensis* tras su exposición a CYN en un estanque de acuicultura conteniendo 589 μg CYN/L. En el cangrejo de río, la toxina se detectó tanto en músculo (900 μg CYN/kg de tejido liofilizado) como en hepatopáncreas (4,300 μg CYN/kg de tejido liofilizado), mientras que en el pez arcoíris se detectó en vísceras una concentración máxima de 1200 μg CYN/kg de tejido liofilizado. En un estudio posterior se observó la acumulación de CYN (130-560 μg CYN/kg) en el mejillón *Alathyria pertexta* después de estar expuesto a niveles < 0,8 μg CYN/L en estanques naturales (Anderson y col., 2003).

A escala de laboratorio, estudios llevados a cabo en *Daphnia magna* expuestos a *C. raciborskii*, no mostraron bioacumulacion evidente (Nogueira y col., 2004b), pero sí se observó en el mejillón *Anodonta cygnea* expuesto a concentraciones de CYN entre 14-90 µg/L durante 16 días, acumulándose en la hemolinfa y en las vísceras (61500 y 5900 µg CYN/kg de peso seco de tejido, respectivamente) a los dos días de exposición, y manteniéndose el 50% de la toxina en los tejidos tras un período de 14 días de depuración (Saker y col., 2004).

También se ha demostrado la acumulación de CYN en el caracol *Melanoides tuberculata* tras 7 y 14 días de exposición con diferentes concentraciones de CYN (91-406 µg/L) (White y col., 2006). En estudios realizados por el mismo grupo sobre el renacuajo *Bufo marinus*, la toxina se acumuló en los tejidos pero no en cantidades suficientes como para demostrar su bioacumulación (White y col., 2007). Sin embargo, Kinnear y col. (2007b), no observaron cambios en este caracol tras su exposición a CYN a través de extractos de células de *C. raciborskii* y cultivos de la misma. En estudio de campo, Berry y Lind (2010), observaron acumulación de CYN en el caracol *Tegogolo* (*Pomecea patula catemacensis*) expuestos a concentraciones de 20 ng CYN/L.

Los peces son generalmente más tolerantes a los efectos de estas toxinas que los mamíferos y tienden a acumularlas en el tiempo (Ross, 2000). Las cianotoxinas pueden acumularse en peces a través de diferentes vías: por ingesta directa del fitoplancton, por captación de la cianotoxina disuelta a través del epitelio (branquias, piel), o a través de la cadena alimentaria. Generalmente se cree que la vía oral es la más importante (Ernst y col., 2001) y la acumulación de cianotoxinas suele depender del tiempo de exposición (Vasconcelos, 1995; Sipiä y col., 2002; Ozawa y col., 2003). En peces, la acumulación de CYN se demostró por primera vez en las truchas (*Salmo trutta*) de un lago natural de Italia, detectándose en las vísceras y músculo (2,7 ng CYN/g tejido y 0,8 ng CYN/g de tejido, respectivamente) (Messineo y col., 2010). Berry y col. (2012) llevaron a cabo un estudio sobre la acumulación de CYN en peces, particularmente en el músculo. Con este fin se recolectaron múltiples especies de peces en el lago de Catemaco (México) y los resultados mostraron que CYN se acumuló en el músculo entre 0,09 y 1,26 µg/ kg, no superando la IDT.

Aunque la mayoría de las floraciones de cianobacterias se producen en sistemas acuáticos abiertos tales como lagos, lagunas, ríos, océanos, etc., también pueden aparecer en los depósitos de agua para el riego de plantas. Por esta razón, las plantas pueden estar

expuestas a CYN y ésta acumularse en sus tejidos, incluidas las partes comestibles, como hojas, raíces, frutas, etc. En este sentido, Prieto y col. (2011) encontraron acumulación de CYN en las hojas y raíces de arroz (*Oryza sativa*) expuestas a un extracto de *A. ovalisporum* conteniendo 2,5 µg CYN/L durante 48 h, hallando 12,2 µg/g peso seco (p.s.) y 14,8 µg/g p.s., respectivamente para las hojas y raíces.

En plantas del género *Brassica* expuestas a diferentes concentraciones de CYN a través de un sistema de cultivo hidropónico se detectaron concentraciones de CYN proporcionales a los niveles de toxina utilizados durante la exposición (Kittler y col., 2012). Además, los cultivos pueden también verse reducidos si las cianotoxinas están presentes en altas concentraciones en el agua de riego (Pflugmacher y col., 2006). En general, se necesitan más estudios, especialmente en condiciones reales de cultivo, para evaluar los riesgos para los seres humanos con más precisión.

A partir de los pocos datos que existen en la bibliografía sobre la toxicidad de CYN, se ha establecido una IDT provisional para esta toxina de 0,03 µg/kg/día, la cual se ha obtenido a partir de un valor de NOAEL (Non Observed Adverse Effects Level) de 30 µg/kg/día, obtenido a partir de ensayos en ratones y al cual se le aplica un factor de incertidumbre de 1000 (Humpage y Falconer, 2003). Además, se ha calculado que el nivel de alerta sanitaria para CYN en diferentes alimentos marinos debe ser de 158 µg/kg de peso húmedo en peces, 720 µg/kg de peso húmedo en gambas, y 933 µg/kg de peso húmedo en mejillones (Saker y col., 2004).

Además, para la protección de los ecosistemas acuáticos, Seifert y col. (2007) sugirieron un nivel umbral provisional de 100 µg/L de CYN total (cantidad extracelular e intracelular), basándose en un trabajo de ecotoxicidad que mostraba que es poco frecuente la toxicidad subletal y letal por debajo de este nivel. De acuerdo con estos autores, concentraciones de CYN superiores a 100 µg/L han sido evaluadas como de riesgo particularmente alto (Kinnear y col., 2009). Sin embargo, las últimas investigaciones indican que la bioacumulación de CYN puede ocurrir incluso a niveles muy bajos de exposición, y por lo tanto, debemos ser cautelosos en el desarrollo de directrices para la evaluación de riesgo para la salud de los ecosistemas acuáticos (Kinnear, 2010).

3.7. Tratamiento y prevención del daño ocasionado por la CYN

Los daños producidos por las cianotoxinas además de tener consecuencias directas sobre la salud humana y animal, también tienen repercusiones económicas, ya que pueden afectar a la producción de las piscifactorías, donde pueden desarrollarse floraciones tóxicas que afectan al desarrollo de los peces. Por eso es interesante identificar sustancias que permitan prevenir o recuperar los efectos tóxicos inducidos por la CYN. Así, por ejemplo, sustancias capaces de eliminar del organismo o de inactivar las ERO por distintos mecanismos de defensa antioxidante podrían ser interesantes.

El sistema de defensa antioxidante está constituido por dos partes: enzimático y no enzimático. Los antioxidantes enzimáticos constituyen la llamada primera línea de defensa, y se encargan de eliminar las ERO, especialmente el radical superóxido ($O_2^{-\cdot}$) y el peróxido de hidrógeno (H_2O_2), ya que a partir de estos se generan otras ERO que son más reactivas y, por tanto, más peligrosas para el organismo. De esta forma, los radicales $O_2^{-\cdot}$ presentes en los tejidos serán transformados hasta H_2O_2 mediante una reacción catalizada por la enzima superóxido dismutasa (SOD). Estos peróxidos de hidrógeno pueden ser reducidos hasta moléculas de agua (H_2O) por diferentes reacciones en las que intervienen las enzimas catalasa (CAT) y glutatión peroxidasa (GPx). Esta última requiere, para su actuación, dos moléculas de glutatión reducido (GSH) el cual a su vez será transformado hasta glutatión oxidado (GSSG) en el transcurso de la reacción. La enzima encargada de regenerar estas moléculas de GSH consumidas será la glutatión reductasa (GR), mediante el gasto de una molécula de NADPH gracias a la enzima glucosa-6-fosfato deshidrogenasa (**Figura 6**).

Entre las moléculas que conforman el sistema de defensa no enzimático de los organismos, podemos encontrar el GSH, las vitaminas A, E y C, el selenio (Se), la L-carnitina (LC), la N-acetilcisteína (NAC), carotenos, flavonoides, melatonina, albúmina, ácido úrico y ubiquinol10 (Wilhelm Filho, 1996; Batcioglu y col., 2005; Puerto y col., 2009; Puerto y col., 2010; Gutiérrez-Praena y col., 2012d).

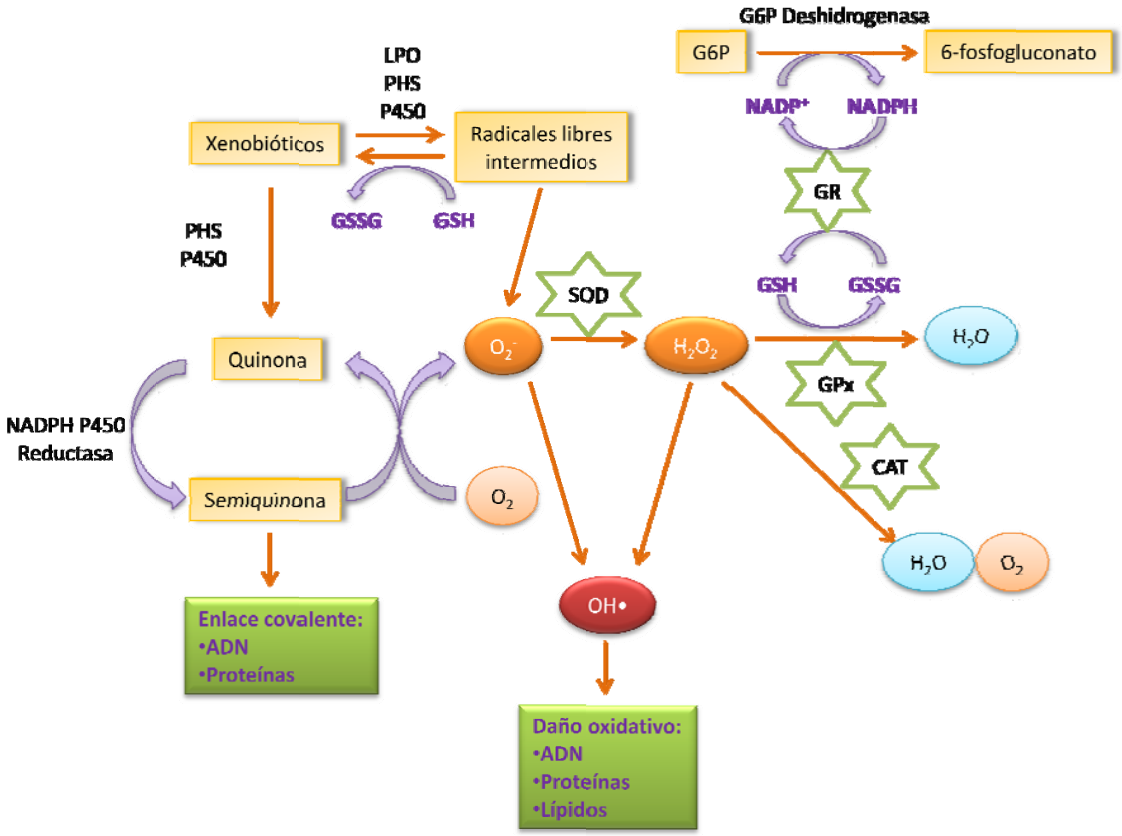

Figura 6. Partes principales del sistema antioxidante celular enzimático y no enzimático (adaptado de Núñez, 2011).

Como es bien sabido, en la biotransformación de los xenobióticos, las principales reacciones son catalizadas por la CYP450 oxidasa y la glutatión-S-transferasa (GST), que introducen grupos reactivos o polares en los xenobióticos, y los conjugan con el GSH. De esta manera, las toxinas modificadas pueden ser más fácilmente eliminadas por parte de las células, se favorece su conjugación con sales biliares o se favorece su biotransformación en el interior de las células para reducir su toxicidad y favorecer su posterior eliminación del organismo.

La enzima más estudiada relacionada con la biotransformación de cianotoxinas es la GST (Campos y Vasconcelos, 2010). A pesar de ello, ni la vía de biotransformación ni la de excreción se conocen con total certeza. Existen tres grandes familias de GST en mamíferos que han sido claramente identificadas: las GST citosólicas (α, μ, π, δ, ω y □), la GST mitocondrial (κ) y la GST microsomal (Hayes y col., 2005). Otras isoformas de GST (ρ) sin homología en los mamíferos, se encuentran en peces, como por ejemplo *Pagrus major* (Konishi y col., 2005). Estos autores verificaron que la transcripción de las distintas isoformas

de GST variaba entre los distintos órganos expuestos a otras cianotoxinas como las MCs, e incluso dentro del propio órgano.

Por tanto, los estudios sobre las alteraciones de los niveles del ARNm de las enzimas implicadas en la prevención del estrés oxidativo, así como de la abundancia relativa de proteínas de dichas enzimas, pueden ser eficaces para la comprensión y posterior prevención de los efectos de las cianotoxinas en la salud de cada especie en cuestión. Por la importancia que tienen en la presente Tesis Doctoral, a continuación se muestran algunas características de las principales sustancias con capacidad antioxidante conocida, dedicando especial atención a la L-carnitina y la vitamina E.

3.7.1. Sustancias antioxidantes

3.7.1.1. L-carnitina

La L-carnitina (β-hidroxi-γ-N-trimetilamonio-butirato) es una amina cuaternaria, con estructura similar a la colina (**Figura 7**), soluble en agua y sintetizada por la mayor parte de los organismos eucarióticos a partir de los aminoácidos esenciales metionina y lisina (Stephens y col., 2007).

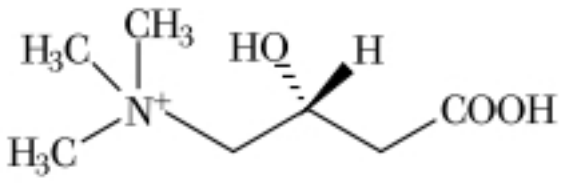

Figura 7. Estructura de la L-carnitina.

La LC participa en diversas funciones fisiológicas, entre las cuales está la de facilitar la entrada de ácidos grasos al interior de la mitocondria para su β-oxidación (Bilinski y Jonas, 1970; Belay y col., 2006), a la vez que exporta derivados acilos no metabolizados en la mitocondria, evitando su acumulación dentro del orgánulo (Schereiber, 2005). También ayuda a restaurar los niveles de CoA libre, que pueden ser utilizados en próximas funciones metabólicas (Suzuki y col., 1981; Kobayashy y Fujisawa, 1994) y juega un papel importante en la cetogénesis (Kelly, 1998; Tein, 2003) y en el ejercicio físico (Roepstorff y col., 2005). Se conoce que actúa como antioxidante, gracias a su papel protector frente a las ERO (Derin y col., 2004; Gómez-Amores y col., 2007), es capaz de modificar la apoptosis y ha demostrado

disminuir el daño orgánico oxidativo en modelos animales con fallo renal crónico (Caló y col., 2006). Por tanto, la LC no sólo desempeña un papel esencial en el metabolismo de los ácidos grasos para la obtención de energía metabólica, sino que además parece ejercer una acción antioxidante y neuromoduladora.

El 75% de la LC requerida por el organismo proviene de la dieta (carne roja, huevos y productos lácteos), mientras que el 25% restante es sintetizado de forma endógena en el hígado y en menor cantidad en el riñón y cerebro a partir de los L-aminoácidos lisina y metionina (Calabrese y col., 2006). Asimismo, gran parte de las reservas de LC están en el músculo esquelético y cardíaco (hasta el 98%), y en el hígado tan solo queda del 1 al 6%. El fluido extracelular de un individuo normal contiene menos del 6% del total de la LC del organismo. La mayor parte se encuentra en forma libre, mientras que el resto se presenta esterificada en forma de acilcarnitinas de cadena corta y cadena larga. Por todo esto, la LC no se considera normalmente como un nutriente esencial, debido a que el organismo es capaz de sintetizar las cantidades necesarias; sin embargo, sí se requiere un aporte exógeno de LC en condiciones de demanda de ácidos grasos o deficiencia de carnitina, por ejemplo en pacientes con tratamientos de hemodiálisis o enfermedades cardiovasculares (Schereiber, 2005).

En cuanto a la capacidad antioxidante de la LC, los datos en animales y humanos apoyan la teoría de que el tratamiento con esta sustancia ejerce efectos beneficiosos en varios trastornos relacionados con el estrés oxidativo: envejecimiento (Kalaiselvi y Panneerselvam, 1998), aterosclerosis (Dayanandan y col., 2001), hipercolesterolemia (Sayed-Ahmed y col., 2001), hipertensión (Rajasekar y col., 2007) y enfermedades renales crónicas (Emami Naini y col., 2012; Fatouros y col., 2010). Recientemente, se observó cómo la capacidad antioxidante de la LC en la hipertensión arterial está mediada por la modulación de factores de transcripción dependientes del estado redox, como el factor nuclear kappa B (NF-κB), el factor nuclear eritroide Nrf2, y receptores activados por proliferadores de peroxisomas (PPAR) (Zambrano y col., 2013).

Por otro lado, la LC ha demostrado ser un eficaz secuestrador de radicales hidroxilo a través de su acción sobre los grupos carbonilo. También juega un papel en la quelación de iones libres Fe^{2+} mediante la interacción con los grupos hidroxilo y carboxilato, resultando en la formación de complejos (Derin y col., 2004; Gülçin y col., 2006), disminuyendo así la generación de radicales libres (Reznick y col., 1992).

La LC endógena puede ser suficiente para las necesidades normales de los animales de granja, aunque en determinadas condiciones son necesarios suplementos exógenos de carnitina, como en dietas con un alto nivel de grasas (Ozorio, 2009). En estudios realizados en peces, suplementos de LC podrían aumentar la eficiencia de utilización de energía a costa de la oxidación lipídica (Harpaz, 2005; Ozorio, 2009).

Hoy en día, el uso recomendado de suplementos de LC en las dietas de peces en acuicultura es:

- Como promotor del crecimiento, en particular para ayudar en la utilización de acúmulos de grasa en la dieta y proporcionar así un efecto ahorrador de proteínas (Jayaprakas y col., 1996; Torreele y col., 1993).
- Como protección frente a niveles tóxicos de amoníaco y xenobióticos (Schreiber y col., 1997).
- Como alivio del estrés relacionado con temperaturas extremas del agua, facilitando una mejor aclimatación a los cambios de temperatura del agua (Harpaz y col., 1999).

3.7.1.2. Vitamina E

La vitamina E es un nutriente esencial cuyas formas naturales son d-estereoisómeros consistentes en la sustitución de un anillo aromático y una larga cadena lateral isoprenoide (Prieto y col., 2009) (**Figura 8**). En concreto, existen ocho compuestos naturales con actividad vitamina E, siendo el α-tocoferol el de mayor potencia biológica.

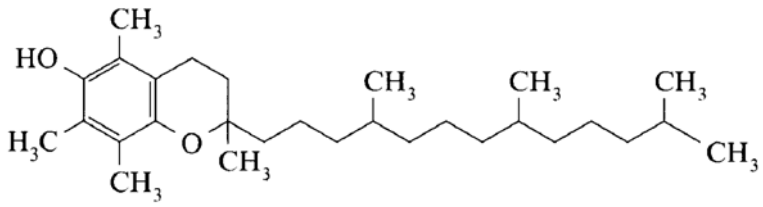

Figura 8. Estructura de la vitamina E.

Es un antioxidante liposoluble que puede interactuar directamente con las ERO y proteger las membranas biológicas del estrés oxidativo (Jones y col., 1995). Actúa donando un átomo de hidrógeno a un radical libre y así el radical pasa a ser inactivo. Debido a la estabilidad de su estructura de resonancia, el α-tocoferol oxidado no es reactivo y por tanto no es capaz de continuar propagando en cadena las reacciones radicalarias (Chen y col., 1993).

La vitamina E se encuentra en muchos alimentos, principalmente en los de origen vegetal, entre ellos el brócoli, las espinacas, la soja, el germen de trigo y la levadura de cerveza (Parker, 1989). También puede encontrarse en alimentos de origen animal como la yema de huevo.

El nivel de vitamina E en el plasma humano es de 22 μmol/L; también puede encontrarse en el hígado, riñón y tejido adiposo. La ingestión diaria recomendada para un adulto es de 10-12 mg/día de α-tocoferol, y de 3 mg/día para un niño recién nacido (Peña y col., 2005). Los requerimientos de vitamina E dependen de varios factores como son: el nivel de lípidos en la dieta, el estado de oxidación o la presencia de otros antioxidantes, existiendo un margen variable según la bibliografía consultada. Así, Satoh y col. (1987) delimitan en 50-100 mg vitamina E/kg de dieta como suficiente si ésta tiene un 5% de lípidos. Sin embargo, Roem y col. (1990) sugieren unos requerimientos de 10-25 mg vitamina E/kg de dieta con un contenido del 3-6% en aceite de maíz.

Existen tres situaciones específicas en las que puede darse la deficiencia de vitamina E: en personas que no pueden absorber dietas ricas en grasas, en niños prematuros con muy bajo peso corporal (nacidos con menos de 1,5 kg), y en individuos con extraños desórdenes en el metabolismo de las grasas. La carencia aislada de vitamina E es muy rara, aunque sí se conoce una forma de deficiencia familiar caracterizada clínicamente por presentar trastornos del tracto espinocerebral, distrofia muscular, oftalmoplejia y esterilidad (Peña y col., 2005).

Existen diversos estudios realizados con vitamina E en animales, por ejemplo en eritrocitos de rata tratadas con el insecticida organofosforado fentión se demostró el efecto protector de la vitamina E junto con la vitamina C; ambas disminuyeron la LPO e indujeron los sistemas enzimáticos antioxidantes (Altuntas y col., 2002). Asimismo, se ha comprobado este efecto protector sobre el sistema de defensa antioxidante enzimático y no enzimático en muestras de sangre de ratas intoxicadas con cadmio, y sobre la LPO (Ognjanovic y col., 2003).

Los requerimientos dietéticos de tilapias para la vitamina E pueden verse afectados por diversos factores, como el nivel lipídico, fuente, y otros antioxidantes o enzimas antioxidantes presentes en la dieta o en el organismo, el grado de insaturación de la fuente lipídica o el estado oxidativo del lípido (Huang y col., 2004; Shiau y Lin, 2006). En peces, además, la vitamina E promueve el crecimiento, inhibiendo la oxidación lipídica (Thorarinsson y col., 1994). En varias especies de peces, se ha demostrado que un descenso

en el aporte dietético de vitamina E provoca un aumento generalizado en la actividad de las enzimas antioxidantes hepáticas y mayores niveles de peróxidos lipídicos (Tocher y col., 2002). Se ha demostrado que el suplemento con vitamina E reducía la actividad de las enzimas antioxidantes hepáticas y prevenía el aumento de LPO y GSH hepático en peces alimentados previamente con aceite oxidado (Tocher y col., 2003; Huang y Huang, 2004). Al mismo tiempo, se observó que suplementos de 63-206 mg vitamina E/kg generaban un aumento de la capacidad de respuesta antioxidante, dando lugar a una disminución de los niveles de LPO en hígado y músculo de tilapias (Huang y col., 2003).

En cuanto a cianotoxinas, se ha observado que la administración de suplementos de vitamina E (a una dosis de 33,3 U/ratón/día) ofrecía cierta protección frente a la exposición crónica de ratones expuestos a dosis repetidas de extractos de MC-LR, demostrado por los valores de LPO y la actividad GST (Gehringer y col., 2003). Por otra parte, Pinho y col. (2005) encontraron que el pretratamiento con vitamina E era capaz de modular las respuestas antioxidantes tras la administración de MCs en agallas del cangrejo de estuario *Chasmagnathus granulatus*.

Estudios previos realizados por nuestro grupo de investigación demostraron que la vitamina E, a una dosis principalmente de 700 mg vit E/kg dieta, protegía del estrés oxidativo en tilapias expuestas por vía oral a células de MCs, atendiendo principalmente a los resultados en los niveles de LPO y actividad de CAT (Prieto y col., 2008). Posteriormente, observaron cómo a esta misma dosis, la vitamina E fue capaz de modular de forma tiempo-dependiente alteraciones en los biomarcadores de estrés oxidativo (LPO, oxidación de proteínas, actividades SOD, CAT, GPx, GR y GST, y GSH/GSSG), así como las lesiones histopatológicas causadas por MCs (120 μg MC-LR/pez) en tilapias expuestas a células de *M. aeruginosa* durante 24, 48 y 72 horas (Prieto y col., 2009).

Teniendo en cuenta el recientemente demostrado daño oxidativo inducido por la CYN en peces, unido al aumento de su aparición en las aguas, su ubicuidad y el creciente uso comercial de la tilapia en la acuicultura, es de gran interés encontrar compuestos con potencial antioxidante para prevenir estos daños, sin que causen toxicidad *per sé*. Hasta la fecha, aún no se ha probado el efecto protector de antioxidantes como L-carnitina y vitamina E en peces expuestos a CYN, por lo que presentan un gran interés de estudio constituyendo parte de la presente Tesis Doctoral.

3.7.1.3. Otras sustancias antioxidantes

Además de la L-carnitina y la vitamina E, existen otras sustancias de probada capacidad antioxidante como son el glutatión, la *N*-acetilcisteína y el selenio.

El **glutatión (GSH)** es un tripéptido formado por los aminoácidos L-glutamato, cisteína y glicina, y su forma reducida y activa (GSH) es la predominante. Junto con el selenio, tiene la función de regenerar la vitamina C gastada, y las tres son capaces de regenerar la vitamina E (Kelly y col., 1998). Por esto, concentraciones adecuadas de glutatión en el organismo ayudan a mantener un equilibrio óptimo en la eliminación de radicales libres, destacando asimismo su papel en las células hepáticas debido a su gran capacidad para reaccionar con sustancias tóxicas y favorecer su eliminación. Los niveles de GSH en el organismo dependen, por tanto, del balance entre su síntesis (catalizada por la γ-GCS), su conjugación (por las GSTs), su oxidación (no enzimática o por GPx) y la reducción de GSSG a GSH (por GR) (Peña-Llopis y col., 2003).

Es una sustancia que se produce de forma natural en las células animales, estando presente en nuestra dieta diaria en alimentos como: frutas y vegetales frescos o congelados, pescados, carnes, espárragos, aguacate y nueces.

En tilapias tratadas con altas dosis de cadmio, se observó una disminución en la síntesis de GSH por inhibición de γ-GCS, enzima implicada en su síntesis, al mismo tiempo que se aumentaba su consumo por estimulación indirecta de la actividad GST (Hui y col., 2001).

La importancia de la actividad antioxidante del GSH quedó demostrada en células HepG2 expuestas a MC-LR, al inhibir la actividad de las cianotoxinas mediante la formación de conjugados GSH-MCs (Zegura y col., 2006). Estos autores verificaron también la expresión aumentada de la glutamato-cisteína ligasa (GCL), una de las enzimas implicadas en su síntesis, lo que indica un aumento en la tasa de la síntesis *de novo* de GSH.

Como ya se ha comentado anteriormente en el apartado 3.3. ("Mecanismos de acción tóxica de la CYN"), varios estudios *in vitro* e *in vivo* observaron una reducción de la actividad de la enzima γ-GCS, induciendo una bajada en los niveles de GSH (Runnegar y col., 1994, 1995; Gutiérrez-Praena y col., 2011a, b). Estos últimos autores lo demostraron en hígado de tilapias expuestas a una única dosis de CYN pura (200 μg/kg p.c.) por vía oral y vía i.p. y

sacrificadas a las 24 h. Por el contrario, se experimentó un aumento en esta actividad enzimática junto con el agotamiento de GSH/GSSG en hígado y riñón de tilapias expuestas a dosis repetidas de CYN (10 μg/L) mediante inmersión en biomasa de *A. ovalisporum* y por vía oral con sus células liofilizadas durante 7, 14 y 21 días (Ríos y col., 2013). Estos resultados, por tanto, señalan como mecanismo predominante en la eliminación de ERO la reducción con GSH.

La **N-acetilcisteína (NAC)**, un derivado del aminoácido natural L-cisteína que no se encuentra en los alimentos, es un antioxidante tiólico que puede actuar por diferentes mecanismos y que colabora en los siguientes procesos fisiológicos:

- Estimula la síntesis de GSH (ya que es fácilmente desacetilado a L-cisteína), y la actividad de enzimas como la glutatión reductasa (GR), contribuyendo a la regeneración del GSH.
- Protege frente al estrés oxidativo al unirse directamente mediante su grupo tiol a las ERO, como el radical hidroxilo ($OH^{\bullet}$) y el peróxido de hidrógeno (H_2O_2) (Banaclocha, 2001).
- Previene la muerte celular programada en células neuronales.

En clínica, la NAC se usa para el tratamiento de enfermedades relacionadas con estrés oxidativo y/o deficiencia de GSH, como la sobredosis por paracetamol, virus de inmunodeficiencia humana (VIH) y afectaciones hepáticas y cardíacas (Peña-Llopis y col., 2003).

Sevgiler y col. (2007) comprobaron la efectividad de la NAC para prevenir o reducir el estrés oxidativo en hígado de peces (*Cyprinus carpio)* expuestos a pesticidas. Posteriormente, nuestro grupo de investigación demostró que la NAC ejerce un efecto protector frente al estrés oxidativo y los daños histopatológicos producidos en tilapias por las MCs y la CYN, resultando pro-oxidante a la dosis mayor empleada (96,8 mg NAC/pez/día) en ensayos con MCs (Puerto y col., 2009, 2010), pero ofreciendo mejor protección frente a los efectos causados por CYN a la dosis de 44 mg NAC/pez/día) (Gutiérrez-Praena y col., 2012d, 2014a). Por lo tanto, este antioxidante ha demostrado su utilidad para la profilaxis y tratamiento de intoxicaciones de peces por ambas cianotoxinas.

El **selenio** (Se) es un oligoelemento que se encuentra de forma natural en la tierra, agua y algunos alimentos como carnes, pescados, cereales y algunos vegetales. Es un micronutriente esencial para peces, aves, humanos y muchos microorganismos al formar parte

de cada una de las cuatro subunidades que forman la GPx (Batcioglu y col., 2002). Por otro lado, el Se también puede generar ERO (Miller y col., 2007), ya que el margen entre sus requerimientos nutricionales y niveles tóxicos es relativamente estrecho, y se bioacumula directamente en la cadena alimentaria (Ohlendorf, 1996; Eisler, 2000).

Este elemento, junto con la vitamina E, ejerce una acción sinérgica y complementaria. Mientras que la vitamina E previene la formación de peróxidos grasos por secuestro de radicales libres antes de que ellos inicien la LPO, el Se (como parte esencial de GPx) reduce peróxidos ya formados a alcoholes menos reactivos (Thorarinsson y col., 1994).

Estudios realizados en nuestro laboratorio comprobaron la influencia protectora del Se en el estrés oxidativo y en las lesiones histopatológicas producidas en peces intoxicados con MCs. Los resultados mostraron protección dosis dependiente, viéndose mejorados diferentes parámetros de estrés oxidativo a diferentes dosis de Se. Además, previnieron los cambios histopatológicos inducidos por MCs en diferentes los órganos estudiados (hígado, riñón, corazón y tracto G.I.) (Atencio y col., 2009).

3.7.2. Depuración de las aguas

Varios estudios han demostrado la efectividad de la depuración en la mejora de los niveles de los biomarcadores de estrés oxidativo en peces y almejas expuestos a diversos contaminantes, como PCBs, los insecticidas DDT y clorpirifós, HAP y metales, entre otros (Ferreira y col., 2005, 2007; Freitas y col., 2012; Gagnaire y col., 2013; Özcan Oruç, 2010). Todo esto indica que los animales son capaces de reprogramar su respuesta celular al ser transferidos a un medio no contaminado, consiguiendo así una recuperación de su estado normal y una eliminación del tóxico por sí mismos.

Los estudios de depuración de cianotoxinas en organismos acuáticos, especialmente en peces, son muy importantes para la economía pesquera y la salud pública (Mohamed y Hussein, 2006). Sólo unos pocos estudios se han centrado en el mecanismo de depuración de cianotoxinas en organismos acuáticos. Así, se ha estudiado la acumulación y depuración de MCs en caracoles de agua dulce (Ozawa y col., 2003), en el cangrejo de río *Procambarus clarkii* (Tricarico y col., 2008) y la dinámica de destoxicación de MC-LR en el camarón *Palaemonetes argentinus* (Galanti y col., 2013). Por otra parte, el mecanismo de depuración de la nodularina en mejillones (*Mytilus edulis*) también ha sido estudiado (Kankaanpaa y col., 2007).

En el caso de la CYN, en un primer lugar se investigó su acumulación y depuración en el mejillón de agua dulce *Anodonta cygnea* (Saker y col., 2004). Con respecto a peces, nuestro grupo de investigación ha demostrado la efectividad de dos períodos de depuración (3 y 7 días) en la recuperación de los efectos tóxicos a nivel molecular en tilapias expuestas durante 14 días a CYN. Estos resultados mostraron que a los tres días de depuración, tanto la actividad GPx como la abundancia relativa sGST volvían a los valores basales, mientras que para la recuperación de la actividad GST y de la expresión génica de GPx fueron necesarios 7 días (Ríos y col., 2014).

Por todo ello, la profundización en los estudios de depuración de CYN en peces es de gran interés, por lo que forman parte también de la presente Tesis Doctoral.

4. MÉTODOS DE ANÁLISIS PARA LA DETERMINACIÓN DE CIANOTOXINAS

El desarrollo de diferentes técnicas analíticas para la identificación y cuantificación de las cianotoxinas MCs y CYN es imprescindible para poder evaluar los riesgos que la exposición a las mismas supone para la salud humana, así como los derivados de su presencia en distintas muestras medioambientales (Dai y col., 2008). En este sentido, se han empleado diferentes técnicas analíticas que lo permiten en diferentes matrices: aguas, cultivos de cianobacterias y tejidos de animales contaminados (Moreira y col., 2012).

A continuación se describen los principales métodos, tanto cualitativos como cuantitativos, para la determinación de estas cianotoxinas, recogidos en la bibliografía.

4.1. Métodos cualitativos y semicuantitativos

4.1.1. Métodos inmunológicos

Se han propuesto varias técnicas analíticas de screening para la determinación de estas toxinas entre las que se incluyen la determinación por ELISA (Enzyme-linked immunosorbent assay) para MCs (Magalhães y col., 2003; Moreno y col., 2004) y para CYN en muestras de agua y de tejido (Masten y Carson, 2000; Prieto y col., 2011). El test ELISA es un kit de competencia directa que utiliza anticuerpos policlonales para el cribado rápido de toxinas (MCs y CYN). Es una herramienta rápida de detección, sensible y de bajo costo, que requiere un procesamiento mínimo de la muestra (Gurbuz y col., 2009). No obstante, debido a la alta variabilidad de los compuestos que pueden encontrarse en las muestras biológicas, a ciertas variables físico-químicas (salinidad, pH, etc.), así como a las interferencias causadas

por los efectos de la matriz, pueden producir falsos positivos y/o negativos (Metcalf y col., 2000).

Además, ha sido desarrollado un método para la inmuno-detección simultánea de varias cianotoxinas (MC-LR, CYN y STX) utilizando un chip de microfluidos (Zhang y col., 2011), aunque el dispositivo detector no está aún disponible comercialmente.

Con el fin de evaluar la distribución de estas toxinas en los tejidos, una técnica interesante es la inmunohistoquímica (IHC), basada en el principio de anticuerpos que se unen específicamente a los antígenos. Esta técnica ha resultado útil en la detección de la distribución tisular de MC-LR en ratones (Yoshida y col., 1998; Guzmán y Solter, 2002), en la trucha arco iris (Fischer y col., 2000) y en diversos órganos del gasterópodo *Lymnaea stagnalis* (Lance y col., 2010). Además, se ha empleado en la detección de diferentes virus en cultivos celulares (Niedobitek y col., 1997) y en tejidos (Risalde y col., 2013), así como en el diagnóstico de enfermedades (Oosterwijk y col., 1986).

Hasta la fecha, solo un trabajo llevado a cabo por Gutiérrez-Praena y col. (2014a) ha estudiado la IHQ como técnica para detectar y monitorizar la presencia y distribución de CYN en diversos tejidos de tilapias expuestas de forma aguda a la toxina.

4.1.2. Métodos moleculares

Se han desarrollado distintos métodos moleculares para la detección de CYN mediante el uso de la reacción en cadena de la polimerasa (PCR) y la caracterización del ADN y ARN de cianobacterias productoras de CYN (Rasmussen y col., 2008; Głowacka y col., 2011; Moreira y col., 2011; Marbun y col., 2012). Sus principales aplicaciones en las investigaciones de campo son la detección e identificación de las cianobacterias, evaluación de la toxicidad y enumeración (Moreira y col., 2014).

Existen **métodos de screening multi-detección** que ahorran en volumen de muestra y número de análisis, acelerando los resultados. Recientemente se ha desarrollado un método semicuantitativo multi-detección basado en microesferas como herramienta de screening para la detección de MCs y CYN, entre otras cianotoxinas, en aguas dulces y salobres (Fraga y col., 2014). De hecho, la coocurrencia y aparente bioacumulación de diferentes tipos de cianotoxinas apoyan la necesidad del desarrollo de esta clase de métodos múltiples de detección (Berry y Lind, 2010; Berry y col., 2012).

4.2. Métodos confirmativos: cromatográficos

Las técnicas cromatográficas son las más utilizadas hasta la fecha para la identificación y cuantificación de congéneres de MCs y de CYN, basándose en la cromatografía líquida (LC) con diferentes detectores (Gurbuz y col., 2009).

Para el análisis de MCs se ha utilizado la LC acoplada a detector de ultravioleta (UV) (Ann y Carmichael, 1994; Lawrence y Menard, 2001; Moreno y col., 2005a), de diodo-array (LC-DAD ó LC-PDA) (Gago-Martínez y col., 2003), acoplada a espectrometría de masas (LC-MS) (Barco y col., 2002; Moreno y col., 2005b) y a espectrometría de masas en tándem (LC-MS/MS) (Dai y col., 2008). Estas dos últimas técnicas se han convertido en las de elección para el análisis de MCs en una gran variedad de matrices (Moreno y col., 2004; Bogialli y col., 2005; Chen y col., 2006; Lei y col., 2008). Requieren de la extracción previa de MCs de las cianobacterias con diferentes disolventes mediante extracción en fase sólida (SPE), técnicas alternativas como la cromatografía de inmunoafinidad (Lawrence y Menard, 2001; Kondo y col., 2002; Aguete y col., 2003) o la electroforesis capilar (Gago-Martínez y col., 2003).

Debido a la amplia distribución y toxicidad de la CYN y a su elevada presencia extracelular, es necesario desarrollar métodos de confirmación validados para la determinación de la toxina disuelta en muestras de agua reales y células liofilizadas de cultivos de cianobacterias. Para ello se han utilizado distintas técnicas cromatográficas como: la electroforesis capilar, LC-DAD, LC-MS y/o LC-MS/MS.

El primer método para la determinación de CYN usando LC/DAD fue desarrollado por Harada y col. (1994). Posteriormente, Welker y col. (2002) desarrollaron un método alternativo para analizar el contenido de CYN en muestras ambientales por la misma técnica, extrayendo la toxina con agua pura. Sin embargo, aunque el uso de agua para la extracción de CYN resulta eficiente, al aplicar el método a muestras más complejas, se observaba un efecto matriz que enmascaraba la toxina. En un ensayo comparativo entre laboratorios para cuantificar CYN en las células de cianobacterias liofilizadas, en el que participaron seis laboratorios de cinco países diferentes, se demostró que el método de extracción más eficaz se conseguía empleando ácido fórmico acuoso al 5%, proporcionando una extracción eficiente y picos menos contaminados en comparación con el método de extracción que utilizaba sólo agua (Törökné y col., 2004).

El método de extracción empleado en la preparación de muestras se ha mejorado con la aplicación de la extracción en fase sólida (SPE) (Norris y col., 2001b; Metcalf y col., 2002),

incluyendo un sistema de doble columna: una con relleno de un polímero de estireno y otra con relleno de intercambio aniónico (Kubo y col., 2005). Posteriormente, con el empleo de cartuchos de carbón grafitizado, se obtuvieron resultados fiables en la extracción de CYN a partir del medio de cultivo (*A. ovalisporum*) o de diversas muestras ambientales, usando una mezcla acidificada de diclorometano:metanol como disolvente, y la cuantificación por LC/DAD (Wormer y col., 2009).

La espectrometría de masas también se ha acoplado a la cromatografía líquida de interacción hidrófila (HILIC-MS) y ha demostrado ser un método eficaz para la detección de CYN incluso cuando hay presencia de otras cianotoxinas, sin requerir limpieza previa o preconcentración de la muestra (Dell'Aversano y col., 2004). La cromatografía líquida de ultra rendimiento acoplada a espectrometría de masas en tándem (UPLC-MS2) también ha sido aplicada para la detección tanto de CYN como de 7-desoxi-CYN (Haande y col., 2008).

La LC-MS/MS es una técnica muy sensible y capaz de detectar adecuadamente cantidades traza de CYN (Welker y col., 2002). De esta manera, la LC-MS/MS ha sido elegido como el método ideal y de referencia para detectar y cuantificar pequeñas cantidades de toxina en muestras de agua (Eaglesham y col., 1999; Bogialli y col., 2006; Oehrle y col., 2010) y en muestras más complejas, como tejidos de de peces (Gallo y col., 2009) o muestras de suero y orina humanas (Foss y Aubel, 2013).

Generalmente, los enfoques clásicos para la validación de métodos analíticos sólo se basaban en la comparación entre los valores medidos y los de referencia, sin tener en cuenta la importancia de la precisión y reproducibilidad del método intra- e interlaboratorio, incluyendo estudios de robustez, todo ello imprescindible para la "transferencia del método" (González y col., 2010). Además, aunque la CYN se haya extraído y detectado en diferentes matrices, incluyendo algunas más complejas como organismos acuáticos (Gallo y col., 2009; Saker y col., 2004; Berry y Lind, 2010; Messineo y col., 2010), no se han llevado a cabo hasta el momento estudios de validación y robustez en ellos. Por todo ello, se pone de manifiesto la necesidad de validar métodos cuantitativos para la determinación de CYN en todas estas matrices, y poder llevar a cabo así una correcta evaluación del riesgo medioambiental y humano, siendo también objetivo importante del presente trabajo, como se explica a continuación.

III. JUSTIFICACIÓN Y OBJETIVOS / SIGNIFICANT AND PURPOSES

Una vez realizada la revisión bibliográfica, se deduce que la capacidad que tienen las microcistinas (MCs) de bioacumularse en distintos órganos de animales acuáticos, destacando los peces de consumo humano, puede suponer un gran riesgo para la salud pública. Así, una correcta evaluación de riesgos de la exposición humana a MCs a través de los alimentos requiere de un detallado conocimiento acerca de la influencia del procesado y cocinado del alimento sobre estas toxinas. En este sentido, se planteó el estudio del efecto de distintas técnicas de cocinado sobre la concentración de MCs libres en músculo de pescado contaminado.

Teniendo en cuenta la ubicuidad de la CYN, sus altos niveles detectados en aguas y floraciones tóxicas en distintos puntos del planeta, y su capacidad de acumulación en diversos organismos acuáticos, se pone de manifiesto la necesidad de poner a punto y validar métodos analíticos que permitan la identificación y cuantificación de esta toxina en diferentes matrices: aguas, cultivos de cianobacterias y tejidos de peces contaminados, y poder llevar a cabo así una correcta evaluación del riesgo correspondiente.

A diferencia de otras cianotoxinas, los estudios existentes con respecto a la producción de estrés oxidativo como uno de los mecanismos de acción tóxica de la Cilindrospermopsina (CYN) son escasos, particularmente en peces, a pesar de compartir hábitat y tener una alta probabilidad de exposición. Es por ello que se decidió investigar *in vivo* las modificaciones inducidas por CYN en las actividades de diversas enzimas implicadas en el estrés oxidativo, a nivel bioquímico y molecular, así como a nivel histopatológico, para esclarecer el impacto de dicha cianotoxina en peces. Se han considerado para ello distintas vías y tiempos de exposición, así como la administración de la CYN pura frente a las células liofilizadas de un cultivo productor de la toxina. Por otro lado, aunque haya algunos estudios al respecto en mamíferos y algunos animales acuáticos, la neurotoxicidad de la CYN es tema de controversia aún, y no se conocen estudios histopatológicos en cerebro de peces contaminados con CYN, por lo que se vio necesario ahondar más en este posible mecanismo de toxicidad en peces como modelo experimental.

Una vez comprobada la inducción de estrés oxidativo por la CYN, nos resultó interesante identificar sustancias que permitiesen prevenir y/o recuperar los efectos tóxicos observados. En este sentido, quisimos abordar el estudio de la efectividad de los antioxidantes

L-carnitina (LC) y vitamina E con este fin, así como la posible reversión de estos efectos en hígado, riñón y cerebro tras diferentes períodos de depuración que, aunque haya sido demostrada en algunos organismos acuáticos expuestos a otros contaminantes, no ha sido probada en el caso de la CYN.

Por otra parte, consideramos de interés evaluar la distribución de la CYN en tejidos de peces contaminados, mediante inmunohistoquímica (IHC).

Para la realización de esta Tesis Doctoral, la doctoranda realizó tres estancias de investigación en el extranjero, en la que se abordaron dos objetivos: a) investigar mediante herramientas proteómicas la diversidad proteica de las GSTs en bivalvos, siendo esta la enzima más estudiada en relación a la biotransformación de cianotoxinas, y utilizada habitualmente como biomarcador de estrés oxidativo en organismos acuáticos expuestos a contaminantes en su hábitat natural; b) además, por ser escasos los estudios que relacionan a la CYN con plantas, se consideró interesante el estudio del efecto de la CYN sobre organismos vegetales destinados al consumo humano, ya que ésta puede encontrarse en los reservorios de aguas destinadas al riego, y por tanto pasar a las plantas, constituyendo con ello un importante riesgo para la salud humana.

Por todo ello, los objetivos específicos establecidos en la presente Tesis Doctoral han sido:

1. Estudiar la influencia de distintas técnicas de cocinado (microondas y hervido) sobre la estabilidad de las MCs puras (MC-LR, MC-RR y MC-YR) en músculo de tilapias (*Oreochromis niloticus*).
2. Poner a punto y validar diferentes métodos cuantitativos, incluyendo ensayos de robustez, para la determinación de CYN en distintas matrices (aguas, cultivos de cianobacterias y tejidos de peces contaminados con CYN), por cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS).
3. Investigar la inducción de estrés oxidativo como mecanismo de toxicidad de CYN en hígado y riñón de tilapias (*O. niloticus*) expuestas de forma subcrónica a CYN mediante inmersión en aguas con células liofilizadas de *Aphanizomenon ovalisporum*, evaluando la influencia de la concentración de la toxina (10 y 100 µg CYN/L) y del

tiempo de exposición (7 y 14 días). Del mismo modo, determinar los posibles cambios histopatológicos que se produzcan bajo las mismas condiciones.

4. Evaluar la potencial neurotoxicidad de la CYN, mediante la medida de la actividad de la acetilcolinesterasa (AChE), la peroxidación lipídica (LPO) y los estudios histopatológicos en el cerebro de tilapias (*O. niloticus*) expuestas por inmersión a dosis repetidas de CYN contenidas en células liofilizadas de *A. ovalisporum* durante 14 días.

5. Investigar el efecto protector de la L-carnitina (en hígado y riñón) y de la vitamina E (en hígado, riñón y cerebro) de tilapias (*O. niloticus*) expuestas a CYN, para su potencial aplicación como suplemento en la dieta, con la finalidad de prevenir la inducción de estrés oxidativo, y/o los cambios histopatológicos y moleculares (mediante técnicas de Western blotting y RT-PCR) producidos por esta toxina.

6. Explorar la eficacia de dos períodos de depuración (3 y 7 días) en la mejora de los niveles de diferentes biomarcadores de estrés oxidativo en hígado y riñón, así como de las alteraciones observadas a nivel neurológico, en tilapias (*O. niloticus*) expuestas a dosis repetidas de CYN (10 µg CYN/L), por inmersión en aguas con células liofilizadas de *A. ovalisporum* durante 14 días.

7. Estimar la utilidad de la inmunohistoquímica (IHC) como técnica para estudiar la distribución de la CYN en diferentes tejidos de peces contaminados por la toxina, teniendo en cuenta distintas dosis, vías y tiempos de exposición.

8. Profundizar en la diversidad proteica de las Glutatión-S-transferasas (GSTs), caracterizando el conjunto de sus isoformas presentes en las branquias del mejillón marino *Mytilus galloprovincialis*, mediante técnicas proteómicas.

9. Conocer los efectos de la CYN sobre las condiciones fisiológicas, capacidad fotosintética y valor nutritivo (contenido en minerales) de zanahorias (*Daucus carota*) expuestas a diferentes dosis de la toxina durante 30 días.

El trabajo experimental se ha realizado en el Área de Toxicología de la Facultad de Farmacia de la Universidad de Sevilla, haciendo uso así mismo de las instalaciones del Servicio de Biología del Centro de Investigación, Tecnología e Innovación de la Universidad

de Sevilla (CITIUS). Asimismo, los estudios histopatológicos se han realizado en el Área de Toxicología y en el Departamento de Anatomía y Anatomía Patológica Comparadas, ambos de la Universidad de Córdoba, gracias a la colaboración de la Dra. Rosario Moyano y del Dr. Alfonso Blanco, respectivamente. Todo ello se corresponde con los objetivos 1-7. También se integran (en los objetivos 8 y 9) las investigaciones llevadas a cabo en el Centro Interdisciplinar de Investigación Marina y Ambiental (CIIMAR) de la Universidad de Oporto, bajo la dirección del Dr. Vitor Vasconcelos.

Siguiendo la normativa de la Universidad de Sevilla, el resumen, la justificación y objetivos, y las conclusiones se redactan tanto en castellano como en inglés para optar a la "Mención Internacional en el Título de Doctor".

Based on the literature review, it appears that the ability of microcystins (MCs) to bioaccumulate in different organs of aquatic animals, including fish intended for human consumption, may constitute a high risk to public health. Thus, a proper risk assessment of human exposure to MCs through food requires a detailed knowledge about the influence of food processing and cooking on these toxins. In this sense, we aimed to study the effects of different cooking techniques on the concentration of free MCs in contaminated fish muscle.

Taking into account the ubiquity of CYN, together with the high levels of the toxin detected in waters and toxic blooms in different parts of the world, and its ability to accumulate in several aquatic organisms, the need for developing and validating analytical methods that allow us to quantify CYN in real samples (waters, cyanobacterial cultures and tissues of contaminated fish) is highlighted, in order to carry out an adequate and appropriate risk assessment.

Unlike other cyanotoxins, current studies regarding the production of oxidative stress as one of the mechanisms of toxic action of Cylindrospermopsin (CYN) are scarce, particularly in fish, despite sharing habitat and, therefore, having a high probability of exposure. For that reason, we decided to investigate *in vivo* the alterations induced by CYN in the activities of several enzymes involved in oxidative stress, as well as analyzing the molecular and histopathological changes, to clarify the impact of such cyanotoxin in fish. For this purpose, we have assayed different routes and periods of exposure, as well as the administration of pure CYN or the lyophilized cells of a CYN-producing culture. Moreover, despite the existence of some studies on mammals and aquatic animals, the neurotoxicity of CYN is somewhat controversial yet, and no histopathological studies have been carried out in fish brain contaminated with CYN. Therefore, we thought it was necessary to gain insight into this possible mechanism of toxicity in fish as the experimental model.

Once the production of oxidative stress by CYN was demonstrated, we found it interesting to identify substances that allowed prevention and/or recovering the observed effects. In this sense, we decided to study the effectiveness of the antioxidants L-carnitine (LC) and vitamin E for this purpose, and the possible reversion of the toxic effects after different periods of depuration using clean water, which has been demonstrated in some aquatic organisms exposed to other contaminants, but not in the case of CYN.

We also decided to study the distribution of CYN in contaminated fish tissues by immunohistochemistry (IHC).

For the fulfillment of this thesis, the PhD student performed three internships abroad, in which two objectives were addressed: a) to investigate the protein diversity of GSTs in bivalves, using proteomic tools, as GSTs are the most studied enzymes in relation to the biotransformation of cyanotoxins, and are usually used as a biomarker of oxidative stress in aquatic organisms exposed to contaminants in their natural habitat; b) to study of the effect of CYN on plant organisms intended for human consumption, since this toxin can be found in water reservoirs for irrigation, thereby constituting a significant risk to human health.

Therefore, the specific objectives of this PhD thesis were:

1. To study the influence of different cooking techniques (microwave oven and boiling) on the concentration of pure MCs (MC-LR, MC-RR and MC-YR) in muscle of tilapia (*Oreochromis niloticus*).

2. To develop and validate different quantitative methods, including robustness assays, for determining CYN in different matrices (waters, cyanobacterial cultures and CYN-contaminated fish tissues), by liquid chromatography tandem mass spectrometry (LC-MS/MS).

3. To investigate the induction of oxidative stress as a toxic mechanism of CYN in liver and kidney from tilapia subchronically exposed by immersion to CYN contained in *Aphanizomenon ovalisporum* lyophilized cells, evaluating the influence of the concentration of the toxin (10 and 100 µg CYN/L) and the exposure time (7 and 14 days). In this context, to identify the potential histopathological changes produced under the same conditions.

4. To evaluate the potential neurotoxicity of CYN, by measuring the activity of acetylcholinesterase (AChE), lipid peroxidation (LPO) and the brain histopathology of tilapia by immersion exposed to repeated doses of CYN contained in *A. ovalisporum* lyophilized cells for 14 days.

5. To investigate the protective effect of L-carnitine (in liver and kidney), and of vitamin E (in liver, kidney and brain) from tilapia exposed to CYN, for their potential

application as dietary supplements, in order to prevent the induction of oxidative stress, and/or histopathological and molecular changes (by western blotting and RT-PCR) produced by this toxin.

6. To explore the efficiency of two depuration periods (3 and 7 days) in improving the levels of oxidative stress biomarkers in liver and kidney, as well as the neurological alterations, in tilapia exposed by immersion to repeated doses of CYN (10 µg/L) contained in *A. ovalisporum* lyophilized cells for 14 days.

7. To estimate the usefulness of immunohistochemistry (IHC) to study the distribution of CYN in different tissues of fish contaminated with a toxin, assaying different doses, routes and periods of exposure.

8. To further investigate the protein diversity of Glutathione-S-transferases (GSTs), characterizing its isoforms in gills from the marine mussel *Mytilus galloprovincialis*, using proteomic techniques.

9. To analyze the effects of CYN on the physiological conditions, photosynthetic capacity and nutritional value (mineral content) in carrots (*Daucus carota*) exposed to different concentrations of the toxin for 30 days.

The experimental work has been performed in the Area of Toxicology, Faculty of Pharmacy, University of Sevilla, using also the Biology Facility from Centro de Investigación, Tecnología e Innovación from the University of Sevilla (CITIUS). Histopathological studies have been conducted in the Area of Toxicology and in the Department of Anatomy and Comparative Pathology and Anatomy, both from the University of Córdoba, thanks to the collaboration of Dra. Rosario Moyano and Dr. Alfonso Blanco, respectively. This corresponds to the objectives 1-7. Research conducted in the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) from the University of Porto, under the direction of Dr. Vitor Vasconcelos, has also been included, in the objectives 8 and 9.

Following the regulations from the University of Sevilla, the summary, significance and purposes, and conclusions are written both in Spanish and in English to aim for a PhD with International Mention.

IV. RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION

CAPÍTULO 1 / CHAPTER 1

Remedios Guzmán-Guillén, Ana I. Prieto, Isabel Moreno, Mª Eugenia Soria, Ana M. Cameán

EFFECTS OF THERMAL TREATMENTS DURING COOKING, MICROWAVE OVEN AND BOILING, ON THE UNCONJUGATED MICROCYSTIN CONCENTRATION IN MUSCLE OF FISH (OREOCHROMIS NILOTICUS).

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Effects of thermal treatments during cooking, microwave oven and boiling, on the unconjugated microcystin concentration in muscle of fish (*Oreochromis niloticus*)

Remedios Guzmán-Guillén, Ana I. Prieto, Isabel Moreno, M[a] Eugenia Soria, Ana M. Cameán *

Nutrición y Bromatología, Toxicología y Medicina Legal Department. Faculty of Pharmacy, University of Sevilla, C/ Profesor García González 2, 41012 Sevilla, Spain

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ABSTRACT

Understanding the factors that contribute to the risk from fish consumption is a relevant public health concern due to potential adverse effects of cyanobacterial toxins. The aim of this work was to study the influence of two usual cooking practices, microwave oven and boiling, on the microcystin (MCs) concentration in fish muscle (Tilapia, *Oreochromis niloticus*) spiked with a stock solution (500 μL) containing a mixture of three toxins (MC-LR, MC-RR, and MC-YR) (1.5 μg/mL of each toxin). Two different variables were investigated: time of cooking in the microwaves treatment (1 or 5 min), and way of boiling, "boiled muscle" or "continuously heated muscle". All samples were then lyophilized and MCs were extracted and purified (Oasis HLB cartridge) and quantified by HPLC-MS. Furthermore, the waters in which the samples boiled were also analyzed after their purification. The results suggest a reduction on MC-LR (36%) and MC-YR (24.6%) in samples cooked in the microwave for 5 min. Major changes were found when the fish was cooked by the continuous boiling, with a decrease of 45.0% (MC-RR), 56.4% (MC-YR) and 59.3% (MC-LR). More studies are necessary to elucidate the mechanisms involved when aquatic food is submitted to usual cooking practices.

1. Introduction

Microcystins (MCs) are potent hepatotoxins and tumor promoters produced by freshwater cyanobacteria (blue-green algae) belonging from at least five genera worldwide: *Anabaena*, *Microcystis*, *Planktothrix* (*Oscillatoria*), *Nostoc*, and *Anabaenopsis*, that often forms blooms in eutrophic freshwater ecosystems, representing a health risk to aquatic organisms, wild life, domestic animals, and humans (Ueno et al., 1996; Sivonen and Jones, 1999; Li et al., 2009).

There are more than 80 MCs that have a ring structure of seven amino acids, which comprise one unique phenyl deca-dienoic acid (named Adda), four invariable D-amino acids and two variable L-amino acids which give a name to the molecule. Microcystin-LR (MC-LR, leucine–arginine) is the most widely investigated cyanobacterial peptide toxin because it is frequently present in cyanobacterial blooms in rivers and lakes (Vasconcelos et al., 1996). Other variants that also occur frequently are MC-RR (arginine-arginine) and MC-YR (tyrosine-arginine) (De Figueiredo et al., 2004).

Chronic toxic effects in humans from exposure through water and food contaminated by cyanotoxins need to be considered, especially if there is long-term frequent exposure (Chen et al., 2009a), because they are associated with increased incidences of primary human liver and colorectal cancer (Ueno et al., 1996; Zhou et al., 2002), and MC-LR has been considered as "possibly carcinogenic to humans" (group 2B) by the International Agency for Research on Cancer (IARC) (http://www.iarc.fr/).

Recognizing its potential adverse health effects, the World Health Organization (WHO) proposed a provisional guideline value in drinking water of 1 μg/L for MC-LR and established 0.04 μg/kg body weight/day as a Tolerable Daily Intake (TDI) of MC-LR (WHO, 1998). It was assumed that 80% of the MCs that were ingested on a daily basis came from contaminated drinking water and that the remaining 20% was intaken from food or inhalation (Dietrich and Hoeger, 2005). In some cases, the daily intake of MC from fisheries product (0.86–2.57 μgMC-LR eq) even exceeds the contribution of the drinking water (1.31 μg MC-LR eq) for high exposure population like fishermen (Chen et al., 2009a).

In natural environments, MCs are found to bioaccumulate in a wide range of aquatic animals such as fish, and this fact has been extensively studied (Magalhães et al., 2001, 2003; Mohamed et al., 2003; Xie et al., 2005; Chen et al., 2007; Moreno et al., 2010). Accumulation was demonstrated in liver, intestine, kidneys, gallbladder, gills, muscle and brain. From several laboratory and field studies, it is established that MCs also accumulate in muscle of different fish species, although the concentration of MCs in muscle is usually lower than in other tissues (Martins and Vasconcelos, 2009; Moreno et al., 2011). Although in low doses, overall MCs uptake through consumption of freshwater fish may in many cases exceed the TDI

* Corresponding author. Tel.: +34 954 55 67 62; fax: +34 954 55 64 22.
E-mail address: camean@us.es (A.M. Cameán).

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and were unsafe for human consumption (Ibelings and Chorus, 2007; Peng et al., 2010). It seems that any organism which is harvested for human consumption from waterbodies (lakes, rivers or estuaries) containing blooms of toxic cyanobacteria might enclose cyanobacterial toxins (Ibelings and Chorus, 2007). The extent to which the MCs concentrations found in these aquatic organisms could present a risk to humans eating them has been discussed by different authors (Codd et al., 2005; Ibelings and Chorus, 2007; Morais et al., 2008). In fact, as described by Chen et al. (2009a), daily intake of MCs by a fisherman could be 2.2–3.9 μg MC-LR eq, close to exceeding the TDI of 2.4 μg for an adult (body weight 60 kg) proposed by WHO.

Previous studies from our group have described the occurrence of toxic cyanobacteria along the Guadiana river between Mérida and Badajoz (Extremadura, Spain) (Moreno et al., 2004, 2005; Cameán et al., 2004), the bioaccumulation of MCs in the native freshwater fish, tench (*Tinca tinca*) from natural ponds in Extremadura (Moreno et al., 2011), and their effects in tench and in Tilapia (*Oreochromis niloticus*) have also been investigated (Jos et al., 2005; Atencio et al., 2008).

Microcystins, being cyclic peptides, are extremely stable and resistant to chemical hydrolysis or oxidation near neutral pH (Harada et al., 1996), and changes from pH 1 to pH 12 had little impact on MC-LR (Yu et al., 2009). These authors also demonstrated that MC-LR was stable from 10 to 150 °C.

On the other hand, qualitative and quantitative knowledge of the influence of food processing, including cooking, on the substances and xenobiotics in food is needed, as they are present in food. A better understanding of processing on food contaminants is necessary, as it affects exposure evaluation, and consequently has implications for risk evaluation and risk assessment, because most consumption studies report consumption on the basis of cooked fish. However, the number of studies is rather limited, being mainly focused on a reduced number of contaminants, such as metals (Devesa et al., 2008; Perelló et al., 2008), polychlorinated biphenyls (PCBs) (Hori et al., 2005), polybrominated diphenyl ethers (PBDEs), hexachlorobenzene (HCB) and polycyclic aromatic hydrocarbons (PAHs) (Perelló et al., 2009), radionuclides (Burger et al., 2004), and some pesticides, but in the case of MCs the studies are very scarce (Morais et al., 2008; Zhang et al., 2010).

Our overall objective in this work was to determine whether microwave oven and boiling affects MCs concentration in contaminated tilapia (*O. niloticus*), in order to know how the inclusion of cooking effects could clarify risks associated with their consumption. This study was carried out on muscle of fish added with a dose of pure standards of the three more common MCs, MC-LR, MC-RR and MC-YR present in natural waters, under laboratory conditions. To study the influence of the microwaves treatment, we investigated MCs levels in fish after being cooked for 1 and 5 min. To test the effects of boiling cooking, two procedures were assayed: fish introduced in boiling water (boiled muscle) and fish introduced in cool water, heated to boiling and boiled for 2 min (continuously heated muscle). In all experiments, high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) has been employed to determine MCs content in muscle of fish, because of its utility for analyzing MCs in a variety of matrices, waters (McElhiney and Lawton, 2005), and especially fish (Moreno et al., 2005; Bogialli et al., 2005).

2. Materials and methods

2.1. Reagents

Microcystin standards (MC-LR, MC-RR, and MC-YR) were purchased from Alexis (Switzerland) with a purity of 99%. Standard stock solutions of each toxin (500 μg/L for MC-LR and 100 μg/L for MC-RR and MC-YR) in methanol were used to spike muscle of fish, and working standard solutions were prepared from these stock solutions by dilution in methanol prior to analysis.

All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

2.2. Fish samples

Fresh male tilapia (*O. niloticus*, Nile tilapia, Perciformes: Cichlidae) with mean weight 55.2 ± 6.7 g and 12 ± 2 cm length were obtained from a fish hatchery (Valenciana de Acuicultura, Valencia) and transferred to the laboratory where they were kept in aquariums (8 individuals/aquarium) with 96 L of tap-water, and the temperature was kept constant (21 ± 2 °C). The fish were fed daily (0.3 g/day) with commercial fish food free of MCs (Dibaq S.L., Segovia, Spain).

Once the fish were acclimatized (approximately 15 days), they were sacrificed, dissected and each muscle sample was cut into approximately 4 g portions. The fish muscles were spiked with a stock solution (500 μL) containing a mixture of the three studied toxins (MC-LR, MC-RR, and MC-YR), directly injecting the toxin into the muscle, at a concentration of 1.5 μg/mL of each toxin. This concentration was considered environmentally relevant, according to the accumulation of MCs found naturally in fish (Ibelings and Chorus, 2007; Martins and Vasconcelos, 2009).

Two cooking procedures were performed: microwave oven and boiling in water. In each procedure two groups were considered: cooked versus non-cooked fish. Moreover, two different variables for each cooking method were investigated: time of cooking in the microwaves treatment, and way of boiling the fish in the other procedure. The assays were always carried out by quintuplicate ($n = 5$). In each experiment, control fish were prepared from fortified MC-muscle fish (500 μL of a solution containing 1.5 μg/mL of each toxin), and were not submitted to any cooking treatment.

Samples of fish muscle were cooked in a conventional household microwave oven (Samsung M17-13, 300 W, 2450 MHz) using two periods of time: 1 and 5 min, in the regular way as when preparing fish, but no salt or any other additional ingredients were added.

To test the boiling method, two different procedures were considered: one group of fish muscle (4 g) was introduced in boiling water (50 mL) for 2 min (“boiled muscle”) and another group was put into cool water, heated to boiling, and then continued to boil for 2 min (“continuously heated muscle”).

Muscle fish weights were recorded before (approximately 4 g) and after cooking. The final weights of fish muscle after the microwaves treatment were 3.47 ± 0.23 g and 1.56 ± 0.16 g, for 1 and 5 min, respectively. Mean weight values for boiled muscle and continuously boiled muscle were 3.42 ± 0.17 g and 2.99 ± 0.24 g, respectively. All samples groups were frozen at −80 °C and lyophilized (Cryodos 80 model, Telstar, Tarrasa, Spain) before MCs were extracted. Once the lyophilization process was over, the samples were weighed, and the final lyophilized weight was, approximately, 1.0 g dry weight (DW) in all cases. Thus, the loss of weight due to the lyophilization process was between 78% and 80%. All results of unconjugated MCs determined were expressed as ng/g dry weight of lyophilized tissue (DW).

In addition, we also analyzed MCs concentration in the waters from the two boiling procedures, and values obtained were expressed as μg of each MC per liter of water.

2.3. Extraction of MCs from fish and clean up procedures

The lyophilized muscle (1 g) of fish were extracted using a modified version of Dai et al.'s method (2008), with some modifications, in relation to volumes of extraction solvents employed, and simplifying the cleanup procedure with the replacement of the second solid-phase extraction (SPE) sorbent from a Sep-Pak silical gel, by filtration through an ultrafree-MC PVDF filter. Briefly, the muscle was extracted with 10 mL of water with EDTA-Na_2 (0.01 M)–5% acetic acid, sonicated for 3 min at 0 °C and then centrifuged at 5000 rpm at room temperature. This procedure was repeated twice, using for the other two extractions 5 mL of the extractant. Once the extracts of each sample had been obtained, a purification step was applied. The extracts (20 mL) were applied to an Oasis HLB cartridge (500 mg/6 mL, Waters, Milford, MA, USA), which had been preconditioned with 100% methanol (10 mL) and distilled water (10 mL). The column containing the sample was washed with 20% methanol (20 mL) and then eluted with 100% methanol (20 mL). The extracts were evaporated to dryness and redissolved in 1 mL of 100% methanol. Then, they were transferred to an ultrafree-MC PVDF (0.45 μm Millipore Corporation, Bedford, MA) and centrifuged (10 min, 5000 rpm). Finally, the extracts were evaporated to dryness and redissolved in 200 μL of methanol 100%. The aliquots (10 μL) were injected into the LC–MS system. To quantify the changes in MCs concentrations (ng/g dry weight) in fish samples after each treatment, the peak areas obtained for each toxin were compared to those obtained in the uncooked samples.

MCs determination in water samples in which fish muscles were boiled was carried out following the method of Dai et al. (2008) with slight modifications, by the replacement of the second solid-phase extraction (SPE) sorbent from a Sep-Pak silical gel, by filtration through an ultrafree-MC PVDF filter. Briefly, after conditioning an Oasis HLB cartridge (500 mg/6 mL, Waters, Milford, MA, USA) with 100% methanol (10 mL) and Milli-Q water (10 mL), the extract obtained from a

centrifugated aliquot of boiled water (50 mL) for 10 min at 4500 rpm, was transferred to the cartridge. The cartridge was washed with 20 mL of 20% methanol (v/v) and the MCs were eluted with 20 mL 100% methanol. The eluate was evaporated to dryness and the residue was dissolved in 100% methanol (1000 μL). Then, the samples were transferred to an ultrafree-MC PVDF (0.45 μm Millipore Corporation, Bedford, MA) and centrifuged (10 min, 5000 rpm). Finally, the extracts were evaporated to dryness (N_2) and redissolved in 200 μL of methanol 100%, prior their determination (10 μL) by HPLC/MS.

2.4. Chromatographic conditions

Chromatographic separation was performed using a Perkin–Elmer Series 200 HPLC system (Wellesley, MA, USA) coupled to an Applied Biosystems QTRAP LC–MS/MS system (Foster City, CA, USA) consisting of a quadrupole-linear ion trap mass spectrometer equipped with an electrospray ion source. The analytical column was a LiChroCART 250-4 reversed-phase column with a particle size of 5 μm (Merck kGaA). The flow rate was 0.4 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) acetonitrile. Both components contained 0.05% trifluoroacetic acid (v/v). The elution profile was: 35% B (2 min), 35% up to 65% B (15 min), 65% B (5 min), 100% B (3 min). The injection volume was 10 μl. Selected-ion monitoring (SIM) was used to detect MCs, in which the ions were monitored at quadrupole Q1 (m/z = 519.8 for MC-RR, m/z 995.6 for MC-LR and m/z 1045.6 for MC-YR). The ion source was operated in positive mode. The capillary voltage and declustering potential were set to 5500 V and 105 V, respectively. Curtain gas, nebulizer gas (gas 1) and heater gas (gas 2) were 35 psi, 60 psi and 60 psi, respectively, and the Turboprobe temperature was maintained at 350 °C.

2.5. Statistics

Results are subjected to one-way analysis of variance (ANOVA) with the statistical package INSTAT, Graph Pad™, and represent mean ± standard deviation (SE) of 5 samples per group. Differences in mean values between groups were assessed by the Tukey's test and were considered statistically significant at $p < 0.05$ level.

3. Results

In this work fish spiked with pure standard solutions of three MCs, MC-RR, MC-YR, and MC-LR, have been submitted to the usual cooking methods, such as microwave oven and boiling, so that the possible transference to water (in the case of boiled fish) caused for both procedures can be assessed. Table 1 summarizes the recovery percentages (%) of MCs obtained in fish after their submission to both cooking procedures. It is important to emphasize that MCs concentration detected in the fish muscles refers to free toxins, once the method applied to analyze these samples is not suitable to detect MCs bound to protein phosphatases or glutathione.

After microwave treatment of spiked fish for 1 min, the time usually employed to heat food, the three MCs tested appeared to be stable, and not significant differences were found in comparison with uncooked samples (Fig. 1). However, when muscles of fish with the mixture of MCs were cooked after 5 min, MC-LR and MC-YR were significantly affected, with decreases of 36% and 24.6%, respectively. MC-RR concentrations found in cooked fish were slightly higher (6–17%), and not considered significant ($p > 0.05$) in any case. Fig. 2(A–C) shows an example of the chromatograms obtained for the unconjugated MCs extracted from fish muscle heated for 5 min in the microwave treatment.

Table 1
Recovery percentages (%) of pure MC (MC-RR, MC-LR and MC-YR), after spiking fish muscle and their submission to the different cooking procedures.

Toxin	Type of cooking			
	Microwave 1 min	Microwave 5 min	Direct boiling	Continuous boiling
MC-RR	117.0	106.0	65.6	55.0
MC-LR	95.4	64.0	49.2	40.7
MC-YR	100.0	75.4	46.8	43.6

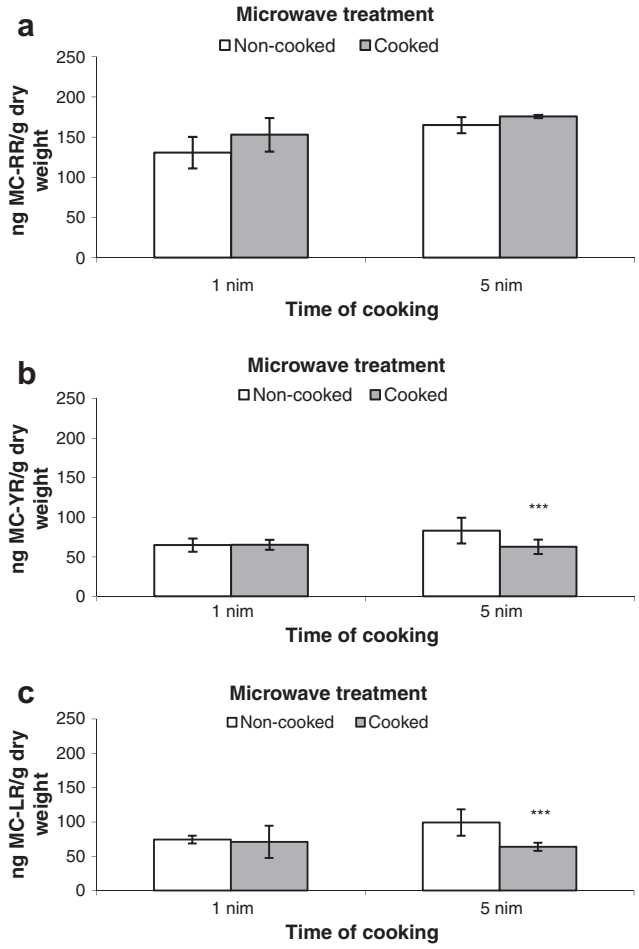

Fig. 1. MC concentrations (ng MC/g dry weight) in cooked and non-cooked fish muscle (*Oreochromis* sp.) spiked with a mixture of pure standard solution of (a) MC-RR, (b) MC-YR and (c) MC-LR (1.5 mg/L of each toxin) and submitted to microwave treatment for 1 or 5 min. Values are expresses as means ± standard deviations for five replicates. The significance levels observed are $^{***}p < 0.001$ in comparison to their respective control group values.

Microcystin concentrations in the fish muscle spiked with the mixture containing the three variants of MCs, were significantly reduced ($p < 0.001$) after the application of both boiling procedures, "continuously heated muscle" (put in cool water, and continued to be boiling), and "boiled muscle" (directly introduced in boiling water) (Fig. 3). In the case of MC-YR and MC-LR, the mean concentration in fish muscle decreased more than 50%, in comparison to uncooked fish, and no differences between both boiling procedures were found. Specifically, the percentages of reduction of MC-YR and MC-LR concentrations in boiled muscle fish were 53.2% and 50.8%, respectively; in the case of continuously heated muscle they were 56.4% and 59.3%, respectively. However, for MC-RR, the type of boiling procedure applied significantly changed its contents in cooked fish muscle ($p < 0.05$), with a major decrease in MC concentration in the continuously heated muscle (45.0%) compared to the direct boiled muscle fish (34.4%). Chromatograms showing the effects of both boiling methods on the MCs concentration in fish muscle are shown in Fig. 4.

Unconjugated MCs from the waters in which the muscles were boiled were analyzed quantitatively (Fig. 5), and the results obtained demonstrated the transference of each toxin from the food to water. No free MCs were detected in boiled water without fish (results not shown). Taking into account the determined levels of MCs, the highest transference was found in the case of MC-RR, followed by MC-LR, and finally MC-YR. The values of free MCs

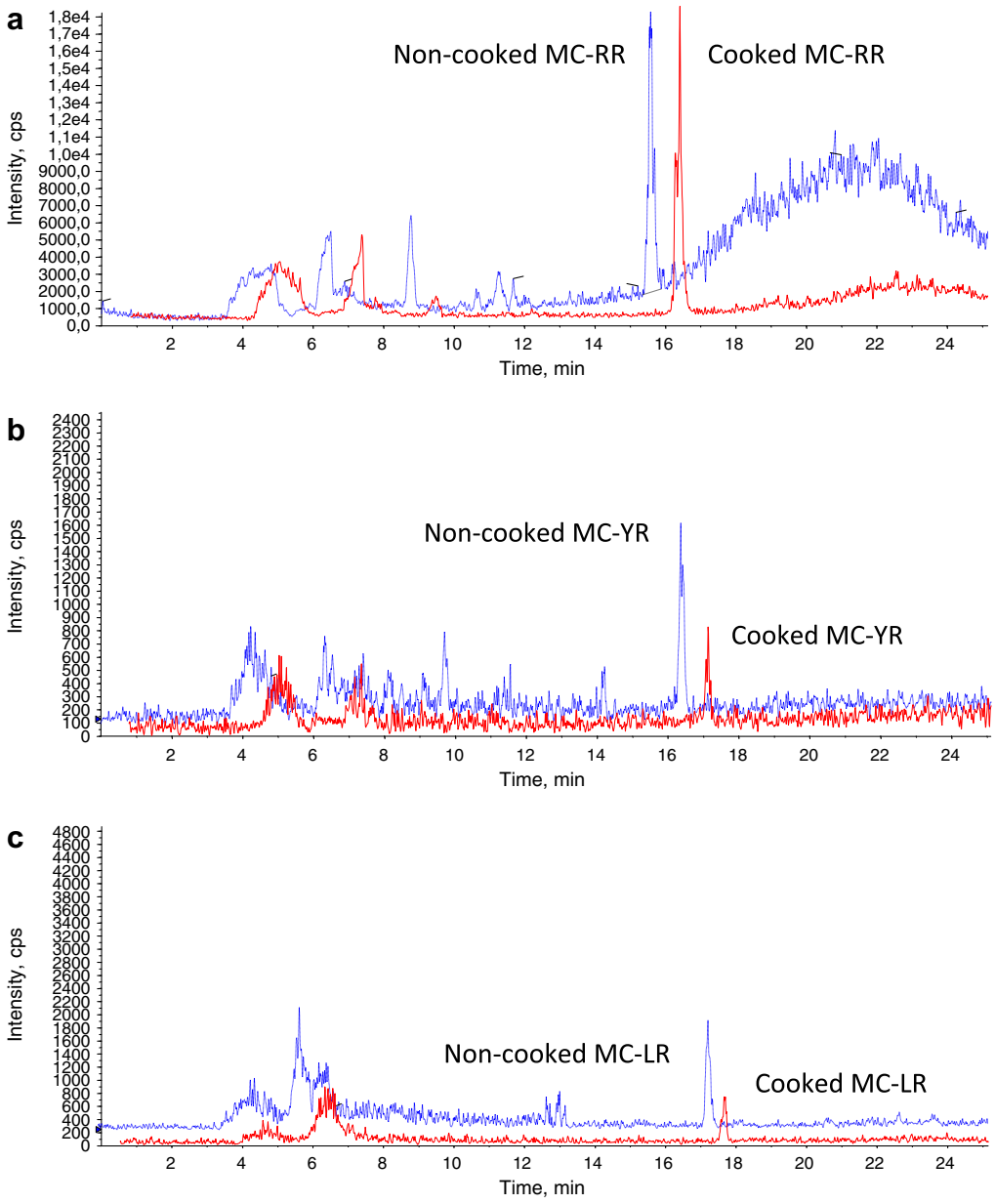

Fig. 2. HPLC–MS chromatograms of microwave treatment of fish contaminated with a mixture of (a) MC-RR, (b) MC-YR and (c) MC-LR (1.5 mg/L of each toxin). (- - -) Non-cooked fish muscle; (——) Cooked fish muscle (5 min).

found in waters oscillated between 5.25 ± 0.76 μg MC-RR/L (boiled water) and 3.40 ± 0.47 μg MC-YR/ L water (continuously heated water).

4. Discussion

Bioaccumulation of MCs through the aquatic food chain/web has been demonstrated in natural conditions (Ibelings and Chorus, 2007; Chen et al., 2009b). Currently, the safety assessment on MCs in aquatic products is carried out according to the free MCs concentration in the edible part of them (Zhang et al., 2010; Codd et al., 2005). In general, for a correct risk assessment, qualitative and quantitative knowledge of the influence of food processing (including cooking practices) on the substances is needed to adequately address whether processing results in a significant change of chemical/physicochemical identity and/or chemical and microbiological specification as compared to the non-processed counterpart (Renwich et al., 2003). The effects of cooking practices depend on the type of method applied (microwaving, boiling, oven-roasting, etc.), on the heating time and temperature employed, and also on the physicochemical characteristics of the nutrient/contaminant, because they influence their stability. It seems that the influence of cooking on the levels of toxic metals and various organic environmental pollutants depends not only on the particular cooking process, but even more on the specific food item (Domingo, 2011).

In relation to the stability of MCs, they are chemically stable compounds, resistant to heat and chemical hydrolysis at near neutral pH (Harada et al., 1996), and changes from pH 1 to pH 12 had little impact on MC-LR (Yu et al., 2009). At high temperatures (40°) and at elevated or low pH, slow hydrolysis has been observed, with the times to achieve greater than 90% breakdown being approximately 10 weeks at pH 1 and greater than 12 weeks at pH 9

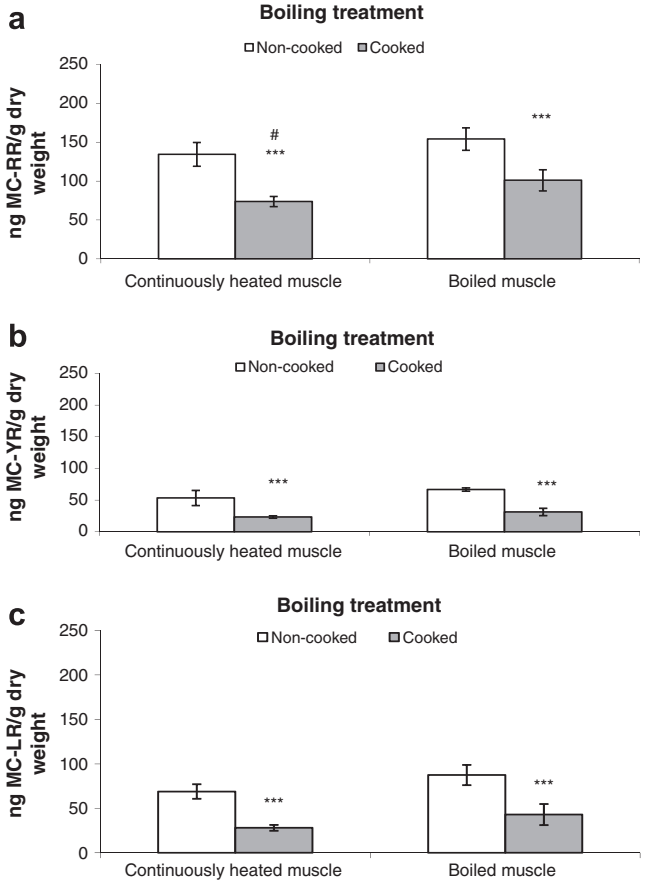

Fig. 3. MC concentrations (ng MC/g dry weight) in cooked and non-cooked fish muscle (*Oreochromis* sp.) spiked with a mixture of pure standard solution of (a) MC-RR, (b) MC-YR and (c) MC-LR (1.5 mg/L of each toxin) and submitted to boiling treatment for 2 min. Values are expresses as means ± standard deviations for 5 replicates. The significance levels observed are $^{***}p < 0.001$ in comparison to their respective control group values, and $^{\#}p < 0.05$ continuously heated muscle vs. boiled muscle.

(Harada et al., 1996). Rapid chemical hydrolysis occurs only under conditions that are unlikely to be attained outside the laboratory, e.g. 6 M HCl at high temperature (Sivonen and Jones, 1999). Previous studies in which MCs were submitted to a dissolution test that used gastric and intestinal fluid according to U.S. Pharmacopeia conditions demonstrated that MC-RR, MC-YR and MC-LR suffered hydrolysis under gastric and acidic conditions (pepsin and pH 1.2), being MC-RR the toxin most affected; however, none was degraded by intestinal digestion (pancreatin and pH 7.5) (Moreno et al., 2004). These results suggest that the application of different domestic cooking techniques cannot remove or produce alterations of MCs, as it had been indicated by some authors (Ibelings and Chorus, 2007).

In this work, we have investigated possible changes in the concentration of three MCs, MC-RR, MC-YR and MC-LR in fish muscle, submitted to two common practices of domestic cooking, namely microwaving and boiling. In the first case, microwave heating of food could result in losses of nutrients, such as water soluble proteins from codfish samples (Yowell and Flurkey, 1986), although it has been shown that the nutritive value of proteins in foods treated by conventional and microwave heating are comparable (Petrucelli and Fisher, 1994); it could also affect the proximate composition and fatty acid composition of fish (Türkkan et al., 2008) and may even result in the production of new derived toxicants (Deshpande, 2002).

As far as we know, only Morais et al. (2008) reported that microwave treatment significantly reduced the global free MCs content in mussels of the species *M. galloprovinciallis* contaminated with MCs from a strain of *M. aeruginosa*-MG, for the two periods used (1 and 5 min), but effects on individual MC-congener have not been previously reported. In our study, whereas MC-YR and MC-LR have been affected after 5 min of treatment, MC-RR was resistant for both periods of time. Earlier studies about microwave hydrolysis of MCs for 10 min at 160 °C and 650 W resulted in complete cleavage of peptide bonds and high recoveries of amino acids, similar to results obtained by application of conventional hydrolysis for 24 h at 110 °C, being MC-RR more stable against the radiation (Reichelt et al., 1999). Furthermore, microwave oven and boiling waterbath extraction methods have been also applied to extract MCs from laboratory strains of *Microcystis* and environmental samples of cyanobacteria (Metcalf and Codd, 2000). These authors demonstrated that exposure of purified MC-LR solutions to microwaves for up to 9 min, or to boiling waterbath incubation for up to 1 min, revealed no differences in the structure and toxicity when compared with controls. These results contrast with the less recovery of MC-LR found in our experiment, and it could be explained by differences due to complexity of the matrices (fish instead of water or cyanobacterial cells), and laboratory conditions. The mechanism of action is not known, but may be similar to proteins, which would be denatured with the modification in molecular structure upon heating; some MCs could be affected. Some investigations should be addressed in this direction.

Moreover, Wannemacher (1989) reported that MC-LR is stable even at temperatures up to 300° in laboratory conditions. In addition, Microcystins are reported to withstand several hours of boiling (van Apeldoorn et al., 2007). In a recent study, Yu et al. (2009) studied the effect of various temperatures (10, 20, 30, 40, 60, 80, 100, 150 and 200) on the stability of MC-LR. After incubation of 6 h, MC-LR was stable when the temperature was less than 100 °C; from 10° to 150° MC-LR was not degraded, whereas the half-life at 200 °C was less than half an hour (8%).Metcalf and Codd (2000) demonstrated that the application of boiling for 1 min followed by microwave oven resulted in the extraction of four MCs (MC-LR, MC-LY, MC-LW, and MC-LF) from cultures of cyanobacteria. In contrast, Bogialli et al. (2005), extracted MCs from fish muscle tissue with hot water acidified to pH 2 and heated at a maximum of 80 °C, because at 100 °C low extraction yields were obtained, presumably due to decomposition of cyanotoxins by pH 2 acidified water.

In aquatic food, two previous studies showed that temperature might have an impact on MCs concentration. Morais et al. (2008) did not reported changes in the bioavailability of the toxins in mussels contaminated with MCs and cooked for 5 and 30 min. More recently, Zhang et al. (2010) studied the stability of MCs (MC-RR and MC-LR) after boiling for 5 min, in fish muscle of bighead carp treated intraperitoneally with 400 and 580 μg MC-LR eq bw, and sacrificed at 3 and 6 h post-injection. They reported that free MCs concentration in the boiled muscle was significantly higher than in the control fish, and concluded that the potential threat of aquatic animals contaminated with microcystins to human health was underestimated. These authors thought that the increase in MCs occurs by the release of phosphatase-bound microcystins by boiling. The possible reason is that the interaction of MCs with protein phosphatases was still in the second step during the study, and boiling may accelerate the MCs release from phosphatases. By contrast, in our study, a significant decrease (around 40–50%) on assayed MCs concentration in fish muscle has been observed in both boiling procedures. We spiked fish muscle with pure standards of three MCs previously sacrificed, similar to a natural contamination of fish, for example by rinsing fish with MC-contaminated water. The interaction of MCs with protein phosphatases in dead fish is different; it could be possible that the interaction corresponds to the first step (Craig et al., 1996), in

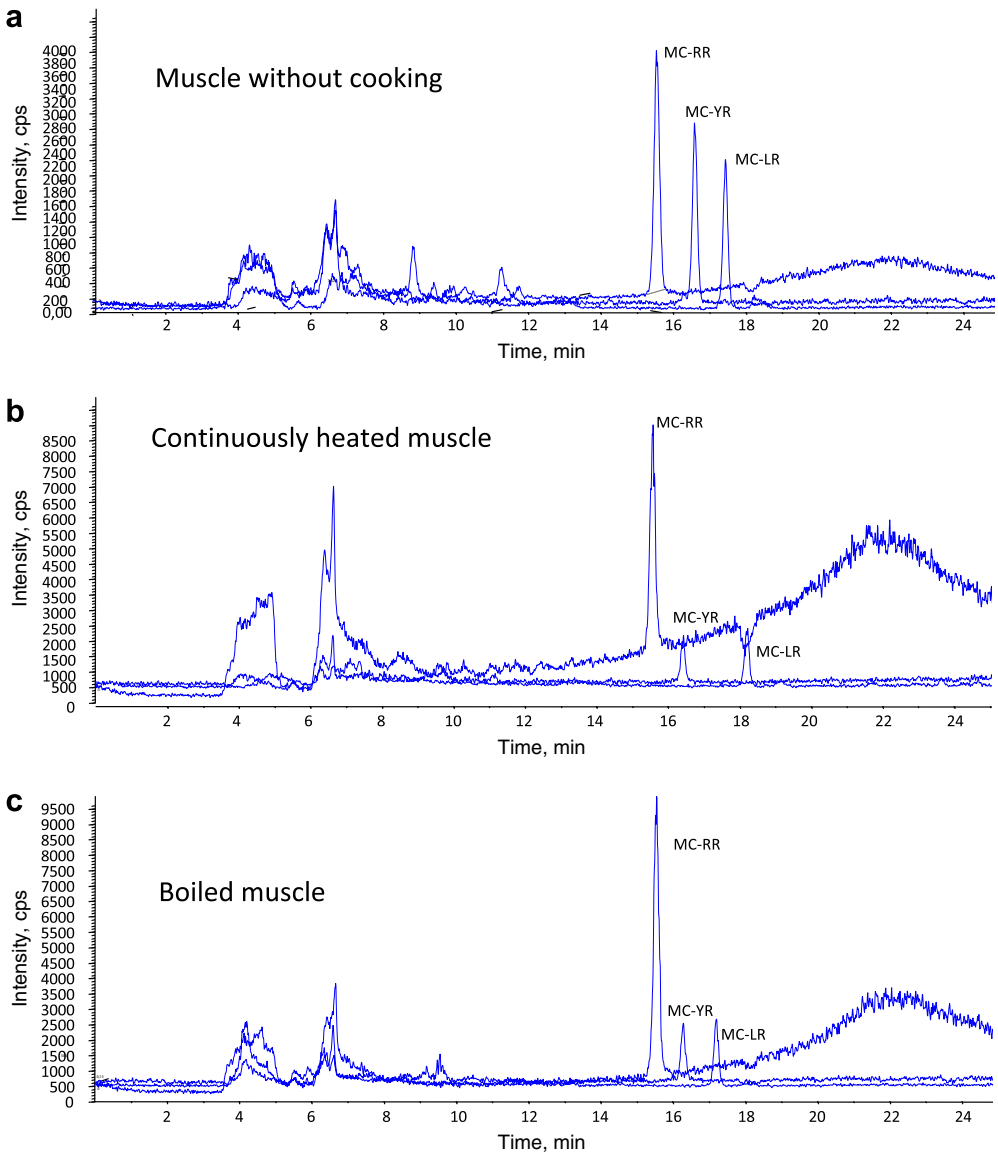

Fig. 4. HPLC–MS chromatograms of boiling treatments (2 min) of fish contaminated with a mixture of MC-RR, MC-YR and MC-LR (1.5 mg/L of each toxin).

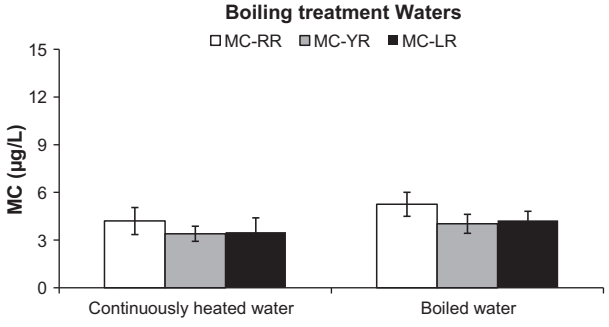

Fig. 5. Unconjugated MC concentrations found in water samples collected (µg MC/L) from boiling treatments assayed.

which a rapid but reversible binding took place. Consequently, the decreased concentrations of MCs in muscle suggests a transference of MCs in hot water, similar to an extraction process, as some authors had previously assayed (Metcalf and Codd, 2000; Bogialli et al., 2005). In the case of MC-RR, there were differences in the transfer rate in both boiling treatments, being higher in the case of "continuously heated muscle"; this result could be explained by a longer contact time muscle-water for this congener, which is more hydrophilic.

In our case, small but significant quantities of free MCs were also found in waters from boiling experiments; consequently, we agree with the suggestion made by Zhang et al. (2010), in the sense that attention should be paid to the combined risks from both MC-contaminated fish and soup to human health.

Globally, when the fish contaminated with a pure solution of three MCs (MC-RR, MC-YR and MC-RR) were submitted to usual cooking practices, such as microwave oven, and boiling, a decrease in MCs concentration in fish muscle was found in both processes, reaching a higher transference of toxins in the last cooking method tested. Microwave oven only produced a significant degradation of unconjugated MC-LR and MC-YR (25–36%) after 5 min of practice. The two boiling treatments examined, continuously heated muscle and boiled muscle for 2 min, seemed more effective in order to extract MCs, with a decrease in concentrations of around 50% for MC-LR and MC-YR; in the case of MC-RR more toxin was extracted (45%) in the continuously boiling process in comparison to the direct boiled muscle fish (35%). In the case of the boiling practice,

the phenomenon could be explained as a better extraction efficiency of MCs in aquatic animals by hot water, with the corresponding risks to human health in cases of consumption of soups and boiled fish sauces.

In conclusion, the innovative results obtained in the present work demonstrate that microwave oven and boiling treatments are able to reduce unconjugated MCs levels in cooked fish in a range between 25% and 50%, so they could be considered as potentially effective control measures for a better dietary estimation of these toxins after human consumption of cooked food. Nevertheless, MCs transference to water was observed in the case of boiled fish. More studies are necessary in order to elucidate the possible mechanisms involved in these variations of unconjugated MCs concentrations in aquatic food when it is submitted to usual cooking practices.

Conflict of Interest

The authors declare that there are no conflicts of interest

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References

Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M., 2008. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to Microcystis under laboratory conditions. Toxicon 52, 1–12.

Bogialli, S., Bruno, M., Curini, R., Di Corcia, A., Laganá, A., Mari, B., 2005. Simple assay for analyzing five microcystins and nodularin in fish muscle tissue, hot water extraction followed by liquid chromatography–tandem mass spectrometry. J. Agric. Food Chem. 53, 6586–6592.

Burger, J., Gaines, K.F., Boring, C.S., Snodgrass, J., Stephens, W.L.J., Gochfeld, M., 2004. Effects of cooking on Radiocesium in fish from the Savannah river, exposure differences for the public. Arch. Environ. Contam. Toxicol. 46, 231–235.

Cameán, A., Moreno, I.M., Ruiz, M.J., Picó, Y., 2004. Determination of microcystins in natural blooms and cyanobacterial strain cultures by matrix solid-phase dispersion and liquid chromatography–mass spectrometry. Anal. Bioanal. Chem. 380, 537–544.

Chen, J., Xie, P., Zhang, D., Lei, H., 2007. In situ studies on the distribution patterns and dynamics of microcystins in a biomanipulation fish bighead carp (*Aristichthys nobilis*). Environ. Pollut. 147, 150–157.

Chen, J., Xie, P., Li, L., Xu, J., 2009a. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. Toxicol. Sci. 108, 81–89.

Chen, J., Zhang, D., Xie, P., Wang, Q., Ma, Z., 2009b. Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic Microcystis blooms. Sci. Total Environ. 407, 3317–3322.

Codd, G.A., Morrison, L.F., Metcalf, J.S., 2005. Cyanobacterial toxins, risk management for health protection. Toxicol. Appl. Pharmacol. 203, 264–272.

Craig, M., Luu, H.A., McCready, T., Williamd, D.E., Andersen, R.J., Holmes, C.F.B., 1996. Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and 2A protein phosphatases. Biochem. Cell. Biol. 74, 569–578.

Dai, M., Xie, P., Liang, G., Chen, J., Lei, H., 2008. Simultaneous determination of microcystin-LR and its gluthatione conjugate in fish tissue by liquid chromatography-tandem mass spectrometry. J. Chromat. B Anal. Technol. Biomed. Life Sci. 862, 43–50.

De Figueiredo, D., Azeiteiro, U.M., Esteves, S.M., Goncalves, F.J.M., Pereira, M.J., 2004. Microcystin-producing blooms – a serious global public health issue. Ecotoxicol. Environ. Saf. 59, 151–163.

Deshpande, S.S., 2002. Handbook of Food Toxicology. Marcel Dekker, New York.

Devesa, V., Vélez, D., Montoro, R., 2008. Effect of thermal treatments on arsenic species contents in food. Food Chem. Toxicol. 46, 1–8.

Dietrich, D., Hoeger, S., 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue green algal supplements), a reasonable or misguided approach? Toxicol. Appl. Pharmacol. 203, 273–289.

Domingo, J.L., 2011. Influence of cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food: a review of the published literature. Crit. Rev. Food Sci. Nutr. 51, 29–37.

Harada, K., Tsuji, K., Watanabe, M.F., Kondo, F., 1996. Stability of microcystins from cyanobacteria – III. Effect of pH and temperature. Phycologia 35, 83–88.

Hori, T., Nakagawa, R., Tobiishi, K., Jida, T., Tsutsum, T., Sasaki, K., Toyoda, M., 2005. Effects of cooking on concentrations of Polychlorinated Dibenzo-p-dioxins and related compounds in fish and meat. J. Agric. Food Chem. 53, 8820–8828.

IARC, 2010. Monographs Database on Carcinogenic Risks to Human. Last updated, October 22, 2010. http://www.iarc.fr/ (last accessed, December 15, 2010).

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health, a review. Environ. Pollut. 150, 177–192.

Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquat. Toxicol. 72, 261–271.

Li, H., Xie, P., Li, G., Hao, L., Xiong, Q., 2009. In vivo study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (c-fos, c-jun and c-myc) in liver, kidney and testis of male Wistar rats injected i.v. with toxins. Toxicon 53, 169–175.

Magalhães, V.F., Soares, R., Azevedo, S., 2001. Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil), ecological implication and human health risk. Toxicon 39, 1077–1085.

Magalhães, V.F., Marinho, M.M., Domingos, P., Oliveira, A.C., Costa, S.M., Azevedo, L.O., Azevedo, S.M.F.O., 2003. Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). Toxicon 42, 289–295.

Martins, J.C., Vasconcelos, V.M., 2009. Microcystinn dynamics in aquatic organisms. J. Toxicol. Environ. Health B 12, 65–82.

McElhiney, J., Lawton, L.A., 2005. Detection of the cyanobacterial hepatotoxins microcystins. Toxicol. Appl. Pharmacol. 203, 219–230.

Metcalf, F.J.S., Codd, G.A., 2000. Microwave oven and boiling waterbath extraction of hepatotoxins from cyanobacterial cells. FEMS Microbiol. Lett. 184, 241–246.

Mohamed, Z.A., Carmichael, W.W., Hussein, A.A., 2003. Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a Microcystis bloom. Environ. Toxicol. 18, 137–141.

Morais, J., Augusto, M., Carvalho, A.P., Vale, M., Vasconcelos, V.M., 2008. Cyanobacteria hepatotoxins, microcystins, bioavailability in contaminated mussels exposed to different environmental conditions. Eur. Food Res. Technol. 227, 949–952.

Moreno, I., Herrador, A., Atencio, L., Puerto, M., González, A.G., Cameán, A.M., 2011. Differentiation between microcystin contaminated and uncontaminated fish by determination of unconjugated MCs using an ELISA Anti-Adda test based on Receiver-Operating Characteristic Curves threshold values, application to *Tinca tinca* from natural ponds. Environ. Toxicol. 26, 45–56.

Moreno, I., Repetto, G., Carballal, E., Gago, A., Cameán, A.M., 2005. Cyanobacteria and microcystins occurrence in the Guadiana river (SW Spain). Int. J. Environ. Anal. Chem. 85, 461–474.

Moreno, I.M., Pereira, P., Franca, S., Cameán, A.M., 2004. Toxic cyanobacteria strains isolated from blooms in the Guadiana river (Southwest of Spain). Biol. Res. 37, 405–417.

Moreno, I.M., Pichardo, S., Moyano, R., González, A.G., Cameán, A.M., 2010. Determination of microcystins in biological samples from freshwater fish. Int. J. Environ. Anal. Chem. 90, 1000–1013.

Peng, L., Liu, Y., Chen, W., Liu, L., Kent, M., Song, L., 2010. Health risks associated with consumption of microcystin-contaminated fish and sellfish in three Chinese lakes: Significance for freshwater aquacultures. Ecotoxicol. Environ. Saf. 73, 1804–1811.

Perelló, G., Martí-Cid, R., Llobet, J.M., Domingo, J.L., 2008. Effects of various cooking processes on the concentrations of arsenic, cadmium, mercury and lead in foods. J. Agric. Food Chem. 56, 11262–11269.

Perelló, G., Martí-Cid, R., Castell, V., Llobet, J.M., Domingo, J.L., 2009. Concentrations of polybrominated diphenyl ethers, hexachlorobenzene and polycyclic hydrocarbons in various foodstuffs before and after cooking. Food Chem. Toxicol. 47, 709–715.

Petrucelli, L., Fisher, G.H., 1994. D-Aspartate and D-glutamate in microwaved versus conventional heated milk. J. Am. Coll. Nutr. 13 (2), 209–210.

Reichelt, M., Hummert, C., Luckas, B., 1999. Hydrolysis of Micocystins and Nodularin by microwave radiation. Chromatografia 49, 671–677.

Renwich, A.G., Barlow, S.M., Hertz-Picciotto, I., Boobis, A.R., Dybing, E., Edler, L., Eisenbrand, G., Greig, J.B., Kleiner, J., Lambe, J., MÜller, D.J.G., Smith, M.R., Trischer, A., Tuijtelaars, S., Van den Brandt, P.A., Walker, R., Kroes, R., 2003. Risk characterisation of chemicals in food and diet. Food Chem. Toxicol. 41, 1211–1271.

Sivonen, K., Jones, G., 1999. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring and Management. WHO, E and FN Spon, New York, pp. 41–111.

Türkkan, A.U., Cakli, S., Kilinc, B., 2008. Effects of cooking methods on the proximate composition, fatty acids of seabass (*Dicentrarchus labrax*, Linnaeus, 1758). Food Bioprod. Process. 86, 163–166.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G., Yu, S.Z., 1996. Detection of microcystin, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17, 1317–1321.

Van Apeldoorn, M.E., Van Egmond, H.P., Speijers, G.J., Bakker, G.J., 2007. Toxins of cyanobacteria. Mol. Nutr. Food Res. 51, 7–60.
Vasconcelos, V.M., Sivonen, K., Evans, W.R., Carmichael, W.W., Namikoshi, M., 1996. Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. Water Res. 30, 2377–2384.
Wannemacher, R.W., 1989. Chemical stability laboratory safety of naturally occurring toxins. US Army Medical Research, Institute of infection diseases. Frot Detrick, pp. 9–11.
WHO, 1998. Guidelines for Drinking Water Quality. Health Criteria and Other Supporting Information. World Health Organization, Geneva.
Xie, L.Q., Xie, P., Guo, L.G., Li, L., Miyabara, Y., 2005. Organ distribution and bioaccumulation of microcystins in freshwater fish at different trophic levels from the eutrophic Lake Chaohu. China. Environ. Toxicol. 20, 293–300.
Yowell, K., Flurkey, W.H., 1986. Effect of freezing and microwave heating on proteins from codfish fillets, analysis by SDS polyacrylamide gel electrophoresis. J. Food Sci. 51, 508–509.
Yu, T., Xie, P., Dai, M., Liang, G., 2009. Determinations of MC-LR and [Dha^7] MC-LR concentrations and physicochemical properties by liquid chromatography–tandem mass spectrometry. Bull. Environ. Contam. Toxicol. 83, 757–760.
Zhang, D., Xie, P., Chen, J., 2010. Effects of temperature on the stability of microcystins in muscle of fish and its consequences for food safety. Bull. Environ. Contam. Toxicol. 84, 202–207.
Zhou, L., You, H., Chen, K., 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed. Environ. Sci. 15, 166–171.

CAPÍTULO 2 / CHAPTER 2

Remedios Guzmán-Guillén, Ana I. Prieto, A. Gustavo González, M. Eugenia Soria-Díaz, Ana M. Cameán

CYLINDROSPERMOPSIN DETERMINATION IN WATER BY LC-MS/MS: OPTIMIZATION AND VALIDATION OF THE METHOD AND APPLICATION TO REAL SAMPLES

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CYLINDROSPERMOPSIN DETERMINATION IN WATER BY LC-MS/MS: OPTIMIZATION AND VALIDATION OF THE METHOD AND APPLICATION TO REAL SAMPLES

REMEDIOS GUZMÁN-GUILLÉN,† ANA I. PRIETO,† A. GUSTAVO GONZÁLEZ,‡
M. EUGENIA SORIA-DÍAZ,§ and ANA M. CAMEÁN*†
†Area of Toxicology, Faculty of Pharmacy, University of Seville, Seville, Spain
‡Department of Analytical Chemistry, University of Seville, Seville, Spain
§Mass Spectrometry Facility, Centro de Investigación Tecnológica e Investigación, University of Seville, Seville, Spain

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Abstract—A new method for determining dissolved cylindrospermopsin (CYN) in waters using solid-phase extraction (SPE) with graphitized carbon cartridges and quantification by liquid chromatography coupled with tandem mass spectrometry is described and discussed. The method has been suitably validated: the linear range covered is from 0.900 to 125 μg CYN/L. Limits of detection and quantification were 0.5 and 0.9 μg CYN/L, respectively, and allow CYN determination at concentrations below the guideline proposed of 1 μg CYN/L in natural waters. The method exhibits mean recoveries from 83 to 95%, and intermediate precision (relative standard deviation (%)) values from 5 to 12%, ensuring adequacy against the Association of Official Analytical Chemists guidelines. The method is robust against the following three influential factors considered in the cleanup stage: the batch of the graphitized carbon cartridges, the flow rate of the water sample through the cartridge, and the final redissolved water volume after SPE treatment. The method has been successfully applied to detection and quantification of CYN in water samples from aquaria of a toxicological in vivo laboratory experiment. Environ. Toxicol. Chem. 2012;31:2233–2238. © 2012 SETAC

Keywords—Cylindrospermopsin Waters Method validation Liquid chromatography coupled with tandem mass spectrometry

INTRODUCTION

Cylindrospermopsin (CYN) is a cyanobacterial toxin of particular scientific interest over the last decade because of its spread from tropical to temperate environments, in apparent correlation with the occurrence of global warming phenomena [1]. Structurally, CYN is a toxic alkaloid-like compound produced by some strains of cyanobacteria, such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum,* and *Anabaena bergii* [2]. The presence of CYN in drinking water has caused human poisonings in Australia and Brazil [3]. Cylindrospermopsin is receiving increased attention by toxicologists and health authorities, because the geographic expansion of the main producer, *C. raciborskii*, occurs at a considerable pace [4], and the toxin has been found on nearly every continent [5].

Cylindrospermopsin is a potent inhibitor of eukaryotic protein synthesis [6,7]; therefore, it causes inhibition of glutathione synthesis [8]. In vivo, CYN causes severe toxicity in the liver, with variable lesions in a range of other organs, including the kidney, intestine, lungs, and thymus, according to several experimental models [6,9–11]. This toxin has been shown to induce cytotoxicity and genotocity both in vivo and in vitro [12,13]. A no-observed-adverse-effect level value of 30 μg/kg body weight per day was reported for CYN [14]; accordingly, the authors proposed a guideline safety value of 1 μg/L in drinking water. Cylindrospermopsin may accumulate through the food chain in animals and plants by direct contact and irrigation water, which is an additional risk to public health. A tolerable daily intake of 0.02 μg/kg body weight/d for human exposure was calculated based on acute toxicity studies in mice [15]. Moreover, CYN is a highly water-soluble molecule [16] and, unlike many other cyanotoxins, it is often present in the extracellular (noncell-bound) form, reaching up to 90% of total CYN [16–19]. Because of its chemical stability and slow degradation [20], CYN shows a high level of persistence in many water bodies [21]. Few studies have investigated the degradation of CYN in water bodies, and some authors have reported slow CYN decomposition in waters at temperatures ranging from 4 to 50°C at pH 7, and variation in pH did not affect its degradation [20]. These findings may have important implications for water authorities if concentrations in surface waters are in a health-relevant range.

Field studies based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have found maximum concentrations of CYN in waters of different countries, reaching values of 1.1 [22] and 800 μg/L in Australia [16], 18.4 μg/L in Italy [4], 97.1 μg/L in Florida, USA [23], and 12.1 μg/L in Germany [19]. For Spain, Quesada et al. [24] found amounts of 9.4 μg CYN/L by using liquid chromatography–diode array detection (LC-DAD). All these concentrations exceed the proposed guideline value for CYN in drinking water.

Different methods for CYN analysis have been reported in the literature, including mouse bioassay and enzyme-linked immunosorbent assay (ELISA) for fast screening [25,26], liquid chromatography [27], and capillary electrophoresis [28]. Moreover, methods for the sensitive and rapid analysis of water samples are currently being investigated using polymerase chain reaction and characterization of DNA and RNA unique to CYN-producing cyanobacteria [25]. Methods based on reversed-phase high-performance LC-DAD have been proposed for analyzing CYN in real water samples and lyophilized cyanobacterial cells [17,29,30]. Wormer et al. [30] have

* To whom correspondence may be addressed (camean@us.es).
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developed a reliable and simple solid-phase extraction (SPE) method that uses graphitized carbon cartridges, paying special attention to both the selection of the most suitable solvent and the need of sample preparation prior to SPE. Although excellent utility of this method in the analysis of CYN was also confirmed in environmental samples, the quantification of CYN was performed by LC-DAD. However, these methods do not offer unequivocal and definitive analyte identification, which is a necessary feature in confirmatory assays. Liquid chromatography–mass spectrometry protocols, including triple quadrupole LC-MS/MS, have been established as a standard method for the identification and quantification of CYN in freshwaters and fish muscle [2,4,31]. Although quantification methods are well developed [31], concentration and extraction of dissolved CYN from natural samples has not been widely developed, and SPE methods selecting graphitized carbon cartridges seem to be effective [30]. Nevertheless, more studies are necessary to elucidate optimal solvent conditions, including robustness assays, which permit their validation, as have been carried out in the present work.

Because of the wide distribution and toxicity of CYN and its common presence in the dissolved fraction of waters (as a result of its hydrophilic nature, the dissolved fraction can contribute more than 99% of total CYN [19]), it is necessary to develop validated confirmation methods for dissolved CYN determination in real water samples. The analysis method must provide a limit of quantification (LOQ) similar to the concentrations found in natural waters in different countries and must be robust so that it can be used as a reference. Classical approaches to analytical method validation rarely consider the stage corresponding to the robustness study, which is primary in the sense of "method transfer," according to harmonization purposes [32].

In the present study, we present the optimization and validation of a method for dissolved CYN detection and quantification by LC-MS/MS in water samples used for drinking water or recreational purposes. The analysis by MS/MS has been optimized according to the guidelines established by the European Commission [33]. The procedure has been validated by using the holistic approach, according to González and Herrador [32].

MATERIALS AND METHODS

The cyanotoxin CYN standard (purity >95%) was supplied by the Alexis Corporation. Standard stock solutions of CYN (100 μg/ml) in Milli-Q water were used for preparing instrument calibration (working) standards and to suitably spike natural waters for obtaining validation standard samples. All chemicals and reagents used in the present study were analytical-grade materials. High-performance liquid chromatography (HPLC)-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid were purchased from Merck. Deionized water (>18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore).

Solid-phase extraction procedure

Natural water samples (200 ml, acidified with formic acid 1% v/v) were spiked with a working standard solution of CYN (5–500 μg/L) and subjected to freeze–thaw cycles twice as a cell disruption method, in combination with ultrasonication (15 min) and stirring (15 min). Afterward, the samples were centrifuged (4500 rpm, 15 min) and filtered through a glass fiber filter (45 mm). Cylindrospermopsin was determined using a modified version of the methods of Metcalf et al. [17] and Wormer et al. [30], with some modifications in relation to the proportion of solvents employed, dichloromethane:methanol [DCM:MeOH; 10:90, v/v]) and its detection and quantification (LC-MS/MS instead of LC-DAD). Briefly, graphitized carbon cartridges were packed with Bond Elut (500 mg; Varian) and were activated with 10 ml of a solvent mixture of DCM:MeOH (10:90, v/v) acidified with 5% formic acid (v/v) and then rinsed with 10 ml Milli-Q water. Subsequently, the sample was passed through the cartridges, washed with 10 ml Milli-Q water, and eluted with 10 ml DCM:MeOH (10:90 v/v). To concentrate the sample, it was evaporated in a rotary evaporator and resuspended in 250 μl Milli-Q water. Thus, a concentration factor of 800 was achieved. Acidification of the water sample was carried out following Wormer et al.'s method [30] to obtain a robust recovery of CYN in environmental samples.

Analysis

Chromatographic separation was performed using a Perkin-Elmer Series 200 HPLC system coupled to an Applied Biosystems QTRAP LC-MS/MS system consisting of a hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. The analytical column was a Zorbax Sb-Aq column (150 × 2.1 mm) with a particle size of 3.5 μm (Agilent Technologies). The flow rate was 0.2 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of water and methanol. Both components contained 0.05% trifluoroacetic acid (v/v). The injection volume was 20 μl, and the elution profile was 0% methanol (1 min), linear gradient to 90% methanol (10 min), 90% methanol (5 min), and finally 0% methanol (5 min).

A multiple reaction monitoring experiment was applied in which the parent ions and fragment ions were monitored at Q1 and Q3, respectively. The transitions for the identification of CYN were 416.2/194.0, 416.2/274.0, and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For high-pressure liquid chromatography–electrospray tandem mass spectrometry analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, source gas 60 psi, and ion spray voltage 5,500 V, and the Turboprobe temperature was maintained at 350°C.

Preparation of CYN in waters from aquaria under laboratory conditions

Five aquaria (96 L fresh water) were used to perform the present study. Fish were obtained from a fish hatchery (Aquaculture Valencia) and maintained at the University of Córdoba to reach an average weight of 50 ± 8 g. Afterward, fish were transferred to the laboratory, where they were held in aquaria (eight individuals/aquarium). Exposure to chlorine was minimized by filling the tanks at least 3 d before the fish were introduced. Aquaria were also set up with a continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges), and the temperature was kept constant (22 ± 3°C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg/L. Mean values for additional parameters of water quality were pH 7.6 ± 0.3, conductivity 287 μs/cm, Ca^{2+} 0.60 mM/L, and Mg^{2+} 0.3 mM/L. Lyophilized *A. ovalisporum* (LEGE X-001; CYN+ producer) isolated strain culture was kindly supplied by Dr. Vitor Vasconcelos (Marine Research Center, Porto, Portugal). Cylindrospermopsin extraction from the lyophilized culture of *A. ovalisporum* (CYN+) was performed based on the work of Guzmán-Guillén et al. [34]. Cylindrospermopsin was detected (retention time of 7.55 min)

and quantified by LC-MS/MS, and the concentration obtained was 8.7 μg CYN/mg. After the acclimation period, an aquarium was used as the control group (without CYN). To simulate natural conditions, fish were introduced into four aquaria, and an adequate quantity of cyanobacterial cells (CYN+) was added in the beginning of the experiment to reach either 10 or 100 μg CYN/L. Fish were maintained in tanks for 7 or 14 d and were fed with commercial fish food (0.3 g/d; Dibaq) for each exposure time. Water samples were collected from the aquaria at three time points (beginning, middle, and end of the exposure period) and were analyzed by the proposed method.

Statistical criteria for method validation

The study of intermediate precision and trueness was performed by applying a one-factor analysis of variance (ANOVA) between days. Three validation standards covering the optimal working range were used (5, 50, and 125 μg CYN/L). Each validation standard was measured in triplicate for three different days. From the ANOVA results, as explained in the *Results and Discussion* section, both the intermediate precision and the recovery are obtained. The values are compared with tabulated reference values.

The robustness study was carried out by using an intermediate validation standard (100 μg CYN/L) according to the Youden procedure. The influential factors (batch of the graphitized carbon cartridges, flow rate of the water sample through the cartridge, and final redissolved water volume) were tested with Student's *t* test as indicated below.

RESULTS AND DISCUSSION

General aspects

To develop the LC-MS/MS method for detection of dissolved CYN in water, commercially available standard solutions of CYN were assayed to acquire mass spectra and adjust mobile-phase strength. Figure 1 shows the MS/MS product ion spectrum and fragmentation scheme of CYN. The spectrum was obtained on collision of *m/z* 416, corresponding to the pseudomolecular ion $[M + H]^+$. The signals at *m/z* 336 and 318 were assigned to the loss of SO_3 and H_2O from the pseudomolecular ion, respectively. Another fragment ion at *m/z* 274 corresponds to the loss of the [6–(2–hydroxy–4–oxo–3–hydropyrimidyl)] hydroxymethinyl moiety of the molecule. Finally, the ions at *m/z* 194 and 176 correspond to the loss of SO_3 and H_2O from the fragment ion at *m/z* 274 [35]. Quantitation of CYN was achieved using the 416/194 transition, with other transitions monitored as confirmation ions.

Figure 2 shows the LC-MS/MS chromatograms for a standard of the toxin CYN (500 μg/L) compared with an aquarium-water sample, with a retention time of 8.90 min.

Method validation

Linear range. Response linearity was established according to Huber [36] by plotting the called response factors (signal response/analyte concentration) against their concentrations. The studied concentration interval was from 1.25 to 125 μg/L CYN. Responses were obtained from natural waters (Montijo water reservoir, Badajoz, Spain) suitably spiked with working standards of CYN, by applying in triplicate the SPE and LC-MS/MS procedure proposed. The Huber plot is shown in Figure 3. The target line has zero slope, and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. No intersections with the lines were found, so the linear range of the method applies to the full range studied. However, from a qualimetric point of view, the linear range cannot start from concentrations less than the LOQ [32]. Accordingly, in our case (see the LOQ under the section *Detection limit and quantitation limit*) the linear range is 0.900 to 125 μg/L.

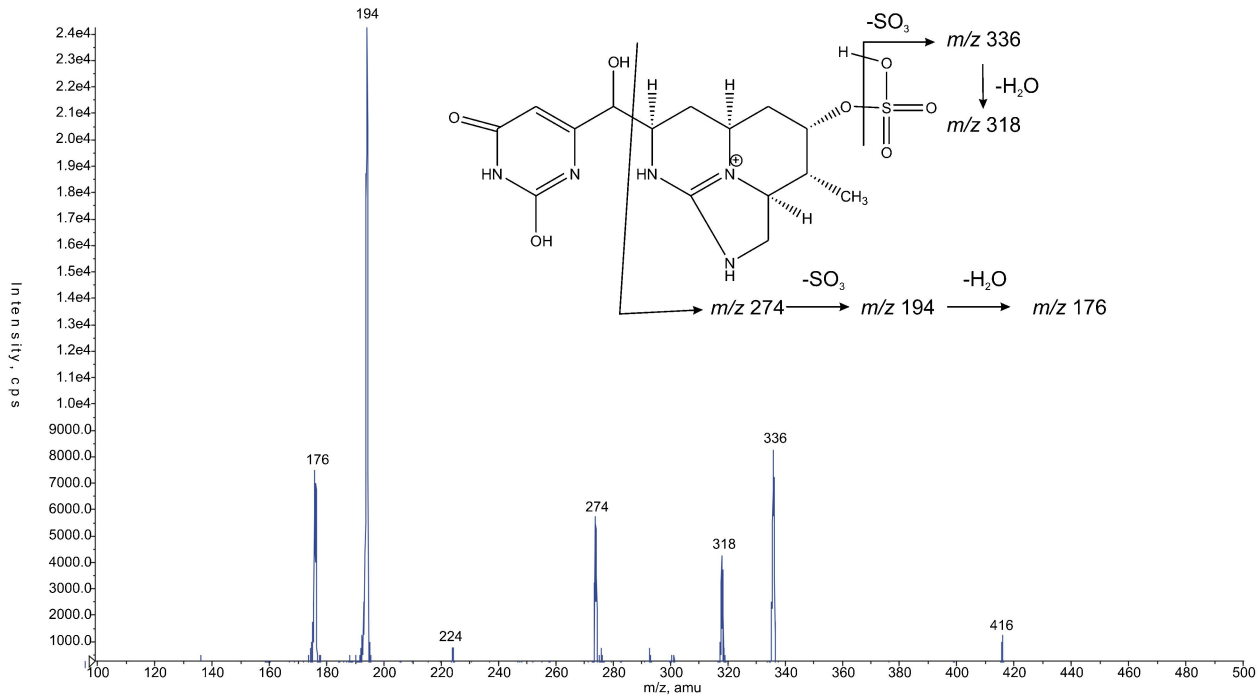

Fig. 1. Tandem mass spectrometry (MS/MS) spectrum of the product ions of cylindrospermopsin $[M + H]^+$ ion at *m/z* 416. The ion at *m/z* 194 was monitored for quantitation using the multiple-reaction monitoring mode. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

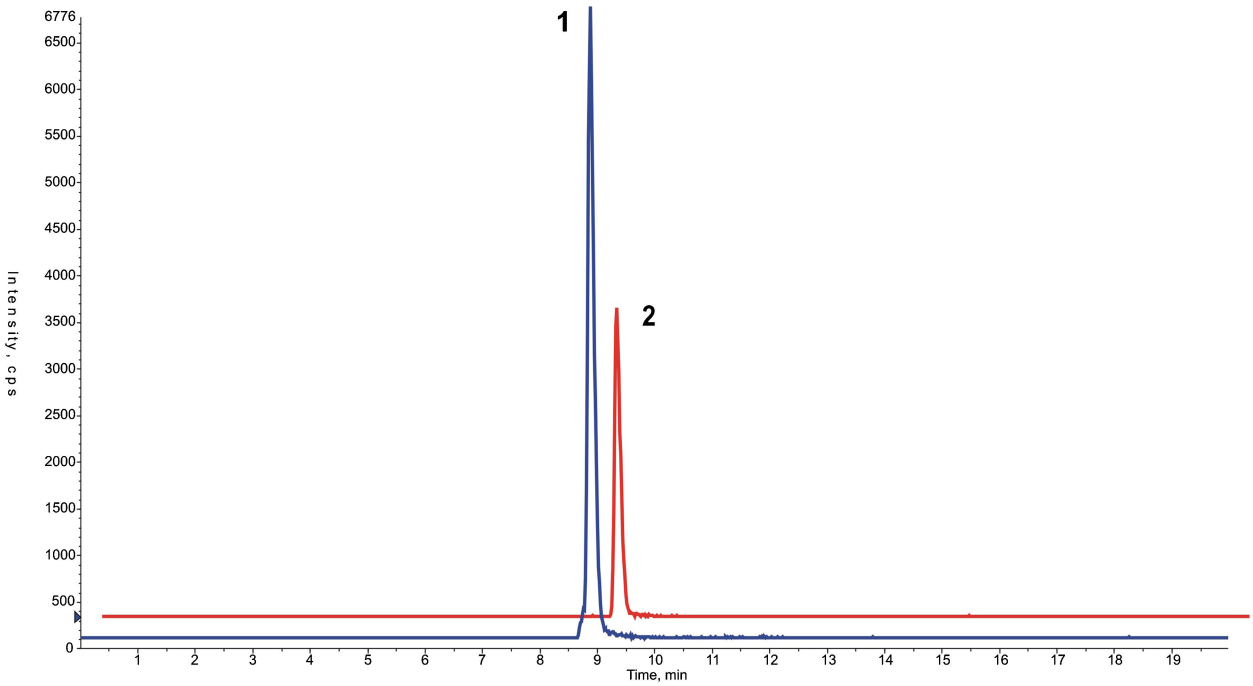

Fig. 2. High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) chromatograms of cylindrospermopsin (CYN) from extract from an aquarium water sample (1) and standard sample of CYN (500 μg/L; 2). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com]

Goodness of fit. The linear calibration function was obtained by preparing six calibration standards in natural waters from Montijo reservoir, in triplicate, from 1.25 to 125 μg/L and recording the signal response according to the proposed SPE and LC-MS/MS procedure. Here, drinking or recreational processed waters are taken as a blank sample, and the analyte is spiked in the natural environment for future samples. Therefore, these calibration standards can also be considered as validation standards. The calibration line has a correlation coefficient of 0.9999, and the corresponding ANOVA of the regression line indicates a lack-of-fit F ratio of 0.48 against a critical F value of 19.0. Consequently, there is no lack of fit, and the calibration function can be considered as linear.

Detection limit and quantitation limit. The limit of detection (LOD) and the LOQ were determined by measuring 10 independent sample blanks. The LOD was estimated using the expression $Y_{LOD} = Y_{blank} + 3S_{blank,}$ where Y_{blank} and S_{blank} are the average value of the blank signal and its corresponding standard deviation. Limit of detection values are then converted into concentration by using the calibration function. The procedure for evaluating LOQ was equivalent to that for LOD, but used the factor 10 instead of 3 for calculations. The LOD and LOQ obtained were 0.5 and 0.9 μg/L, respectively. Both parameters were lower than the proposed guideline safety value of 1 μg/L [14], so the method enables us to quantify trace levels of the toxin in natural waters.

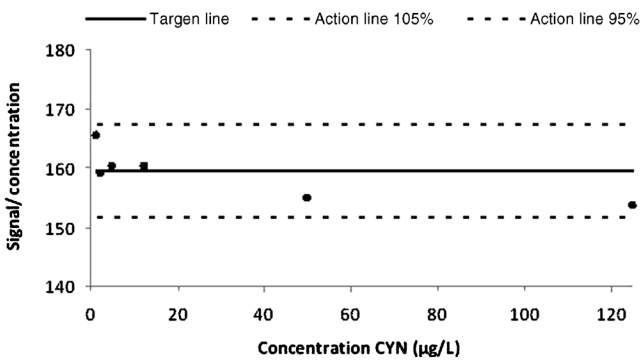

Fig. 3. Huber plot for assessing linear range.CYN = cylindrospermopsin.

Intermediate precision and trueness studies. According to the International Conference on Harmonisation (ICH) guidelines (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf), precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as "intraassay" or "within run." Intermediate precision applies to within-laboratory variations (different days, different analysts, or different equipment) and is sometimes called "between run" or "interassay precision" [37]. The third level, reproducibility, expresses the between-laboratories precision such as in collaborative studies, and it will not be considered in the present study.

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and a conventional value or an accepted reference value such as validation standards [37].

To perform the intermediate precision study, we have considered samples of natural waters spiked at three validation standards of CYN (low, medium, and high) covering the dynamic working range 0.9–125 μg/L, with three replicates at each concentration, as recommended by the ICH guidelines. Calculations of intermediate precision must be carried out on results instead of responses. With three different days considered as the main source of variation, an ANOVA was performed for each concentration. From the ANOVA results, we can obtain estimations of within-condition variance (S_w^2), also known as

Table 1. Estimations of within-condition repeatability (S_w), between-condition repeatability (S_B), intermediate precision (intralaboratory reproducibility; S_{IP}), and recoveries of CYN assayed in waters at three validation standards on three different days

	CYN concentration level (validation standards)		
	5 μg/L	50 μg/L	125 μg/L
Mean CYN concentration estimated (μg/L)	4.75	41.50	116.30
S_w	0.19	4.01	7.66
S_B	0.31	6.64	13.15
S_{IP}	0.24	5.05	9.84
RSD_{IP} (%)	5.00	12.00	8.40
Recovery (%)	95 ± 3	83 ± 8	93 ± 6
1/2 RSD_{AOAC} (%)	11–15	8–11	6–8

CYN = cylindrospermopsin; RSD = relative standard deviation; AOAC = Association of Official Analytical Chemists.

repeatability (S_r^2), and between-condition variance (S_B^2). In addition, the intralaboratory reproducibility or intermediate precision is obtained as $S_{IP}^2 = S_r^2 + S_B^2$ according to González and Herrador [32] and González et al. [37]. All these parameters are shown in Table 1.

From these data, the corresponding relative standard deviations (RSD), RSD_R, are calculated (Table 1). These values were compared with the acceptable RSD percentages obtained from the AOAC Peer-Verified Methods program [36,37]. As a quick rule [37], the RSD_{IP} results should be compared with one-half the corresponding RSD_{AOAC} values tabulated. Our results, at the three concentration levels considered, were lower than or of the same order as the one-half $\%RSD_{AOAC}$ tabulated.

The assessment of trueness can be performed according the same ANOVA results. Trueness can be expressed as the bias or recovery obtained for each validation standard assayed [38]. The recovery term has a more intuitive meaning, and it has been tested in this work. The total recovery for any validation standards is defined as the ratio between the observed estimation of the validation standards concentration, and the true value, T, expressed as a percentage or as fraction. The recoveries (%) computed for the three validation standards considered are shown in Table 1. We checked them for suitability by comparison with the published acceptable recovery ranges as a function of the analyte concentration [36,37]. In our method, with the CYN concentration of the three validation standards ranging between 5 and 125 μg/L, the recovery range (%) could oscillate between 60 and 115% for 5 μg/L and between 80 and 110%, for 50 and 125 μg/L. The recoveries obtained were 95 ± 3%, 83 ± 8%, and 93 ± 6% for 5, 50, and 125 μg/L, respectively. All the recovery data fulfill the rule previously mentioned, and the method can be considered bias free. In summary, this procedure has been successfully assessed for trueness and intermediate precision.

Robustness study. Robustness, considered in the sense of internal validation, deals with the effect of experimental variables, called "factors," inherent in the analytical procedure (e.g., temperature, mobile-phase composition, pH) on the analytical result. A robustness study examines the alteration of these factors, as expected in a transfer between laboratories, so it is of the utmost importance in the uncertainty budget. The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [39], according to the practical guide of González and Herrador [32]. Three influential factors in the SPE cleanup procedure were identified: (X_1), the batch of the graphitized carbon cartridges employed; (X_2), the flow rate of the water sample through the cartridge; and (X_3), the final redissolved water volume after SPE treatment. The levels are coded according to the rule: high value = +1 ($X_1 = 1$ (batch 1), $X_2 = 1$ min, $X_3 = 250$ μl) and low level = −1 ($X_1 = 2$ (batch 2), $X_2 = 2$ min, $X_3 = 260$ μl). The effect of every factor is estimated as the difference of the mean result obtained at the level +1 from that obtained at the level −1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance t test is used [32], and the t values (X_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming from the precision study for each concentration.

In the present study, the experiments were carried out using water samples spiked with 100 μg/L, and each factor was analyzed in triplicate on three different days. Thus, for 8 *df*, the t values obtained were 0.540, 0. 564, and 0.629, for X_1, X_2, and X_3 factors, respectively. In all cases, $t(X_k) < t_{tab}$ (2.306), and then the procedure can be considered as robust against the three factors considered (at the levels fixed in the study).

Table 2. Cylindrospermopsin (CYN) concentrations found in waters from aquaria (96 L) spiked with lyophilized cells from an *Aphanizomenon ovalisporum* culture

	10 μg CYN/L	100 μg CYN/L
Aquaria maintained for 7 d		
Day 1	4.7 ± 0.2	69.0 ± 5.5
Day 3	5.5 ± 0.1	68.2 ± 4.0
Day 7	5.4 ± 0.1	71.5 ± 4.6
Aquaria maintained for 14 d		
Day 1	5.3 ± 0.2	73.0 ± 3.2
Day 7	6.6 ± 0.2	72.5 ± 3.0
Day 14	6.0 ± 0.2	65.0 ± 5.2

Evaluation of CYN in waters from aquaria under laboratory conditions

No CYN was detected in the control aquarium. Waters from the aquaria spiked with the lyophilized *A. ovalisporum* cells presented lower CYN values (Table 2) in comparison with the initial concentrations (10 and 100 μg/L), and they were kept nearly constant over 7 and 14 d.

CONCLUSIONS

We have developed and validated a method for determining dissolved CYN in waters, using SPE with graphitized carbon cartridges and quantification by LC-MS/MS. Limits of detection and quantification were 0.5 and 0.9 μg/L, respectively, and allow CYN determination at concentrations below the proposed guideline of 1 μg CYN/L in natural waters. The method was successfully applied to detection and quantification of CYN in water samples from aquaria under laboratory conditions. The proposed method is suitable for monitoring this toxin in surface waters and also for routine human health assessment in relation to the provisional guideline and tolerable daily intake.

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REFERENCES

1. Messineo V, Melchiore S, Di Corcia A, Gallo P, Bruno M. 2010. Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizo-*

menon ovalisporum blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. *Environ Toxicol* 25:18–27.
2. Gallo P, Fabbrocino S, Cerulo MG, Ferranti P, Bruno M, Serpe L. 2009. Determination of cylindrospermopsin in freshwaters and fish tissue by liquid chromatography coupled to electrospray ion trap mass spectrometry. *Rapid Commun Mass Spectrom* 23:3279–3284.
3. Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR. 2005. Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress. *J Toxicol Environ Health Part A* 68:739–753.
4. Bogialli S, Bruno M, Curini R, Corcia AD, Fanali C, Laganà A. 2006. Monitoring algal toxins in lake water by liquid chromatography tandem mass spectrometry. *Environ Sci Technol* 40:2917–2923.
5. Briand JF, Leboulanger C, Humbert JF, Bernard C, Dufour P. 2001. *Cylindrospermopsis raciborskii* (cyanobacteria) invasion at mid-latitudes: Selection, wide physiological tolerance, or global warming? *J Phycol* 40:231–238.
6. Terao K, Ohmori S, Igarashi K, Ohtani I, Watanabe MF, Harada KI. 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue–green alga *Umezakia natans*. *Toxicon* 32:833–843.
7. Froscio S, Humpage AR, Wickramasinghe W, Shaw G, Falconer IR. 2008. Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. *Toxicon* 51:191–198.
8. Runnegar M, Kong SM, Zhong YZ, Ge JL, Lu SC. 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem Biophys Res Commun* 201:235–241.
9. Hawkins PR, Chandrasena NR, Jones GJ, Humpage AR, Falconer IR. 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* 35:341–346.
10. Falconer I, Hardy SJ, Humpage A, Froscio S, Tozer GJ, Hawkins PR. 1999. Hepatic and renal toxicity of the blue–green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. *Environ Toxicol* 14:143–150.
11. Gutierrez-Praena D, Pichardo S, Jos A, Camean AM. 2011. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. *Ecotoxicol Environ Saf* 74:1567–1572.
12. Humpage AR, Fenech M, Thomas P, Falconer IR. 2000. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. *Mutat Res* 472:155–161.
13. Bazin E, Mourot A, Humpage AR, Fessard V. 2010. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. *Environ Mol Mutagen* 51:251–259.
14. Humpage AR, Falconer IR. 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male swiss albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline value. *Environ Toxicol* 18:94–103.
15. Duy TN, Lam PKS, Shaw GR, Connell DW. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue–green algal) toxins in water. *Rev Environ Contam Toxicol* 163:113–186.
16. Shaw GR, Seawright AA, Moore MA, Lam PKS. 2000. Cylindrospermopsin, a cyanobacterial alkaloid: Evaluation of its toxicological activity. *Ther Drug Monit* 22:89–92.
17. Metcalf JS, Beattie KA, Saker ML, Codd GA. 2002. Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. *FEMS Microbiol Lett* 216:159–164.
18. Saker ML, Griffith DJ. 2000. Effects of temperature on growth and cylindrospermopsin content of seven isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from water bodies in northen Australia. *Phycologia* 39:349–354.
19. Rucker J, Stuken A, Nixdorf B, Fastner J, Chorus I, Wiedner C. 2007. Concentration of particulate and dissolved cylindrospermopsin in 21 *Aphanizomenun*-dominated temperate lakes. *Toxicon* 50:800–809.
20. Chiswell R, Shaw R, Eaglesham G, Smith MJ, Norris RL, Seawright AA, Moore MR. 1999. Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, temperature, and sunlight on decomposition. *Environ Toxicol* 14:155–161.
21. Wormer L, Cires S, Carrasco D, Quesada A. 2007. Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study. *Harmful Algae* 7:206–213.
22. Hoeger SJ, Schmid D, Bloom JF, Dietrich DR. 2004. Analytical and functional characterizacion of microcystin [Asp^3] MC-RR and [Asp^3, Dhb^7] MC-RR: Consequences for risk assessment? *Environ Sci Technol* 41:2609–2616.
23. Burns J, Williams C, Chapman A. 2002. Cyanobacteria and their toxins in Florida surface waters. In *Proceedings of Health Effects of Expòsure to Cyanobacterial Toxins: State of the Science*. Florida Department Of Health, Gainesville, FL, USA, pp 16–21.
24. Quesada A, Moreno E, Carrasco D, Paniagua T, Wormer L, De Hoyos C, Sukenik A. 2006. Toxicity of *Aphanizomenon ovalisporum* (cyanobacteria) in a Spanish water reservoir. *Eur J Phycol* 41:39–45.
25. Masten S, Carson B. 2000. *Toxicological Summary for Cylindropermopsin, Final Report. Rev Toxicol Lit T*. Integrated Laboratory System, Research Triangle Park, NC, USA, pp i–viii.
26. Prieto AI, Campos A, Caméan AM, Vasconcelos VM. 2011. Effects on growth and oxidative stress status of rice plants (*Oriza sativa*) exposed to two extracts of toxin-producing cyanobacteria *(Aphanizomenun ovalisporum*) and *Microcystis aeruginosa*). *Ecotoxicol Environ Saf* 74:1973–1980.
27. Welker M, Bickel H, Fastner J. 2002. HPLC-PDA detection of cylindrospermopsin–Opportunities and limits. *Water Res* 36:4659–4663.
28. Vasas G, Gáspár A, Surányi G, Batta G, Gyémant G, Hamvas M, Máthé C, Molnár E, Borbély G. 2002. Capillary electrophorectic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenun ovalisporum*, by plant test (blue–green Sinapsis test). *Anal Biochem* 302:95–103.
29. Törökné A, Aszatols M, Bánkinné M, Bickel H, Borbély G, Carmeli S, Codd GA, Fastner J, Huang Q. 2004. Interlaboratory comparison trial on cylindrospermopsim measurement. *Anal Biochem* 332:280–284.
30. Wormer L, Carrasco D, Cirés S, Quesada A. 2009. Advances in solid phase extraction of the cyanobacterial toxin cylindrospermopsin. *Limnol Oceanogr Methods* 7:568–575.
31. Eaglesham G, Norris KR, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA, Moore BS. 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue–green algal toxin, for public health purposes. *Environ Toxicol* 14:151–155.
32. González AG, Herrador MA. 2007. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends Anal Chem* 26:227–238.
33. The Commission of the European Communities. 2002. Commission Decision (EC) No 2002/657 of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Comm*, 221:8–36.
34. Guzman-Guillen R, Rios V, Prieto AI, Moreno I, Camean AM. 2010. Optimisation of the extraction, purification and quantification of cylindrospermopsin form different matrices. *Toxicol Lett* 196S:S346–S346.
35. Dell'Aversano C, Eaglesham GK, Quilliam MA. 2004. Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography–mass spectrometry. *J Chromatogr A* 1028:155–164.
36. Huber L. 1998. *Validation and Qualification in Analytical Laboratories*. Interpharm, East Englewood, CO, USA.
37. González AG, Herrador MA, Asuero AG. 2010. Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta* 82:1995–1998.
38. Association of Official Analytical Chemists. 1998. *Peer Verified Methods Program. Manual on Policies and Procedures*. AOAC, Arlington, VA, USA.
39. Youden WY. 1967. *Statistical Techniques for Collaborative Tests*. Association of Official Analytical Chemists, Washington DC, USA.

CAPÍTULO 3 / CHAPTER 3

Remedios Guzmán-Guillén, Ana I. Prieto Ortega, I. Moreno, Gustavo González, M. Eugenia Soria-Díaz, Vitor Vasconcelos, Ana M. Cameán

DEVELOPMENT AND OPTIMIZATION OF A METHOD FOR THE DETERMINATION OF CYLINDROSPERMOPSIN FROM STRAINS OF APHANIZOMENON CULTURES: INTRA-LABORATORY ASSESSMENT OF ITS ACCURACY BY USING VALIDATION STANDARDS

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Development and optimization of a method for the determination of Cylindrospermopsin from strains of *Aphanizomenon* cultures: Intra-laboratory assessment of its accuracy by using validation standards

Remedios Guzmán-Guillén [a], Ana I. Prieto Ortega [a], I. Moreno [a], Gustavo González [b], M. Eugenia Soria-Díaz [c], Vitor Vasconcelos [c,d,e], Ana M. Cameán [a,*]

[a] *Area of Toxicology, Faculty of Pharmacy, University of Seville, Spain*
[b] *Department of Analytical Chemistry, University of Seville, Spain*
[c] *Mass spectrometry Facility, Centro de Investigación Tecnológica e Investigación (CITIUS), University of Seville, Spain*
[d] *Marine and Environmental Research Center (CIIMAR/CIMAR), University of Porto, Rua dos Bragas, m289, 4050-123 Porto, Portugal*
[e] *Biology Department, Faculty of Sciences, University of Porto. Rua do Campo Alegre, Porto, 4169-007, Portugal*

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ABSTRACT

The occurrence of cyanobacterial blooms in aquatic environments is increasing in many regions of the world due to progressive eutrophication of water bodies. Because of the production of toxins such as Cylindrospermopsin (CYN), contamination of water with cyanobacteria is a serious health problem around the world. Therefore it is necessary to develop and validate analytical methods that allow us to quantify CYN in real samples in order to alert the public of this toxin. In this work, an analytical method has been developed an optimized for the determination of CYN from *Aphanizomenon ovalisporum* cultures. The analytical procedure is based on solvent extraction followed by a purification step with graphitized cartridges and CYN quantification by LC–MS/MS. The extraction and purification steps were optimized using a two-level full factorial design with replications. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards. The method has been suitably validated: the regression equation was calculated from standards prepared in extracts from lyophilized *M. aeruginosa* PCC7820 ($r^2 \geq 0.9999$) and the linear range covered is from 5 to 500 μg CYN/L, equivalent to 0.18–18.00 μg CYN/g dry weight lyophilized cells. Limits of detection and quantification were 0.04 and 0.15 μg CYN/g, respectively, the recovery range (%) oscillated between 83 and 94% and intermediate precision (RSD %) values from 5.6 to 11.0%. Moreover, the present method showed to be robust for the three factors considered: the batch of the graphitized carbon cartridges, the flow rate of the sample through the cartridge, and the final re-dissolved water volume after SPE treatment, which permits its validation. The validated method has been applied to different lyophilized cultures of *A. ovalisporum* (LEGE X-001) to evaluate CYN content. This procedure can be used for determining CYN in lyophilized natural blooms samples in environmental studies.

1. Introduction

Harmful cyanobacterial blooms are occurred in eutrophicated freshwater lakes and reservoirs throughout the world and can present a public safety hazard through contamination of drinking water supplies [1–2]. This hazard results from the production of harmful secondary metabolites, otherwise known as cyanotoxins. There are over 40 species representing 20 genera from three cyanobacterial orders known to produce cyanotoxins which include both cyclic peptides and alkaloids [3]. Cylindrospermopsin (CYN) is a hepatotoxic alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethiluracil which as originally described as being produced by *Cylindrospermopsis raciborskii*. At present, CYN is known to also be produced by *Umezakia natans*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata*, *Lyngbia wollei*, *Anabaena bergii*, *Aphanizomenos flos-aquae* [4] and more recently by *Oscillatoria* sp. and *Raphidiopsis mediterranea* [3,5]. Two naturally occurring analogs of CYN, 7-epicylindrospermopsin (7-Epi-CYN) and deoxy-cylindrospermopsin (7-deoxy-CYN) have been also identified [6]. 7-Deoxy-CYN was found in *C. raciborskii* as minor metabolite and in *Raphidiopsis curvata* and *Lyngbia wollei* as the major metabolite.

* Corresponding author. Área de Toxicología, Facultad de Farmacia, Universidad de Sevilla, C/Profesor García González, 2, 41012, Sevilla, España.
Tel.: +34 954 556762; fax: +34 954 233765.
E-mail address: camean@us.es (A.M. Cameán).

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7-Epi-CYN has so far been detected only in *A. ovalisporum* as minor metabolite. These three closely related molecules were shown previously to be toxic using in vivo assays, and they were shown to inhibit protein synthesis with similar potency [5].

In Palm Island outbreak of hepatoenteritis, Australia 1979, in which 148 people were hospitalized with hepatitis, the organisms in the original bloom of the water supply dam were not identified before treatment with copper sulphate. Retrospectively, the alga *C. raciborskii* was subsequently observed as a seasonally dominant species in the domestic water supply reservoir on Palm Island. Its severe hepatotoxic but also wide-ranging effects in mice make it an organism capable of producing the clinical disease seen at Palm Island, and following the suggestions of Hawkins et al. [7] *C. raciborskii* blooms should be considered as one possible cause. CYN is a highly biologically active molecule that interferes with several metabolic pathways. CYN was shown to be a potent inhibitor of protein synthesis in an in vitro rabbit reticulocyte globin synthesis assay [8] CYN also inhibits glutathione synthesis and induce stress responses using both in vivo and in vitro experimental models [9–11]. CYN is a potent hepatotoxin but it can cause also damage to the kidney, lungs, thymus and heart in several experimental models [8,10,12,13]. Genotoxic effects of CYN include DNA adduction and strand breakage in mouse liver [14] and micronuclei formation in a lymphoblastoid cell line [15]. No dose-response or other available information is available regarding the carcinogenicity of pure CYN [16].

The observation that CYN is generated by a range of cyanobacteria species has initiated efforts to define the spatial distribution and sources of CYN [17]. This toxin is widely distributed in tropical and subtropical freshwaters, e.g., Australia [18], and Florida [19], but is also found in temperate regions such as Europe. CYN was detected in Europe for the first time in Germany in 2002 [20], then in Italy [21,22], Spain [23], Finland [24], Poland [25] and Czech Republic [26]. Due to the toxicity of CYN, a derivation of a guideline value for CYN is in progress by the WHO [27], and a guideline safety value (GV) of 1 μg/L in drinking water has been proposed by Humpage and Falconer [28].

The monitoring of drinking water supplies for the presence of this toxin is of critical importance for the assessment of environmental and health risks. Compared to microcystins and saxitoxins, relatively little work has been done on methods for the detection of CYN. Common methods for quantitative determination of this toxin are liquid chromatography coupled with photo diode array detection (LC-PDA) with a C18 reverse phase column, and both, isocratic and gradient mobile phase methods have been developed. Harada et al. [29] developed the first screening method for CYN using reverse phase high performance (LC/PDA), and CYN has an easily identifiable peak and maximum UV absorbance at 262 nm. Welker et al. [30] developed an alternative method to analyze environmental samples for their content of CYN based on LC/DAD; the application of the protocol to natural samples proved to be hampered by the fact that the extraction with pure water, though very efficient for CYN, gave a considerable matrix background and occasionally covered CYN completely in chromatograms. In an interlaboratory comparison trial on CYN measurement in lyophilized cyanobacterial cells that involved six laboratories of five countries, it was shown that the most effective extraction method employed 5% aqueous formic acid, providing efficient extraction and fewer contaminants peaks than the extraction method using water only, when analyzed by LC-PDA employing an isocratic mobile phase of 5% (v/v) methanol plus 0.1% (v/v) TFA [31]. Due to its hydrophilic nature, some authors indicated that CYN cannot be extracted and concentrated from water samples with SPE cartridges such as C18, but SPE with graphitized carbon, has been used successfully [32,33]. Wormer et al. [34] obtained a reliable method by the sole use of graphitized carbon cartridges for the concentration of CYN from culture medium (*A. ovalisporum*) or from diverse environmental samples, using a previous sample preparation, a combination of dichloromethane:-methanol (DCM:MeOH, 1:4) with 5% formic acid as solvent, and quantification of CYN by LC-PDA.

Whilst the use of LC/DAD is less expensive alternative to MS/MS, it not adequately detect trace quantities of CYN [30]. Liquid chromatography–mass spectrometry (LC–MS) protocols, including triple quadrupole LC–MS/MS, have been established as a standard method for the identification and quantification of CYN in freshwaters and fish muscle [35–38]. The combination of hydrophilic interaction liquid chromatography with electrospray mass spectrometry (HILIC–MS) was applied to the analysis of field and cultured samples of *A. circinalis* and *C. raciborskii* [1]. Kubo et al. [2]proposed a fractionation method for CYN analysis from cells of *C. raciborskii* using a double-cartridges column (styrene polymer+anion exhange) with 0.1 M carbonate buffer at pH 10.5 followed by LC-PDA or LC–MS analysis of the extracts. In order to determine CYN at traces levels from cultures of cyanobaterial cells, the development of highly sensitive and quantitative validated method is essential. Classical approaches to analytical method validation consisted of checking the conformity of a performance measure to a reference value, but this does not reflect the end–user of the data or the consumer's needs [39]. Assessment of accuracy of analytical methods is a fundamental stage in method validation, and some procedures of intra-laboratory testing of method accuracy have been discussed by González et al. [40].

The aim of this work was to develop an analytical procedure based on solvent extraction followed by a purification step with graphitized cartridges and LC–MS/MS technique for the CYN determination from lyophilized cultures. The extraction and purification steps were optimized using a two-level full factorial design with replications. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards, according to González et al. [40]. The present procedure has been intended for routine determination of aquatic samples (water, blooms, cultures) in order to detect CYN at trace levels.

2. Materials and methods

2.1. Reagents and materials

Cylindrospermposin (CYN) standards (purity > 95%) were supplied by Alexis Corporation (Lausen, Switzerland). The chemical structures of CYN and 7-deoxi-CYN are shown in Fig. 1. Standard solutions of CYN were prepared in water milli Q (100 μg/mL) and diluted as required for their use as working solutions (5–500 μg/L).

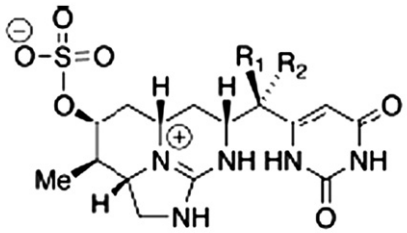
All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (> 18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

2.2. Cyanobacterial cultures samples

2.2.1. Microcystis aeruginosa

PCC7820, a non-CYN-producing strain (CYN-), was obtained from the Pasteur Culture Collection (Paris, France). The culture was maintained in sterilized 250 mL Erlenmeyer flask containing 100 mL of BG11 medium (+1 M $K_2HPO_4 \cdot 3 \cdot H_2O$ + 5 mM $NaNO_3$ + 12 mM $NaHCO_3$) at 30 °C under continuous illumination with an



Compound name	R_1	R_2	Mw
Cylindrospermopsin (7-*R*)	OH	H	415.42
7-Epi-cylindrospermopsin (7-*S*)	H	OH	415.42
7-Deoxy-cylindrospermopsin	H	H	399.42

Fig. 1. Structures of Cylindrospermopsin (CYN) and its analogs ([5]).

intensity of 28 μmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes, and later transferred to bottles containing 20 L of BG11 medium. After the period of 21 days, cultures were harvested and concentrated by centrifugation in continuous (14000 rpm).

2.2.2. Aphanizomenon ovalisporum

(LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was supplied by the Marine Research Center (Porto, Portugal). Being isolated from Lake Kinneret, Israel [41]. Two cultures of this strain were maintained in Z8 medium at 25 °C under continuous illumination with an intensity of 28 μmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. After 33 days, cultures were harvested by decantation with a plankton net (20 μm diameter).

Both concentrated biomass were preserved at −80 °C until lyophilization (Telstar Cryodos, Madrid).

2.3. Solvent extraction and purification procedures

Both the extraction (SPE) and the purification (Clean-up) steps were optimized, according to the methods from Welker et al. [30], intended to modify final volume of milliQ water for extracting CYN, as well as the procedure of Wormer et al. [34], regarding to the proportion of solvents employed and its detection and quantification (LC–MS/MS instead of LC-DAD used in this work). After optimization of several variables through a full factorial design 2^3, the following extraction procedure was adopted: CYN content was extracted from the lyophilized cells of *A. ovalisporum* culture (14 mg) with 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated again for 15 min. The resulting mixture was centrifuged at 4500 rpm. for 10 min, after which the supernatant was collected and 6 μL of 0.1% trifluoroacetic acid (TFA) were added. Then, it was stirred for 1 h and allowed to stand for 3 h. The supernatant was taken for further purification/concentration.

For the clean-up procedure, graphitized carbon cartridges are packed Bond Elut® which were activated with 10 mL of a solvent mixture of DCM/MeOH (10/90) and rinsed with 10 mL of MilliQ water. Subsequently, the sample is passed through the cartridges, washed with 10 mL of MilliQ water and eluted with 10 mL DCM/MeOH (10/90). For concentration of the sample, extract is evaporated in a rotary evaporator and resuspended in 500 μL MilliQ water, prior to its LC–MS/MS analysis.

Extraction efficiencies were performed in triplicate by spiking the matrix, lyophilized cells of *M. aeruginosa* PCC7820 strain (CYN-) with CYN standard solutions at three concentration levels: 20, 200 and 500 μg/L. Besides, a robustness study was carried out by spiking the matrix with a standard solution of 200 μg CYN/L.

2.4. Chromatographic conditions

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 × 2.1 mm Zorbax Sb-Aq column. The flow rate was 0.2 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90%B (5 min) and finally 0% B (5 min).

Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions for the detection of CYN are: 416.2/194.0, 416.2/ 274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For the detection of 7-deoxy-CYN the following transitions are: 400.0/194.0, 400.0/320.0, and 400.0/274.0. For LC-ESI–MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

2.5. Evaluation of CYN in different lyophilized cultures of Aphanizomenon ovalisporum (LEGE X-001)

Two different cultures of *A. ovalisporum* were used for our study. They were cultured under conditions referred to in paragraph 2.2.2. Levels of CYN from those samples of lyophilized *A. ovalisporum* cultures were analyzed according to the proposed and validated method. The total time for carrying out the complete procedure (including the freezing and lyophilization steps) oscillated between 2 and 3 days.

3. Results and discussion

3.1. General aspects

In order to develop the LC–MS/MS for the detection of CYN, commercially available standards solutions of CYN were assayed to acquire mass spectra and adjust mobile phase strength. Fig. 2 shows the MS/MS product ion spectrum and fragmentation scheme of CYN. The spectrum was obtained on collision of m/z 416, corresponding to the pseudomolecular ion $[M+H]^+$. The signals at m/z 336 and 318 were assigned to the loss of SO_3 and H_2O from the pseudomolecular ion, respectively. Another fragment ion, at m/z 274 corresponds to the loss of the [6-(2-hydroxy-4-oxo-3-hydropyrimidyl)]hydroxymethinyl moiety of the molecule. Finally, the ions at m/z 194 and 176 correspond to the loss of SO_3 and H_2O from the fragment ion at m/z 274 [1]. Quantitation of CYN was achieved using the 416/194 transition with other transitions monitored as confirmation ions.

3.2. Results from the optimization of the extraction procedure

The extraction procedure was optimized by a full factorial 2^3 design with replications. The considered factors were: amount of lyophilized cells of *A. ovalisporum* (X_1), volume of TFA (X_2) to precipitate dissolved cell components, and dichloromethane

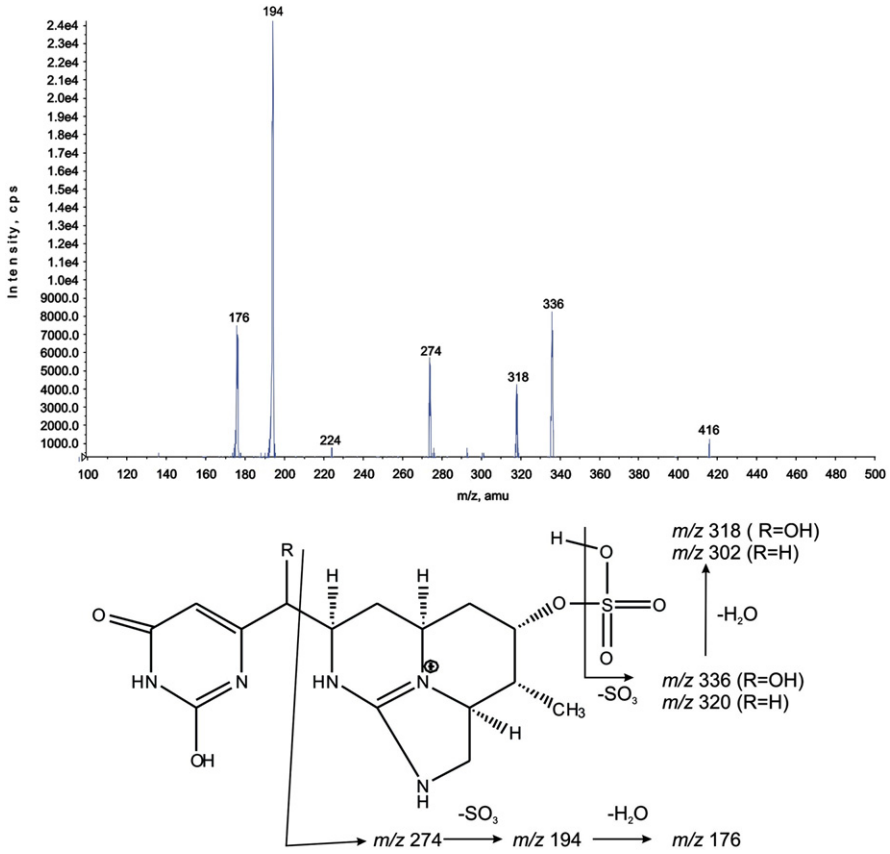


Fig. 2. MS/MS spectrum of the product ions of Cylindrospermopsin $[M+H]^+$ ion at m/z 416. The ion at m/z 194 was monitored for quantitation using the multiple reaction monitoring mode (a). Assignments of labeled fragment ions of CYN and its deoxy-derivative (7-deoxy-CYN) are shown (b).

Table 1
Tested values in the full factorial design for the variables related to the extraction and purification step in CYN determination lyophilized culture.

Variable	Tested value	Coded	Tested value	Coded	Tested value	Coded
Amount of lyophilized (mg) (X_1)	14	−1	28	0	42	+1
TFA volume (mL) (X_2)	2	−1	6	0	10	+1
Solvent proportion (mL) (X_3) (DCM/MeOH)	10/90	−1	20/80	0	30/70	+1

/methanol (DCM/MeOH) proportion used for the clean up procedure (X_3) to elute the toxin from the SPE cartridges. The levels are coded according to the rule: high level = +1, central level = 0 and low level = −1 as it is depicted in Table 1.

The results of the three factors, two-level full factorial design with replications of the extraction procedure of CYN from the lyophilized culture (explained in Section 2.3), are shown in Table 2. The significant factors were: amount of lyophilized material (X_1) and solvent proportion (X_3), as well as the interactions X_{23} and X_{13}, because their corresponding coefficients are significant. According to this, the best results were obtained taking 14 mg of lyophilized cells of *A. ovalisporum*, 6 μL of TFA, and using the proportion 10/90 of the solvent mixture DCM/MeOH. Thus, the experiment should be performed at −1 level for the factors X_1 and X_3, the other variable (X_2) is not affecting the results. This would lead to the best CYN recovery.

3.3. Calibration study

The response as a function of concentration was measured by a 6-point calibration curve with a linear range within 5–500 μg/L, equivalent to 0.18–18.00 μg/g. The regression equation was calculated from standards prepared in extracts from lyophilized *M. aeruginosa* PCC7820 ($r^2 \geq 0.9999$) Fig. 3.

3.3.1. Linear range

Response linearity was established according to Huber, 1998 [44] by plotting the called response factors (signal response/analyte concentration) against their respective concentrations, obtained from six lyophilized *M. aeruginosa* PCC7820 (CYN-) extracts spiked with standards ranging in concentrations from 5 to 500 μg CYN/L (equivalent to 0.18–18.00 μg CYN/g dry weight) and submitted to the proposed method (by triplicate). Fig. 4 shows the Huber plot. The target line has zero slopes and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. As no intersections with the lines were found, the linear range of the method applies to the full range studied.

3.3.2. Goodness of the fit

Linear calibration function was obtained by preparing six calibration standards in extracts of lyophilized *M. aeruginosa*

Table 2
Results from the three factors, two-level full factorial design with replications.

	Estimate	Standard error	*t*-Value df=3	Significance
b0	19.12273	0.309066	61.8726	YES
b1	−4.27250	0.362412	−11.7891	YES
b2	−1.04500	0.362412	−2.8835	NO
b3	−2.02750	0.362412	−5.5945	YES
b12	1.08750	0.362412	3.0007	NO
b13	−1.51000	0.362412	−4.1665	YES
b23	2.84250	0.362412	7.8433	YES
b123	−0.02000	0.362413	−0.0552	NO

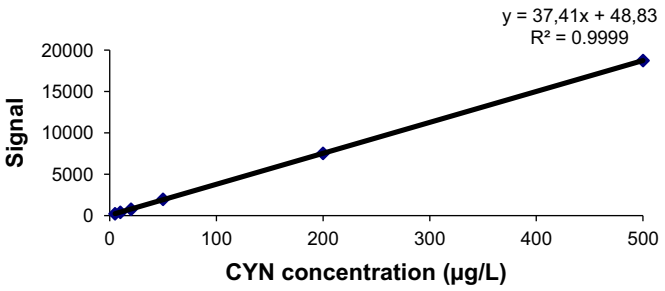


Fig. 3. Linear calibration function for the proposed procedure.

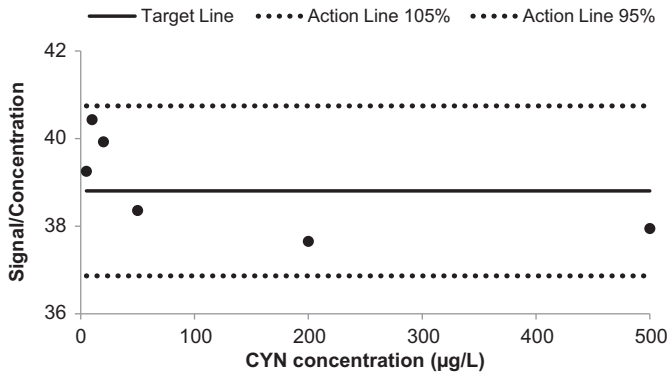


Fig. 4. Huber plot for assessing linear range.

PCC7820 (CYN-) cells (in triplicate) from 5 to 500 µg/L (equivalent to 0.18–18.00 µg CYN/g dry weight), and recording the signal response according to the proposed procedure. Here, drinking or recreational processed waters are taken as "placebo" and the analyte is spiked in the natural environment required for future samples. So, these calibration standards can be also considered as validation standards (VS). The calibration line has a correlation coefficient of 0.9999 and the corresponding ANOVA of the regression line indicates a lack-of-fit F ratio of 0.82 (Fig. 3). Consequently, there is not lack-of fit and the calibration function can be considered as linear.

3.3.3. Detection limit and quantification limit

The limits of detection (LOD) and quantification (LOQ) were calculated from the expression $Y_{LOD/LOQ}=Y_{blank}+nS_{blank}$, where Y_{blank} and S_{blank} are the average value of 10 independent blank samples and its corresponding standard deviation. In these expressions, $n=3$ in the case of LOD and $n=10$ in the case of LOQ. Afterwards, Y_{LOD} and Y_{LOQ} values are converted in concentration units by using the calibration function. The LOD and LOQ obtained were 0.04 µg CYN/g and 0.15 µg/ g, respectively. These values are similar to those found by Fastner et al. [42] (around 0.1 µg/g dry weight) when they analyzed lyophilized crude extracts using the MRM for CYN analysis by LC–MS/MS. Higher values of LOD and LOQ, 16 and 52 µg CYN/g, respectively, were reported by Liu and Scott (2011) [43] in algal food supplements extracts.

3.4. Accuracy study

3.4.1. Intermediate precision and recovery studies

According to ICH guidelines, 2005 [45], precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. Intermediate precision applies to within-laboratory variations: different days, different analysts or equipments, and is sometimes called between-run or inter-assay precision [40]. The third level, reproducibility, expresses the between-laboratories precision like in collaborative studies, and it will not be considered in this work.

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted, either a conventional value or an accepted reference value like validation standard (VS) [40].

Repeatability and intermediate precision were calculated analyzing five replicates of *M. aeruginosa* PCC7820 strain (CYN-) extracts spiked with different concentrations of standard CYN (20, 200 and 500 µg/L) on the same day and in two different days, respectively.

Considering two different days, as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-condition variance (S_w^2), also known as repeatability (S_r^2), and between-condition variance (S_B^2). Also, the intra laboratory reproducibility or intermediate precision, is obtained as $S^2_{IP}=S_r^2+S_B^2$ [39,40]. All these parameters are shown in Table 3.

From these data, the corresponding relative standard deviations, RSD_R are calculated, and were compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [40,44]. As a quick rule [40], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results, at the three concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated (11–15%) (Table 3).

Trueness can be expressed as the bias or recovery obtained for each validation VS assayed [46]. The total recovery for VS is defined as the ratio between the observed estimation of the VS concentration, and the "true" value T, expressed as percentage or as fraction. The recoveries (%) computed for the three VS considered are show in Table 3. We checked them for suitability by comparison with the published acceptable recovery range as a

Table 3
Estimations of within-condition (repeatability), between-condition, intermediate precision (intra laboratory reproducibility) and recoveries of CYN assayed in lyophilized cells of *M. aeruginosa* PCC7820, at three concentrations levels, in two different days.

	CYN concentration level		
	20 µg/L	200 µg/L	500 µg/L
S_w	1.73	16.62	10.16
S_B	2.49	24.98	37.31
S_{IP}	2.01	19.80	23.09
RSD_{IP} (%)	10.70	11.00	5.60
Recovery (%)	94	89	83

function of the analyte concentration [40,44]. In our method, as the CYN concentration of the three VS ranged between 20 and 500 μg/L, the recovery range (%) could oscillate between 80 and 110% for the three of them. The recoveries obtained oscillated between 94% for 20 μg/L and 83% for 500 μg/L. Thus, the method can be considered as acceptable in terms of recoveries.

These recoveries are higher than those obtained by Kubo et al. [2] ($75 \pm 1\%$) in extracts of *C. raciborskii* analyzed by LC/MS. Authors suggested that some compounds in the algal extract could interfere with the isolation of CYN. Recently, Liu and Scott (2011) [43] have determined CYN in algal food supplements extracts by LC–UV and the recoveries ranged between 70 and 90%.

3.4.2. Robustness study

The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [47] and García et al. [48], according to the practical guide of González and Herrador (2007) [39]. These factors, relative to the SPE procedure,

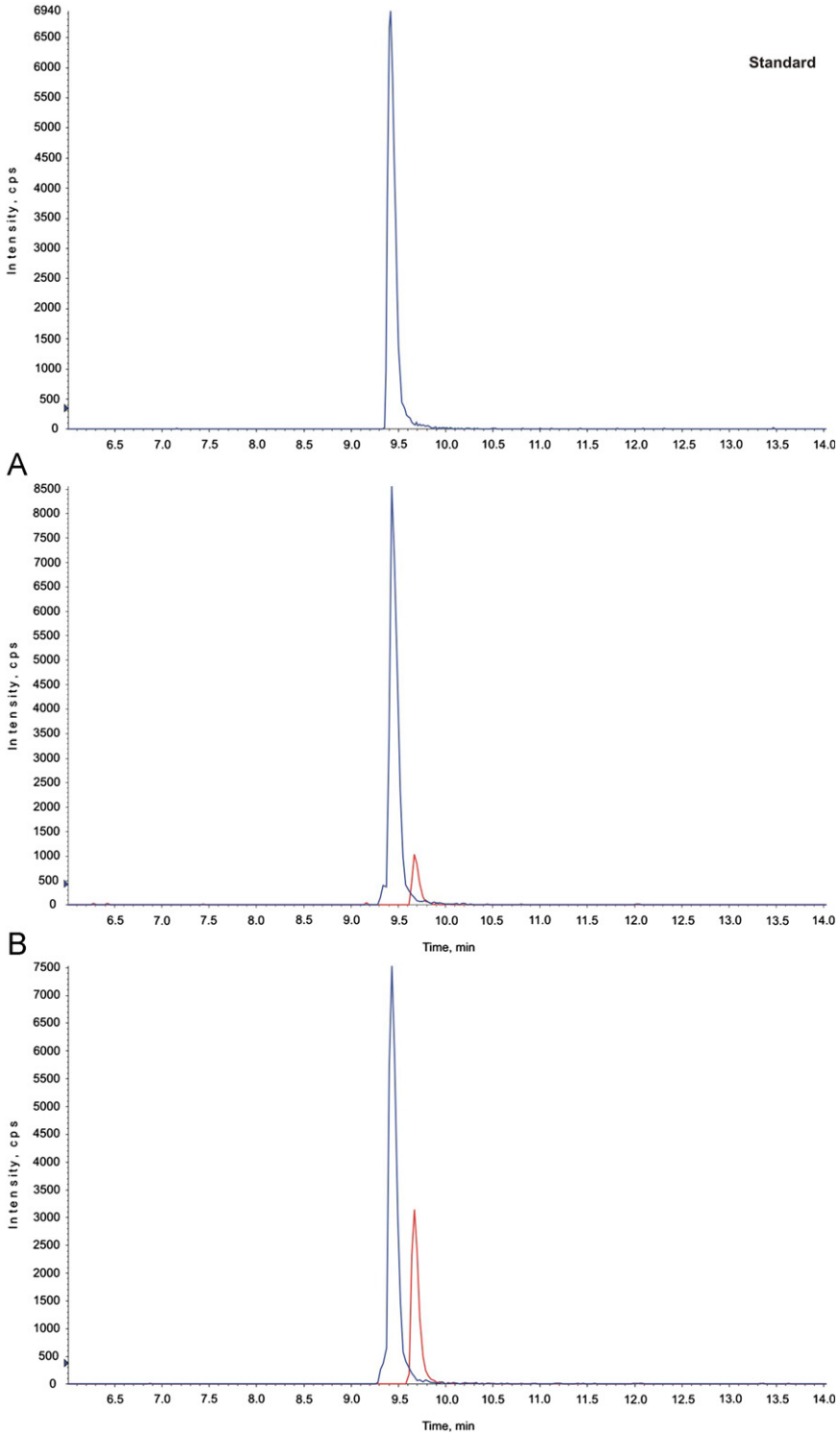


Fig. 5. LC–MS/MS Chromatograms of CYN standard and CYN and 7-deoxy-CYN from diluted extracts from two different samples of *Aphanizomenon ovalisporum* cultures (sample A and sample B, respectively).

were: (Z_1) the batch of the graphitized carbon cartridges employed; (Z_2) flow rate of the water sample through the cartridge, and (Z_3) final redissolved water volume after SPE treatment. The levels are coded according to the rule: high value$=+1$ ($Z_1=1$ (batch no. 1); $Z_2=1$ min; $Z_3=500$ μL), and low level$=-1$ ($Z_1=2$(batch no. 2); $Z_2=2$ min; $Z_3=520$ μL). The effect of each considered factor is estimated as the difference of the mean result obtained at the level$+1$ from that obtained at the level -1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance *t*-test is used [49], and the *t*-values (Z_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming the precision study for each concentration. In our study, the experiments were carried using culture extracts of cyanobacterial cells (CYN-) solutions spiked with 200 μg/L, and each factor was analyzed by quintuplicate in two different days. So, for 9 degrees of freedom, the *t*-values obtained were 0.768, 6.869, and 0.409, for Z_1, Z_2 and Z_3 factors, respectively. For the Z_1 and Z_3 $t(X_k) < t_{tab}$ (2.262), and then the procedure can be considered as robust against these two factors (at the levels fixed in the study). Accordingly, the flow rate in this case is an important factor to take into account when carrying out this method. Thus, a new assay was performed by selecting new levels of this factor: high value$=+1$($Z_2=60$ s) and low level$=-1$($Z_2=75$ s). Now, the *t*-values obtained were 0.910, 0.803, and 0.589 for Z_1, Z_2 and Z_3 factors, respectively, and $t(Z_k) < t_{tab}$ (2.262) always. Hence, the procedure can be considered as robust against the three factors (at the levels fixed in the study).

3.5. Evaluation of CYN in different lyophilized cultures of Aphanizomenon ovalisporum (LEGE X-001)

Levels of CYN from two different samples of *A. ovalisporum* cultures were analyzed according to the proposed and validated method, previously diluting the extracts 1/100 in milli-Q water. CYN was detected (retention time of 7.55 min) and quantified, and the results were 3675 (sample A) and 3979 μg CYN/g (sample B) (Fig. 5). Moreover, its deoxy-derivative (7-deoxy-CYN) has been also detected in both samples, with a retention time of 7.76 min. In this case, the full scan and tandem mass spectra of 7-deoxy-CYN were very similar, the only difference being the shift of ions at m/z 336 and 318 in CYN down 16 mass units in 7-deoxy-CYN (Fig. 2). As no pure standard of this isomer was available, the quantification of 7-deoxy-CYN was made using the CYN calibration curve (equivalent of CYN).The values obtained were 1405 μg 7-deoxy-CYN/g and 427 μg 7-deoxy-CYN/g, for samples A and B, respectively. The ratio between both toxins (CYN/7-deoxy-CYN) were 3/1 and 9/1 in both samples, respectively. The predominance of CYN agrees with previous results found by Li et al. [50] in a *C. raciborskii* strain (CY-Thai); they detected CYN and Deoxy-CYN in a ratio of 10/1 (CYN/7-deoxy-CYN) when they analyzed both toxins by HPLC–MS/MS. Nevertheless, other authors analyzed the production of CYN and Deoxy-CYN by HPLC–MS/MS from *Raphidiopsis mediterranea* showing a production of 917 and 1065 μg/g of CYN and Deoxy-CYN, respectively, reporting a ratio 1/1 [3]. Therefore, the ratio CYN/7-deoxy-CYN may depend on the cyanobaterial producing strain and/or the culture conditions.

4. Conclusion

This report presents a sensitive, reproducible, accurate, and robust method for extraction and determination of CYN in lyophilized cells, using SPE with graphitized carbon cartridges and quantification by LC–MS/MS. The recoveries (83–94%) and intermediate precision values obtained (5.6–11.0%), as well as the robustness of the method for the three factors considered, permit its validation. This method provides detection and quantification limits acceptable for environmental studies and proves its utility for determining CYN in lyophilized natural blooms samples. The total time for carrying out the complete procedure (including the freezing and lyophilization steps) oscillated between 2 and 3 days. Consequently, this LC–MS/MS method is appropriate to confirm and quantify CYN in natural samples previously assayed using screening methods (e.g. ELISA). Therefore, its usefulness would be as a confirmatory method to those employed in monitoring water for public health protection.

Novelty statement

We have developed and validated a sensitive, reproducible, accurate, and robust method for extraction and determination of CYN from *A. ovalisporum* lyophilized cells. This method is based on SPE with graphitized carbon cartridges for the extraction and quantification by LC–MS/MS. The recoveries (83–94%) and intermediate precision values obtained (5.6–11.0%), as well as the robustness of the method for the three factors considered relative to the SPE procedure (the batch of the graphitized carbon cartridges employed; flow rate of the water sample through the cartridge, and final redissolved water volume), permit its validation. This method provides detection and quantification limits acceptable for environmental studies.

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References

[1] C. Dell'Aversano, G.K. Eaglesham, M.A. Quilliam, J. Chromatogr. A 1028 (2004) 155–164.
[2] T. Kubo, T. Sanob, K. Hosoyac, N. Tanakac, K. Kayaa, Toxicon 46 (2005) 104–107.
[3] G.B. McGregor, B.C. Sendall, L.T. Hunt, G.K. Eaglesham, Harmful Algae 10 (2011) 402–410.
[4] S. Kikuchi, T. Kubo, K. Kaya, Anal. Chim. Acta 583 (2007) 124–127.
[5] R. Mazmouz, F. Chapuis-Hugon, S. Mann, V. Pichon, A. Mejean, O. Ploux, Appl. Environ. Microbiol. 76 (2010) 4943–4949.
[6] S. Kinnear, Mar. Drugs 8 (2010) 542–564.
[7] P.R. Hawkins, M.T.C. Runnegar, A.R.B. Jackson, I.R. Falconer, Appl. Environ. Microbiol. 50 (1985) 1292–1295.
[8] K. Terao, S. Ohmori, K. Igarashi, I. Ohtani, M.F. Watanabe, K.I. Harada, et al., Toxicon 32 (1994) 833–843.
[9] P. Bain, G. Shaw, B. Patel, J. Toxicol. Environ. Health A 70 (2007) 1687–1693.
[10] D. Gutierrez-Praena, S. Pichardo, A. Jos, A.M. Camean, Ecotoxicol. Environ. Saf. 74 (2011) 1567–1572.
[11] M. Puerto, A. Campos, A.I. Prieto, A.M. Cameán, A. Almeida, A. Varela Coelho, V.M. Vasconcelos, Aquat. Toxicol. 101 (2011) 109–116.
[12] P.R. Hawkins, N.R. Chandrasena, G.J. Jones, A.R. Humpage, I.R. Falconer, Toxicon 35 (1997) 341–346.
[13] I. Falconer, S.J. Hardy, A. Humpage, S. Froscio, G.J. Tozer, P.R. Hawkins, Environ. Toxicol. 14 (1999) 143–150.
[14] G.R. Shaw, A.A. Seawright, M.A. Moore, P.K.S. Lam, Ther. Drug Monit. 22 (2000) 89–92.
[15] A.R. Humpage, M. Fenech, P. Thomas, I.R. Falconer, Mutat. Res. 472 (2000) 155–161.
[16] S. Masten, B. Carson, Toxicological Summary for Cylindropermopsin, Final Report. Integrated Laboratory System i–viii, Review of Toxicology Literature, North Carolina, 2000.
[17] M. Yılmaz, E.J. Phlipsa, N.J. Szabob, S. Badylaka, Toxicon 51 (2008) 130–139.
[18] G.B. McGregor, L.D. Fabbro, Lakes Reservoirs Res. Manage. 5 (2000) 195–205.
[19] J. Burns, C. Williams, A. Chapman, Cyanobacteria and their toxins in Florida surface waters, in: Proccedings of Health Effects of Exposure to

Cyanobacterial Toxins: State of the Sciencie, Florida Department Of Health, Gainesville, 2002, pp. 16–21.
[20] J. Fastner, R. Heinze, A.R. Humpage, U. Mischke, G.K. Eaglesham, I. Chorus, Toxicon 42 (2003) 313–321.
[21] G. Manti, D. Mattei, V. Messineo, S. Melchiorre, S. Bogialli, N. Sechi, P. Casiddu, L. Luglio, M. Di Brizio, M. Bruno, Harmful Algal News 28 (2005) 8–9.
[22] V. Messineo, S. Melchiore, A. Di Corcia, P. Gallo, M. Bruno, Environ. Toxicol. 25 (2008) 18–27.
[23] A. Quesada, E. Moreno, D. Carrasco, T. Paniagua, L. Wormer, C. De Hoyos, A. Sukenik, Eur. J. Phycol. 41 (2006) 39–45.
[24] L. Spoof, K.A. Berg, J. Rapala, K. Lahti, L. Lepisto, J.S. Metcalf, G.A. Codd, J. Meriluoto, Environ. Toxicol. 21 (2006) 552–560.
[25] M. Kokocinski, D. Dziga, L. Spoof, K. Stefaniak, T. Jurczak, J. Mankiewicz-Boczek, J. Meriluoto, Chemosphere 74 (2009) 669–675.
[26] L. Bláhová, M. Oravec, B. Marsalek, L. Sejnohova, Z. Simek, L. Bláha, Toxicon 53 (2009) 519–524.
[27] M. Welker, G. Christiansen, H. von Döhren, Arch. Microbiol. 182 (2004) 288–298.
[28] A.R. Humpage, I.R. Falconer, Environ. Toxicol. 18 (2003) 94–103.
[29] K.I. Harada, I. Ohtani, K. Iwamoto, M. Suzuki, M.F. Watanabe, M. Watanabe, K. Terao, Toxicon 32 (1994) 73–84.
[30] M. Welker, H. Bickel, J. Fastner, Water Res. 36 (2002) 4659–4663.
[31] A. Törökné, M. Aszatols, M. Bánkinné, H. Bickel, G. Borbély, S. Carmeli, G.A. Codd, J. Fastner, Q. Huang, et al., Anal. Biochem. 332 (2004) 280–284.
[32] J.S. Metcalf, K.A. Beattie, M.L. Saker, G.A. Codd, FEMS Microbiol. Lett. 216 (2002) 159–164.
[33] L. Wormer, S. Cires, D. Carrasco, A. Quesada, Harmful Algae 7 (2007) 206–213.
[34] L. Wormer, D. Carrasco, S. Cirés, A. Quesada, Limnol.Oceanogr. Methods 7 (2009) 568–575.
[35] G. Eaglesham, K.R. Norris, G.R. Shaw, M.J. Smith, R.K. Chiswell, B.C. Davis, G.R. Neville, A.A. Seawright, B.S. Moore, Environ. Toxicol. 14 (1999) 151–155.
[36] S. Bogialli, M. Bruno, R. Curini, A.D. Corcia, C. Fanali, A. Laganà, Environ. Sci. Technol. 40 (2006) 2917–2923.
[37] P. Gallo, S. Fabbrocino, M.G. Cerulo, P. Ferranti, M. Bruno, L. Serpe, Rapid Commun. Mass Spectrom. 23 (2009) 3279–3284.
[38] S.A. Oehrle, B. Southwell, J. Westrick, Toxicon 55 (2010) 965–972.
[39] A.G. González, M.A. Herrador, Trends Anal. Chem. 26 (2007) 227–238.
[40] A. González, M. Herrador, A.G. Asuero, Talanta 82 (1010) (1995–1998).
[41] R. Banker, S. Carmeli, O. Hadas, B. Teltsch, R. Porat, A. Sukenik, Identification of cylindropsermopsin in *Aphanizomenon ovalisporum* (cyanophyceae) isolated from Lake Kinneret, Isr. J. Phycol. 33 (1997) 613–616.
[42] J. Fastner, J. Rucker, A. Stuken, K. Preußel, B. Nixdorf, I. Chorus, A. Kohler, C. Wiedner, Environ. Toxicol. 22 (2007) 26–32.
[43] H. Liu, P.M. Scott, Food Addit. Contaminant 28 (2011) 786–790.
[44] L. Huber (Ed.), Validation and Qualification in Analytical Laboratories, Interpharm Press, East Englewood, CO, USA, 1998.
[45] ICH Harmonised Tripartite Guideline, Validation of Analytical procedures: Text and Methodology, ICH Working Group, November 2005. 〈http://www.ich.org/LOB/media/MEDIA417.pdf〉.
[46] A.O.A.C. Peer Verified Methods Program. Manual on Polices and Procedures, AOAC Inter., 1998. 〈http://www.aoac.org/vmeth/PVM.pdf〉.
[47] W.Y. Youden, Statistical Techniques for Collaborative Tests, AOAC Inter, Washington DC, USA, 1967.
[48] I. García, M.C. Ortiz, L. Sarabaia, C. Vilches, E.J. Gredilla, Chromatogr. A 992 (2003) 11.
[49] Y. Vander Heyden, K. Luypaert, C. Hartmann, D.L. Nassart, J. Hoogmartens De Beer, Anal. Chim. Acta (1995) 245–312.
[50] R. Li, W.W. Carmichael, S. Brittain, G.K. Eaglesham, G.R. Shaw, A. Mahakhant, N. Noparatnaraporn, W. Yongmanitchai, K. Kaya, M.M. Watanabe, Toxicon 39 (2001) 973–980.

CAPÍTULO 4 / CHAPTER 4

Remedios Guzmán-Guillén, I. Moreno, Ana I. Prieto Ortega, M. Eugenia Soria-Díaz, Vitor Vasconcelos, Ana M. Cameán

CYN DETERMINATION IN TISSUES FROM FRESHWATER FISH BY LC-MS/MS: VALIDATION AND APPLICATION IN TISSUES FROM SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS)

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CYN determination in tissues from freshwater fish by LC–MS/MS: Validation and application in tissues from subchronically exposed tilapia (*Oreochromis niloticus*)

Remedios Guzmán-Guillén [a,*], I. Moreno [a], Ana I. Prieto Ortega [a], M. Eugenia Soria-Díaz [b], Vitor Vasconcelos [c,d], Ana M. Cameán [a]

[a] Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Sevilla, Spain
[b] Mass Spectrometry Facility, Centro de Investigación Tecnológica e Investigación (CITIUS), University of Sevilla, Sevilla, Spain
[c] Biology Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4169-007, Portugal
[d] Marine and Environmental Research Center (CIIMAR/CIMAR), University of Porto, Rua dos Bragas, m289, 4050-123 Porto, Portugal

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ABSTRACT

Harmful cyanobacterial blooms are occurring in eutrophic freshwater lakes and reservoirs throughout the world and, because of the production of toxins such as cylindrospermopsin (CYN), they can present a public safety hazard through contamination of seafood and fish for human consumption. Therefore it is important to develop methods to determine CYN at trace levels in those organisms. A new method for unconjugated CYN determination in tissues (liver and muscle) of tilapia (*Oreochromis niloticus*) is herein described and discussed; it is based on solvent extraction and purification with C18 and graphitized carbon cartridges, and quantification by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The method was optimized and suitably validated, with a linear range from 0.125–12.5 μg CYN/g dry weight (dw) in the case of the liver, and 0.02–1 μg CYN/g dw for the muscle. Limits of detection and quantitation were 0.07 and 0.12 μg/g dw for the liver, and 0.002 and 0.007 μg/g dw for the muscle, respectively. Mean recoveries ranged 80–110% in liver, and 94–104% in muscle, and intermediate precision values from 6 to 11%. The method is robust against the three factors considered for purification (batch of the graphitized carbon cartridges, time for the sample to pass through the cartridge, and final dissolving water volume). Furthermore, it has been successfully applied to the extraction and quantification of CYN in tissue samples from tilapia subchronically exposed to CYN in the laboratory. This represents a sensitive, reproducible, accurate, and robust method for extraction and determination of unconjugated CYN in tissues of fish exposed to the toxin. This procedure can be used for confirmatory routine monitoring of CYN in fish samples in environmental studies.

1. Introduction

Cyanobacteria are procariotic organisms growing in eutrophic freshwaters and lakes, and they are able to produce toxic secondary metabolites called cyanotoxins. Cylindrospermopsin (CYN) is an emerging toxin known to be produced by eleven species of cyanobacteria [1], *Aphanizomenon ovalisporum* among them. It is a polyketide-derived alkaloid with a central functional guanidine moiety combined with hydroxymethyluracil attached to its tricyclic carbon skeleton. CYN has a relatively low molecular weight of 415 Da and is highly soluble in water [2], so CYN concentration dissolved in the fraction of water could be more than 99% of total toxin available [3].

CYN is a potent inhibitor of proteins [4] and glutathione (GSH) synthesis [5,6]. It is known that oxidative stress *in vitro* and *in vivo* [7–9] are involved in CYN mechanism of toxicity. Moreover, CYN may play a potential role as endocrine disruptor [10] and genotoxic [11]. Histopathological lesions have been found in the liver, kidney, intestine, lungs, and thymus of different experimental models exposed to CYN [4,12–14].

A derivation of a guideline value for CYN is in progress by the WHO [15]. A guideline safety value of 1 μg/L in drinking water and a no-observed-adverse-effect level value of 30 μg/kg body weight/day were reported for CYN by Humpage and Falconer [16], who also proposed a tolerable daily intake (TDI) of 0.03 μg/kg body weight/day for human exposure.

* Correspondence to: Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González n° 2, 41012 Sevilla, Spain. Tel.: +34 954 556762; fax: +34 954 233765.
E-mail address: rguzman1@us.es (R. Guzmán-Guillén).

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Many aquatic animals are capable of living under the presence of cyanotoxins, and able to accumulate them in their tissues, becoming toxin reservoirs for animals higher up the trophic chain, including humans. The extent to which the concentrations found in these aquatic animals present a risk to humans after consumption has been discussed and reviewed by some authors [17–19]. Because CYN shows high stability and slow degradation in many water bodies [20], this toxin can bioaccumulate in different aquatic organisms even if they are exposed to trace quantities of the toxin. Moreover, taking into account that many incidents of human poisonings involving CYN may be unnoticed because of complicated toxin detection procedures [1], the development of highly sensitive, quantitative validated methods for its determination in animal tissues is essential for food safety and risk assessment.

Many methods for CYN analysis in different matrices are reported in the literature: from enzyme-linked immunosorbent assay (ELISA) for fast screening [21,22] and capillary electrophoresis [23,24], to confirmation tests by liquid chromatography (LC) with diode-array detection (DAD) [25,26], with single quadrupole LC/MS [27,28], or triple quadrupole LC/MS/MS [29–34]. LC–MS/MS is the ideal method for small amounts of toxin in water samples [29,32] and for complicated sample types (fish muscle) [35], as well as the addition of a clean-up step by solid phase extraction (SPE) helps to obtain a clean background from animal tissue extracts. Although CYN has been extracted and detected in different aquatic organisms [35–38] validation studies and robustness assays are scarce and have not been widely developed. Gallo et al. [35] developed a method for CYN determination in freshwaters and fish muscle by LC/ESI–MS/MS, but the method was not applied to those muscle samples, as has been carried out in the present work.

Analytical method validation strategies used to compare a performance measure with a reference value, not reflecting the consumer's needs, and they rarely consider the robustness study, which is essential for the "method transfer", following harmonization purposes [39]. Besides, the assessment of intra-laboratory accuracy is a fundamental stage in method validation [40]. Following all these considerations, we have previously validated two methods in our laboratory for determining CYN in waters [33] and in cyanobacterial cultures [34] by LC–MS/MS, which were applied to samples from aquaria of an in vivo experiment and to lyophilized cultures of *A. ovalisporum* to evaluate CYN content.

In the present study, we aimed to develop an analytical procedure based on solvent extraction and purification with C18 and graphitized carbon cartridges, followed by LC–MS/MS for CYN detection and quantification in fish tissue samples (liver and muscle). Optimization and validation of the proposed method was carried out according to the holistic approach [39,40]. The present procedure has been intended for routine determination in fish samples (*Oreochromis niloticus*) for human consumption with risk assessment purposes.

2. Materials and methods

2.1. Chemicals and reagents

Cylindrospermposin (CYN) standards (purity $>95\%$) were supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in Milli-Q water (100 μg/mL) and diluted as required for their use as working solutions (5–500 μg/L). All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, dichloromethane, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA). Bakerbond® C18 cartridges (500 mg, 6 mL) and BOND ELUT® Carbon cartridges (PGC column) (500 mg, 6 mL) were supplied by Dicsa (Andalucía, Spain) and Agilent Technologies (The Netherlands, Europe), respectively.

2.2. Extraction and clean up procedures

In order to study the efficiency of the extraction and purification procedures, control lyophilized fish liver (0.04 g) and muscle (0.5 g) samples were spiked with CYN standard solutions at three concentration levels: 20, 200 and 400 μg/L, equivalent to 0.5, 5.0 and 10.0 μg CYN/g dry liver, or 0.04, 0.4 and 0.8 μg CYN/g dry muscle. Afterward, the toxin was extracted from the samples using a modified version of Gallo et al.'s method [35] intended for CYN determination in freshwaters and fish muscle, by increasing the proportion of TFA contained in the extraction solvents and introducing 15 min of sonication for tissue disruption. The liquid-liquid partitioning with *n*-hexane in the original method was eliminated. Briefly, the liver was extracted with 10 mL Milli-Q water/acetonitrile (30:70, v/v), containing 0.5% TFA (v/v); after ultraturrax homogenization for 2 min, the sample was sonicated for 15 min. The resulting mixture was centrifuged at 20,000 rpm for 10 min. This procedure was repeated one more time. In the case of the muscle, 20 mL of the same extraction solvent was used, following the above-mentioned procedure. Regarding sample preparation prior SPE, Foss and Aubel [41] presented a methodology for the extraction of CYN from human serum and urine by ELISA and LC/MS/MS. For the first matrix, they explored different extraction techniques: sonication (probe and bath) and protein precipitation with four different solutions (100% methanol, 100% acetone, 1% formic acid in methanol, and 1% formic acid in acetonitrile). Finally, they suggested sample homogenization (water bath sonication), followed by protein precipitation with 100% methanol and centrifugation, and SPE with graphitized carbon cartridges. In the case of the urine analysis, pH manipulation was used ($pH>10$) as a means to precipitate salts and other interfering agents before SPE.

Once the extracts of each sample had been obtained (liver and muscle), a purification step was applied. These extracts (≈ 20 mL from liver or ≈ 40 mL from muscle extracts) were passed through a combined SPE system consisting of a C18 column and a PGC column, following the method of Liu and Scott [42] for algal food supplements optimizing it in our laboratory for biological samples (liver and muscle). The C18 columns were preconditioned with 10 mL of methanol containing 0.1% TFA (v/v), followed by 10 mL of water. The extracts were passed through the columns and washed with 10 mL of Milli-Q water; both fractions were collected together and concentrated to ≈ 10 mL for further purification with the second cartridge, the PGC column. This column was activated with 10 mL of methanol containing 0.1% TFA (v/v) and rinsed with 10 mL of Milli-Q water. Subsequently, the sample was passed through the cartridge, washed with 10 mL of Milli-Q water and eluted with 16 mL of methanol containing 0.1% TFA (v/v). The extracts were evaporated to dryness and redissolved in 1 mL of Milli-Q water. Then, they were transferred to a microcentrifuge tube and centrifuged (10 min, 13200 rpm). The supernatant was centrifuged again using ultrafree-MC PVDF (0.45 μm Millipore Corporation, Bedford, MA) (10 min, 11400 rpm). Finally, the extract was passed through a syringe filter (0.22 μm) before injection into the Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) system.

2.3. Chromatographic conditions

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA)

consisting of an hybrid triple quadrupole linear ion trap (QqQlit) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 × 2.1 mm Zorbax Sb–Aq column. The flow rate was 0.2 mL/min.

Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90% B (5 min) and finally 0 % B (5 min). Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragments ions were monitored at *Q*1 and *Q*3, respectively. The transitions for the detection of CYN are: 416.2/194.0, 416.2/274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For LC/ESI–MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: curtain gas 35 psi, 185 source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

2.4. Evaluation of CYN in fish tissues

2.4.1. Experimental set up and fish acclimation

In order to apply the validated method to real samples, six groups of fish ($n=8$) were used to perform the present study. Fish (Male *O. niloticus*, Nile tilapia, Perciformes: Cichlidae) were obtained from a fish hatchery "Aquaculture Valencia" and maintained at the laboratory to reach the average weight of 25 ± 7 g and 8 ± 2 cm length. Fish were held in aquaria with 96 L of fresh water, minimizing exposure to chlorine by filling the tanks at least 3 days before the fish were introduced. The aquaria were also set up with a continuous system of water filtration and aeration (Eheim Liberty and Bio-Espumador cartridges (Bio-Espumador)). The temperature was kept constant (21 ± 2 °C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg/L. Mean values for additional parameters of water quality were: pH 7.6 ± 0.3, conductivity 287 μS/cm, Ca^{2+} 0.60 mM/L and Mg^{2+} 0.3 mM/L. Fish were fed only with 0.3 g/d of commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) being left to acclimatize for 15 days before the beginning of the experiment.

2.4.2. Experimental exposure and application of the validated method

The *A. ovalisporum* (LEGE X-001) cyanobacterial CYN− producing strain (CYN+) was isolated from Lake Kinneret, Israel [43] and kindly supplied by Dr. Vitor Vasconcelos (Marine Research Centre, Porto, Portugal). Once cultivated in our laboratory, the biomass obtained was frozen at −80 °C, for lyophilization (Cryodos-80, Telstar, Tarrasa, Spain), and CYN extraction from the lyophilized cells of *A. ovalisporum* (CYN+) was performed according to the method of Guzmán-Guillén et al. [34]. Extracts were injected into the LC–MS/MS system, detecting CYN at a retention time of 9.55 min, and with a concentration of 2.14 μg CYN mg^{-1} of lyophilized cells.

After the acclimation period, three groups of fish were exposed to CYN by a manually-crushed mixture of fish food and lyophilized cyanobacterial cells (to obtain 30 μg CYN/fish), resulting in small, sticky pellets that were placed in the aquaria and allowed to drift to the bottom, ensuring the fish ate them within an hour. This procedure was repeated every 2 days along the exposure periods of 7, 14 or 21 d, adding to the aquaria the same quantity of lyophilized cells every time. Other three aquaria were used as the control groups (without CYN), being only fed with the commercial fish food for the whole experiment and sacrificed after their respective exposure times (7, 14 or 21 d).

At the end of the experiment, fish muscle of each specimen was portioned and lyophilized, together with the entire fish liver (Cryodos-80, Telstar, Tarrasa, Spain), for unconjugated, free CYN extraction. Samples were weighed and the median water losses were 78–80% for fish muscle and ≈ 87% for fish liver. In addition, CYN levels in the aquaria were analyzed every 48 h throughout the exposure periods by LC–MS/MS [33].

2.5. Statistical criteria for method validation

Once the method had been developed, it must be validated, in order to verify that it satisfies the requirements of the application domain. Thus, the proposed method was validated taking into account the ICH Guidelines for linearity, sensitivity, precision and recovery [44].

Three validation standards covering the optimal working range were used, which were measured in triplicate for three different days. One mL of three different concentrations (20, 200, and 400 μg CYN/L) was added to the matrices to obtain 0.5, 5.0 and 10.0 μg CYN/g dry liver, or 0.04, 0.4 and 0.8 μg CYN/g dry muscle. By applying a one-factor analysis of variance (ANOVA), as explained in the *Results and Discussion* section, both the precision and the recovery are obtained, and then they are compared with tabulated reference values.

Besides, a robustness study was performed by spiking the matrices with an intermediate validation standard of 200 μg CYN/L (equivalent to 5 μg CYN/g dry liver or 0.4 μg CYN/g dry muscle), according to the Youden procedure [45]. The influential factors (batch of the graphitized carbon cartridges, time for the sample to pass through the cartridge, and final dissolving water volume) were tested with Student's *t* test as indicated below.

3. Results and discussion

Before starting the analysis of CYN extraction efficiency, the LC–MS/MS method needed to be set up for this use. In order to do that, commercially available standard solutions of CYN were assayed, acquiring mass spectra and adjusting mobile phase strength, as previously developed in our laboratory [33,34].

3.1. Method validation

The responses as a function of concentration were calculated from CYN standards prepared in extracts from lyophilized liver and muscle, and were measured by a 6-point calibration curve with a linear range within 5–500 μg/L, equivalent to 0.125–12.5 μg CYN/g dry tissue in the case of the liver, and by a 5-point calibration curve with a linear range of 10–500 μg/L, equivalent to 0.02–1 μg CYN/g dry tissue for the muscle. The regression equations obtained were $y=88.36x-43.97$ ($r^2=0.999$) and $y=23.67x+9.656$ ($r^2=0.995$), for the liver and the muscle, respectively.

3.1.1. Linearity and goodness of the fit

The linearity of an analytical method represents the range of analyte concentrations over which the method gives test results proportional to the concentration of the analyte [46]. Six and five different concentrations of CYN were spiked to blank extracts of fish liver (0.125–12.5 μg CYN/g dw) and muscle (0.02–1 μg CYN/g dw), respectively, submitting them to the proposed method. The calibration plot (signal response/analyte concentration against their concentrations) was established according to Huber [47] by replicate analysis ($n=3$) at all concentration levels (Fig. 1). The target line has zero slopes, representing the median of the response factors obtained. Then, two parallel horizontal lines are

drawn in the graph at 0.95 and 1.05 times the median value. As can be observed, no intersections with the lines were found in neither of the matrices, thus the linear ranges of the methods apply to the full ranges studied.

With the same signal responses, we carried out the corresponding ANOVA of the regression lines, indicating a lack-of-fit F ratio of 0.72 for the liver and 7.79 for the muscle, compared to a tabulated F value of 19.4. As the calculated ratios are below the reference value, there is no lack of fit and the calibration functions can be considered as linear.

3.1.2. Sensitivity

For validation purposes it is normally sufficient to provide an indication of the level at which detection becomes problematic and quantitation is acceptable in terms of repeatability precision and trueness. For this purpose, the limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the blank, by measuring 10 independent sample blanks once each, and were estimated according to the equation $Y_{\text{LOD or LOQ}}=Y_{\text{blank}}+nS_{\text{blank}}$, where Y_{blank} and S_{blank} are the average value of the blank signals and its corresponding standard deviation, and n is a constant (3 for LOD and 10 for LOQ). These values are then converted into concentration by using the calibration functions obtained before. The LOD and LOQ obtained are 0.07 μg/g dw and 4.66 μg/g dw for the liver, and 0.002 μg/g dw and 0.007 μg/g dw for the muscle, respectively, and are recorded in Table 1. Other authors obtained values of 0.6 and 1.0 ng/g for LOD and LOQ, respectively, for CYN determination in fish muscle by LC coupled to electrospray ion trap mass spectrometry (LC/ESI–MS/MS), but they did not show any values of CYN detected in those tissue samples [35]. Our values were lower or of the same order than those obtained by Ríos et al. [48] for MCs determination in tench (*Tinca tinca*).

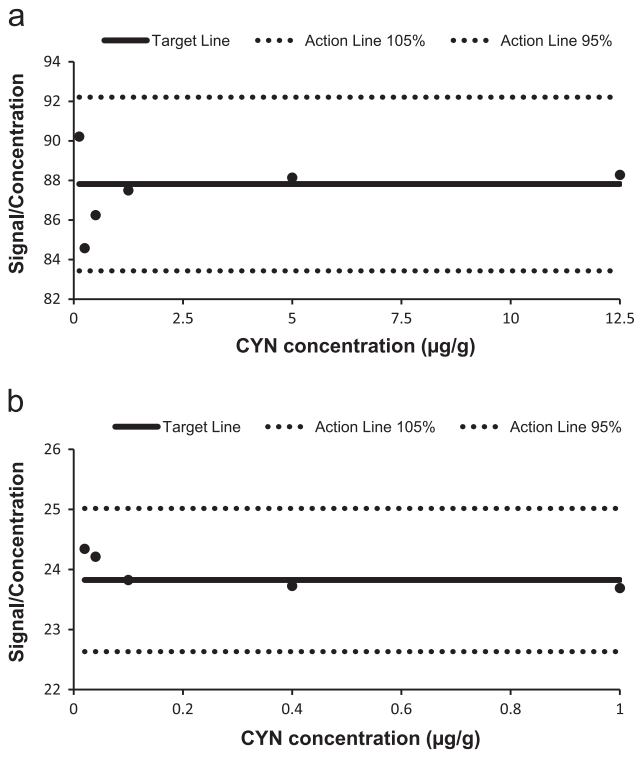

Fig. 1. Huber plot for assessing the linear range in (a) liver and (b) muscle of tilapia.

3.1.3. Precision

Precision refers to the closeness of agreement between independent test results obtained under stipulated conditions and, according to the International Conference on Harmonisation Guidelines [44], the measure of precision is usually expressed in terms of three concepts: repeatability, intermediate precision and reproducibility. The first one represents the closeness of the agreement between the results of successive measurements of the same measurand carried out in the same conditions, and it is called "within run" [49]. On the other hand, intermediate precision, also termed "between run", "inter-assay precision" or "intra-laboratory reproducibility", expresses within-laboratories variation: different days, different analysts, different equipment, etc. [44].

To assess the precision study, we spiked blank extracts from lyophilized liver or muscle at three concentrations of CYN standard (20, 200 and 400 μg/L, equivalent to 0.5, 5 and 10 μg CYN/g dry liver, or 0.04, 0.4 and 0.8 μg CYN/g dry muscle), in triplicate ($n=3$) within the same day, as recommended by the ICH guidelines, and over a period of three days; afterward, they were subjected to the proposed method and results were obtained. Considering three different days as the main source of variation, an analysis of variance (ANOVA) was performed for each validation standard and, operating with those results according to González and Herrador [39] and González et al. [40], the estimations of within-day repeatability (S_w), between-day repeatability (S_B) and intermediate precision (intra-laboratory reproducibility, S_{IP}) are obtained. From this last parameter and the mean

Table 1
Estimations of within-condition repeatability (S_w), between-condition repeatability (S_B), intermediate precision (intra-laboratory reproducibility, S_{IP}) and its relative standard deviations (%RSD_{IP}), and recoveries of CYN assayed in liver and muscle of fish, at three concentration levels, in three different days. Reference RSD values and recovery percentages by AOAC. Limits of detection (LOD) and quantitation (LOQ) for both matrices.

	CYN concentration level (validation standards)					
	20 μg/L		200 μg/L		400 μg/L	
	Liver (0.5 μg/g)	Muscle (0.04 μg/g)	Liver (5.0 μg/g)	Muscle (0.4 μg/g)	Liver (10.0 μg/g)	Muscle (0.8 μg/g)
S_w	2.42	1.88	2.92	8.64	13.94	26.81
S_B	1.33	3.72	15.02	27.52	25.21	22.10
S_{IP}	2.12	2.64	8.99	17.38	18.47	25.34
RSD_{IP} (%)	9.60	11.00	6.00	8.10	6.00	6.77
Recoveries (%)	110	104	80	103	81	94
1/2 RSD_{AOAC} (%)	8–11		6–8		6–8	
Acceptable Recovery Range (%)	80–110					
	Liver		Muscle			
LOD	0.07 μg/g dw[a]		0.002 μg/g dw[a]			
LOQ	0.12 μg/g dw[a]		0.007 μg/g dw[a]			

[a] dw: Dry weight.

concentration values obtained for the validation standard, the corresponding relative standard deviation (RSD_{IP}) is computed and checked with the expected value issued by the AOAC Peer Verified Methods Program [40,47]. The RSD_{IP} are compared with one-half the corresponding RSD_{AOAC} tabulated values, and they should be lower or of the same order than 8–11% for 20 μg/L, and lower or by 6–8% for 200 and 400 μg/L. In our case, this requirement is fulfilled at the three concentration levels assayed, exhibiting suitable values, so the proposed method can be considered as precise. The results for all these parameters are summarized in Table 1.

3.1.4. Trueness and recovery

The trueness of an analytical assay expresses the closeness of agreement between the average value obtained from a series of measurements and an accepted reference value. It and can be obtained from the same ANOVA results previously described for the intermediate precision, and it is normally expressed in terms of bias or recovery obtained for each validation standard considered [46]. These recoveries are defined as the ratio between the mean concentration of analyte measured in the fortified sample and the concentration of analyte added ("true" reference value, not determined by method) in the fortified sample, expressed as a percentage. The recoveries obtained for the three validation standards are shown in Table 1.

Once recovery is computed, it can be checked for suitability by comparison with the published acceptable recovery percentages as a function of the analyte concentration by the AOAC Peer Verified Methods Program. As observed in Table 1, very satisfactory mean recoveries were calculated: 110%, 80% and 81% (liver), and 104%, 103% and 94% (muscle) for the three concentrations assayed, respectively. For our analyte concentrations, the acceptable recovery range (%) could oscillate between 80 and 110%; accordingly, the collected results indicate that the method can be considered as bias-free and reliable in terms of recoveries. These values are higher than the recoveries obtained by Gallo et al. [35] in fish muscle by LC/ESI–MS/MS (63.6%).

Taking into account these considerations, the analytical procedure developed in this work can be considered suitably validated.

3.1.5. Robustness

This concept represents the capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [44]. The assay is based on the procedure suggested by Youden [45], according to the practical guide of González and Herrador [39]. Youden robustness test can be performed either during the optimization of the separation technique or during the optimization of sample preparation, investigating potential factors important for the analytical procedure; for the SPE, these factors may be the sorbent type or manufacturer, the wash or elution solvent or the evaporation temperature, for example [50]. In this work, we identified three influential factors in the SPE clean-up procedure to consider for the robustness study: the batch of the graphitized carbon cartridges employed (X_1); time for the sample to pass through the cartridge (X_2); and the final dissolving water volume after the SPE (X_3). By combination of those parameters, we obtained 8 different possibilities that were all tested. The levels were coded according to the rule represented in Table 2. The effect of every factor is calculated by the difference between the mean result obtained at the level +1 and that obtained at the level −1. Once the effects have been estimated, to determine whether variations have a significant impact on the results, a significance t test is applied [39], and the t values obtained (X_k) are compared with the 95% confidence level two-tailed tabulated value (t_{tab}) corresponding to the degrees of freedom from the precision study for the concentration assayed.

In our study, the experiments were carried out using tissue samples fortified with a medium validation standard of 200 μg CYN/L, and the amount of CYN added was 5 μg/g dry liver and 0.4 μg/g dry muscle. Each factor was analyzed in triplicate on three different days, thus obtaining 8 degrees of freedom (df). The tabulated t value (t_{tab}) for an experiment with 8 df is 2.306 and, as can be observed in Table 2, $t(X_k) < t_{tab}$ in all cases, so then the procedure can be considered as robust against the three factors considered for both matrices (at the levels fixed in the study).

Table 2
Coding rules for combination of the parameters in the robustness study. X_1: batch of the graphitized carbon cartridges employed; X_2: time for the sample to pass through the cartridge; and X_3: the final dissolving water volume after the SPE. t values obtained for each parameter after the significance t test applied.

Condition	X_1		X_2		X_3	
High (+)	Batch 1		1 min		1000 μL	
Low (−)	Batch 2		1.5 min		950 μL	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
t Values	1.07	2.13	0.36	0.76	0.80	0.19

3.2. CYN determination in tissues from freshwater fish by the proposed validated method

The developed and validated method was applied to detect and quantify the unconjugated CYN fraction in tissues of fish submitted

Table 3
CYN (μg) detected in water samples from aquaria (96 L) after 7, 14 or 21 days of exposure to lyophilized cells of *Aphanizomenon ovalisporum*. CYN concentrations (μg/kg dry weight) detected in liver and muscle of fish exposed to lyophilized cyanobacterial cells of *Aphanizomenon ovalisporum* (LEGE X-001) for 7, 14 and 21 days under laboratory conditions, showing the yield (%) in each case, compared to the total CYN (μg) added to aquaria after exposure periods.

CYN exposure periods (days)	Waters from aquaria (average ± SD[a])		Tissue (average ± SD[a])	
	Theoretical CYN (μg) added to aquaria after exposure periods	Detected CYN (μg) in aquaria (Yield %)[b]	μg CYN/kg dw[c] (Yield %)[b]	
			Liver	Muscle
7	3840	3456 (90 ± 5.0)[b]	83 ± 4.0 (2.2 ± 0.1)[b]	9 ± 5.0[d] (0.23 ± 0.1)[b]
14	6720	5568 (83 ± 1.4)[b]	240 ± 103.0[d] (3.6 ± 1.5)[b]	n.d.[e]
21	9600	8928 (93 ± 0.4)[b]	703 ± 548.0[d] (7.3 ± 5.7)[b]	4 ± 0.4 (0.04 ± 0.0)[b]

[a] SD: standard deviation.
[b] Yield: in relation to theoretical CYN added to aquaria.
[c] dw: Dry weight.
[d] Above Limit of Quantitation (LOQ).
[e] n.d.: Not detected.

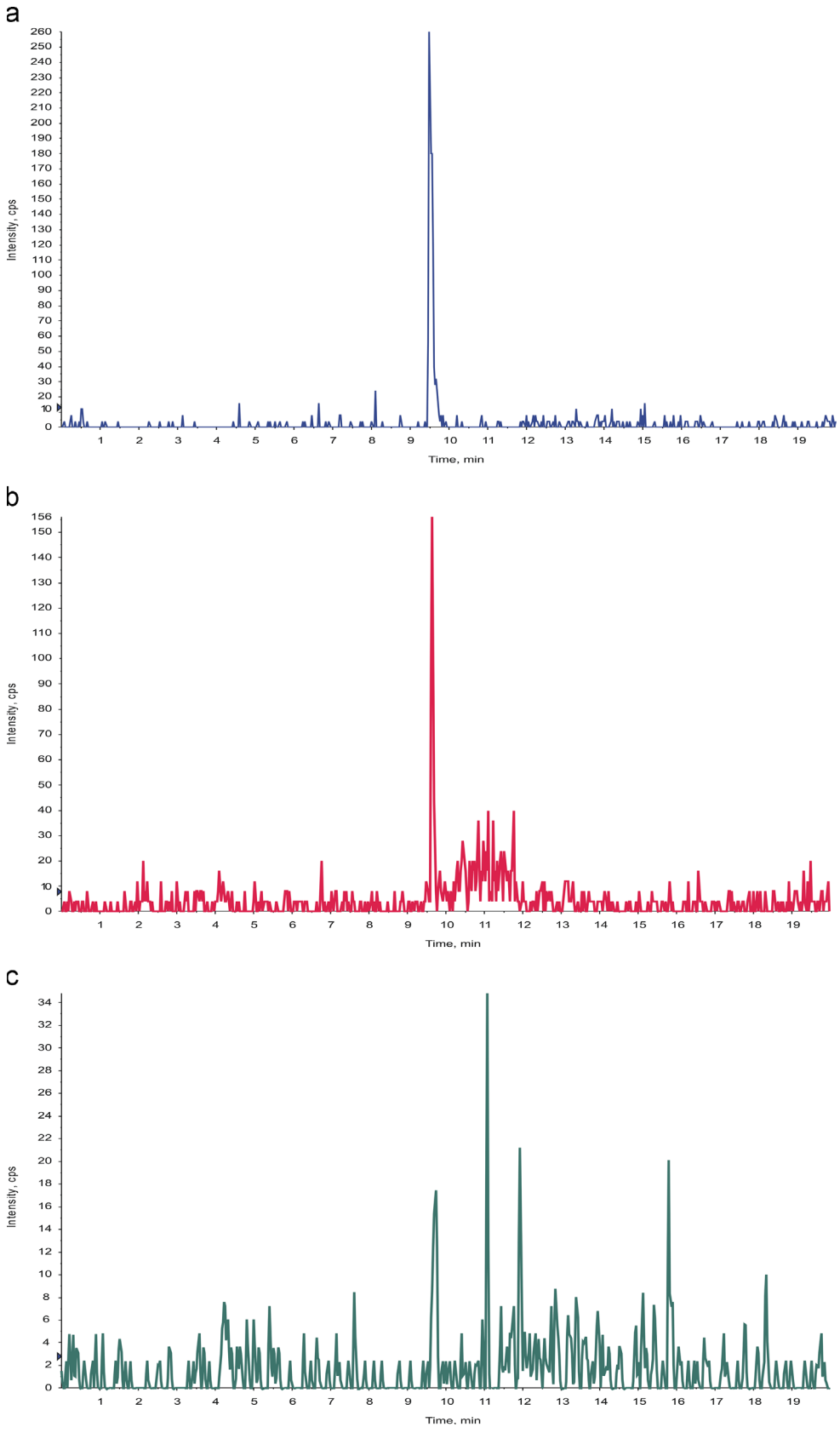

Fig. 2. LC–MS/MS chromatograms of a CYN standard (10 μg/L) (A), and CYN from extracts from a liver sample (B) and a muscle sample (C).

to the experimental conditions previously described (see Section 2.4.2) and Table 3 shows these results. The LC–MS/MS chromatograms for a CYN standard (10 μg/L), a liver sample and a muscle sample from tilapia intoxicated with the toxin in the laboratory are displayed in Fig. 2. No free CYN was detected in control tissue samples. In the case of the liver, it was possible to quantify the toxin

above the LOD after 7 days of exposure to the lyophilized cyanobacterial cells, whereas we were able to determine CYN above the LOQ after 14 or 21 days. On the other hand, CYN quantification was possible above the LOQ even after a short-term exposure (7 d) in the case of the muscle. Muscle presented much lower CYN values in comparison with the liver, which is an important consideration taking into account human consumption of possibly contaminated fish, as the muscle is usually the edible portion. These results are in accordance with previous studies reporting that concentrations of cyanotoxins accumulated in muscle are usually lower than in other tissues, being mainly high in the less edible parts [51].

A few experimental studies have been made on cyanotoxins accumulation in aquatic organisms such as bivalves, crustaceans, snails and amphibian [36,52–55] under laboratory conditions. However, laboratory and field studies on presence and accumulation of CYN in these aquatic organisms are scarce, especially in the case of fish. Gallo et al. [35] developed a method for determination of CYN both in fish muscle and in freshwaters by LC/ESI–MS/MS, but they only successfully applied it to freshwater samples, not to tissues.

On the other hand, some field studies demonstrated presence and accumulation of CYN not only in fish, but also in other aquatic species [19,37,56,57]. In fish, Messineo et al. [38] demonstrated for the first time that CYN was present in trouts from a deep natural lake during an *A. ovalisporum* bloom (2.7 μg/kg dw in liver and 0.8 μg/kg dw in muscle). More recently, Berry et al. [58] performed a study on CYN accumulation in fish muscle, showing CYN values between 0.09 and 1.26 μg/kg of wet weight (ww) in different fish species. However, in both cases, CYN concentration was determined by ELISA. Although previous studies have shown that ELISA correlates with other analytical methods, it is known the fact that it possibly overestimates concentrations relative to other techniques such as HPLC-PDA and LC/MS [28,59] for CYN determination in water reservoirs, and LC/MS for measurement of MCs in strains of *Microcystis aeruginosa* [60]. Indeed, tissue concentration values given by the above-mentioned authors may be overestimated, indicating the need for using more sensitive techniques, such as LC–MS/MS. In our study, CYN concentrations found were of the same order or higher than the reported by those authors, especially in the case of the liver, where we detected a much higher amount of the toxin (83.1–703.4 μg CYN/kg dry liver and 3.7–9.2 μg CYN/kg dry muscle). Although relatively little data exists for CYN safety limits in tissues, a maximum allowable intake of 18 μg CYN/day can be calculated based on a TDI of 0.03 μg/kg/d proposed by Humpage and Falconer [16]. Besides, taking into account a fish consumption of 1600 g over a fortnight period obtained from surveys [61], a derived health alert level in fish flesh of approximately 158 μg/kg ww is obtained [36]. Based on these guidelines, none of the concentrations of CYN found in fish tissue in our study would exceed the maximum allowable values indicated, thus suggesting there would be no potential human risk. What is important for human consumption of potential contaminated fish is the muscle (edible fraction), nevertheless, it is noteworthy to mention that the higher accumulation of CYN in the fish liver contributes additionally to the total toxin intake. That would not represent a higher risk in the case of fish, as viscera are removed, but it would be of particular interest with other seafood which is eaten whole. Moreover, according to Ibelings and Chorus [17], although consumption of aquatic animals is often restricted to muscle tissue, especially with fish, this is not always the case everywhere.

3.3. CYN determination in water samples from aquaria

In our study, we also applied a LC–MS/MS method previously validated in our laboratory [33] to analyze CYN levels in waters from the aquaria every 48 h throughout the exposure periods of 7, 14 or 21 d, and the actual CYN concentrations found are recorded in Table 3. CYN was not detected in waters from control aquaria, and the total amount of toxin found in waters plus liver and muscle, after the respective exposure periods, tends to correspond to the total theoretical CYN concentrations added to the aquaria.

4. Conclusions

In this study, a LC–MS/MS method was developed and validated for the determination of unconjugated, not cell-bound CYN in fish tissues (liver and muscle), proving to be sensitive, reproducible, accurate and robust. Its recoveries (80–110% in liver and 94–104% in muscle) and intermediate precisions obtained (6.0–9.6% in liver and 6.8–11.0% in muscle) permit its validation. Moreover, it has been possible to apply the present method for detection and quantification of CYN in liver and muscle of fish exposed to the toxin under laboratory conditions, showing that CYN can also accumulate in the edible portion of fish which may be consumed by humans. Hence, these results emphasize the need for monitoring CYN in fish tissues so that the associated potential health risks can be reduced or prevented, and the proposed method would be of usefulness for that purpose.

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References

[1] B. Poniedziałek, P. Rzymski, M. Kokociński, Environ. Toxicol. Pharmacol 34 (2012) 651–660. http://dx.doi.org/10.1016/j.etap.2012.08.005.
[2] K. Sivonen, G. Jones, Cyanobacterial toxins, in: I. Chorus, M.J. Bartram (Eds.), Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management, E & FN Spon, London, 1999, pp. 41–111.
[3] J. Rücker, A. Stüken, B. Nixdorf, J. Fastner, I. Chorus, C. Wiedner, Toxicon 50 (2007) 800–809.
[4] K. Terao, S. Ohmori, K. Igarashi, I. Ohtani, M.F. Watanabe, K.I. Harada, E. Ito, M. Watanabe, Toxicon 32 (1994) 833–843.
[5] M.T. Runnegar, S.M. Kong, Y.Z. Zhong, J.L. Ge, S.C. Lu, Biochem. Biophys. Res. Commun. 201 (1994) 235–241.
[6] M.T. Runnegar, C. Xie, B.B. Snider, G.A. Wallace, S.M. Weinreb, J. Kuhlenkamp, Toxicol. Sci. 67 (2002) 81–87.
[7] D. Gutiérrez-Praena, S. Pichardo, Á. Jos, A. María Cameán, Ecotoxicol. Environ. Saf. 74 (2011) 1567–1572.
[8] D. Gutiérrez-Praena, A. Jos, S. Pichardo, A.M. Cameán, Aquat. Toxicol. 105 (2011) 100–106.
[9] R. Guzmán-Guillén, A.I. Prieto, V.M. Vasconcelos, A.M. Cameán, Chemosphere 90 (2013) 1184–1194.
[10] F.M. Young, J. Micklem, A.R. Humpage, Reprod. Toxicol. 25 (2008) 374–380.
[11] B. Zegura, A. Straser, M. Filipic, Mutat. Res. 727 (2011) 16–41.
[12] D. Gutiérrez-Praena, A. Jos, S. Pichardo, R. Moyano, A. Blanco, J.G. Monterde, A.M. Cameán, Ecotoxicol. Environ. Saf. 76 (2012) 102–113.
[13] M. Puerto, A. Jos, S. Pichardo, R. Moyano, A. Blanco, A.M. Cameán, Environ. Toxicol. 29 (2014) 371–385.
[14] R. Guzmán-Guillén, A.I. Prieto, I. Moreno, V.M. Vasconcelos, R. Moyano, A. Blanco, A.M. Cameán, Environ. Toxicol. (2013), http://dx.doi.org/10.1002/tox.21904.
[15] M. Welker, G. Christiansen, H. von Döhren, Arch. Microbiol. 182 (2004) 288–298.
[16] A.R. Humpage, I.R. Falconer, Environ. Toxicol. 18 (2003) 94–103.
[17] B.W. Ibelings, I. Chorus, Environ. Pollut. 150 (2007) 177–192.
[18] T. Papadimitriou, I. Kagalou, C. Stalikas, G. Pilidis, I.D. Leonardos, Ecotoxicology 21 (2012) 1155–1166.

[19] D. Gutiérrez-Praena, A. Jos, S. Pichardo, I.M. Moreno, A.M. Cameán, Food Chem. Toxicol. 53 (2013) 139–152.
[20] R.K. Chiswell, G.R. Shaw, G. Eaglesham, M.J. Smith, R.L. Norris, A.A. Seawright, M.R. Moore, Environ. Toxicol. 14 (1999) 155–161.
[21] Masten, S., Carson, B., Cylindrospermopsin-Review of Toxicological Literature. Toxicological Summary for Cylindrospermopsin. CASRN 143545-90-8; National Institute of Environmental Health Sciences: Research Triangle Park, NC, USA, 2000.
[22] A.I. Prieto, A. Campos, A.M. Cameán, V.M. Vasconcelos, Ecotoxicol. Environ. Saf. 74 (2011) 1973–1980.
[23] G. Vasas, A. Gáspár, G. Surányi, G. Batta, G. Gyémant, M. Hamvas, C. Máthé, E. Molnár, G. Borbély, Anal. Biochem. 302 (2002) 95–103.
[24] G. Vasas, A. Gáspár, C. Páger, G. Surányi, C. Máthé, M.M. Hamvas, G. Borbély, Electrophoresis 25 (2004) 108–115.
[25] M. Welker, H. Bickel, J. Fastner, Water Res. 36 (2002) 4659–4663.
[26] J. Meriluoto, G.A. Codd (Eds.), Toxic Cyanobacterial Monitoring and Cyanotoxin Analysis, Abo Akademi University Press, Abo, Finland, 2005, p. 149.
[27] S. Kikuchi, T. Kubo, K. Kaya, Anal. Chim. Acta 583 (2007) 124–127.
[28] L. Bláhová, M. Oravec, B. Marsalek, L. Sejnohova, Z. Simek, L. Blaha, Toxicon 53 (2009) 519–524.
[29] G. Eaglesham, K.R. Norris, G.R. Shaw, M.J. Smith, R.K. Chiswell, B.C. Davis, G. R. Neville, A.A. Seawright, B.S. Moore, Environ. Toxicol. 14 (1999) 151–155.
[30] D.J. Stirling, M.A. Quilliam, Toxicon 39 (2001) 1219–1222.
[31] C. Dell'Aversano, G.K. Eaglesham, M.A. Quilliam, J. Chromatogr. A 1028 (2004) 155–164.
[32] S. Bogialli, M. Bruno, R. Curini, A.D. Corcia, C. Fanali, A. Laganà, Environ. Sci. Technol. 40 (2006) 2917–2923.
[33] R. Guzmán-Guillén, A.I. Prieto, A.G. González, M.E. Soria-Díaz, A.M. Cameán, Environ. Toxicol. Chem. 31 (2012) 2233–2238.
[34] R. Guzmán-Guillén, A.I. Prieto Ortega, I. Moreno, A.G. González, M.E. Soria-Díaz, V. Vasconcelos, A.M. Cameán, Talanta 100 (2012) 356–363.
[35] P. Gallo, S. Fabbrocino, M.G. Cerulo, P. Ferranti, M. Bruno, L. Serpe, Rapid Commun. Mass Spectrom. 23 (2009) 3279–3284.
[36] M.L. Saker, J.S. Metcalf, G.A. Codd, V.M. Vasconcelos, Toxicon 43 (2004) 185–194.
[37] J.P. Berry, O. Lind, Toxicon 55 (2010) 930–938.
[38] V. Messineo, S. Melchiorre, A. Di Corcia, P. Gallo, M. Bruno, Environ. Toxicol. 25 (2010) 18–27.
[39] A.G. González, M.A. Herrador, Trends Anal. Chem. 26 (2007) 227–238.
[40] A.G. González, M.A. Herrador, A.G. Asuero, Talanta 82 (2010) 1995–1998.
[41] A.J. Foss, M.T. Aubel, Toxicon 70 (2013) 54–61. http://dx.doi.org/10.1016/j.toxicon.2013.04.007.
[42] H. Liu, P.M. Scott, Food Addit. Contam. 28 (2011) 786–790.
[43] R. Banker, S. Carmeli, O. Hadas, B. Teltsch, R. Porat, A. Sukenik, J. Phys. 33 (1997) 613–616.
[44] ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology, ICH Working Group, November 2005. ⟨http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf⟩.
[45] W.Y. Youden, Statistical Techniques for Collaborative Tests, AOAC Inter, Washington DC, USA, 1967.
[46] Association of Official Analytical Chemists. Peer Verified Methods Program, Manual on Policies and Procedures, AOAC, Arlington, VA, USA, 1998.
[47] L. Huber, Validation and Qualification in Analytical Laboratories, Interpharm, East Englewood, CO, USA, 1998.
[48] V. Ríos, I. Moreno, A.I. Prieto, M. Puerto, D. Gutiérrez-Praena, M.E. Soria-Díaz, A.M. Cameán, Food Chem. Toxicol. 57 (2013) 170–178.
[49] IUPAC Analytical Chemistry Division, Compendium of Analytical Nomenclature (The IUPAC 'Orange Book'), Prepared for Publication by Inczédy, in: J. Lengyel, T. Ure, A.M. (Eds.), third ed.,Blackwell Science, Ltd., Oxford, UK, 1998.
[50] E. Karageorgou, V. Samanidou, J. Chromatogr. A (2014), http://dx.doi.org/10.1016/j.chroma.2014.01.050 (in press).
[51] J.C. Martins, V.M. Vasconcelos, J. Toxicol. Environ. Health B: Crit. Rev. 12 (2009) 65–82.
[52] V.M. Vasconcelos, Aquat. Toxicol. 32 (1995) 227–237.
[53] V. Vasconcelos, S. Oliveira, F.O. Teles, Toxicon 39 (2001) 1461–1470.
[54] S.H. White, L.J. Duivenvoorden, L.D. Fabbro, G.K. Eaglesham, Toxicon 47 (2006) 497–509.
[55] S.H. White, L.J. Dulvenvoorden, L.D. Fabbro, G.K. Eaglesham, Environ. Pollut. 147 (2007) 158–167.
[56] S. Kinnear, Mar. Drugs 8 (2010) 542–564.
[57] C. Moreira, J. Azevedo, A. Antunes, V. Vasconcelos, J. Appl. Microbiol. 114 (3) (2013) 605–620.
[58] J.P. Berry, A. Jaja-Chimedza, L. Dávalos-Ling, O. Lind, Food Addit. Contam. Part A: Chem. Anal. Control. Exp. Risk Assess. 29 (2012) 314–321.
[59] M. Yilmaz, E.D. Philips, N.J. Szabo, S. Badylak, Toxicon 51 (2008) 130–139.
[60] V. Ríos, I. Moreno, A.I. Prieto, M.E. Soria-Díaz, J.E. Frías, A.M. Cameán, J. Water Health 12 (1) (2014) 69–80.
[61] P.G. Van Buynder, T. Oughtred, B. Kirby, S. Phillips, G. Eaglesham, K. Thomas, M. Burch, Environ. Toxicol. 16 (2001) 468–471.

CAPÍTULO 5 / CHAPTER 5

R. Guzmán-Guillén, A. I. Prieto, V. M. Vasconcelos, A. M. Cameán

CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES OXIDATIVE STRESS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUB-CHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS)

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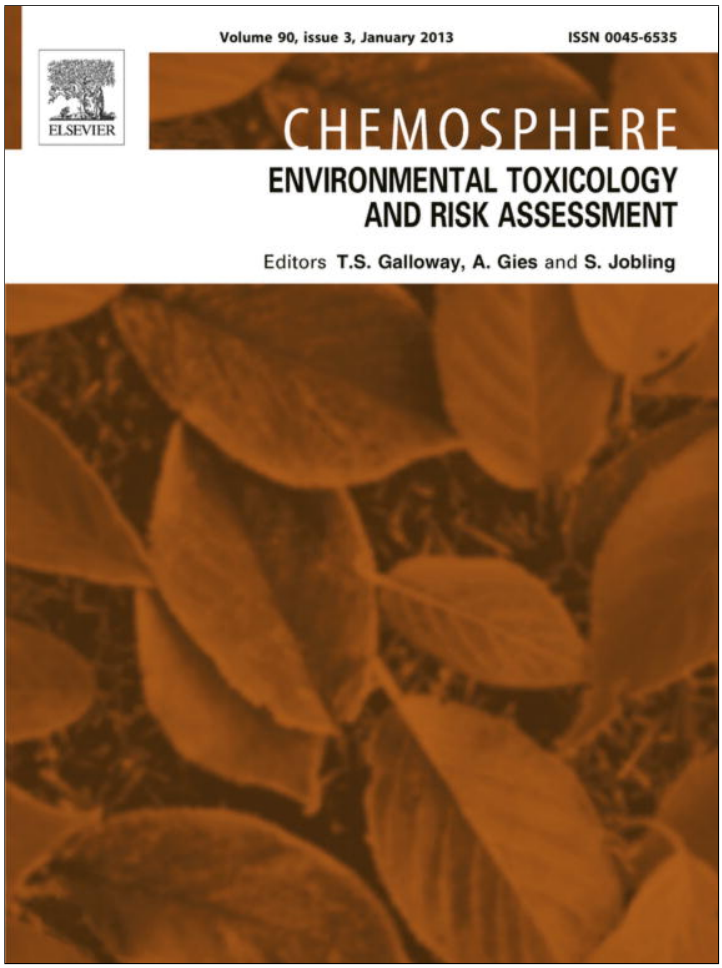

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Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*)

R. Guzmán-Guillén [a], A.I. Prieto [a], V.M. Vasconcelos [b,c], A.M. Cameán [a,*]

[a] *Area of Toxicology, Faculty of Pharmacy, University of Seville, Spain*
[b] *Centro Interdisciplinar de Investigação Marinha e Ambiental, CIIMAR/CIMAR, Universidade do Porto, Portugal*
[c] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal*

HIGHLIGHTS

- Cyanobacterial cells containing CYN and deoxy-CYN induce oxidative stress in fish.
- Kidney of tilapia exposed to cells was more affected than the liver.
- The lowest concentration assayed (10 μg L^{-1}) caused damages after 7 d of exposure.
- Toxicity of CYN- and deoxy-CYN-containing cells was time and dose-dependent.
- Exceeding 10 μg CYN or deoxy-CYN L^{-1} would be considered of high risk for fish.

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ABSTRACT

Cylindrospermopsin (CYN) is a potent cyanobacterial cytotoxin produced by certain freshwater cyanobacteria. Structurally, it is an alkaloid with a tricyclic guanidine moiety combined with hydroxymethyluracil. It has proved to be a potent inhibitor of protein synthesis, and to deplete hepatic glutathione. Recently, some studies have shown that CYN produces changes in some oxidative stress biomarkers in fish acutely exposed to pure CYN by oral and intraperitoneal (i.p.) routes. In the present study tilapia (*Oreochromis niloticus*) were exposed by immersion to lyophilized *Aphanizomenon ovalisporum* cells added to the aquaria using two concentration levels, 10 or 100 μg CYN L^{-1}, during two different exposure times: 7 and 14 d. Fish were sacrificed and liver and kidney were extracted. The oxidative status of fish was evaluated by analyzing in both organs the following biomarkers: lipid peroxidation (LPO), protein oxidation, DNA oxidation, reduced-oxidized glutathione ratio (GSH/GSSG), and changes in the activity of Glutathione-S-transferase (GST), Glutathione Peroxidase (GPx), Superoxide dismutase (SOD), Catalase (CAT), and γ-Glutamyl-cysteine synthetase (GCS). In general, major changes were observed in tilapia treated with 100 μg CYN L^{-1} after 14 d of exposure. However, some endpoints were altered at the lowest concentration assayed only after 7 d of exposure, such as DNA oxidation and γ-GCS in kidney, and CAT and GSH/GSSG decrease in the liver and kidney. The kidney was the most affected organ. These findings confirm that the oxidative stress play a role in the pathogenicity induced by CYN in this fish species, and the results obtained could be useful for future ecotoxicological risks assessment studies, for the protection of fish and aquatic ecosystems. To our knowledge this is the first study dealing with the oxidative stress changes induced by cyanobacterial cells containing CYN and its derivative deoxy-CYN on fish exposed sub-chronically under laboratory conditions.

1. Introduction

The occurrence of cyanobacterial blooms in aquatic environments is increasing in many regions of the world with progressive eutrophication of water bodies and climate change (Zegura et al., 2011). The presence of the cyanotoxin cylindrospermopsin (CYN) in drinking water sources from different countries has caused

* Corresponding author. Address: Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González no. 2, 41012 Seville, Spain. Tel.: +34 954 556762; fax: +34 954 233765.
E-mail address: camean@us.es (A.M. Cameán).

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human poisonings in Australia and Brazil (Humpage et al., 2005). CYN is rapidly being recognized as one of the most globally important freshwater cyanobacterial toxins. The ever-expanding distribution of CYN producers into temperate zones is increasing the concern about the risks on the human and environmental health across many countries (Kinnear, 2010).

CYN is a toxin that can be produced by particular strains of *Aphanizomenon ovalisporum* (Banker et al., 2001), *Cylindrospermopsis raciborskii* (Padisák, 1997) *Umezakia natans* (Harada et al., 1994), and other freshwater cyanobacterial species belonging to the genera *Anabaena*, *Raphidiopsis* and *Lyngbya* (Li et al., 2001; Seifert et al., 2007; Pearson et al., 2010). Structurally, CYN is a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992), highly soluble in water and with a relatively low molecular weight – 415 Da (Sivonen and Jones, 1999). Recently, structural variants of CYN have been isolated from *C. raciborskii* and *A. ovalisporum* strains. The first variant was identified as deoxy-cylindrospermopsin (deoxy-CYN), which appears to be non-toxic (Norris et al., 1999); while 7-epicylindrospermopsin, isolated from *A. ovalisporum*, shows toxicity (Banker et al., 2001). Nevertheless, both variants need to be considered in CYN risk assessment process.

CYN, unlike many other cyanotoxins, it is often present in the extracellular (non-cell bound) form, reaching up to 90% of total CYN. Field studies have reported maximum concentrations of CYN in water reaching values of 1.1 and 800 $\mu g\ L^{-1}$ in Australia (Shaw et al., 2000; Hoeger et al., 2004), 18.4 $\mu g\ L^{-1}$ in Italy (Bogialli et al., 2006), 9.4 $\mu g\ L^{-1}$ in Spain (Quesada et al., 2006), 97.7 $\mu g\ L^{-1}$ in USA (Florida) (Burns et al., 2002), and 12.1 $\mu g\ L^{-1}$ in Germany (Rücker et al., 2007).

The CYN mechanism of toxicity is not fully elucidated yet, although it is known that it is mediated by protein synthesis inhibition, as well as genotoxicity by DNA fragmentation (Humpage, 2008; Bazin et al., 2010). Moreover, a depletion of hepatic glutathione has been proved in mouse and fish in vivo (Gutierrez-Praena et al., 2011a; Norris et al., 2002) and in vitro (Gutierrez-Praena et al., 2011b). More recently, some studies have shown that pure CYN is able to induce oxidative stress in tilapia fish (*Oreochromis niloticus*) when they are exposed to a single dose of CYN (Puerto et al., 2011; Gutiérrez-Praena et al., 2011a) by oral route and intraperitoneal (i.p.) injection (Gutiérrez-Praena et al., 2012). However, there are still very few studies in aquatic animals in order to show the involvement of oxidative stress as a mechanism of action of CYN.

The main target of CYN is the liver, but other organs such as the kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and the heart might be affected (Terao et al., 1994; Falconer et al., 1999; Humpage et al., 2000). Generally, exposure to the toxin results in delayed toxicity (Ohtani et al., 1992). The effects of CYN have been studied in mammalian species, and more recent studies of CYN have expanded toxicity models to show effects in phytoplankton, zooplankton and other invertebrates (Kinnear, 2010). There is considerable variability amongst the toxicity of CYN between different animal models (Saker et al., 1999) and even between different individuals of the same species (Seawright et al., 1999).

The possibility of CYN accumulation in the aquatic fauna of natural lakes is a serious concern, especially when the organisms can be used to human consumption. In fact, a high level of variability exists in CYN accumulation by different aquatic animals and, also, the relationship between accumulation of CYN and toxic effects seems to be inversely proportional (Kinnear et al., 2009). Thus, the general order of bioaccumulation capacity would be gastropods > bivalves > crustaceans > amphibians > fish. And the reverse relationship appears to be true for the susceptibility of organisms to CYN toxicity (Kinnear, 2010). The toxicological studies of CYN in fish are very scarce (Berry et al., 2009), and LD50 data have been not reported, in spite of their likely exposure to CYN. In fact, CYN bioaccumulation has been demonstrated in freshwater mussels (*A. cygnea)* (Saker et al., 2004), redclaw crayfish (*Cherax quaricarinatus*) and rainbow fish (*Melanotaenia eachamensis*) from both aquaculture ponds and laboratory conditions (Saker and Eaglesham, 1999). Thus, there is a need to study more closely the effects that CYN has on a wide range of target organisms (Kinnear, 2010).

Recently, we have demonstrated that acute exposure by oral route (gavage) to 200 and 400 μg pure CYN kg^{-1} bw fish induced dose-dependent histopathological effects, in the liver, kidney, heart, intestines, and gills of tilapia (*Oreochromis* sp.) (Puerto et al., in press). Moreover, we have shown the influence of the exposure route (gavage and i.p. injection) and the time of sacrifice (24 h and 5 d) on the effects of pure CYN on oxidative stress biomarkers using tilapia (*O. niloticus*) as experimental model (Gutierrez-Praena et al., 2011a). Tilapia exposed to pure CYN showed histopathological changes in different tissues (liver, kidney, heart, gills) more severe after 5 d of exposure in comparison to 24 h (Gutiérrez-Praena et al., 2012). In all these reports, tilapia were acutely exposed to a single dose of pure CYN.

Other important issue to consider is the differential toxicity between pure CYN and CYN extracted or contained in cyanobacterial material (cultures, blooms). Toxicity differences in potency have been shown when comparing the toxicity of the pure CYN with the extracts from natural blooms. Thus, Seifert et al. (2007) demonstrated that significant adverse effects were rarely recorded for CYN exposure concentrations below 100 $\mu g\ L^{-1}$ of pure toxin, whereas greater sublhetal toxicities were found after exposure to *C. raciborskii* extracts. This suggests that cell extracts – and hence, field populations of CYN-producing blooms – are likely to contain one or more bioactive compounds other than CYN, increasing the risk of toxic effects (Kinnear, 2010). This situation has also been documented by other authors (Hawkins et al., 1997; Falconer et al., 1999; Norris et al., 1999). On natural conditions, therefore, fish are exposed to cyanobacterial blooms for sub-chronic periods, but on the other hand, CYN toxicological studies for long exposure periods are very scarce at the time (Masten and Carson, 2000), and non-existent in the case of fish.

Taking all these data into account, the aim of the present study was test the hypothesis that tilapia exposed to CYN containing cells at environmentally relevant concentrations suffer from oxidative stress and that the effects are time and concentration dependant. We exposed tilapia sub-chronically to *A. ovalisporum* lyophilized cells from a natural bloom, by immersion route under laboratory conditions, at two periods, 7 or 14 d. Two cyanobacterial cells densities have been assayed, equivalent to 10 and 100 μg CYN L^{-1} water, in order to know the sensitivity of the fish to this toxin. The following biomarkers were assayed: LPO, protein and DNA oxidation, and the response of the antioxidant enzymes GST, GPx, SOD, CAT, GCS, and the GSH/GSSG ratio, in liver and kidney of the fish. This investigation would allow to determine the involvement of oxidative stress as a mechanism of toxicity, and the results may help us to evaluate if there is a slow and gradual poisoning in fish induced by CYN by this route of exposure.

2. Materials and methods

2.1. Chemicals

CYN standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in water milli Q (100 $\mu g\ mL^{-1}$) and diluted as required for their use as working solutions (0.08–5.0 $\mu g\ mL^{-1}$). All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 $M\Omega\ cm^{-1}$ resistivity) was obtained from a Milli-

Q water purification system (Millipore, Bedford, USA). BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

2.2. A. ovalisporum culture and determination of cyanobacterial toxins

A. ovalisporum (LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was originally isolated from Lake Kinneret (Banker et al., 1997) and supplied by the Marine Research Center – CIIMAR (Porto, Portugal). A culture of this strain was maintained in Z8 medium at 25 °C under continuous illumination with an intensity of 28 μmol photons m^{-2} s^{-1} provided by cool white fluorescent tubes. After 33 d, cultures were harvested by decantation with a plankton net (20 μm diameter). The biomass obtained was frozen at −80 °C until lyophilization (Telstar Cryodos, Madrid).

CYN extraction from the lyophilised culture of *A. ovalisporum* (CYN+) was performed based on Guzmán-Guillén et al. (2010). Briefly, the lyophilized cells (14 mg) were extracted three times with 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated for 15 min. The resulting mixture was centrifuged at 4500 r.p.m. for 10 min, after which the supernatant was collected and 6 μL of 0.1% trifluoroacetic acid (TFA) were added. Then, it was stirred for 1 h and allowed to stand for 3 h. The supernatant was taken for further purification/concentration. For the clean-up procedure, graphitized carbon cartridges (Bond Elut®) were activated with 10 mL of a solvent mixture of DCM/MeOH (10/90) and rinsed with 10 mL of MilliQ water. Subsequently, the sample was passed through the cartridges, washed with 10 mL of MilliQ water and eluted with 10 mL DCM/MeOH (10/90). For concentration of the sample, extract was evaporated in a rotary evaporator and resuspended in 500 μL MilliQ water, prior to its LC–MS/MS analysis.

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 × 2.1 mm Zorbax Sb-Aq column. The flow rate was 0.2 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90% B (5 min) and finally 0% B (5 min).

Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions for the detection of CYN are: 416.2/194.0, 416.2/274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For LC–ESI–MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

CYN was detected (retention time of 7.55 min) and quantified (Fig. 1), and the concentration of CYN obtained was 8.7 μg CYN mg^{-1}. Moreover, its deoxy-derivative (deoxy-CYN) has been also detected in the sample culture, with a retention time of 7.76 min. In this case, as no pure standard of this isomer was available, the quantification of deoxy-CYN was made using the CYN calibration curve (equivalent of CYN), and the concentration obtained was 0.4 μg deoxy-CYN mg^{-1}.

2.3. Experimental setup and acclimation of fish

Male *O. niloticus* (Nile tilapia, Perciformes: *Cichlidae)* were used to conduct our studies. They were obtained from a fish hatchery "Aquaculture Valencia" and maintained at the University of Córdoba to reach the average weight of 50 ± 8 g and 12 ± 2 cm length. Fish were transferred to the laboratory, where they were held in aquariums (eight individuals/aquarium) with 96 L of freshwater. Exposure to chlorine was minimized by filling the tanks at least 3 d before the fish were introduced. The aquariums were also set up with continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges (Bio-Espumador)) and the temperature was kept constant (21 ± 2 °C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg L^{-1}. Mean values for additional parameters of water quality were: pH 7.6 ± 0.3, conductivity 287 μS cm^{-1}, Ca^{2+} 0.60 mM L^{-1} and Mg^{2+} 0.3 mM L^{-1}. Fish were fed with commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) and were acclimatized for 15 d before the beginning of the experiments.

2.4. Experimental exposure

After the acclimation period, fish were held in six aquaria ($n = 8$) and treated as follows: for each period of exposure time considered (7 or 14 d), fish in two aquaria were fed with commercial fish food, and were exposed by immersion once to an adequate quantity of cyanobacterial cells in the beginning of the experiment, in order to obtain 10 or 100 μg CYN L^{-1} water (0.46 or 4.6 μg deoxy-CYN L^{-1}), respectively. Due to the scarce toxicity data of CYN on fish, these concentrations were selected in accordance with our previous experiments carried out in this fish species, in which 200 μg kg^{-1} bw fish/day administered by gavage induced damage (Gutiérrez-Praena et al., 2011a; Puerto et al., 2011). Moreover, both concentrations assayed are relevant from an environmental point of view (Kinnear, 2010; Seifert et al., 2007). According to the concentration of CYN in the culture analyzed, 110 and 1103 mg of lyophilized *A. ovalisporum* cells (CYN+) were taken, which were previously mixed with 100 mL of water and sonicated for 15 min to promote cell lysis. CYN levels in the aquaria throughout both exposure periods were controlled based on Guzmán-Guillén et al. (2012) using Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). After 24 h of exposure the initial concentrations of CYN decreased approximately 40% and 30% for 10 or 100 μg L^{-1}, respectively, and they were maintained until the end of the experiment (7 or 14 d). All fish were fed with 0.3 g d^{-1} of commercially prepared fish food for each exposure period, and were sacrificed at the two periods of time indicated, 7 or 14 d.

Two control groups of fish ($n = 8$) were administered only the commercial fish food during the whole experiment and were sacrificed in two different periods of time, 7 d and 14 d, at the same time as exposed fish.

2.5. Preparation of postmitochondrial supernatant (PMS)

After the exposure fish were sacrificed by transection of the spinal cord, previously anesthetized in ice. The liver and kidney were removed, weighed, rinsed with ice-cold saline and kept at −85 °C until analysis. Enzyme extracts from each tissue were prepared from each individual (not pooled) according to the method described by Puerto et al. (2009). Briefly, tissues were homogenized using 0.1 M potassium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetra-acetic acid, and 1.4 mM dithioerythritol. After removal of cell debris (10 min at 13 000*g*), the membrane fraction was separated by centrifugation at 105 000*g* for 60 min. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for subsequent determination of the oxidative stress parameters.

2.6. Lipid peroxidation

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman, 1990).

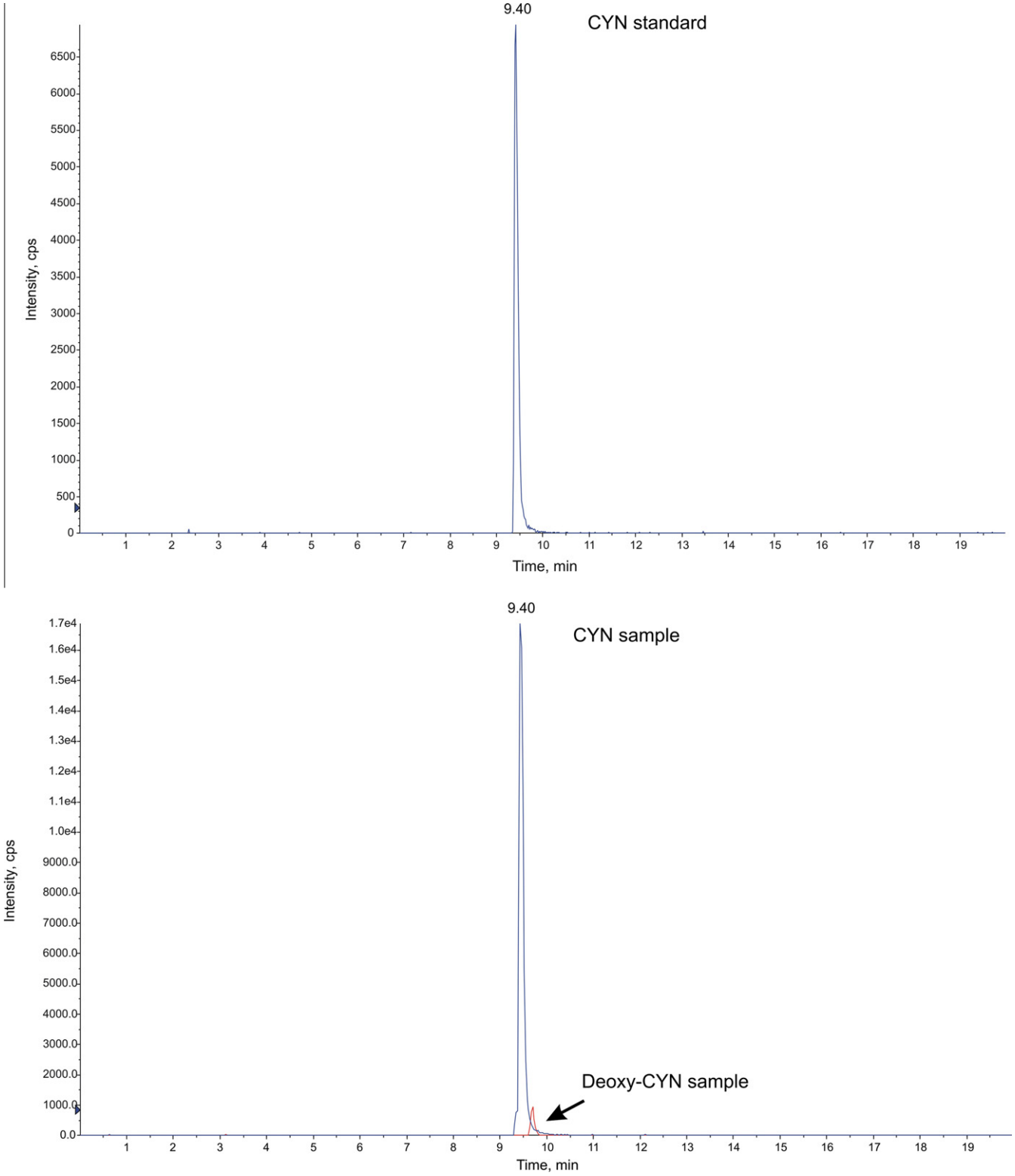

Fig. 1. HPLC–MS/MS Chromatograms of CYN standard, and CYN and deoxy-CYN results from a diluted extract of the *Aphanizomenon ovalisporum* culture analyzed.

Malondialdehyde (MDA) is formed like an end lipid peroxidation product with reacts with TBA reagent under acidic conditions to generate a pink colored product. Briefly, the homogenized tissue (0.5 mL), previously treated with 25 μL of butylhydroxytoluene 1% vol/vol in glacial acetic, was mixed with 0.2 mL of sodium laurylsulphate (8%), 1 mL of acetic acid (20% vol/vol) and 1 mL of 0.8% thiobarbituric acid. This mixture was then heated at 95 °C for 30 min. The resulting chromogen was extracted with 3 mL of *n*-butyl alcohol and, after centrifugation (1500*g* for 10 min), the absorbance of the organic phase was determined at 532 nm. 1,1,3,3-tetraethoxypropane (TEP) was used as a standard. Values were presented as nmol TBARS g^{-1} tissue.

2.7. Protein oxidation: quantification of carbonyl groups

Carbonyl groups of proteins were quantified by the method described by Levine et al., 1990. The purpose of this method is to measure the amount of carbonyl groups derived from the oxidation of proteins present in the sample, where the 2,4-dinitrophenylhydrazine reacts with the carbonyl groups of proteins forming 2,4-dinitrophenylhydrazone, a colored complex that absorbs at 366 nm. From the regression curve and the Lambert–Beer law, the concentration of carbonyl groups present in the sample is calculated, using a molar extinction coefficient (ε) of 22 000 M^{-1} cm^{-1}. The result is expressed as nmoles of carbonyl groups mg^{-1} protein.

2.8. DNA oxidation

For DNA oxidation the OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP sites) (Cell Biolabs, INC., San Diego, CA, USA) was used. Previously the DNA was isolated from the samples using DNAzol® following the manufacturer's instructions (Invitrogen, CA, USA). Results are expressed as apurinic/apyrimidinic (AP) sites per 100 000 bp.

2.9. Antioxidant enzymes

Glutathione-S-transferase activity (sGST; EC 2.5.1.18) was measured in the liver and renal homogenates according to the method described by Habig et al. (1974), by monitoring at 340 nm the formation of a conjugate between 16 mM GSH and 16 mM 1-chloro-2,4-dinitrobenzene (CDNB). Assays were performed in a reaction mixture containing 1.75 mL of phosphate buffer 0.2 M, 100 μL CDNB 16.4 mM, 100 μL GSH 16.4 mM and 50 μL tissue homogenate. The enzymatic activity was expressed in nkat mg^{-1} protein.

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Puerto et al., 2011). The specific activity was determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using the xanthine-oxidase-cytochrome *c* method as described by McCord and Fridovich (1969). The reactions between xanthine and xanthine oxidase, and 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) led to superoxide radicals which reacted to form a red colored formazan. In the presence of SOD in the medium, superoxide radicals were removed and the formation of formazan was therefore inhibited. SOD activity was measured spectrophotometrically at 505 nm, calculated as inhibition percent of formazan formation and expressed as nkat mg^{-1} protein.

Catalase (CAT; EC 1.11.1.6) activity was assayed by the method of Beers and Sizer (1952). The reduction of hydrogen peroxide was followed spectrophotometrically at 240 nm, using 1.0 mL quartz cuvettes with a light path of 1.0 cm. Results are expressed in terms of nkat mg^{-1} protein.

Gamma-glutamyl-cysteine synthetase (EC 6.3.2.2.) is the rate limiting enzyme in GSH synthesis. Its activity was measured using the method described by Seelig and Meister (1985), adapted to fish samples (Gutiérrez-Praena et al., 2011a). The formation of ADP was monitored spectrophotometrically by a coupled assay with pyruvate kinase and lactate dehydrogenase. The result of enzyme activity is expressed as nkat mg^{-1} protein.

2.10. Non-enzymatic antioxidant parameters

GSH/GSSG ratio GSH/GSSG ratio was determined in liver and kidney homogenates using a commercial kit (Bioxytech GSH/GSSH-412, Oxis Research, Foster City, CA, USA) adapted to fish tissues as in Atencio et al. (2008a).

2.11. Protein estimation

Protein contents in the samples were estimated by the method of Bradford (1976) using bovine γ-globulin as standard. Briefly, 5 μl of the diluted samples were mixed with 95 μl H_2O and 5 ml Coomassie Brilliant blue dye (Biorad Laboratories, Hercules, USA) and the absorbance was read at 595 nm in the spectrophotometer (Cary100, Varian, Madrid, Spain).

2.12. Statistical analysis

All results were subjected to one-way analysis of variance (ANOVA), and represent means ± SE of eight animals per group. Differences in mean values between groups were assessed by the Tukey's test and were considered statistically different from $p < 0.05$.

3. Results

There was no mortality or visual changes in any of the fish during the performance of the experiment. Waters from the aquaria spiked with the lyophilized *A. ovalisporum* cells presented lower CYN values in comparison with the initial concentrations (10 and 100 μg CYN L^{-1}), and they were kept nearly constant over 7 d (5.5 and 71.5 μg CYN L^{-1}) and 14 d (6.0 and 65 μg CYN L^{-1}).

3.1. Effects of cyanobacterial cells on lipid peroxidation

LPO showed a significant increase in liver of fish treated with 10 and 100 μg CYN L^{-1} only for a exposure period of 14 d (1.7-fold and 1.5-fold, respectively) (Table 1), while in kidney LPO significantly increased in fish treated with the highest dose of CYN after 7 d (1.2-fold) and 14 d (1.4-fold). Furthermore, in the 14 d exposed groups, there was a significant increase in LPO in kidney of fish treated with the highest concentration in comparison to the group treated with the lowest concentration (Table 1).

3.2. Effects of cyanobacterial cells on protein oxidation

Liver and kidney of fish exposed to 100 μg CYN L^{-1} for 14 d showed significant increases in protein oxidation (2.0-fold, and 1.6-fold, respectively) in comparison with the control groups (Table 1). In both organs, these increases are influenced by the time of exposure and the concentration of CYN.

3.3. Effects of cyanobacterial cells on DNA oxidation

In fish treated with 10 μg CYN L^{-1} there was a significant increase in DNA oxidation only in the kidney, after 7 d or 14 d of exposure (Table 1). In addition, the liver and kidney of fish exposed to 100 μg CYN L^{-1} showed significant increases in DNA oxidation in comparison with the control groups after exposure to CYN for 7 d or 14 d (Table 1). In the liver, significant and concentration-dependent increases of this parameter were found in fish exposed at the same time of exposure (7 or 14 d).

3.4. Effects of cyanobacterial cells on antioxidant enzymes

After 7 d of exposure, there was a significant increase of GST activity (1.7-fold) in the liver of fish treated with the highest concentration (Fig. 2A). Similar and significant increases of GST activities were observed after 14 d of exposure at both concentrations assayed in comparison to their respective control groups (2.1-fold and 1.8-fold, for 10 and 100 μg CYN L^{-1}, respectively). However, in kidney of fish treated after 14 d with the highest concentration

Table 1
Lipid peroxidation (nmol TBARS g^{-1} tissue), protein oxidation (nmol Carbonyls mg^{-1} protein) and DNA oxidation (AP sites/100 000 bp) in liver and kidney of tilapia fish (*Oreochromis* sp.) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). The values are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ or $^{**}p < 0.01$ in comparison to control group values, $^{\#\#\#}p < 0.001$ or $^{\#}p < 0.05$ when fish were exposed with different doses (10 or 100 μg CYN L^{-1}) at the same time, and $^{\&\&\&}p < 0.001$ when fish were intoxicated with the same dose (100 μg CYN L^{-1}) at different times of exposure (7 d or 14 d).

Tissue	Time of exposure	Experimental groups	nmol TBARS g^{-1} tissue	nmol Carbonyl mg^{-1} protein	AP sites/100 000 bp
Liver	7 d	Control	2521 ± 361	14.4 ± 5.1	11.6 ± 1.5
		10 μg CYN L^{-1}	3294 ± 889	19.2 ± 3.9	12.5 ± 0.5
		100 μg CYN L^{-1}	2873 ± 155	19.9 ± 3.3	14.1 ± 0.3$^{***\#}$
	14 d	Control	2076 ± 498	15.1 ± 3.3	12.0 ± 1.5
		10 μg CYN L^{-1}	3528 ± 601***	19.1 ± 0.3	13.4 ± 0.3
		100 μg CYN L^{-1}	3210 ± 674**	29.8 ± 5.9$^{***\#\#\#\&\&\&}$	15.0 ± 0.7$^{***\#}$
Kidney	7 d	Control	1368 ± 198	84.9 ± 8.6	13.0 ± 1.1
		10 μg CYN L^{-1}	1535 ± 169	68.1 ± 4.8	14.2 ± 0.2**
		100 μg CYN L^{-1}	1679 ± 126**	85.1 ± 8.3	15.0 ± 0.3***
	14 d	Control	1566 ± 200	99.7 ± 0.7	13.2 ± 0.8
		10 μg CYN L^{-1}	1732 ± 136	86.5 ± 7.5	14.9 ± 0.3***
		100 μg CYN L^{-1}	2240 ± 173$^{***\#\#\#}$	162.1 ± 4.8$^{***\#\#\#\&\&\&}$	15.0 ± 0.3***

there was a significant decrease in GST activity (1.6-fold) compared with the control group, and with the group treated with 10 μg CYN L^{-1} (Fig. 2A).

No changes in GPx activity were observed in the liver of fish exposed (Fig. 2B), whereas a significant increase was observed in kidney of fish treated with the lowest concentration after 14 d (1.9-fold). In this organ, GPx activity significantly increased in fish treated with 100 μg CYN L^{-1} after 7 d (2.2-fold) and 14 d (3.1-fold) in comparison to control group values. In addition, significant differences in GPx activity were observed in fish treated with different concentrations of CYN at the same exposure period.

SOD enzyme activity increased significantly in the liver of fish treated with 100 μg CYN L^{-1} for both exposure times (7 or 14 d) in comparison to fish control groups (1.9-fold and 3.2-fold, respectively) (Fig. 3A). After 14 d of exposure, fish exposed to the 10 μg CYN L^{-1} also showed a significant increase of SOD activity (1.4-fold). The differences were concentration-dependent in both periods (7 or 14 d) and time-dependent for the highest concentration. Kidney showed increased SOD activity (1.2-fold) in fish treated with the highest concentration for a period of 7 d in comparison to control group. By contrast, SOD decreased significantly in fish treated for 14 d, at both concentrations assayed (1.2 and 1.7-fold at 10 and 100 μg CYN L^{-1}, respectively) in a concentration- and time-dependent way.

Regarding to CAT activity, liver and kidney showed a different response. In the liver of fish treated with 100 μg CYN L^{-1} for 7 d, CAT activity increased significantly (1.9-fold) in comparison to control group, but decreased (1.5-fold) after 14 d of exposure in comparison to control fish (Fig. 3B). Liver showed statistical differences between fish subjected to different concentrations at the same time of exposure (7 d), and between fish subjected to 100 μg CYN L^{-1} at different times. In kidney, CAT activity showed significant decreases (ranging between 1.4 and 1.9-fold) in both periods of exposure at the two concentrations of CYN assayed. Furthermore, after 14 d of exposure, CAT activity declined as the concentration of CYN increased.

A significant 2-fold increase of GCS activity was observed in the liver tissue of fish treated with 100 μg CYN L^{-1} for 7 d (Fig. 4A). In the kidney, GCS activity showed major changes (Fig. 4A): an enhanced GCS activity (2.5–3.3-fold) was observed in all groups of fish treated with both concentrations (10 and 100 μg CYN L^{-1}), after exposure to 7 and 14 d in comparison to control fish.

3.5. Effects of cyanobacterial cells on GSH/GSSG ratio

The relationship GSH/GSSG decreased 1.7–1.8-fold in the liver (Fig. 4B) of fish treated with 10 μg CYN L^{-1} for both periods of exposure (7 and 14 d) in comparison to control fish. For groups

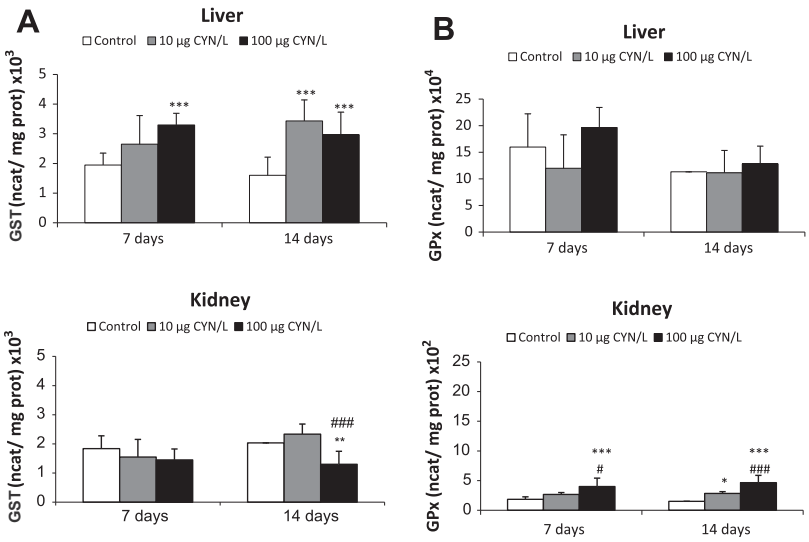

Fig. 2. Glutathione S-transferase (GST) (A) and Glutathione peroxidase (GPx) (B) activities in the liver and kidney of tilapia fish (*Oreochromis* sp.) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). The values are expressed as mean ± SE ($n = 8$). GST and GPx values are expressed as nkat mg^{-1} protein. The significance levels observed are $^{***}p < 0.001$, $^{**}p < 0.01$ or $^{*}p < 0.05$ in comparison to control group values, and $^{\#\#\#}p < 0.001$ or $^{\#}p < 0.05$ when fish were exposed to different doses of CYN at the same time.

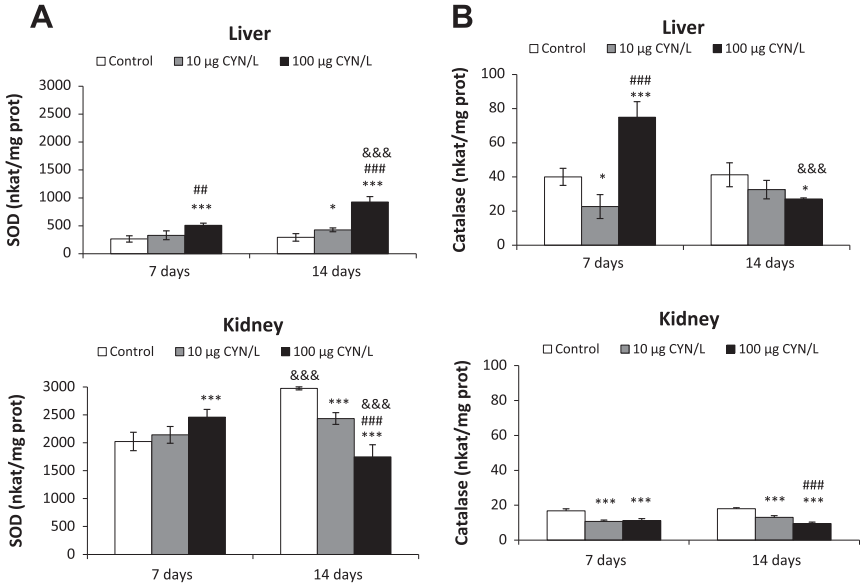

Fig. 3. Superoxide dismutase (SOD) (A) and catalase (CAT) (B) activities in the liver and kidney of tilapia fish (*Oreochromis* sp.) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 µg CYN L^{-1}). The values are expressed as mean ± SE ($n = 8$). SOD and CAT values are expressed as nkat mg^{-1} protein. The significance levels observed are $^{***}p < 0.001$ or $^{*}p < 0.05$ in comparison to control group values; $^{###}p < 0.001$, $^{##}p < 0.01$ or $^{#}p < 0.05$ when fish were exposed to different doses of CYN at the same time, and $^{&&&}p < 0.001$ or $^{&}p < 0.05$ when fish were intoxicated with the same dose of CYN at different times of exposure (7 d or 14 d).

of fish treated with 100 µg CYN L^{-1}, this parameter decreased after a period of 14 d (2.6-fold). The kidney was the most affected organ, showing the highest decrease of GSH/GSSG ratio in all treated groups (10 and 100 µg CYN L^{-1}) and for both periods (7 d and 14 d) in comparison to their control groups (Fig. 4B). These GSH/GSSG ratio changes were concentration-dependent for both periods of time considered (7 d and 14 d), and also time-dependent in fish treated with the same concentration of toxin (10 or 100 µg CYN L^{-1}).

4. Discussion

The emergence and detection of toxic cyanobacteria blooms worldwide lead to an increased need for risk assessment procedures for pollution and toxicity evaluation studies (Bogialli et al., 2006). CYN is being recognized as one of the most globally important of the freshwater cyanobacterial toxins. The effects of CYN have been studied in mammalian species, or their target organs and cells (Seawright et al., 1999; Froscio et al., 2003; Humpage and Falconer, 2003; Rogers et al., 2007), and more recent studies have expanded toxicity models to show effects in fish, such as tilapia (*O. niloticus*) (Gutiérrez-Praena et al., 2011a; Puerto et al., 2011, in press). The comparison of the data available for both mammalian and piscine systems demonstrates that the toxic and adaptive stress are similar across species, and that suggests that fish may serve as good biomonitoring tools, and may be considered useful models for further research to understand oxidative stress (Kelly et al., 1998). Research progress with respect to

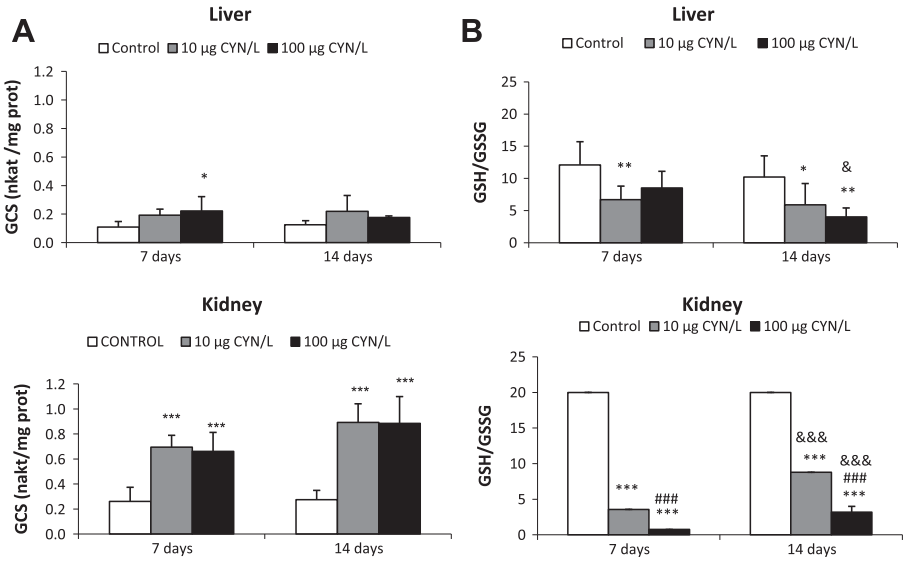

Fig. 4. γ-Glutamyl-cysteine synthetase (GCS) activity (A) and Ratio reduced glutathione-oxidized glutathione (GSH/GSSG) (B) in the liver and kidney of tilapia fish (*Oreochromis* sp.) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 µg CYN L^{-1}). The values are expressed as mean ± SE ($n = 8$). GCS values are expressed as nkat mg^{-1} protein. The significance levels observed are $^{***}p < 0.001$, $^{**}p < 0.01$ or $^{*}p < 0.05$ in comparison to control group values; $^{###}p < 0.001$ when fish were exposed to different doses of CYN at the same time, and $^{&&&}p < 0.001$ or $^{&}p < 0.05$ when fish were intoxicated with the same dose of CYN at different times of exposure (7 d or 14 d).

the mechanism of toxicity of CYN has been also summarized (Humpage, 2008).

Humpage et al. (2005) suggest that oxidative stress and reactive oxygen species (ROS) do not have a role in the toxicity of CYN. This was stated on the basis of the determination of LPO levels without considering other biomarkers such as protein oxidation and antioxidant enzymes activity. More recently, Gutiérrez-Praena et al. (2011b) have shown a relationship between the toxic effects produced by pure CYN and the production of ROS in fish cell lines (PLHC-1) exposed to it. We have previously shown that in vivo acute exposure to pure CYN (200–400 μg kg^{-1} fish) induces stress in tilapia fish exposed by gavage and i.p. injection (Gutiérrez-Praena et al., 2011a,b; Puerto et al., in press), but these results cannot be extrapolated to a sub-chronic exposure. Consequently, oxidative stress should not be excluded as a possible mechanism of toxic action of CYN, and nowadays, there are very few studies in aquatic animals in order to show the involvement of oxidative stress as a mechanism of action of CYN.

For the protection of aquatic ecosystems, Seifert et al. (2007) suggested that an interim trigger level of 100 μg L^{-1} total CYN (extracellular plus intracellular quantities) was an appropriate value. This was based on an ecotoxicity work showing that sublethal and lethal toxicities are rarely significant below this level. In agreement with this author, toxin concentrations exceeding 100 μg L^{-1} were assessed as being of particularly high risk (Kinnear et al., 2009). However, the emerging research indicates that bioaccumulation of CYN can occur even at trace quantities of exposure to the toxin, and consequently, we must be cautious when developing risk assessment guidelines for aquatic ecosystem health (Kinnear, 2010). Thus, in vivo studies to clarify the mechanism and extent of CYN toxicity in aquatic organisms, such as fish, are very useful, especially using environmentally realistic test concentrations in laboratory applications, lower than 100 μg CYN L^{-1}. Furthermore, the toxicity of different analogs, such as CYN compared with deoxy-CYN is quite different (Neumann et al., 2005), hence the need to include both CYN and do-CYN in human and ecological risks assessments (Orr et al., 2010).

In this sense, the present study tries to simulate the natural exposure of tilapias to cyanobacterial cells (*A. ovalisporum*) containing CYN and do-CYN, at two realistic concentrations, 10 and 100 μg CYN L^{-1}. In order to achieve that, tilapia were exposed sub-chronically to the lyophilized material (*A. ovalisporum*) by direct contact in the water. To the extend of our knowledge this is the first study in which cyanobacterial cells containing both toxin variants, are able to induce oxidative stress in fish, after 7 d or 14 d of exposure. The results obtained confirm the oxidative damage induced by CYN in fish sub-chronically expose to the toxin under laboratory conditions, as LPO, protein oxidation, and DNA oxidation levels increased, and significant changes in the studied antioxidant enzymes activities (GST, GPx, SOD, CAT and γ-GCS) were observed. In addition, there was a decrease in the GSH/GSSG ratio for the treated groups.

In this work, we observed a significant increase in LPO levels in liver and kidney mainly with the concentration of 100 μg CYN L^{-1} for 14 d of exposure, although this parameter also increased in kidney for 7 d of exposure. Similarly, Puerto et al. (2011) have shown a significant increase in LPO levels measured in liver and kidney of tilapia exposed by gavage to a single dose of 200 or 400 mg kg^{-1} bw pure CYN, and Gutierrez-Praena et al. (2011a) also found LPO increased in both organs of fish acutely exposed to pure CYN (200 μg kg^{-1}) by gavage and sacrificed after 24 h and 5 d. In vitro, Gutiérrez-Praena et al. (2011b) observed an increase of this biomarker in the PLHC-1 cell line derived from *Poeciliopsis lucida* liver. Humpage et al. (2005) did not observe an increase in malondialdyde (MDA) in rat isolated hepatocytes exposed to 5 μM CYN for 12 h. Jos et al. (2005) evaluated this biomarker in fish exposed sub-chronically to cyanobacterial cells containing Microcystins (MCs) for 14 and 21 d, and found an increase in a time-dependent manner in both organs, and no changes were observed in MDA after 14 d, at the dose assayed (60 μg MC-LR/fish day). Prieto et al. (2007) showed similar results in tilapia treated with a dose of 120 mg MC-LR/fish by oral route, or by i.p. route (Prieto et al., 2006).

Protein oxidation increased significantly in the liver and kidney of tilapia exposed to 100 μg CYN L^{-1} but only after 14 d of exposure. Both organs exhibit a similar response, although Gutiérrez-Praena et al. (2011a) showed more effects on this biomarker in kidney than in liver in fish, after acute exposure to pure CYN.

Similarly to lipid peroxidation, oxidative damage to DNA has received increasing attention in aquatic organisms (Kelly et al., 1998). In the present study, it is remarkable that DNA oxidation was a more sensible biomarker, because significant increases were observed in the kidney at the lowest concentration assayed after 7 d of exposure, and they were maintained after 14 d of exposure. The liver was also affected at the highest concentration assayed after both periods of time considered. These results confirm that although CYN was primarily considered a hepatotoxin (Norris et al., 2002; Berry et al., 2009), it can also affect other organs such as kidney, and some authors considered that kidney appeared to be the more sensitive organ to this toxin in mice. However, no changes in DNA oxidation were observed in the liver and kidney of tilapias acutely exposed to pure CYN by oral route (Gutiérrez-Praena et al., 2011a). This might be due to the different exposure periods and CYN doses used: in the experiment of Gutiérrez-Praena et al. (2011a) tilapias were exposed to a single dose of 200 μg CYN kg^{-1} fish and sacrificed 24 h or 5 d after the exposure, whereas in the present study fish were in contact with cyanobacterial cells containing CYN, do-CYN and other bioactive substances for 7 d or 14 d. Lower CYN doses, given for a longer period of time, may induce DNA damage. Falconer and Humpage (2001) suggest that the presence of the uracil group into CYN could interact with adenine groups in RNA and DNA, interfere with DNA synthesis and therefore could induce mutations and acts as a carcinogen. Some studies show that mixed function oxidases (MFO) are involved in CYN metabolism in the liver, which may generate more toxic metabolites that can attack DNA (Bazin et al., 2010). Another study shows that CYN is genotoxic and probably more hazardous to human and animal health than MCs (Zegura et al., 2011). But today, there is no evidence of the influence of oxidative stress as a mechanism of genotoxicity (Humpage et al., 2005). In this study, the significant increase in DNA oxidation shows, for the first time, the involvement of oxidative stress as a possible genotoxic mechanism of lyophilized cyanobacterial cells containing CYN and do-CYN.sGST are the most widely studied conjugation enzymes in vertebrates. GST and GSH participate in the processes of conjugation of electrophilic substances for subsequent direct excretion or metabolism to mercapturic acids (catabolism) (Habig et al., 1974). This process prevents toxic substances generated (MDA, 4-HNE and short-chain 2-alquenals) from binding proteins and nucleic acids causing cellular damage or mutation, respectively. In this study, GST activity increased significantly in the liver of tilapia exposed to CYN for both periods, and these changes agree with a detoxifying mechanism towards the peroxidised lipids, detected in the liver. Similarly, Puerto et al. (2011) found increased GST activity, gene expression and protein abundance of this enzyme in tilapia after exposure to 200 μg CYN kg^{-1}. By contrast, GST activity decreased significantly in kidney of fish treated with 100 μg CYN L^{-1} for 14 d. This can be explained by a high and more important hepatic conjugation activity with respect to the kidney (Jos et al., 2005), showing the kidney more sensitivity to CYN, although to date, it is not known if sGST is involved in CYN metabolism.

Lipid peroxides are known to be reduced to alcohols by GSH via the activity of the enzyme GPx (Nordberg and Arner, 2001). In the present work, although an increase in LPO products was observed in the liver, GPx activity in this organ did not show any change respect to the control at both doses and periods of time assayed. Similar results were shown in other studies conducted in aquatic organisms exposed to MCs (Li et al., 2003; Jos et al., 2005). The increased activity of GPx in kidney was dose-dependent for both periods considered (7 and 14 d), and these changes could be an adaptive response to compensate the higher injuries induced in the kidney in comparison to the liver.

Among all the antioxidant enzymes studied, the most important changes were generally observed for SOD and CAT activities in the liver and the kidney, as in previous studies performed in tilapia exposed to toxic cyanobacterial cells containing MCs (Prieto et al., 2007). These major changes in both enzymes can be explained because the SOD-CAT system provides the first defence against oxygen toxicity (Atencio et al., 2008a). It is remarkable to indicate that there are not previous studies investigating the effects of cyanobacterial cells (A. ovalisporum) containing CYN on both enzymatic activities. Li et al. (2005) reported a greater variation of SOD and CAT activities in comparison to GPx in the liver when loaches were orally exposed to a Microcystis bloom for 28 d. SOD catalyzes the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide, which is detoxified by the CAT activity, and usually a simultaneous induction response in the activities of both enzymes is observed when exposed to contaminants, and particularly to cyanobacterial cells containing MCs (Jos et al., 2005; Prieto et al., 2007). In the present study, SOD increased in the liver after 7 d of exposure at the highest dose of CYN (100 μg CYN L^{-1}), and maintained its activity increased after 14 d, to counteract ROS formation. The liver has been described as the most important organ involved in the regulation of redox metabolism and it is a target organ of this toxin; therefore, its oxidative status might be affected extensively by CYN. In the kidney, after an initial increase, SOD activity decreased at 14 d. This correlates with an increased susceptibility of the kidney to CYN (higher LPO, protein and DNA oxidation). CAT and GPx act cooperatively as scavengers of hydrogen peroxide (both enzymes) and other hydroperoxides (GPx). In our study, CAT activity seems more actively trying to control those oxyradicals after 7 d of CYN exposure, being GPx activity not induced in the liver. After 14 d, CAT activity decreased, in correlation with an increased oxidative damage. Cazenave et al. (2006) found a dual response of CAT activity in the liver of Corydoras paleatus after exposure to dissolved MC-RR, with an increase at low concentrations and a depletion at higher level. In the kidney, CAT activity diminished at even the lowest concentration of 10 μg CYN L^{-1} after 7 d, and remains decreased under greater exposure to the toxin.

We observed a decrease in GSH/GSS ratio in both organs of fish exposed to the lowest concentration of cyanobacterial cells containing 10 μg CYN L^{-1} after 7 d of exposure, with greater changes in kidney than in liver, and these depletions were maintained after 14 d. Runnegar et al. (1994) observed cell damage preceded by a severe drop in GSH levels associated with CYN toxicity. Norris et al. (2002) demonstrated in vivo that GSH depletion either by conjugation with CYN or by inhibition of its synthesis is unlikely to be of primary importance in hepatic toxicity of CYN in mouse. The more pronounced response observed in the kidney could be explained by increased levels of ROS and its effects (LPO, protein and DNA oxidation) in this organ in comparison with the liver, and consequently, an increased detoxification of ROS that implies the oxidation of GSH to GSSG. Recently, Puerto et al. (in press) have studied the response of GSH levels after acute exposure to pure CYN at different doses, so that at the lowest dose of 200 μg CYN kg^{-1} bw fish, the response was variable depending on the organ, an increase was found in the liver (defensive response with attempts to reduce the oxidative damage by ROS scavenging), while it decreased in the kidney. With the highest dose of 400 μg CYN kg^{-1} bw fish, significant decreases of GSH contents were recorded in both organs. This situation is similar to that presented in this work, after repeated exposure to cyanobacterial cells, at even only 10 μg CYN L^{-1} water, indicating a higher damage induced by CYN.

γ-Glutamylcysteine synthetase activity increased mainly in the kidney of tilapia exposed to CYN, similarly to changes observed in GPx activities. This increased activity of γ-GCS, together with the low GSH/GSSG ratio mostly in the kidney, showed that GSH is not affected in its synthesis. Instead of this, the decreases could be explained because of its role in the antioxidant defense mechanism, such as increased consumption, increased formation of oxidized GSH, increased GSH efflux, etc. By contrast, γ-GCS activity was inhibited in the liver of tilapia exposed to a single dose of 200 μg pure CYN kg^{-1} bw fish by oral and i.p. routes and sacrificed at 24 h; 5 d after the exposure, no statistical differences with respect to the control groups were observed (Gutiérrez-Praena et al., 2011a). Thus, a higher and single dose of pure CYN administered may damage the enzymes, such as GCS activity, while lower doses of cyanobacterial cells (equivalent to 10 or 100 μg CYN L^{-1}), given for a longer period of time in this study, may induce a defensive response. This pattern of behavior has been demonstrated in antioxidant enzyme activities in tilapia depending on whether fish were sub-chronically exposed to MCs (60.0 μg MC-LR/fish/day) for 21 d (Jos et al., 2005), or to a single dose (120.0 μg MC-LR/fish) and sacrificed 24 or 72 h after exposure (Prieto et al., 2007).

Globally, the observed susceptibility of the kidney to the cyanobacterial cells containing CYN and deoxy-CYN compared to liver could be related to the high hydrophilicity of both toxins. In fact, in the case of CYN, Humpage and Falconer (2003) considered that kidney appeared to be the more sensitive organ to this toxin in mice, and studies of the body distribution of ^{14}C-labeled CYN in mice have shown that the main excretory route is through the kidneys, with nearly 50% of an intraperitoneally (i.p.) administered dose appearing in the urine in 6 h, when 20% of the dose was present in the liver (Norris et al., 2001). When comparing to other cyanotoxins, MC-LR administered i.p. caused in tilapia more severe general damage in the liver, whereas the effect of MC-RR (more hydrophilic) was more severe in the kidney (Atencio et al., 2008b). The proximal renal tubular damage in mice (Humpage and Falconer, 2003), in fish (Gutiérrez-Praena et al., 2012) as well as the clinical findings of renal insufficiency in the Palm Island human poisoning incident (Byth, 1980; Griffiths and Saker, 2003), suggest that cytotoxic mechanisms may predominate in the kidney at higher doses. But, the mechanisms behind the kidney damage remain to be explored (Falconer et al., 1999).

5. Conclusion

The present study shows an involvement of oxidative stress as a mechanism of toxic action of CYN in tilapia after sub-chronic exposure to cyanobacterial cells containing cylindrospermopsin (10 and 100 μg CYN L^{-1}) and deoxy-CYN (0.46 and 4.6 μg deoxy-CYN L^{-1}), for 7 and 14 d by immersion route. Alterations in oxidative stress parameters were more pronounced as the concentration of toxins increased, and also in a time-dependent way. In general, more significant changes were observed in tilapia treated with the highest concentration of 100 μg CYN L^{-1} for 14 d. But it is noteworthy that some biomarkers have been changed at the lowest concentration used (equivalent to 10 μg CYN L^{-1}) after only 7 d of exposure, such as increased DNA oxidation and γ-GCS activity (only in the kidney) and decreased CAT activity and GSH/GSSG in the liver and kidney, or after 14 d of exposure (LPO and GST in liver, GPx in kidney, and SOD in liver and kidney). Moreover, in general, the kidney was the main affected organ by exposure to these toxins under laboratory

conditions. Finally, according to the results of this study, we can suggest that oxidative stress could be involved as genotoxic mechanism of CYN. Changes in these oxidative stress biomarkers, therefore, are valuable tools in the assessment of early responses of fish to the increasing occurrence of cyanobacterial blooms containing CYN and its derivatives worldwide. The results obtained could be taken into account in ecotoxicological risks assessments, for the protection of fish and aquatic ecosystems, as it has been demonstrated that cyanobacterial cells containing CYN and deoxy-CYN exceeding 10 μg L^{-1} should be considered of particularly high risk for fish.

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References

Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Camean, A.M., 2008a. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to microcystis under laboratory conditions. Toxicon 52, 1–12.

Atencio, L., Moreno, I., Prieto, A.I., Moyano, R., Molina, A.M., Cameán, A.M., 2008b. Acute effects of Microcystins MC-LR and MC-RR on acid and alkaline phosphatase activities and pathological changes in intraperitoneally exposed tilapia fish (Oreochromis sp.) Toxicol. Pathology 36, 449–458.

Banker, R., Carmeli, S., Hadas, O., Teltsch, B., Porat, R., Sukenik, A., 1997. Identification of cylindropsermopsin in *Aphanizomenon ovalisporum* (cyanophyceae) isolated from Lake Kinneret. Israel J. Physcol. 33, 613–616.

Banker, R., Carmeli, S., Werman, M., Teltsch, B., Porat, R., Sukenik, A., 2001. Uracil moiety is required for toxicity of the cyanobacterial hepatotoxin cylindrospermopsin. J. Toxicol. Environ. Health A 62, 281–288.

Bazin, E., Mourot, A., Humpage, A.R., Fessard, V., 2010. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environ. Mol. Mutagen. 51, 251–259.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L., 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53, 289–299.

Bogialli, S., Bruno, M., Curini, R., Corcia, A.D., Fanali, C., Laganà, A., 2006. Monitoring algal toxins in lake water by Liquid chromatography tandem mass spectrometry. Environ. Sci. Technol. 40, 2917–2923.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burns, J., Williams, C., Chapman, A., 2002. Cyanobacteria and their toxins in Florida surface waters. In: Procceedings of Health Effects of Exposure to Cyanobacterial Toxins: State of the Science. Florida Dep. Of Health, Gainesville, pp. 16–21.

Byth, S., 1980. Palm Island mystery disease. Med. J. Aust. 2, 40–42.

Cazenave, J., Bistoni, M.A., Pesce, S.F., Wunderlin, D.A., 2006. Differential detoxification and antioxidant response in diverse organs of *Corydoras paleatus* exposed to Microcystin-RR. Aquat. Toxicol. 76, 1–12.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonetal. Methods Enzymol. 186, 407–421.

Falconer, I.R., Humpage, A.R., 2001. Preliminary evidence for in vivo tumour initiation by oral administration of extracts of the blue–green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. Environ. Toxicol. 16, 192–195.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J., Hawkins, P.R., 1999. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ. Toxicol. 14, 143–150.

Froscio, S.M., Humpage, A.R., Bucham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ. Toxicol. 18, 243–251.

Griffiths, D.J., Saker, M.L., 2003. The Palm Island mystery disease 20 years on: a review of research on the cyanotoxin cylindrospermopsin. Environ. Toxicol. 18, 78–93.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Cameán, A.M., 2011a. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory condition: Influence of exposure route and time of sacrifice. Aquat. Toxicol. 105, 100–106.

Gutiérrez-Praena, D., Pichardo, S., Jos, M.A., Cameán, A.M., 2011b. Toxicity and glutathione implication in effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotoxicol. Environ. Saf. 74, 1567–1572.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M., 2012. Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral and intraperitoneal route. Ecotoxicol. Environ. Saf. 76, 102–113.

Guzmán-Guillén, R., Rios, V., Prieto, A.I., Moreno, I., Camean, A.M., 2010. Optimisation of the extraction, purification and quantification of cylindrospermopsin form different matrices. Toxicol. Lett. 196S, S346-S346.

Guzmán-Guillén, R., Prieto, A.I., González, A.G., Doria-Díaz, M.E., Cameán, A.M., 2012. Cylindrospermopsin determination in water by LC–MS/MS: Optimization and validation of the method and application to real samples. Environ. Toxicol. Chem 12, 1–6.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Harada, K., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 32, 73–84.

Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R., Falconer, I.R., 1997. Isolation and toxicity of *Cylindrospermosis raciborskii* from an ornamental lake. Toxicon 35, 341–346.

Hoeger, S.J., Shaw, G., Hitzfeld, B.C., Dietrich, D.R., 2004. Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plats. Toxicon 43, 639–649.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice. Determination of No observed adverse effect level fro deriving a drinking water guideline. Environ. Toxicol. 18, 94–103.

Humpage, A.R., Fenech, M., Thomas, P., Falconer, I.R., 2000. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat. Res./Genet. Toxicol. Environ. Mutagen. 472, 155–161.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress. J. Toxicol. Environ. Health A 68, 739–753.

Humpage, A., 2008. Toxin types, toxicokinetics and toxicodynamics. In: Hudnel, H.K. (Ed.), Cyanobacerial harmful algal blooms state of the science and research needs, vol. 619. Springer Press, New York. pp. 383–415.

Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquat. Toxicol. 72, 261–271.

Kelly, S.A., Havrilla, C.M., Brady, T.C., Abramo, K.H., Levin, E.D., 1998. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ. Health Perspect. 106, 375–384.

Kinnear, S.H.W., Duivenvoorden, L.J., Fabro, L.D., 2009. Ecotoxicity and bioaccumulation of toxin from *Cylindrospermopsis raciborskii*: towards the development of environmental protection guidelines for contaminated water bodies. In: Miranda, F.R., Bernards, L.M. (Eds.), Lake Pollution Research Progress. Nova Science Publishers, Inc., New York, pp. 81–105.

Kinnear, S., 2010. Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar. Drugs 8, 542–564.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shatiel, S., Stadman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.

Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y., Watanabe, M.M., 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata*. J. Phycol. 37, 1121–1126.

Li, X., Liu, Y., Song, L., Liu, J., 2003. Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. Toxicon 42, 85–89.

Li, X., Chung, I., Kim, J., Lee, J., 2005. Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and has no effect on lipid peroxidation. Comp. Biochem. Physiol. C 141, 292–296.

Masten, S., Carson, B., 2000. Cylindrospermopsin. National Institute of Environmental Health Sciences P.O. Box 12233 and Integrated Laboratory Systems. P.O. Box 13501. Research Triangle Park, North Carolina 27709.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Neumann, C., Bain, P., Shaw, G., 2005. The in vitro toxicology of deoxycylindrospermopsin. In: Australian Research Network for Algal Toxin Workshop, Moreton Bay Research Station, Australia, 9–11th.

Nordberg, J., Arner, E.S.J., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system free radical. Biol. Med. 31, 1287–1312.

Norris, R.L., Eaglesham, G.K., Pierens, G., Shaw, G.R., Smith, M.J., Chiswell, R.K., Seawright, A.A., Moore, M.R., 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *cylindrospermopsis raciborskii*. Environ. Toxicol. 14, 163–165.

Norris, R.L., Seawright, A.A., Shaw, G.R., Smith, M.J., Chiswell, R.K., Moore, M.R., 2001. Distribution of ^{14}C-cylindrospermopsin in vivo in the mouse. Environ. Toxicol. 16, 498–505.

Norris, R.L.G., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., Chiswell, R.K., Moore, M.R., 2002. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 40, 471–476.

Ohtani, I., Moore, R.E., Runnegar, M., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. Am. Chem. Soc. 114, 7941–7942.

Orr, P.T., Rasmussen, J.P., Burford, M.A., Eaglesham, G.K., Lennox, S.M., 2010. Evaluation of quantitative real-time PCR to characterize spatial and temporal variations in cyanobacteria, *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya et Subba Raju and cylindrospermopsin concentrations in three subtropical Australian reservoirs. Harmful Algae 9, 243–254.

Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynka) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Archiv. Fur. Hydrobiologie 107, 563–593.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar. Drugs 8, 1650–1680.

Prieto, A.I., Jos, A., Pichardo, S., Moreno, I., Cameán, A.M., 2006. Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (*oreochromis* sp.). Aquat. Toxicol. 77, 314–321.

Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M., 2007. Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. Aquat. Toxicol. 84, 337–345.

Puerto, M., Prieto, A.I., Pichardo, S., Moreno, I., Jos, A., Moyano, R., Cameán, A.M., 2009. Effects of dietary N-acetylcysteine (NAC) on the oxidative stress induced in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Environ. Toxicol. Chem. 28, 1679–1686.

Puerto, M., Jos, M.A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852–1860.

Puerto M., Jos A., Pichardo S., Moyano R., Blanco A., Cameán A.M., in press. Acute exposure to pure Cylindorspermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environ Toxicol. http://dx.doi.org/10.1002/tox.21764.

Quesada, A., Moreno, E., Carrasco, D., Paniagua, T., Wormer, L., de Hoyos, C., Sukenik, A., 2006. Toxicity of *Aphanizomenon ovalisporum* (Cyanobacteria) in a Spanish water reservoir. Eur. J. Phycol. 41, 39–45.

Rogers, E.H., Zehr, rD., Gage, M.I., Hupage, A.R., Falconer, I.R., Marr, M., Chernoff, N., 2007. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49, 855–864.

Rücker, J., Stüken, A., Nixdorf, B., Fastner, J., Chorus, I., Wiedner, C., 2007. Concentrations of particulate and dissolved cylindrospermopsin in 21 Aphanizomenon-dominated temperate lakes. Toxicon 50, 800–809.

Runnegar, M., Kong, S.M., Zhong, Y.Z., Ge, J.L., Lu, S.C., 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Biophys. Res. Commun. 201, 235–241.

Saker, M., Eaglesham, G.K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. Toxicon 37, 1065–1077.

Saker, M.L., Thomas, A.D., Norton, J.H., 1999. Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of north queensland. Environ. Toxicol. 14, 179–182.

Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. Toxicon 43, 185–194.

Seawright, A.A., Nolan, C.C., Shaw, G.R., Chiswell, R.K., Norris, R.L., Moore, M.R., Smith, M.K., 1999. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). Environ. Toxicol. 14, 135–142.

Seelig, G.F., Meister, A., 1985. Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. Methods. Enzymol. 113, 379–392.

Seifert, M., McGregor, G., Eaglesham, G., Wickramasinghe, W., Shaw, G., 2007. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. Harmful Algae 6, 73–80.

Shaw, G.R., Seawright, A.A., Moore, M.R., Lam, P.K.S., 2000. Cylindrospermopsin, a cyanobacterial alkaloid, evaluation of its toxicologic activity. Ther. Drug Monit. 22, 89–92.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, M.J. (Eds.), Toxic Cyanobacteria in Water: A guide to Their Public Health Consequences, Monitoring and Management. E & FN Spon, London, pp. 41–111.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M., 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32, 833–843.

Zegura, B., Straser, A., Filipic, M., 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutat. Res. 727, 16–41.

CAPÍTULO 6 / CHAPTER 6

Remedios Guzmán-Guillén, Ana I. Prieto, Isabel Moreno, Vitor M. Vasconcelos, Rosario Moyano, Alfonso Blanco, Ana M. Cameán

CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN CAUSES HISTOPATHOLOGICAL CHANGES AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS IN SUBCHRONICALLY EXPOSED TILAPIA (OREOCHROMIS NILOTICUS)

Environmental Toxicology (in press), **2013**

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Editor Environmental Toxicology
paul.b.tchounwou@jsums.edu

Cyanobacterium Producing Cylindrospermopsin Cause Histopathological Changes at Environmentally Relevant Concentrations in Subchronically Exposed Tilapia (*Oreochromis niloticus*)

Remedios Guzmán-Guillén,[1] Ana I. Prieto,[1] Isabel Moreno,[1] Vitor M. Vasconcelos,[2,3] Rosario Moyano,[4] Alfonso Blanco,[5] Ana M. Cameán Fernandez[1]

[1]Faculty of Pharmacy, Area of Toxicology, University of Seville, Spain

[2]Laboratório de Ecotoxicologia, Genómica e Evoluçã (LEGE), Centro Interdisciplinar de Investigação Marinha e Ambiental, CIIMAR/CIMAR, Universidade do Porto, Portugal

[3]Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

[4]Department of Pharmacology, Toxicology and Legal and Forensic Medicine, University of Córdoba, Campus de Rabanales Carretera Madrid-Cádiz s/n, Córdoba 14071, Spain

[5]Department of Anatomy and Comparative Pathology and Anatomy, University of Córdoba, Campus de Rabanales Carretera Madrid-Cádiz s/n, Córdoba 14071, Spain

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ABSTRACT: The acute toxicity of cylindrospermopsin (CYN) has been established in rodents, based on diverse intraperitoneal an oral exposure studies and more recently in fish. But no data have been reported in fish after subchronic exposure to cyanobacterial cells containing this cyanotoxin, so far. In this work, tilapia (*Oreochromis niloticus*) were exposed by immersion to lyophilized *Aphanizomenon ovalisporum* cells added to the aquaria using two concentration levels of CYN (10 or 100 μg CYN L^{-1}) and deoxy-cylindrospermopsin (deoxy-CYN) (0.46 or 4.6 μg deoxy-CYN L^{-1}), during two different exposure times: 7 or 14 d. This is the first study showing damage in the liver, kidney, hearth, intestines, and gills of tilapia after subchronic exposure to cyanobacterial cells at environmental relevant concentrations. The major histological changes observed were degenerative processes and steatosis in the liver, membranous glomerulopathy in the kidney, myofibrolysis and edema in the heart, necrotic enteritis in the gastrointestinal tract, and hyperemic processes in gill lamellae and microhemorrhages. Moreover, these histopathological findings confirm that the extent of damage is related to the CYN concentration and length of exposure. Results from the morphometric study indicated that the average of nuclear diameter of hepatocytes and cross-sections of proximal and distal convoluted tubules are useful to evaluate the damage induced by CYN in the main targets of toxicity. Environ Toxicol 00: 000–000, 2013.

Correspondence to: A. M. C. Fernández; e-mail: camean@us.es

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INTRODUCTION

Cyanobacteria are ancient organisms that have evolved to adapt to almost every environment on the planet, particularly to freshwater (Ho et al., 2012), which can bloom under favorable conditions. Cyanobacteria produce bioactive metabolites such as potent hepatotoxins, neurotoxins, cytotoxins, and inflammatory agents that can pose serious threats to human and environmental health via drinking water, recreational exposure, and possible accumulation of toxins in the food web (Berry et al., 2009). The presence of the cyanotoxin cylindrospermopsin (CYN) in drinking water sources from different countries has caused human poisonings in Australia and Brazil (Humpage et al., 2005). The first human poisoning episode caused by CYN was recorded in Palm Island (Australia) in November 1979, when an aboriginal community was affected by an outbreak of hepatoenteritis (Griffiths and Saker, 2003). In this outbreak, the organisms in the original bloom of the water supply dam were not identified before treatment with copper sulphate. Retrospectively, the alga *Cylindrospermopsis raciborskii* was subsequently observed as a seasonally dominant species in the domestic water supply reservoir on Palm Island. Its severe hepatotoxic and also wide-ranging effects in mice make it an organism capable of producing the clinical disease seen at Palm Island, and following the suggestions of Hawkins et al. (1985), *C. raciborskii* blooms should be considered as one possible cause. CYN is rapidly being recognized as one of the most globally important freshwater cyanobacterial toxins. The ever-expanding distribution of CYN producers into temperate zones is increasing the concern about the human and environmental health risks worldwide (Kinnear, 2010).

CYN can be produced by strains of *Aphanizomenon ovalisporum* (Banker et al., 2001), *C. raciborskii* (Padisak, 1997), *Umezakia natans* (Harada et al., 1994), and other freshwater cyanobacterial species belonging to the genera *Anabaena, Raphidiopsis*, and *Lyngbya* (Li et al., 2001; Seifert et al., 2007; Pearson et al., 2010). Structurally, CYN is a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992) and is highly soluble in water (Sivonen and Jones, 1999). Structural variants of CYN have been isolated from *C. raciborskii* and *A. ovalisporum* strains. The first variant was identified as deoxy-cylindrospermopsin (deoxy-CYN), which appears to be nontoxic (Norris et al., 1999), whereas 7-epicylindrospermopsin, isolated from *A. ovalisporum*, shows toxicity (Banker et al., 2001). Nevertheless, both variants need to be considered in CYN risk assessment process.

In mammals, it has been demonstrated that the main target of CYN is the liver, but other organs such as the kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, immune system, and heart might also be affected (Terao et al., 1994; Falconer et al., 1999; Humpage et al., 2000). Mutagenicity of CYN was shown *in vitro*, and strong evidence also exists for its carcinogenicity *in vivo* (Fastner et al., 2003; Rücker et al., 2007). The CYN mechanism of toxicity is not fully elucidated yet, although it is known that it is mediated by protein synthesis inhibition, as well as genotoxicity by DNA fragmentation (Humpage, 2008; Bazin et al., 2010). Moreover, a depletion of hepatic glutathione has been proved in mouse and fish *in vivo* (Norris et al., 2002; Gutiérrez-Praena, 2011a) and *in vitro* (Gutiérrez-Praena, 2011b). Recently, some studies have shown that pure CYN is able to induce oxidative stress in tilapia fish (*Oreochromis niloticus*) when they are exposed to a single dose of CYN (Gutierrez-Praena et al., 2011a; Puerto et al., 2011) orally and by intraperitoneal (i.p.) injection (Gutierrez-Praena et al., 2012a).

In general, the risks presented by cyanotoxins in aquatic environments continue to be characterized; however, currently, the quantification of these risks is constrained due to a lack of mammalian oral toxicity data for cyanotoxins other than microcystins (MCs) (Metcalf et al., 2012). There is considerable variability amongst the toxicity of CYN between different animal models and even between different individuals of the same species (Kinnear, 2010). Ecotoxicity studies and bioassays have shown CYN exposure to result in adverse effects on zooplankton and phytoplankton populations, brine shrimp, locusts, and amphibians (Kinnear et al., 2007). However, the toxicological studies on CYN in fish are very scarce (Berry et al., 2009), and LD50 data have not been reported, in spite of their likely exposure to CYN. Recently, we have demonstrated that acute exposure by oral route (gavage) to 200 and 400 μg pure CYN per kilogram bw fish induced dose-dependent histopathological effects, in the liver, kidney, heart, intestines, and gills of tilapia (*Oreochromis sp.*) (Puerto et al., in press). Tilapia

Abbreviations

CYN	cylindrospermopsin
deoxy-CYN	deoxy-cylindrospermopsin
EM	electron microscopy
HPLC	high-performance liquid chromatography
i.p.	intraperitoneal
MC	microcystins
PAS	periodic acid Schiff
TFA	trifluoroacetic acid

exposed to pure CYN showed histopathological changes in different tissues (liver, kidney, heart, and gills) more severe after 5 d of exposure in comparison to 24 h (Gutierrez-Praena et al., 2012a). In all these reports, tilapia was acutely exposed to a single dose of pure CYN.

Other important issue to consider is the differential toxicity between pure CYN and CYN extracts (cultures, blooms). Seifert (2007) demonstrated that significant adverse effects were rarely recorded for CYN exposure concentrations below 100 μg L^{-1} of pure toxin, whereas greater sublethal toxicities were found after exposure to *C. raciborskii* extracts. This suggests that cell extracts—and hence, field populations of CYN-producing blooms—are likely to contain one or more bioactive compounds other than CYN, increasing the risk of toxic effects (Kinnear, 2010). This situation has also been documented by other authors (Hawkins et al., 1997; Falconer et al., 1999; Norris et al., 1999). Fish are exposed in nature to cyanobacterial blooms for subchronic and chronic periods, but on the other hand, CYN toxicological studies for long exposure periods are very scarce at the time (Masten and Carson, 2000), and nonexistent in the case of fish.

Taking all these data into account, the aim of this study was to evaluate the histopathological alterations induced in the liver, kidney, heart, intestinal mucosa, and gills of tilapia (*O. niloticus*) exposed subchronically to cyanobacterial cells containing CYN at environmentally relevant concentrations. We exposed tilapia to *A. ovalisporum* lyophilized cells, by immersion route under laboratory conditions, at two periods, 7 or 14 d. Two cyanobacterial cell densities have been assayed, equivalent to 10 or 100 μg CYN L^{-1} water, to know the sensitivity of this species to this toxin. The results may help us to evaluate if there is a slow and gradual poisoning in tilapia induced by CYN by this route of exposure, and if the effects are concentration and time dependant.

MATERIALS AND METHODS

Chemicals

CYN standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in water milli Q (100 μg mL^{-1}) and diluted as required for their use as working solutions (0.08–5.0 μg mL^{-1}). All chemicals and reagents used in this study were analytical grade materials. high-performance liquid chromatography (HPLC)-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ·cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Bond Elut® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

Aphanizomenon ovalisporum Culture and Determination of Cyanobacterial Toxins

A. ovalisporum (LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was originally isolated from Lake Kinneret, Israel (Banker et al., 1997) and supplied by the Marine Research Center—CIIMAR (Porto, Portugal). A culture of this strain was maintained in Z8 medium at 25°C under continuous illumination with an intensity of 28 μmol photons·m^{-2}·s^{-1} provided by cool white fluorescent tubes. After 33 d, cultures were harvested by decantation with a plankton net (20 μm diameter). The biomass obtained was frozen at −80°C until lyophilization (Telstar Cryodos, Madrid).

CYN extraction from the lyophilized culture of *A. ovalisporum* (CYN+) was performed based on the study by Guzmán-Guillén et al. (2012). Briefly, the lyophilized cells (14 mg) were extracted with 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated for 15 min. The resulting mixture was centrifuged at 4500 r.p.m. (3280 rcf) for 10 minutes, after which the supernatant was collected and 6 μL of 0.1% TFA were added. Then, it was stirred for 1 h and allowed to stand for 3 h. The supernatant was taken for further purification/concentration. For the clean-up procedure, graphitized carbon cartridges (Bond Elut®) were activated with 10 mL of a solvent mixture of DCM/MeOH (10/90) and rinsed with 10 mL of MilliQ water. Subsequently, the sample was passed through the cartridges, washed with 10 mL of MilliQ water, and eluted with 10 mL DCM/MeOH (10/90). For concentration of the sample, extract was evaporated in a rotary evaporator and resuspended in 500 μL MilliQ water, prior to its LC-MS/MS analysis.

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, MA, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, CA, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 × 2.1 mm Zorbax Sb-Aq column. The flow rate was 0.2 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0 % B (1 min), linear gradient to 90% B (10 min), 90 %B (5 min), and finally 0 % B (5 min).

Multiple reaction monitoring experiment was applied where the parent ions (Q_1) and fragments ions (Q_3) were monitored at Q1 and Q3, respectively. For the detection of CYN, the transitions *m/z* are 416.2/194.0, 416.2/274.0, 416.2/336.0, and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For LC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe

temperature was maintained at 350°C. CYN was detected (retention time of 7.55 min) and quantified by LC-MS/MS, and the concentration of CYN obtained was 8.7 μg CYN mg^{-1}. Moreover, its deoxy-derivative (deoxy-CYN) has been also detected in the sample culture (with a retention time of 7.76 min). In this case, as no pure standard of this isomer was available, the quantification of deoxy-CYN was made using the CYN calibration curve (equivalent of CYN), and the concentration obtained was 0.4 μg deoxy-CYN mg^{-1}.

Experimental Setup and Acclimation of Fish

Male *O. niloticus* (Nile tilapia, Perciformes: *Cichlidae)* were used to conduct our studies. The fish were obtained from a fish hatchery "Aquaculture Valencia" and maintained at the University of Córdoba to reach the average weight of 50 ± 8 g and 12 ± 2 cm length. Afterward, fish were transferred to the laboratory (University of Sevilla), where they were held in aquaria (five individuals/aquarium) with 96 L of tap water. Exposure to chlorine was minimized by filling the tanks at least 3 d before the fish were introduced. Aquaria were also set up with continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges (Bio-Espumador)), and the temperature was kept constant (22 ± 3°C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg L^{-1}. Mean values for additional parameters of water quality were as follows: pH 7.6± 0.3, conductivity 287 μS cm^{-1}, Ca^{2+} 0.60 mM L^{-1} and Mg^{2+} 0.3 mM L^{-1}. Fish were fed with commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) and were acclimatized for 15 d before the beginning of the experiments.

Experimental Exposure

After the acclimation period, fish were held in six aquaria ($n = 5$) and treated as follows: for each period of exposure time considered (7 and 14 d), fish in two aquaria were fed with commercial fish food (0.3 g day^{-1} $fish^{-1}$) and were exposed by immersion to an adequate quantity of lyophilized cyanobacterial cells in the beginning of the experiment, to obtain 10 or 100 μg CYN L^{-1} water (and 0.46 or 4.6 μg deoxy-CYN L^{-1}), respectively. According to the concentration of CYN in the culture analyzed, 110 and 1103 mg of lyophilized *A. ovalisporum* cells (CYN+) were taken, which were previously mixed with 100 mL of water and sonicated for 15 min to promote cell lysis. Due to the scarce toxicity data of CYN on fish, these concentrations were selected in accordance with our previous experiments carried out in this fish species, in which 200 μg kg bw $fish^{-1}$ day administered by gavage-induced damage (Gutiérrez-Praena, 2011a; Puerto et al., 2011). Moreover, both concentrations assayed are relevant from an environmental point of view (Seifert et al., 2007; Kinnear, 2010). All fish were sacrificed at the two periods of time indicated, 7 or 14 d. Two control groups of fish ($n = 5$) were administered only the commercial fish food during the whole experiment and were sacrificed at the same time as exposed fish, 7 or 14 d.

After 7 d or 14 d of the exposure, all fish were anaesthetized by immersion in iced water for 5–10 min before they were sacrificed by transection of the spinal cord. The organs, liver, kidney, heart, gastrointestinal tract, and gills were removed and weighed. Waters from the aquaria spiked with the lyophilized *A. ovalisporum* cells presented lower CYN values in comparison with the initial concentrations (10 and 100 μg CYN L^{-1}), and they were kept nearly constant over 7 (5.5 and 71.5 μg CYN L^{-1}) and 14 d (6.0 and 65.0 μg CYN L^{-1}).

Light Microscopy and Electron Microscopy

Tissue samples for histological examination were taken from the liver, kidney, heart, intestines, and gills of control and exposed fish. For light microscopy, samples were first fixed in 10% buffered formalin for 24 h at 4°C, and then immediately dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax by using an automatic processor. Sections of 3–5 μm were mounted. After they had been deparaffinized, the sections were rehydrated, stained with hematoxylin and eosin (H&E), and mounted with Cristal/Mount (Paraplast, Oxford Labware, St. Louis, MO). Liver tissue sections were also stained with periodic acid Schiff (PAS) (Sigma-Aldrich Química, SA, Madrid, Spain) for glycogen content assessment.

For electron microscopy (EM), samples were prefixed in 2% (v/v) glutaraldehyde fixative (in pH 7.4 phosphate buffer for 10 h at 4°C) and postfixed in 1% osmium tetroxide fixative (in pH 7.4 phosphate buffer for 0.5 h at 4°C). Subsequently, they were dehydrated in a graded ethanol series and embedded in epon. Ultra thin sections, 50–60 nm, were cut with a LKB microtome. The sections were mounted on a copper grid and stained with uranylacetate and lead citrate. The tissue sections were examined in a Philips CM10 electron microscope. Gill samples were fixed in glutaraldehyde 2.5% in 0.1 M phosphate buffer for ultrastructural study, postfixed in 2% osmium tetroxide, dehydrated in acetone, critical point dried, ion-sputter coated with gold, and viewed through a scanning electron microscope (JEOL JSM 6300).

Quantitative Study

For the structural quantifications, the fixed liver and kidney were cut into three sections, and each portion was then histologically processed, dehydrated in a graded series of ethanol, immersed in xylol, and embedded in paraffin wax. The first section (4-μm thick) of each block was stained with H&E.

The quantitative study was performed using an image analysis system consisting of a Leitz Ortholux trinocular microscope connected by means of a SONY SSC-C370P (Sony, Spain) and Visilog 5 software (Noesis, S.A., Les Ulis)® color video camera to an IBM-compatible personal

computer equipped with a frame grabber board. Each specimen was sampled systematically for the selection of microscopic images that were then digitized; a 100× lens (N.A. 1.25) was used for this procedure. An average of 20 microscopic fields per slide was chosen in each specimen. Each microscopic image was processed using Visilog 5® software. Quantification was performed by an observer experienced in the use of the analysis system (J.G.M) but with no previous knowledge of which group was being analysed. The system was initially, and regularly, calibrated using a millimeter slide, and then the images were measured to obtain morphometric parameters.

To quantify any alterations on hepatocytes size, the average diameter of the nuclei was employed; in the case of the proximal and distal convoluted tubules, the average of the tubule cross-sections was estimated. The morphometric data

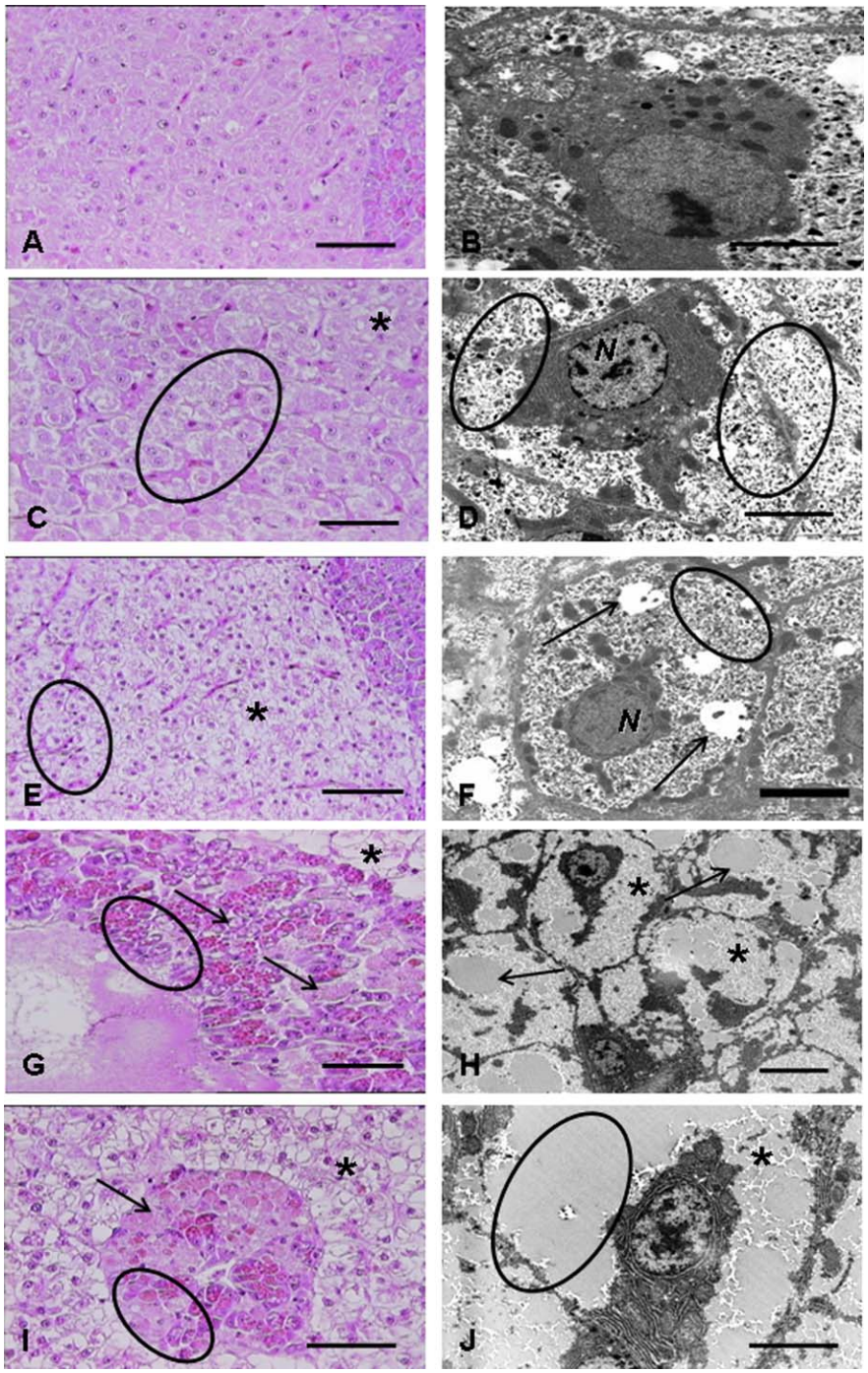

Fig. 1. (Continued)

were obtained by tracing the outline of the profiles of hepatocytes, and the boundary of the tubule cross-sections, with the cursor of the analysis system previously calibrated in the images.

Statistical Analysis

Data were analyzed by applying bivariate comparisons considering nonparametric methods. Differences among groups, with respect to hepatocyte nuclear diameters and convoluted tubules cross-sections diameters values, were tested using the Kruskal–Wallis test, whereas pair-wise differences were compared by the Tukey–Kramer test. The analyses were conducted using the Statistical software (Statistica, version 6. Statsoft inc). All reported p values were two-tailed with $p < 0.05$ considered as significant.

RESULTS

No fish died during the experiment, and no noticeable pathological changes were observed in the organs studied in control fish after microscopic examinations and ultrastructural studies [Figs. 1–5(A,B)].

Globally, the microscopic examination of the H&E-stained liver section of fish intoxicated with CYN revealed degenerative glucogenic processes and steatosis. The liver of fish treated with 10 μg CYN L^{-1} for 7 d showed hepatocytes with clear nucleus and acidophil cytoplasm, with irregular content and lipids [Fig. 1(C)]. Ultrastuctural study [Fig 4(D)] showed hepatocyte with rounded nucleus surrounded by scarce cytoplasm and organelles, and abundant glycogen. These alterations were more pronounced in fish treated with the same concentration for 14 d [Fig 1(G,H)], and also degranulated cells together vacuolization of cytoplasm were observed in the pancreatic area. Moreover, in fish intoxicated with 100 μg CYN L^{-1}, all these histopathological changes were more severe, especially in fish sacrificed after 14 d of exposure [Fig. 1(E,F,I,J)]. They showed hepatocytes with clear cytoplasm full of glycogen, confirmed by PAS staining, and central nucleus with some lipids accumulation. Moreover, pancreatic cells appeared degranulated and suffered necrosis [Fig.1(I)]. Ultrastructural study clearly showed hepatocytes with central nucleus, scarce cytoplasm with predominant rough endoplasmic reticulum, full of amorphous glycogen, and presence of lipids [Fig. 1(J)].

In general, the kidney of fish intoxicated with CYN revealed the presence of a membranous glomerulopathy, with glomerular capillary atrophy, hyperemia, and interstitial microhemorrhages. Fish have showed tumefacted proximal and distal convoluted tubules after 7 d of exposure, whereas after 14 d of CYN treatment, degeneration and atrophy of tubular cells were observed. Fish intoxicated with 10 μg CYN L^{-1} for 7 d showed dilated fenestrated capillaries and interstitial hemorrhages [Fig. 2(C)]; these lesions were more pronounced when fish were intoxicated for 14 d (with the same concentration), which also showed densifications of fenestrated capillaries of the glomerulus and hyperemia [Fig. 2(G)]. Ultrastructurally, the glomerulus revealed irregular basal membrane and densification in podocyte primary foot processes [Fig. 2(D)]. After 14 of exposure, these lesions were more evident, with partial loss of basal membrane and tumefacted podocyte foot processes [Fig. 2(H)]. In fish intoxicated with the highest concentration of 100 μg CYN L^{-1}, all these histopathological changes were more severe [Fig. 2(E,I)], especially after 14 d of exposure, and were characterized by glomerular atrophy, with dilatation of

Fig. 1. Histopathological changes in liver and pancreas of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). (A, C, E, G, and I) Hematoxylin and eosin–stained liver section. Bars, 100 μm. (B, D, F, H, and J) Ultrastructural observations. Bars, 10 μm. (A and B) Control fish: (A) normal hepatic cords, normal polyhedral hepatocytes appeared with central nucleus and clear cytoplasm; (B) normal hepatocyte with cytoplasmic organelles, and mitochondrias. (C and D) Tilapia exposed to 10 μg CYN L^{-1} for 7 d: (C) parenchymal architecture with necrotic hepatocytes (circle) and presence of lipids (asterisk); (D) hepatocyte with nucleus (N) with rounded edges and cytoplasm with abundant organelles and glycogen accumulation (circle). (E and F) Tilapia exposed to 100 μg CYN L^{-1} for 7 d: (E) liver parenchyma with disperses necrosis of hepatocytes (circle) and presence of glycogen (asterisk); (F) hepatocyte with very dense nucleus (N), surrounded by a scarce cytoplasm full of glycogen (circle) and presence of lipids (arrow). (G and H) Tilapia exposed to 10 μg CYN L^{-1} for 14 d: (G) liver parenchyma with hepatocytes with clear cytoplasm (asterisk), and degranulated cells in the pancreatic zone (arrow) and cytoplasmic vacuoles (circle); (H) hepatocytes with central nucleus surrounded by scarce cytoplasm with few cytoplasmic organelles, full of glycogen (asterisk), and presence of small liposomes (arrow). (I and J) Tilapia exposed to 100 μg CYN L^{-1} for 14 d: (I) hepatopancreas with hepatocytes with clear cytoplasm (asterisk) and degranulated pancreatic cells (arrow) with necrosis (circle); (J) hepatocyte with central nucleus surrounded by scarce cytoplasm with predominant rough endoplasmic reticulum; most of the cytoplasm full of amorphous glycogen (asterisk) and lipids (circle). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the Bowman's capsule, microhemorrhages, and a decrease in the width of proximal and distal convoluted tubules [Fig. 2(I)]. Electronic microscopy revealed glomerulus with irregular and thickened basal membrane [Fig. 2(F)], with even partial loss of basal membrane, irregular distribution of fenestrated epithelium, and tumefacted podocyte foot processes [Fig. 2(J)].

Heart lesions of fish treated with the lowest concentration of CYN (10 μg CYN L^{-1}) were myofibrolysis and edema, especially after 14 d of treatment (Fig. 3). Ultrastructural study showed amorphous substances from the lysis of muscle fibers, edemas, hyperemia, and microhemorrhages. These processes were maintained in the highest concentration (100 μg CYN L^{-1}), although they were more evident, with strong destruction of cardiac fibers, especially after 14 d of exposure to lyophilized cyanobacterial cells.

Overall, the gastrointestinal tract of fish treated with CYN showed a necrotic enteritis with desquamation areas

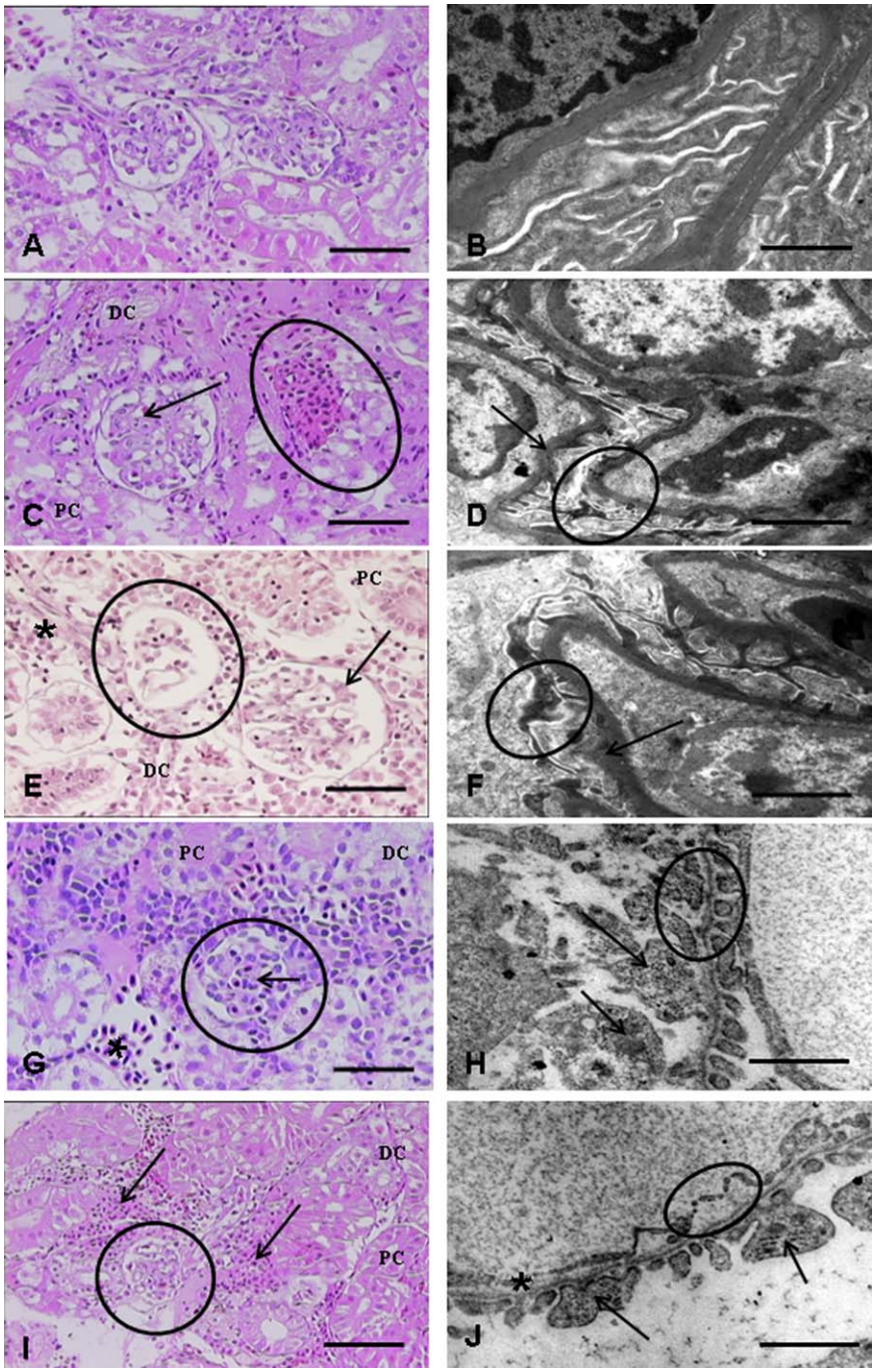

Fig. 2. (Continued)

(Fig. 4). Fish treated with 10 μg CYN L^{-1} showed intestinal villi with necrosis of enterocytes and severe edema of lamina propria. The EM revealed microvilli at the basal zone, cells showing irregular nucleus and detachment of their nuclear membrane, indicative of necrosis process. Fish treated with the highest concentration of 100 μg CYN L^{-1} showed more necrotic enterocytes and desquamation areas, mainly after 14 of exposure to the toxin.

Morphological alterations observed in gills consisted in hyperemic processes in gill lamellae, hemorrhagic cell infiltrates, and microhemorrhages (Fig. 5). Tilapia exposed to 10 μg CYN L^{-1} showed strong hyperemia in branchial arch; the scanning EM images showed irregular lamellae structure, tumefacted and sometimes with loss of their integrity, microhemorrhages, and hemorrhagic cell infiltrates. These lesions were more pronounced in fish treated with the highest concentration of 100 μg CYN L^{-1}.

Regarding the results obtained in the morphometric study, in the liver, average hepatocyte nuclear diameters were augmented in all CYN exposure periods especially at the highest dose assayed (Fig. 6). A significant increase of this parameter was observed in fish exposed to 10 μg CYN L^{-1} after 14 d of exposure (1.4-fold in comparison to its respective control group). At 100 μg CYN L^{-1}, the respective increases of this parameter were 1.2-fold and 1.6-fold after 7 or 14 d of exposure, respectively. Statistical differences were observed between fish subject to the same concentration of CYN and exposed for different times, and between fish subject to either concentration of CYN and sacrificed after 14 d of exposure.

In the kidney, the data concerning the average cross-sections of the proximal convoluted tubules showed a significant increase in CYN-exposed fish to 100 μg CYN L^{-1}, when they were exposed for 7 d [Fig. 7(a)], in comparison to control fish. After 14 d of exposure to CYN, a significant decrease was observed in this parameter, for the highest concentration of CYN assayed. The average cross-section of distal convoluted tubules showed significant increases in fish administered CYN for 7 d (1.3-fold for 100 μg CYN L^{-1}) in comparison to control fish [Fig. 7(b)]. By contrast, when the exposure time was the highest, 14 d, significant decreases of the average cross-sections of these tubules were observed, in comparison to control fish. Overall the effects of CYN were more pronounced when fish were subject to 100 μg CYN L^{-1} for 14 d.

DISCUSSION

CYN is being recognized as one of the most globally important freshwater cyanobacterial toxins, but the data based on its oral toxicity are limited due to a small and insufficient number of studies (EPA, 2011). The effects of CYN have been studied in mammalian species, or their target organs and cells (Seawright et al., 1999; Froscio et al., 2003; Humpage and Falconer, 2003; Rogers et al., 2007), and more recent studies showed effects in fish, such as tilapia (*O. niloticus*) (Gutiérrez-Praena, 2011a; Puerto et al., 2011, in press). We have previously shown that *in vivo* acute exposure to pure CYN (200–400 μg kg^{-1} fish) induces stress and histopathological effects in tilapia fish exposed by gavage and i.p. injection (Gutierrez-Praena et al., 2011a, 2012a; Puerto et al., in press), but these results cannot be extrapolated to a subchronic exposure.

Seifert et al. (2007) suggested that an interim trigger level of 100 μg L^{-1} total CYN (extracellular plus intracellular quantities) was an appropriate value for the protection of aquatic ecosystems,. This was based on an ecotoxicity approach showing that sublethal and lethal toxicities are

Fig. 2. Histopatohological changes in kidney of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). (A, C, E, G, and I) Hematoxylin and eosin–stained kidney section. Bars, 100 μm. (B, D, F, H, and J) Ultrastructural observations. Bars, 10 μm. (A and B) Control fish: (A) normal renal parenchyma; (B) renal parenchyma with normal membrane, endothelium, and podocytes. (C and D) Tilapia exposed to 10 μg CYN L^{-1} for 10 d: (C) detail of the renal parenchyma showing dilated fenestrated capillaries (arrow) and interstitial hemorrhages (circle); (D) glomerulus with irregular basal membrane (arrow) and densification in podocyte primary foot processes (circle). (E and F) Tilapia exposed to 100 μg CYN L^{-1} for 7 d: (E) glomerular atrophy (circle) and dilated Bowman's capsule (arrow) and microhemorrhages (asterisk); (F) detail of renal glomerulus showing irregular basal membrane (arrow) and thickening, especially densification of podocyte primary foot processes. (G and H) Tilapia exposed to 10 μg CYN L^{-1} for 14 d: (G) renal parenchyma showing densifications of fenestrated capillaries of the glomerulus (circle), hyperemia (arrow) and microhemorrhages (asterisk), and (H) partial loss of basal membrane (arrow) with tumefacted podocyte foot processes (circle). (I and J) Tilapia exposed to 100 μg CYN L^{-1} for 14 d: (I) atrophied glomerulus (circle), abundant hemorrhages (arrow); (J) detail of the glomerulus with partial loss of basal membrane (asterisk), irregular distribution of fenestrated epithelium (circle), and tumefacted podocyte foot processes (arrow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

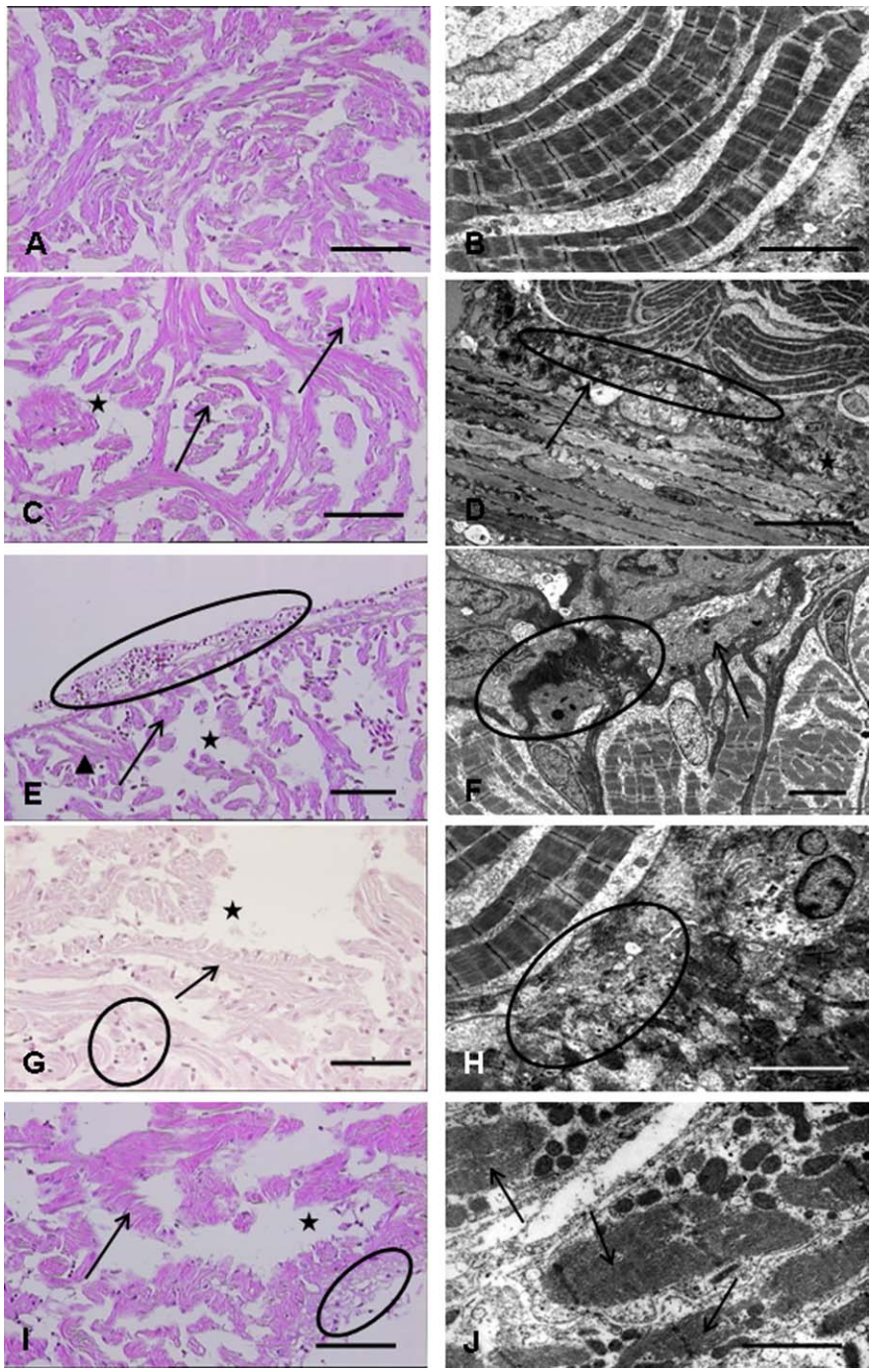

Fig. 3. Histopathological changes in heart of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). (A, C, E, G, and I) Hematoxylin and eosin–stained heart section. Bars 100 μm. (B, D, F, H, and J) Ultrastructural observations. Bars, 10 μm. (A and B) Control fish: (A) detail of normal heart fibers; (B) normal myofibrils and abundant mitochondrias. (C and D) Tilapia exposed to 10 μg CYN L^{-1} for 10 d: (C) myofibrolysis (arrow) and revealed edema (asterisk); (D) the tissue surrounding the myofibrils showed amorphous substances from the lysis of the fibers (circle), edemas (arrow), and microhemorrhages (asterisk). (E and F) Tilapia fish exposed to 100 μg CYN L^{-1} for 7 d: (E) destruction of cardiac fibers (arrow), abundant microhemorrhages (triangle), hyperemia (circle), and edema (asterisk), and (F) edema (arrow), accumulation of high electron density amorphous substances (circle). (G and H) Tilapia exposed to 10 μg CYN L^{-1} for 14 d: (G) strong destruction of cardiac fibers (arrow), severe edema (asterisk), and microhemorrhages (circle); (H) detail of destruction of myofibrils with amorphous substances (circle). (I and J) Tilapia exposed to 100 μg CYN L^{-1} for 14 d: (I) strong destruction of cardiac fibers (arrow), severe edema (asterisk), and microhemorrhages (circle); (J) destruction of myofibrils (arrow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

rarely significant below this level. In agreement with this author, toxin concentrations exceeding 100 μg L^{-1} were considered as being of particularly high risk (Kinnear et al., 2009). However, the emerging research indicates that bioaccumulation of CYN can occur even at trace quantities of the toxin, and consequently, we must be cautious when developing risk assessment guidelines for aquatic ecosystem health (Kinnear, 2010). Thus, *in vivo* studies to clarify the mechanism and extent of CYN toxicity in aquatic organisms, such as fish, are very useful, especially using environmentally realistic test concentrations in laboratory applications, lower than 100 μg CYN L^{-1}. Furthermore, the toxicity of different analogs, such as CYN compared with deoxy-CYN is quite different (Neumann et al., 2005), hence the need to include both CYN and deoxy-CYN in human and ecological risks assessments (Orr et al., 2010).

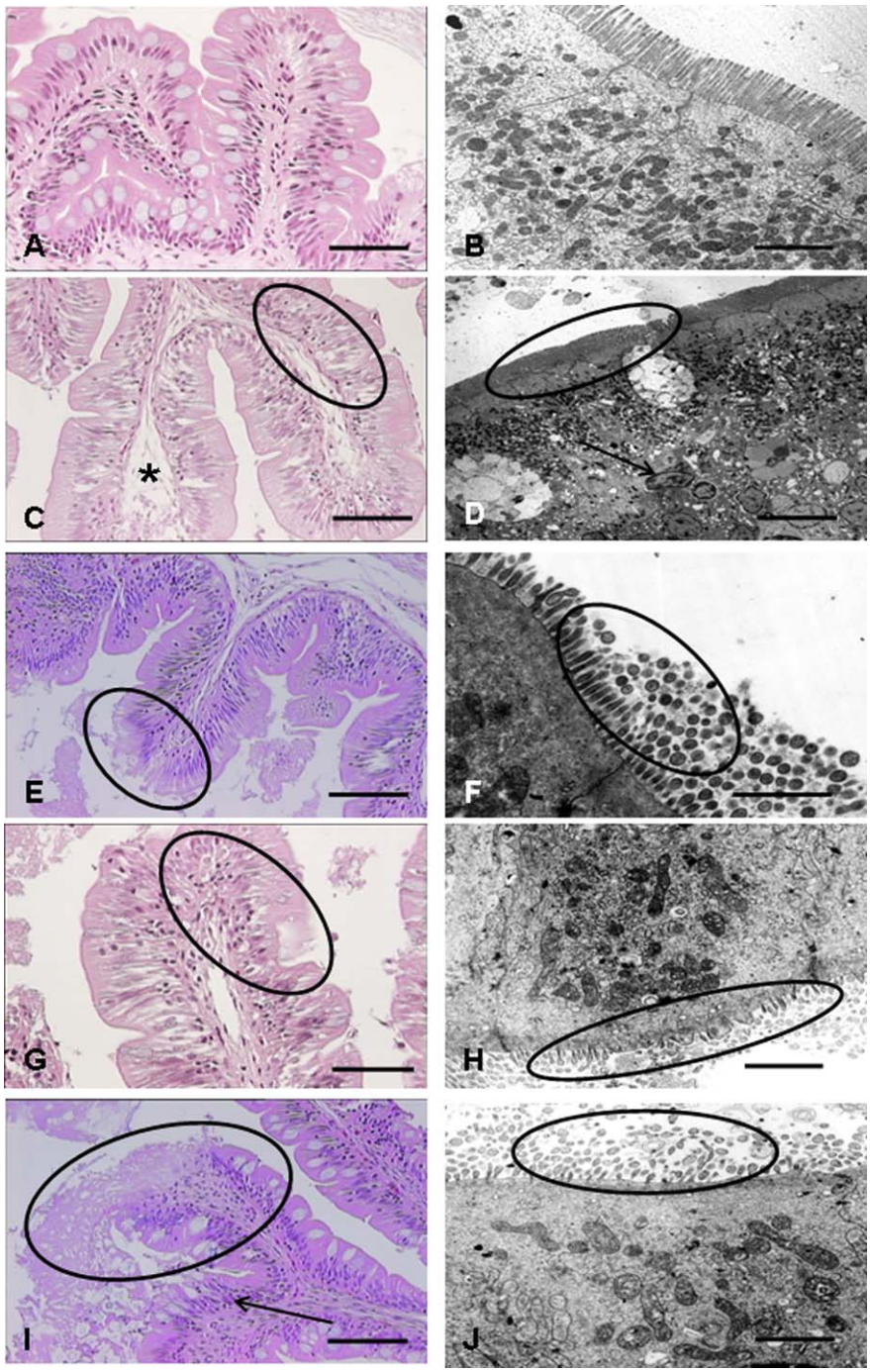

Fig. 4. (Continued)

In this regard, this study tries to mimmic the natural exposure of tilapias to cyanobacterial cells (*A. ovalisporum*) containing CYN and deoxy-CYN, at two realistic concentrations, 10 and 100 μg CYN L^{-1}. To achieve that, tilapia were exposed subchronically to the lyophilized material (*A. ovalisporum*) by direct contact in the water. To the extend of our knowledge, this is the first study in which cyanobacterial cells containing both toxin variants are able to induce histopahotlogical changes in fish, after 7 or 14 d of exposure. The results obtained confirm that liver and kidney were the main targets of toxicity, but histopathological changes have been also observed in the heart, intestines, and gills. Moreover, in this study, on chronic exposure to CYN, the organs exhibited pathological changes proportional to the exposure period (14 d in comparison to 7 d) and to the concentration of cyanobacterial cells assayed (10 or 100 μg CYN L^{-1}). It has been demonstrated that subchronic exposure to low concentrations of cyanobacterial cells exceeding 10 μg CYN L^{-1} should be considered of particularly high risk for fish, because evident histopathological changes were found from this realistic environmental concentration.

The histological findings were similar and more intense by subchronic immersion exposure to CYN, in comparison to fish exposed to an acute dose of 200 μg CYN kg^{-1} by oral route and euthanized after 24 h and 5 d (Gutiérrez-Praena et al., 2012a).

The major histological findings in the liver were a loss of the parenchymal architecture, general glucogenic degeneration, and steatosis. Similar alterations were found in the same fish species exposed to pure CYN (200 μg^{-1}kg fish) by oral and i.p. routes and sacrificed after 24 h or 5 d after exposure (Gutierrez-Praena et al., 2012a), although in this case, were less pronounced after subchronic exposure by immersion route. After 14 d of exposure with the highest concentration of 100 μg CYN L^{-1}, a generalized degeneration in the pancreatic acinus with necrotic cells has been detected. These results are in agreement with those obtained in tilapia exposed acutely to pure CYN, especially by i.p. injection. The lower potency of CYN after immersion exposure in comparison to i.p. injection could be due to the lack of efficient uptake from the gastrointestinal lumen, and also, it can be explained due to its reduction by presystemic hepatic elimination, as it was reported by Carbis et al. (1996) in carp exposed to MCs. Results from the quantitative study revealed increased size of the nuclear diameter of hepatocytes, especially in the case of the highest concentration of 100 μg CYN L^{-1} and exposed during 14 d, supporting microscopic and ultrastructural findings. These increases could suggest higher hepatocellular metabolic activity for adapting and avoiding toxicity (Madureira et al., 2012). Moreover, these results could be due to the high liver glycogen content in fish after 7 d of exposure and also by lipid accumulation resulted from the degenerative processes suffered by the hepatocytes after 14 d. Thophon et al. (2003) reported hydropic swelling of hepatocytes in the liver of Cd-exposed seabass (*Lates calcarifer*), and lipid vacuoles and abundant glycogen were observed only after subchronic exposure to the metal. Vacuolization of hepatocytes and increased lipid content could not be the result of uptake of abundant lipid precursors, but a problem of removing, or a failure to mobilize, lipid stores from the hepatocytes (van Dick et al., 2007), due to the inhibition of glycoprotein synthesis by CYN, as it is a well-known protein synthesis inhibitor and glutathione production (Terao et al., 1994; Runnegar et al., 1995). High liver glycogen content in fish may be due to the inhibition of glycogenosis for the same reason mentioned above, or due to the initiation of gluconeogenesis and/or glycogenesis (Bakthavathsalam and Reddy, 1982).

In kidney, histological changes include glomerulopathy with glomerular capillary atrophy, dilated Bowman's capsule, hyperemia of capillaries, and interstitial microhemorrhages. CYN had a concentration-dependent deleterious

Fig. 4. Histopathological changes in gastrointestinal tract of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). (A, C, E, G, and I) Hematoxylin and eosin–stained intestine section. Bars, 100 μm. (B, D, F, H, and J) Ultrastructural observations. Bars, 10 μm. (A and B) Control fish: (A) intestinal villi with abundant apparently normal enterocytes; (B) enterocytes with abundant normal microvilli. (C and D) Tilapia exposed to 10 μg CYN L^{-1} for 10 d: (C) intestinal villi with necrosis of enterocytes (circle) and severe edema of lamina propria (asterisk); (D) areas in the epithelial lamina maintaining microvilli at the apical zone (circle) and necrosis of cells at the basal zone (arrow). (E and F) Tilapia fish exposed to 100 μg CYN L^{-1} for 7 d: (E) detail of intestinal villi showing particularly in the apical zone necrosis of enterocytes and their detachment; (F) apical zone of the enterocyte showing anomalous microvilli (circle). (G and H) Tilapia exposed to 10 μg CYN L^{-1} for 14 d: (G) intestinal villi with necrosis of enterocytes and desquamation areas (circle); (H) detail of enterocyte with altered microvilli, which appeared fractionated and with small size (circle). (I and J) Tilapia exposed to 100 μg CYN L^{-1} for 14 d: (I) intestinal villi with extensive areas of necrotic enterocytes (arrow) and large areas of desquamation of the mucosa (circle); (J) detail of enterocyte with altered microvilli, which were scarce and small (circle). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

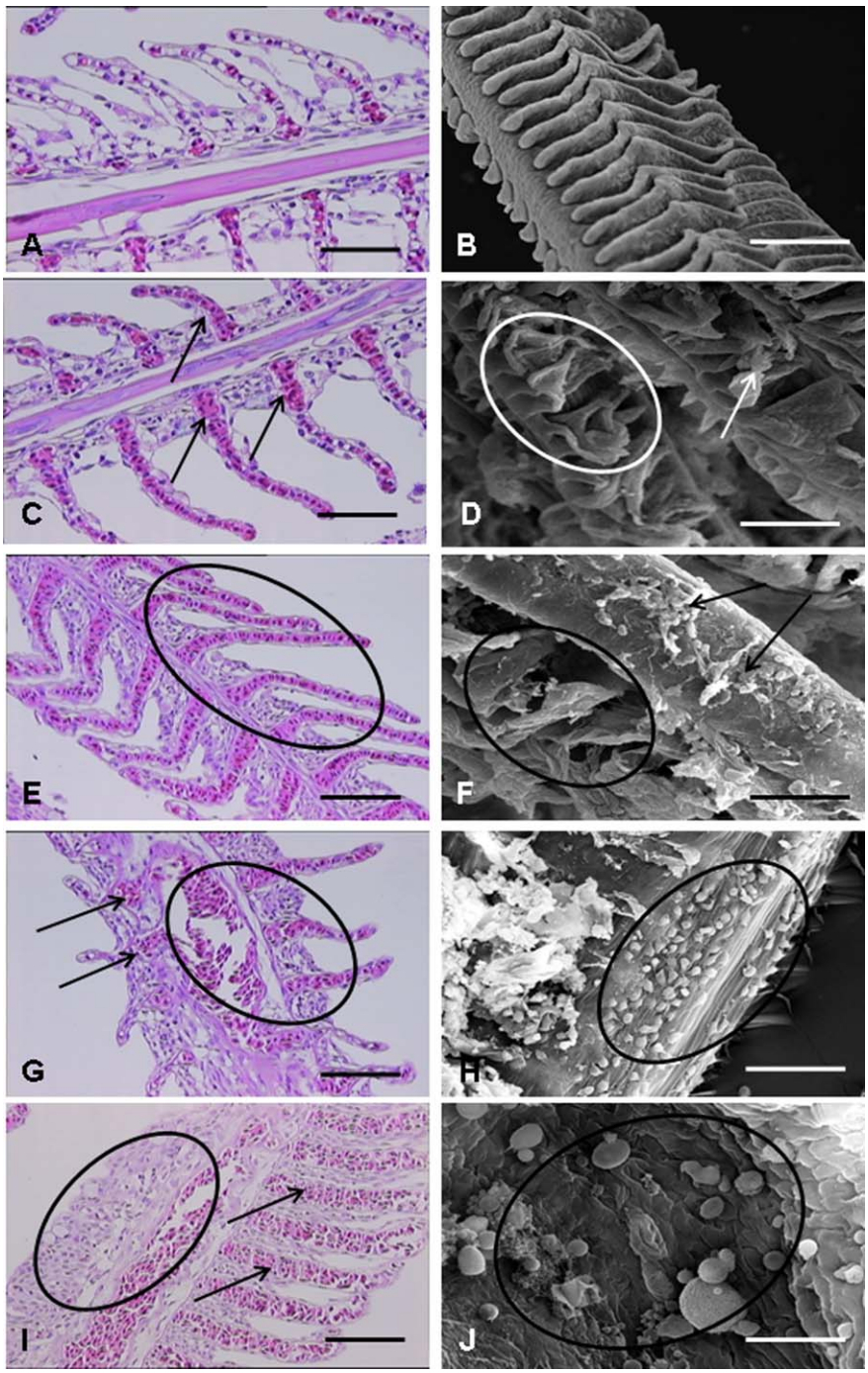

Fig. 5. Histopathological changes in gills of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). (A, C, E, G, and I) Hematoxylin and eosin–stained gill section. Bars, 100 μm. (B, D, F, H, and J) Ultrastructural observations. Bars, 100 μm. (A and B) Control fish. (C and D) Tilapia exposed to 10 μg CYN L^{-1} for 10 d: (C) branchial arch with lamellae well developed, but with a strong hyperemia in capillaries (arrow); (D) irregular lamellae structure, tumefacted (circle), and sometimes with loss of their integrity (arrow). (E and F) Tilapia fish exposed to 100 μg CYN L^{-1} for 7 d: (E) hyperemic processes in lamellae and branchial arch (circle): (F) branchial arch zone showing blood cells, microhemorrhages (arrow), and tumefacted lamellae (circle).(G and H) Tilapia exposed to 10 μg CYN L^{-1} for 14 d: (G) very hyperemic lamellae (arrow), and hyperemic branchial arch with microhemorrhages (circle); (H) detail of lamellae structure with hemorrhagic cell infiltrates, microhemorrhages (circle). (I and J) Tilapia exposed to 100 μg CYN L^{-1} for 14 d: (I) thickening of lamellae with severe hyperemia (arrow), cell infiltrates and microhemorrhages (circle); (J) detail of lamellae with very evident microhemorrhages (circle). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

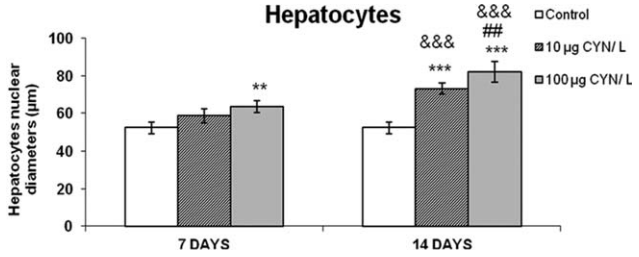

Fig. 6. Hepatocyte nuclear diameters values (micrometers) of tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). The values are expressed as mean $\pm$ sd ($n = 5$). The significance levels observed are $^{**}p < 0.01$ or $^{***}p < 0.001$ in comparison to control group values, $\#\#p < 0.01$ when fish were sacrificed at the same time and exposed to different doses and $\&\&\&p < 0.001$ when fish were exposed to the same dose and sacrificed at different times.

effect (100 or 10 μg CYN L^{-1}), and the extent of pathological findings varied with the duration of exposure, being more severe after 14 d of the exposure. These changes were consistent with ones observed in tilapia exposed acutely to pure CYN (oral and i.p. routes) (Gutierrez-Praena et al., 2012a), although a more severe atrophy of the tubules has been observed in this study, subchronic exposure by immersion route, mainly after 14 d of exposure. CYN is highly hydrophilic, and it could result in a greater damage to the kidney, as the time of exposure increased. In the case of CYN, Humpage and Falconer (2003) considered that kidney appeared to be the more sensitive organ to this toxin in mice, and studies of the body distribution of ^{14}C-labeled CYN in this species have shown that the main excretory route is through the kidneys, with nearly 50% of i.p. administered dose appearing in the urine in 6 h, when 20% of the dose was present in the liver (Norris et al., 2001).

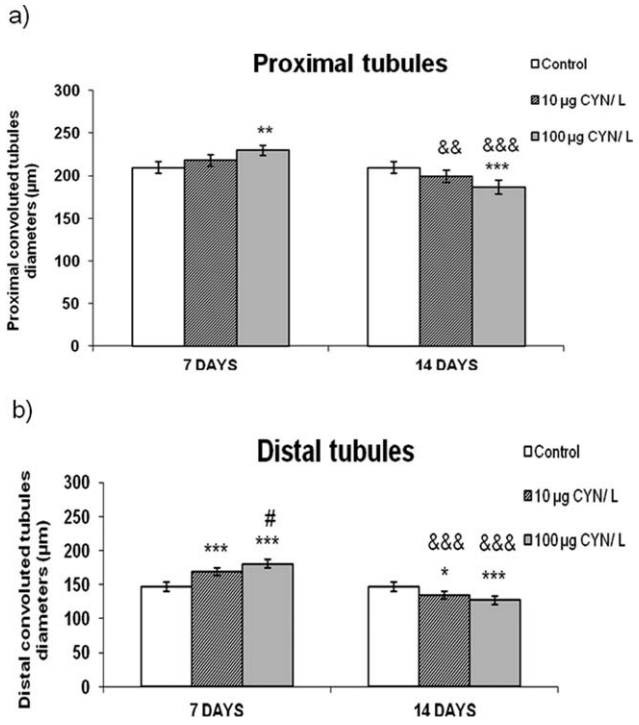

Fig. 7. Proximal (a) and distal (b) convoluted tubules cross sections (micrometers) of fish tilapia fish (*Oreochromis niloticus*) after 7 or 14 d of exposure to cyanobacterial cells (10 or 100 μg CYN L^{-1}). The values are expressed as mean $\pm$ sd ($n = 5$). The significance levels observed are $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ in comparison to control group values, $\#p < 0.05$ when fish were sacrificed at the same time and exposed to different doses and $\&\&p < 0.01$ or $\&\&\&p < 0.001$ when fish were exposed to the same dose and sacrificed at different times.

Results from the quantitative morphometric study revealed a biphasic response in the cross-sections of the urinary tubules of tilapia exposed to CYN, depending on the exposure time assayed. Increased cross-sections of the urinary tubules of fish have been detected after 7 d of exposure, and these increases could be explained by the tumefaction and swelling of tubular cells. Cell injuries may result in a fall in the level of intracellular ATP, which in turn would impair the action of the cation pump of the cell, allowing the influx of sodium, chloride, calcium, and water, causing an increase in the cell volume and damage to the cell membrane, with the efflux of some ions (K^+), enzymes, and other proteins (Silva and Martinez, 2007). Falconer et al. (1999) reported that the i.p. administration in mice of extracts of the cyanobacterium *C. raciborskii* strain AWT 205 induced an increase in the space around the glomerulus, increased diameter of the tubula lumina, proximal tubule epithelial necrosis, and the presence of proteinaceous material in the distal tubules. By contrast, after 14 d of exposure, decreases in the cross-sections of the urinary tubules of tilapia were found in our experiment, which supported the histological findings mentioned above (atrophy of tubular cells). Similarly, Gutiérrez-Praena et al. (2012a) demonstrated decreased cross-sections of the urinary tubules when fish were exposed to a single dose of CYN pure (200 μg CYN kg^{-1} fish), even after 24 h after administration of the toxin. These differences might be due to the different exposure periods and CYN doses considered: single dose of pure toxin 200 μg CYN kg^{-1} (Gutierrez-Praena et al., 2012a), whereas in this study, fish were in contact with cyanobacterial cells containing CYN, deoxy-CYN and other bioactive substances for 7 or 14 d.

Ultimately, lower CYN concentrations given for a longer period of time may induce an initial response characterized by tumefaction of tubular cells, and therefore, as time progresses (14 d), cell atrophy was induced. This biphasic response on histological findings has been also reported in fish exposed to mercurial compounds (Kirubagaran and Joy, 1988). According to these authors, during short-term exposure with sublethal concentrations of Hg compounds, the diameter of the proximal tubules of the catfish *Clarias*

batrachus increased with hypertrophy and activation of epithelial cells, suggesting active tubular elimination of the xenobiotic. However, some of the glomeruli of the fish treated for 14 d exhibited degenerative changes, which suggest also an impairment of glomerular function as well, and severe damage of the proximal tubules with vacuolated and atrophied cells was found after 90 d.

Our results confirm that the cross-sections of proximal and distal convoluted tubules may be useful for quantifying the extent of damage or the severity of CYN in this organ. The proximal renal tubular damage in mice (Humpage and Falconer, 2003), in fish (Gutiérrez-Praena et al., 2012a) as well as the clinical findings of renal insufficiency in the Palm Island human poisoning incident (Blyth, 1980; Griffiths and Saker, 2003) suggest that cytotoxic mechanisms may predominate in the kidney at higher doses. But, the mechanisms behind the kidney damage remain to be explored (Falconer et al., 1999).

Heart lesions of fish exposed to cyanobacterial cells containing CYN and deoxy-CYN were myofibrolisis and interstitial edema after 7 d of exposure, and they were more pronounced after 14 d, with strong destruction of cardiac fibers, abundant edema, and presence of microhemorrhages. These pathological changes were more evident in comparison to cardiac lesions detected in tilapia exposed to an acute dose of pure CYN (200 or 400 μg CYN kg^{-1} fish) (Puerto et al., in press) and sacrificed after 24 h, or exposed to a single dose of 200 μg CYN kg^{-1} fish by oral or i.p. route and euthanized after 5 d (Gutiérrez-Praena et al., 2012a). Subsequently, a subchronic exposure by immersion to CYN even at low concentrations (10 or 100 μg CYN (per kilogram of fish) induced more intense lesions in fish in comparison to an acute administration of CYN by oral or i.p. routes. Vascular damage in the heart muscle, and subepicardial and myocardial hemorrhages were also described in mice (Seawright et al., 1999; Falconer and Humpage, 2006). The degenerative process of the cardiac muscular fibers observed could be attributed to the direct action of CYN on the hearth tissue, coupled with the indirect effects on the kidney. Recently, Gutierrez-Praena et al. (2012b) have demonstrated *in vitro* morphological alterations (nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases of granules, and apoptosis) in human vascular endothelium cells exposed to pure CYN. MCs also induced similar histological lesions in tilapias exposed i.p. to pure MCs (Atencio et al., 2008), or by oral route to MC-LR from *Microcystis* cells (Molina et al., 2005).

In this study, intestines and gills were also affected in a concentration and time-dependent manner. After 14 d of exposure (10 or 100 μg CYN L^{-1}), both organs suffered more remarkable injuries in comparison to those reported in tilapia exposed to a single dose of 200 μg CYN kg $fish^{-1}$ by oral or i.p. administration (Gutierrez-Praena et al., 2012a). According to these authors, the gastrointestinal tract of fish treated with CYN by oral route showed a catarrhal enteritis process, whereas in this study, after subchronic exposure by immersion route, the changes included necrotic changes with edemas, loss of microvilli, and hemorrhages, mainly after 14 d with the highest concentration. In mice, the administration of a single oral dose of freeze-dried *C. raciborskii* culture containing 0.2% CYN induced multiple alterations of the esophageal part of the gastric mucosa, usually with moderate edema of the associated stomach wall; occasionally, there was also hyperemia of this mucosa (Seawright et al., 1999). *In vitro*, pure CYN is able to induce degenerative processes in human Caco cells, including damage to nuclei, segregated nucleoli, degraded components in cell cytoplasm, and mitochondrial injury, which were greatest in cells exposed to the highest concentration at the longest exposure time assayed (Gutierrez-Praena et al., 2012c). In gills, the alterations consisted in generalized hyperemic lamellae, big areas of desorganization, microhemorrhages, especially after 14 d of exposure, were more evident in comparison to those reported after oral administration of CYN (Gutierrez-Praena et al., 2012a), although in both cases, cell infiltrates were observed. The gill lesions in fish induced by toxicants are mediated through inflammation on central regulatory systems (Part et al., 1982). Gill is the primary site of osmoregulation and respiration in aquatic vertebrates, and it could be main target organ, which gets affected when the organism is exposed to dissolved xenobiotics, such as heavy metals (Athikesavan et al., 2006), or cyanotoxins in this particular case.

CONCLUSION

This study showed damage in the liver, kidney, heart, intestines, and gills of tilapia after subchronic exposure to cyanobacterial cells containing cylindrospermopsin (10 or 100 μg CYN L^{-1}) and deoxy-CYN (0.46 or 4.6 μg deoxy-CYN L^{-1}), for 7 and 14 d by immersion route. Histopathological findings confirm that the extent of damage is related to the CYN concentration and length of exposure. In comparison to previous studies from our laboratory, the variability in the response of the fish to the toxic insult induced by CYN might be due to the different concentrations and exposure periods considered (single dose of 200 or 400 μg kg $fish^{-1}$ vs. subchronic concentrations of 10 or 100 μg CYN L^{-1}), the route assayed (oral or i.p. administration vs. immersion route), and the material administered (pure standard CYN vs. contact with cyanobacterial cells containing CYN, deoxy-CYN, and other bioactive substances). It has been demonstrated that subchronic exposure to low concentrations of cyanobacterial cells containing CYN and deoxy-CYN exceeding 10 μg L^{-1} should be considered of particularly high risk for fish, because evident histopathological changes were found from this concentration. Results from the morphometric study indicated that the average of nuclear diameter of hepatocytes, and cross-sections of proximal and distal

convoluted tubules are useful to evaluate the damage induced by CYN in the main targets of toxicity. Further research should be performed to gain insight into the pathogenic mechanisms of CYN.

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REFERENCES

Atencio L, Moreno I, Jos A, Pichardo S, Moyano R, Blanco A, Cameán AM. 2008. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to microcystis under laboratory conditions. Toxicon 52:1–12.

Athikesavan S, Vincent S, Ambrose T, Velmurugan B. 2006. Nickel induced histopathological changes in the different tissues of freshwater fish, *Hypophthalmchthys molitrix* (Valenciennes). J Environ Biol 27:391–395.

Bakthavathsalam R, Reddy YS. 1982 Changes in the content of glycogen and its metabolites during acute exposure of Anabas testudineus (Bloch) to furadan. J Biosci 4:19–24.

Banker R, Carmeli S, Hadas O, Teltsch B, Porat R, Sukenik A. 1997. Identification of cylindrospermopsin in. *Aphanizomenon ovalisporum* (cyanophyceae) isolated from Lake Kinneret, Israel. J Phycol. 33:613–616.

Banker R, Carmeli S, Werman M, Teltsch B, Porat R, Sukenik A. 2001. Uracil moiety is required for toxicity of the cyanobacterial hepatotoxin cylindrospermopsin. J Toxicol Environ Health A 62:281–288.

Bazin E, Mouro A, Humpage AR, Fessard V. 2010. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environ Molec Mutag 51:251–259.

Berry JP, Gibbs PDL, Schmale MC, Saker ML. 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53:289–299.

Blyth S. 1980. Palm Island mystery disease. Med J Aust 2:40–42.

Carbis CR, Rawlin GT, Mitchell GF, Anderson JW, McCauley I. 1996. The histopathology of carp, *Cyprinus carpio* L., exposed to microcystins by gavage, immersion and intraperitoneal administration. J Fish Dis 19:199–207.

EPA. 2011. Environmental Protection Agency of the United States of America. Toxicological Review of Cyanobacterial Toxins: Cylindrospermopsin. http://cfpub2.epa.gov/ncea/cfm/recordisplay.cfm?deid=160547#Download. Accessed July 23, 2012.

Falconer IR, Hardy SJ, Humpage AR, Froscio SM, Tozer GJ, Hawkins PR. 1999. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ Toxicol 14:143–150.

Falconer IR, Humpage AR. 2006. Cyanobacterial (Blue-green Algal) toxins in water supplies: Cylindrospermopsins. Environ Toxicol 21:299–304.

Fastner J, Heinze R, Humpage AR, Mischle U, Ealesham GK, Chorus I. 2003. Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborslii* (Cyanobacteria) isolates. Toxicon 42:313–321.

Froscio SM, Humpage AR, Bucham PC, Falconer IR. 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ Toxicol 18:243–251.

Griffiths DJ, Saker ML. 2003. The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. Environ Toxicol 18:78–93.

Gutiérrez-Praena D, Jos MA, Pichardo S, Cameán AM. 2011a. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory condition: Influence of exposure route and time of sacrifice. Aquat Toxicol 105:100–106.

Gutiérrez-Praena D, Pichardo S, Jos MA, Cameán AM. 2011b. Toxicity and glutathione implication in effects observed by exposure of the liver fish cell line PLHC-1 to pure Cylindrospermopsin. Ecotoxicol Environ Safe 74:1567–1572.

Gutiérrez-Praena D, Jos MA, Pichardo S, Moyano R, Blanco A, Monterde JG, Cameán AM. 2012a. Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure Cylindrospermopsin by oral and intraperitoneal route. Ecotoxicol Environ Safe 76:102–113.

Gutiérrez-Praena D, Pichardo S, Jos A, Moreno FJ, Cameán AM. 2012b. Alterations observed in the endothelial HUVEC cell line exposed to purê Cylindrospermopsin. Chemosphere 89: 1151–1160.

Gutiérrez-Praena D, Pichardo S, Jos A, Moreno FJ, Cameán AM. 2012c. Biochemical and pathological toxic effects induced by the cyanotoxin cylindrospermopsin on the human cell line Caco-2. Water Res 46:1566–1575.

Guzmán-Guillen R, Prieto Ortega AI, Moreno I, González G, Soria-Díaz ME, Vasconcelos V, Camea'n AM. 2012. Development and optimization of a method for the determination of cylindrospermopsin from strains of Aphanixomenon cultures: Intralaboratory assessment of its accuracy by using validation standards. Talanta 100:356–363.

Harada K, Ohtani I, Iwamoto K, Suzuki M, Watanabe MF, Watanabe M, Terao K. 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 32:73–84.

Hawkins PR, Runnegar MTC, Jackson ARB, Falconer IR. 1985. Severe hepatotoxicity caused by the tropical Cyanobacterium (blue Green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. Appl Environ Microbiol 50:1292–1295.

Hawkins PR, Chandrasena NR, Jones GJ, Humpage AR, Falconer IR. 1997. Isolation and toxicity of *Cylindrospermosis raciborskii* from an ornamental lake. Toxicon 35:341–346.

Ho L, Tang T, Monis PT, Hoefel D. 2012. Biodegradation of multiple cyanobacterial metbolites in drinking waters supplies. Chemosphere 87:1149–1154.

Humpage AR, Fenech M, Thomas P, Falconer IR. 2000. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat Res/Genet Toxicol Environ Mutagen 472:155–161.

Humpage AR, Falconer IR. 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss Albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline. Environ Toxicol 18:94–103.

Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR. 2005. Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress. J Toxicol Environ Health A 68:739–753.

Humpage A. 2008. Toxin types, toxicokinetics and toxicodynamics. In: Hudnel HK, editor. Cyanobacterial Harmful Algal Blooms. State of the Science and Research Needs. New York: Springer Press. Volume 619, pp 383–415.

Kinnear S. 2010. Cylindrospermopsin: A decade of progress on bioaccumulation research. Mar Drugs 8:542–564.

Kinnear SHW, Fabbro LD, Duivenvoorden LJ, Hibberd EMA. 2007. Multiple-organ toxicity resulting from Cylindrospermopsin exposure in tadpoles of the cane toad (*Bufo marinus*). Environ Toxicol 22:550–558.

Kinnear SHW, Duivenvoorden LJ, Fabro LD. 2009. Ecotoxicity and bioaccumulation of toxin from Cylindrospermopsis raciborskii: Towards the development of environmental protection guidelines for contaminated water bodies. In: Miranda FR, Bernards LM, editors. Lake Pollution Research Progress. New York: Nova Science Publishers, Inc. pp 81–105.

Kirubagaran R, Joy KP. 1988. Toxic effects of three mercurial compounds on survival, and histology of the kidney of the catfish *Clarias batrachus* (L.). Ecotox Environ Safe 15:171–179.

Li R, Carmichael WW, Brittain S, Eaglesham GK, Shaw GR, Liu Y, Watanabe MM. 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata*. J Phycol 37:1121–1126.

Madureira TV, Rocha MJ, Cruzeiro C, Rodrigues I, Monteiro RAF, Rocha E. 2012. The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): Evaluation of impacts on fish liver, by hystopathology, stereology, vitellogenin and CYP1A immunohistochemistry, after sub-acute exposures of the zebrafish model. Environ Toxicol Pharmacol 34:34–45.

Masten S, Carson B. 2000. Cylindrospermopsin. National Institute of Environmental Health Sciences P.O. Box 12233 and Integrated Laboratory Systems. P.O. Box 13501. Research Triangle Park, North Carolina 27709.

Metcalf JS, Richer R, Cox PA, Codd GA. 2012. Cyanotoxins in desert environments may present a risk to human health. Sci Total Environ 421–422:118–123.

Molina R, Moreno I, Pichardo S, Jos A, Moyano R, Monterde JG, Cameán A. 2005. Acid and alkaline phosphatase activities and pathological changes induced in Tilapia fish (*Oreochromis* sp.) exposed subchronically to microcystins from toxic cyanobacterial blooms under laboratory conditions. Toxicon 46:725–735.

Neumann C, Bain P, Shaw G. 2005. The *in vitro* toxicology of deoxycylindrospermopsin. In: Australian Research Network for Algal Toxin Workshop, Moreton Bay Research Station, Australia, 9–11, 2005.

Norris RL, Eaglesham GK, Pierens G, Shaw GR, Smith MJ, Chiswell RK, Seawright AA, Moore MR. 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from C*ylindrospermopsis raciborskii*. Environ Toxicol 14:163–165.

Norris RL, Seawright AA, Shaw GR, Smith MJ, Chiswell RK, Moore MR. 2001. Distribution of ^{14}C cylindrospermopsin in vivo en the mouse. Environ Toxicol 16:498–505.

Norris RLG, Seawright AA, Shaw GR, Senogles P, Eaglesham GK, Smith MJ, Chiswell RK, Moore MR. 2002. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 40:471–476.

Ohtani I, Moore RE, Runnegar M. 1992. Cylindrospermopsin: A potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. Am Chem Soc 114: 7941–7942.

Orr PT, Rasmussen JP, Burford MA, Eaglesham GK, Lennox SM. 2010. Evaluation of quantitative real-time PCR to characterize spatial and temporal variations in cyanobacteria, *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya et Subba Raju and cylindrospermopsin concentrations in three subtropical Australian reservoirs. Harmful Algae 9: 243–254.

Padisák J. 1997. *Cylindrospermopsis raciborskii* (Woloszynka) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: Worldwide distribution and review of its ecology. Archiv Fur Hydrobiologie 107:563–593.

Part PH, Tuurala H, Soivio A. 1982. Oxygen transfer, gill resistance and structural changes in rainbow trout (*Salmo gairdneri* Richardson) gills perfused with vasoactive agents. Comp Biochem Physiol 71C:7–13.

Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B. 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar Drugs 8:1650–1680.

Puerto M, Jos MA, Pichardo S, Gutiérrez-Praena D, Cameán AM. 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in Tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20:1852–1860.

Puerto M, Jos A, Pichardo S, Moyano R, Blanco A, Cameán AM. Acute exposure to pure cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environ Toxicol (in press).

Rogers EH, Zehr RD, Gage MI, Humpage AR, Falconer IR, Marr M, Chernoff N. 2007. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49:855–864.

Rücker J, Stüken A, Nixdorf B, Fastner J, Chorus I, Wiedner C. 2007. Concentrations of particulate and dissolved cylindrospermopsin in 21 Aphanizomenon-dominated temperate lakes. Toxicon 50:800–809.

Runnegar MT, Kong SM, Zhong YZ, Lu SC. 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Pharmacol 49:219–225.

Seawright AA, Nolan CC, Shaw GR, Chiswel RK, Norris RL, Moore MR, Smith MK. 1999. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). Environ Toxicol 14: 135–142.

Seifert M, McGregor G, Eaglesham G, Wickramasinghe W, Shaw G. 2007. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. Harmful Algae 6:73–80.

Silva AG, Martinez CBR. 2007. Morphological changes in the kidney of a fish living in an urban stream. Environ Toxicol Pharmacol 23:185–192.

Sivonen K, Jones G. 1999. Cyanobacterial toxins. In: Chorus I, Bartram MJ, editors. Toxic Cyanobacteria in Water: A guide to Their Public Health Consequences, Monitoring and Management. London: E & FN Spon. p 41–111.

Terao K, Ohmori S, Igarashi K, Ohtani I, Watanabe MF, Harada KI, Ito E, Watanabe M. 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32:833–843.

Thophon S, Kruatrachue M, Upatham ES, Pokethitiyook P, Sapaphong S, Jaritkhuan S. 2003. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure. Environ Pollut 121: 307–320.

van Dick JC, Pieterse GM, van Vuren JHJ. 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. Ecotox Eviron Safe 66: 432–44.

CAPÍTULO 7 / CHAPTER 7

Remedios Guzmán-Guillén, Inmaculada Lomares Manzano, Isabel M. Moreno, Ana I. Prieto Ortega, Rosario Moyano, Alfonso Blanco, Ana M. Cameán

CYLINDROSPERMOPSIN INDUCES NEUROTOXICITY IN TILAPIA FISH (OREOCHROMIS NILOTICUS) EXPOSED TO APHANIZOMENON OVALISPORUM

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Cylindrospermopsin induces neurotoxicity in tilapia fish (*Oreochromis niloticus*) exposed to *Aphanizomenon ovalisporum*

Remedios Guzmán-Guillén[a*], Inmaculada Lomares Manzano[a], Isabel M. Moreno[a], Ana I. Prieto Ortega[a], Rosario Moyano[b], Alfonso Blanco[c], Ana M. Cameán[a]

[a] Area of Toxicology. Faculty of Pharmacy. University of Sevilla. C/Profesor García González 2, 41012 Sevilla. Spain.
[b] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, University of Córdoba. Campus de Rabanales Carretera Madrid-Cádiz s/n, 14071 Córdoba. Spain.
[c] Department of Anatomy and Comparative Pathology and Anatomy, University of Córdoba. Campus de Rabanales Carretera Madrid- Cádiz s/n, 14071 Córdoba. Spain.

***Corresponding author**:
Remedios Guzmán Guillén
Area of Toxicology. Faculty of Pharmacy. University of Sevilla
C/ Profesor García González 2, 41012 Sevilla, Spain.
e-mail address: rguzman1@us.es
Tel: +34 954 556762
Fax: +34 954 233765

Abstract

Cylindrospermopsin (CYN) is a cytotoxic cyanotoxin produced by several species of freshwater cyanobacteria, such as *Aphanizomenon ovalisporum*. CYN is a tricyclic alkaloid known for its ability to inhibit both protein and glutathione synthesis, and the alteration of different oxidative stress biomarkers in mammals and vertebrates. Although the liver and kidney appear to be the main CYN targets for this toxin, it also affects other organs. In fish, there is no evidence about the neurotoxicity of CYN yet. In the present study, we aimed to study the potential neurotoxicity of CYN, based on the measure of Acetylcholinesterase (AChE) activity, lipid peroxidation (LPO) levels and histopathological studies in brain of tilapia (*O. niloticus*) exposed by immersion to repeated doses of a CYN-containing culture of *A. ovalisporum* for 14 days. The results showed significant inhibition of AChE activity and increases in LPO levels, as well as relevant histopathological alterations in brain of fish (*O. niloticus*) subchronically exposed to the toxin. Moreover, we also investigated the potential recovery of these parameters by subjecting the fish to two depuration periods (3 and 7 days) in clean uncontaminated water, showing a recovery of the biochemical parameters since 3 days of depuration, and being necessary 7 days to recover the histopathological changes. In order to support these results, CYN was detected and quantified by enzyme-linked immunosorbent assay (ELISA) in brain of all the exposed fish and the effects of the depuration periods were also observed. Based on these results, it was demonstrated for the first time the neurotoxicity of CYN and its presence in brain of tilapia fish subchronically exposed to CYN.

Keywords: Cylindrospermopsin; tilapia; brain; Acetylcholinesterase; LPO; histopathology.

1. Introduction

Cyanobacteria are procariotic organisms growing in eutrophic freshwaters and lakes, and they are able to produce toxic secondary metabolites called cyanotoxins. Cylindrospermopsin (CYN) is an emerging toxin known to be produced by eleven species of cyanobacteria, *Aphanizomenon ovalisporum* among them (Poniedziałek et al., 2012).

CYN is a potent inhibitor of proteins (Terao et al., 1994) and glutathione (GSH) synthesis (Runnegar et al., 1994; 2002), and oxidative stress has resulted to play a significant role in CYN toxicity *in vitro* (Gutiérrez-Praena et al., 2011a; 2012a,b) and *in vivo*. Thus, a significant increase in LPO levels was observed in liver and kidney of tilapia acutely exposed by gavage to 200 or 400 $\mu g\, kg^{-1}$ bw pure CYN, or subchronically to cyanobacterial cells containing CYN (10 and 100 μg CYN L^{-1}) for 7 and 14 d by immersion route (Puerto et al., 2011; Gutiérrez-Praena et al., 2011b; Guzmán-Guillén et al., 2013a). It has been demonstrated that CYN induces several histopathological effects depending on the dose, time of exposure and route of exposition in various organs of tilapia (Gutiérrez-Praena et al., 2012c; Guzmán-Guillén et al., 2013b; Puerto et al., 2014). Moreover, CYN may act as endocrine disruptor (Young et al., 2008) and genotoxic (Zegura et al., 2011). More limited knowledge exists on potential neuro-, immuno-, dermato-, cyto-, and fetal toxicity in human (Poniedziałek et al., 2012).

There is some controversy regarding CYN neurotoxicity to date. Studies on snails, alligators and mice have revealed typical symptoms of neurotoxicity in those animals exposed to CYN (Poniedziałek et al., 2012). In mammalian species, CYN has demonstrated to induce neurotoxic symptoms after acute and chronic exposures to diverse *C. raciborskii* strains and *Umezakia natans* (Hawkins et al., 1985; Terao et al., 1994; Bernard et al., 2003). However, there are no studies involving human cell lines and no neurotoxicity was observed in the two confirmed cases of human poisoning. Moreover, few studies have investigated the histopathological effects of xenobiotics on fish brain, particularly of cyanotoxins, with only two authors encompassing the PSTs

(Zhang et al., 2013) and MCs (Berillis et al., 2014), showing different degrees of injury. Nevertheless, as far as we are concerned, no studies regarding histopathological effects induced by CYN in fish brain have been carried out to date.

Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating nerve impulses by catalyzing hydrolysis of the neurotransmitter acetylcholine in acetate and choline. It is reported to be a specific biomarker of exposure to some pesticides, which can inhibit the enzyme. This inhibitory action results in the accumulation of acetylcholine in the synapses of the central nervous system, neuromuscular junctions, sympathetic and parasympathetic nerve endings, with the subsequent excessive stimulation of cholinergic nerves, resulting in behavioral alterations (tremors, convulsions, and erratic or lethargic swimming) (Pretto et al., 2010). A variety of works have proved the sensitivity of brain AChE from various fish species against different xenobiotics, thus supporting the idea of using this enzyme activity as a biomarker of early exposure to pollutants that are able to inhibit it. Such is the case of AChE activity in brain from silver catfish (*Rhamdia quelen*) exposed to cadmium (Pretto et al., 2010), from tench (*Tinca tinca*) exposed to the insecticides carbofuran and deltamethrin (Hernández-Moreno et al., 2010), from *Prochilodus lineatus* exposed to the herbicide clomazone (5 and 10 mg/L) (Pereira et al., 2013), and peacock bass (*Cichla ocellaris*) exposed to several organophosphates (OPs), carbamates and heavy metals (Silva et al., 2013). On the other hand, brain AChE activation has also been demonstrated as a consequence of exposure to MC-LR (Kist et al., 2012). Despite this, in some cases no significant changes were observed in this parameter, as in *Geophagus brasiliensis* exposed to paralytic shellfish toxins (PSTs) (Clemente et al., 2010), and adult guppies (*Poecilia vivipara*) exposed to the herbicide Roundup (Harayashiki et al., 2013).

Thorough reviews about CYN presence and accumulation processes in aquatic invertebrate and vertebrate organisms have been fulfilled by Kinnear (2010) and Gutiérrez-Praena et al. (2013). CYN has been detected and quantified in liver and muscle of different fish species (Messineo et al., 2010; Berry et al., 2012; Guzmán-Guillén et al., 2015). Nevertheless, studies regarding accumulation of CYN in fish brain are non-existing up to now (Berry et al., 2012), thus taking into account the known neurotoxic effects of other cyanotoxins and CYN possible crossing of the blood-brain

barrier, it would be of interest at least being able to detect the toxin in this organ by a rapid technique. To meet this end, enzyme-linked immunosorbent assay (ELISA) for the quantitative and sensitive detection of CYN is of usefulness. Besides, depuration of CYN was observed in liver and kidney from tilapia transferred to clean waters for 3 and 7 days after being exposed to the toxin for 7 and 14 days (Guzmán-Guillén et al., 2014; Ríos et al., 2014).

Based on the background studies, our aim was:

1. To determine the potential neurotoxicity of Cylindropermopsin, by the measure of Acetylcholinesterase (AChE) activity, lipid peroxidation (LPO) and histopathological studies in brain of tilapia (*O. niloticus*) exposed by immersion to repeated doses of a CYN-containing culture of *A. ovalisporum* for 14 days.

2. To test the potential recovery of these parameters by subjecting the fish to two depuration periods in clean uncontaminated water (3 and 7 days).

To support the results obtained regarding CYN neurotoxicity we tried to detect its presence in brains of the exposed tilapia by ELISA, and to investigate the possible depuration of the toxin from this organ.

2. Material and Methods

2.1 Chemicals

CYN standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in water milli Q (100 µg/mL, Millipore, Bedford, MA, USA) and diluted as required for their use as working solutions (0.08–5.0 µg/mL). All chemicals and reagents used for the different assays and analysis were purchased from Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab S.L. (Sevilla, Spain). Deionized water (>18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (Amstelveen, The Netherlands). Protein assay reagent was obtained from BioRad Laboratories (Hercules, CA, USA).

2.2 Aphanizomenon ovalisporum culture

Aphanizomenon ovalisporum (LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was isolated from Lake Kinneret, Israel (Banker et al., 1997) and supplied by the Marine Research Center (Porto, Portugal), and it was grown as the conditions explained in Guzmán-Guillén et al. (2014). The extraction and purification of CYN from the culture was performed by LC-MS/MS, based on Guzmán-Guillén et al. (2012), detecting 0.1 μg CYN/mg and 0.012 μg deoxy-CYN/mg of culture.

2.3 Experimental set up, fish acclimation and exposure to CYN

Fifty four fish (male *O. niloticus,* Nile tilapia, Perciformes: *Cichlidae*) were obtained from a fish hatchery "Aquaculture Valencia" and maintained at the laboratory in the same conditions as Guzmán-Guillén et al. (2014). After the acclimation period, six groups of tilapia (8 individuals per group) were established as explained below. The experiment included a 14-day exposure period followed either by no depuration (Group 2), 3 days (Group 4) or 7 days of depuration (Group 6). Controls were included for each experimental group for the same time period (Groups 1, 3, and 5, respectively). In addition, fish from Groups 2, 4, and 6 were exposed to a CYN-containing culture of *A. ovalisporum* LEGE X-001 by immersion during 14 days. Exposure to the toxin was carried out according to the conditions referred to in Guzmán-Guillén et al. (2014). The procedure was designed to simulate the fish diet during and after a toxic *A. ovalisporum* bloom, whereas other feeding sources (represented by the fish food) were available at all times as well.

2.4 Enzyme extraction, Protein estimation and Biochemical assays

The fishes were anesthetized in an ice bath and sacrificed by transection of the spinal cord. The brains were immediately removed, weighed, frozen in liquid nitrogen and kept at -85ºC until use. Enzyme extracts from brain were prepared from each individual (not pooled) according to the method of Pereira et al., (2013) with slight modifications. Briefly, samples of brain tissue were homogenized (IKA®-WERKE T25 Basic Ultra-Turrax® Homogenizer, Staufen, Germany) in potassium phosphate buffer (0.1 M, pH 8) in a proportion of 1:10 (w/v). The homogenate was centrifuged (10.000 g, 10 min, 4°C) and the supernatants (crude extracts) were frozen at -85ºC for further assays.

Protein contents in the samples were estimated by the method of Bradford (1976), and Lipid Peroxidation Levels by Esterbauer and Cheeseman (1990), as referred to in Guzmán-Guillén et al. (2014).

AChE activity was measured as described by Ellman et al. (1961) and adapted for microplates, as described by Guimarães et al. (2007). Acetylthiocholine iodide (AcSCh) 9 mM was used as substrate and 5,5'-dithio-bis (2-nitrobenzoic) 0.75 mM (DTNB) acid as chromogen. The optical density at 415 nm was measured each 30 seconds for 3 min using an ELISA plate reader (TECAN, Infinite® M200, Männedorf, Switzerland). Enzyme activity is expressed as micromoles of AcSCh hydrolyzed per minute per milligram of protein.

2.5 Histopathology

Brain samples for histological examination were taken from control and exposed fish. For optical and electron microscopy studies, blocks of 3 mm^3 and 1 mm^3 of tissue were taken out, respectively.

For light microscopy, samples were first fixed in 10% buffered formalin for 24 h at 4°C, and then immediately dehydrated in graded series of ethanol, immersed in xylol and embedded in paraffin wax by using an automatic processor. Sections of 3 to 5 µm were mounted. After they had been deparaffinized, sections were rehydrated, stained with hematoxylin and eosin (HE), and mounted with Cristal/Mount (Paraplast, Oxford Labware, St. Louis, MO).

For electron microscopy, samples were prefixed in 2% glutaraldehyde fixative (in pH 7.4 phosphate buffer for 10 h at 4°C) and postfixed in 1% osmium tetroxide fixative (in pH 7.4 phosphate buffer for 0.5 h at 4°C). Subsequently, they were dehydrated in graded ethanol series and embedded in epon. Ultra thin sections, 50 to 60 nm, were cut with a LKB microtome. Sections were mounted on copper grid and stained with uranylacetate and lead citrate. Tissue sections were examined in a Philips CM10 electron microscope.

2.6 CYN analysis in fish brain

Brain extracts (supernatants) were analyzed using an Enzyme-Linked Immunosorbent Assay for the determination of CYN (Abraxis ELISA kit). Average absorbance values from non-exposed fish were considered as background for CYN quantification. Spectrophotometric measurements were performed at 450 nm with a microtiter plate reader (TECAN, Infinite® M200, Männedorf, Switzerland). Results are expressed as nanograms of CYN per grams of tissue.

2.7 Statistical analysis

All results were subjected to one-way analysis of variance (ANOVA), and represent means ± SE of 8 animals per group (GraphPad Instat Sofware, La Jolla, USA). Differences in mean values between groups were assessed by the Tukey's test and were considered statistically different from $p<0.05$.

3. Results

No fish died during the experiment, either in the exposure or in the depuration periods.

3.1. AChE activity

The enzymatic analysis of *O. niloticus* brain exposed to CYN (group 2) indicated a significant inhibition of 35% in AChE activity, in comparison with its respective control group ($^{***}p<0.001$) (Figure 1). AChE activity showed a recovery from 3 d of depuration (group 4) with a 12% inhibition in comparison with its control. There was a significant increase in the enzymatic activity after 7 d of depuration (group 6), when comparing with CYN-intoxicated fish for 14 days ($^{\#\#}p<0.01$).

3.2. Effects of CYN on Lipid Peroxidation

Levels of LPO in the brain of control and CYN-exposed groups are expressed in terms of nmol TBARs/g tissue and are shown in Figure 2. It was observed that LPO levels were significantly increased (71%, $^{*}p<0.05$) in fish exposed to CYN during 14 d (group 2) compared to its control group. Three days of depuration were enough to recover this parameter.

3.3 Brain histopathology

No gross pathological changes were macroscopically observed in the brains of fish treated with CYN (groups 2, 4 and 6). Brain histopathology of the control fish showed normal histological architecture without any indication of deformities by optical microscopy. Microscopic examinations showed in the irregular structure of the neuropil, molecular neuron cell bodies, with vesicular and large nuclei, as well as in the cytoplasm without apparent alterations. In the neuropil, nor vascular or degenerative changes were observed (Figure 3A).

The histological study of the brain sections from fish exposed to CYN and not depurated (group 2) showed by light microscopy a degenerative process and signs of necrosis, together with vascular processes. These alterations were observed in both the neurons bodies and the neuropil. Small necrotic and basophilic neurons with irregular borders were severely observed (Figure 3C), together with hyperemic processes and haemorrhagia (Figure 3E). Brain of CYN-intoxicated fish and depurated for 3 d (group 4) also showed the same pathologies as above but to a lesser degree. Specifically, degenerative lesions still persisted, with presence of necrotic neurons, not observing either hyperemia or microhaemorrhages (Figure 3G). After 7 d of depuration (group 6), the brain tissue of CYN-exposed fish exhibited an apparently normal structure in comparison with control groups (Figure 3I).

Ultrastructural examination of control fish showed apparently normal neurons with vesicular nucleus, abundant cytoplasmic organelles, highlighting the Golgi apparatus and mitochondria (Figure 3B). Electron microscopy of brain from CYN-exposed and not depurated fish revealed ultrastructural changes that were absent in controls. These changes included alterations of neurons both in the nucleus and cytoplasm, showing densified chromatin and vacuoles in the nucleus with irregular edges. Regarding the cytoplasm, the matrix appears homogeneous, dense and dark, with a strong vacuolization of all the membranous organelles and mitochondrial swelling (Figure 3D). Moreover, the neuropil also showed vascular modifications**,** evident hyperemic processes, edema and microhaemorrhages (Figure 3F). After 3 days of depuration there was a significant recovery of cell structure and most signs of cell damage had receded (Figure 3H), although a full recovery was observed only after 7 days of depuration (Figure 3J).

3.3 CYN determination in fish brain

Tilapia exposed to CYN for 14 days showed accumulation in brain in a range from 0.83-5.48 ng/g tissue, and the toxin was detected and quantified in 100% of the samples analyzed. After 3 and 7 days of depuration, CYN values were found in lower concentrations only in 40% of the samples, with a maximum of 0.75 and 1.04 ng/g tissue, respectively, indicating elimination of the toxin in that organ after both depuration periods (Table 1).

4. Discussion

The lack of studies on CYN effects in brain with human cell lines, and the absence of clinical signs of neurotoxicity in the two confirmed cases of CYN human intoxication, lead to some debate when stating the possible neurotoxicity of CYN in humans (Poniedziałek et al., 2012). Moreover, although neurotoxicity is considered as one important effect of some cyanotoxins, *in vitro* and *in vivo* studies about CYN neurotoxicity are very scarce, having observed some neurologic symptoms only in snails (Kiss et al., 2002), mice, *Daphnia* and fish (Zagatto et al., 2012) after CYN exposure.

This study shows for the first time the neurotoxic effects of CYN in fish, with alterations in AChE activity and LPO levels in brain from tilapia (*O. niloticus*) exposed by immersion to repeated doses of CYN for 14 days. The study is completed by subjecting the CYN-exposed fish to two depuration periods (3 and 7 days), detecting from day 3 a recovery of both parameters. However, for a full recovery of the histopathological changes observed, 7 days of depuration were necessary.

AChE is considered as a specific biomarker of exposure to toxicants because they often inhibit its activity causing cholinergic overstimulation and therefore behavioral disturbances (Pretto et al., 2010). Our results show an inhibition of 35% in AChE activity in tilapia exposed to CYN by repeated doses for 14 days. This result is in agreement with previous results by Kiss et al. (2002), who studied the membrane effects

of toxins isolated from a *C. raciborskii* culture on the neurons of two snail species. Based on the high performance liquid chromatography (HPLC) analysis, they found that the isolated purified fraction could be identical to CYN, and this extract decreased the ACh response of the neurons, interacting with the acetylcholine (Ach) receptor. They suggested that similarly to other cyanotoxins (anatoxin, saxitoxins), CYN may have a neurotoxic effect, apart from its known hepatotoxicity. And this is not unexpected since CYN alkaloid structure is more alike to neurotoxins than to hepatotoxins. Whereas anatoxin was demonstrated to be a specific agonist at the vertebrate nicotinic ACh receptors (Kiss et al., 2002), they were not able to clarify if the effects of this purified extract on the molluscan neurons were specific for a particular type of receptor, or if it could have a wider spectrum of biological action.

We have not found previous literature connecting the inhibition of AChE activity to fish exposure to CYN. Generally, the degree of overstimulation of postsynaptic neurons and therefore the gravity of the induced disorders depend on the level of AChE inhibition. In this sense, some authors have established an inhibition of 50% in AChE as a marker of intoxication (Dembélé et al., 2000). According to the Food and Agriculture Organization (2007), 20% inhibition of AChE activity is the point from which the presence of an anticholinesterasic agent can be considered harmful, above 50% inhibition signals and symptoms appear and death occurs after 90%. However, the relation between AChE inhibition and severe changes or mortality is relative, since some fish species are able to survive higher percentages of AChE inhibition (90-95%) due to exposure to toxic substances, such as OPs (Fulton and Key, 2001). The carbamate carbofuran managed to induce an inhibition of 60% in brain AChE from *Tinca tinca* exposed to the pesticide for 20 days, with no clinical symptoms or behavioral alterations observed (Hernández-Moreno et al., 2010). Pereira et al. (2013) observed a significant AChE inhibition (60%) in the fish *Prochilodus lineatus* after exposure to Clomazon (10 mg/L) for 96 h, noticing behavioral alterations with hyperactivity, tremors and convulsions. There are studies with other contaminants such as cadmium (Pretto et al., 2010), which caused significant inhibition in AChE activity in brain of silver catfish after 14 days of exposure, remaining even after 14 days of a recovery period. By contrast, Hernández-Moreno et al. (2010) observed inhibition of AChE activity in tench exposed to carbofuran during the first 30 days of exposition,

returning to basal levels after this initial period. After a reactivation assay, they suggested the recovery of the enzyme activity was due to its superproduction, just in agreement with Soler et al. (1998) in quail brain AChE after exposure to chlorpyrifos. In the present work we found a recovery in AChE activity from 3 days of depuration, which may be due to: 1) an overproduction of the enzyme to counteract the toxic effect of CYN, similarly to the above-mentioned studies; 2) the removal of the toxin from the organism during the depuration process, which could induce a reactivation of the enzyme; 3) a combination of both situations.

Several studies have observed increases in LPO levels in primary hepatocytes, human cell lines and different organs of fish exposed to CYN (Liebel et al., 2011; Gutiérrez-Praena et al., 2011b, 2012b; Guzmán-Guillén et al., 2013a). However, the oxidative stress (measured by LPO levels) induced by CYN in fish brain had not been previously investigated, and it is important to take it into account since neutralizing lipid peroxides and repairing cell structures in this organ is very difficult. Our results show an increase of 71% in LPO in the brain of CYN-exposed fish, similarly to several studies with other contaminants (Kavitha and Venkateswara Rao, 2009; Hernández-Moreno et al., 2010; Pretto et al., 2010). Kavitha and Venkateswara Rao (2009) proved that exposure of *O. mossambicus* to the pesticide profenofos for 28 days induced a LPO enhancement by 93% in the brain. They noticed a recovery of this parameter after 7 days of post exposure, whereas in our study a full recuperation was observed after 3 days of depuration. Besides, Pretto et al. (2010) observed significant increases (37-75%) in TBARS contents in brain of silver catfish (*Rhamdia quelen*) exposed to cadmium for 7 and 14 days and followed by the same periods of recovery. This parameter did not return to control values after the recovery periods studied. On the contrary, Hernández-Moreno et al. (2010) did not show any alterations in LPO in brain of tench exposed for 60 days to sublethal concentrations of two pesticides, whereas MDA contents were higher on the recovery periods. Our increase in LPO could probably be associated to the continuous generation of free radicals due to the subchronic exposure by repeated doses of the toxin, in agreement with other authors who reported grater LPO levels in chronically exposed animals (Hernández-Moreno et al., 2010). It is worth to mention that fish brain contains low levels of antioxidants and higher levels of peroxidizable unsaturated lipids and it consumes a high percentage of the body's oxygen, hence

facilitating to imbalance the proportion between antioxidants and ROS, and leading to oxidative stress (Üner et al., 2006).

Some authors have found a negative correlation between brain AChE activity and LPO. It was significant in *O. niloticus* exposed for 7 and 15 days to the pesticide diazinon (1 and 2 mg/L, respectively) (Üner et al. 2006), in silver catfish (*Rhamdia quelen*) exposed to cadmium (Pretto et al., 2010) and in brain of tench following exposure to the insecticide deltamethrin (Hernández-Moreno et al., 2010). The increase in brain TBARS levels can indicate damage to molecules like lipids, interfere in membrane fluidity and induce AChE enzymatic alterations in this tissue, as it is a membrane bound enzyme with lipid dependence in brain. In accordance, in our case we found an inverse relationship between both parameters studied.

Several authors have studied the histopathological effects in fish brain caused by different contaminants. Thus, Benli and Özkul (2010) showed that fenitrothion caused hyperemia in brain tissue of Nile tilapia (*Oreochromis niloticus*). Afterwards, Navaraj and Yasmin (2012) addressed the histopathological impact of paper mill wastewater to *Oreochromis mossambicus*, which induced in the brain enlarged pyramidal cells, binucleated nuclei, vacuolation, and necrosis, similar to the effects observed in the present study. Rainbow trout (*Oncorhynchus mykiss*) exposed to waterborne copper nanoparticles and copper sulphate displayed alterations in the nerve cell bodies in the telencephalon, in the thickness of the mesencephalon layers, and enlargement of blood vessel on the ventral surface of the cerebellum (Al-Bairuty et al., 2013). With regard to cyanobacterial toxins, low doses of PSTs in zebrafish brain induced apoptosis (chromatin condensation, cell-membrane blebbing, and the appearance of apoptotic bodies) and higher doses induced necrosis (cytoplasmic vacuolization, mitochondrial swelling, and expansion of the endoplasmic reticulum) (Zhang et al., 2013). In contrast, examination of brain of *Carassius gibelio* from a lake contaminated with MCs-producing cyanobacteria did not show any histopathological changes in this organ; the pyramidal layer appeared normal with no signs of necrosis and no haemorrhagic symptoms (Berillis et al., 2014). Our study reveals signs of necrosis and abundant vacuoles in CYN-exposed fish, with chromatin condensation, cytoplasmic edema, vacuolization and mitochondrial swelling, similar to the effects described by Zhang et

al. (2013) in brain of zebrafish exposed to PSTs. Besides, in the present work, a recovery was already noticeable after 3 days of depuration, being complete after 7 days. These changes could be related to possible inhibition or decreased cholinergic activity also induced by the toxin exposure, as also supported by other authors (Chamarthi et al., 2014).

There is evidence that CYN bioaccumulates in organs of fish. Messineo et al. (2010) detected 2.7 ng CYN/g dry weight (dw) in liver and 0.8 ng CYN/g dw in muscle from trouts, and Berry et al. (2012) found between 0.09 and 1.26 ng CYN/g wet weight (ww) in muscle of different fish species. Both analyses were carried out by ELISA. More recently, we reported CYN concentrations of up to 83.1-703.4 ng/g dry liver and 3.7-9.2 ng/g dry muscle from subchronically exposed tilapia by LC-MS/MS (Guzmán-Guillén et al., 2015). In addition, depuration of CYN was observed in liver and kidney from tilapia transferred to clean water for 3 and 7 days, after 7 and 14 days of exposure to CYN (Guzmán-Guillén et al., 2014; Ríos et al., 2014). ELISA for CYN detection has been used in this study with to confirm the presence of the toxin in brain, supporting the neurotoxic effects demonstrated by AChE activity reduction and increased LPO levels. In the present study, CYN was detected in all the samples analyzed, and the toxin levels in the brain of tilapia exposed to CYN for 14 days were quantified in a range from 0.83 to 5.48 ng/g tissue. Furthermore, it has been observed that despite CYN being still detectable in 40% of the fish subjected to depuration periods, the values show a tendency to decrease by this process. CYN ability to cross the blood-brain barrier could be supported by other studies with MCs (Kagalou et al., 2008; Papadimitriou et al., 2012), that detected MCs by ELISA in the brain of *C. gibelio* and *Cyprinus carpio*, respectively, suggesting their ability to cross this barrier. Some more authors have studied the accumulation and consequent effects of MCs in fish brain, such in *C. carpio* after gavage (Fischer and Dietrich, 2000) or after exposure to naturally concentrations of the toxins (Moutou et al., 2012), and in *Jenynsia multidentata* brain exposed to MC-RR dissolved in water for 24 h (Cazenave et al., 2005). Due to cyanotoxin exposure, changes in the locomotor behavior of MC-LR-exposed zebrafish, and neurotoxicity together with dysfunction of cytoskeleton have also been observed (Baganz et al., 2004; Wang et al., 2010). Hence taking into account this ability of MCs to pass through the blood-brain barrier and the effects caused by CYN in the present study, which also

indicates that fish brain is another target of the toxin, CYN crossing this barrier to exert its effects at neurological level could be explained by interaction with and affection of specific transporters, but further investigations would help to clarify it.

5. Conclusions

CYN neurotoxic effects have been demonstrated for the first time in brain of tilapia (*Oreochromis niloticus*) subchronically exposed by immersion to repeated doses of a CYN-containing culture of *A. ovalisporum* for 14 days, according to the significant inhibition of AChE activity and increases in LPO levels, as well as the histopathological alterations found in this organ. In addition, we have demonstrated the presence of the toxin, detecting it in 100% of the brains of exposed fish. Hence the use of biomarkers such as AChE activity or levels of LPO (their decrease or increase, respectively), is a good way of assessing the exposure and potential effects of CYN. After subjecting the fish to depuration processes, a recovery of the biochemical parameters studied (AChE and LPO) was observed from 3 days of depuration; however, for complete recovery of histopathological changes, 7 days of depuration were necessary. Furthermore, CYN was detected in only 40% of brain samples of depurated fish, indicating that the depuration process would serve as a good detoxification mechanism in brain of fish that have been exposed to toxic cyanobacterial blooms.

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References

1. Al-Bairuty, G.A., Shaw, B.J., Handy, R.D., Henry, T.B. 2013. Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 126, 104-115.
2. Baganz, D., Staaks, G., Pflugmacher, S., Steinberg, C.E. 2004. Comparative study of microcystin-LR-induced behavioral changes of two fish species, *Danio rerio* and *Leucaspius delineatus*. Environ. Toxicol. 19, 564–570.
3. Banker, R., Carmeli, S., Hadas, O., Teltsch, B., Porat, R. and Sukenik, A. 1997. Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* isolated from Lake Kinneret, Israel. J. Phycol. 33, 613–616.
4. Benli, A.C.K., Özkul, A. 2010. Acute toxicity and histopathological effects of sublethal fenitrothion on Nile tilapia, *Oreochromis niloticus*. Pestic. Biochem. Physiol. 97, 32-35.
5. Berillis, P., Papadimitriou, T., Petridou, E., Konstantinos, K., Ifigenia, K. 2014. Brain and liver histopathological examination of *Carassius gibelio* from a newly reconstructed lake with toxic cyanobacteria. Turk. J. Fish. Aquat. Sci. 14, 213-219.
6. Bernard, C., Harvey, M., Briand, J.F., Bire, R., Krys, S., Fontaine, J.J. 2003. Toxicological comparison of diverse *Cylindrospermopsis raciborskii* strains: evidence of liver damage caused by a French *C. raciborskii* strain. Environ. Toxicol. 18, 176–186.
7. Berry, J.P., Jaja-Chimedza, A., Dávalos-Lind, L., Lind, O. 2012. Apparent bioaccumulation of cylindrospermopsin and paralytic shellfish toxins by finfish in Lake Catemaco (Veracruz, Mexico). Food Addit. Contam. 2, 314–321.
8. Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.
9. Cazenave, J., Wunderlin, D.A., Bistoni, M.A., Amé, M.V., Krause, E., Pflugmacher, S.,Wiegand, C. 2005. Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis*: a field and laboratory study. Aquat. Toxicol. 75, 178–190.
10. Chamarthi, R.R., Bangeppagari, M., Gooty, J.M., Mandala, S., Tirado, J.O., Marigoudar, S.R. 2014. Histopathological alterations in the gill, liver and brain of C*yprinus carpio* on exposure to quinalphos. Am. J. Life Sci. 2, 211-216.

11. Clemente, Z., Busato, R.H., Oliveira Ribeiro, C.A., Cestari, M.M., Ramsdorf, W.A., Magalhães, V.F., Wosiack, A.C., Silva de Assis, H.C. 2010. Analyses of paralytic shellfish toxins and biomarkers in a southern Brazilian reservoir. Toxicon 55, 396-406.
12. Dembélé, K., Haubruge, E., Gaspar, C. 2000. Concentration effects of selected insecticides on brain Acetylcholinesterase in the common carp (*Cyprinus carpio L.*). Ecotox. Environ. Safe. 45, 49-54.
13. Ellman, G.L., Courtney, K.D., Anders, V., Featherstone, R.M. 1961. Biochem. Pharmacol. 7, 88–95.
14. Esterbauer, H., Cheeseman, K.H. 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonetal. Methods Enzymol. 186, 407–421.
15. FAO, Food and Agriculture Organization, 2007. Pesticides in Food Report 2007. FAO Plant Production and Protection Paper 191. Food and Agriculture Organization, Rome, Italy.
16. Fischer, W.J., Dietrich, D.R. 2000. Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). Toxicol. Appl. Pharmacol. 164, 73–81.
17. Fulton, M.H., Key, V. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organofophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37-45.
18. Guimarães, A.T.B., Silva de Assis, H.C., Boeger, W. 2007. The effect of trichlorfon on acetylcholinesterase activity and histopathology of cultivated fish *Oreochromis niloticus*. Ecotox. Environ. Safe. 68, 57-62.
19. Gutiérrez-Praena, D., Pichardo, S., Jos, A., Cameán, A.M. 2011a. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure Cylindrospermopsin. Ecotox. Environ. Safe. 74, 1567–1572.
20. Gutiérrez-Praena, D., Jos, A.,Pichardo, S., Cameán, A.M. 2011b. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory conditions: Influence of exposure route and time of sacrifice. Aquat. Toxicol. 105, 100-106.

21. Gutiérrez-Praena, D., Pichardo, S., Jos, Á., Moreno, F.J., Cameán, A.M. 2012a. Alterations observed in the endothelial HUVEC cell line exposed to pure Cylindrospermopsin. Chemosphere 89, 1151-1160.
22. Gutiérrez-Praena, D., Pichardo, S., Jos, Á., Moreno, F.J., Cameán, A.M. 2012b. Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2. Water Res. 46, 1566-1575.
23. Gutiérrez-Praena, D., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M. 2012c. Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure Cylindrospermopsin by oral and intraperitoneal route. Ecotox. Environ. Safe. 76, 102–113.
24. Gutiérrez-Praena, D., Jos, A., Pichardo, S., Moreno, I.M., Cameán, A.M. 2013. Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review. Food. Chem. Toxicol. 53, 139-152.
25. Guzmán-Guillén, R., Prieto, A.I., González, A.G., Soria-Díaz, M.E., Cameán, A.M., 2012. Cylindrospermopsin determination in water by LC–MS/MS: optimization and validation of the method and application to real samples. Environ. Toxicol. Chem. 31, 2233–2238.
26. Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán, A.M. 2013a. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed Tilapia (*Oreochromis niloticus*). Chemosphere 90, 1184–1194.
27. Guzmán-Guillén, R., Prieto, A.I., Moreno, I., Vasconcelos, V.M., Moyano, R., Blanco, A., Cameán Fernandez, A.M. 2013b. Cyanobacterium producing cylindrospermopsin cause histopathological changes at environmentally relevant concentrations in subchronically exposed tilapia (*Oreochromis niloticus*). Environ. Toxicol. doi: 10.1002/tox.21904.
28. Guzmán-Guillén, R., Prieto, A.I., Moreno, I., Rios, V., Vasconcelos, V.M., Camean, A.M. 2014. Effects of depuration on oxidative biomarkers in Tilapia (*Oreochromis niloticus*) after subchronic exposure to Cyanobacterium producing Cylindrospermopsin. Aquat. Toxicol. 149, 40-49.
29. Guzmán-Guillén, R., Moreno, I., Prieto Ortega, A.I., Soria-Díaz, M.E., Vasconcelos, V., Cameán, A.M. 2015. CYN determination in tissues from

freshwater fish by LC–MS/MS: Validation and application in tissues from subchronically exposed tilapia (*Oreochromis niloticus*). Talanta 131, 452-459.

30. Harayashiki, C.A.Y., Varela Jr, A.S., Machado, A.A.S., Cabrera, L.C., Primel, E.G., Bianchini, A., Corcini, C.D. 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquat. Toxicol. 142–143, 176–184.
31. Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B., Falconer, I.R. 1985. Severe hepatotoxicity caused by the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raja isolated from a domestic water supply reservoir. Appl. Environ. Microbiol. 50, 1292-1295.
32. Hernández-Moreno, D., Soler, F., Míguez, M.P., Pérez-López, M. 2010. Brain acetylcholinesterase, malondialdehyde and reduced glutathione as biomarkers of continuous exposure of tench, *Tinca tinca*, to carbofuran or deltamethrin. Sci. Total Environ. 408, 4976-4983.
33. Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M. 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquat. Toxicol. 72, 261–271.
34. Kagalou, I., Papadimitriou, T., Bacopoulos, V., Leonardos, I. 2008. Assessment of microcystins in lake water and the omnivorous fish (*Carassius gibelio*, Bloch) in Lake Pamvotis (Greece) containing dense cyanobacterial bloom. Environ. Monit. Assess. 137, 185-195.
35. Kavitha, P., Venkateswara Rao, J. 2009. Sub-lethal effects of profenofos on tissue-specific antioxidative responses in a Euryhyaline fish, *Oreochromis mossambicus*. Ecotox. Environ. Safe. 72, 1727-1733.
36. Kinnear, S. 2010. Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar. Drugs 8, 542-564.
37. Kiss, T., Vehovszky, A., Hiripi, L., Kovacs, A. and Voros, L. 2002. Membrane effects of toxins isolated from a cyanobacterium, *Cylindrospermopsis raciborskii*, on identified molluscan neurones. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 131, 167–176.
38. Kist, L.W., Rosemberg, D.B., Pereira, T.C., de Azevedo, M.B., Richetti, S.K., de Castro Leão, J., Yunes, J.S., Bonan, C.D., Bogo, M.R. 2012. Microcystin-LR acute

exposure increases AChE activity via transcriptional ache activation in zebrafish *(Danio rerio*) brain. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 155, 247-252.

39. Laurén, D.J., The, S.J., Hinton, D.E. 1990. Cytotoxicity phase of diethylnitrosamine-induced hepatic neoplasia in medaka. Cancer Res. 50, 5504–5514.
40. Liebel, S., Oliveira, C.A., Silva, R.C., Ramsdorf, W.A., Cestari, M.M., Magalhaes, V.F., García, J.R., Esquivel, B.M., Filipak Neto, F. 2011. Cellular responses of *Prochilodus lineatus* hepatocytes after cylindrospermopsin exposure. Toxicol. in Vitro. 25, 1493-1500.
41. Messineo, V., Melchiore, S., Di Corcia, A., Gallo, P., Bruno, M. 2010. Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. Environ. Toxicol. 25, 18–27.
42. Moutou, K.A., Tsikogias, S., Papadimitriou, T., Kagalou, I. 2012. Oxidative stress in *Cyprinus carpio* to analyze microcystin impact in eutrophic shallow lakes: a preliminary study. J. Environ. Monit. 14, 2195-2203.
43. Navaraj, P.S., Yasmin, J. 2012. Histopathological impact of indrustrial wastewater on the vital organs of *Oreochromis mossambicus*. Toxicol. Environ. Chem. 94, 574-582.
44. Papadimitriou, T., Kagalou, I., Stalikas, C., Pilidis, I., Leonardos, I.D. 2012. Assessment of microcystins distribution and biomagnifications in tissues of aquatic food web compartments from a shallow lake and evaluation of potential risks to public health. Ecotoxicology 21, 1155-1166.
45. Pereira, L., Fernandes, M.N., Martínez, C.B.R. 2013. Hematological and biochemical alterations in the fish *Prochilodus lineatus* caused by the insecticide clomazone. Environ. Toxicol. Pharmacol. 36, 1-8.
46. Poniedziałek, B., Rzymski, P., Kokociński, M. 2012. Cylindrospermopsin: Water-linked potential threat to human health in Europe. Environ. Toxicol. Pharmacol. 34, 651-660.
47. Pretto, A., Loro, V.L., Morsch, V.M., Moraes, B.S., Menezes, C., Clasen, B., Hoehne, L., Dressler, V. 2010. Acetylcholinesterase activity, lipid peroxidation,

and bioaccumulation in silver catfish (*Rhamdia quelen*) exposed to cadmium. Arch. Environ. Contam. Toxicol. 58, 1008–1014.

48. Puerto, M., Jos, A., Pichardo, S., Gutiérrez-Praena, D.,Cameán, A.M. 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage Ecotoxicology 20, 1852-1860.
49. Puerto, M., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M. 2014. Acute exposure to pure cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environ. Toxicol. 29, 371–385.
50. Ríos, V., Guzmán-Guillén, R., Moreno, I., Prieto, A.I., Puerto, M., Jos, A., Cameán, A.M. 2014. Influence of Two Depuration Periods on the Activity and Transcription of Antioxidant Enzymes in Tilapia Exposed to Repeated Doses of Cylindrospermopsin under Laboratory Conditions. Toxins 6, 1062-1079.
51. Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Ge, J.L. and Lu, S. C. 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Biophys. Res. Commun. 201, 235–241.
52. Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J. 2002. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol. Sci. 67, 81-87.
53. Soler, F., Míguez, M.P., Reja, A., Roncero, V., García-Cambero, J.P. 1998. Recovery of brain acetylcholinesterase and plasma cholinesterase activities in quail (*Coturnix coturnix*) after chlorpyriphos administration and effect of pralidoxime treatment. Environ. Toxicol. Chem. 17, 1835–1839.
54. Silva, K.C.C., Assis, C.R.D., Oliveira, V.M., Carvalho, L.B. Jr., Bezerra, R.S. 2013. Kinetic and physicochemical properties of brain acetylcholinesterase from the peacock bass (*Cichla ocellaris*) and *in vitro* effect of pesticides and metal ions. Aquat. Toxicol. 126, 191-197.
55. Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M. 1994. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32, 833–843.

56. Üner, N., Oruc¸ E.Ö., Sevgiler, Y., Sahin, N., Durmaz, H., Usta, D. 2006. Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of *Oreochromis niloticus*. Environ. Toxicol. Pharmacol. 21, 241–245.
57. Wang, M., Wang, D., Lin, L., Hong, H. 2010. Protein profiles in zebrafish (*Danio rerio*) brains exposed to chronic microcystin-LR. Chemosphere 81, 716–724.
58. Young, F.M., Micklem, J., Humpage, A.R. 2008. Effects of the blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells in vitro. Reprod. Toxicol. 25, 374–380.
59. Zagatto, P.A., Buratini, S.V., Aragão, M.A., Ferrão-Filho, A.S. 2012. Neurotoxicity of two *Cylindrospermopsis raciborskii* (cyanobacteria) strains to mice, *Daphnia* and fish. Environ. Toxicol. Chem. 31, 857–862.
60. Zegura, B., Straser, A., Filipic, M. 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutat. Res. 727, 16–41.
61. Zhang, D., Hu, C., Wang, G., Li, D., Li, G., Liu, Y. 2013. Zebrafish neurotoxicity from aphantoxins-cyanobacterial paralytic shellfish poisons (PSPs) from *Aphanizomenon flos-aquae* DC-1. Environ. Toxicol. 28, 239-254.

Table 1. Cylindrospermopsin (CYN) range values (ng/g tissue) in brain of *O. niloticus* after exposure to a CYN-containing culture of *A. ovalisporum* for 14 days and after 3 and 7 days of depuration.

	ng/g tissue (range)	% positive samples
14 days of exposure	0.83-5.48	100%
3 days of depuration	ND[a]-0.75	40%
7 days of depuration	ND[a]-1.04	40%

[a] ND: not detected

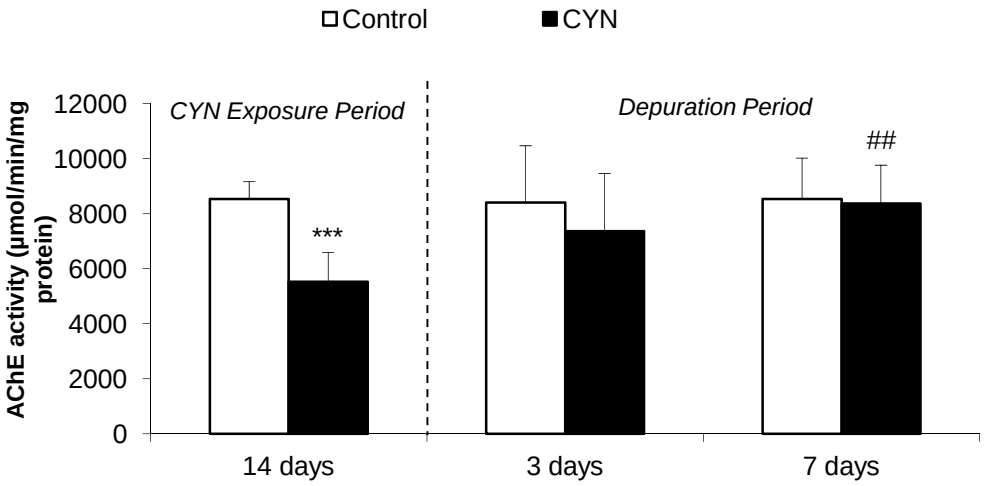

Figure 1.

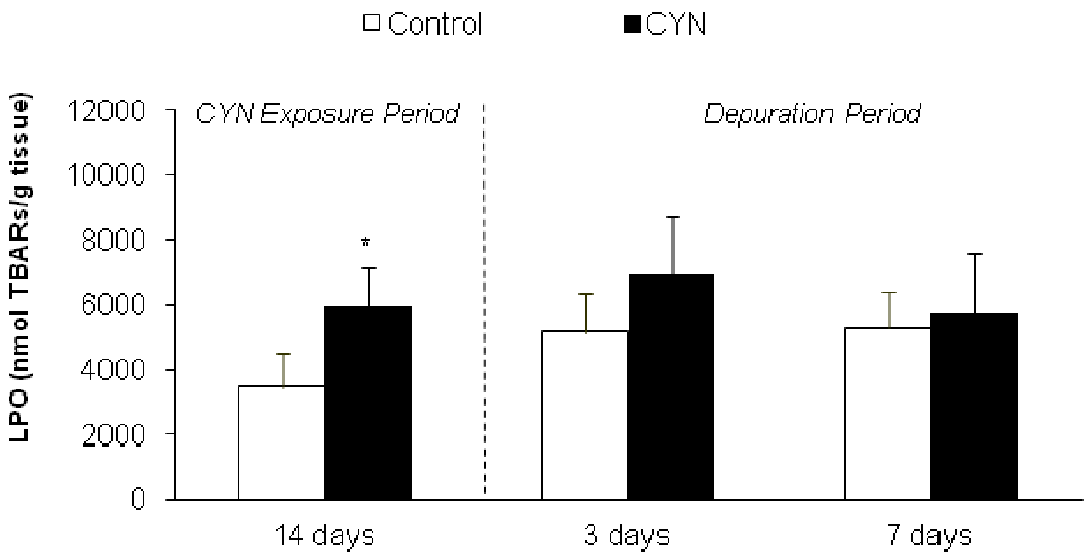
Control
CYN
LPO (nmol TBARs/g tissue)
12000
10000
8000
6000
4000
2000
0
CYN Exposure Period
Depuration Period
*
14 days
3 days
7 days

Figure 2.

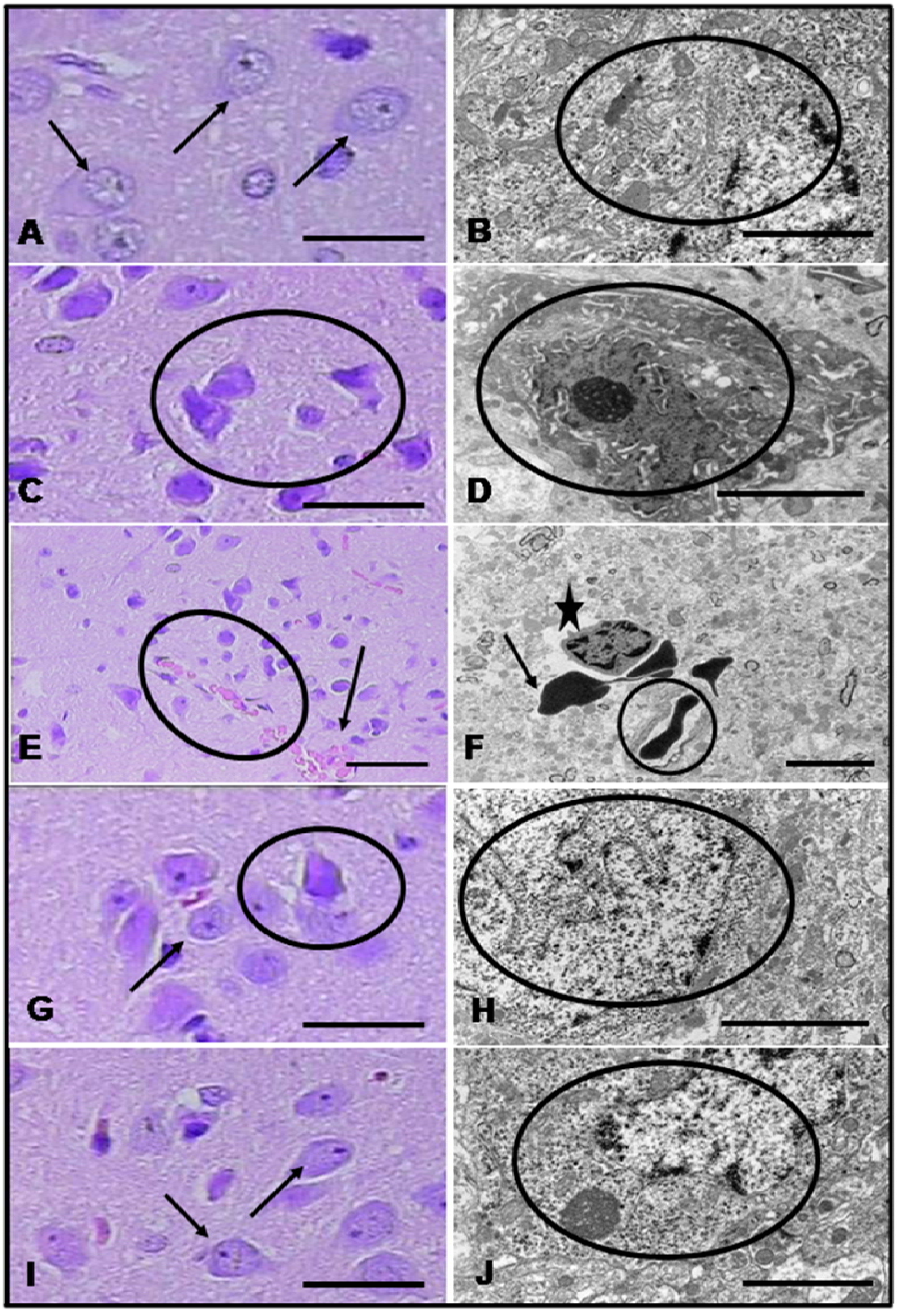

Figure 3.

Figure Captions

Fig. 1. AChE activity in brain of tilapia fish (*Oreochromis niloticus*) exposed to repeated doses of CYN (10 µg CYN/L) by immersion in an *A. ovalisporum* culture for 14 days, and submitted to two depuration periods (3 and 7 days). Enzyme activity is expressed as µmol/min/mg protein. The significance levels observed are *** $p<0.001$ in comparison to the respective control group, and $^{\#\#}$ $p<0.01$ when comparing CYN-intoxicated fish for 14 days to depurated fish.

Fig. 2. Lipid peroxidation values in brain of tilapia fish (*Oreochromis niloticus*) exposed to repeated doses of CYN (10 µg CYN/L) by immersion in an *A. ovalisporum* culture for 14 days, and submitted to two depuration periods (3 or 7 days). LPO values are expressed as nmol TBARS/g tissue. The significance levels observed are * $p<0.05$ in comparison to the respective control group.

Fig. 3. Histopathological changes in brain of tilapia fish (*Oreochromis niloticus*) exposed to repeated doses of CYN (10 µg CYN/L) by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, C, E, G)**: HE-stained brain sections. Bars: 100 µm. (**B, D, F, H)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** Detail of neuronal bodies apparently normal (arrows); **(B)** detail of neuron with high content of cytoplasmic organelles (circle). **(C, D, E, F)** Tilapia exposed to CYN for 14 days: **(C)** Small degenerated neurons with signs of necrosis and basophilia (circle); **(D)** detail of neuron with dense nucleus and abundant vacuoles in the cytoplasm (circle); **(E)** neurons with hyperemic processes (circle) and some haemorrhagia (arrow); **(F)** evident hyperemic processes (circle), edema (star) and microhaemorrhages (arrow). **(G, H)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(G)** Degenerated neurons with basophilic cytoplasm (circle) together with other normal neurons (arrow); **(H)** apparently normal neuron but with abundant lysosomes (circle). **(I, J)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(I)** Apparently normal neurons (arrows); **(J)** apparently normal neuron with abundant cytoplasmic organelles (circle).

CAPÍTULO 8 / CHAPTER 8

R. Guzmán-Guillén, A. I. Prieto, C. M. Vázquez, V. Vasconcelos, A. M. Cameán

THE PROTECTIVE ROLE OF L-CARNITINE AGAINST CYLINDROSPERMOPSIN-INDUCED OXIDATIVE STRESS IN TILAPIA (OREOCHROMIS NILOTICUS)

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The protective role of L-carnitine against cylindrospermopsin-induced oxidative stress in tilapia (*Oreochromis niloticus*)

R. Guzmán-Guillén[a], A.I. Prieto[a], C.M. Vázquez[b], V. Vasconcelos[c,d], A.M. Cameán[a,*]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Professor García González n°2, 41012 Seville, Spain
[b] Department of Physiology, Faculty of Pharmacy, University of Seville, Professor García González n°2, 41012 Seville, Spain
[c] Biology Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto 4169-007, Portugal
[d] Marine and Environmental Research Centre (CIIMAR/CIMAR), University of Porto, Rua dos Bragas, m289, 4050-123 Porto, Portugal

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ABSTRACT

Cylindrospermopsin (CYN) is one of the most important cyanotoxins in terms of both human health and environmental quality and is produced by several different species of cyanobacteria, including *Aphanizomenon ovalisporum*. The principal mechanisms of action of CYN involve inhibition of protein and glutathione synthesis. In addition, CYN-mediated genotoxicity results from DNA fragmentation. The results of both in vivo and in vitro studies suggest that oxidative stress also plays a significant role in CYN pathogenesis in fish. We investigated the protective effects of L-carnitine (LC) pre-treatment on *A. ovalisporum*-induced oxidative stress in cells containing CYN and deoxy-CYN, or pure standard CYN, in tilapia (*Oreochromis niloticus*) that had been acutely exposed via oral administration. Various oxidative stress markers, including lipid peroxidation (LPO), protein oxidation, DNA oxidation, and the ratio of reduced glutathione to oxidised glutathione (GSH/GSSG), and the activities of NADPH oxidase, superoxide dismutase (SOD), catalase (CAT), and gamma-glutamyl-cysteine synthetase (γ-GCS), were evaluated in the livers and kidneys of fish in the absence and presence of 400 or 880 mg LC/kg fish/day during a 21 day period prior to CYN-intoxication. The results of our study demonstrated for the first time the beneficial antioxidant effects of LC dietary supplementation on oxidative stress status in fish. No pro-oxidant effects were detected at any of the LC doses assayed, suggesting that LC is a chemoprotectant that reduces hepatic and renal oxidative stress and may be effective when used for the prophylaxis and treatment of CYN-related intoxication in fish.

1. Introduction

Mass populations of cyanobacteria can create significant water quality problems. Many cyanobacterial species synthesise a wide range of odorous molecules, noxious compounds or potent toxins (Sivonen and Jones, 1999). Cylindrospermopsin (CYN) is one of the most important toxins produced by cyanobacteria in terms of both human health and environmental quality (Rogers et al., 2007). CYN has been isolated from several cyanobacterial species, including *Cylindrospermopsis raciborskii* (Ohtani et al., 1992), *Umezakia natans* (Harada et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Raphidiopsis curvata* (Li et al., 2001), *Anabaena bergii* (Schembri et al., 2001), and *Aphanizomenon flos-aquae* (Preußel et al., 2006). In contrast to other cyanotoxins, CYN is mainly produced as an extracellular toxin, and it is common to find approximately 70–98% of the total toxin dissolved in the water column (Van Apeldoorn et al., 2007). Literature regarding the presence and ecological effects of CYN is growing, in part because reports regarding the expansion of the geographical range of cyanobacteria are increasing (Ibelings and Chorus, 2007).

Human exposure to CYN may occur as a result of the ingestion of toxin-contaminated water during recreational activities, and may also occur as a result of ingestion of food and water that has been contaminated with the toxin. In fact, a bloom of toxic *C. raciborskii* in a water supply reservoir may have been the cause of a severe outbreak of hepatoenteritis in Queensland, Australia in 1979 that resulted in the hospitalisation of 138 children and 10 adults (Hawkins et al., 1985).

Several studies have demonstrated the ability of CYN to bioaccumulate in aquatic animals (Kinnear, 2010; Saker et al., 2004). The effects of this cyanotoxin in piscine models have not been thoroughly studied. Berry et al. (2009) suggested that CYN was lethal to zebrafish embryos. In addition, histopathological changes have been reported to be induced by CYN in fish (Gutiérrez-Praena et al., 2012a; Puerto et al., in press).

The principal mechanisms of action of CYN are the inhibition of protein (Froscio et al., 2003; Runnegar et al., 2002) and glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1994,

* Corresponding author. Tel.: +34 954 556762; fax: +34 954 233765.
E-mail address: camean@us.es (A.M. Cameán).

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1995), suggesting that oxidative stress may play a significant role in CYN pathogenesis in fish (Gutiérrez-Praena et al., 2011a,b; Puerto et al., 2011). In addition, CYN-mediated genotoxicity results from DNA fragmentation (Bazin et al., 2009; Zegura et al., 2011). However, the toxic mechanism of CYN has not yet been fully elucidated.

Another important issue to consider is the differential toxicity observed between pure CYN and CYN extracts (including cultures and blooms). *C. raciborskii* extracts have been demonstrated to have greater sublethal toxicities than the pure toxin (at exposure levels below 100 μg/L) (Seifert, 2007), indicating that cell extracts, and hence field populations of CYN-producing blooms, may contain one or more bioactive compounds other than CYN that increase the toxic effects (Falconer et al., 1999; Hawkins et al., 1997; Kinnear, 2010; Norris et al., 1999). In fact, tilapia (*Oreochromis niloticus*) that were exposed to cyanobacterial cells containing CYN suffered from higher levels of toxic effects than tilapia that had been exposed to pure CYN (Gutiérrez-Praena et al., 2012b). Moreover, some authors reported the simultaneous presence of CYN and paralytic shellfish toxins (PSTs) in freshwater systems, field samples (Berry and Lind, 2010; Berry et al., 2012). These authors described the presence of both CYN and PSTs in the Lake Catemano (Veracruz, Mexico) and in fish tissues from the lake.

The protective effects of pre-treatment with some antioxidants, including vitamin E (Pinho et al., 2005a,b; Prieto et al., 2008), selenium (Atencio et al., 2009) and N-acetylcysteine (NAC) (Puerto et al., 2009, 2010), against the toxic action of cyanotoxins, specifically microcystins (MCs), have been studied in aquatic organisms. Nevertheless, little is known about the protective effects of antioxidants against the toxic action of CYN. To our knowledge, the only substance that has shown a protective effect against CYN-induced oxidative stress in fish is NAC (Gutiérrez-Praena et al., 2012b). N-acetylcysteine is a precursor of GSH synthesis that functions as a cysteine supplier and stimulates the activities of cytosolic enzymes that are involved in the GSH cycle, such as glutathione reductase (GR), which enhances the rate of GSH generation. Moreover, NAC also protects the cell by reacting directly with reactive oxygen species (ROS) (Aruoma et al., 1989).

L-Carnitine (β-hydroxy-γ-N-trimethylammonium-butyrate) is a vital component that is required for the production of ATP through the β-oxidation of long-chain fatty acids during lipid metabolism (Bilinski and Jonas, 1970). This water-soluble quaternary amine is synthesised from the amino acids lysine and methionine, and the presence of vitamin C and other secondary compounds is necessary for L-carnitine to be produced in animal bodies (Rebouche, 1991). The supplementation of LC in fish diets has been advocated in aquaculture for multiple reasons: LC can be used to promote growth by providing a protein sparing effect (Jayaprakas et al., 1996; Torreele et al., 1993), to protect against toxic levels of ammonia and xenobiotics (Schreiber et al., 1997), to ameliorate stress that is related to water temperature extremes and to facilitate better acclimation to water temperature changes (Harpaz et al., 1999). However, contradictory results have been reported even within the same fish species. Regarding the antioxidant capacity of LC, human and animal data support the notion that LC treatment exerts beneficial effects in several disorders that are related to oxidative stress, including aging (Kalaiselvi and Panneerselvam, 1998), atherosclerosis (Dayanandan et al., 2001), hypercholesterolaemia (Sayed-Ahmed et al., 2001), hypertension (Rajasekar et al., 2007) and chronic kidney diseases (Emami Naini et al., 2012; Fatouros et al., 2010). The antioxidant capacity of LC in arterial hypertension was recently reported to be mediated by modulation of redox-sensitive transcription factor proteins, such as nuclear factor-kappa B (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf2), and peroxisome proliferator-activated receptors (PPARs) (Zambrano et al., 2012).

Based on these background studies, the aim of the present study was to investigate the protective role of L-carnitine against cylindrospermopsin-induced oxidative stress in tilapia (*O. niloticus*). The levels of different oxidative stress markers, including LPO, protein oxidation, DNA oxidation and the ratio of reduced glutathione to oxidised glutathione (GSH/GSSG), were determined in the livers and kidneys of fish. In addition, to obtain a better understanding of the mechanisms involved in the antioxidant effects of LC, the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), NADPH oxidase (the main enzyme involved in superoxide anion production) and γ-glutamylcysteine synthetase (γ-GCS), were also evaluated.

2. Materials and methods

2.1. Chemicals

Pure CYN (purity > 95%) was supplied by the Alexis Corporation (Lausen, Switzerland). LC was provided by Fragon Ibérica (Barcelona, Spain). Chemicals for the different assays were provided by Sigma–Aldrich and VWR International Eurolab. HPLC-grade methanol (MeOH), dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionised water (>18 MΩ cm^{-1} resistivity) was obtained using a Milli-Q water purification system (Millipore, Bedford, USA). BOND ELUT® carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe). Protein assay reagents were obtained from BioRad Laboratories.

2.2. Collection of *A. ovalisporum* strain cultures and determination of cyanobacterial toxins

The CYN-producing strain (CYN+) *A. ovalisporum* (LEGE X-001) was isolated from Lake Kinneret (Banker et al., 1997) and supplied by the Marine Research Centre (Porto, Portugal). Cultures of this strain were maintained in Z8 medium at 25 °C under continuous illumination with an intensity of 28 μmol photons m^{-2} s^{-1} that was provided by cool white fluorescent tubes. After 33 days, the cultures were harvested by decantation and filtration with a plankton net (20 μm diameter). The biomass obtained was frozen at −80 °C prior to lyophilisation (Telstar Cryodos, Madrid).

CYN extraction from the lyophilised culture of *A. ovalisporum* (CYN+) was performed according to the procedure outlined by Guzmán-Guillén et al. (2012). Briefly, the lyophilised cells (14 mg) were extracted using 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated again for 15 min. The resulting mixture was centrifuged at 4500 rpm for 10 min, after which point the supernatant was collected and 6 μL of 0.1% trifluoroacetic acid (TFA) were added. The mixture was then stirred for 1 h and allowed to stand for 3 h. The supernatant was collected for further purification/concentration. For the clean-up procedure, graphitised carbon cartridges packed with Bond Elut® were activated using 10 mL of a solvent mixture of dichloromethane/MeOH (10/90) and rinsed with 10 mL of MilliQ water, after which point the samples were passed through the cartridges, washed with 10 mL of MilliQ water and eluted with 10 mL DCM/MeOH (10/90). To concentrate the samples, the extracts were evaporated in a rotary evaporator and resuspended in 500 μL of MilliQ water prior to LC–MS/MS analysis.

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 150 mm × 2.1 mm Zorbax Sb-Aq column.

Table 1
Feeding conditions[a] of *Oreochromis niloticus* and exposure conditions to *A. ovalisporum* lyophilized cells containing cylindrospermopsin (CYN) and deoxy-CYN, or pure CYN.

Condition	Treatment								
	1	2	3	4	5	6	7	8	9
Lyophilized cells with CYN and deoxy-CYN	–	+	–	–	+	–	–	+	–
Pure CYN	–	–	+	–	–	+	–	–	+
21 d LC 400 mg/kg fish	–	–	–	+	+	+	–	–	–
21 d LC 880 mg/kg fish	–	–	–	–	–	–	+	+	+

[a] With or without L-carnitine (LC) for 21 d.
CYN = cylindrospermopsin.

The flow rate was 0.2 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of: (A) water and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90% B (5 min) and finally 0% B (5 min). Multiple Reaction Monitoring (MRM) experiment was applied in which the parent ions and fragment ions were monitored at Q1 and Q3, respectively. The transitions that can be used for the detection of CYN are: 416.2/194.0, 416.2/274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for the quantification of CYN. For LC–ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

CYN was detected (retention time of 7.55 min) and quantified, and the concentration of CYN obtained was 5.6 μg CYN/mg. Moreover, its deoxy-derivative (deoxy-CYN) was also detected in the sample culture, with a retention time of 7.76 min. In this case, as no pure standard of this isomer was available, the quantification of deoxy-CYN was made using the CYN calibration curve (equivalent of CYN), and the concentration obtained was 0.3 μg deoxy-CYN/mg.

The cyanobacterial culture (LEGE X-001) was tested for production of MCs (Moreno et al., 2004), and PSTs toxins, both by chemical and molecular methods (Nogueira et al., 2004; Brito et al., 2012) and the results were negative.

2.3. Experimental setup and acclimation of fish

Seventy-two male *O. niloticus* (Nile tilapia, Peciformes: Cichlidae) were used to conduct our studies. They were obtained from the Aquaculture Valencia fish hatchery and were maintained at the laboratory until they reached an average weight of 50 ± 8 g and an average length of 12 ± 2 cm. The fish were held in aquaria (8 individuals/aquarium) containing 96 L of fresh water. Exposure to chlorine was minimised by filling the tanks at least 3 days prior to the introduction of the fish. The aquaria were also set up with a continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges (Bio-Espumador)) and the temperature was kept constant (21 ± 2 °C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg/L. Mean values for additional parameters of water quality were: pH = 7.6 ± 0.3, conductivity of 287 μS/cm, Ca^{2+} = 0.60 mM/L and Mg^{2+} = 0.3 mM/L. The fish were fed with commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) and were acclimatised for 15 days prior to the beginning of the experiments.

2.4. Experimental exposures

After the acclimation period, nine groups of tilapia (n = 8) were established and are shown in Table 1. The fish were fed a mixture of the different components (fish food, toxic lyophilised cells, pure CYN, and different doses of LC, depending on the treatment group that the fish had been assigned to) that had been manually crushed using a mortar. This procedure was carried out daily and resulted in small, sticky pellets. The procedure was designed to replicate the type of exposure that may occur when a bloom of cyanobacteria undergoes lysis under field conditions, releasing its CYN content. The pellets were placed in the tank and were allowed to drift to the bottom in order to allow the fish to access them. All of the pellets were visually ensured to have been eaten within an hour. The amount of commercial fish food administered per fish was 0.5 g/day. All groups of fish were sacrificed within 24 h after the administration of the toxin.

The fish housed in aquaria 1 through 3 were fed daily with commercial fish food for 21 days. The tilapia housed in the LC groups (aquaria 4–9) were fed in a similar manner to those housed in aquaria 1 through 3, but were supplemented daily with LC (400 mg of LC/kg of body weight (b.w.) or 880 mg LC/kg of b.w.) for 21 d, as shown in Table 1. Both doses of LC were selected based on the data obtained by Harpaz (2005), who reported that levels of dietary LC supplementation in fish ranging from a few hundred to over 4000 mg LC/kg of diet over long periods of time (e.g., 120 days) played a protective role during exposure to xenobiotics. When focusing specifically on tilapia, Jayaprakas et al. (1996) found beneficial effects on growth and reproductive performance of fish fed a 900 mg LC/kg diet for a period of 252 days. Moreover, previous experiments from our laboratory have demonstrated the antioxidant activity of L-carnitine to be 300 mg/kg of b.w./day (Miguel-Carrasco et al., 2010). After 21 d, the fish housed in aquaria 5, 6, 8, and 9 received single doses of 400 μg CYN/kg of fish (either that isolated from lyophilised *A. ovalisporum* cells or pure CYN) as described in Table 1. The fish housed in aquarium 1 served as a control (no CYN exposure). The fish housed in aquaria 4 and 7 did not receive the toxin and were used as controls for the fish receiving both doses of LC. The CYN dose used in the present study was selected based on the results of previous experiments (Gutiérrez-Praena et al., 2011a,b; Puerto et al., 2011).

2.5. Preparation of postmitochondrial supernatants (PMS)

Fish were sacrificed by transection of the spinal cord subsequent to anesthetisation in ice. The livers and kidneys were extracted, weighed, rinsed with ice-cold saline and kept at −85 °C prior to analysis. Tissues from each individual (not pooled) were homogenised according to the methods outlined by Puerto et al. (2009), using a 0.1 M potassium phosphate buffer (pH = 6.5) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetra-acetic acid, and 1.4 mM dithioerythritol. After the removal of cellular debris (10 min at 13,000 × g), the membrane fraction was separated by centrifugation at 105,000 × g for 60 min. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for subsequent determinations of oxidative stress parameters. A different homogenisation technique was performed in order to determine NADPH oxidase activity.

2.6. Lipid peroxidation, protein carbonyl content and DNA oxidation

Lipid peroxidation products were quantified using the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman, 1990), with slight modifications (Jos et al., 2005). Malondialdehyde (MDA) is formed as an end lipid peroxidation product that reacts with the TBA reagent under acidic conditions to generate a pink coloured product. The values obtained are presented as nmol TBARS/g tissue.

The carbonyl groups of proteins were quantified using the method described by Levine et al. (1990). The purpose of this method is to measure the amounts of the carbonyl groups derived from the oxidation of the proteins present in the sample, where 2,4-dinitrophenylhydrasine reacts with the carbonyl groups of the proteins forming 2,4-dinitrophenylhydrasone, a coloured complex that absorbs light at 366 nm. The concentration of carbonyl groups present in the sample is calculated using the regression curve and the Lambert–Beer law with a molar extinction coefficient (ε) of 22,000 M^{-1} cm^{-1}, as has been previously described by Atencio et al. (2008). The results are expressed as nmoles of carbonyl groups/mg of protein.

To measure DNA oxidation, the OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP sites) (Cell Biolabs, Inc., San Diego, CA, USA) was used according to the procedure outlined by Gutiérrez-Praena et al. (2011a). The results are expressed as apurinic/apyrimidinic (AP) sites per 100,000 bp.

2.7. NADPH oxidase enzyme activity

To determine NADPH oxidase (EC 1.6.3.1) activity, tissues were homogenised in phosphate buffer containing protease inhibitors and centrifuged at 2000 × *g* for 10 min. NADPH oxidase activity was analysed according to the procedures outlined by Ohara et al. (1993) and Zambrano et al. (2012), with slight modifications (Puerto et al., in press). Lucigenin (Sigma–Aldrich, Madrid, Spain) was used to measure superoxide anion production. Measurements are expressed as relative light units (RLUs) per second.

2.8. Other enzyme activities

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed using the xanthine-oxidase-cytochrome c method as described by McCord and Fridovich (1969), and measuring its activity spectrophotometrically at 505 nm, as described in Prieto et al. (2008). Catalase (CAT; EC 1.11.1.6) activity was assayed according to the method described by Beers and Sizer (1952) by measuring the initial rate of hydrogen peroxide (10 mM) decomposition at 240 nm (Prieto et al., 2008). The activity of gamma-glutamylcysteine synthetase (EC 6.3.2.2.) (γ-GCS) was measured using the method described by Seelig and Meister (1985), which was modified for use in fish samples (Gutiérrez-Praena et al., 2011a). The results of all of these enzyme activity assays are expressed as nkat/mg of protein.

2.9. Non-enzymatic antioxidant parameters: GSH/GSSG ratio

The GSH/GSSG ratio was determined in liver and kidney homogenates using a commercial kit (Bioxytech GSH/GSSH-412, Oxis Research, Foster City, CA, USA). The protocol was modified for use in fish samples (Atencio et al., 2008).

2.10. Protein estimations

The protein concentrations in the samples were estimated using the method outlined by Bradford (1976). Bovine γ-globulin was used as a standard. Briefly, 5 μL of the diluted samples were mixed with 95 μL of H_2O and 5 mL of Coomassie Brilliant blue dye (Bio-rad Laboratories, Hercules, USA) and the absorbances were read at 595 nm using a spectrophotometer (Cary100, Varian, Madrid, Spain).

2.11. Statistical analyses

All results were subjected to one-way analysis of variance (ANOVA), and represent the means ± SE of 8 animals per group. Differences in mean values between groups were assessed using Tukey's test and were considered to be significantly different when a $p < 0.05$ was obtained.

3. Results

No mortality or visual changes were observed in any of the fish during these experiments.

3.1. Effects on lipid, protein and DNA oxidation

LPO values were increased significantly in the livers of fish that had been exposed to lyophilised *A. ovalisporum* cells as compared to those in the control group (1.6-fold), although there were no significant differences in LPO levels in fish that had been exposed to pure CYN as compared to those in the control group (group 1) (Fig. 1). In the kidneys, there was a significant induction of LPO in fish that had been exposed to lyophilised *A. ovalisporum* cells (group 2) and pure CYN (group 3) as compared to those in the control group (1.4-fold and 1.5-fold, respectively). Both doses of L-carnitine (400 and 880 mg/kg fish) resulted in effective protection against

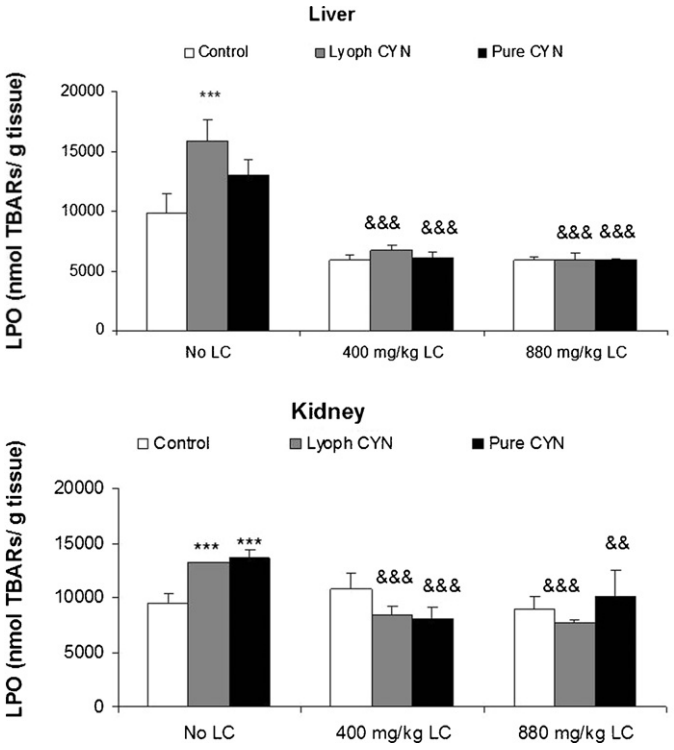

Fig. 1. Lipid peroxidation (LPO) in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values are expressed as mean ± SE ($n = 8$). LPO values are expressed as nmol TBARS/g tissue. The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, and $^{\&\&\&}p < 0.001$ or $^{\&\&}p < 0.01$ when 400 or 880 mg LC versus no LC supplementation groups are compared.

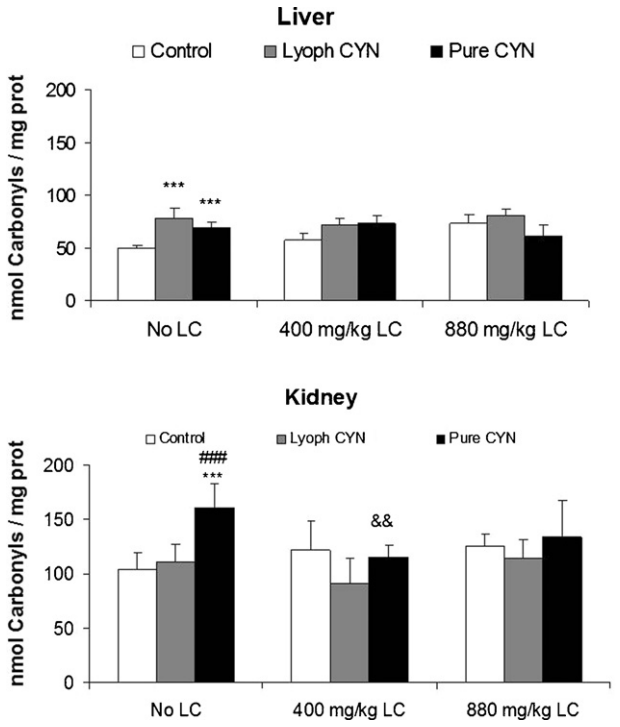

Fig. 2. Protein oxidation values (nmol carbonyl/mg protein) in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg fish/d) or without it. The values are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ when fish were exposed to *A. ovalisporum* lyophilized cells culture versus pure CYN, and $^{\&\&}p < 0.01$ when 400 or 880 mg LC versus no LC supplementation groups are compared.

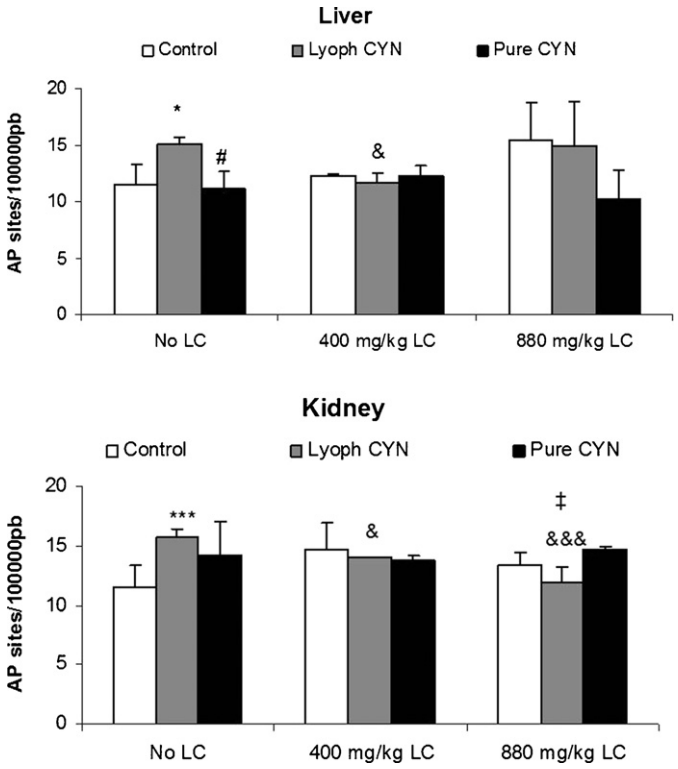

Fig. 3. DNA oxidation in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values are expressed as mean ± SE ($n = 8$). DNA oxidation values are expressed as AP sites/100,000 bp. The significance levels observed are $^{***}p < 0.001$ or $^{*}p < 0.05$ in comparison with their respective control group, $^{\#}p < 0.05$ when fish were exposed to *A. ovalisporum* lyophilized cells culture versus pure CYN, $^{\&\&\&}p < 0.001$ or $^{\&}p < 0.05$ when 400 or 880 mg LC versus no LC supplementation groups are compared, and $^{‡}p < 0.05$ when 880 mg LC versus 400 mg LC supplementation groups are compared.

CYN-induced LPO alterations in the livers and kidneys of fish (groups 5–6, or 8–9). The LPO values in both organs were significantly lower than those in fish that had not been pretreated with L-carnitine (groups 2–3).

Protein oxidation levels in fish that had been exposed to lyophilised *A. ovalisporum* cells or pure CYN (groups 2 and 3) were significantly increased (1.6-fold and 1.4-fold, respectively) as compared to those in the control group (group 1) (Fig. 2). However, no significant changes in protein oxidation levels were observed in fish that had been pretreated with 400 or 880 mg LC/kg of b.w. and CYN (groups 5–6 or 8–9, respectively) as compared to their respective control groups (groups 4 and 7). In the kidneys, significant increases in the protein oxidation levels of fish that had been exposed to pure CYN (group 3) were observed as compared with both the control group (group 1) (1.6-fold) and the group that had been exposed to lyophilised *A. ovalisporum* cells (group 2) (1.6-fold). A significant decrease was observed between the group that had been exposed to pure CYN and pretreated with 400 mg LC/kg (group 6) versus the group that had only been exposed to pure CYN (group 3) (1.4-fold).

DNA oxidation (Fig. 3) was significantly increased in the livers and kidneys of fish that had been exposed to lyophilised *A. ovalisporum* cells (group 2) in comparison with their respective control group (group 1) (1.3-fold and 1.4-fold, respectively). In contrast, no changes were observed in the group that had been exposed to pure CYN (group 3) in comparison to the control fish (group 1), although significant differences regarding the type of CYN employed (pure versus cyanobacterial cells) were found in the livers (1.4-fold). Treatment with both doses of LC (400 or 880 mg/kg) resulted in effective protection in the livers and kidneys. No significant increases in DNA oxidation in the fish that had been pre-treated with LC (groups 5 and 8) were observed in comparison to their respective controls groups (groups 4 and 7). In the kidneys, treatment with the highest dose of LC (880 mg LC/kg of fish) decreased DNA oxidation in the fish that had been exposed to lyophilised *A. ovalisporum* cells (group 8) in comparison to both the fish that had not been pretreated (group 2) and intoxicated fish (cyanobacterial cells) that had been pretreated with 400 mg LC/kg (group 5).

3.2. Effects on NADPH oxidase activity

NADPH oxidase activity was significantly increased in the livers of fish that had been exposed to either *A. ovalisporum* (group 2) (2.7-fold) or pure CYN (group 3) (2.0-fold) as compared to the control group (group 1) (Fig. 4). Moreover, significant differences in this parameter were detected in fish that had been exposed to CYN via *A.ovalisporum* versus pure CYN (group 2 versus 3). Both doses of LC resulted in similarly effective protection, restoring NADPH oxidase activity levels to those observed in control fish. In fact, significantly lower values were found in CYN-exposed fish that had been pre-treated with 400 or 880 mg LC/kg of fish (groups 5–6 or 8–9) than in CYN-exposed fish that had not been pre-treated with LC (groups 2–3). No significant changes were observed in the activity of this enzyme in the kidneys of fish from any of the treatment groups.

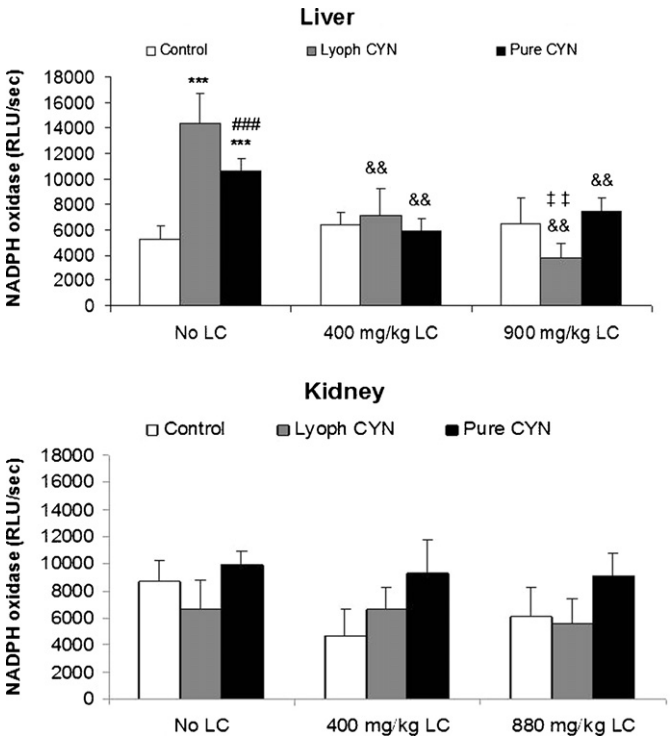

Fig. 4. NADPH oxidase activity (relative light units per second, RLU/s) in liver and kidney of control fish and fish acutely exposed to to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values are expressed as mean ± SE ($n = 8$). NADPH oxidase activity is expressed as relative light units per second, RLU/s. The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ when fish were exposed to *A. ovalisporum* lyophilized cells culture versus pure CYN, $^{\&\&}p < 0.01$ when 400 or 880 mg LC versus no LC supplementation groups are compared, and $^{‡‡}p < 0.01$ when 880 mg LC versus 400 mg LC supplementation groups are compared.

3.3. Effects on other enzyme activities

Different effects on SOD enzyme activity were observed in the livers and kidneys. In the livers, SOD enzyme activity was not modified by any of the toxic treatments and LC supplementation also had no effects (Fig. 5A). In contrast, significant increases in SOD activity were found in the kidneys of fish that had been exposed to lyophilised *A. ovalisporum* cells or pure CYN (groups 2–3) as compared to the control group (1.8-fold and 2.0-fold, respectively). Fish exposed to CYN that had been pretreated with 400 mg LC/kg of fish (groups 4–6) did not show significant changes in comparison to control fish, and a significant reduction in NADPH oxidase activity was detected in pretreated fish that had been exposed to pure CYN when compared to fish that had not been pretreated with LC.

LC pre-treatment did not alter CAT activity in the livers of fish that had been exposed to lyophilised *A. ovalisporum* cells or pure CYN (groups 2 and 3) (Fig. 5B). However, the kidneys of fish that had been exposed to lyophilised *A. ovalisporum* cells (group 2) displayed significant increases in CAT activity when compared to the control group, which were reversed after the administration of both doses of LC (groups 1–3).

The activity of γ-GCS (Fig. 6) in the livers of fish that had been exposed to CYN (from lyophilised *A. ovalisporum* cells or pure CYN, groups 2–3) was not significantly altered as compared to the control group (group 1). In the kidneys, decreases in this parameter were observed in CYN-exposed fish (2.9-fold and 1.9-fold for lyophilised *A. ovalisporum* cells and pure CYN, respectively), and exposure to both LC doses prevented the reduction induced by CYN in comparison to fish that did not receive it (groups 2–3).

3.4. Effects on the GSH/GSSG ratio

The GSH/GSSG ratio was significantly decreased in the livers and kidneys of fish that had been exposed to lyophilised *A. ovalisporum* cells (group 2) (1.9-fold and 1.6-fold, respectively) as compared to the control group (group 1) (Fig. 7). Moreover, fish that had been exposed to pure CYN (group 3) only showed significant GSH depletion in the kidneys (1.8-fold) when compared with control fish (group 1). Both doses of LC were shown to have beneficial effects, and the LC treatments enhanced the GSH/GSSG ratios when compared with the CYN-exposed groups that had not been treated with the chemoprotectant.

4. Discussion

Cylindrospermopsin can be found worldwide in drinking-water sources, including lakes and reservoirs, and has been implicated to be the cause of human intoxications and animal mortality (Carmichael et al., 2001; Hawkins et al., 1985). Under natural conditions, fish are exposed to cyanobacterial blooms, which may contain CYN and other unknown bioactive compounds. Oxidative stress is one of the mechanisms that has been reported to be involved in CYN pathogenicity in fish (Gutiérrez-Praena et al., 2011a,b; Guzmán-Guillén et al., 2013; Puerto et al., 2011, in press). Therefore, an ROS-decreasing treatment may ameliorate the adverse effects of oxygen-derived free radicals. NAC is the only substance that has been shown to have a protective effect against CYN-induced oxidative stress in fish (Gutiérrez-Praena et al., 2012b). In the present study, we explored the usefulness of dietary pretreatment with L-carnitine (400 or 880 mg LC/kg of fish over a 21 day period) as prophylaxis for CYN-induced oxidative stress in fish that have been acutely exposed to lyophilised *A. ovalisporum* cells or pure CYN (at a dose of 400 μg CYN/kg of fish) for the first time.

Exposure to CYN from lyophilised cyanobacterial cells induced higher toxic effects than pure CYN, as indicated by increases in LPO, protein oxidation, DNA oxidation, NADPH oxidase activity, and decreases in the GSH/GSSG ratio in the livers of treated fish. Moreover, the kidneys also displayed alterations in the activities of SOD, CAT and γ-GCS, as well as significant decreases in the GSH/GSSG ratio. These results are in agreement with the results of similar studies that were carried out in different animal models in which no significant changes were found between pure CYN and extracts from cyanobacterial cells (Falconer et al., 1999; Seifert, 2007). LC supplementation per se did not affect any of these parameters in fish that were not exposed to the toxin, indicating that LC only ameliorates or prevents CYN-induced toxic effects.

Decreases in CYN-induced antioxidant markers, such as LPO, protein oxidation, DNA oxidation, and changes in antioxidant enzyme activities, following LC pretreatment reflect the antioxidant properties of this compound, which combine both free radical scavenging of ROS and metal-chelating properties (Muthuswamy et al., 2006). Thus, LC has been demonstrated to be an effective scavenger of hydroxyl radicals via its action on carbonyl groups. LC also plays a role in the chelation of free Fe^{2+} ions by interacting with the hydroxyl and carboxylate groups, resulting in complex formation (Derin et al., 2004; Gülçin, 2006), thereby decreasing free radical generation (Reznick et al., 1992). Moreover, LC has been demonstrated to protect against lipid peroxidation in disorders that are related to oxidative stress (Loster and Bohm, 2001; Luo et al., 1999; Miguel-Carrasco et al., 2010; Şıktar et al., 2011). These effects were thought to be associated with both the cell membrane stabilising ability of LC and its antioxidant properties (Canbaz et al., 2007).

In the present study, pretreatment with both doses of LC decreased the presence of carbonyl groups in CYN-intoxicated fish, thereby decreasing oxidant hydroxyl groups. These results are in

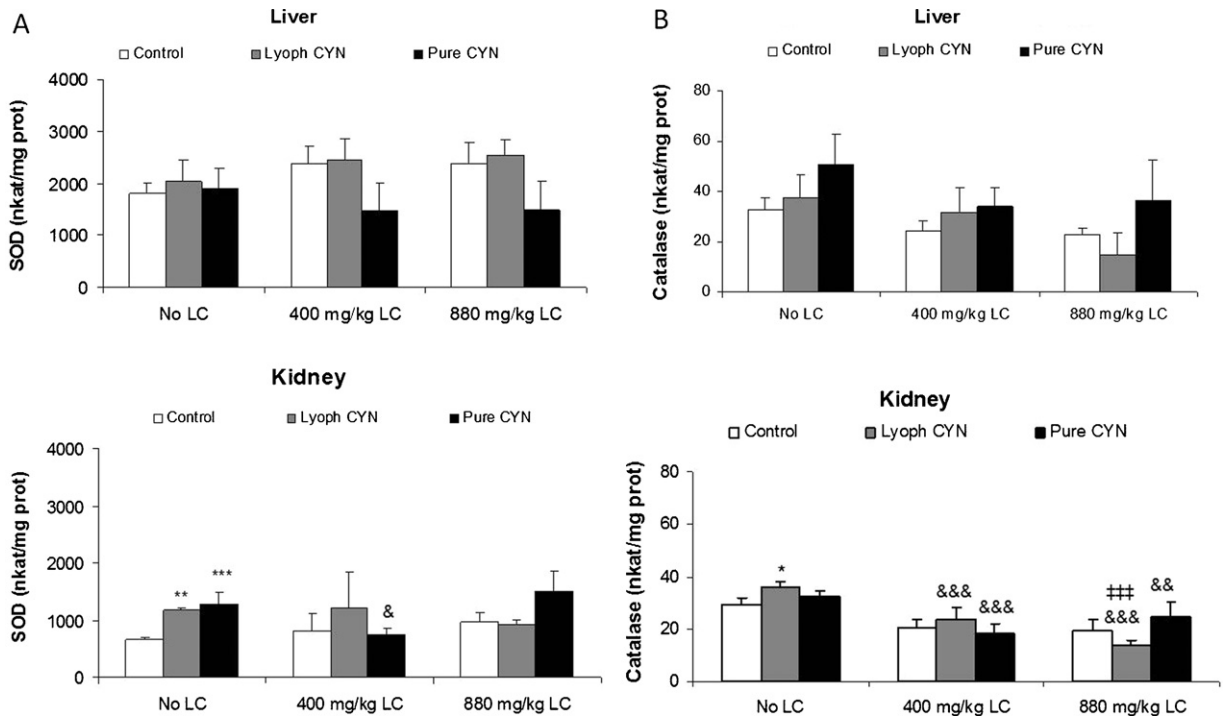

Fig. 5. (A) Superoxide dismutase activity (SOD) and (B) catalase activity (CAT) in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values (nkat/mg protein) are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$, $^{**}p < 0.01$ or $^{*}p < 0.05$ in comparison with their respective control group, $^{\&\&\&}p < 0.001$, $^{\&\&}p < 0.01$ or $^{\&}p < 0.05$ when 400 or 880 mg LC versus no LC supplementation groups are compared, and $^{\#\#\#}p < 0.001$ when 880 mg LC versus 400 mg LC supplementation groups are compared.

agreement with the results of previous studies that demonstrated that LC administration significantly protected proteins against oxidative modifications in the serum and livers of rats that had been intoxicated with ethanol (Augustyniak and Skrzydlewska, 2009).

Cylindrospermopsin significantly increased DNA oxidation in the livers and kidneys of fish that were exposed to lyophilised *A. ovalisporum* cells, although no changes were observed in fish that were exposed to pure CYN. These results suggest that oxidative stress plays a role in the underlying genotoxic mechanism of lyophilised cyanobacterial cells containing CYN (Guzmán-Guillén et al., 2013). According to the results of a study conducted by Falconer and Humpage (2001), the uracil group contained within the structure of CYN may interact with adenine groups in RNA and DNA, thereby interfering with DNA synthesis, and may in turn promote mutations, acting as a carcinogen. In the present study, treatment with LC protected DNA against ROS-induced damage. Some authors have demonstrated a marked and reproducible reduction in H_2O_2-induced chromosome damage in mitotic CHO cells that involves an LC-mediated capacity to buffer the intracellular formation of ROS (Santoro et al., 2005).

NADPH oxidases are important sources of ROS and play key roles in oxidative stress-mediated pathology (Griendling et al., 2000). Significant increases in NADPH oxidase activity were observed in the livers of CYN-intoxicated fish, which is in agreement with the results obtained in the livers and kidneys of tilapia that had been acutely exposed to pure CYN (Gutiérrez-Praena et al., 2011a; Puerto et al., in press). The relationship between ROS and LPO, protein and DNA oxidation could explain the parallel increases in these parameters that were induced by CYN. As previously demonstrated in other pathologic situations that are characterised by increases in NADPH oxidase activity (Zambrano et al., 2012), our results showed that NADPH oxidase activity was restored to basal levels in the livers of CYN-exposed fish that had been pre-treated with doses of 400 mg LC/kg of fish. This effect of LC seems to be mediated by

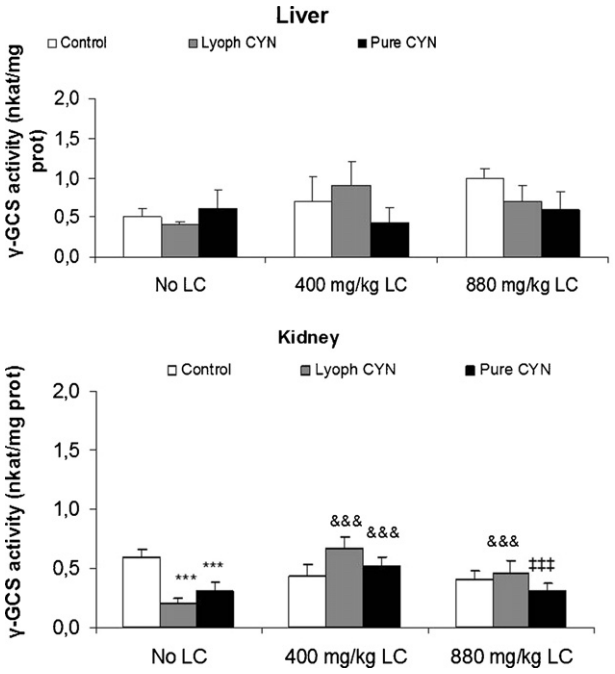

Fig. 6. γ-Glutamyl-cysteine synthetase (GCS) in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values (nkat/mg protein) are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\&\&\&}p < 0.001$ when 400 or 880 mg LC versus no LC supplementation groups are compared, and $^{\#\#\#}p < 0.001$ when 880 mg LC versus 400 mg LC supplementation groups are compared.

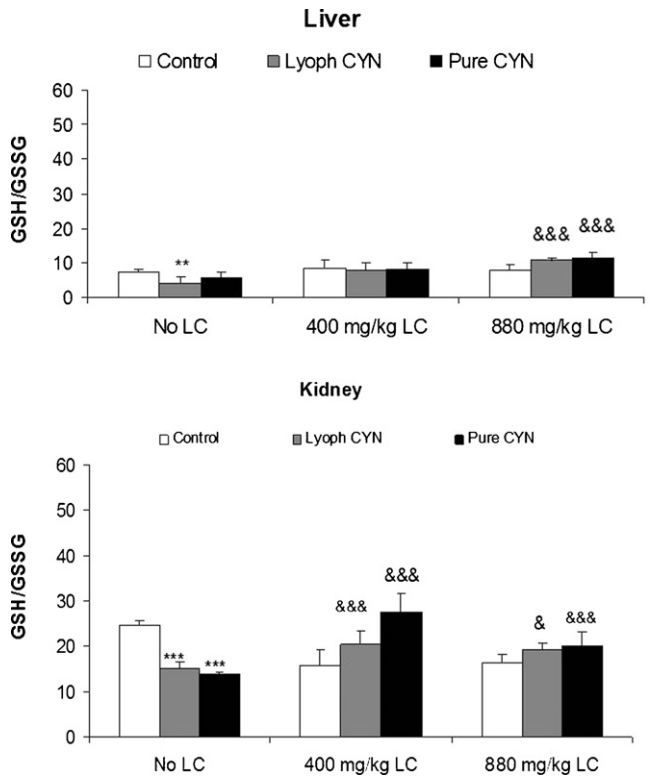

Fig. 7. Ratio between reduced and oxidized glutathione (GSH/GSSG) in liver and kidney of control fish and fish acutely exposed to 400 μg CYN/kg fish from *A. ovalisporum* lyophilized cells culture or pure CYN, and two different levels of L-carnitine (LC) supplementation (400 or 880 mg LC/kg b.w. fish/d) or without it. The values are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ or $^{**}p < 0.01$ in comparison with their respective control group, and $^{\&\&\&}p < 0.001$ or $^{\&}p < 0.05$ when 400 or 880 mg LC versus no LC supplementation groups are compared.

modulation of transcription factors, such as NF-κB, Nrf2 and PPARs (Chen et al., 2009).

In general, antioxidant enzyme activities showed greater changes in the kidneys than in the livers of fish that had been exposed to CYN. SOD and CAT enzymatic activities were increased only in the kidneys of fish that had been acutely exposed to lyophilised *A. ovalisporum* cells. Subchronic studies in tilapia also revealed changes in these biomarkers (Guzmán-Guillén et al., 2013). The SOD-CAT system provides the first defence against oxygen toxicity (Atencio et al., 2008), and the increases detected in the kidneys of the fish studied in this work may indicate the detoxification activity of this tissue. Supplementation with LC ameliorates SOD-CAT activity levels, due its ability to scavenge superoxide anions (Gülçin, 2006), thereby conferring protection against damage induced by hydrogen peroxide (Abdelrazik et al., 2009).

The activity of γ-GCS and the GSH/GSSG ratio were affected by exposure to CYN from both sources (pure CYN or that obtained from cyanobacterial cells). These effects were more pronounced in the kidneys of intoxicated fish. Reductions in GSH levels may be the result of decreases in GSH synthesis or increases in its availability to reduce oxidative damage. Because the largest decrease in the GSH/GSSG ratio in the kidneys versus the liver occurred in parallel with a greater decrease in the activity of the γ-GCS enzyme, GSH depletion may be due to inhibition of GSH synthesis. These results are in agreement with those of Runnegar et al. (1994, 1995) and Gutiérrez-Praena et al. (2011b) which demonstrated in vitro in rat hepatocytes and in PLHC-1 fish cells, respectively, the ability of CYN to reduce GSH synthesis. In the kidneys, the administration of the chemoprotectant (400 or 880 mg/kg) resulted in a significant increase in γ-GCS activity. N-acetylcysteine was also able to prevent pure CYN-induced reductions in γ-GCS (Gutiérrez-Praena et al., 2012b).

In regard to the GSH/GSSG ratio, the more pronounced response observed in the kidneys may be explained by increased ROS levels and, consequently, detoxification in this organ, implying that oxidation of GSH to GSSG occurred, as has been previously demonstrated (Gutiérrez-Praena et al., 2011a,b; Guzmán-Guillén et al., 2013; Puerto et al., in press). LC pre-treatment prevented significant GSH depletion in the livers and kidneys of fish that had been exposed to lyophilised *A. ovalisporum* cells or pure CYN. Elevations of GSH levels after LC therapy may occur as a result of increased NADPH generation due to enhanced G6PDH activity. NADPH is used by GSH reductase to reduce GSSG to GSH (Kumaran et al., 2003). In accordance with these results, some authors have shown that LC induced significant increases in GSH levels in the liver and serum in rats that had been chronically exposed to ethanol, and partially protected nonenzymatic antioxidants against oxidative stress (Augustyniak and Skrzydlewska, 2009). Moreover, LC administration has been shown to raise GSH levels during ischemia (Sushamakumari et al., 1989), and in hypertensive rats (Gómez-Amores et al., 2007; Miguel-Carrasco et al., 2010). In comparison to other antioxidants, Gutiérrez-Praena et al. (2012b) showed that doses of 45 mg NAC/fish/day prevented the toxic effects induced by pure CYN in fish. However, LC administration could not counteract the decreases completely due to cyanobacterial cell exposure.

Both LC doses studied (400 or 880 mg/kg fish) were effective at preventing CYN-induced oxidative stress. In general, the lowest dose was sufficient to counteract the toxic effects. One advantage of LC versus other antioxidants is that none of the LC doses tested induced pro-oxidant effects per se. In contrast, NAC can be considered to be a useful chemoprotectant in the prophylaxis and treatment of cyanotoxin-related intoxications in fish, but the dose used requires special attention due to the pro-oxidant activity of NAC (Puerto et al., 2009). Cost effectiveness is an important issue that should be considered in LC supplementation in fish, and according to Harpaz (2005), the levels of this rather expensive substance that would be required may not be economically justifiable. More studies should be undertaken in order to gain a better understanding of the mechanism(s) involved in the protective effects of LC in CYN-induced oxidative stress in fish, as well as to identify the lowest possible levels of dietary LC supplementation in fish culture, in order to alleviate stress related to CYN exposure.

5. Conclusion

The present study demonstrated for the first time the beneficial antioxidant effects of LC dietary supplementation at doses over 400 mg/kg of fish on the oxidative stress status of different organs in tilapia (*O. niloticus*) that had been exposed to lyophilised *A. ovalisporum* cells or pure CYN, indicating the potential use of this natural antioxidant to prevent oxidative damage in fish that have been exposed to CYN. Supplementation with LC decreased LPO, protein oxidation, DNA oxidation and NADPH oxidase activity, and significantly increased the activities of some of the antioxidant enzymes studied (SOD, CAT and γ-GCS). In addition, LC prevented the depletion of free radical scavengers, such as reduced glutathione, indicating that these effects of LC may ameliorate oxidative status in fish that have been exposed to cyanobacterial cells. The occurrence of cyanobacterial blooms in aquatic environments is increasing in many regions of the world due to progressive eutrophication of water bodies and climate change. Therefore, it is vital that more studies be carried out in order to establish the activity of new substances that can be used to treat and prevent cyanotoxin-induced injury in fish.

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References

Abdelrazik, H., Sharma, R., Mahfouz, R., Agarwal, A., 2009. L-Carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertility and Sterility 91, 589–596.

Aruoma, O.I., Halliwell, B., Hoey, B.M., Butler, J., 1989. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radical Biology and Medicine 6, 593–597.

Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Camean, A.M., 2008. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to microcystis under laboratory conditions. Toxicon 52, 1–12.

Atencio, L., Moreno, I., Jos, A., Prieto, A.I., Moyano, R., Blanco, A., Cameán, A.M., 2009. Effects of dietary selenium on the oxidative stress and pathological changes in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Toxicon 53, 269–282.

Augustyniak, A., Skrzydlewska, E., 2009. L-Carnitine in the lipid and protein protection against ethanol-induced oxidative stress. Alcohol 43, 217–223.

Banker, R., Carmeli, S., Hadas, O., Teltsch, B., Porat, R., Sukenik, A., 1997. Identification of cylindropsermopsin in *Aphanizomenon ovalisporum* (cyanophyceae) isolated from Lake Kinneret, Israel. Journal of Phycology 33, 613–616.

Bazin, E., Mourot, A., Humpage, A.R., Fessard, V., 2009. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environmental and Molecular Mutagenesis 51, 251–259.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. Journal of Biological Chemistry 195, 133–140.

Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L., 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53, 289–299.

Berry, J.P., Lind, O., 2010. First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in tegogolo snails (*Pomacea patula catemacensis*). Toxicon 55, 930–938.

Berry, J.P., Jaja-Chimedza, A., Dávalos-Lind, L., Lind, O., 2012. Apparent bioaccumulation of cylindrospermopsin and paralytic shellfish toxins by finfish in Lake Catemaco (Veracruz, México). Food Additives and Contaminants 29, 314–321.

Bilinski, E., Jonas, R.E.E., 1970. Effects of coenzyme A and carnitine on fatty acid oxidation in rainbow trout mitochondria. Journal of the Fisheries Research Board of Canada 27, 857–864.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Brito, A., Ramos, V., Santos, A., Seabra, R., Martins, R., Frazão, B., Santos, C.L., Lopo, M., Ferreira, S., Martins, A., Vasconcelos, V., Tamagnini, P., 2012. Diversity of cyanobacteria in the intertidal zones of the Portuguese coast: a polyphasic study. Systematic and Applied Microbiology 35, 110–119.

Canbaz, H., Akca, T., Tataroglu, C., Caglikulekci, M., Dirlik, M., Ayaz, L., Ustunsoy, A.B., Tasdelen, B., Aydin, S., 2007. The effects of exogenous L-carnitine on lipid peroxidation and tissue damage in an experimental warm Hepatic Ischemia-Reperfusion Injury Model. Current Therapeutic Research 68, 32–46.

Carmichael, W.W., Azevedo, S.M., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environmental Health Perspectives 109, 663–668.

Chen, H.H., Sue, Y.M., Chen, C.H., Hsu, Y.H., Hou, C.C., Cheng, C.Y., Lin, S.L., Tsai, W.L., Chen, T.W., Chen, T.H., 2009. Peroxisome proliferator-activated receptor alpha plays a crucial role in L-carnitine anti-apoptosis effect in renal tubular cells. Nephrology, Dialysis, Transplantation 24, 3042–3049.

Dayanandan, A., Kumar, P., Panneerselvam, C., 2001. Protective role of L-carnitine on liver and heart lipid peroxidation in atherosclerotic rats. The Journal of Nutritional Biochemistry 12, 254–257.

Derin, N., Izgut-Uysal, V.N., Agac, A., Aliciguzel, Y., Demir, N., 2004. L-Carnitine protects gastric mucosa by decreasing ischemiae reperfusion induced lipid peroxidation. Journal of Physiology and Pharmacology 55, 595–606.

Emami Naini, A., Moradi, M., Mortazavi, M., Amini Harandi, A., Hadizadeh, M., Shirani, F., Basir Ghafoori, H., Emami Naini, P., 2012. Effects of oral L-carnitine supplementation on lipid pro-file, anemia, and quality of life in chronic renal disease patients under hemodialysis: a randomized, double-blinded, placebo-controlled trial. Journal of Nutrition and Metabolism, http://dx.doi.org/10.1155/2012/510483.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonetal. Methods in Enzymology 186, 407–421.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J., Hawkins, P.R., 1999. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environmental Toxicology 14, 143–150.

Falconer, I.R., Humpage, A.R., 2001. Preliminary evidence for in vivo tumour initiation by oral administration of extracts of the blue-green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. Environmental Toxicology 16, 192–195.

Fatouros, I.G., Douroudos, I., Panagoutsos, S., Pasadakis, P., Nikolaidis, M.G., Chatzinikolaou, A., Sovatzidis, A., Michailidis, Y., Jamurtas, A.Z., Mandalidis, D., Taxildaris, K., Vargemezis, V., 2010. Effects of L-carnitine on oxidative stress responses in patients with renal disease. Medicine and Science in Sports and Exercise 42, 1809–1818.

Froscio, S.M., Humpage, A.R., Bucham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environmental Toxicology 18, 243–251.

Gómez-Amores, L., Mate, A., Miguel-Carrasco, J.L., Jiménez, L., Jos, A., Cameán, A.M., Revilla, E., Santa-María, C., Vázquez, C.M., 2007. L-Carnitine attenuates oxidative stress in hypertensive rats. The Journal of Nutritional Biochemistry 18, 540–553.

Griendling, K.K., Sorescu, D., Ushio-Fukai, M., 2000. NAD(P)H oxidase: role in cardiovascular biology and disease. Circulation Research 86, 494–501.

Gülçin, I., 2006. Antioxidant and antiradical activities of L-carnitine. Life Sciences 78, 803–811.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Cameán, A.M., 2011a. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory condition: influence of exposure route and time of sacrifice. Aquatic Toxicology 105, 100–106.

Gutiérrez-Praena, D., Pichardo, S., Jos, M.A., Cameán, A.M., 2011b. Toxicity and glutathione implication in effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotoxicology and Environmental Safety 74, 1567–1572.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M., 2012a. Time-dependent histopathological changes induced in Tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral route and intraperitoneal route. Ecotoxicology and Environmental Safety 76, 102–113.

Gutiérrez-Praena, D., Puerto, M., Prieto, A.I., Jos, A., Pichardo, S., Vasconcelos, V.M., Cameán, A.M., 2012b. Protective role of dietary N-acetylcysteine on the oxidative stress induced by cylindrospermopsin in tilapia (*Oreochromis niloticus*). Environmental Toxicology and Chemistry 31, 1548–1555.

Guzmán-Guillén, R., Prieto Ortega, A.I., Moreno, I., González, A.G., Soria-Díaz, M.E., Vasconcelos, V., Cameán, A.M., 2012. Development and optimization of a method for the determination of cylindrospermopsin from strains of *Aphanizomenon* cultures: intra-laboratory assessment of its accuracy by using validation standards. Talanta 100, 356–363.

Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán, A.M., 2013. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed Tilapia (*Oreochromis niloticus*). Chemosphere 90, 1184–1194.

Harada, K., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 32, 73–84.

Harpaz, S., Becker, K., Blum, R., 1999. The effect of dietary lcarnitine supplementation on cold tolerance and growth of ornamental cichlid fish (*Pelvicachromis pulcher*)—preliminary results. Journal of Thermal Biology 24, 57–62.

Harpaz, S., 2005. Carnitine and its attributed functions in fish culture and nutrition—a review. Aquaculture 249, 3–21.

Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B., Falconer, I.R., 1985. Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju isolated form a domestic supply reservoir. Applied and Environment Microbiology 50, 1292–1295.

Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R., Falconer, I.R., 1997. Isolation and toxicity of *Cylindrospermosis raciborskii* from an ornamental lake. Toxicon 35, 341–346.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. Journal of Toxicology and Environmental Health, Part A 68, 739–753.

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: a review. Environmental Pollution 150, 177–192.

Jayaprakas, V., Sambhu, C., Sunil Kumar, S., 1996. Effect of dietary L-carnitine on growth and reproductive performance of male *Oreochromis mossambicus* (Peters). Fishery Technology 33, 84–90.

Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquatic Toxicology 72, 261–271.

Kalaiselvi, T., Panneerselvam, C., 1998. Effect of L-carnitine on the status of lipid peroxidation and antioxidants in aging rats. The Journal of Nutritional Biochemistry 9, 575–581.

Kinnear, S., 2010. Cylindrospermopsin: a decade of progress on bioaccumulation research. Marine Drugs 8, 542–564.
Kumaran, S., Deepak, B., Naveen, B., Panneerselvam, C., 2003. Effects of levocarnitine on mitochondrial antioxidant systems and oxidative stress in aged rats. Drugs R&D 4, 141–147.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shatiel, S., Stadm, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology 186, 464–478.
Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y., Watanabe, M.M., 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata*. Journal of Phycology 37, 1121–1126.
Loster, H., Bohm, U., 2001. L-Carnitine reduces malondialdehyde concentrations in isolated rat hearts in dependence on perfusion conditions. Molecular and Cellular Biochemistry 217, 83–90.
Luo, X., Reichetzer, B., Trines, J., Benson, L.N., Lehotay, D.C., 1999. L-Carnitine attenuates doxorubicin-induced lipid peroxidation in rats. Free Radical Biology and Medicine 26, 1158–1165.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 244, 6049–6055.
Miguel-Carrasco, J.L., Monserrat, M.T., Mate, A., Vázquez, C.M., 2010. Comparative effects of captopril and L-carnitine on blood pressure and antioxidant enzyme gene expression in the heart of spontaneously hypertensive rats. European Journal of Pharmacology 632, 65–72.
Moreno, I.M., Pereira, P., Franca, S., Cameán, A., 2004. Toxic cyanobacteria strains isolated from blooms in the Guadiana river (southwest of Spain). Biological Research 37, 405–417.
Muthuswamy, A.D., Vedagiri, K., Ganesan, M., Chinnakannu, P., 2006. Oxidative stress-mediated macromolecular damage and dwindle in antioxidant status in aged rat brain regions: role of L-carnitine and DL-alpha-lpoic-acid. Clinica Chimica Acta 368, 84–92.
Nogueira, I., Pereira, P., Dias, E., Pflugmacher, S., Wiegand, C., Franca, S., Vasconcelos, V., 2004. Accumulation of Paralytic Shellfish Toxins (PST) from the cyanobacterium *Aphanizomenon issatschenkoi* by the cladoceran *Daphnia magna*. Toxicon 44, 773–780.
Norris, R.L., Eaglesham, G.K., Pierens, G., Shaw, G.R., Smith, M.J., Chiswell, R.K., Seawright, A.A., Moore, M.R., 1999. Deoxycylindrospermopsin an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. Environmental Toxicology 14, 163–165.
Ohara, Y., Peterson, T.E., Harrison, D.G., 1993. Hypercholesterolemia increases endothelial superoxide anion production. Journal of Clinical Investigation 91, 2546–2551.
Ohtani, I., Moore, R.E., Runnegar, M., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. Journal of the American Chemical Society 114, 7941–7942.
Pinho, G.L.L., Moura da Rosa, C., Maciel, F.E., Bianchini, A., Yunes, J.S., Proença, L.A.O., Monserrat, J.M., 2005a. Antioxidant responses after microcystin exposure in gills of an estuarine crab species pre-treated with vitamin E. Ecotoxicology and Environmental Safety 61, 361–365.
Pinho, G.L.L., Moura da Rosa, C., Macie l, F.E., Bianchini, A., Yunes, J.S., Proença, L.A.O., Monserrat, J.M., 2005b. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicology and Environmental Safety 61, 353–360.
Preußel, K., Stüken, A., Wiedner, C., Chorus, I., Fastner, J., 2006. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. Toxicon 47, 156–162.
Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M., 2008. Protective role of vitamin E on the Microcystin-induced oxidative stress in tilapia fish (*Oreochromis niloticus*). Environmental Toxicology and Chemistry 27, 1152–1159.
Puerto, M., Prieto, A.I., Pichardo, S., Moreno, I., Jos, A., Moyano, R., Cameán, A.M., 2009. Effects of dietary N-acetylcysteine (NAC) on the oxidative stress induced in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Environmental Toxicology and Chemistry 28, 1679–1686.
Puerto, M., Prieto, A.I., Jos, A., Moreno, I., Moyano, R., Blanco, A., Cameán, A.M., 2010. Dietary N-Acetylcysteine (NAC) prevents histopathological changes in tilapias (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Aquaculture 306, 35–48.
Puerto, M., Jos, M.A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in Tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852–1860.
Puerto, M., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M. Acute exposure to pure cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environmental Toxicology, in press
Rajasekar, P., Palanisamy, N., Anuradha, C.V., 2007. Increase in nitric oxide and reductions in blood pressure, protein kinase C beta II and oxidative stress by L-carnitine: a study in the fructose-fed hypertensive rat. Clinical and Experimental Hypertension 29, 517–530.
Rebouche, C.J., 1991. Ascorbic acid and carnitine biosynthesis. American Journal of Clinical Nutrition 54, 1147S–1152S.
Reznick, A.K., Kagen, V.E., Ransey, R., Tsuchiya, M., Serbinova, E.A., Packer, L., 1992. Antiradical effect in L-propionyl carnitine. Protection of the heart against ischemic reperfusion injury. The possible role of ion chelation. Archives of Biochemistry and Biophysics 296, 349–401.
Rogers, E.H., Zehr, R.D., Gage, M.I., Humpage, A.R., Falconer, I.R., Marr, M., Chernoff, N., 2007. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49, 855–864.
Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Ge, J.L., Lu, S.C., 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical and Biophysical Research Communications 201, 235–241.
Runnegar, M.T., Kong, S., Zhong, Y.A., Lu, S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical Pharmacology 49, 219–225.
Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J., 2002. *In vitro* hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicological Sciences 67, 81–87.
Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. Toxicon 43, 185–194.
Santoro, A., Lioi, M.B., Monfregola, J., Salzano, S., Barbieri, R., Ursini, M.V., 2005. L-Carnitine protects mammalian cells from chromosome aberrations but not from inhibition of cell proliferation induced by hydrogen peroxide. Mutation Research 587, 16–25.
Sayed-Ahmed, M.M., Khattab, M.M., Gad, M.Z., Mostafa, N., 2001. L-Carnitine prevents the progression of atherosclerotic lesions in hypercholesterolaemic rabbits. Pharmacological Research 44, 235–242.
Schembri, M.A., Neilan, B.A., Saint, C.P., 2001. Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermoptis raciborskii*. Environmental Toxicology 16, 413–421.
Schreiber, S., Becker, K., Bresler, V., Fishelson, L., 1997. Dietary L-carnitine protects the gills and skin of guppies (*Poecilia reticulata*) against anionic xenobiotics. Comparative Biochemistry and Physiology 117C, 99–102.
Seelig, G.F., Meister, A., 1985. Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. Methods in Enzymology 113, 379–392.
Seifert, M., 2007. The ecological effects of the cyanobacterial toxin cylindrospermopsin. PhD Thesis. University of Queensland, Brisbane, Australia.
Şıktar, E., Ekinci, D., Şıktar, E., Beydemir, S., Gülçin, I., Günay, M., 2011. Protective role of L-carnitine supplementation against exhaustive exercise induced oxidative stress in rats. European Journal of Pharmacology 668, 407–413.
Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, M.J. (Eds.), Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. E & FN Spon, London, pp. 41–111.
Sushamakumari, S., Jayadeep, A., Kumar, J.S., Menon, V.P., 1989. Effect of carnitine on malondyaldehyde, taurine and glutathione levels in the heart of rats subjected to myocardial stress by isoprotenol. Indian Journal of Experimental Biology 27, 134–137.
Torreele, E., Van der Sluizen, A., Verreth, J., 1993. The effect of dietary L-carnitine on the growth performance in fingerlings of the African catfish *Clarias gariepinus* in relation to dietary lipid. British Journal of Nutrition 69, 289–299.
Van Apeldoorn, M.E., van Egmond, E.P., Speijers, G.J.A., Bakker, G.J.I., 2007. Toxins of cyanobacteria. Molecular Nutrition & Food Research 51, 7–60.
Zambrano, S., Blanca, A.J., Ruiz-Armenta, M.V., Miguel-Carrasco, J.L., Revilla, E., Santa-María, C., Mate, A., Vázquez, M.C., 2012. The renoprotective effect of L-carnitine in hypertensive rats is mediated by modulation of oxidative stress-related gene expression. European Journal of Nutrition, http://dx.doi.org/10.1007/s00394-012-0470-x.
Zegura, B., Gajski, G., Straser, A., Garaj-Vrhovac, V., 2011. Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress. Toxicon 58, 471–479.

CAPÍTULO 11/ CHAPTER 11

R. Guzmán-Guillén, A. I. Prieto, I. Moreno, V. Ríos, V. M. Vasconcelos, A. M. Cameán

EFFECTS OF DEPURATION ON OXIDATIVE BIOMARKERS IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER SUBCHRONIC EXPOSURE TO CYANOBACTERIUM PRODUCING CYLINDROSPERMOPSIN

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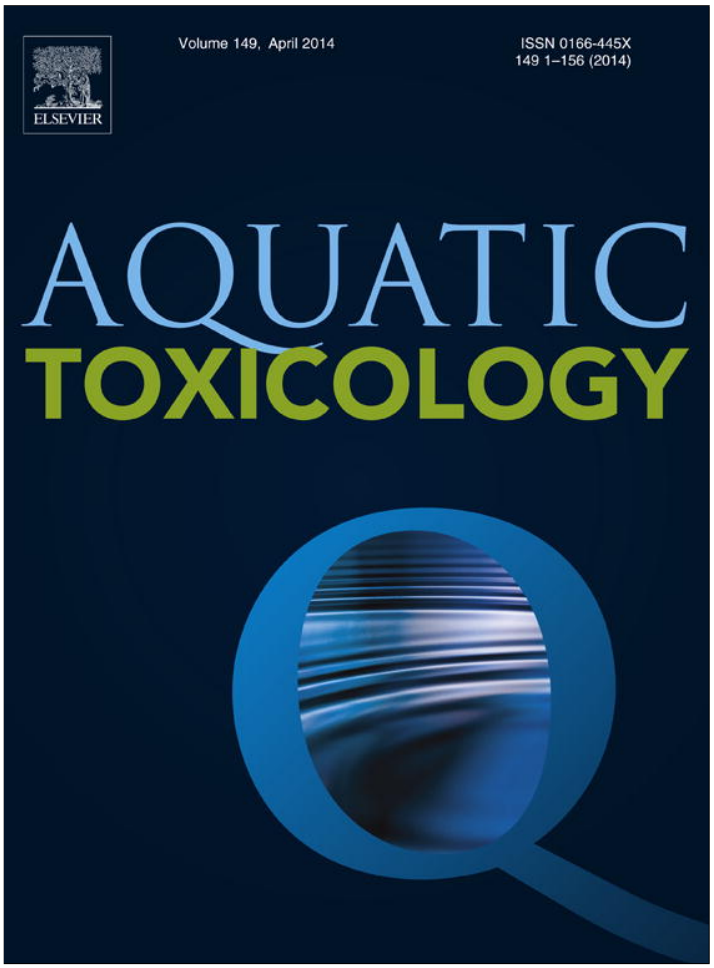

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Effects of depuration on oxidative biomarkers in tilapia (*Oreochromis niloticus*) after subchronic exposure to cyanobacterium producing cylindrospermopsin

R. Guzmán-Guillén[a], A.I. Prieto[a], I. Moreno[a], V. Ríos[a], V.M. Vasconcelos[b,c], A.M. Cameán[a,*]

[a] Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Spain
[b] Centro Interdisciplinar de Investigação Marinha e Ambiental, CIIMAR/CIMAR, Universidade do Porto, Portugal
[c] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

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ABSTRACT

Cylindrospermopsin (CYN) is a cytotoxic polyketide-derived alkaloid produced by several freshwater cyanobacterial species. It is now considered the second most studied cyanotoxin worldwide. Among the toxic mechanisms suggested for CYN pathogenicity are inhibition of protein and glutathione synthesis, genotoxicity by DNA fragmentation, and oxidative stress. The study of depuration of cyanobacterial toxins by aquatic organisms, particularly by fish, is important for fish economy and public health, but in the case of CYN is practically nonexistent. In this work, we investigated the efficiency of two distinct depuration periods, 3 or 7 d, in a clean environment, as a mean of restoring the levels of several oxidative stress biomarkers in tilapia (*Oreochromis niloticus*) subchronically exposed to CYN by immersion in an *Aphanizomenon ovalisporum* culture (by adding 10 μg CYN/L every two days during 14 d). Lipid peroxidation (LPO) and DNA oxidation returned to normal values after 7 d of depuration, whereas the time needed for restoring of the oxidatively damaged proteins was longer. Superoxide dismutase (SOD) and gamma-glutamyl-cysteine-synthetase (γ-GCS) activities recovered after just 3 d of depuration, while catalase (CAT) activity needed up to 7 d to return to control values. Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) returned to control levels after 7 d of depuration in both organs. These results validate the depuration process as a very effective practice for detoxification in fish contaminated with these toxins.

1. Introduction

Massive proliferations of cyanobacteria occur in many water bodies in all continents as a consequence of increasing eutrophication (Whitton and Potts, 2000) and climate change (Paerl and Huisman, 2008). One of the major concerns related with blooms is that many cyanobacteria may produce cyanotoxins, which pose a risk not only to the integrity of aquatic ecosystems but also to human health (Yılmaz et al., 2008). Cylindrospermopsin (CYN) is one of the most important cyanotoxins in terms of human health and environmental quality (Rogers et al., 2007) and it is produced by some fresh water cyanobacterial species such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon* sp., *Raphidiopsis curvata*, *Lyngbya wollei*, and *Anabaena bergii* (Padisák, 1997; Rücker et al., 2007). CYN has been found in eight different cyanobacterial species in four of the five continents so far (Moreira et al., 2012). Structurally, CYN is an alkaloid consisting of a sulfate ester of a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992). The compound is zwitterionic, it has a relatively low molecular weight of 415 Da, and is stable in varying heat, light and pH conditions (Sivonen and Jones, 1999). It is also highly water soluble. Moreover, the fraction of CYN dissolved in the water can constitute as much as 90% of total CYN available (Chiswell et al., 1999; Rücker et al., 2007). CYN also has an epimere called 7-epiCYN (Banker et al., 2000) and another variant called deoxyCYN (Li et al., 2001; Norris et al., 1999). Both variants have proved to be less toxic than CYN (Sukenik et al., 2001).

The first reported human outbreak of hepatoenteritis associated with CYN occurred in Palm Island, northern Queensland, Australia, in 1979 (Bourke et al., 1983). 148 cases were reported including 10 adults, 138 children and the majority required hospital treatment. 69% of the patients required intravenous therapy, 85 cases were flown to a hospital with intensive care facilities, and recovery occurred within 26 d (Byth, 1980). Similar to some other

* Corresponding author at: Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González nº 2, 41012 Sevilla. Spain. Tel.: +34 954 556762; fax: +34 954 233765.
E-mail address: camean@us.es (A.M. Cameán).

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cyanotoxins (e.g., microcystins (MCs) and nodularins), human exposure to CYN may occur during recreational activities in toxin contaminated lakes, by ingestion of contaminated water or food, such as agricultural products that have been irrigated with water containing CYN or by the consumption of aquatic organisms (Gutiérrez-Praena et al., 2013; Poniedziałek et al., 2012). Humpage and Falconer (2003) proposed 1 μg/L as a guideline safety value (GV) of CYN in drinking water and a Tolerable Daily Intake (TDI) of 0.02 μg/kg body weight/day for human exposure was calculated based on acute toxicity studies in mice (Duy et al., 2000).

The potential for cyanobacterial toxins to bioaccumulate has received some attention in the last decade, particularly with respect to accumulation in fish, crustacean or other seafood species with recreational or commercial importance (Ibelings and Chorus, 2007). However, until now few studies have been made on the bioaccumulation of CYN in aquatic organisms (Gutiérrez-Praena et al., 2013; Kinnear, 2010). Fish are generally more tolerant to cyanobacterial toxins than mammals and tend to accumulate them over time (Ross, 2000). For instance, current evidence suggests the general order of bioaccumulation capacity being gastropods > bivalves > crustaceans > amphibians > fish (Kinnear, 2010). As bioaccumulation and trophic transfer of CYN is possible, toxicity may also affect first, second and higher-order consumers in aquatic food webs. Curiously, the reverse relationship appears to be true for the susceptibility of organisms to CYN toxicity. Thus, the need to study the effects that CYN has on a wide range of organisms is clearly evident (Kinnear, 2010).

CYN is widely cytotoxic and generally toxin exposure is characterized by delayed toxicity involving multiple organ systems, showing histopathological damage in different organs of mammals (Falconer et al., 1999; Falconer and Humpage, 2006; Harada et al., 1994; Hawkins et al., 1985) and fish (Gutiérrez-Praena et al., 2012; Puerto et al., 2012), mainly liver and kidney. Various toxic mechanisms have been suggested in order to explain the pathogenicity of CYN. The principal mode of action is the inhibition of protein synthesis (Froscio et al., 2003; Runnegar et al., 2002) and glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1994, 1995), as well as genotoxicity by DNA fragmentation (Bazin et al., 2009; Žegura et al., 2011a,b). Recently, in vivo and in vitro studies have shown that oxidative stress also plays a significant role in CYN pathogenesis on fish (Gutiérrez-Praena et al., 2011a,b; Puerto et al., 2011). These studies showed damage after acute exposure to pure CYN. However, in natural conditions, fish are exposed to cyanobacterial blooms for long periods, during which toxins are produced continuously. Despite this, toxicological studies with fish exposed to cyanobacterial blooms containing CYN for long periods are very scarce (Guzmán-Guillén et al., 2013a). Alterations in oxidative stress biomarkers were more pronounced as the concentration of CYN and time of exposure increased.

Depuration studies on cyanobacterial toxins in aquatic organisms, particularly fish, are important for fish economy and public health (Mohamed and Hussein, 2006). Only a few studies have discussed the depuration mechanism of cyanotoxins in aquatic organisms. Ozawa et al. (2003) studied the accumulation and depuration of MCs in freshwater snails, Tricarico et al. (2008) in the crayfish *Procambarus clarkii* and Galanti et al. (2013) the detoxification dynamic of MC-LR in the shrimp *Palaemonetes argentinus*. Moreover, Kankaanpää et al. (2007) studied the depuration mechanism of nodularin in mussel (*Mytilus edulis*). Saker et al. (2004) investigated the accumulation and depuration of CYN in the freshwater mussel *Anodonta cygnea*.

In this context the aim of this study was to assess the impact of subchronic exposure to CYN on the oxidative status in tilapia (*Oreochromis niloticus*) and to investigate the efficiency of two distinct depuration periods, 3 or 7 d, as a mean of reducing the levels of several oxidative stress biomarkers. For this purpose tilapia (*O. niloticus*) were repeatedly exposed by immersion (every 2 d) to an *Aphanizomenon ovalisporum* culture (10 μg CYN/L) for two different periods of time (7 and 14 d), before being transferred to a clean environment without toxin. The biomarkers analyzed included lipid peroxidation (LPO), protein oxidation, DNA oxidation, activities of superoxide dismutase (SOD), catalase (CAT), γ-glutamylcysteine synthetase (GCS), and reduced to oxidized glutathione ratio (GSH/GSSG) in the liver and kidney of fish. Moreover, CYN determination in samples of waters from aquaria was carried out during the experiment.

2. Materials and methods

2.1. Chemicals

CYN standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in MilliQ water (100 μg mL^{-1}) and diluted as required for their use as working solutions (0.08–5.0 μg mL^{-1}). All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol (MeOH), dichloromethane (DCM), formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). MilliQ deionized water (>18 MΩ cm^{-1} resistivity) was obtained using a MilliQ water purification system (Millipore, Bedford, USA). BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe). Chemicals for the different assays were provided by Sigma–Aldrich and VWR International Eurolab. Protein assay reagent was obtained from BioRad Laboratories.

2.2. A. ovalisporum culture

A. ovalisporum (LEGE X-001) CYN-producing cyanobacterial strain (CYN+) was isolated from Lake Kinneret, Israel (Banker et al., 1997) and supplied by the Marine Research Center (Porto, Portugal). This strain was cultured in Z8 medium, temperature of 25 °C, 28 μmol photons m^{-2} s^{-1} of light intensity in continuous illumination. After 33 d, cultures were harvested by decantation and filtration with a plankton net (20 μm diameter). The biomass obtained was frozen at −80 °C.

2.3. Quantitation of CYN produced by LEGE X-001 strain

For this study, a LEGE X-001 extract was analyzed by LC–MS/MS in order to determine the CYN present in the extract and produced per cell. CYN extraction from the culture of *A. ovalisporum* (CYN+) was performed based on Guzmán-Guillén et al. (2012a). Briefly, the biomass (134 mg) was extracted with 3 mL of MilliQ water, sonicated for 15 min, stirred for 1 h and sonicated again for 15 min. The resulting mixture was centrifuged at 3281 × *g* for 10 min, after which the supernatant was collected and 6 μL of 0.1% TFA were added. Then, it was stirred for 1 h and allowed to stand for 3 h. The supernatant was taken for further purification/concentration. For the clean-up procedure, graphitized carbon cartridges (Bond Elut®) were activated with 10 mL of a solvent mixture of DCM/MeOH (10/90) and rinsed with 10 mL of MilliQ water. Subsequently, the sample was passed through the cartridges, washed with 10 mL of MilliQ water and eluted with 10 mL DCM/MeOH (10/90). For sample concentration the extract was evaporated in a rotary evaporator and resuspended in 500 μL MilliQ water, prior to its LC–MS/MS analysis.

Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQlit) mass spectrometer equipped with an electrospray ion source. LC

analyses were performed on a 150 × 2.1 mm Zorbax Sb-Aq column. The flow rate was 0.2 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) methanol. Both components contained 0.05% TFA (v/v). The injection volume was 20 μL. The elution profile was: 0% B (1 min), linear gradient to 90% B (10 min), 90%B (5 min) and finally 0% B (5 min). Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragment ions were monitored at Q1 and Q3, respectively. The transitions for the detection of CYN are: 416.2/194.0, 416.2/274.0, 416.2/336.0 and 416.2/175.9. The transition 416.2/194.0 was chosen for quantification of CYN. For LC–ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, source gas 60 psi, ion spray voltage 5500 V, and the Turboprobe temperature was maintained at 350 °C.

CYN was detected (retention time of 9.55 min) and quantified, and the concentration of CYN obtained was 0.1 μg CYN mg^{-1} of culture. Moreover, its deoxy-derivative (deoxy-CYN) was also detected in the sample culture, with a retention time of 9.77 min. In this case, since no pure standard of this isomer was available, the quantification of deoxy-CYN was made using the CYN calibration curve (equivalent of CYN), and the concentration obtained was 0.012 μg deoxy-CYN mg^{-1}.

2.4. Fish capture and maintenance

Sixty four male *O. niloticus* (Nile tilapia, Perciformes: Cichlidae), were used in our studies. They were obtained from a fish hatchery "Aquaculture Valencia" and maintained in the laboratory to reach the average weight of 20 ± 8 g and length of 7.8 ± 2 cm. Fish were held in aquaria (8 individuals/aquarium) with 96 L of fresh water. Exposure to chlorine was minimized by filling the tanks at least 3 d before the fish were introduced. The aquaria were also set up with a continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges (Bio-Espumador)). The temperature was kept constant (21 ± 2 °C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg/L. Mean values for additional parameters of water quality were: pH 7.6 ± 0.3, conductivity 287 μS/cm, Ca^{2+} 0.60 mM/L and Mg^{2+} 0.3 mM/L. Fish were fed with commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) and were acclimatized for 15 d before the beginning of the experiment.

2.5. Experimental exposure

After the acclimation period, eight groups of tilapia (n = 8) were established and are shown in Table 1. Fish in all these aquaria were fed only with 0.3 g d^{-1} of commercial fish food. The experiment was performed during 7 and 14 d of exposure to CYN (groups 2 and 4, respectively) followed by transferring the fish to clean water (groups 6 and 8). Intoxicated fish were exposed to a CYN-containing culture of *A. ovalisporum* LEGE X-001 and 10 μg CYN L^{-1} was added every two days to the aquaria holding the fish during 7 d (group 2) or 14 d (groups 4, 6 and 8). This concentration was selected in accordance with our previous experiment carried out in this fish species, in which 10 and 100 μg/L induced damage when fish were sub-chronically exposed by immersion (Guzmán-Guillén et al., 2013a). After 14 d of exposure, fish were transferred into clean aerated water aquaria for 3 (group 6) or 7 d (group 8) (see schematic diagram, Fig. 1). Four control groups of fish (no CYN) were administered only with the commercial fish food during the whole experiment and were sacrificed in two different times (7 or 14 d, groups 1 and 3, corresponding to the exposures, respectively) and simultaneously with depuration sampling (3 or 7 d, groups 5 and 7).

CYN levels in the aquaria throughout both exposure and depuration periods were determined every 48 h based on Guzmán-Guillén et al. (2012b) using Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), and the actual CYN concentration after 14 d of exposure was 42.4 μg CYN L^{-1} (see Section 3.4).

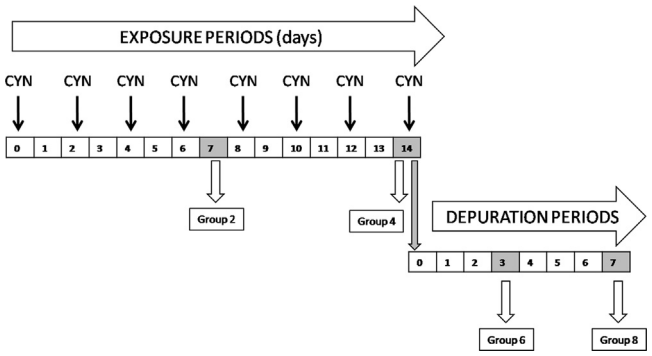

Fig. 1. Schematic diagram of the experimental exposure.

2.6. Preparation of post-mitochondrial supernatant (PMS)

Fish were anesthetized in ice, and sacrificed by transection of the spinal cord. The livers and kidneys were quickly removed, weighed, rinsed with ice-cold saline, frozen in liquid nitrogen, and kept at −85 °C until use. Enzyme extracts from each tissue were prepared from each individual (not pooled) according to the method described by Puerto et al. (2009) using 0.1 M potassium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetra-acetic acid, and 1.4 mM dithioerythritol. After removal of cell debris (10 min at 13,000 × g), the membrane fraction was separated by centrifugation at 105,000 × g for 60 min. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for biochemical measurements.

2.7. Lipid peroxidation, protein carbonyl content and DNA oxidation

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman, 1990) with slight modifications (Jos et al., 2005). Malondialdehyde (MDA) is formed as an end-lipid peroxidation product which reacts with the TBA reagent under acidic conditions to generate a pink colored product. Values were presented as nmol TBARS g^{-1} tissue.

Protein carbonyl content, a biomarker of protein oxidation, was assayed with the method described by Levine et al. (1990) using 2,4-dinitrophenylhydrazine prepared in 2 M HCl, 20% (w/v) trichloroacetic acid, and 6 M guanidine hydrochloride, as described by Atencio et al. (2008). Results are expressed as nmol carbonyl mg^{-1} protein, using the extinction coefficient of 22,000 M^{-1} cm^{-1}.

For DNA oxidation the OxiSelectTM Oxidative DNA Damage Quantitation Kit (AP sites) (Cell Biolabs, INC., San Diego, CA, USA) was used, following the Gutiérrez-Praena et al. (2011a) work. Results are expressed as apurinic/apyrimidinic (AP) sites per 100,000 bp.

2.8. Antioxidant enzymes

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using the xanthine oxidase-cytochrome c method as described by McCord and Fridovich (1969). SOD activity was measured spectrophotometrically at 505 nm, as has been described in Prieto et al. (2008). Catalase (CAT; EC 1.11.1.6) activity was assayed by the method of Beers and Sizer (1952) measuring the initial rate of hydrogen peroxide (10 mM) decomposition at 240 nm (Prieto et al.,

Table 1
Treatment conditions of tilapia fish (*Oreochromis niloticus*) exposed to *A. ovalisporum* (CYN+) culture by immersion.

Groups of tilapia fish									
Treatment condition	Period of treatment	1	2	3	4	5	6	7	8
Exposure period to *A. ovalisporum*	7 days	–	+	–	–	–	–	–	–
(CYN+) culture	14 days	–	–	–	+	–	+	–	+
Depuration	3 days	–	–	–	–	+	+	–	–
period	7 days	–	–	–	–	–	–	+	+

CYN+ = cells containing cylindrospermopsin.

Table 2
Changes of oxidative stress biomarkers in liver and kidney of tilapia fish exposed to *A. ovalisporum* (CYN+) culture by immersion compared to their respective control groups after two depuration periods (3 d or 7 d).

Biomarker	CYN depuration periods in liver		CYN depuration periods in kidney	
	3 d	7 d	3 d	7 d
Lipid peroxidation (LPO)	No	No	Yes	No
Protein oxidation	Yes	Yes	Yes	No
DNA oxidation	No	No	No	No
Superoxide dismutase (SOD)	No	No	No	No
Catalase (CAT)	Yes	No	Yes	No
γ-Glutamylcysteine synthetase (GCS)	No	No	No	No
GSH/GSSG ratio	Yes	No	No	No

Yes: significant changes on the biomarker of exposed fish to *A. ovalisporum* cells (CYN +) in comparison to their respective control group.
No: no significant changes on the biomarker of exposed fish to *A. ovalisporum* cells (CYN +) in comparison to their respective control group.

2008). Gamma-glutamyl-cysteine synthetase (EC 6.3.2.2) (GCS) is the rate limiting enzyme in GSH synthesis. Its activity was measured using the method described by Seelig and Meister (1985), adapted to fish samples (Gutiérrez-Praena et al., 2011a). The formation of ADP was monitored spectrophotometrically by coupled assay with pyruvate kinase and lactate dehydrogenase. The results of all these enzyme activities were expressed as nkat mg^{-1} protein.

2.9. Non-enzymatic antioxidant parameters: GSH/GSSG ratio

GSH/GSSG ratio was determined in liver and kidney homogenates using a commercial kit (Bioxytech GSH/GSSH-412, Oxis Research, Foster City, CA, USA) adapted for fish tissues as in Atencio et al. (2008).

2.10. Protein estimation

Protein contents in the samples were estimated by the method of Bradford (1976) using bovine γ-globulin as standard. Briefly, 5 μL of the diluted samples were mixed with 95 μL H_2O and 5 mL Coomassie Brilliant blue dye (Biorad Laboratories, Hercules, USA) and the absorbance was read at 595 nm in the spectrophotometer (Cary100, Varian, Madrid, Spain).

2.11. Statistical analysis

All results were subjected to one-way analysis of variance (ANOVA), and represent means ± SE of 8 animals per group. Differences in mean values between groups were assessed by the Tukey's test and were considered statistically different from $p < 0.05$.

3. Results

There was no mortality or visual changes in any of the fish during the performance of the experiment.

3.1. Effects on lipid, protein and DNA oxidation

LPO values significantly increased in liver of fish exposed to *A. ovalisporum* culture (CYN+) (groups 2 or 4) in comparison with their respective control groups after 7 or 14 d of exposure (aquaria 1 or 3) (5.4 or 4.5-fold, respectively). Both depuration periods (3 or 7 d) were able to restore the basal values of this parameter, showing significant decreases in depurated fish compared to non-depurated fish (8.5-fold for 3 day-depurated fish and 5.9-fold for 7 day-depurated fish) (Fig. 2a). In kidney, a significant induction of LPO in fish exposed to CYN for 7 and 14 d (groups 2 or 4) in comparison with their respective control groups (1.6 or 2.6-fold, respectively) was observed. Moreover, LPO significantly increased with the time of exposure 14 d vs. 7 d (1.6-fold) (Fig. 2b). LPO levels in fish submitted to both depuration periods (3 or 7 d) showed

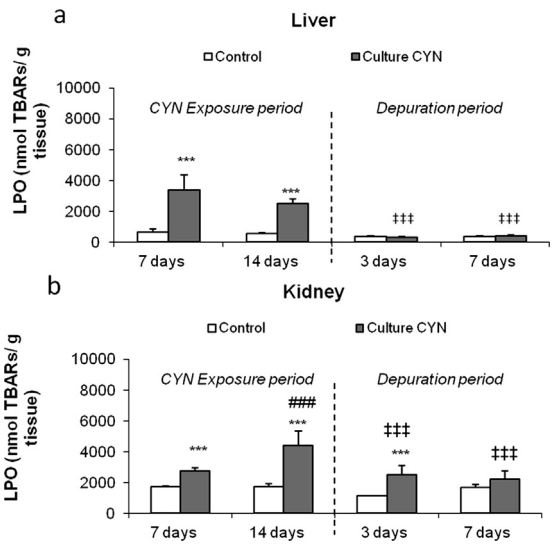

Fig. 2. Lipid peroxidation (LPO) in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 d or 14 d) and depuration (3 d or 7 d). The values are expressed as mean ± SE ($n = 8$). LPO values are expressed as nmol TBARS/g tissue. The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ when CYN-intoxicated fish at different times of exposure (7 d or 14 d) are compared, and $^{‡‡‡}p < 0.001$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

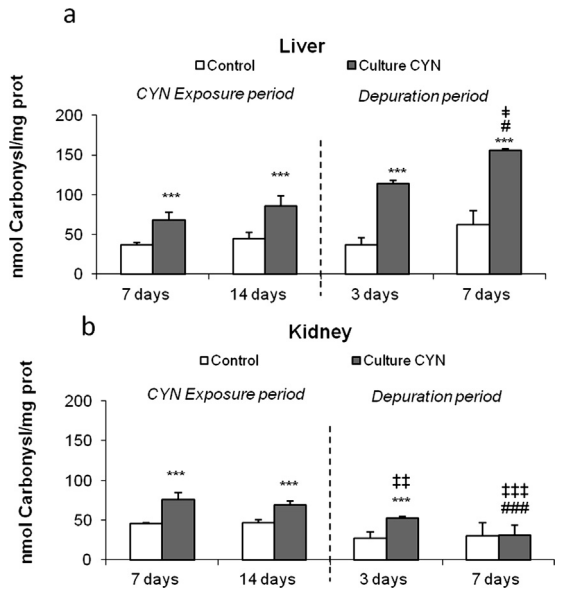

Fig. 3. Protein oxidation values (nmol carbonyl/mg protein) in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 d or 14 d) and depuration (3 d or 7 d). The values are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ or $^{\#}p < 0.05$ when comparing CYN-exposed fish depurated at different times (3 d or 7 d) and $^{‡‡‡}p < 0.001$, $^{‡‡}p < 0.01$ or $^{‡}p < 0.05$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

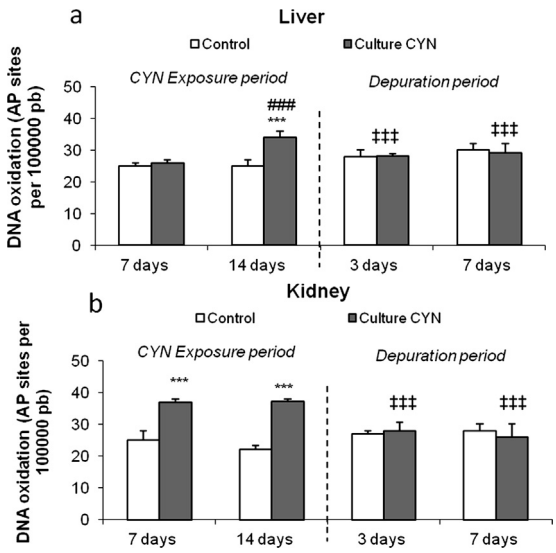

Fig. 4. DNA oxidation in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 d or 14 d) and depuration (3 d or 7 d). The values are expressed as mean ± SE ($n = 8$). DNA oxidation values are expressed as AP sites/100,000 bp. The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ when comparing CYN-intoxicated fish at different times of exposure (7 d or 14 d) and $^{‡‡‡}p < 0.001$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

significant decreases in comparison to non-depurated fish exposed for 14 d (up to 2.0-fold). The 3 day-depuration period was not able to restore LPO values to control levels, showing a significanty higher value than its control group (2.2-fold). A longer depuration period (7 days) was required to restore LPO values to values similar to those in the control group.

After both periods of exposure to cyanobacterial cells (7 or 14 d), a significant increase was recorded in the protein oxidation level of liver (1.8-fold or 1.9 fold after 7 d or 14 d, respectively) and kidney (1.7-fold or 1.5-fold for 7 d or 14 d, respectively) of exposed fish in comparison with their respective control groups (Fig. 3a and b). A depuration period of 3 d was not enough to restore this parameter to the respective control group values in either organ. In the liver, protein oxidation values continued to increase over time even during depuration (7 d depuration values were 1.3 fold higher than the 3 d depuration ones) (Fig. 3a). In kidney of fish after 7 d of depuration, the protein oxidation levels were lower than in the 14-d CYN-exposed fish without depuration (2.2-fold), and the values were similar to those in the controls. Significant differences were obtained between the depuration periods (1.7-fold).

No changes were found in DNA oxidation in liver of fish exposed to culture of *A. ovalisporum* (group 2) for a period of 7 d in comparison to control group (group 1) (Fig. 4a). Nevertheless, DNA oxidation showed a significant increase (1.4-fold) in fish exposed to CYN for a period of 14 d (group 4) compared to its control group. Moreover, significant differences (1.3-fold) were observed in non-depurated fish over time (7 d vs. 14 d). In kidney, DNA oxidation showed a significant increase in both groups of CYN exposed fish without depuration (1.5 or 1.7-fold for 7 d or 14 d, respectively) compared to their respective control groups (Fig. 4b). After 3 d or 7 d of depuration, the values of DNA oxidation decreased significantly (up to 1.4-fold) in comparison to CYN-exposed fish for 14 d and not depurated, and were similar to the ones measured in control groups.

3.2. Effects on antioxidant enzymes

Regarding to SOD enzyme activity (Fig. 5a), liver and kidney showed a different response. In liver, this biomarker was increased in CYN-exposed fish without depuration (groups 2 and 4) (1.7 or 2.1-fold) in comparison with the respective control groups, and was higher after the longer exposure time. By contrast, in kidney SOD activity decreased in CYN-exposed fish in both studied exposure times (up to 1.7-fold after 14 d of CYN exposure). In both organs SOD activity recovered fully after 3 d or 7 d of depuration: the activities were similar to the respective control values. Moreover, hepatic and renal SOD activities of depurated fish (3 d or 7 d) were significantly different from those of CYN-exposed fish without depuration.

Catalase activity showed a different response according to the exposure period (7 d or 14 d) in both organs of CYN-exposed fish (Fig. 5b). CAT activities at an early CYN-exposure period (7 d) showed an initial significant increase (1.6-fold in liver and 2.7-fold in kidney) compared to the respective control groups. After 14 d of exposure, this enzyme activity experienced a significant decrease (1.3-fold or 1.5-fold in liver and kidney, respectively). A depuration period of 3 d was not able to restore this biomarker level in any of the organs studied, and only after 7 days of depuration was CAT activity restored to the control levels.

The activity of GCS experienced significant increases in the liver (3.0 and 5.0-fold for 7 d or 14 d, respectively) and kidney (5.0 and 4.0-fold for 7 d or 14 d, respectively) of fish exposed to CYN in comparison with the respective control groups. In the liver also the exposure time (7 vs. 14 d) affected the activity (Fig. 6). Liver and renal GCS activities decreased after both depuration periods, and were restored to their respective control values, showing significant differences between depurated and non-depurated fish.

3.3. Effects on GSH/GSSG ratio

For all periods of exposure (7 and 14 d), the GSH/GSSG ratio significantly decreased in liver (1.5-fold for both times) and kidney

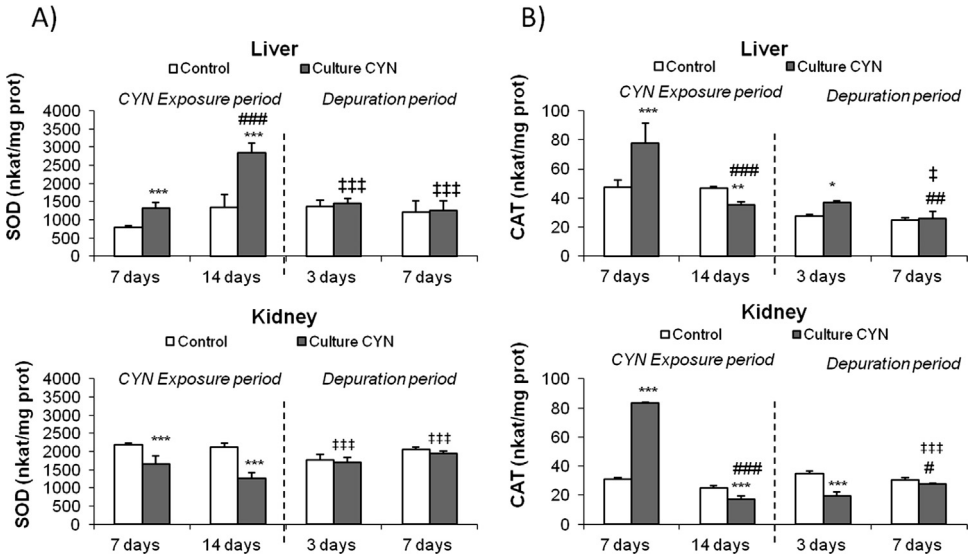

Fig. 5. (a) Superoxide dismutase activity (SOD) and (b) catalase activity (CAT) in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 d or 14 d) and depuration (3 d or 7 d). The values (nkat/mg protein) are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$, $^{**}p < 0.01$ or $^{*}p < 0.05$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$ when CYN exposed fish for 14 d or 7 d and depurated at different times (3 d or 7 d) are compared, and $^{\ddagger\ddagger\ddagger}p < 0.001$ or $^{\ddagger}p < 0.05$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

(5.8 and 2.3-fold for 7 and 14 d, respectively) of CYN-exposed fish in comparison with the respective control groups (Fig. 7). Three days of depuration was not adequate to restore GSH/GSSG ratio in the liver to control values (it was 1.8-fold lower than in the controls). However, after 7 d of depuration this parameter was increased to control values. On the other hand, in the kidney, the GSH/GSSG ratio was restored already by 3 d of depuration (Table 2).

3.4. Determination of CYN in samples of water from aquaria and fish tissue

Cylindrospermopsin values detected in water samples were increasing along the exposure periods, from 11.2 μg CYN/L at the beginning of the experiment to 21.1 μg CYN/L or 42.4 μg CYN/L after 7 d or 14 d. Once the fish were changed to aquaria without toxin, CYN was not detected in any of the water samples (Table 3).

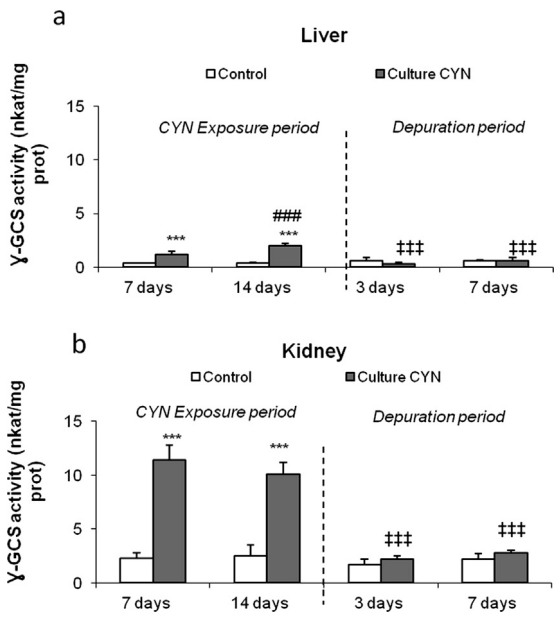

Fig. 6. γ-Glutamyl-cysteine synthetase (GCS) activity in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 d or 14 d) and depuration (3 d or 7 d). The values (nkat/mg protein) are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ when comparing CYN-intoxicated fish at different times of exposure (7 d or 14 d), and $^{\ddagger\ddagger\ddagger}p < 0.001$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

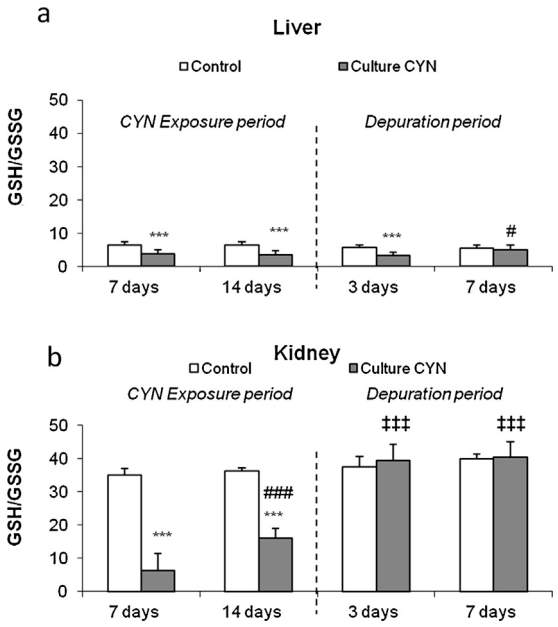

Fig. 7. Ratio between reduced glutathione and oxidized glutathione (GSH/GSSG) in liver and kidney of fish exposed to repeated doses of CYN (10 μg CYN/L, added into aquaria every two days) for two different periods of treatment (7 or 14 d) and depuration (3 d or 7 d). The values are expressed as mean ± SE ($n = 8$). The significance levels observed are $^{***}p < 0.001$ in comparison with their respective control group, $^{\#\#\#}p < 0.001$ or $^{\#}p < 0.05$ when CYN exposed fish for 14 d or 7 d and fish depurated at different times (3 d or 7 d) are compared, and $^{\ddagger\ddagger\ddagger}p < 0.001$ when comparing CYN-intoxicated fish for 14 d to non-depurated fish.

Table 3
CYN (μg/L) analysis in water samples harvested from aquaria after exposure and depuration periods.

CYN analysis in water samples		
	Total theoretical CYN (μg/L) in aquaria after the final exposure periods	Detected CYN (μg/L) in aquaria
CYN exposure periods (days)		
0 d	10	11.2 ± 0.5
7 d	40	21.1 ± 0.14
14 d	80	42.4 ± 0.2
CYN depuration periods (days)		
0 d	–	–
3 d	–	<LOD
7 d	–	<LOD

4. Discussion

There are very few studies in aquatic animals that demonstrate the involvement of oxidative stress as a mechanism of action of CYN. We have previously reported that CYN causes oxidative stress in fish when the pure toxin is applied in a single dose (Gutiérrez-Praena et al., 2011a,b; Puerto et al., 2012) or sub-chronically exposed by immersion to lyophilized cells of *A. ovalisporum* containing CYN and deoxy-CYN (10 or 100 μg CYN L^{-1}) (Guzmán-Guillén et al., 2013a). However, the changes on oxidative stress biomarkers and the ability to recover from damages by transferring the fish to a cleaner environment without the toxin, has not been analyzed. Several authors have demonstrated a reduction of some stress biomarkers levels in fish and clams exposed to contaminants during depuration (Ferreira et al., 2005, 2007; Freitas et al., 2012; Gagnaire et al., 2013; Özcan Oruç, 2010), indicating that the animals could reprogram the cell response when transferred to an unpolluted environment. Kankaanpää et al. (2007) studied the oxidative response in mussels exposed to nodularin, showing an increased in CAT activity during the depuration period, representing a stimulation of the antioxidant enzyme defence. The present work presents for the first time the biochemical responses of tilapia when subjected to 14 d exposure to CYN contained in cyanobacterial cells (*A. ovalisporum*) and the effect of transferring the fish to clean water (3 d or 7 d) on their oxidative status.

The significant increases in LPO levels in liver and kidney observed after both periods of exposure (7 or 14 d) were lowered to basal concentrations just after 3 d of depuration in the liver. However, this reversion was not observed in the kidney under a depuration period of 7 d, indicating that this organ takes longer to recover from oxidative stress than liver. Freitas et al. (2012) observed a reduction in the cellular oxidative damage in two clam species (*Ruditapes decussates* and *Ruditapes philippinarum*) subjected to anthropogenic pressures, as indicated by the reduction in the LPO levels just after 2 and 7 d of depuration. A decrease in LPO was also detected during a long-term depuration study (1, 4 and 8 months) in mullets (*Mugil cephalus*) chronically exposed to contaminants in River Douro estuary (Ferreira et al., 2007).

Protein oxidation can be increased in fish after xenobiotic exposure (Fessard and Livingstone, 1998; Ferreira et al., 2005). In this work, increased protein oxidation was statistically more significant in the kidney in comparison to the liver at the end of the exposure phase (14 d). When fish were maintained for short periods in unpolluted water (3 or 7 d) a tendency of oxidized proteins to a decrease is observed. While in kidney this biomarker recovered to control levels after 7 d of depuration, liver needed more than 7 d for repair or replacement of the damaged proteins. According to Ferreira et al. (2007), considering that the formation of carbonyl derivatives is a non-reversible process we should expect a longer time to replace the damaged proteins.

In the present study it is remarkable that DNA oxidation was a sensitive biomarker, confirming that oxidative stress could cause DNA damage (Guzmán-Guillén et al., 2013a). Fish subjected to both depuration periods decreased DNA oxidation to basal values, even just in 3 d. These results agree with those reported by Emmanouil et al. (2008) which showed that mussels (*M. edulis*) from a contaminated site (Merseyside, UK) exhibited higher oxidative DNA damage compared to those from a less contaminated site, and their 1-month maintenance under laboratory conditions abolished these differences.

The antioxidant defence can be induced by a slight oxidative stress due to compensatory response; however, a severe oxidative stress suppresses the activities of the antioxidant enzymes due to the oxidative damage and a loss in compensatory responses (Sun et al., 2006). A simultaneous induction response in the activities of SOD and catalase is observed in fish exposed to Microcystins (MCs) (Jos et al., 2005; Prieto et al., 2007), although in the case of CYN the results are variable (Guzmán-Guillén et al., 2013a,b). In the present work the activity of hepatic SOD was significantly induced with the time of exposure, and this increase indicates there is $O_2^{\bullet -}$ generation, which implies that SOD elimination capacity is still working (Sun et al., 2006). After both depuration periods (3 or 7 d) SOD activity did not show alterations compared to controls. These results are in agreement with those obtained by Sun et al. (2006) who observed in fish exposed to phenanthrene and transferred to clean water (1, 3 and 7 d) that hepatic SOD levels returned to the control ones. Ferreira et al. (2005, 2007) detected a decrease in SOD activities in mullets collected in a polluted site and subjected to long periods of depuration. Otherwise, in kidney the SOD activity showed a significant inhibition in comparison with controls, and this fact could be explained because the kidney is more sensitive to CYN, and the generation of $O_2^{\bullet -}$ in this organ exceeds the function of SOD elimination. This radical as well as other oxyradicals can inactivate the enzyme (Sun et al., 2006). In kidney and liver the SOD activity returned to the control levels after only 3 d of depuration. Freitas et al. (2012) reported in two clams (*R. decussates*, *R. philippinarum*) different SOD activity responses between the depuration periods assayed (2 and 7 d) depending on the specie considered: in *R. decussates* no significant changes were obtained, while in *R. philippinarum* a steady decrease was observed over time.

CAT activities were significantly inhibited both in liver and kidney compared to the control group just after 14 d of exposure, which suggests that the accumulation of H_2O_2 was enough to make CAT damaged. After 3 d of depuration, increased CAT activity in liver may suggest a recovery of the enzyme which retrieves its H_2O_2 elimination capacity. Kankaanpää et al. (2007) found increased CAT activity in homogenates of digestive glands of the mussel *M. edulis* exposed to nodularin, during the depuration period (144 h), showing that biotransformation reactions generate oxygen radical and the antioxidant enzyme defence is stimulated. In kidney after a depuration period of 3 d the CAT activity was still significantly inhibited in comparison to its control group, confirming the higher susceptibility of the kidney to CYN. Finally, after 7 d of depuration, CAT activity returns to basal levels in both organs. A similar pattern of CAT response has been reported by Sun et al. (2006) in fish after 7 d of depuration, and by Galanti et al. (2013) in shrimps (*P. argentinus)* exposed to the cyanotoxin MC-LR (3 d) and relocated in fresh water (3 d).

In this work we observed a decrease in GSH/GSSG ratio in both organs of CYN-exposed fish as we have previously reported in vivo (Gutiérrez-Praena et al., 2011a; Guzmán-Guillén et al., 2013a; Puerto et al., 2012) and in vitro (Gutiérrez-Praena et al., 2011b). Runnegar et al. (1995) suggested that the hepatotoxicity of CYN resulted from the inhibition of GSH synthesis. Later, Norris et al. (2002) demonstrated in vivo that GSH depletion either by conjugation with CYN or by inhibition of its synthesis is unlikely

to be of primary importance in hepatic toxicity of CYN in mouse. Moreover, levels of GSH/GSSG achieve the control values after 7 d of clean water treatment in both organs, although in kidney only 3 d of clean water treatment were enough. This could be due to the action of detoxification and recovery mechanisms, which were able to neutralize the alteration in this biomarker. In Tilapia from a fish farm exposed to treated sewage water GSH levels were significantly raised in liver and muscle and, following depuration in fresh water (6 weeks), elevated GSH levels were restored to control values, while remained unchanged in muscle (Al-Ghais, 2013). In fish (*Brachydanio rerio*, Cyprinidae) exposed to sublethal concentrations of copper sulphate (14 d) and then replaced for 14 d in clean water, an increased GSH content in liver was observed and, at the end of the depuration period higher GSH levels were measured and the hepatic alterations were not reversed (Paris-Palacios et al., 2000).

γ-Glutamylcysteine synthetase activity increased in both liver and kidney of tilapia exposed to CYN for 7 or 14 d of exposure. This increased activity of γ-GCS, together with the low GSH/GSSG ratio, showed that GSH is not affected in its synthesis. Instead of this, the decreases could be explained because of its role in the antioxidant defence mechanism, such as increased consumption, increased formation of oxidized GSH, increased GSH efflux, etc. (Guzmán-Guillén et al., 2013a). These increased levels of γ-GCS were reverted to basal levels after only 3 d of depuration, situation maintained during the 7-day depuration period. No results about the effects of a depuration period in levels of this enzyme have been found either in fish or other aquatic organisms exposed to pollutants.

Globally, after fish were transferred to clean water (3 or 7 d), they showed a reduction in the cellular oxidative damage, as indicated by the reduction in the injury effects (LPO, protein and DNA oxidation), and the antioxidant systems returned to the control levels. According to Sun et al. (2006), this may be explained by several reasons: (1) some of the toxin which bioconcentrated in fish tissue was released to water again and its decreased concentration in fish would diminish many harmful effects; (2) hydroxyl radical and other free radical concentrations had been diminished by the antioxidant defence such as GSH, or metabolized to other less harmful radicals in vivo; (3) when the hydroxyl radical and other free radical levels are reduced back to the control levels, it could indicate that fish had returned to the normal physiological state, and cellular antioxidant defences need not be activated, with the consequence that, e.g., the changes in SOD and CAT activities ceased. In relation to the point (1) no CYN released to water has been detected in our experiment (Table 3) during the depuration periods (3 or 7 d); it would be necessary to have longer depuration periods to confirm this issue. Concerning the points (2) and (3) relative to biomarkers of oxidative stress, the values of some endpoints such as LPO (liver), DNA oxidation, SOD and GCS activities, and GSH content (kidney) decreased in the shortest depuration period (3 d), while other parameters such as LPO in kidney, CAT activity and GSH content in liver needed until 7 d to return to control values. Protein oxidation needs a longer depuration period for a full recovery. Again, it would be interesting to extend the depuration period to investigate if this effect is reversible over a longer period of time. In addition, the present work showed a higher susceptibility of the kidney to the cyanobacterial cells containing CYN and deoxy-CYN compared to the liver, which could be related to the high hydrophilicity of both toxins.

5. Conclusion

The present study brings key information about the capacity of tilapias (*O. niloticus*) to recover from oxidative damages induced by the exposure to *A. ovalisporum* cells containing CYN and deoxy-CYN for 7 and 14 d. LPO and DNA oxidation returned to normal values after 7 d of depuration, whereas the time needed for replacing the oxidatively damaged proteins was longer. Antioxidant enzyme activities showed different responses, with SOD and GCS activities recovering already after 3 d of depuration, while CAT activity needed up to 7 d to return to control values. Levels of GSH/GSSG returned to the control levels after 7 d of depuration in both organs, although in kidney just 3 d of depuration were enough to restore to the basal levels. These results support the notion that the depuration can be an effective detoxification process when fish could be potentially exposed to CYN.

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References

Al-Ghais, S.M., 2013. Acetylcholinesterase, glutathione and hepatosomatic index as potential biomarkers of sewage pollution and depuration in fish. Mar. Pollut. Bull. 74, 183–186.

Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M., 2008. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to microcystis under laboratory conditions. Toxicon 52, 1–12.

Banker, R., Carmeli, S., Hadas, O., Teltsch, B., Porat, R., Sukenik, A., 1997. Identification of cylindropsermopsin in *Aphanizomenon ovalisporum* (cyanophyceae) isolated from Lake Kinneret Israel. J. Phycol. 33, 613–616.

Banker, R., Carmeli, S., Teltsch, B., Sukenik, A., 2000. 7-epicylindrospermopsin, a toxic minor metabolite of the cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel. J. Nat. Prod. 63, 387–389.

Bazin, E., Mourot, A., Humpage, A.R., Fessard, V., 2009. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines, Caco-2 and HepaRG. Environ. Mol. Mutagen. 51, 251–259.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Bourke, A.T.C., Hawes, R.B., Neilson, A., Stallman, N.D., 1983. An outbreak of hepato-enteritis (the Palm Island mystery disease) possibly caused by algal intoxication. Toxicon 21, 45–48.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Byth, S., 1980. Palm Island mystery disease. Med. J. Aust. 2, 40–42.

Chiswell, R.K., Shaw, G.R., Eaglesham, G., Smith, M.J., Norris, R.L., Seawright, A.A., Moore, M.R., 1999. Stability of cylindrospermopsin, the toxin from cyanobacterium, *Cylindropsermopsis raciborskii*: effect of pH, temperature, and sunlight on decomposition. Environ. Toxicol. 14, 155–161.

Duy, T.N., Lam, P.K.S., Shaw, G., Connell, D.W., 2000. Toxicology and risk assessment of freshwater cyanobacterial (bluegreen algal) toxins in water. Rev. Environ. Contam. Toxicol. 163, 113–186.

Emmanouil, C., Green, R.M., Willey, F.R., Chipman, J.K., 2008. Oxidative damage in gill of *Mytilus edulis* from Merseyside, UK, and reversibility after depuration. Environ. Pollut. 151, 663–668.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonetal. Methods Enzymol. 186, 407–421.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J., Hawkins, P.R., 1999. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ. Toxicol. 14, 143–150.

Falconer, I.R., Humpage, A.R., 2006. Cyanobacterial (Blue-green Algal) toxins in water supplies: cylindrospermopsins. Environ. Toxicol. 21, 299–304.

Ferreira, M., Moradas-Ferreira, P., Reis-Henriques, M.A., 2005. Oxidative stress biomarkers in two resident species, mullet *(Mugil cephalus)* and flounder (*Platichthys flesus*), from a polluted site in River Douro Estuary, Portugal. Aquat. Toxicol. 71, 39–48.

Ferreira, M., Moradas-Ferreira, P., Reis-Henriques, M.A., 2007. The effect of long-term depuration on levels of oxidative stress biomarkers in mullets (Mugil cephalus) chronically exposed to contaminants. Mar. Environ. Res. 64, 181–190.

Fessard, V., Livingstone, D.R., 1998. Development of western analysis of oxidised proteins as a biomarker of oxidative damage in liver of fish. Mar. Environ. Res. 46, 407–410.

Freitas, R., Ramos Pinto, L., Sampaio, M., Costa, A., Silva, M., Rodrigues, A.M., Quintino, V., Figueira, E., 2012. Effects of depuration on the element concentration in bivalves: comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. Estuar. Coast. Shelf Sci. 110, 43–53.

Froscio, S.M., Humpage, A.R., Bucham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ. Toxicol. 18, 243–251.

Galanti, L.N., Ame, M.V., Wunderlin, D.A., 2013. Accumulation and detoxification dynamic of cyanotoxins in the freshwater shrimp *Palaemonetes argentinus*. Harmful Algae 27, 88–97.

Gagnaire, B., Cavalie, I., Camilleri, V., Adam-Guillermin, C., 2013. Effects of depleted uranium on oxidative stress, detoxification, and defence parameters of zebrafish danio rerio. Arch. Environ. Contam. Toxicol. 64, 140–150.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Cameán, A.M., 2011a. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory condition: influence of exposure route and time of sacrifice. Aquat. Toxicol. 105, 100–106.

Gutiérrez-Praena, D., Pichardo, S., Jos, M.A., Cameán, A.M., 2011b. Toxicity and glutathione implication in effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotoxicol. Environ. Saf. 74, 1567–1572.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M., 2012. Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral and intraperitoneal route. Ecotoxicol. Environ. Saf. 76, 102–113.

Gutiérrez-Praena, D., Jos, Á., Pichardo, S., Moreno, I.M., Cameán, A.M., 2013. Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: a review. Food Chem. Toxicol. 53, 139–152.

Guzmán-Guillén, R., Prieto Ortega, A.I., Moreno, I., González, A.G., Soria-Díaz, M.E., Vasconcelos, V., Cameán, A.M., 2012a. Development and optimization of a method for the determination of Cylindrospermopsin from strains of Aphanizomenon cultures: intra-laboratory assessment of its accuracy by using validation standards. Talanta 100, 356–363.

Guzmán-Guillén, R., Prieto, A.I., González, A.G., Soria-Díaz, M.E., Cameán, A.M., 2012b. Cylindrospermopsin determination in water by LC–MS/MS: optimization and validation of the method and application to real samples. Environ. Toxicol. Chem. 31, 2233–2238.

Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán, A.M., 2013a. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). Chemosphere 90, 1184–1194.

Guzmán-Guillén, R., Prieto, A.I., Vázquez, C.M., Vasconcelos, V., Cameán, A.M., 2013b. The protective role of l-carnitine against cylindrospermopsin-induced oxidative stress in tilapia (*Oreochromis niloticus*). Aquat. Toxicol. 132–133, 141–150.

Harada, K., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 32, 73–84.

Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B., Falconer, I.R., 1985. Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju isolated form a domestic supply reservoir. Appl. Environ. Microbiol. 50, 1292–1295.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94–103.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. J. Toxicol. Environ. Health A 68, 739–753.

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: a review. Environ. Pollut. 150, 177–192.

Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis sp.*) under laboratory conditions. Aquat. Toxicol. 72, 261–271.

Kankaanpää, H., Leinio, S., Olin, M., Sjövall, O., Meriluoto, J., Lehtonen, K.K., 2007. Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68, 1210–1217.

Kinnear, S., 2010. Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar. Drugs 8, 542–564.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shatiel, S., Stadman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.

Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y., Watanabe, M.M., 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata*. J. Phycol. 37, 1121–1126.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Mohamed, Z.A., Hussein, A.A., 2006. Depuration of microcystins in tilapia fish exposed to natural populations of toxic cyanobacteria: a laboratory study. Ecotoxicol. Environ. Saf. 63, 424–429.

Moreira, C., Azevedo, J., Antunes, A., Vasconcelos, V., 2012. Cylindrospermopsin: occurrence, methods of detection and toxicology. J. Appl. Microbiol. 114, 605–620.

Norris, R.L., Eaglesham, G.K., Pierens, G., Shaw, G.R., Smith, M.J., Chiswell, R.K., Seawright, A.A., Moore, M.R., 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *cylindrospermopsis raciborskii*. Environ. Toxicol. 14, 163–165.

Norris, R.L.G., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., Chiswell, R.K., Moore, M.R., 2002. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 40, 471–476.

Ohtani, I., Moore, R.E., Runnegar, M., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J. Am. Chem. Soc. 114, 7941–7942.

Ozawa, K., Yokoyama, A., Ishikawa, K., Kumagai, M., Watanabe, M.F., Park, H.D., 2003. Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. Limnology 4, 131–138.

Özcan Oruç, E., 2010. Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in *Oreochromis niloticus* exposed to chlorpyrifos. Pestic. Biochem. Physiol. 96, 160–166.

Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Arch. Hydrobiol. 107, 563–593.

Paerl, H.W., Huisman, J., 2008. Climate: Blooms like it hot. Science 320, 57–58.

Paris-Palacios, S., Biagianti-Risbourg, S., Vernet, G., 2000. Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulphate. Aquat. Toxicol. 50, 109–124.

Poniedziałek, B., Rzymski, P., Kokociński, M., 2012. Cylindrospermopsin: water-linked potential threat to human health in Europe. Environ. Toxicol. Pharmacol. 34, 651–660.

Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M., 2007. Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. Aquat. Toxicol. 84, 337–345.

Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M., 2008. Protective role of vitamin E on the Microcystin-induced oxidative stress in tilapia fish (*Oreochromis niloticus*). Environ. Toxicol. Chem. 27, 1152–1159.

Puerto, M., Prieto, A.I., Pichardo, S., Moreno, I., Jos, A., Moyano, R., Cameán, A.M., 2009. Effects of dietary N-acetylcysteine (NAC) on the oxidative stress induced in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Environ. Toxicol. Chem. 28, 1679–1686.

Puerto, M., Jos, A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure Cylindrospermopsin on the activity and transcription of antioxidant enzymes in Tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852–1860.

Puerto, M., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M., 2012. Acute exposure to pure Cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environ. Toxicol., http://dx.doi.org/10.1002/tox.21764 (in press).

Rogers, E.H., Zehr, R.D., Gage, M.I., Humpage, A.R., Falconer, I.R., Marr, M., Chernoff, N., 2007. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49, 855–864.

Ross, J.P., 2000. The effect of toxic algae on alligators and alligator egg development. Program Report, Florida Water Resources Research Center. In: Federal Grant No. 1434-HQ-96-GRO2663, Available online at: http://www.ce.ufl.edu/~wrrc/annual.htm [last accessed 15.12.00, last updated 26.06.00].

Rücker, J., Stüken, A., Nixdorf, B., Fastner, J., Chorus, I., Wiedner, C., 2007. Concentrations of particulate and dissolved cylindrospermopsin in 21 *Aphanizomenon*-dominated temperate lakes. Toxicon 50, 800–809.

Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Ge, J.L., Lu, S.C., 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Biophys. Res. Commun. 201, 235–241.

Runnegar, M.T., Kong, S.-M., Zhong, Y.A.-Z., Lu, S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem. Pharmacol. 49, 219–225.

Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J., 2002. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol. Sci. 67, 81–87.

Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. Toxicon 43, 185–194.

Seelig, G.F., Meister, A., 1985. Glutathione biosynthesis; γ-glutamylcysteine synthetase from rat kidney. Meth. Enzymol. 113, 379–392.

Sivonen, K., Jones, G., 1999. Cyanobacterial Toxins. In: Chorus, I., Bartram, M.J. (Eds.), Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. E & FN Spon, London, pp. 41–111.

Sukenik, A., Banker, R., Carmeli, S., Werman, M., Teltsch, B., Porat, R., 2001. Proposed toxicity mode of cylindrospermopsin (cyanobacterial hepatotoxin) based on its structurally related derivatives. In: Towards a Global Perspective on Toxic Cyanobacteria. Fifth International Conference on Toxic Cyanobacteria, Noosa, Queensland, Australia, pp. 16–20.

Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi, H., Wang, X., 2006. Bioaccumulation, depuration and oxidative stress in fish *Carassius auratus* under phenanthrene exposure. Chemosphere 63, 1319–1327.

Tricarico, E., Bertocchi, S., Brusconi, S., Casalone, E., Gherardi, F., Giorgi, G., Mastromei, G., Parisi, G., 2008. Depuration of microcystin-LR from the red swamp crayfish *Procambarus clarkii* with assessment of its food quality. Aquaculture 285, 90–95.

Whitton, B.A., Potts, M., 2000. The Ecology of Cyanobacteria. Their Diversity in Time and Space. Academic Publishers, Kluwer.

Yılmaz, M., Phlips, E.J., Szabob, N.J., Badylak, S., 2008. A comparative study of Florida strains of *Cylindrospermopsis* and *Aphanizomenon* for cylindrospermopsin production. Toxicon 51, 130–139.

Žegura, B., Gajski, G., Štraser, A., Garaj-Vrhovac, V., 2011a. Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress. Toxicon 58, 471–479.

Žegura, B., Štraser, A., Filipic, M., 2011b. Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutat. Res. 727, 16–41.

CAPÍTULO 12/ CHAPTER 12

Remedios Guzmán-Guillén, Daniel Gutiérrez-Praena, María de los Ángeles Risalde, Rosario Moyano, Ana Isabel Prieto, Silvia Pichardo, Ángeles Jos, Vitor M. Vasconcelos, Ana María Cameán

IMMUNOHISTOCHEMICAL APPROACH TO STUDY CYLINDROSPERMOPSIN DISTRIBUTION IN TILAPIA (OREOCHROMIS NILOTICUS) UNDER DIFFERENT EXPOSURE CONDITIONS

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Article

Immunohistochemical Approach to Study Cylindrospermopsin Distribution in Tilapia (*Oreochromis niloticus*) under Different Exposure Conditions

Remedios Guzmán-Guillén [1], Daniel Gutiérrez-Praena [1,*], María de los Ángeles Risalde [2], Rosario Moyano [3], Ana Isabel Prieto [1], Silvia Pichardo [1], Ángeles Jos [1], Vitor Vasconcelos [4,5] and Ana María Cameán [1]

[1] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González 2, Seville 41012, Spain; E-Mails: rguzman1@us.es (R.G.-G.); anaprieto@us.es (A.I. P.); spichardo@us.es (S. P.); angelesjos@us.es (A. J.); camean@us.es (A.M. C.)

[2] Department of Anatomy and Comparative Pathology and Anatomy, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, Córdoba 14071, Spain; E-Mail: risalde10@hotmail.com

[3] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, University of Córdoba, Campus de Rabanales, Carretera Madrid-Cádiz s/n, Córdoba 14071, Spain; E-Mail: ft1mosam@uco.es

[4] Laboratory of Ecotoxicology, Genomics and Evolution, Interdisciplinary Center of Marine and Environmental Research—CIIMAR/CIMAR, University of Porto, Rua dos Bragas 289, Porto 4050-123, Portugal; E-Mail: vmvascon@fc.up.pt

[5] Department of Biology, Faculty of Sciences, Porto University, Porto 4069-007, Portugal

* Author to whom correspondence should be addressed; E-Mail: dgpraena@us.es; Tel.: +34-954-556-762; Fax: +34-954-556-422.

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Abstract: Cylindrospermopsin (CYN) is a cytotoxic cyanotoxin produced by several species of freshwater cyanobacteria (*i.e.*, *Aphanizomenon ovalisporum*). CYN is a tricyclic alkaloid combined with a guanidine moiety. It is well known that CYN inhibits both protein and glutathione synthesis, and also induces genotoxicity and the alteration of different oxidative stress biomarkers. Although the liver and kidney appear to be the main target organs for this toxin based on previous studies, CYN also affects other organs. In the present study, we studied the distribution of CYN in fish (*Oreochromis niloticus*) under

two different exposure scenarios using immunohistochemical (IHC) techniques. In the first method, fish were exposed acutely by intraperitoneal injection or by gavage to 200 µg pure CYN/Kg body weight (bw), and euthanized after 24 h or five days of exposure. In the second method, fish were exposed by immersion to lyophilized *A. ovalisporum* CYN-producing cells using two concentration levels (10 or 100 µg/L) for two different exposure times (7 or 14 days). The IHC was carried out in liver, kidney, intestine, and gills of fish. Results demonstrated a similar pattern of CYN distribution in both experimental methods. The organ that presented the most immunopositive results was the liver, followed by the kidney, intestine, and gills. Moreover, the immunolabeling signal intensified with increasing time in both assays, confirming the delayed toxicity of CYN, and also with the increment of the dose, as it is shown in the sub-chronic assay. Thus, IHC is shown to be a valuable technique to study CYN distribution in these organisms.

Keywords: Cylindrospermopsin; *Aphanizomenon ovalisporum*; *Oreochromis niloticus*; immunohistochemistry; distribution; cyanobacteria; cyanotoxin

1. Introduction

Cyanobacteria, also known as blue-green algae, have the ability to form dense blooms in certain situations, decreasing the water quality. Some bloom-forming species of cyanobacteria are able to produce harmful secondary metabolites called cyanotoxins, causing undesirable effects with implications in human and animal health via drinking and recreational waters or consumption of contaminated food through the food web [1].

Cylindrospermopsin (CYN) is one cyanotoxin present in freshwater bodies around the world and has caused environmental concern. CYN is produced by several species of cyanobacteria, among them *Aphanizomenon ovalisporum* [2]. Structurally, it is an alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil [3]. Due to its physicochemical properties, CYN is highly water-soluble [4] and very stable under different environmental conditions [5].

Funari and Testai [6] established that the main targets of CYN are the liver and kidney, but it is considered a cytotoxin since other organs may also be affected after an exposure to the toxin [7–9]. Toxicity of CYN is mediated by the inhibition of protein [7] and glutathione synthesis [10], genotoxicity mediated by DNA fragmentation [11], and also by the induction of oxidative stress [12]. Cylindrospermopsin may also inhibit progesterone production, recognizing that the toxin has some potential for endocrine disruption [13].

The possibility of CYN accumulation in aquatic organisms through the trophic web is a serious concern, since many aquatic organisms are for human consumption. Field and laboratory studies on presence and accumulation of CYN in these aquatic organisms are scarce [14]. White *et al*. [15] studied the accumulation of CYN in the aquatic snail *Melanoides tuberculata* exposed to an extract from a CYN-producer *Cylindrospermopsis raciborskii* strain and also to a living culture of these cyanobacteria. Results showed that CYN from live cells presented higher tissue accumulation. In addition, Berry and Lind [16] evaluated the presence of CYN in the snail *Pomacea patula catemacensis*.

Regarding bivalves, accumulation up to 560 μg CYN/kg fresh weight was found in the mussel *Alathyria pertexta pertexta*, collected from a reservoir containing <0.8 μg CYN/L [17]. Saker *et al*. [18] also demonstrated CYN accumulation in different tissues from the swan mussel, *Anodonta cygnea*, exposed to 14–90 μg CYN/L for 16 days. Saker and Eaglesham [19] reported the accumulation of CYN in the hepatopancreas and muscle of the redclaw crayfish *Cherax quadricarinatus* collected in an aquaculture pond with a severe bloom of *C. raciborskii*. They also demonstrated that this accumulation occurred to a lesser extent in *in vivo* studies in the laboratory. White *et al*. [20] studied the accumulation of CYN in tadpoles of *Bufo marinus* exposed to an extract of a CYN-producer *C. raciborskii* culture or to a culture of *C. raciborskii*, indicating the bioaccumulation of CYN, mainly via grazing. Finally, fish are able to accumulate CYN by direct feeding, by the uptake of the dissolved toxin through gills or skin, or by exposure through the food web. For the first time, Messineo *et al*. [21] found a moderate CYN accumulation in wild trout tissues. More recently, Berry *et al*. [22] showed that CYN accumulation in several fish species mainly occurred in the muscle. Thus, the general order for organisms' bioaccumulation ability has been established by Kinnear [23] as follows: gastropods > bivalves > crustaceans > amphibians > fish.

When referring to CYN toxicity, there are different issues to consider, such as the variability between animal species and even between the individuals of the same species [23] because of their idiosyncrasies. In addition, the different toxicity induced by pure CYN or CYN cyanobacterial extracts, is another matter to take into account. Results show that the toxicity induced by the toxin from the extracts is higher than that produced by the pure CYN. This could be explained by the presence of other compounds in the cyanobacterial extract [8,24,25]. Hawkins *et al*. [26] showed that pure CYN mainly affected the liver, while crude extracts of *C. raciborskii* administered by intraperitoneal (i.p.) injection or oral route to mice also induced pathological symptoms in kidney, lungs, stomach, spleen, thymus, heart, and the vascular and lymphatic systems. Berry *et al*. [27] concluded that direct immersion of zebrafish embryos in extracts from several isolates of *C. raciborskii* and *A. ovalisporum* resulted in high toxicity, suggesting that these extracts could contain some toxic metabolites other than CYN. Recently, our group has demonstrated that acute exposure to pure CYN by oral route (gavage) and i.p. injection induced a dose- and a time-dependent oxidative stress and histopathological effects in different organs of tilapia (*Oreochromis niloticus*) [28–30]. It has also been proved that CYN from a cyanobacterial extract produced severe injuries in comparison to the pure toxin [31]. As Kinnear [23] described, the reverse order regarding the bioaccumulation has been established for the susceptibility of the organisms to CYN.

In order to assess CYN distribution in tissues, immunohistochemistry (IHC) is an interesting technique based on the principle of antibodies binding specifically to antigens. IHC has been useful for detecting tissue distribution of Microcystin-LR (MC-LR) in mice [32,33], rainbow trout [34] and in various organs of the gastropod *Lymnaea stagnalis* [35]. Furthermore, it has been employed in the detection of different viruses in cell cultures [36] and tissues [37], as well as in the diagnosis of illnesses [38]. In any case, these studies only refer to acute toxicity but, taking into account that fish are naturally exposed to cyanobacterial blooms for sub-chronic and chronic periods, it would be of interest to compare the effects of acute and sub-chronic exposures to CYN by different routes.

Therefore, the aim of this study was to examine and compare the tissue distribution of CYN in various organs from tilapia (*O. niloticus*) by IHC. Two different exposure scenarios were used:

(a) acute exposure by oral route (gavage) and i.p. injection to a single dose of 200 µg/Kg bw of pure CYN for 24 h and five days; (b) sub-chronic exposure to different concentrations of CYN (10 and 100 µg CYN/L) contained in a lyophilized *A. ovalisporum* culture obtained from a natural cyanobacterial bloom by immersion for 7 and 14 days.

2. Results and Discussion

2.1. Results

2.1.1. Acute Dose Assay

In the positive control tissue samples, CYN-specific labeling appeared as evenly distributed dark red granules or as diffuse homogeneous staining distributed in the cytoplasm of the cells. The distribution of CYN, mainly associated with the organs studied, is summarized in Table 1.

Table 1. Distribution of immunolabeled cells for CYN in different organs from tilapia (*Oreochromis niloticus*) exposed to a single dose of 200 µg/Kg bw of pure CYN and euthanized 24 h or five days after the exposure. Results are expressed as number of immunolabeled cells per area of 0.2 mm^2: - absent, + scarce (0–10), ++ moderate (10–50), +++ intense (>50). **OC:** Control fish treated with a saline solution (0.9% NaCl) by the oral route; **IPC:** Control fish treated with a saline solution (0.9% NaCl) by the i.p. route; **OI24h:** Fish exposed to a single dose of 200 µg/Kg bw of pure CYN by the oral route and euthanized after 24 h; **IPI24h:** Fish exposed to a single dose of 200 µg/Kg bw of pure CYN by the i.p. route and euthanized after 24 h; **OI5d:** Fish exposed to a single dose of 200 µg/Kg bw of pure CYN by the oral route and euthanized after five days; **IPI5d:** Fish exposed to a single dose of 200 µg/Kg bw of pure CYN by the i.p. route and euthanized after five days.

	OC	IPC	OI24h	IPI24h	OI5d	IPI5d
Liver						
Hepatocytes	-	-	-	+	+	++
Pancreatic acini	-	-	+	++	+	+
Erythrocytes	-	-	-	-	+	++
Kidney						
Tubules	-	-	-	-	+	+
Glomeruli	-	-	-	-	+	++
Erythrocytes	-	-	-	-	-	+++
Intestine						
Epithelium	-	-	-	-	-	++
Gills						
Secondary lamellae	-	-	-	-	++	+
Erythrocytes	-	-	-	-	-	+

There was no CYN-specific labeling in any control samples when rabbit anti-CYN serum was replaced by rabbit non-immune serum or PBS in the immunohistochemical study (Figure 1). CYN antigen was not detected in any of the tissue sections analyzed from fish not exposed to the toxin (Figure 2).

Figure 1. Photomicrographs of the immunohistochemistry of liver (**A**) and gills (**B**) from positive tissue sections of tilapia exposed to CYN where the primary antibody was replaced by PBS or by rabbit non-immune serum, respectively. Bars: 25 µm. No labeling and residual endogenous peroxidase activity were observed in any of these technical negative controls.

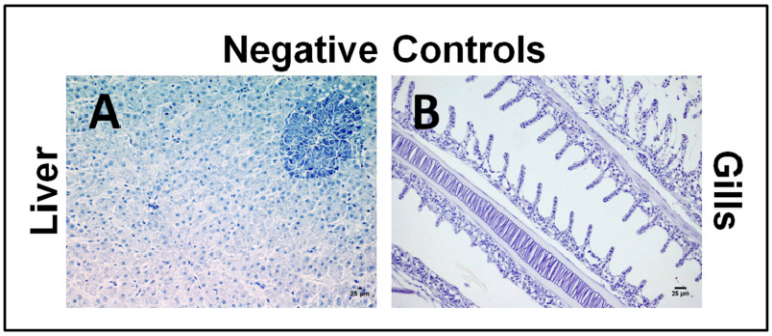
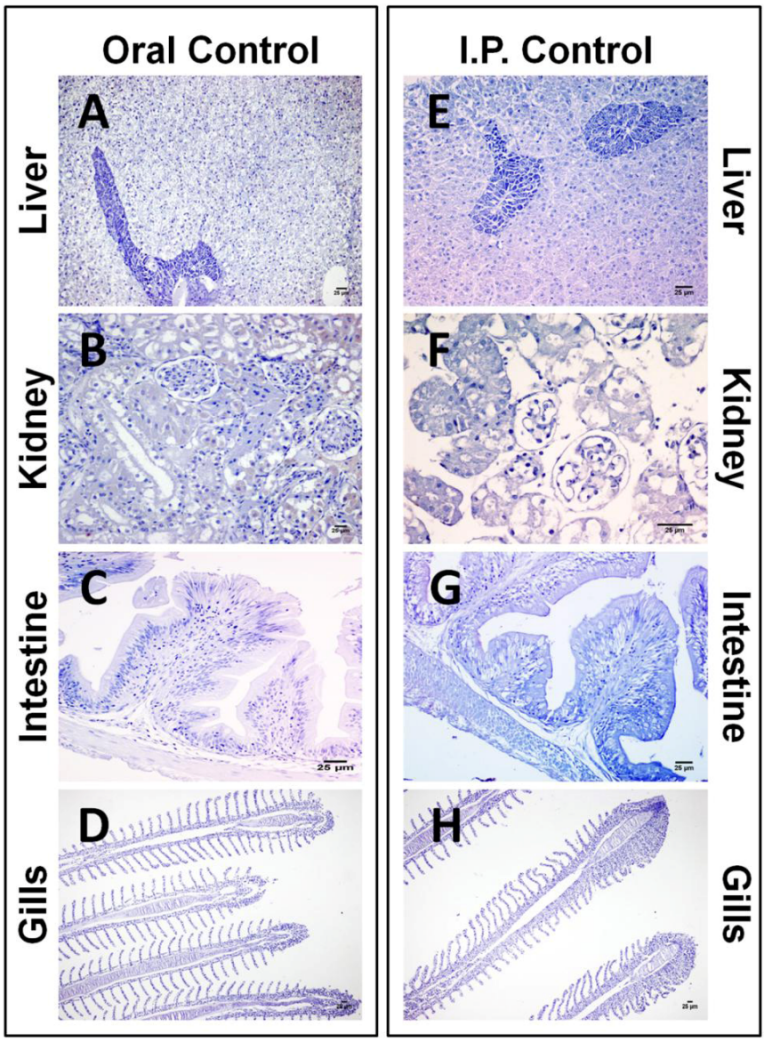


Figure 2. Photomicrographs of the immunohistochemistry of CYN in liver (**A**,**E**), kidney (**B**,**F**), intestine (**C**,**G**), and gills (**D**,**H**) from tilapia used as control in the acute assay. Bars: 25 µm. No labeling was observed in any of the control tissues.



After 24 h of exposure to 200 µg/Kg bw of pure CYN, the liver from those fish exposed by gavage showed fewer immunopositive cells against the cyanotoxin (Figure 3A) in comparison to those from fish exposed by i.p. injection (Figure 3E). The immunopositivity appeared mainly in the pancreatic acini (both routes) and the hepatocytes (i.p. route). After five days of exposure, CYN immunostaining appeared to be more intense in the centrilobular hepatocytes and also appeared in the erythrocytes, this staining being higher in those animals exposed by the i.p. route (Figure 4E) in comparison to the oral route (Figure 4A). The CYN-positive hepatocytes exhibited a stronger granular staining in the cytoplasm than in the nuclei, together with a cytoplasmic eosinophilic condensation aggregated along the outer nuclear membrane.

Figure 3. Photomicrographs of immunohistochemistry of CYN in liver (**A**,**E**), kidney (**B**,**F**), intestine (**C**,**G**), and gills (**D**,**H**) from tilapia exposed orally (**A**–**D**) or intraperitoneally (**E**–**H**) to 200 µg/kg bw of pure CYN and euthanized after 24 h. Bars: 25 µm. (**A**) Liver with scarce CYN-immunopositive staining; (**B**) Normal renal parenchyma without CYN-positive staining; (**C**) No immunostaining in the intestine; (**D**) Normal gills parenchyma after the exposure; (**E**) CYN-positive staining of the cytoplasm of the hepatocytes from tilapia exposed by i.p. injection; (**F**) Renal parenchyma with an intense immunostaining; (**G**) Absence of staining in the epithelium of the intestine; (**H**) Isolated CYN-immunopositivity in gills from fish intraperitoneally exposed. Arrows indicate CYN-immunopositive cells.

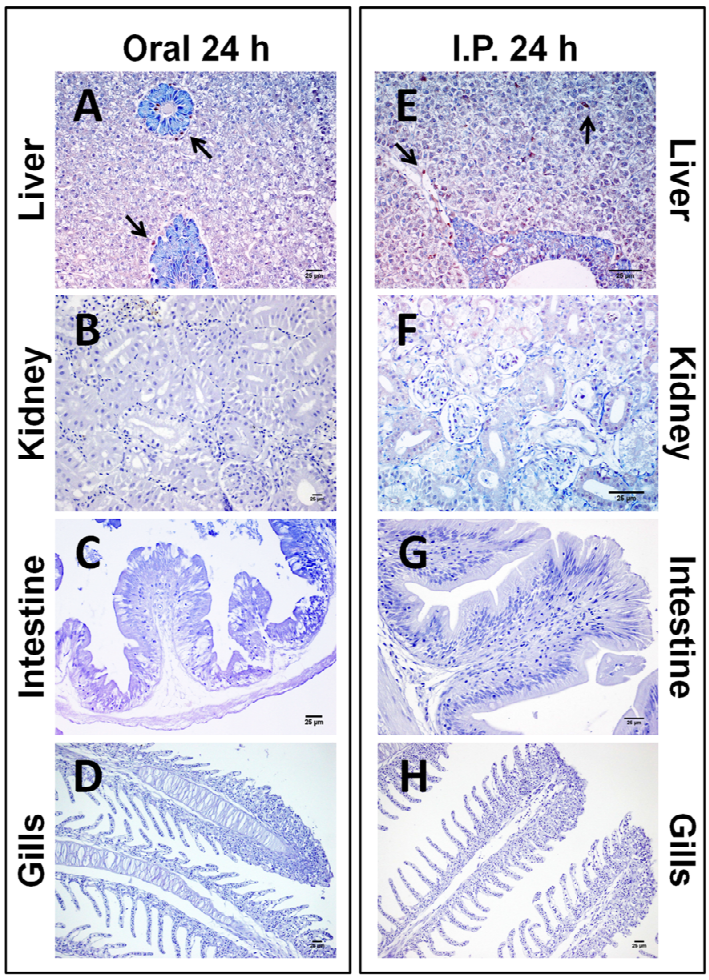
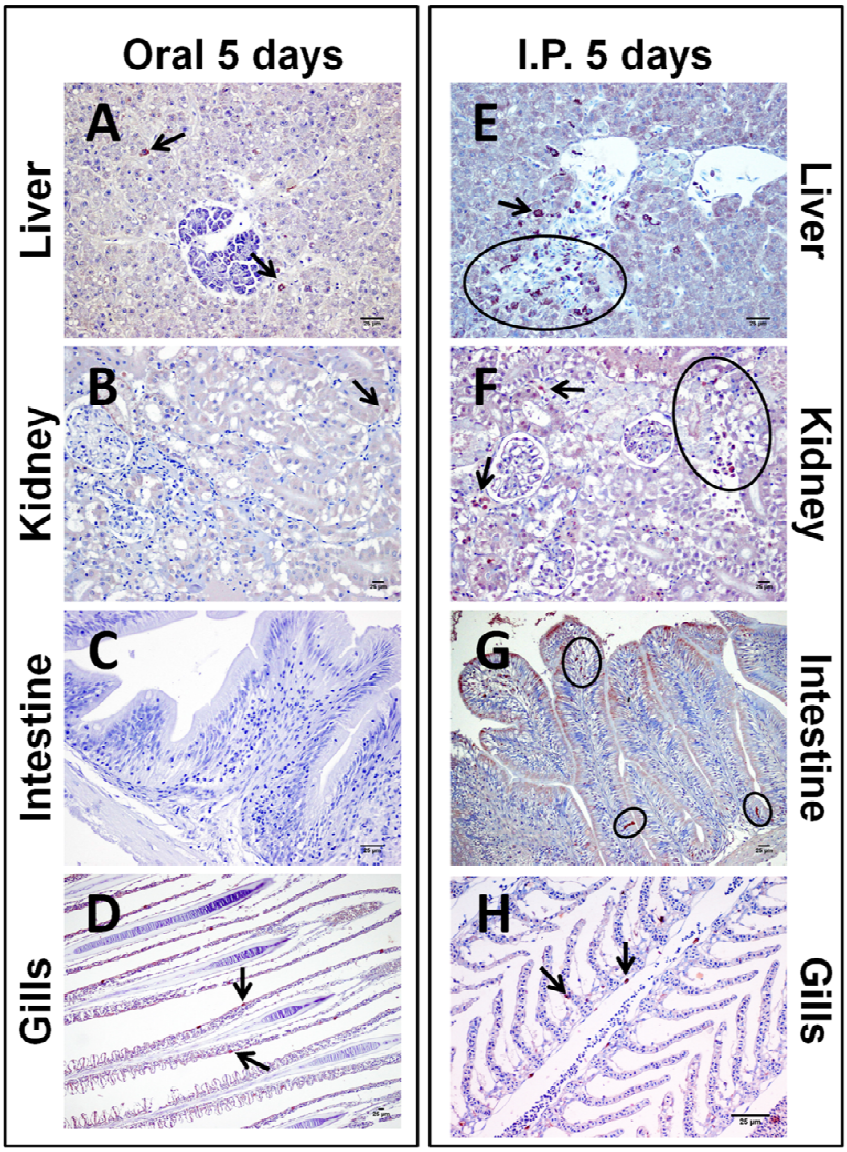


Figure 4. Photomicrographs of immunohistochemistry of CYN in liver (**A**,**E**), kidney (**B**,**F**), intestine (**C**,**G**), and gills (**D**,**H**) from tilapia exposed orally (**A–D**) or intraperitoneally (**E–H**) to 200 µg/kg bw of pure CYN and euthanized after five days. Bars: 25 µm. (A) Hepatocytes from liver with a light CYN-immunostaining; (**B**) Positive staining of the renal parenchyma mainly localized in proximal and convoluted tubules; (**C**) No immunostaining in the intestine; (**D**) Normal gills parenchyma after the exposure; (**E**) Moderate CYN-positive staining the hepatocytes; (**F**) Intense immunostaining in cells from the kidney principally localized in glomeruli and erythrocytes; (**G**) Slight immunostaining in the intestine, mainly localized in the epithelial and globet cells; (**H**) Weak immunoreactivity of the cells from the gills against the CYN. Arrows and circles indicate isolated or groups of CYN-immunopositivity cells, respectively.



In the kidney, no presence of CYN-positive cells was detected 24 h after the exposure to the toxin by any of the intoxication routes assayed (Figure 3B,F). However, the i.p. administration of CYN gave rise to the appearance of more CYN-immunoreactive cells in comparison to the oral administration at five days post-exposition. The staining in fish exposed by the oral route was primarily localized in

proximal and distal convoluted tubules (Figure 4B), whereas by the i.p. route, CYN was mainly present in glomeruli and erythrocytes (Figure 4F).

Intestine samples after 24 h and five days from fish exposed orally appeared totally clear in both cases (Figures 3C and 4C), as well as the intestine from those fish exposed i.p. and euthanized after 24 h (Figure 3G). Only the samples from fish exposed by i.p. injection and euthanized after five days presented immunopositive results (Figure 4G). In this case, CYN distribution was mainly confined to the epithelium, showing a moderate presence of CYN-positive epithelial and goblet cells.

The immunohistochemical study of the gills revealed that the targeted cells for CYN were both epithelial and goblet cells, and in a lesser extent, erythrocytes from the secondary lamellae. Staining had a cytoplasmic location with a dark granular appearance homogeneously distributed. This immunolabeling was only evident in both groups of animals euthanized at 5 days post-exposition (Figure 4D,H).

2.1.2. Sub-Chronic Dose Assay

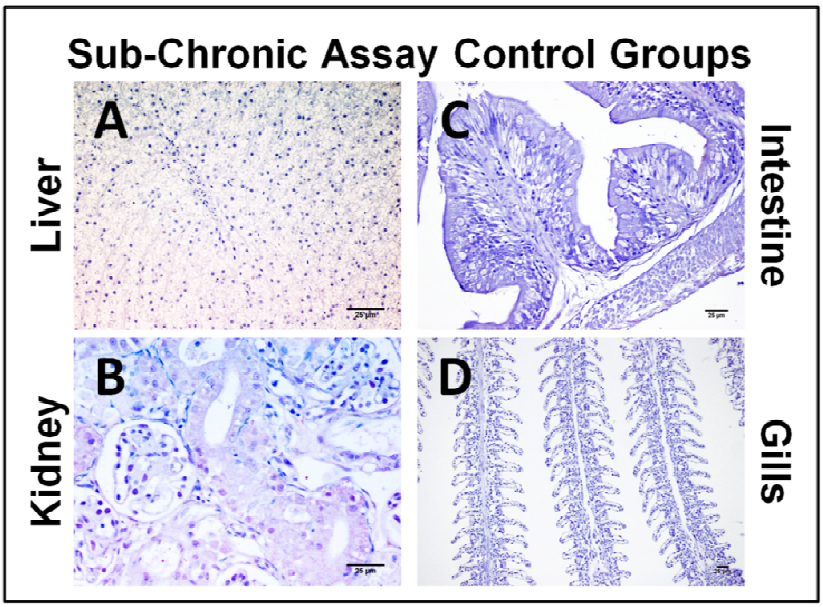
In the positive control tissue samples, CYN-specific labeling appeared as evenly distributed dark red granules or as diffuse homogeneous staining distributed in the cytoplasm of the cells. The distribution of CYN, mainly associated with the organs studied, is summarized in Table 2.

Table 2. Distribution of immunolabeled cells for CYN in different organs from tilapia (*Oreochromis niloticus*) exposed by immersion to 10 or 100 µg/L of CYN contained in a lyophilized CYN-producer *A. ovalisporum* strain during 7 or 14 days. Results are expressed as number of immunolabeled cells per area of 0.2 mm^2: - absent, + scarce (0–10), ++ moderate (10–50), +++ intense (>50). **C7d:** Control fish euthanized after 7 days; **10C7d:** Fish exposed to 10 µg/L of CYN from a CYN-producer *A. ovalisporum* strain and euthanized after 7 days; **100C7d:** Fish exposed to 100 µg/L of CYN from a CYN-producer *A. ovalisporum* strain and euthanized after 7 days; **C14d:** Control fish euthanized after 14 days; **10C14d:** Fish exposed to 10 µg/L of CYN from a CYN-producer *A. ovalisporum* strain and euthanized after 14 days; **100C14d:** Fish exposed to 100 µg/L of CYN from a CYN-producer *A. ovalisporum* strain and euthanized after 14 days.

	C7d	10C7d	100C7d	C14d	10C14d	100C14d
Liver						
Hepatocytes	-	+++	+++	-	+++	+++
Pancreatic acini	-	++	+++	-	-	+++
Erythrocytes	-	-	++	-	+	+
Kidney						
Tubules	-	-	+++	-	++	+++
Glomeruli	-	-	++	-	+++	+++
Erythrocytes	-	-	++	-	+	+
Intestine						
Epithelium	-	+	+	-	+++	+++
Gills						
Secondary lamellae	-	+	++	-	++	+++
Erythrocytes	-	+	+	-	-	+

There was no CYN-specific labeling in any control samples when rabbit anti-CYN serum was replaced by rabbit non-immune serum in the immunohistochemical study. CYN antigen was not detected in any of the tissue sections analyzed from fish not exposed to the toxin (Figure 5).

Figure 5. Photomicrographs of the immunohistochemistry of CYN in liver (**A**), kidney (**B**), intestine (**C**), and gills (**D**), from tilapia used as control in the sub-chronic assay. Bars: 25 µm. No labeling was observed in any of the organs studied.



The immunohistochemical study of fish exposed by immersion to two different concentrations of CYN from an *A. ovalisporum* strain for 7 or 14 days revealed that the liver was the organ with most CYN-positive cells. This staining was concentration-dependent for both considered periods of time (Figure 6A,E). Thus, a slight minor quantity of CYN-positive cells was detected in the animals exposed to 10 µg CYN/L, without the influence of the exposure time. At 7 and 14 days, in fish exposed to 100 µg CYN/L the same areas were stained, and erythrocytes, pancreatic islet cells and, most frequently, hepatocytes were CYN-positives. Such cytoplasmic granular staining observed in hepatocytes was detected in almost all the lobules, these cells being enlarged and distributed especially adjacent to the central vein (Figure 7A,E).

Immunolabeling in kidney displayed differences between fish subjected to different concentrations of CYN at the same time of exposure, and between fish subjected to the same concentration of CYN at different times. In those fish exposed to 10 µg CYN/L for seven days, the toxin was not immunohistochemically detectable in tissue sections (Figure 6B). On the contrary, when fish were exposed for 14 days, a strong staining in the cytoplasm of proximal and distal convoluted tubules, as well as in the glomeruli was observed (Figure 6F). In those fish exposed to the highest concentration of CYN (100 µg/L), there was an increase in the number of CYN-positive cells at both exposure times (Figure 7B,F)

In the intestine, the presence of CYN-immunopositive cells was time-dependent. Thus, after seven days of the exposure, the intestine of fish intoxicated with both doses of CYN showed a slight quantity of epithelial and goblet cells with granular staining in the epithelium (Figures 6C and 7C), while fish

exposed to both concentrations for 14 days showed an increase of the CYN-specific labeling, mainly in the goblet cells (Figures 6G and 7G).

Figure 6. Photomicrographs of immunohistochemistry of liver (**A**,**E**), kidney (**B**,**F**), intestine (**C**,**G**), and gills (**D**,**H**) from tilapia exposed by immersion during seven days (**A**–**D**) or 14 days (**E**–**H**) to 10 µg/L of CYN contained in a lyophilized *Aphanizomenon ovalisporum* culture. Bars: 25 µm. (**A**) Moderate staining of the hepatocytes from fish exposed during seven days to CYN; (**B**) Absence of immunoreactive cells against CYN in the renal parenchyma; (**C**) Slight immunostaining in the intestine, mainly localized in the epithelial and globet cells; (**D**) Weak immunolabeling in cells from gills; (**E**) Mild CYN-positive staining of the hepatocytes from liver exposed during 14 days; (**F**) Strong immunostaining in cells from the proximal and distal convoluted tubules, and in cells from the glomeruli of the kidney; (**G**) Considerable immunostaining of the intestinal cells, mainly localized in the epithelial and globet cells; (**H**) Positive staining of cells from gills. Arrows and circles indicate isolated or groups of CYN-immunopositivity cells, respectively.

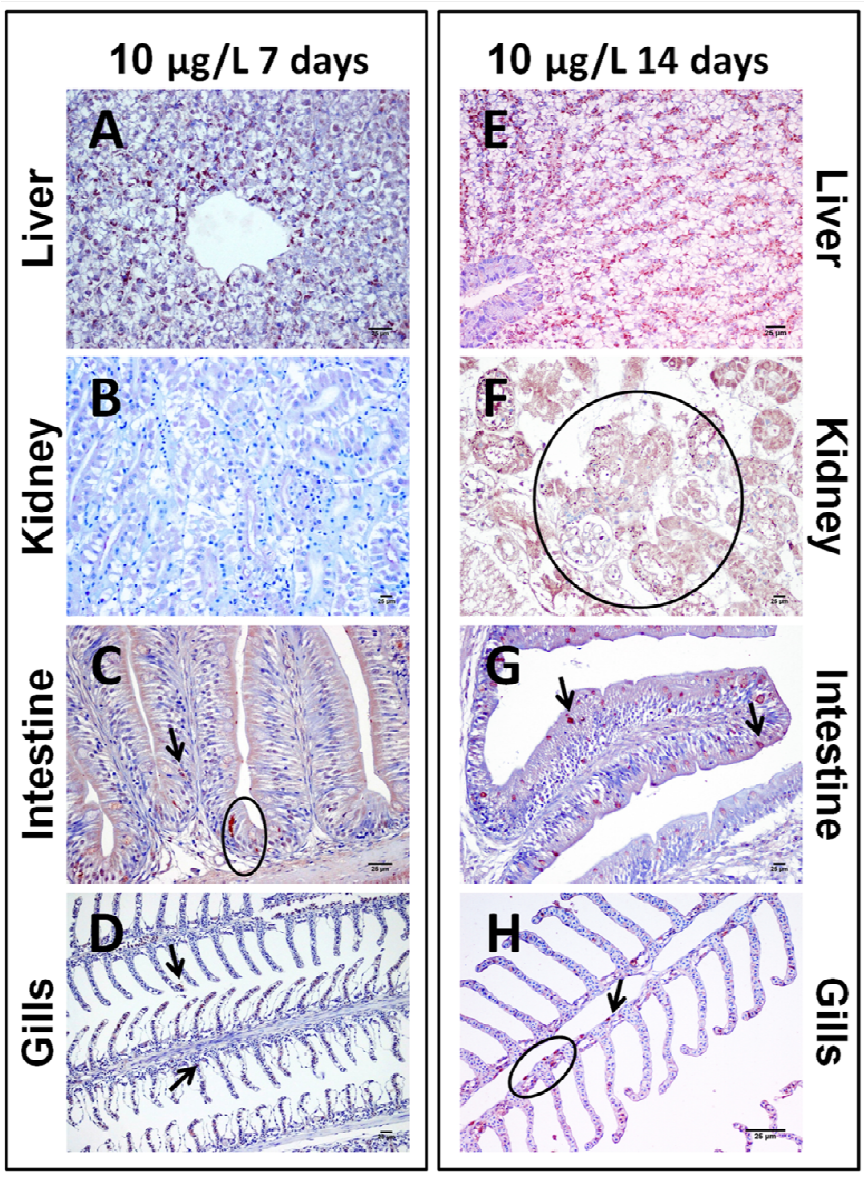
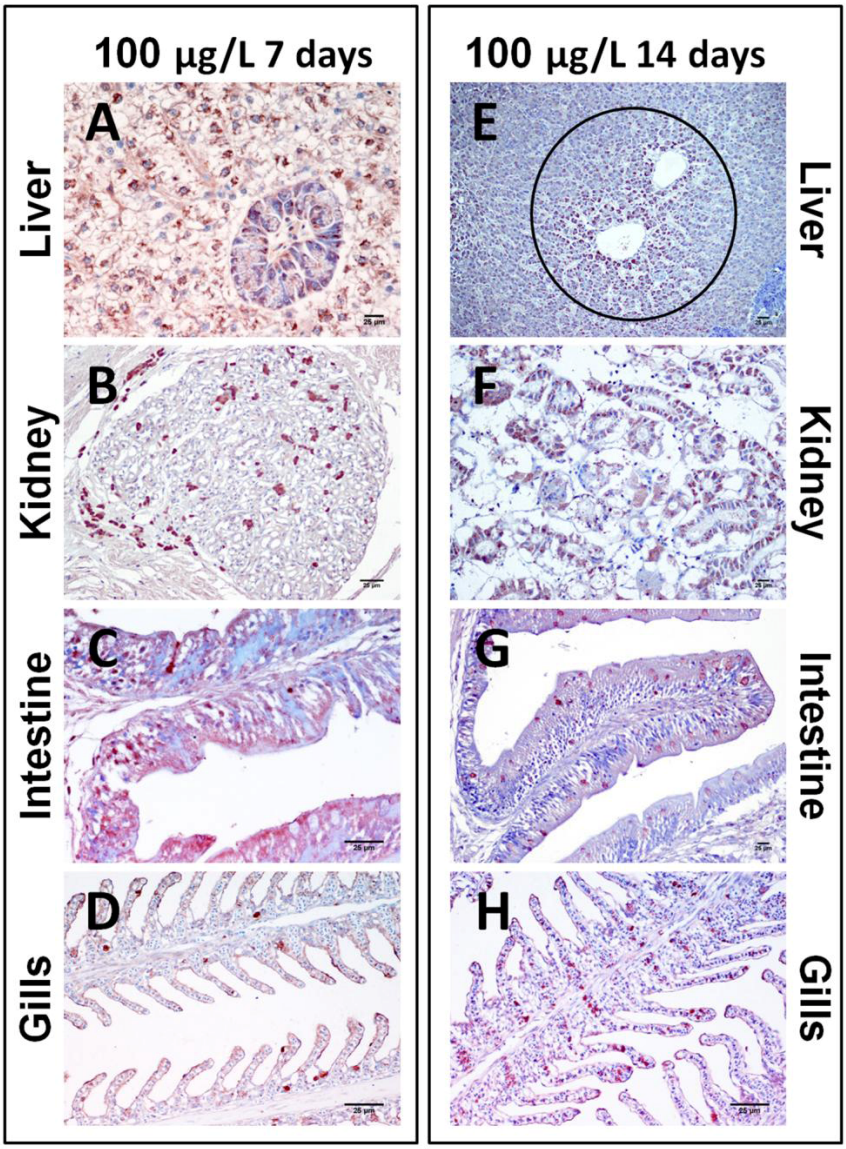


Figure 7. Photomicrographs of immunohistochemistry of liver (**A**,**E**), kidney (**B**,**F**), intestine (**C**,**G**), and gills (**D**,**H**) from tilapia exposed by immersion during seven days (**A**–**D**) or 14 days (**E**–**H**) to 100 µg/L of CYN contained in a lyophilized *Aphanizomenon ovalisporum* culture. Bars: 25 µm. (**A**) Intense staining of the hepatocytes from fish exposed during seven days to CYN; (**B**) Immunoreactive cells against CYN in the renal parenchyma; (**C**) Slight immunostaining in the intestine, mainly localized in the epithelial and globet cells; (**D**) Immunolabeling in cells from gills; (**E**) Strong CYN-positive staining of the hepatocytes from liver exposed during 14 days; (**F**) Intense positive immunostaining in cells from the proximal and distal convoluted tubules, and in cells from the glomeruli of the kidney; (**G**) CYN-positive cells of the intestinal lumen, mainly in the epithelial and globet cells; (**H**) Positive staining of cells from gills. Circles indicate CYN-immunopositive cells.



Finally, the immunohistochemical study of the gills showed that the main CYN-positive cells were epithelial and goblet cells from the secondary lamellae and erythrocytes. The immunolabeling was more pronounced with the highest concentration of CYN used (100 µg/L) and the longest time of exposure (14 days) (Figure 7H).

2.2. Discussion

Studies concerning CYN are increasing due to its recent increase in freshwater bodies all over the world. There are several studies regarding the CYN-toxic effects in different animal models [12,23], but studies related to its distribution in organisms and the relation with the damage induced are still scarce. To our knowledge, this is the first study that investigates by an immunohistochemical technique the distribution of CYN in fish acutely (gavage and i.p. injection) or sub-chronically (immersion) exposed to pure CYN or to a CYN-producing culture, respectively.

Regarding our work, CYN-immunostaining appeared in all the organs studied (liver, kidney, intestine, gills) in both the acute and the sub-chronic assays, with the exception of the heart. In both experiments, the order of the intensity of the CYN-positive staining in all the organs was as follows: liver > kidney > intestine > gills.

The highest liver CYN-immunopositivity correlates with the histopathological lesions described by light and electron microscopy in tilapia (*O. niloticus*) acutely and sub-chronically exposed to the same conditions as described in this work [30,39]. These histopathological alterations were also consistent with the exposure route and the time of euthanization. The liver appeared to be the main target organ, as previously stated by Fischer *et al*. [34]. This study also indicated that the liver was the main target organ for the cyanotoxin MC-LR in rainbow trout (*Oncorrhynchus mykiss*) orally-exposed to 5.7 mg/Kg bw of MC-LR from a cyanobacterial strain. In the same way, Ito *et al*. [40] also found that liver from mice exposed by the same route to 0.5 mg/Kg bw of MC-LR was the organ presenting the most intense immunolabeling, especially the cytoplasm from the hepatocytes located around the central vein, as we also found in the experiments carried out in the present work. Yoshida *et al*. [32] correlated the immunopositivy in the liver with some injuries, such as hemorrhage and apoptosis, in mice i.p. administered with MC-LR.

Following the liver, the kidney was the organ with more CYN-immunopositive cells, corroborating the selectivity of the CYN for this organ, as other authors stated before in mice and fish [29,41]. Gutiérrez-Praena *et al*. [30] and Guzmán-Guillén *et al*. [39] also corroborated these findings through histopathology of the kidney of *O. niloticus* treated under the same conditions as in the present experiments. The distribution of CYN described in this work correlates, mostly, with the histopathological findings described. Moreover, studies of the body distribution of ^{14}C-labeled CYN in mice showed that CYN was mainly excreted through the kidney, as nearly 50% of an i.p. administered dose of CYN appeared in the urine in 6 h, whereas 20% of the dose was present in the liver [42].

Concerning the intestine, we observed that staining appeared in a low range in comparison to liver and kidney, and mainly in the epithelium. This is in agreement with the histopathological results presented by Gutiérrez-Praena *et al*. [30] and Guzmán-Guillén *et al*. [39] as the localization of CYN in the epithelium correspond with the main damage induced in the intestine. Ito *et al*. [40] described a MC-LR-immunopositivity in the epithelial and globet cells from the intestine of mice. However, Lance *et al*. [35] found that the intestine of the snail *Lymnaea stagnalis* presented an intense MC-LR-immunopositive staining derived from pure MC-LR or MC-LR extracted from a toxic cyanobacteria strain. This fact could indicate a barrier function of the intestine, reducing the damage produced by the toxins in other organs. This has not been observed in either of our two studies.

When comparing both assays, some differences appeared in relation to the immunostaining of the organs. Thus, the positive staining in liver and kidney was more intense in those fish from the sub-chronic assay than in those from the acute assay, being even absent in those fish euthanized after 24 h. Although these differences could be due to the kind of CYN administered to fish (pure or producing cells), it is also probable that the exposure time plays a role in them. Thus, fish exposed for 14 days to CYN presented in general the most intense signal in both organs. The differences were also noticeable in the intestine, where a more intense staining appeared in fish from the sub-chronic assay. In this organ, these differences could also be explained by the exposure route. Hence, when CYN was i.p. administered, the intestine was not a usual absorption route, and when it comes to gavage it is probably that the unique dose of pure toxin crosses the membranes and enters into the blood stream. However, CYN from the cyanobacterial strain appeared evident from seven days of administration, which could be due to the continuous presence of the toxin in the water. This last statement could be also applied to gills, since fish from the sub-chronic assay are continuously filtrating water containing CYN, whereas fish from the acute assay only presented CYN-positive cells after a five-day exposure.

3. Experimental Section

3.1. Chemicals

Pure Cylindrospermopsin (purity ≥ 95%) was supplied by Alexis Corporation (Lausen, Switzerland). All chemicals were provided by Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab (Seville, Spain).

3.2. Aphanizomenon Ovalisporum *Culture and Determination of Cyanobacterial Toxins*

A. ovalisporum (LEGE-X001) strain was supplied by the Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR, Porto, Portugal). A culture of this strain was maintained in Z8 medium at 25 °C under continuous illumination with an intensity of 28 μmol photons m^{-2} s^{-1} provided by cool white fluorescent tubes. After 33 days, cultures were harvested by decantation with a plankton-net (20 μm diameter). The biomass obtained was frozen at −80 °C until lyophilization (Telstar Cryodos, Madrid, Spain).

Cylindrospermopsin was extracted from the dried cell material following the Guzmán-Guillén *et al*. [43] method. The liquid chromatography (LC) system used to analyze the toxin content was a Varian 9012 equipped with a Varian ProStar 330 Diode Array Detector (DAD, Varian Technologies, Palo Alto, CA, USA). Chromatographic data were processed with a Star Chromatography Workstation (Varian Technologies, Palo Alto, CA, USA). Chromatographic separation of CYN was performed according to Guzmán-Guillén *et al*. [43] on a 250 mm × 4.6 mm internal diameter, 5 μm, LiChrosphere C18 column purchased from Merck (Darmstadt, Germany). The standard solutions of CYN were prepared in water (100 μg/mL) and diluted as required with water for their use as working solutions (0.08–5.0 μg/mL). After analysis, the concentration of CYN obtained from the lyophilized cells was 8.7 μg/mg.

3.3. Experimental Setup and Acclimation of Fish

The experiment had the approval of the Ethic Committee of the University of Seville. *Oreochromis niloticus* (Nile tilapia, Cichlidae) were obtained from Valenciana de Acuicultura (Valencia, Spain). Fish were transferred to the laboratory, where they were held in glass aquaria, with 96 L of dechlorinated tap water, a continuous system of water filtration and aeration (Eheim Liberty 150 Bio-Espumador cartridges (Eheim, Stuttgart, Germany), and a 12:12-h light:dark photoperiod. Temperature was maintained at 21 ± 2 °C and dissolved oxygen at 7.0 ± 0.5 mg/L. Mean values for additional water-quality parameters were pH 7.6 ± 0.3, conductivity of 287 µS/cm, 0.60 mM Ca^{2+}, and 0.3 mM Mg^{2+}. Fish were fed with commercial fish food (Dibaq, Segovia, Spain) containing 6% lipids, 31% proteins, 37% carbohydrates, 2.5% fiber, 1.5% total phosphorus, 12% ash, 200 mg α-tocopherol/kg, 1700 IU vitamin D3/kg feed, and 10,000 IU vitamin A/kg feed. Fish were acclimatized for 15 days before the beginning of the experiments. For the acute assay sixty-four fish (53.4 ± 5.2 g; length: 12 ± 3 cm) were used, whereas forty-eight fish were needed in for the sub-chronic experiment (50 ± 8 g; length: 12 ± 2 cm). In both cases, fish were randomly distributed in aquaria (n = 8 individuals/aquarium).

3.4. Experimental Exposure

For the acute assay (Table 3), fish in aquaria 3 and 7 were exposed by gavage to a single dose of 200 µg/Kg of pure CYN in 0.5 mL of 0.9% (*w*/*v*) NaCl solution and were euthanized after 24 h (aquarium 3) and five days (aquarium 7). Control fish were allocated to aquaria 1 and 5, received only the vehicle solution (0.5 mL of 0.9%, *w*/*v*, NaCl), and were euthanized after 24 h (aquarium 1) and five days (aquarium 5). Similarly, two other groups of fish were exposed by i.p. injection to 200 µg/Kg bw of pure CYN in 0.5 mL of 0.9% (*w*/*v*) NaCl solution and were euthanized after 24 h (aquarium 4) and five days (aquarium 8). The corresponding control groups were injected with the vehicle only and were euthanized after 24 h (aquarium 2) and five days (aquarium 6).

Table 3. Feeding conditions of *Oreochromis niloticus* and exposure pathways to pure Cylindrospermopsin (CYN). Numbers from 1 to 8 correspond with the aquaria where the different treatments were carried out. (+ With; - Without).

	24 h				5 days			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
NaCl solution by gavage	+	-	-	-	+	-	-	-
NaCl solution by i.p. injection	-	+	-	-	-	+	-	-
Pure CYN by gavage	-	-	+	-	-	-	+	-
Pure CYN by i.p. injection	-	-	-	+	-	-	-	+

For the sub-chronic assay (Table 4), fish in aquaria 2 and 5 were fed with commercial fish food and exposed by immersion to an adequate quantity of cyanobacterial cells at the beginning of the experiment in order to obtain 10 µg CYN/L. Fish in aquaria 3 and 6 were fed similarly but with enough cyanobacterial cells to obtain 100 µg CYN/L. Fish from aquaria 1 and 4 were fed only with the commercial food. After 7 days (aquaria 1, 2, and 3) and 14 days (aquaria 4, 5, and 6), fish were euthanized.

Table 4. Feeding conditions of *Oreochromis niloticus* and exposure to Cylindrospermopsin (CYN) contained in a lyophilized *Aphanizomenon ovalisporum* strain. Numbers from 1 to 6 correspond with the aquaria where the different treatments were carried out. (+ With; - Without).

	7 days			14 days		
	1	**2**	**3**	**4**	**5**	**6**
10 µg/L of CYN	-	+	-	-	+	-
100 µg/L of CYN	-	-	+	-	-	+

3.5. Immunization with Cylindrospermopsin (CYN) and Preparation of Polyclonal Antiserum

Polyclonal antiserum was prepared according to the method of Hancock and O'Reilly [44]. Rabbit females (*Orhyctolagus cuniculus*, breed New Zealand White) weighing 1.8 to 2.0 kg were maintained in sterile conditions a week before the start-up of the immunization protocol to become accustomed. Then, they received two intravenous administrations into the marginal ear vein with seven days apart of a solution of 100 µg of pure CYN in 0.5 mL of phosphate buffered saline solution **(**PBS). Blood samples were taken from the central ear artery at 14 days post-inoculation, allowed to clot at room temperature for 1 h and then centrifuged at 3000 rpm for 10 min to obtain the serum, which was stored at −20 °C until assayed.

3.6. Indirect ELISA

In order to elucidate the specificity of the anti-CYN antibody, an indirect ELISA was carried out. First of all, since CYN is not a good immunogen, it was coupled to keyhole limpet hemocyanin (KLH) from *Megathura crenulata*, following the procedure described by Elliot *et al*. [45]. After that, the same competitive indirect ELISA protocol followed by these authors was performed. Different dilutions of the positive serum (containing anti-CYN antibody) and the negative serum (without anti-CYN antibody) were screened in order to study the sensitivity of the anti-CYN antibody. The dilutions assayed were 0, 1/10, 1/20, 1/50, 1/100, 1/200, 1/500, and 1/1000. The study demonstrated the specificity of the anti-CYN antibody of the serum for the CYN, as well as a good sensitivity at the dilution 1/100 (data not shown).

3.7. Immunohistochemical Analyses for the Detection of CYN in Tissues

For the immunohistochemical examinations, tissue samples (0.5–1 cm thick) were taken from the organs of control and exposed fish. These samples were fixed in 10% neutral buffered formalin for 24 h at 4 °C, and then they were immediately dehydrated in a graded series of ethanol, immersed in xylol, and embedded in paraffin wax using an automatic processor. The immunohistochemical studies were always performed by the same person using the avidin-biotin-peroxidase complex (ABC) method, where the samples of each organ were analyzed the same day and with the same reagents. Briefly, tissue sections (3 µm) were dewaxed and rehydrated through a graded ethanol descendent series. Although endogenous peroxidase activity is almost completely destroyed during formalin fixation, this was exhausted by incubation of the samples with H_2O_2 3% in methanol for 45 min at

room temperature (RT) to abolish pseudoperoxidase activity of red blood cells and peroxidase activity in myeloid cells, avoiding as well the presence of background in order to facilitate the interpretation of the immunologic reaction [37,46]. To achieve satisfactory results, tissue sections were subjected to different unmasking methods for retrieving the antigen masked by the formalin fixation and increasing the permeability of tissues to the anti-toxin serum, being the most effective the 0.01 M citrate buffer (pH = 6) for 30 min at 37 °C in oven (Table 5). Then, samples were submitted to three rinses of 10 min each one in PBS and covered with 30% normal goat serum (ICN Biomedicals, Aurora, OH, USA) in PBS for 30 min at RT to avoid background. Tissue detection of CYN was carried out by incubation of the samples with different rabbit anti-CYN serum dilutions in PBS at 4 °C overnight, obtaining the best results with the 1:100 serum dilution (Table 5).

Table 5. Immunoreactivity of the serum containing anti-CYN in tilapia tissues. The different pre-treatments used were: (**a**) sections not subjected to antigen retrieval; (**b**) Tween 20® (Merck, München, Germany) 0.1% in 0.01M PBS, pH 7.2, for 10 min at RT; (**c**) protease type XIV (Sigma-Aldrich Chemie, Steinheim, Germany) 0.1%, pH 7.2, for seven min at RT; incubation with 0.1 M citrate buffer (CB) in oven for 30 min at 37 °C; (**d**) microwave heating in 0.01 M CB, pH 3.2, 6 or 9 (6 or 15 min from the beginning of boiling). † (-) no positivity; (+) light positivity and light background; (++) positive reaction and light background; (+++) positive reaction without background; (Bs) positive reaction but intense background of the staining.

Antigen retrieval method		**Dilutions of anti-CYN rabbit serum** †			
		1:10	**1:50**	**1:100**	**1:200**
None		-	-	-	-
Tween 20®		-	-	-	-
Protease		-	-	-	-
CB pH 3.2	oven	+	+	+	+
	microwave (6 min)	+	+	+	-
	microwave (15 min)	-	-	-	-
CB pH 6	oven	Bs	Bs	+++	+
	microwave (6 min)	Bs	++	++	+
	microwave (15 min)	+	+	+	-
CB pH 9	oven	-	-	-	-
	microwave (6 min)	-	-	-	-
	microwave (15 min)	-	-	-	-

After primary incubation, slides were washed in PBS (three times for 10 min each) and incubated with the biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in PBS for 30 min at RT. After three further washes in PBS, samples were incubated with the avidin-biotin-peroxidase complex (Vectastain® ABC Kit Elites, Vector Laboratories, Peterborough, United Kingdom) for 1 h at RT in the dark. All tissue sections were finally rinsed in 0.05 M Tris buffered saline (TBS), pH 7.6, and labeling was visualized by application of a chromogen solution (NovaRED® Substrate Kit, Vector Laboratories, Peterborough, United Kingdom). Slides were counterstained with Mayer's haematoxylin.

As a negative control of the primary antibody, this was replaced in positive tissue sections by rabbit non-immune serum (DakoCytomation, Glostrup, Denmark) or by PBS followed by incubation with secondary antibodies and detection reagents. Moreover, tissue negative controls were included using tissue samples from different organs of fish not submitted to CYN exposure. Positive control tissues were from both control fish used on each experiment.

The identification of target cells for the toxin was based on morphological features, location, and size of the cells. Assessment of the immunolabeled cells was performed in 25 fields of 0.2 mm^2 randomly chosen, being the results expressed as number of immunolabeled cells per area of 0.2 mm^2, as well as: - (absent), + or scarce (0–10), ++ or moderate (10–50), +++ or intense (>50). These quantifications were performed by two experienced observers but with no previous knowledge of which group was being analyzed.

4. Conclusions

The present immunohistochemical study demonstrates a similar pattern of CYN distribution in both experiments, presenting most immunopositive results in the liver, followed by the kidney, intestines, and gills. Moreover, it suggests that fish organs subjected to a sub-chronic exposure of CYN, presented a stronger staining in comparison to the acute exposure. In general, immunolabeling intensified with increasing time in the acute assay, confirming the delayed toxicity of CYN, and also with the increment of the dose, as it is shown in the sub-chronic assay, where the staining was more pronounced with the highest concentration of CYN assayed (100 µg/L) and the longest time of exposure (14 days). Noteworthy are the differences in the immunolabeling in the intestine and gills between both assays, with more CYN-immunopositive cells appearing in fish from the sub-chronic assay, which is explained by the exposure route, as the fish are constantly immersed in the water containing the toxin. Finally, we also conclude that immunohistochemistry may be a useful tool for detecting and monitoring CYN in tissues, helping to establish the target organs for this toxin.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Moreira, C.; Azevedo, J.; Antunes, A.; Vasconcelos, V. Cylindrospermopsin: Occurrence, methods of detection and toxicology. *J. Appl. Microbiol.* **2012**, *114*, 605–620.
2. Shaw, G.; Sukenik, A.; Livne, A.; Chiswell, R.K.; Smith, M.J.; Seawright, A.A.; Norris, R.L.; Eaglesham, G.K.; Moore, M.R. Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti), in newly constructed lakes, Queensland, Australia. *Environ. Toxicol.* **1999**, *14*, 167–177.
3. Ohtani, I.; Moore, R.E.; Runnegar, M. Cylindrospermopsin: A potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *Am. Chem. Soc.* **1992**, *114*, 7941–7942.
4. Sivonen, K.; Jones, G. Cyanobacterial Toxins. In *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*; Chorus, I., Bartram, M.J., Eds.; E & FN Spon: London, UK, 1999; pp. 41–111.
5. Chiswell, R.K.; Shaw, G.R.; Eaglesham, G.; Smith, M.J.; Norris, R.L.; Seawright, A.A.; Moore, M.R. Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, temperature, and sunlight on decomposition. *Environ. Toxicol.* **1999**, *14*, 155–161.
6. Funari, E.; Testai, E. Human health risk assessment related to cyanotoxins exposure. *Crit. Rev. Toxicol.* **2008**, *38*, 97–125.
7. Terao, K.; Ohmori, S.; Igarashi, K.; Ohtani, I.; Watanabe, M.F.; Harada, K.I.; Ito, E.; Watanabe, M. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. *Toxicon* **1994**, *32*, 833–843.
8. Falconer, I.R.; Hardy, S.J.; Humpage, A.R.; Froscio, S.M.; Tozer, G.J.; Hawkins, P.R. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. *Environ. Toxicol.* **1999**, *14*, 143–150.
9. Seawright, A.A.; Nolan, C.C.; Shaw, G.R.; Chiswell, R.K.; Norris, R.L.; Moore, M.R.; Smith, M.K. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). *Environ. Toxicol.* **1999**, *14*, 135–142.
10. Runnegar, M.T.; Kong, S.M.; Zhong, Y.Z.; Lu, S.C. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid Cylindrospermopsin in cultured rat hepatocytes. *Biochem. Pharmacol.* **1995**, *49*, 219–225.
11. Humpage, A.R.; Fontaine, F.; Froscio, S.; Burcham, P.; Falconer, I.R. Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress. *J. Toxicol. Environ. Health A* **2005**, *68*, 739–753.
12. Puerto, M.; Jos, A.; Pichardo, S.; Gutiérrez-Praena, D.; Cameán, A.M. Acute effects of pure Cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. *Ecotoxicology* **2011**, *20*, 1852–1860.
13. Young, F.M.; Micklem, J.; Humpage, A.R. Effects of the blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells in vitro. *Reprod. Toxicol.* **2008**, *25*, 374–380.

14. Gutiérrez-Praena, D.; Jos., A.; Pichardo, S.; Moreno, I.M.; Cameán, A.M. Presence and bioaccumulation of Microcystin and Cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review. *Food Chem. Toxicol.* **2013**, *53*, 139–152.
15. White, S.H.; Duivenvoorden, L.J.; Fabbro, L.D.; Eaglesham, G.K. Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). *Toxicon* **2006**, *47*, 497–509.
16. Berry, J.P.; Lind, O. First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (*Pomacea patula catemacensis*). *Toxicon* **2010**, *55*, 930–938.
17. Anderson, L.; Fabbro, L.D.; Cowden, K. *Assessment of Blue-Green Algal Toxins in Barramundi, Red Clay and Mussels from Awoonga Dam*; Central Queensland University: Gladstone, Australia, 2003.
18. Saker, M.L.; Metcalf, J.S.; Codd, G.A.; Vasconcelos, V.M. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon* **2004**, *43*, 185–194.
19. Saker, M.L.; Eaglesham, G.K. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. *Toxicon* **1999**, *37*, 1065–1077.
20. White, S.H.; Duivenvoorden, L.J.; Fabbro, L.D.; Eaglesham, G.K. Mortality and toxin bioaccumulation in *Bufo marinus* following exposure to *Cylindrospermopsis raciborskii* cell extracts and live cultures. *Environ. Pollut.* **2007**, *147*, 158–167.
21. Messineo, V.; Melchiorre, S.; Di Corcia, A.; Gallo, P.; Bruno, M. Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizomenon Ovalisporum* blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. *Environ. Toxicol.* **2010**, *25*, 18–27.
22. Berry, J.P.; Jaja-Chimedza, A.; Dávalos-Lind, L.; Lind, O. Apparent bioaccumulation of cylindrospermopsin and paralytic shellfish toxins by finfish in Lake Catemaco (Veracruz, Mexico). *Food Addit. Contam.* **2012**, *2*, 314–321.
23. Kinnear, S. Cylindrospermopsin: A decade of progress on bioaccumulation research. *Mar. Drugs* **2010**, *8*, 542–564.
24. Norris, R.L.G.; Eaglesham, G.K.; Pierens, G.; Shaw, G.R.; Smith, M.J.; Chiswell, R.K.; Seawright, A.A.; Moore, M.R. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environ. Toxicol.* **1999**, *14*, 163–165.
25. Seifert, M.; Mcgregor, G.; Eaglesham, G.; Wickramasinghe, W.; Shaw, G. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. *Harmful Algae* **2007**, *6*, 73–80.
26. Hawkins, P.R.; Runnegar, M.T.C.; Jackson, A.R.B.; Falconer, I.R. Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated form a domestic supply reservoir. *Appl. Environ. Microbiol.* **1985**, *50*, 1292–1295.

27. Berry, J.P.; Gibbs, P.D.L.; Schmale, M.C.; Saker, M.L. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. *Toxicon* **2009**, *53*, 289–299.
28. Puerto, M.; Jos, A.; Pichardo, S.; Moyano, R.; Blanco, A.; Cameán, A.M. Acute exposure to pure Cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). *Environ. Toxicol.* **2012**, doi:10.1002/tox.21764.
29. Gutiérrez-Praena, D.; Jos, A.; Pichardo, S.; Cameán, A.M. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure Cylindrospermopsin under laboratory conditions: Influence of exposure route and time of sacrifice. *Aquat. Toxicol.* **2011**, *105*, 100–106.
30. Gutiérrez-Praena, D.; Jos, A.; Pichardo, S.; Moyano, R.; Blanco, A.; Monterde, J.G.; Cameán, A.M. Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure Cylindrospermopsin by oral and intraperitoneal route. *Ecotoxicol. Environ. Saf.* **2012**, *76*, 102–113.
31. Gutiérrez-Praena, D.; Puerto, M.; Prieto, A.I.; Jos, A.; Pichardo, S.; Vasconcelos, V.; Cameán, A.M. Protective role of dietary *N*-Acetylcysteine on the oxidative stress induced by Cylindrospermopsin in tilapia (*Oreochromis niloticus*). *Environ. Toxicol. Chem.* **2012**, *31*, 1548–1555.
32. Yoshida, T.; Makita, Y.; Tsutsumi, T.; Nagata, S.; Tashiro, F.; Yoshida, F.; Sekijima, M.; Tamura, S.I.; Harada, T.; Maita, K.; *et al*. Immunohistochemical localization of Microcystin-LR in the liver of mice: A study on the pathogenesis of Microcystin-LR-induced hepatotoxicity. *Toxicol. Pathol.* **1998**, *26*, 411–418.
33. Guzmán, R.E.; Solter, P.F. Characterization of sublethal microcystin-LR exposure in mice. *Vet. Pathol.* **2002**, *39*, 17–26.
34. Fischer, W.J.; Hitzfeld, B.C.; Tencalla, F.; Eriksson, J.E.; Mikhailov, A.; Dietrich, D.R. Microcystin-LR toxicodynamics, induced pathology, and immunohistochemical localization in livers of blue-green algae exposed Rainbow trout (*Oncorhynchus mykiss*). *Toxicol. Sci.* **2000**, *54*, 365–373.
35. Lance, E.; Josso, C.; Dietrich, D.; Ernst, B.; Paty, C.; Senger, F.; Bormans, M.; Gérard, C. Histopathology and Microcystin distribution in *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacterial or dissolved microcystin-LR exposure. *Aquat. Toxicol.* **2010**, *98*, 211–220.
36. Niedobitek, G.; Kremmer, E.; Herbst, H.; Whitehead, L.; Dawson, C.W.; Niedobitek, E.; von Ostau, C.; Rooney, N.; Grässer, F.A.; Young, L.S. Immunohistochemical detection of the Epstein-Barr Virus-encoded latent membrane protein 2A in Hodkin's disease and infectious mononucleosis. *Blood* **1997**, *90*, 1664–1672.
37. Risalde, M.A.; Molina, V.; Sánchez-Cordón, P.J.; Pedrera, M.; Romero-Palomo, F.; Bautista, M.J.; Moreno, A.; Gómez-Villamandos, J.C. Comparison of pathologic changes and viral antigen distribution in tissues of calves with and without pre-existing bovine viral diarrhea virus infection following challenge with bovine Herpesvirus-1. *Am. J. Vet. Res.* **2013**, *74*, 598–610.
38. Oosterwijk, E.; Ruiter, M.D.; Wakka, J.C.; Huiskens-van der Meij, J.W.; Jonas, U.; Fleuren, G.J.; Zwartendijk, J.; Hoedemaeker, P.; Warnaar, S.O. Immunohistochemical analysis of monoclonal antibodies to renal antigens. Application in the diagnosis of renal cell carcinoma. *J. Am. Pathol.* **1986**, *123*, 301–309.

39. Guzmán-Guillén, R.; Prieto, A.I.; Moreno, I.; Vasconcelos, V.M.; Moyano, R.; Blanco, A.; Cameán, A.M. Cyanobacterium producing Cylindrospermopsin cause histopathological changes at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). *Environ. Toxicol.* **2013**, doi:10.1002/tox.21904.
40. Ito, E.; Kondo, F.; Harada, K.H. First report of the distribution of orally administered Microcystin-LR in mouse tissue using an immunostaining method. *Toxicon* **2000**, *38*, 37–48.
41. Humpage, A.R.; Falconer, I.R. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline value. *Environ. Toxicol.* **2003**, *18*, 94–103.
42. Norris, R.L.G.; Seawright, A.A.; Shaw, G.R.; Smith, M.J.; Chiswell, R.K.; Moore, M.R. Distribution of 14C Cylindrospermopsin *in vivo* in the Mouse. *Environ. Toxicol.* **2001**, *16*, 498–505.
43. Guzmán-Guillén, R.; Prieto, A.I.; Moreno, I.M.; González, G.; Soria-Díaz, M.E.; Vasconcelos, V.; Cameán, A.M. Development and optimization of a method for the determination of Cylindrospermopsin from strains of *Aphanizomenon* cultures: Intra-laboratory assessment of its accuracy by using validation standards. *Talanta* **2012**, *100*, 356–363.
44. Hancock, D.C.; O'Reilly, N.J. Production of Polyclonal Antibodies in Rabbits. *Methods Mol. Biol.* **2005**, *295*, 27–40.
45. Elliot, C.T.; Redshaw, C.H.; George, S.E.; Campbell, K. First development and characterization of polyclonal and monoclonal antibodies to the emerging fresh water toxin Cylindrospermopsin. *Harmful Algae* **2013**, *24*, 10–19.
46. Straus, W. Cleavage of heme from horseradish peroxidase by methanol with inhibition of enzyme activity. *J. Histochem. Cytochem.* **1974**, *22*, 908–911.

V. RESULTADOS Y DISCUSIÓN GENERAL/ GENERAL RESULTS AND DISCUSSION

Tal y como se ha expuesto previamente en la Introducción, las cianotoxinas se han convertido en un importante problema a nivel mundial en materia de contaminación ambiental, toxicológica, sanitaria y económica, ya que pueden afectar tanto a animales y plantas como a seres humanos. Es por esta razón por la que decidimos realizar una serie de estudios que nos permitieran conocer, por una parte, métodos prácticos para disminuir las MCs en el pescado contaminado destinado al consumo humano. En el caso de la CYN, en primer lugar, nos pareció de importante la puesta a punto y validación de métodos analíticos para cuantificar la toxina en diferentes matrices, y posteriormente decidimos profundizar en su mecanismo de toxicidad y sus efectos tóxicos, así como estudiar alternativas que nos permitieran contrarrestar los daños producidos por la CYN. Asimismo, también estudiamos su distribución en tejidos de peces contaminados. Todo ello en una de las especies acuáticas de consumo humano que pueden verse afectadas por esta toxina en su hábitat natural, como es el pez tilapia (*Oreochromis niloticus*). Por otra parte, también se ha abordado, mediante proteómica, el estudio del repertorio de Glutatión-S-transferasas (GSTs) en bivalvos, y los efectos de la CYN sobre plantas de zanahorias (*Daucus carota*) expuestas a la toxina mediante el agua de riego.

1. ESTUDIOS SOBRE MICROCISTINAS

La bioacumulación de las MCs a través de la cadena alimentaria ha quedado demostrada previamente por otros autores (Ibelings y Chorus, 2007; Chen y col., 2009b). Se ha sugerido que las técnicas domésticas de cocinado no son capaces de eliminarlas o producir alteraciones en la concentración de estas toxinas en alimentos, en base a la estabilidad de las MCs a elevadas temperaturas y diferentes condiciones de pH (Ibelings y Chorus, 2007).

A pesar de que el estudio de la influencia de estas técnicas con dicho fin se ha llevado a cabo con otros contaminantes como metales (Devesa y col., 2008; Perelló y col., 2008), bifenilos policlorados (PCBs) (Hori y col., 2005), difeniléteres polibromados (PBDEs), hexaclorobenceno (HCB) e hidrocarburos aromáticos policíclicos (HAPs) (Perelló y col., 2009), radionúclidos (de Burger y col., 2004), y algunos pesticidas; sin embargo, en el caso de las MCs, los estudios son muy escasos (Morais y col., 2008; Zhang y col., 2010; Freitas y col., 2014).

Es por ello que nos propusimos investigar los posibles cambios en la concentración de MCs libres en músculo de tilapias (*Oreochromis niloticus*) (porción comestible) a los que se les añadió una solución mezcla de MCs puras (MC-LR, MC-RR y MC-YR), conteniendo

1,5 µg/mL de cada toxina, y sometidos posteriormente a dos técnicas de cocinado corrientes, como son el horno microondas y el hervido.

En lo concerniente al microondas, las concentraciones de MC-LR y MC-YR se mostraron disminuidas sólo tras 5 minutos de cocinado; no obstante, la MC-RR permaneció inalterada tras ambos períodos ensayados (1 y 5 minutos). Previamente, Morais y col. (2008) habían observado que el cocinado por microondas disminuía el contenido de MCs en mejillones (*Mytilus galloprovincialis*) expuestos, incluso tras 1 min de cocinado, aunque no hacen referencia a los congéneres individuales de MCs. Por el contrario, los experimentos de Metcalf y Codd (2000) revelaron que al exponer al microondas durante 9 min una solución de MC-LR purificada a partir de células liofilizadas de un cultivo de cianobacterias, no se alteraba la estructura de la toxina. Las diferencias con nuestros resultados podrían explicarse por la diferente complejidad de las matrices, tejidos de peces en nuestro caso y agua o células liofilizadas en el suyo.

También observamos que ambas técnicas de hervido ensayadas consiguieron una disminución significativa (40-50%) en la concentración de MCs en el músculo del pescado, siendo mayor con el hervido continuo en el caso de la MC-RR, que podría explicarse por un mayor tiempo de contacto del alimento con el agua de cocción, junto con la mayor hidrosolubilidad de esta MC en comparación con otros congéneres. En contraste con estos resultados, otros estudios no observaron alteración en los niveles de MCs en mejillones hervidos durante 5 y 30 min (Morais y col., 2008), o incluso demostraron cómo el cocinado provocaba un aumento en la concentración de la toxina en peces hervidos (Zhang y col., 2010) y en mejillones cocinados al microondas y hervidos (Freitas y col., 2014), sugiriendo así que el hervido podría acelerar la liberación de MCs de las fosfatasas de proteínas PP1 y PP2A, aumentando su bioaccesibilidad. Esto último indica que al tener sólo en cuenta los valores detectados en alimentos crudos, estaríamos subestimando la exposición real a estas toxinas a través del consumo de alimentos contaminados que serán cocinados previos a su consumo. Sin embargo, en nuestro estudio podría haber una sobreestimación de la exposición a las mismas. Asimismo, tras detectar MCs en el agua de hervido, mostramos que existe un riesgo adicional inherente a la utilización de esta agua para hacer sopas de pescado, de acuerdo con Zhang y col. (2010).

Es importante hacer hincapié que en todos estos estudios, la concentración de MCs detectada en músculo se refiere a la porción libre, ya que el método aplicado para analizar las

muestras no detecta conjugados de MCs unidas a fosfatasas de proteínas o a GSH. Además, en concordancia con otros autores (Zhang y col., 2010), nuestros resultados muestran la transferencia de las MCs de los alimentos al agua de cocinado.

2. ESTUDIOS SOBRE LA CILINDROSPERMOPSINA

2.1. Estudios realizados en tilapia (*Oreochromis niloticus*)

Por todo lo expuesto a lo largo de esta Tesis Doctoral, la monitorización de la CYN en medios contaminados por cianobacterias es de gran importancia para evaluar los riesgos que supone su exposición para la salud y el medioambiente. Las técnicas analíticas empleadas para detectar y cuantificar esta toxina, tanto en aguas como en otras matrices, son de diverso fundamento y complejidad (ELISA, LC-DAD, LC-MS y LC-MS/MS), sin embargo, no todos estos métodos ofrecen la misma sensibilidad. La LC-MS/MS ha sido elegida como el método ideal y de referencia para detectar y cuantificar pequeñas cantidades de toxina en muestras de agua (Eaglesham y col., 1999; Bogialli y col., 2006; Oehrle y col., 2010) y en muestras más complejas, como tejidos de peces (Gallo y col., 2009) o muestras de suero y orina humanas (Foss y Aubel, 2013). Por ello, nos propusimos poner a punto y validar diferentes métodos cuantitativos para la determinación de CYN en distintas matrices (aguas, cultivos de cianobacterias y tejidos de peces contaminados con dicha toxina) por LC-MS/MS.

En nuestro estudio, con el empleo de cartuchos de carbón grafitizado se obtuvieron resultados fiables en la extracción de CYN a partir de aguas y de cultivos de cianobacterias, usando una mezcla acidificada de diclorometano:metanol como disolvente, de acuerdo con Wormer y col. (2009), con la diferencia de que estos autores llevaban a cabo la cuantificación por LC/DAD.

En el caso de los tejidos de peces, y basándonos en el método propuesto por Gallo y col. (2009) para extracción y determinación de CYN en aguas y tejidos contaminados de peces, conseguimos eliminar la etapa de la extracción líquido-líquido con hexano, obteniéndose extractos limpios que permitieron la recuperación de la toxina en un alto porcentaje (80-110%). Por otra parte, para la limpieza y purificación del extracto, nos basamos en la metodología seguida por Liu y Scott (2011) para detección de CYN en suplementos alimenticios a base de algas, y lo pusimos a punto en matrices más complejas como son el hígado y el músculo de peces, mediante un sistema SPE de doble cartucho (uno

de C18 y uno de carbón grafitizado), lo que nos proporcionó una limpieza óptima de las muestras.

De forma general los enfoques clásicos para la validación de métodos analíticos sólo se basaban en la comparación entre los valores medidos y los de referencia, sin tener en cuenta la importancia de la precisión y reproducibilidad del método intra- e interlaboratorio, incluyendo estudios de robustez, todo ello imprescindible para la "transferencia del método" (González y col., 2010). En los estudios de extracción y detección de CYN en diferentes matrices, incluyendo algunas más complejas como organismos acuáticos (Gallo y col., 2009; Saker y col., 2004; Berry y Lind, 2010; Messineo y col., 2010), no se han llevado a cabo estudios de validación y robustez. Por ello, los métodos que proponemos han sido adecuadamente validados: con amplios rangos de concentraciones de CYN, límites de detección y cuantificación aceptables (que en el caso del agua, permiten la determinación de la toxina por debajo del valor guía propuesto de 1 μg/L), recuperaciones en el intervalo 80-110%, y valores adecuados de precisión según la guía AOAC (Association of Official Analytical Chemists). Asimismo, la combinación de factores variables en la metodología demostró la robustez de los métodos propuestos para poder ser reproducidos bajo diferentes condiciones sin sufrir variaciones significativas en los resultados. Los métodos desarrollados en esta Tesis Doctoral pudieron ser aplicados con éxito y rapidez posteriormente para el análisis de CYN en las diferentes matrices estudiadas (aguas y matrices biológicas), permitiendo un control de la exposición, degradación, transferencia y bioacumulación de la CYN.

Debido a que la CYN es una toxina emergente, su mecanismo de acción tóxica en las distintas especies acuáticas es menos conocido que el de las MCs. Las cianotoxinas pueden acumularse en peces a través de diferentes vías: por ingesta directa del fitoplancton, por captación de la cianotoxina disuelta a través del epitelio (branquias, piel), o a través de la cadena alimentaria. Generalmente se cree que la vía oral es la más importante (Ernst y col., 2001) y la acumulación de cianotoxinas suele depender del tiempo de exposición (Vasconcelos, 1995; Sipiä y col., 2002; Ozawa y col., 2003). Nos propusimos profundizar en el conocimiento del papel que juega el estrés oxidativo en el mecanismo de toxicidad de la CYN, investigando el efecto de diversas variables experimentales como son las dosis administradas (10 y 100 μg CYN/L) y el tiempo de exposición (7 y 14 días) sobre la alteración de biomarcadores de estrés oxidativo en hígado y riñón de peces de consumo humano (*O. niloticus*) expuestos a CYN por inmersión en aguas con un liofilizado de células

de *A. ovalisporum* con 10 y 100 µg CYN/L, y 0,46 y 4,6 µg 7-desoxi-CYN/L, respectivamente. Estas dosis empleadas son semejantes a las encontradas en la naturaleza, ya que fueron escogidas en base a estudios de campo realizados que demuestran la detección de CYN en diferentes partes del planeta en un amplio rango de concentraciones (0,03-202 µg/L). Concentraciones de CYN superiores a 100 µg/L fueron evaluadas de alto riesgo para las comunidades de peces (Seifert y col., 2007), aunque la toxina se puede bioacumular a niveles mucho más bajos de exposición (Kinnear, 2010), lo que concuerda con nuestros resultados al aparecer efectos tóxicos ya a concentraciones de 10 µg CYN/L. Además, al trabajar con células liofilizadas productoras de CYN, se intentó reproducir el escenario de una exposición real de los peces a la toxina, como podría ocurrir en un ecosistema acuático contaminado con una floración de cianobacterias tóxicas.

Nuestros resultados muestran un aumento de la LPO, oxidación de proteínas y del ADN en ambos órganos de peces expuestos a CYN, observándose en general los cambios más significativos en caso de la concentración más alta y durante el mayor período, lo que muestra una toxicidad dependiente de la concentración y del tiempo de exposición. De forma similar, otros autores encontraron generalmente aumentos en estos parámetros de estrés oxidativo en tilapias expuestas a CYN (Puerto y col., 2011; Gutiérrez-Praena y col., 2011b; Ríos y col., 2013). Por el contrario, Humpage y col. (2005) no encontraron cambios en los niveles de malondialdehído (MDA) en hepatocitos de ratas expuestas a CYN purificada. Además, Gutiérrez-Praena y col. (2011b) no observaron alteración en la oxidación del ADN en peces expuestos de forma aguda a 200 µg CYN pura/kg. Esta discrepancia puede deberse a las diferentes dosis, tiempos y formas de exposición ensayados, así como al modelo experimental. Asimismo, estos autores investigaron con CYN pura mientras que nuestro ensayo fue realizado con células de cianobacterias que incluyen, además de CYN, otros posibles compuestos bioactivos (Hawkins y col., 1997; Falconer y col., 1999; Norris y col., 1999) que podrían propiciar una mayor toxicidad. En este sentido, un estudio realizado en varios invertebrados acuáticos demostró que los efectos adversos más importantes rara vez se registraban tras la exposición de CYN pura a concentraciones menores a 100 µg/L, mientras que los efectos eran mayores tras la exposición a extractos de *C. raciborskii* (Seifert, 2007).

El aumento significativo de la oxidación del ADN que observamos en nuestro caso, muestra por primera vez la implicación del estrés oxidativo como un posible mecanismo genotóxico de la CYN y su análogo 7-desoxi-CYN contenidas en células de cianobacterias liofilizadas. Falconer y Humpage (2001) sugirieron que el grupo uracilo de la CYN podría

interactuar con la adenina en el ARN y el ADN, interferir con la síntesis de ADN, y por lo tanto inducir mutaciones y actuar como carcinógeno. Otros autores mostraron que la CYN es genotóxica y probablemente más peligrosa para la salud humana y animal que las MCs (Zegura y col., 2011).

Además, el riñón se mostró como el principal órgano afectado por la exposición a CYN: la LPO aumentó tras 7 días de exposición y la oxidación del ADN se produjo con la concentración más pequeña ensayada tras ambos períodos de exposición. Nuestro estudio confirma que, aunque CYN se consideraba en un principio una hepatotoxina (Norris y col., 2002; Berry y col., 2009), también puede afectar a otros órganos como el riñón, siendo este el órgano señalado como más sensible a esta toxina en ratones (Humpage y Falconer, 2003). El riñón es asimismo el órgano más afectado en relación a la oxidación de proteínas en peces expuestos de forma aguda a CYN pura (Puerto y col., 2011; Gutiérrez-Praena y col., 2011b). En tilapias expuestas a CYN por inmersión en biomasa de *A. ovalisporum*, el hígado resultó más dañado durante una exposición de 7 días, mientras que con el aumento del tiempo de exposición (21 días), el riñón se vio más afectado (Ríos y col., 2013).

Nuestros resultados muestran un aumento en la actividad GST en hígado de los peces a partir de 7 días de exposición, que se mantiene a los 14 días, coincidiendo con los aumentos en la LPO, pudiendo deberse a una necesidad de contrarrestar estos peróxidos lipídicos, en concordancia con otros estudios realizados en tilapias expuestas a CYN durante 24 h (Puerto y col., 2011). Sin embargo, en riñón observamos una inhibición de la actividad GST tras 14 días de exposición a CYN. De forma similar, Ríos y col. (2013) demostraron, en este mismo órgano, que la actividad GST también disminuía en tilapias expuestas a CYN por inmersión en biomasa de *A. ovalisporum* durante 14 y 21 días. Todos estos resultados sugirieren que se requieren mayores períodos de exposición para inducir esta inhibición enzimática.

La función de GPx es reducir los hidroperóxidos lipídicos a sus correspondientes alcoholes, y el peróxido de hidrógeno a agua, por lo que el aumento que observamos en nuestro estudio en la actividad GPx en el riñón de tilapias expuestas a la dosis más alta tras ambos períodos de exposición (7 y 14 días), confirmaría la mayor sensibilidad de este órgano.

Los cambios más importantes se observaron en general en las actividades de SOD y CAT en hígado y riñón, constituyendo el sistema SOD-CAT la primera defensa antioxidante (Atencio et al., 2008). De forma general, tanto CAT como GPx actúan intentando eliminar el exceso de H_2O_2, aunque en nuestro estudio, la GPx no se afecta en hígado de los peces

intoxicados y se observó una respuesta dual de la actividad CAT en dicho órgano, pareciendo estar más activa tras 7 días de exposición al tratar de controlar las ERO, mientras que experimentó un drástico descenso tras 14 días de exposición. Este comportamiento también fue demostrado en hígado de tilapias expuestas a CYN (Ríos y col., 2013), y en hígado de *Corydoras paleatus* expuesta a MC-RR, aumentando la actividad CAT a bajas concentraciones y disminuyendo a las más altas (Cazenave y col., 2006).

En general, los cambios más significativos observados en estas actividades enzimáticas (GST, GPx, SOD, CAT y γ-GCS) y en el cociente GSH/GSSG en el riñón, pueden explicarse como una respuesta adaptativa para compensar los mayores niveles de ERO producidos por CYN y sus efectos (aumentos de LPO, oxidación de proteínas y del ADN) en este órgano en comparación con el hígado. De esta manera, las actividades GST, SOD y CAT se vieron disminuidas posiblemente por una destrucción enzimática, y la GPx sólo se mostró afectada en riñón, incluso tras el período de exposición más corto. En un principio, las investigaciones llevadas a cabo por Norris y col. (2002) en ratón consideraban al CYP450 fundamental en la patogenicidad de CYN, y sugerían que era poco probable que el agotamiento de GSH (por conjugación con CYN o por inhibición de su síntesis) constituyera el principal mecanismo de toxicidad hepática. Por el contrario, nuestros resultados muestran un aumento en la actividad γ-GCS (enzima limitante en la síntesis de GSH), junto con el agotamiento de GSH/GSSG en ambos órganos de tilapias expuestas a CYN, apuntando que el GSH no se encuentra afectado a nivel de síntesis. En lugar de esto, su disminución podría explicarse debido al papel que juega como antioxidante no enzimático, mediante un aumento de su consumo, mayor formación de GSSG, aumento de un eflujo de GSH, etc. Las alteraciones mostradas en estos dos parámetros, principalmente en el riñón, concuerdan con otros experimentos realizados con la misma especie de peces expuestos a CYN pura por sonda nasogástrica (Puerto y col., 2011). Por el contrario, Gutiérrez-Praena y col. (2011b) observaron una reducción significativa en la actividad de la enzima γ-GCS en tilapias expuestas de forma aguda a CYN por vía oral o inyección i.p., y sacrificadas a las 24 horas, aunque no hubo cambios significativos en los peces sacrificados a los 5 días. Esto indicaría que una única dosis más alta de CYN pura administrada puede dañar las enzimas, mientras que dosis más bajas de CYN contenida en células de cianobacterias (10 ó 100 μg/L), y administradas por un período de tiempo más largo, pueden inducir una respuesta defensiva, tal y como muestran nuestros resultados.

Por lo general para la mayoría de los parámetros estudiados, la mayor susceptibilidad del riñón a células de cianobacterias que contienen CYN y 7-desoxi-CYN, en comparación con el hígado, podría estar relacionada con la alta hidrofilia de ambas moléculas. Además, un estudio de la distribución corporal de la CYN marcada con C14 en ratones, demostró que la principal vía de excreción es a través de los riñones, apareciendo en orina a las 6 h casi el 50% de la dosis administrada vía i.p., cuando un 20% de la dosis estaba presente en el hígado (Norris y col., 2001).

Demostrada la alteración de parámetros bioquímicos de estrés oxidativo, nos propusimos estudiar las alteraciones histopatológicas producidas en tilapias bajo las mismas condiciones experimentales. Nuestro equipo de investigación había demostrado anteriormente que la exposición aguda a CYN pura (200-400 μg/kg p.c.) indujo efectos histopatológicos en tilapias expuestas a la toxina por sonda nasogástrica e inyección i.p. (Gutiérrez-Praena y col., 2012c; Puerto et al., 2014). Sin embargo, estos resultados no pueden ser extrapolados a una exposición subcrónica como es nuestro caso. En general, las alteraciones observadas en nuestro estudio son similares pero más intensas en esta exposición subcrónica por inmersión a células liofilizadas productoras de CYN, que en tilapias expuestas por vía oral a CYN pura y sacrificadas a las 24 h y a los 5 días (Gutiérrez-Praena y col., 2012c).

Los principales cambios encontrados en hígado (procesos degenerativos y esteatosis), en riñón (glomerulonefritis membranosa), en corazón (miofibrolisis y edema), en el tracto G.I. (enteritis necrótica) y en las branquias (procesos hiperémicos en las lamelas y microhemorragias), demuestran que los efectos histopatológicos tóxicos son proporcionales a la concentración y al tiempo de exposición. Este hecho también se comprobó en los estudios morfométricos, con un incremento del diámetro nuclear de los hepatocitos (por tumefacción de las células tubulares), de acuerdo con Gutiérrez-Praena y col. (2012c), y una disminución del diámetro de los túbulos renales tras 7 días, debido a la atrofia de los mismos, que aumentaban tras 14 días. Gutiérrez-Praena y col. (2012c) también observaron una disminución del diámetro de los túbulos contorneados proximal y distal tras 24 horas de exposición a CYN.

Una vez demostrado el papel del estrés oxidativo como mecanismo de acción tóxica de la CYN, así como las alteraciones histopatológicas producidas en varios órganos de tilapia, nos preguntamos si esta toxina también sería capaz de inducir alteraciones neurológicas en esta especie, basándonos en algunos estudios al respecto realizados en mamíferos y animales

acuáticos expuestos a CYN (Kiss y col., 2002; Schoeb y col., 2002; Zagatto y col., 2012). Nuestros resultados muestran por primera vez los efectos neurotóxicos de la CYN en peces, con disminución en la actividad de la acetilcolinesterasa (AChE), aumento de los niveles de LPO y alteraciones histopatológicas en el cerebro de tilapias expuestas a dosis repetidas de CYN por inmersión en un cultivo de *A. ovalisporum* durante 14 días. Además, la toxina fue detectada en el 100% de los cerebros de peces expuestos a la toxina.

Otros autores han observado las mismas alteraciones en los niveles de AChE y de LPO en varias especies de peces expuestos a diferentes contaminantes (Hernández-Moreno y col., 2010; Pereira y col., 2013; Pretto y col., 2010; Kavitha y Venkateswara Rao, 2009). La acumulación de CYN en músculo e hígado de peces de consumo humano también había sido probada previamente (Messineo y col., 2010; Berry y col., 2012); sin embargo, no se había confirmado la presencia de CYN y sus efectos en cerebros de especies acuáticas hasta ahora.

Tras comprobar los efectos producidos por la CYN en nuestro modelo experimental bajo las condiciones de ensayo anteriormente discutidas, nos preguntamos sobre la efectividad del uso de algunos antioxidantes como suplementos en la dieta (LC y vitamina E), para prevenir la aparición de estos efectos, así como la utilidad de la depuración como medio para revertirlos.

En primer lugar, se llevó a cabo un pretratamiento de 21 días con LC, utilizándose dos dosis diferentes (400 y 880 mg LC/kg p.c./día, equivalentes a 20 y 40 mg LC/pez/día, respectivamente) administradas junto con la dieta, y se valoró su capacidad de prevenir los efectos tóxicos inducidos por la exposición aguda a 400 μg CYN/kg p.c. (tanto a la toxina pura como a la proveniente de un cultivo de células liofilizadas de *A. ovalisporum* que contiene CYN y 7-desoxi-CYN) administrada en la dieta. Se comprobó que la LC ejerce un efecto protector frente al daño oxidativo producido por CYN, tanto pura como liofilizada, a partir de la menor dosis utilizada (400 mg LC/kg p.c./día), disminuyendo los niveles de LPO, oxidación de proteínas y del ADN, y recuperando los valores control de las actividades enzimáticas NADPH oxidasa, SOD, CAT y γ-GCS, así como del cociente GSH/GSSG. En peces, la suplementación dietética desde unos pocos cientos a más de 4000 mg LC/kg de dieta durante largos períodos de tiempo (por ejemplo, 120 días) jugó un papel protector durante la exposición a xenobióticos (Harpaz, 2005). En tilapias concretamente, Jayaprakas y col. (1996) encontraron efectos beneficiosos sobre su crecimiento y reproducción con una suplementación de 900 mg LC/kg de dieta durante 252 días. Por otra parte, experimentos

previos han demostrado la actividad antioxidante de la LC (300 mg LC/kg p.c./día) en ratas hipertensas (Miguel-Carrasco y col., 2010). Resultados similares fueron obtenidos por nuestro grupo cuando se administró otro antioxidante, la N-acetilcisteína (NAC), a tilapias intoxicadas con MCs y con CYN (Puerto y col., 2009; Gutiérrez-Praena y col., 2012d, 2014a), con la ventaja de que ninguna de las dosis de LC presentó efectos pro-oxidantes *per sé*, a diferencia de la NAC (Puerto y col., 2009). Un aspecto a considerar en la administración de suplementos de LC en cultivos de peces es el precio, ya que de acuerdo con Harpaz (2005), la necesidad de emplear altos niveles de esta sustancia podría no ser muy rentable debido a su coste. El hecho de haber encontrado protección a la dosis más baja ensayada constituye otra ventaja para su uso, aunque podrían llevarse a cabo más estudios con el fin de identificar los niveles más bajos posibles de suplementación dietética con LC. La protección conferida frente a todas estas alteraciones podría deberse a la capacidad de la LC de secuestrar las ERO, así como de estabilización de las membranas celulares (Muthuswamy y col., 2006; Canbaz y col., 2007).

También demostramos la utilidad de la LC para revertir las alteraciones histopatológicas en hígado, riñón, corazón, tracto G.I. y branquias de peces intoxicados con CYN, presentando estos órganos una estructura aparentemente normal en los casos en que se administró el antioxidante como suplemento en la dieta. Además, la LC resultó efectiva a partir de la dosis menor ensayada, equivalente a 20 mg LC/pez/día. Nuestros resultados concuerdan con los obtenidos previamente por nuestro grupo de investigación en tilapias expuestas de forma aguda a CYN pura y pretratadas con NAC (Gutiérrez-Praena y col., 2014a), donde se observó además que la recuperación de los tejidos no era muy diferente entre ambas dosis de NAC empleadas, destacando en algunos casos (corazón) la mayor de las dosis (44 mg NAC/pez/día).

También nos propusimos conocer la prevención que conferían las mismas dosis de LC frente a las alteraciones a nivel molecular inducidas en tilapias expuestas de forma aguda por vía oral a 400 µg CYN/kg p.c. (CYN pura y células liofilizadas de *A. ovalisporum* conteniendo CYN y 7-desoxi-CYN). En primer lugar, observamos un incremento significativo de la actividad GPx y una disminución de su expresión génica, únicamente en hígado de los peces intoxicados. Por otro lado, otros autores no observaron cambios en la actividad GPx en el hígado y sí en su expresión génica, pero las concentraciones de CYN y tiempos de exposición fueron diferentes (Puerto y col., 2011; Gutiérrez-Praena y col., 2013b; Ríos y col., 2014). Sobre estas alteraciones moleculares de CYN, la dosis de LC que consigue restaurar la actividad enzimática a los niveles control fue a partir de 400 mg LC/pez/día, y de

880 mg LC/pez/día para la expresión génica de GPx, necesitándose la mayor de la dosis de pretratamiento para este último parámetro. En cuanto a la actividad GST, se observó un descenso en riñón de los peces expuestos, especialmente en el caso de la toxina pura. De forma similar, Ríos y col. (2014) obtuvieron una disminución de la actividad GST en riñón de tilapias. Sin embargo, mientras que estos autores observaron un aumento de la expresión génica de GST y una disminución de su abundancia proteica, nuestros resultados indican patrones diferentes, sin cambios respecto a su expresión génica ni en la abundancia proteica en el riñón. Resultados similares respecto a la abundancia relativa de GST fueron obtenidos por este mismo grupo aunque las concentraciones de CYN y el tiempo de exposición fueron diferentes (Puerto y col., 2011; Gutiérrez-Praena y col., 2013b). Nuevamente, se observa una recuperación de la actividad enzimática y la abundancia proteica a partir de la menor dosis de LC, mientras que esta misma dosis parece contribuir a la alteración génica de peces expuestos, mostrando un mejor perfil para su uso la dosis mayor de LC ensayada (880 mg LC/pez/día). Asimismo, se observó un aumento de la expresión génica de GST en hígado de los peces suplementados con la dosis menor de LC e intoxicados con CYN pura y liofilizada, de la misma forma que ocurría con experimentos previos realizados con NAC (Gutiérrez-Praena y col., 2014a). La falta de correlación entre actividad y expresión génica tanto de GPx como de GST, así como entre la abundancia relativa de GST y dichos parámetros podrían indicar que existe una regulación a nivel transcripcional y traduccional o modificaciones postraduccionales. Otro factor a tener en cuenta para entender estas diferencias son las distintas isoformas de GST existentes. Además, las respuestas relativas a GST en hígado y riñón pueden ser diferentes debido a que la transcripción de las isoformas de la enzima varía de distinta manera dentro de un órgano y entre órganos, como dedujo Li y col. (2008b) tras realizar un estudio en peces expuestos a MC-LR. A pesar de esta gran variabilidad, la LC se muestra como un protector seguro y eficaz de amplio espectro.

Por otro lado, hemos observado respuestas organoespecíficas, ya que las alteraciones moleculares se produjeron por lo general en el hígado, mientras que en el riñón, ni la actividad GPx, ni la expresión génica de GPx y GST experimentaron cambio alguno, sólo la disminución de la actividad GST. Este patrón de expresión diferencial organoespecífica ya había sido sugerida por otros autores (Gadagbui y James, 2000), y se repite con respecto a resultados previos de nuestro grupo de investigación con MCs (Puerto y col., 2011) y CYN (Gutiérrez-Praena y col., 2014a). Los resultados derivados del uso de LC indican un efecto protector sobre ambos órganos frente al daño oxidativo.

De forma similar, nos propusimos evaluar el potencial antioxidante de la vitamina E sobre las alteraciones inducidas por CYN en tilapias. Para ello, realizamos un pretratamiento de 7 días con 700 mg de vitamina E/kg p.c./día, seguida de la intoxicación aguda de los peces con 400 µg CYN pura/kg p.c. por vía oral, mezclando con la comida ambos componentes. Y se evaluaron los mismos biomarcadores y alteraciones histopatológicas inducidas por CYN que en el experimento anterior realizado con LC. En general, el pretratamiento con la dieta suplementada con vitamina E fue capaz de restaurar las alteraciones causadas en la mayoría de los parámetros estudiados. La protección ofrecida es similar a la observada y discutida anteriormente con la LC, y con la NAC en tilapias (Gutiérrez-Praena y col., 2012d). En este último caso, dosis de NAC similares a las nuestras de vitamina E protegían frente al daño oxidativo en tilapias intoxicadas con 200 µg CYN pura/kg p.c., mientras que la vitamina E fue capaz de ejercer este efecto frente al doble de la dosis de toxina administrada, demostrando mayor efectividad como antioxidante. Del mismo modo, la vitamina E también demostró tener un efecto protector sobre los valores de algunos de estos biomarcadores en el hígado, riñón y branquias de tilapias expuestas a MCs (Prieto y col., 2008, 2009). Al igual que ocurría con la LC, una ventaja del uso de la vitamina E es que, a la dosis ensayada de 700 mg de vitamina E/kg p.c./día, no mostró efectos pro-oxidantes *per sé,* al contrario de lo que sugerían otros autores al emplear dosis altas de la misma, pudiendo inducir aumentos en la LPO en tejidos de peces bajo condiciones de estrés oxidativo (Gao y col., 2014). Dandapat y col. (2000) demostraron la efectividad de la vitamina E para mejorar los niveles *per sé* de LPO, GSH y las mismas enzimas antioxidantes estudiadas por nosotros, en hepatopáncreas y branquias del langostino. Por lo tanto, con este trabajo demostramos que la suplementación con vitamina E ayuda a proteger frente el estrés oxidativo inducido por CYN reforzando el sistema de defensa antioxidante enzimático, explicándose por su capacidad captadora de los radicales peróxidos lipídicos, que suelen ser los responsables de propagar la peroxidación, impidiendo así que las ERO reaccionen con macromoléculas como lípidos, proteínas y ADN.

En los estudios histopatológicos realizados hemos observado cómo el pretratamiento con vitamina E es capaz de prevenir o moderar estos daños en los tejidos estudiados ya mencionados anteriormente, además de los cambios morfométricos en los túbulos hepáticos y renales. Hasta la fecha, la única sustancia que ha demostrado esta habilidad con respecto a la CYN en organismos acuáticos ha sido la NAC (Gutiérrez-Praena y col., 2014a). De forma similar, Prieto y col. (2009) observaron recuperación de daños histopatológicos causados por MCs en tilapias gracias a la suplementación con vitamina E. Su efecto protector también ha

sido probado en diversas especies intoxicadas con otros contaminantes (Anwar y col., 2013), pero no había sido estudiado para esta toxina. Además, y como novedad, presentamos el primer estudio sobre la prevención de alteraciones histopatológicas en cerebro de peces intoxicados con CYN.

Por tanto, de todos los antioxidantes ensayados (NAC, LC y vitamina E), y teniendo en cuenta las dosis de efectividad, costes y efectos secundarios, proponemos el siguiente orden de preferencia para el uso de sustancias preventivas frente a los efectos de la CYN: vitamina E > LC > NAC.

Otro medio para disminuir o contrarrestar los efectos tóxicos inducidos por contaminantes en peces consiste en transferirlos a un medio no contaminado, para su depuración. Por esto, nos interesó estudiar la posible reversión de los efectos tóxicos producidos por CYN tras dos períodos de depuración. Para ello, los peces fueron expuestos a dosis repetidas cada 2 días de 10 μg CYN/L mediante inmersión en cultivo de *A. ovalisporum* durante 7 y 14 días, tras los cuales se pasaron a acuarios libres de CYN para ser depurados durante 3 ó 7 días. Los valores de algunos biomarcadores como la LPO (en hígado), la oxidación del ADN y las actividades γ-GCS y SOD (en ambos órganos) y el contenido de GSH (en riñón) se restauraron con el período de depuración más corto (3 días), mientras que otros parámetros como la LPO (en riñón), la actividad CAT (en ambos órganos) y el contenido de GSH (en hígado) necesitaron hasta 7 días para volver a valores control. No obstante, la oxidación de proteínas necesitaría un período de depuración más largo para una recuperación completa en ambos órganos. Además, nuestros resultados mostraron una mayor susceptibilidad del riñón a las células de cianobacterias que contienen CYN y 7-desoxi-CYN en comparación con el hígado. De forma semejante, Freitas y col. (2012) observaron una reducción en los niveles de LPO en almejas sometidas a presiones antropogénicas, después de 2 y 7 días de depuración. El mismo efecto, así como una disminución de la actividad SOD, también fue detectado durante un estudio de depuración a largo plazo (1, 4 y 8 meses) en salmonetes expuestos de forma crónica a contaminantes (PCBs, DDT, HAP y metales) en el estuario del río Duero (Ferreira y col., 2007). Al igual que en nuestro estudio, estos autores sólo observaron recuperación en la oxidación de proteínas con los mayores tiempos de depuración, y esto podría explicarse debido a que la formación de grupos carbonilos es un proceso irreversible, por lo que cabría esperar un mayor tiempo para el reemplazo de las proteínas dañadas. Asimismo, los mejillones de una zona contaminada del Reino Unido exhibieron mayores daños oxidativos en el ADN en comparación con las de un área menos

contaminada, y su depuración durante 1 mes en agua no contaminada acabó con estas diferencias (Emmanouil y col., 2008). En dicho estudio, y de forma similar a nuestros resultados, se observó un aumento de la actividad de CAT en homogeneizados de las glándulas digestivas de mejillones expuestos a nodularina, y sólo después de 7 días de depuración la actividad CAT retornó a los niveles basales en ambos órganos (Kankaanpää y col., 2007). Un patrón similar ha sido descrito por Sun y col. (2006) en peces tras 7 días de depuración, y por Galanti y col. (2013) en camarones expuestos a MC-LR por 3 días y reubicados en agua no contaminada por el mismo período. En el presente trabajo, se observó una disminución en el cociente GSH/GSSG en ambos órganos de los peces expuestos a CYN, tal y como se ha demostrado anteriormente *in vitro* (Gutiérrez-Praena y col., 2011a) e *in vivo* (Gutiérrez-Praena y col., 2011b; Puerto y col., 2012), y tras ambos períodos de depuración se consiguió restaurar los niveles de GSH hasta valores control. Por el contrario, París-Palacios y col. (2000), demostraron que la depuración durante 14 días no era capaz de revertir las alteraciones hepáticas producidas en peces previamente expuestos a concentraciones subletales de sulfato de cobre durante el mismo período. Lo que sí encontraron estos autores fue un aumento del GSH, de la actividad CAT y GPx como respuesta adaptativa del hígado a lo largo de los 14 días de exposición al tóxico. El aumento que observamos de los niveles de γ-GCS volvió a valores basales tras sólo 3 días de depuración, situación que se mantuvo durante el período de 7 días. Hasta donde sabemos, no existe bibliografía referente a los efectos de la depuración en los niveles de esta enzima, ya sea en peces u otros organismos acuáticos expuestos a los contaminantes.

En nuestro experimento, no se detectó CYN en las muestras de agua de las peceras donde las tilapias fueron sometidas a la depuración, durante ninguno de los dos períodos ensayados. Por tanto, al contrario de lo que sugerían Sun y col. (2006), en nuestro caso no parece probable que la causa de que disminuyera la concentración de CYN en los tejidos y, por consiguiente, la magnitud de los efectos tóxicos, fuera una parcial transferencia de la toxina al agua. Se necesitarían ensayar períodos más largos de depuración para aclarar este aspecto.

Una vez demostrada la utilidad del proceso de depuración sobre los parámetros de estrés oxidativo y teniendo en cuenta la neurotoxicidad de la CYN, discutida anteriormente, nos pareció interesante conocer si este proceso también era capaz de revertir o mitigar los efectos de la toxina a nivel neurológico. Tras someter a los peces a dos períodos de depuración (3 y 7 días), se observó una recuperación de los niveles de LPO y de AChE a

partir del día 3. Sin embargo, fueron necesarios 7 días de depuración para una recuperación completa de los cambios histopatológicos observados, principalmente procesos degenerativos y vasculares con signos de necrosis. Además, mientras que la toxina fue detectada en los cerebros de todos los peces expuestos a CYN durante 14 días, sólo se consiguió detectar en el 40% de las muestras depuradas, lo que indica que el proceso de depuración serviría como un buen mecanismo de destoxicación en el cerebro de peces que han estado expuestos a floraciones de cianobacterias tóxicas productoras de CYN.

Por otra parte, la inmunohistoquímica (IHC) es una técnica que se ha empleado para el estudio de la distribución tisular de otra cianotoxina, como la MC-LR, en ratones (Yoshida y col., 1998; Ito y col., 2000; Guzmán y Solter, 2002), en diversos órganos del gasterópodo *Lymnaea stagnalis* (Lance y col., 2010) y en la trucha arco iris (Fischer y col., 2000). Además, se ha empleado para la detección de diferentes virus en cultivos celulares (Niedobitek y col., 1997) y en tejidos (Risalde y col., 2013), así como en el diagnóstico de enfermedades (Oosterwijk y col., 1986). Teniendo esto en cuenta, nos planteamos estudiar la utilidad de esta técnica para conocer la distribución de la CYN en diferentes tejidos (hígado, riñón, intestinos y branquias) de tilapias expuestas a la toxina, utilizando dos escenarios distintos de exposición: (a) tras exposición aguda por vía oral (por sonda) e inyección i.p. de una única dosis de 200 µg CYN pura/ kg p.c. durante 24 h y cinco días; (b) tras exposición subcrónica por inmersión en aguas con células liofilizadas de *A. ovalisporum* a diferentes concentraciones (10 y 100 µg CYN / L) durante 7 y 14 días. De forma general, los resultados demostraron un patrón similar de distribución de la CYN en ambos ensayos. El órgano que presenta más inmunodetección fue el hígado, seguido del riñón, intestino y branquias. Además, la señal se intensificó con el aumento del tiempo en ambos ensayos, lo que confirma la toxicidad retardada de CYN, y también con el incremento de la dosis, como se muestra en el ensayo de exposición subcrónica. De forma similar, una única exposición por vía oral (junto con la dieta) a 200 µg CYN / kg p.c. durante 24 h (tanto CYN pura como la contenida en un cultivo de *A. ovalisporum*), mostró mayor presencia de la toxina en hígado e intestino, aunque también se detectó en riñón y branquias, siendo más patente en el caso de los peces intoxicados con la toxina pura, en comparación con las células liofilizadas (Gutiérrez-Praena y col., 2014a). Por el contrario, en caracoles expuestos a MC-LR (tanto pura como contenida en un cultivo de cianobacterias) se ha observado una mayor tinción inmunopositiva en el intestino en el caso del cultivo, en comparación con la toxina pura (Lance y col., 2010). En nuestro caso, como se ha comentado, no se observaron diferencias significativas entre los

diferentes tipos de toxina empleada. Igualmente, nuestros resultados concuerdan con estudios llevados a cabo en ratones expuestos por vía oral a MC-LR, que indican que el principal órgano diana era el hígado, ya que la toxina aparecía teñida especialmente alrededor de la vena central de los hepatocitos (Ito y col., 2000).

2.2. Estudios realizados en el mejillón marino *Mytilus galloprovincialis*

El estudio y caracterización de las enzimas involucradas en los procesos de destoxicación resulta de importancia previa valorización de la afectación de dichas enzimas por distintas toxinas. Por ello, fue interesante realizarlo en branquias del mejillón *Mytilus galloprovincialis.* Con respecto a ese estudio, nuestros resultados preliminares muestran la identificación de tres isoformas de GST en las branquias de *M. galloprovincialis* (1 isoforma Pi, 1 Mu y 1 Sigma). Las GSTs-Pi pueden inactivar productos de lipoperoxidación y especies de oxígeno directamente a través de su grupo -SH (Doyen y col., 2008). Están más involucrados que otras clases de GST en la destoxicación de las prostaglandinas y otros compuestos carbonílicos insaturados (Whalen y col., 2008). Las isoformas Pi (Q8MUC3) y Mu (A7LFK0) tienen puntos isoeléctricos cercanos, del 6 al 7, indicando que podrían compartir funciones comunes. La regulación de estas isoformas en ciertos lugares podría indicar la inducción de mayores condiciones de estrés en áreas potencialmente más contaminadas, en comparación con otras más limpias. Por otra parte, el punto isoeléctrico de la isoforma Sigma es más alcalino que el de las isoformas Pi y Mu, y probablemente realice un conjunto diferente de funciones.

Recientemente, Martins y col. (2014) han estudiado el perfil proteómico de las GSTs citosólicas en tres especies de bivalvos por 2DE y MALDI-TOF/TOF. Así, identificaron 4 isoformas Pi y 2 Sigma en *M. galloprovincialis,* 4 Pi y 1 Sigma en *Corbicula fluminea*, y 2 Mu y 1 Pi en *Anodonta cygnea*. Las isoformas de GST fueron identificadas en los extractos purificados de todo el cuerpo de los animales, no haciendo distinción entre ningún órgano en concreto. Sin embargo, nuestros resultados muestran la identificación de isoformas GST específicamente en las branquias de *M. galloprovincialis,* constituyendo la primera caracterización de la familia de GSTs en este órgano, que por estar en contacto directo con el agua de mar, constituyen uno de los órganos más importantes de biotransformación de los xenobióticos (Fernandes y col., 2009).

2.3. Estudios realizados en plantas de zanahoria (*Daucus carota*)

Los efectos de la CYN en plantas han sido mucho menos estudiados en comparación con las MCs. Los escasos estudios al respecto indican la inhibición del crecimiento de *Sinapis alba* (Vasas et al., 2002) y del macrófito *Phragmites australis* (Beyer et al., 2009), la reducción en la germinación del polen en plantas de tabaco (*Nicotiana tabacum*) (Metcalf et al., 2004), así como la inducción de estrés oxidativo (Prieto et al., 2011). Como posible vía de entrada de la CYN en la cadena alimentaria, tienen también gran interés los estudios de los efectos de esta toxina en alimentos vegetales que están en contacto permanente con las aguas que puedan estar potencialmente contaminadas, como es el caso de las zanahorias. Hasta la fecha, solo se han descrito los efectos de la exposición a CYN y a MCs en arroz (Prieto et al., 2011), y en las zanahorias únicamente con MCs (Machado, 2014).

En nuestro estudio, la exposición de zanahorias a 10 µg CYN/L durante 30 días llevó a un aumento del peso fresco de las raíces, observándose la misma tendencia en las plantas expuestas a 50 µg CYN/L. Kinnear y col. (2008) observaron un aumento en el crecimiento de la raíz de *Hydrilla verticillata* tras 14 días de exposición a extractos de *C. raciborskii* conteniendo 400 µg CYN/L. Por el contrario, Machado (2014) observó una disminución del crecimiento de las raíces de zanahorias expuestas a MCs (50 µg/L) durante 28 días, corroborando los resultados reportados por Khalloufi y col. (2012) en el tomate (*Lycopersicon esculentum*), aunque no determinan que la exposición produjera efectos negativos en el crecimiento de la planta, de forma general. En esta línea, otros estudios han sugerido que la exposición a MC-LR no produce efectos sobre el crecimiento de la planta. Así, Järvenpää y col. (2007) informaron de que la exposición de *Brassica oleracea* y *S. alba* a concentraciones de MC-LR entre 1 y 10 µg/L durante 20 y 19 días, respectivamente, no afectó al crecimiento de las raíces. De forma similar a nuestros resultados, Prieto y col. (2011) encontraron un aumento en el peso fresco de las raíces de *Oriza sativa* tras 48 h de exposición a extractos celulares de *A. ovalisporum* conteniendo 2,5 µg CYN/L, mientras que no observaron cambios significativos tras 9 días de exposición al mismo extracto. Sin embargo, comparando con otras cianotoxinas como las MCs, estos mismos autores también encontraron que la exposición de 48 h a un extracto crudo conteniendo 50 µg MC-LR/L no produjo cambios significativos en el peso fresco de las raíces de *O. sativa*. En el caso de las MCs, algunos estudios informan de un aumento de la susceptibilidad de las plantas durante las etapas de germinación e inicio del desarrollo (Saqrane y col., 2009). Por el contrario, nuestros resultados sugieren que las plantas no serían tan resistentes a la toxicidad inducida por CYN, en comparación con estos resultados descritos para MCs, debido a que se observan efectos

sobre el crecimiento ya en una etapa más madura de la planta (tras 30 días de exposición). Varios factores pueden explicar las diferencias existentes entre estos estudios y el nuestro, como son la especie de planta estudiada, la fase de desarrollo en la que se encuentre, la cianotoxina en cuestión, así como el tiempo y las dosis de exposición a la misma.

Además, en nuestro estudio, la capacidad fotosintética de las plantas aumentó con 50 µg CYN/L con respecto al control. En zanahorias expuestas a MCs este parámetro experimentó un aumento a partir de 10 µg/L (Machado, 2014). Nuestros resultados parecen indicar que las plantas no fueron sometidas a un alto nivel de estrés, sino más bien que la exposición a la toxina provocó una estimulación de la fotosíntesis como mecanismo de defensa. Si comparamos con los efectos de las MCs, estos datos no concuerdan con otras especies de plantas, donde se ha podido observar una inhibición fotosintética por la MC-LR (El Khalloufi y col., 2012; Gutiérrez-Praena y col., 2014b), aunque en estos casos la concentración de MC ensayada fue más alta (100 µg/L).

De forma general, se observó una disminución en la cantidad de micronutrientes (Fe, Mn, Cu, Zn y Mo) en las zanahorias expuestas a CYN, especialmente a la concentración de 50 µg CYN/L. El P experimentó igualmente un descenso y el Cu un aumento dosis-dependiente, en concordancia con resultados previos en zanahorias expuestas durante 28 días a MCs en el agua de riego (Machado, 2014). En cuanto a los minerales Na y Mg, se observó una disminución al exponer las plantas a CYN, mientras que el Ca no mostró alteraciones y el K experimentó un aumento con la concentración de 50 µg CYN/L, de acuerdo con los resultados de zanahorias expuestas a MCs durante 28 días (Machado, 2014).

En general, la CYN afecta al peso fresco, a la capacidad fotosintética y contenido en minerales en *D. carota*, pudiendo tener una respuesta más tardía en relación con otras cianotoxinas como las MCs. Para el adecuado crecimiento de las plantas es esencial que los nutrientes sean asimilados en niveles adecuados. Tanto una deficiencia como un aumento de los nutrientes puede afectar al crecimiento de las plantas por los efectos directos sobre el metabolismo celular (como la biosíntesis de macromoléculas o el funcionamiento del sistema fotosintético) (Grusak, 2001). En nuestro estudio, donde se observa una disminución generalizada de los nutrientes, dependiente de la concentración de toxina, este hecho no pareció tener grave consecuencias en el crecimiento de las zanahorias, lo que no es de extrañar ya que normalmente las plantas de cultivo precisan poca cantidad de micronutrientes. No obstante, más estudios son necesarios para profundizar en los efectos de la CYN en vegetales de consumo humano.

VI. CONCLUSIONES / CONCLUSIONS

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones:

PRIMERA. Se ha comprobado que las técnicas de cocinado por horno microondas y hervido son capaces de reducir los niveles de MCs libres en el pescado (*Oreochromis niloticus*) en un rango entre 25-50%. Concretamente, el hervido continuo ha demostrado ser el más eficaz, debido tal vez al mayor tiempo de permanencia de la muestra en el agua, observándose transferencia de MCs al agua. En general, estas técnicas podrían ser consideradas como medidas de control de estas toxinas en la dieta tras el consumo humano de los alimentos cocinados.

SEGUNDA. Hemos desarrollado y validado tres métodos analíticos sensibles, reproducibles, precisos y robustos para determinar CYN en aguas, células liofilizadas y tejidos de peces expuestos (hígado y músculo), usando SPE con cartuchos de carbono grafitizado y cuantificación por LC-MS/MS. Se obtuvieron límites de detección y cuantificación aceptables para estudios ambientales, con recuperaciones en un rango de 80-110%. Posteriormente, los métodos se aplicaron con éxito para la detección y cuantificación de CYN en muestras de agua de acuarios, en muestras liofilizadas de floraciones naturales y en hígado y músculo de peces expuestos a la toxina en condiciones de laboratorio.

TERCERA. Se ha demostrado la implicación del estrés oxidativo como un mecanismo de acción tóxica de la CYN en tilapias expuestas por inmersión de forma subcrónica a células de cianobacterias que contienen CYN (10 y 100 μg/L) y 7-desoxi-CYN (0,46 y 4,6 μg/L), durante 7 y 14 días. Esto se manifiesta por alteraciones en los niveles de LPO, oxidación de proteínas y del ADN, la relación GSH/GSSG, y cambios en las actividades de GST, GPx, SOD, CAT, y γ-GCS. En general, estos cambios han sido dosis y tiempo-dependientes (100 μg CYN/L, 14 días), siendo el riñón el órgano más afectado.

CUARTA. Esta relación dosis y tiempo-dependiente se confirma con las alteraciones histopatológicas observadas en el hígado, riñón, corazón, tracto G.I. y branquias de tilapias expuestas a 10 y 100 μg CYN/L. Los principales cambios histológicos observados fueron procesos degenerativos, esteatosis y aumento de los diámetros nucleares de los hepatocitos en el hígado, glomerulopatía membranosa con alteraciones de los diámetros de los túbulos contorneados en el riñón, miofibrolisis y edema en el corazón, enteritis necrótica en el tracto G.I., e hiperemia y microhemorragias en las lamelas de las branquias.

QUINTA. Se han observado por primera vez los efectos neurotóxicos de CYN en el cerebro de tilapias expuestas por inmersión de forma subcrónica a dosis repetidas de un cultivo de *A. ovalisporum* que contiene CYN, durante 14 días, demostrado por la inhibición significativa de la actividad de la AChE y el aumento de los niveles de LPO, y confirmado por la alteraciones histopatológicas observadas en este órgano, y la detección de la toxina en todos los cerebros de los peces expuestos.

SEXTA. Se ha demostrado la utilidad de la suplementación dietética con L-carnitina (400 o 880 mg LC/kg pez/día, durante 21 días) sobre la defensa antioxidante mediante la restauración de los diferentes biomarcadores de estrés oxidativo a niveles basales (LPO, oxidación de proteínas y ADN, actividades GST, GPx, SOD, CAT y γ-GCS, y relación GSH/GSSG), sobre las alteraciones histopatológicas y los cambios en la expresión génica producidos en hígado y riñón de tilapias expuestas durante 24 h a las células liofilizados *A. ovalisporum* o a CYN pura (400 µg CYN/kg de pez). Se puede considerar, por lo tanto, como un útil quimioprotector para la prevención de los daños producidos por CYN en peces.

SÉPTIMA. El pretratamiento con vitamina E (700 mg vitamina E/kg pez/día, durante 7 días) ha demostrado reducir el estrés oxidativo hepático y renal en tilapias tras exposición aguda a CYN pura por vía oral (400 µg CYN/kg de pez), especialmente en el hígado, mediante la restauración de los valores de biomarcadores oxidativos (LPO, oxidación de proteínas y del ADN, actividades GST, GPx, SOD, CAT y γ-GCS y relación GSH/GSSG) a niveles basales. Por otra parte, la vitamina E ayuda a prevenir o mejorar las lesiones histopatológicas inducidas por CYN pura en el hígado, riñón, corazón, tracto G.I., branquias y cerebro de los peces expuestos. Por lo tanto, se demuestra el uso de la vitamina E como un quimioprotector seguro para la profilaxis de intoxicaciones de tilapias por CYN.

OCTAVA. Se ha comprobado que cortos periodos de depuración (3 ó 7 días) podrían ser efectivos para restaurar hasta los niveles basales algunos marcadores de estrés oxidativo (LPO, oxidación de proteínas y del ADN, actividades SOD, CAT y γ-GCS, y la relación GSH/GSSG), en hígado y riñón de tilapias expuestas a células de *A. ovalisporum* que contienen CYN y 7-desoxi-CYN durante 7 y 14 días. Asimismo, se ha demostrado la mejora de las alteraciones a nivel neurológico (actividad AChE, niveles de LPO, efectos histopatológicos y presencia de CYN en cerebro) en las mismas condiciones. Esto sugiere que la depuración puede ser un proceso de destoxicación efectiva en peces potencialmente expuestos a CYN.

NOVENA. En cuanto al estudio inmunohistoquímico (IHC), el órgano que presentó mayor tinción fue el hígado, seguido por el riñón, el intestino y las branquias de tilapias expuestas a CYN. Además, la señal se intensificó con el aumento del tiempo tanto en el ensayo agudo como en el subcrónico, confirmando la toxicidad retardada de CYN, y también con el aumento de la dosis, como se muestra en el ensayo subcrónico. Las señales en el intestino y las branquias fueron más intensas en el ensayo subcrónico, debido a que los peces están continuamente sumergidos en el agua que contiene la toxina. Por lo tanto, se demuestra la utilidad de la IHC para estudiar la distribución de la CYN en estos organismos.

DÉCIMA. Los resultados preliminares muestran una eficiente extracción, purificación y separación de varias isoformas de GST mediante electroforesis bidimensional (2D) en branquias del mejillón marino *Mytilus galloprovincialis.* Se identificaron 1 isoforma Sigma, 1 Pi y 1 Mu por MALDI-TOF/TOF, constituyendo la primera caracterización de la familia de GSTs en este órgano.

UNDÉCIMA. Los resultados preliminares sugieren que las plantas de zanahorias (*Daucus carota*) son capaces de hacer frente a concentraciones ambientales de CYN (10 y 50 μg/L) contenida en extractos de *A. ovalisporum*, con un mantenimiento y aumento del peso fresco de las raíces, sin afectar negativamente la fotosíntesis, aunque sí afecta a procesos metabólicos tales como la acumulación de minerales.

The main conclusions that can be drawn on the basis of the obtained results in the present Thesis are:

FIRST. Microwave oven and boiling treatments are able to reduce unconjugated MCs levels in cooked fish (*Oreochromis niloticus*) in a range between 25% and 50%. In particular, the continuous boiling process was demonstrated to be the most effective, due perhaps to the longer contact between the sample and the water, leading to MCs transference to water. In general, they could be useful as control measures for a better dietary estimation of these toxins after human consumption of cooked food.

SECOND. We have developed and validated three sensitive, reproducible, accurate, and robust methods for determining CYN in waters, lyophilized cells and fish tissues (liver and muscle), using SPE with graphitized carbon cartridges and quantification by LC-MS/MS. Detection and quantification limits obtained were acceptable for environmental studies, with recoveries in a range 80–110%. Moreover, the methods were successfully applied to detection and quantification of CYN in water samples from aquaria, in lyophilized natural blooms samples and in liver and muscle of fish exposed to the toxin under laboratory conditions.

THIRD. It has been demonstrated the involvement of oxidative stress as a mechanism of toxic action of CYN in tilapia after sub-chronic exposure to cyanobacterial cells containing CYN (10 and 100 μg/L) and 7-deoxy-CYN (0.46 and 4.6 μg/L), for 7 and 14 d by immersion route, by alterations in LPO, protein and DNA oxidation levels, GSH/GSSG ratio, and changes in the activities of GST, GPx, SOD, CAT, and γ-GCS. In general, these changes depend on the concentration and exposure periods assayed (100 μg CYN/L, 14 days), and the kidney was the most affected organ.

FORTH. Histopathological findings in the liver, kidney, heart, G.I. tract and gills of tilapia confirm that the extent of damage is related to the CYN concentration and length of exposure. The major histological changes observed were degenerative processes, steatosis and augmented hepatocyte nuclear diameters in the liver, membranous glomerulopathy with alterations of convoluted tubules diameters in the kidney, myofibrolysis and edema in the heart, necrotic enteritis in the G.I. tract, and hyperemic processes in gill lamellae and microhemorrhages.

FIFTH. CYN neurotoxic effects have been demonstrated for the first time in brain of tilapia subchronically exposed by immersion to repeated doses of a CYN-containing culture of

A. ovalisporum for 14 days, according to the significant inhibition of AChE activity and increases in LPO levels, the histopathological alterations found in this organ, and the presence of the toxin in all of the brains of exposed fish.

SIXTH. It has been demonstrated the usefulness of L-carnitine (LC) dietary supplementation (400 or 880 mg LC/kg fish/day, during a 21 days) in the antioxidant defense by restoring the different oxidative stress biomarkers values to control levels (LPO, protein and DNA oxidation, GST, GPx, SOD, CAT and γ-GCS activities, and GSH/GSSG ratio), the histopathological changes and alterations in gene expression produced in liver and kidney of tilapia exposed for 24 h to lyophilised *A. ovalisporum* cells or pure CYN (400 μg CYN/kg fish). It can be considered, therefore, as a useful chemoprotectant for prevention of the damages produced by CYN in fish.

SEVENTH. Vitamin E pretreatment (700 mg vitamin E/kg fish/day, for 7 days) has been proved to reduce hepatic and renal oxidative stress in tilapia after CYN acute exposure by oral route (400 μg pure CYN/kg fish), especially in the liver, by restoring to control levels the oxidative biomarkers values (LPO, protein and DNA oxidation, GST, GPx, SOD, CAT and γ-GCS activities, and GSH/GSSG ratio). Moreover, vitamin E pretreatment helps to prevent or ameliorate the histopathological lesions induced by pure CYN in liver, kidney, heart, G.I. tract, gills and brain of tilapia acutely exposed to the toxin. The use of vitamin E as a safe chemoprotectant in the prophylaxis of CYN-intoxications in tilapia is therefore demonstrated.

EIGHTH. Short depuration periods (3 and 7 days) managed to recover the oxidative damages induced in tilapia by exposure to *A. ovalisporum* cells containing CYN and 7-deoxy-CYN for 7 and 14 days, by returning some oxidative stress biomarkers (LPO, protein and DNA oxidation, SOD, CAT and γ-GCS activities, and GSH/GSSG ratio) to control levels. Moreover, depuration managed to recover the neurological alterations (AChE activity, LPO levels, histopathological changes and presence of CYN in brain) in the same conditions. This suggests that the depuration can be an effective detoxification process when fish could be potentially exposed to CYN.

NINTH. Regarding the immunohistochemical study (IHC), the organ that presented the most immunopositive results was the liver, followed by the kidney, intestine, and gills of tilapia exposed to CYN. Moreover, the immunolabeling signal intensified with increasing time in both the acute and sub-chronic assays, confirming the delayed toxicity of CYN, and also with

the increment of the dose, as it is shown in the sub-chronic assay. Immunolabeling in the intestine and gills were stronger in fish from the sub-chronic assay, as the fish are constantly immersed in the water containing the toxin. Therefore, IHC is shown to be a valuable technique to study CYN distribution in these organisms.

TENTH. Preliminary results show an efficient extraction, purification and separation of several GST isoforms with 2DE from the gills of the marine mussel *Mytilus galloprovincialis.* 5 Pi, 4 Mu, and 1 Sigma GST isoforms were identified by MALDI-TOF/TOF analysis, constituting the first comprehensive characterization of the GST family in this organ.

ELEVENTH. Preliminary results suggest that carrot plants (*Daucus carota*) are able to cope with environmental CYN concentrations (10 and 50 μg/L) from extracts of *A. ovalisporum*, by ensuring and enhancing root growth, without a negative effect on photosynthesis, although it does affect metabolic processes such as mineral accumulation.

VII. OTROS MÉRITOS / OTHER MERITS

CAPÍTULO DE LIBRO / BOOK CHAPTER

Remedios Guzmán-Guillén

EFECTIVIDAD DE ANTIOXIDANTES EN LA PREVENCIÓN DE INTOXICACIÓN POR MICROCISTINAS Y CILINDROSPERMOPSINA EN PECES

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EFECTIVIDAD DE ANTIOXIDANTES EN LA PREVENCIÓN DE INTOXICACIÓN POR MICROCISTINAS Y CILINDROSPERMOPSINA EN PECES

REMEDIOS GUZMÁN GUILLÉN

Área de Toxicología. Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Becaria FPU. Facultad de Farmacia. Universidad de Sevilla.

RESUMEN

La aparición de floraciones de cianobacterias está aumentando en muchas regiones del mundo debido a la eutrofización progresiva de las aguas y al cambio climático. Las cianobacterias producen una gran variedad de toxinas que pueden causar graves problemas de salud incluyendo la muerte en los seres humanos y animales salvajes o domésticos, pudiendo acumularse a través de la cadena alimentaria. Deben tenerse en cuenta los efectos crónicos en los seres humanos de la exposición a cianotoxinas mediante el agua y alimentos contaminados por éstas, sobre todo en casos de una exposición prolongada y frecuente. La Organización Mundial de la Salud (OMS) ha establecido un valor guía provisional de 1 μg/L de microcistina-LR (MC-LR) en el agua potable. Aunque aún no hay valor de referencia para la cilindrospermopsina (CYN), se ha propuesto un límite en agua de bebida de 1 μg/L de CYN total. En este trabajo se resumen los efectos beneficiosos de la suplementación con diferentes sustancias antioxidantes (vitamina E, selenio, *N*- acetilcisteína y L-carnitina) en la prevención y/o tratamiento del estrés oxidativo provocado por las hepatotoxinas Microcistinas (MC) y la citotoxina CYN. Los avances en la comprensión y el descubrimiento de nuevos antioxidantes pueden ser útiles en la profilaxis y tratamiento de intoxicaciones causadas por cianotoxinas en peces y, por tanto, en los problemas de salud en humanos relacionados con la exposición a cianotoxinas.

Palabras clave: microcistinas, cilindrospermopsina, peces, estrés oxidativo, antioxidantes.

ABSTRACT

The occurrence of cyanobacterial blooms in aquatic environments is increasing in many regions of the world with progressive eutrophication of water bodies and climate change. Cyanobacteria produce an unparalleled variety of toxins that can cause severe health problems or even death in humans, and wild or domestic animals, being able to accumulate in aquatic organisms. Chronic toxic effects in humans from exposure through water and food contaminated by cyanotoxins need to be considered, especially if there is long-term frequent exposure. The World Health Organization (WHO) has set a provisional guideline value of 1 μg/L of microcystin-LR (MC-LR) in drinking water. Although no guideline value has been established for cylindrospermopsin (CYN) yet, there is a proposed drinking water limit of 1 μg/L of total CYN. This work summarizes current knowledge about the beneficial effects of different antioxidants (Vitamin E, Selenium, *N*-acetylcysteine and L-carnitine) to prevent and/or counteract oxidative stress effects caused by the hepatotoxins Microcystins (MC) and the cytotoxin CYN. Advances in the understanding and discovering of new antioxidants may be useful in the prophylaxis and treatment of cyanotoxins-related intoxication in fish and thus, in human health problems related to cyanotoxins-exposure.

Keywords: microcystins, cylindrospermopsin, fish, oxidative stress, antioxidants.

ÍNDICE

1. INTRODUCCIÓN

Las cianobacterias son protofitas pigmentadas, organismos procarióticos similares a las bacterias Gram negativas, pero capaces de realizar fotosíntesis oxigénica (Quesada y col., 2006), que cuentan con aproximadamente 150 géneros y unas 2000 especies.

En aguas eutróficas y bajo determinadas condiciones ambientales pueden crecer excesivamente produciendo floraciones, dispuestas en forma de lámina verde, más o menos viscosa, que puede tener aspecto de espuma (Pizzolon, 1996; Campos y Vasconcelos, 2010). Su distribución es ubicua y hoy en día este crecimiento excesivo pone en compromiso la calidad del agua en muchos países debido a la producción de toxinas que se liberan (Figura 1).

Figura 1. Típica imagen de aguas eutróficas con floración de cianobacterias

Los géneros de cianobacterias que se han detectado en floraciones tóxicas con mayor frecuencia a nivel mundial son: *Microcystis, Anabaena, Aphanizomenon, Planktothrix, Cylindrospermopsis y Nodularia* (Kardinaal y Visser, 2005).

1.1. Cianotoxinas: Microcistinas y Cilindrospermopsina

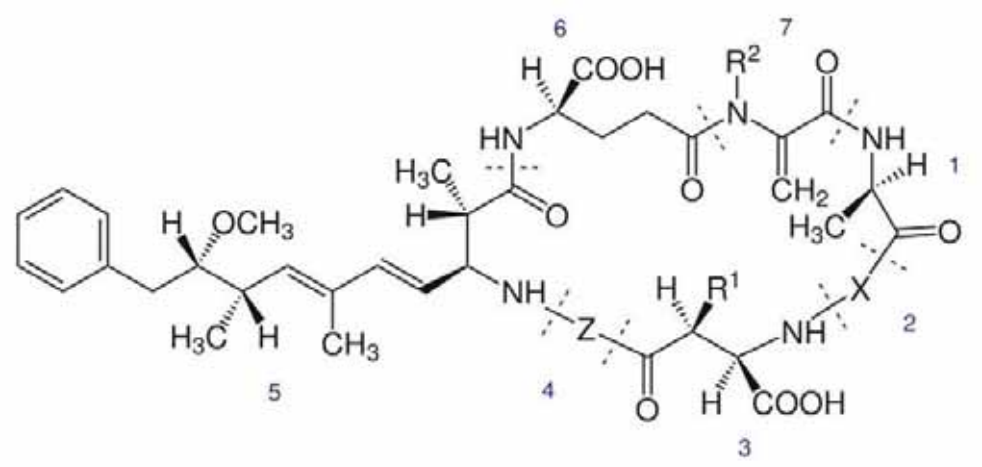
Las cianobacterias producen una gran variedad de compuestos, considerados metabolitos secundarios, por no realizar funciones principales en la cianobacteria que los produce. Entre ellos se encuentran las cianotoxinas, que producen diferentes efectos sobre la salud de los organismos que se ven expuestos a ellas. Se consideran los compuestos más tóxicos y más preocupantes en las masas de agua tanto por su amplia distribución como por su elevada toxicidad.

Estas cianobacterias son un componente importante en la dieta de muchos ciprínidos y cíclidos tropicales, como las Tilapias (Zurawell y col., 2005). Los peces y otros organismos con sistemas metabólicos avanzados tienen mayor riesgo de toxicidad por cianotoxinas (Pearson y col., 2010). Los organismos acuáticos pueden estar expuestos en la naturaleza a cianotoxinas, directamente por ingestión de cianobacterias tóxicas (Li y col., 2004) o pasivamente cuando la toxina pasa a través de las branquias durante la respiración (Zimba y col., 2001), pudiendo sufrir también procesos de bioacumulación (Magalhaes y col., 2003; Mohamed y col., 2003; Soares y col., 2004). Efectivamente, hay evidencias de que estas cianotoxinas se acumulan en los tejidos de los organismos acuáticos que habitan las aguas contaminadas, pudiendo transferirlas a niveles superiores de la cadena alimentaria hasta llegar al hombre. Consecuentemente, la ingestión de alimentos contaminados con cianotoxinas representa un riesgo potencial para la salud humana. Tanto las células intactas como las toxinas liberadas tras la lisis celular pueden ser responsables de los efectos tóxicos observados en animales y seres humanos (Carmichael y Falconer, 1993; Moreno y col., 2004).

Las cianotoxinas son muy diversas, tanto en su estructura química (péptidos cíclicos, alcaloides y lipopolisacáridos) como en su toxicidad (Briand y col., 2003) y tradicionalmente se han clasificado atendiendo a sus efectos tóxicos en: hepatotoxinas, neurotoxinas, citotoxinas, dermatotoxinas y toxinas irritantes (De Figueiredo y col., 2004; Teneva y col., 2005). Nos centraremos en las Microcistinas (hepatotoxinas) y en la Cilindrospermopsina (citotoxina), y en los efectos que estas tienen sobre el estrés oxidativo. La combinación de estos dos tipos de cianotoxinas, MCs y CYN pareció ser la causa de la contaminación del agua usada en un centro de hemodiálisis en Caruaru (Brasil), donde 100 pacientes desarrollaron fallo hepático agudo y 50 de ellos murieron por la intoxicación por cianobacterias (Carmichael y col., 2001).

Entre las cianotoxinas más comúnmente detectadas destacan las Microcistinas (MCs). Son heptapéptidos cíclicos que contienen L- y D-aminoácidos hidrófobos y un D-amino ácido carácterístico en C20 conocido como ácido 3-amino-9-metoxi-2,6,8-trimetil-10-phenyldeca-4,6-dienoico (ADDA) (Figura 2). Hay más de 80 variantes de MCs, que difieren principalmente en los dos L-aminoácidos en las posiciones 2 y 4, y la desmetilación de D-MeAsp y/o Mdha en las posiciones 3 y 7, respectivamente (Sivonen y Jones, 1999). Las más

comunes, y también las más ampliamente estudiadas son MC-LR (2: Leu, 4: Arg), MC-RR (2: Arg, 4: Arg) y MC-YR (2: Tyr, 4: Arg) (Park y Watanabe, 1995). Estas toxinas pueden ser producidas por varios géneros de cianobacterias tales como *Anabaena, Nostoc, Oscillatoria, Nodularia y Microcystis*, principalmente.



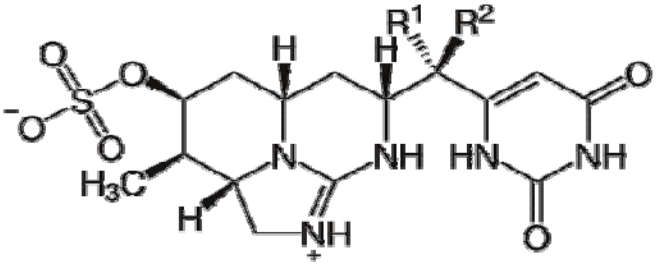
	Posición 4	Posición 2
MC-LR	Arginina	Leucina
MC-RR	Arginina	Arginina
MC-YR	Arginina	Tirosina

Figura 2. Estructura general de MCs (adaptado de Chorus y Bartram, 1999)

En respuesta al aumento de estos problemas de salud a nivel mundial, la Organización Mundial de la Salud (OMS) ha establecido un nivel guía provisional para el agua potable de 1,0 µg MC-LR/L (Falconer y col., 1999). Asimismo, se ha establecido una Ingesta Diaria Tolerable (IDT) provisional de 0,04 µg/Kg/día de equivalentes de MC-LR. (Chorus y Bartram, 1999). La Agencia Internacional de Investigación sobre el Cáncer (IARC) ha clasificado a la MC-LR como posiblemente cancerígeno para los humanos (Grupo 2B), aunque los extractos de *Microcystis* no son clasificables aún por su carcinogenicidad (http:www.iarc.fr/).

La Cilindrospermopsina (CYN) es una cianotoxina frecuentemente implicada en las floraciones (Fessard y Bernard, 2003), producida por cepas de *Aphanizomenon ovalisporum* (Banker y col., 2001), *Cylindrospermopsis raciborskii* (Padisak, 1997) *Umezakia natans*

(Harada y col., 1994), y otras especies de cianobacterias de agua dulce pertenecientes a los géneros *Anabaena, Raphidiopsis y Lyngbya* (Li y col., 2001; Pearson y col., 2010; Seifert y col., 2007). Estructuralmente, CYN es un derivado tricíclico de guanidina combinado con hidroximetiluracilo (Ohtani y col., 1992) (Figura 3), muy soluble en agua y con un peso molecular relativamente bajo de 415 Da (Sivonen y Jones, 1999), que al parecer penetra en las células por difusión pasiva (Chong y col., 2002). La disponibilidad predominantemente extracelular de esta toxina hace que sea fácilmente absorbida por gran variedad de organismos acuáticos (Kinnear, 2010), entre ellos, los peces.



Estructura	
Cilindrospermopsina	R^1: H / R^2: OH
7-epi-cilindrospermopsina	R^1: OH / R^2: H
Deoxycilindrospermopsina	R^1: H / R^2: H

Figura 3. Estructura general de Cilindrospermopsina (adaptado de Hiller y col., 2007).

En relación con su toxicidad en humanos, en el brote de hepatoenteritis de Palm Island (Australia, 1979), en el que 148 personas fueron hospitalizadas, los organismos en la floración original de la fuente de abastecimiento de agua no se identificaron antes del tratamiento con sulfato de cobre. Posteriormente, se observó que *Cylindrospermopsis raciborskii* era una especie estacional dominante en el depósito de agua. Sus graves efectos hepatotóxicos, así como variedad de alteraciones provocadas en ratones lo convierten en un organismo capaz de producir la enfermedad clínica ocurrida en Palm Island y, siguiendo las sugerencias de Hawkins y col. (1985), estas floraciones que contenían *C. raciborskii* deben considerarse como posible causa del brote.

Recientemente, han sido aisladas variantes estructurales de CYN a partir de *C. raciborskii* y *A. ovalisporum*. La primera variante se identificó como deoxy-

cilindrospermopsina (deoxy-CYN), que parecía ser no tóxica (Norris y col., 1999), mientras que 7-epicilindrospermopsina, aislada de *A. ovalisporum*, mostraba toxicidad (Banker y col., 2001). Posteriormente, y en contra de los resultados previamente reportados por Norris, se sugirió que la deoxy-CYN actúa por mecanismos de toxicidad similares a CYN, inhibiendo la síntesis proteica y la viabilidad celular con una potencia similar a esta (Neumann y col., 2007). Por ello, ambas variantes se deben considerar en la evaluación de riesgos de CYN.

Aunque no existe un nivel guía para CYN, Humpage y Falconer (2003) han propuesto un valor límite en agua potable de 1 µg/L de CYN total.

1.2. Antioxidantes

Los organismos aeróbicos generan como resultado del metabolismo oxidativo especies reactivas de oxígeno (ERO), como el radical anión superóxido ($O_2^{-\bullet}$), peróxido de hidrógeno (H_2O_2) y el radical hidroxilo ($OH^{\bullet}$), que puede iniciar la peroxidación lipídica (LPO) en los tejidos (Halliwell y Gutteridge, 1984). Para reducir al mínimo los efectos negativos de las ERO, existen sistemas de defensa antioxidante responsables de eliminar los radicales libres. Estos sistemas comprenden tanto enzimas como compuestos no enzimáticos. Entre los primeros podemos encontrar la superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) y glutatión reductasa (GR). Entre los no enzimáticos están el glutatión (GSH), las vitaminas A, E y C, Selenio (Se), L-carnitina, carotenos, flavonoides, melatonina, albúmina, ácido úrico y ubiquinol10 (Wilhelm Filho, 1996; Batcioglu y col., 2005), y también se han estudiado las propiedades antioxidantes de la *N*-acetilcisteína (NAC) (Puerto y col., 2009; Puerto y col., 2010; Gutiérrez-Praena y col., 2012).

Los antioxidantes que actúan como preventivos, rompiendo la cadena de reacciones radicalarias, están consideradas como protección de primera línea frente al daño oxidativo. En la segunda línea de defensa entran ya en juego los sistemas de reparación, como las enzimas que intentan restaurar directamente las biomoléculas a su conformación inicial, y las enzimas catabólicas que degradan específicamente las proteínas, lípidos y ácidos nucleicos no funcionales (Savanian, 1988; Niki, 1991; Davies, 1991).

El glutatión (GSH) es un tripéptido formado por los aminoácidos L-glutamato, cisteína y glicina, y su forma reducida y activa (GSH) es la predominante. Está involucrado en multitud de procesos esenciales para el funcionamiento normal de las funciones biológicas, como la síntesis de ADN y proteínas (Meister y Anderson, 1983). Contribuye a la eliminación de sustancias electrófilas mediante su conjugación por medio de las glutatión S-transferasas (GSTs). También se encarga de secuestrar directamente las ERO, o bien indirectamente en una reacción catalizada por la enzima glutatión peroxidasa (GPx), oxidándose dos moléculas de GSH a una de GSSG. Los niveles de glutatión en el organismo dependen por tanto del balance entre su síntesis (catalizada por la γ-glutamilcisteína sintetasa, γ-GCS), su conjugación (por las GSTs), su oxidación (no enzimática o por GPx) y la reducción de GSSG a GSH (por la glutatión reductasa, GR) (Peña-Llopis y col., 2003). La vitamina E es un conocido antioxidante que actúa impidiendo la propagación de reacciones radicalarias. Asimismo, el Se es un elemento traza, constituyente de la enzima GPx.

2. CIANOTOXINAS Y ESTRÉS OXIDATIVO

2.1. Microcistinas y estrés oxidativo

Las MCs han demostrado ser potentes hepatotoxinas en diferentes modelos experimentales en condiciones de laboratorio (Ding y col., 1998; Wiegand y col., 1999; Fisher y Dietrich, 2000; Towner y col., 2002; Moreno y col., 2005; Li y col., 2005a; Jos y col., 2005; Cazenave y col., 2006; Prieto y col., 2007), y en condiciones naturales (Qiu y col., 2007; Li y col., 2007). Es bien conocida su capacidad de inhibir las fosfatasas de proteína 1 y 2A, afectando a la señalización intracelular, crecimiento celular y procesos de diferenciación (Runnegar y col., 1995a).

Moreno y col. (2005) fueron los primeros en estudiar los cambios producidos en las actividades de las enzimas antioxidantes tras la administración aguda de MC-LR en hígado y riñón de ratas, lo que sugirió la importancia del estrés oxidativo en la patogénesis de la toxicidad inducida por MC-LR en estos animales.

Muchos experimentos documentan los efectos tóxicos que la exposición a cianobacterias a través del sistema gastrointestinal (GI) puede causar, incluyendo el estrés oxidativo, en diferentes especies de peces como la carpa común (Fischer y Dietrich, 2000), la carpa plateada (Xie y col., 2007), la locha (Li y col., 2005b), la trucha arco iris

(Tencalla y Dietrich, 1996), y la tilapia (Mohamed y col., 2003; Mohamed y Hussein, 2006). Estos efectos tóxicos pueden observase cuando la toxina se administra por vía oral (Jos y col., 2005; Li y col., 2005b; Puerto y col., 2011a), por inmersión (Cazenave y col., 2006), o por vía intraperitoneal (i.p.) (Prieto y col., 2006).

En cuanto a la vía oral, Jos y col. (2005) observaron una mayor toxicidad cuando los peces fueron alimentados con células trituradas de cianobacterias (toxinas liberadas) que contenían 60,0 µg MC-LR/pez/día, comparado con células no trituradas. El sistema de defensa antioxidante endógeno (CAT, SOD, GPx, GR) y el estado de peroxidación lipídica (LPO) se alteraron de manera tiempo-dependiente (ensayo de 14 y 21 días). Estos hallazgos sugirieron que el estrés oxidativo juega un papel importante en la toxicidad de MC-LR en tilapia. Se ha demostrado que estas alteraciones se producen tanto a nivel bioquímico como molecular, por la medida de la actividad y expresión génica de GPx y GST (Puerto y col., 2011a).

Se han observado alteraciones de estrés oxidativo en tencas (*Tinca tinca*) expuestas a dosis únicas de células de *Microcystis* por vía oral (25 y 55 µg MC-LR/pez), induciéndose disminución de las actividades de las enzimas SOD y CAT, y aumento de LPO en hígado y riñón. Además, también se demostraron cambios histopatológicos dosis-dependientes en hígado, riñón, corazón e intestino (Atencio y col., 2008).

2.2. Cilindrospermopsina y estrés oxidativo

La CYN es capaz de interferir con varias rutas metabólicas. El hígado se presenta como su principal órgano diana, aunque a diferencia de las MCs, se ha demostrado en distintos modelos experimentales que esta toxina puede afectar a diversos órganos, como riñón, pulmones, timo, y corazón (Terao y col., 1994; Hawkins y col., 1997; Falconer y col., 1999; Puerto et al., 2012). Incluso estudios recientes realizados en peces demuestran que el riñón parece ser más susceptible que el hígado (Guzmán-Guillén y col., 2012a). También tiene efectos citotóxicos (Runnegar y col., 1994, 1995b, 2002) y neurotóxicos (Kiss y col., 2002), y parece actuar como potencial carcinógeno (Humpage y col., 2000).

El mecanismo de toxicidad de CYN no ha sido totalmente elucidado aún, aunque se sabe que está mediado por la inhibición de la síntesis de proteínas (Terao y col., 1994; Falconer y col., 1999; Seawright y col., 1999), así como genotoxicidad por fragmentación del ADN (Bazin y col., 2010; Humpage y col., 2000; Humpage, 2008). Además, se ha

demostrado una disminución del glutatión hepático (GSH) en ratones y peces in vivo (Norris y col., 2002; Gutiérrez-Praena y col., 2011a) e in vitro (Gutiérrez-Praena y col., 2011b).

En un principio, Humpage y col. (2005) sugirieron que el estrés oxidativo y las especies reactivas de oxígeno (ERO) no median como mecanismo de toxicidad de CYN, basándose en la determinación de los niveles de LPO, sin considerar otros biomarcadores tales como la oxidación de proteínas y la actividad de las enzimas antioxidantes. Experimentos de nuestro equipo de investigación han demostrado que la CYN es capaz de inducir estrés oxidativo en tilapias (*Oreochromis niloticus*) expuestas a dosis única de CYN pura (Gutierrez-Praena y col., 2011a; Puerto y col., 2011b) por vía oral e inyección i.p., tal como revelan las alteraciones observadas en la actividad de NADPH oxidasa, y de γ-GCS, niveles de LPO, oxidación de proteínas, y niveles de GSH. Estos efectos dependen de la vía de exposición y el momento del sacrificio (Gutierrez-Praena y col., 2011a).

Guzmán-Guillén y col. (2012a) intentaron simular la exposición natural de tilapias a células cianobacterianas de *A. ovalisporum*, y han demostrado la inducción de estrés oxidativo en tilapias expuestas de forma subcrónica (7 y 14 días) mediante inmersión a células de cianobacterias que contenían CYN (10 y 100 µg CYN/L) y deoxy-CYN (0,46 y 4,6 µg deoxy-CYN/L). Las alteraciones en los niveles de LPO, la oxidación de proteínas y oxidación de ADN, en las actividades de las enzimas antioxidantes estudiadas (GST, GPx, SOD, CAT y γ-GCS) y en el balance GSH/GSSG, que se encontraba disminuido en los grupos tratados, fueron más pronunciados con 100 µg CYN/L y tras 14 días. Además, en general, el riñón fue el principal órgano afectado. Se puede sugerir que el estrés oxidativo podría estar involucrado como un mecanismo genotóxico de CYN.

3. ANTIOXIDANTES

3.1. Vitamina E

3.1.1. Qué es y cómo funciona

La Vitamina E es un nutriente esencial, un antioxidante liposoluble que puede interactuar directamente con las ERO y proteger las membranas biológicas del estrés oxidativo (Jones y col., 1995). En peces, además, promueve el crecimiento, inhibiendo la oxidación lipídica (Thorarinsson y col., 1994).

Las formas naturales de la vitamina E son todas d-estereoisómeros y consisten en la sustitución de un anillo aromático y una larga cadena lateral isoprenoide (Prieto y col., 2009). Existen ocho compuestos naturales con actividad vitamina E, siendo el α-tocoferol el de mayor potencia biológica. Actúa donando un átomo de hidrógeno a un radical libre y así el radical pasa a ser inactivo. Debido a la estabilidad de su estructura de resonancia, el α-tocoferol oxidado no es reactivo y por tanto no es capaz de continuar propagando en cadena las reacciones radicalarias (Chen y col., 1993).

Los requerimientos dietéticos de tilapias para la vitamina E pueden verse afectados por diversos factores, como el nivel lipídico, fuente, y otros antioxidantes o enzimas antioxidantes presentes en la dieta o en el organismo, el grado de insaturación de la fuente lipídica o el estado oxidativo del lípido (Huang y col., 2004; Shiau y Lin, 2006).

En varias especies de peces, un descenso en el aporte dietético de vitamina E provoca un aumento generalizado en la actividad de las enzimas antioxidantes hepáticas y mayores niveles de peróxidos lipídicos (Tocher y col., 2002). Tocher y col. (2003) demostraron que el suplemento con vitamina E reducía la actividad de las enzimas antioxidantes hepáticas que previamente se vieron afectadas por administración de aceite oxidado a los peces. De igual manera, Huang y Huang (2004) observaron que el suplemento con vitamina E a tilapias híbridas alimentadas previamente con aceite oxidado, prevenía del aumento de LPO y GSH hepático.

3.1.2. Microcistinas y Vitamina E

Gehringer y col. (2003a) estudiaron el efecto protector de la vitamina E en ratones expuestos a dosis repetidas de extractos de MC-LR, y observaron que la administración de suplementos de vitamina E (a una dosis de 33,3 U/ratón/día) ofrecía cierta protección frente a la exposición crónica a esta toxina, demostrado por los valores medidos de LPO y actividad GST.

Por otro lado, Pinho y col. (2005) encontraron que el pretratamiento con vitamina E era capaz de modular las respuestas antioxidantes tras la administración de MCs en agallas del cangrejo de estuario *Chasmagnathus granulatus*.

Estudios de nuestro grupo de investigación demostraron que la vitamina E, a una dosis de 700 mg vit E/kg dieta, protegía del estrés oxidativo en tilapias expuestas por vía oral a células de MCs, atendiendo principalmente a los resultados en los niveles de LPO y actividad

de CAT (Prieto y col., 2008). Además, Prieto y col. (2009) estudiaron el efecto protector del Trolox, nombre comercial dado a un análogo hidrosoluble de la vitamina E, de forma tiempo-dependiente, en tilapias expuestas a una sola dosis de células de *Microcystis aeruginosa* conteniendo MCs. Para ello, determinaron los cambios provocados en diferentes biomarcadores de estrés oxidativo (LPO, oxidación de proteínas, actividades SOD, CAT, GPx, GR y GST, y GSH/GSSG) así como las alteraciones histopatológicas en diferentes órganos, a las 24, 48 y 72 horas de la intoxicación. Observaron que las células de *Microcystis* (120 μg MC-LR/pez) alteraban los biomarcadores de estrés oxidativo y que el Trolox (700 mg Trolox/kg dieta, equivalente a 0,21 mg Trolox/pez/día) durante 7 días, gracias a su capacidad para modular dichos biomarcadores y retirar los radicales libres, contribuye a restaurar sus valores a niveles controles, así como a recuperar las lesiones histopatológicas causadas.

3.2. Selenio

3.2.1. Qué es y cómo funciona

El selenio (Se) es un elemento que está presente de forma natural en muchos suelos y sedimentos y es un micronutriente esencial para peces, aves, humanos y muchos microorganismos. El Se protege contra el estrés oxidativo por ser parte de la GPx, constituida por cuatro subunidades, y cada subunidad contiene un átomo de Se (Batcioglu y col., 2002), pero también puede generar ERO (Miller y col., 2007). Y es que, a pesar de ser un micronutriente esencial, el margen entre sus requerimientos nutricionales y niveles tóxicos es relativamente estrecho, y se bioacumula directamente en la cadena alimentaria hasta niveles tóxicos para la fauna (Ohlendorf, 1996; Eisler, 2000).

El selenio y la Vitamina E ejercen una acción sinérgica para constituir un importante sistema de defensa enzimática en los tejidos animales (Thorarinsson y col., 1994).

Miller y col. (2007) estudiaron los efectos del Se (como selenito de sodio) sobre los mecanismos de defensa antioxidantes de la trucha arco iris (*Oncorhynchus mykiss*) y encontraron que la exposición aguda al Se redujo la LPO en hígado, pero la exposición subcrónica no modificó los niveles de LPO ni actividades antioxidantes (GPx, GSH). Orun y col. (2005) reportaron una disminución de los niveles de LPO en el hígado y otros

órganos de la trucha arco iris expuesta durante 72 horas a 2-6 mg/L de selenito de sodio. Lin y Shiau (2007) estudiaron cómo la suplementación dietética de *Epinephelus malabaricus* con 0,8 y 1,6 mg Se/g de dieta por 8 semanas afectó a los niveles hepáticos de sustancias reactivas del ácido tiobarbitúrico (TBARS). Por otro lado, se observó un aumento en la actividad GPx en la trucha arco iris (Hilton y col., 1980) y salmón del Atlántico (Bell y col., 1987), alimentados con dietas suplementadas con selenio.

3.2.2. Microcistinas y Selenio

Gehringer y col. (2003b) estudiaron el efecto protector del Selenio sobre el daño inducido por MC-LR en hígado de ratón, administrando selenito sódico (1,5 µg/ratón/día) durante dos semanas antes de la inyección i.p. de la toxina. Se reducía el daño hepático causado por las dosis letales y sub-letales de toxina, como demuestran la histopatología, los niveles disminuidos de LPO y el aumento en la actividad GPx en los grupos pretratados con Se.

Atencio y col. (2009) demostraron por primera vez que el Se (selenito de sodio) suministrado en la dieta a tilapias (*O. niloticus*) conseguía conferir cierta protección contra el estrés oxidativo y las lesiones histológicas inducidas por las células de cianobacterias que contienen MCs. Algunos biomarcadores se podían restaurar con la dosis más baja utilizada (1,5 µg Se/g de dieta), tales como CAT, GSH/GSSG, y GR (sólo en el hígado) y SOD (riñón) o en la dosis media (GPx en hígado). Sin embargo, para contrarrestar los cambios inducidos en LPO (hígado y riñón), SOD y GST (hígado), y los daños histopatológicos en varios tejidos, fue necesaria la dosis más alta de 6,0 µg Se/g dieta (equivalente a 1,8 µg Se/pez/día), e incluso esta dosis fue ineficaz en relación a la actividad de GR en el riñón de los peces intoxicados. Sin embargo, la dosis más alta de Se empleada en este estudio afectó a los valores basales de algunos marcadores de estrés oxidativo (CAT en el riñón, GSH/GSSG en el hígado) e indujo la LPO y la oxidación de proteínas en los grupos no intoxicados. Esto concuerda con los efectos observados en trucha arco iris expuesta a Se durante 96 h (Miller y col., 2007). Una posible explicación es que el Se a altas concentraciones puede funcionar como un pro-oxidante, catalizando la oxidación de tioles (como GSH) y la generación de anión superóxido (Rudolf y col., 2004). Según Spallholz y col. (1998) el Se en algunas de sus

formas puede combinarse con el GSH para formar un anión (seleno-persulfuro) que finalmente genera radicales superóxido.

3.3. N-acetilcisteína (NAC)

3.3.1. Qué es y cómo funciona

La N-acetilcisteína (NAC), un derivado del aminoácido natural L-cisteína que no se encuentra en los alimentos, es un antioxidante tiólico que puede actuar por diferentes mecanismos. Es fácilmente desacetilado a L-cisteína, por lo que estimula la síntesis de GSH, y la actividad de enzimas como la glutatión reductasa (GR), responsable de la regeneración del GSH. Asimismo, protege frente al estrés oxidativo al unirse directamente mediante su grupo tiol a ERO, como el radical hidroxilo ($OH^{\bullet}$) y el peróxido de hidrógeno (H_2O_2) (Banaclocha, 2001). En clínica se usa para el tratamiento de enfermedades relacionadas con estrés oxidativo y/o deficiencia de glutatión, como la sobredosis por paracetamol, virus de inmunodeficiencia humana (VIH) y afectaciones hepáticas y cardíacas (Peña-Llopis y col., 2003).

Sevgiler y col. (2007) comprobaron la efectividad de la NAC para prevenir o reducir el estrés oxidativo en hígado de peces (*Cyprinus carpio)* expuestos a pesticidas

3.3.2. Microcistinas, Cilindrospermopsina y NAC

Puerto y col. (2009) estudiaron el efecto dosis-dependiente del pretratamiento con NAC (20, 44 y 96,8 mg NAC/pez/día) en intoxicación de tilapias expuestas por vía oral a un liofilizado de células conteniendo MC-LR. En los peces intoxicados, la suplementación con NAC contribuyó a restaurar los valores que se vieron alterados por la administración de MCs a niveles controles, reduciendo el estrés oxidativo a nivel hepático y renal. Sin embargo, debido a la propia actividad pro-oxidante de la sustancia, se demostró la aparición de efectos oxidantes a las dosis más elevada ensayada, de 96,8 mg NAC/pez/día, concluyéndose en la necesidad de poner especial atención en la dosis administrada.

Las mismas condiciones y modelo experimental sirvieron para el estudio de los cambios patológicos y ultraestructurales producidos en estos peces intoxicados con MCs, y el potencial papel protector de la NAC (Puerto y col., 2010). Los resultados mostraron que la

NAC ayudó a prevenir las lesiones histopatológicas en hígado, riñón, corazón, intestino y branquias de tilapias, de forma dosis-dependiente, alcanzando la mayor protección con 44 mg NAC/pez/día.

Por otro lado, Gutiérrez-Praena y col. (2012) evaluaron el efecto protector de NAC en tilapias intoxicadas por vía oral con CYN pura y CYN procedente de un liofilizado de células de *A. ovalisporum*. De los cambios producidos en los niveles de LPO, oxidación de proteínas, balance GSH/GSSG y GCS, así como en la actividad y expresión génica de GST y GPx, sugieren que el pretratamiento con NAC contribuye a restaurar los valores de estos biomarcadores alterados por la toxina. Por lo tanto, este antioxidante demuestra su utilidad igualmente como profilaxis y tratamiento de intoxicaciones de peces por CYN.

3.4. L-carnitina (LC)

3.4.1. Qué es y cómo funciona

La L-carnitina (β-hidroxi-γ-N-trimetilamonio-butirato; LC) es un cofactor esencial requerido en la β-oxidación lipídica para facilitar la importación de las largas cadenas activadas de ácidos grasos desde el citoplasma hasta el compartimiento de la matriz mitocondrial (Bilinski y Jonas, 1970). Esta amina cuaternaria soluble en agua se sintetiza a partir de lisina y metionina, con la ayuda de la vitamina C y otros compuestos secundarios producidos en organismos animales (Rebouche, 1991).

La cantidad de LC endógena puede ser suficiente para las necesidades normales de los animales de granja, aunque en determinadas condiciones son necesarios suplementos exógenos de carnitina, como en dietas con un alto nivel de grasas (Ozorio, 2009). No obstante, en varios peces los suplementos de LC podrían aumentar la eficiencia de utilización de energía a costa de la oxidación lipídica (Harpaz, 2005; Ozorio, 2009). Además, estos suplementos pueden permitir la utilización de dietas de alta energía con menos acumulación de grasa en el pescado (Burtle y Liu, 1994). La L-carnitina también tiene un papel protector contra las ERO, ejerciendo propiedades antioxidantes (Derin y col., 2004; Gómez-Amores y col., 2007). Hoy en día, el uso de suplementos de LC en las dietas de peces en acuicultura se recomienda: como promotor del crecimiento, en particular al ayudar en la utilización de acúmulos de grasa en la dieta y proporcionar así un efecto ahorrador de proteínas (Jayaprakas y col., 1996; Torreele y col., 1993); como protección contra los niveles tóxicos de amoníaco y

xenobióticos (Schreiber y col., 1997); como alivio del estrés relacionado con temperaturas extremas del agua, facilitando una mejor aclimatación a los cambios de temperatura del agua (Harpaz y col., 1999).

3.4.2. Cilindrospermopsina y L-carnitina

Guzmán-Guillén y col. (2012b) mostraron por primera vez los efectos antioxidantes beneficiosos de la suplementación dietética con LC, a dosis superiores a 400 mg/kg de pescado, sobre el estrés oxidativo inducido en diferentes órganos de tilapia (*O. niloticus*) expuestas a células liofilizadas de *A. ovalisporum* o CYN pura, sugiriendo la posibilidad de utilizar este antioxidante natural en la prevención del daño oxidativo producido en peces expuestos a CYN. La suplementación con L-carnitina redujo los niveles de LPO, la oxidación de proteínas, la oxidación del ADN y actividad de la NADPH oxidasa, y aumentó significativamente las actividades de algunas enzimas antioxidantes estudiadas (SOD, CAT, γ-GCS). Además, la LC previno del descenso de los secuestradores de radicales libres, como el glutatión reducido (GSH). Todos estos efectos darían lugar a una mejora del estado oxidativo en los peces expuestos a las células de cianobacterias conteniendo CYN y deoxy-CYN.

4. CONCLUSIONES

La aparición de floraciones de cianobacterias es un hecho cada vez más frecuente, siendo un problema de la calidad del agua en muchos países, debido a la producción de cianotoxinas que las convierten en un riesgo para la salud, tanto para humanos como para diversas especies animales. Hay evidencias de que estas cianotoxinas se acumulan en los tejidos de los organismos acuáticos que habitan las aguas contaminadas, pudiendo transferirlas a niveles superiores de la cadena alimentaria hasta llegar al ser humano.

Es recomendable la monitorización de la presencia de cianotoxinas y la investigación de las alteraciones inducidas en peces cuando se detecte una floración de cianobacterias, con el fin de conocer los riesgos asociados al consumo de pescado expuesto a estas toxinas, en un contexto de protección de la salud.

Del conjunto de los resultados recogidos en este trabajo, podemos concluir que existe una clara implicación del estrés oxidativo en el mecanismo de acción de ambas toxinas, MCs

y CYN, así como que existen una serie de sustancias antioxidantes con capacidad para prevenir y/o contrarrestar este daño (entre ellas, vitamina E, Selenio, NAC y LC). Por ello es importante llevar a cabo más estudios para establecer el papel de las nuevas sustancias contra las lesiones inducidas por cianotoxinas en peces.

Actualmente, no hay ninguna opinión de las Agencias internacionales o nacionales sobre la caracterización del riesgo derivada de la presencia de MCs y CYN en alimentos y aguas de consumo público. La problemática es mayor especialmente en el caso de la CYN, ya que al ser una toxina emergente, existen menos datos que permitan un correcto análisis del riesgo que puede suponer para el consumidor de aguas y alimentos contaminados. Estos análisis deben incluir estudios toxicológicos (dosis repetidas, crónicos, carcinogenicidad), así como evaluar la exposición de distintos grupos de la población.

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6. BIBLIOGRAFÍA

Atencio L, Moreno I, Jos A, Pichardo S, Moyano R, Blanco A, Cameán AM. Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to *Microcystis* under laboratory conditions. Toxicon 2008; 52: 1-12.

Atencio L, Moreno I, Jos A, Prieto AI, Moyano R, Blanco A, Cameán AM. Effects of dietary selenium on the oxidative stress and pathological changes in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Toxicon 2099; 53: 269-282.

Banaclocha MM. Therapeutic potential of N-acetylcysteine in age-related mitochondrial neurodegenerative diseases. Medical Hypotheses 2001; 5: 472-477.

Banker R., Carmeli S., Werman M., Teltsch B., Porat R., Sukenik A. Uracil moiety is required for toxicity of the cyanobacterial hepatotoxin cylindrospermopsin. Journal Toxicology Environmental Health A 2001; 62: 281-288.

Batcioglu K, Ozturk IC, Karagözler AA, Karatas F. Comparison of selenium level with GSH-Px activity in the liver of mice treated 7,12-DMBA. Cell Biochemistry and Function 2002; 20: 115-118.

Batcioglu K, Karagözler AA, Ozturk IC, Genc M, Bay A, Ozturk F, Aydogdu N. Comparison of chemopreventive effects of Vitamin E plus selenium versus melatonin in 7,12-dimethylbenz(a)anthracene-induced mouse brain damage. Cancer Detection and Prevention 2005; 29:54-58.

Bazin E, Mourot A, Humpage AR, Fessard V. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environmental and Molecular Mutagenesis 2010; 51: 251–259.

Bell JG, Cowey CB, Adrom JW, Pirie BJS. Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in Atlantic salmon parr (*Salmo salar*). Aquaculture 1987; 65: 43-54.

Bilinski E, Jonas REE. Effects of coenzyme A and carnitine on fatty acid oxidation in rainbow trout mitochondria. Journal of the Fisheries Research Board of Canada 1970; 27: 857-864.

Briand JF, Jacket S, Bernard C, Humbert JF. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. Veterinary Research 2003; 34:361-377.

Burtle GJ, Liu Q. Dietary L-carnitine and lysine affect channel catfish lipid and protein composition. Journal of The World Aquaculture Society 1994; 25: 169-174.

Campos A, Vasconcelos V. Molecular mechanisms of microcystin toxicity in animal cells. International Journal of Molecular Sciences 2010; 11: 268-287.

Carmichael WW, Azevedo SM, An JS, Molica RJ, Jochimsen EM, Lau S y col. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environmental Health Perspectives 2001; 109: 663-668.

Carmichael WW, Falconer IR. Diseases related to freshwater blue green algal toxins, and control measures. En: Falconer IR (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press, Londres 1993; 187-209.

Cazenave J, Bistoni MA, Pesce SF, Wunderlin DA. Differential detoxification and antioxidant response in diverse organs of *Corydoras paleatus* experimentally exposed to microcystin-RR. Aquatic Toxicology 2006; 76:1-12.

Chen H, Pellett LJ, Andersen HJ, Tappel AL. Protection by vitamin E, Selenium, and β-carotene against oxidative damage in rat liver slices and homogenate. Free Radical Biology & Medicine 1993; 14: 473-482.

Chong MWK, Wong BSF, Lam PKS, Shaw GR, Seawright AA. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon 2002; 40:205-211.

Chorus I, Bartram J. Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management, World Health Organization; 1999.

Davies KJA. Oxidative damage and repair: Introduction and overview. En: Davies KJA, ed. Oxidative damage and repair: Chemical, biological and medical aspects. Elmsford, HY: Pergamon Press 1991. xvii-xxvii.

De Figueiredo D, Azeiteiro UM, Esteves SM, Goncalves FJM, Pereira MJ. Microcystin-producing blooms- a serious global public health issue. Ecotoxicology and Environmental Safety 2004; 59:151-163.

Derin N, Izgut-Uysal VN, Agac A, Aliciguzel Y, Demir N. L-carnitine protects gastric mucosa by decreasing ischemiae reperfusion induced lipid peroxidation. Journal of Physiology and Pharmacology 2004; 55: 595-606.

Ding WX, Shen HM, Zhu HG, Ong CN. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environmental Research Section A 1998; 78:12-18.

Eisler, R. Handbook of chemical risk assessment: Health hazards to humans, plants, and animals. Boca Raton, FL: Lewis. 2000.

Falconer I, Hardy SJ, Humpage A, Froscio S, Tozer GJ, Hawkins PR. Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss Albino mice. Environmental Toxicology 1999; 14: 143-150.

Fessard V, Bernard C. Cell Alterations but no DNA strand breaks induced in vitro by cylindrospermopsin in CHO K1 cells. Environmental Toxicology 2003; 18:353-359.

Fisher WJ, Dietrich DR. Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). Toxicology and Applied Pharmacology 2000; 16: 73-81.

Gehringer MM, Govender S, Shah M, Downing TG. An investigation of the role of Vitamin E in the protection of mice against microcystin toxicity. Environmental Toxicology 2003a; 18: 142-148.

Gehringer MM, Downs KS, Downing TG, Naudé RJ, Shephard EG. An investigation into the effect of selenium supplementation on microcystin hepatotoxicity. Toxicon 2003b; 41(4):451-8.

Gómez-Amores L, Mate A, Miguel-Carrasco JL, Jiménez L, Jos A, Cameán AM, Revilla E, Santa-María C, Vázquez CM. L-Carnitine attenuates oxidative stress in hypertensive rats. The Journal of Nutritional Biochemistry 2007; 18: 553-540.

Gutiérrez-Praena D, Jos A, Pichardo S, Cameán AM. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory conditions: influence of the exposure way and the time of sacrifice. Aquatic Toxicology 2011a; 105: 100-106.

Gutiérrez-Praena D, Jos A, Pichardo S, Cameán AM. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure Cylindrospermopsin. Ecotoxicology and Environmental Safety 2011b; 74: 1567-1572.

Gutiérrez-Praena D, Puerto M, Prieto AI, Jos Á, Pichardo S, Vasconcelos V, Cameán AM. Protective role of dietary N-acetylcysteine on the oxidative stress induced by cylindrospermopsin in tilapia (*Oreochromis niloticus*). Environmental Toxicology and Chemistry. 2012; 31: 1548-55.

Guzmán-Guillén R, Prieto AI, Vasconcelos VM, Cameán AM. Cyanobacterium producing Cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed Tilapia (*Oreochromis niloticus*). Chemosphere 2012a Oct 13. http://dx.doi.org/10.1016/j.chemosphere.2012.09.027.

Guzmán-Guillén R, Prieto AI, Vázquez CM, Vasconcelos V, Cameán AM. Uso de L-carnitina para proteger a los peces de la intoxicación por cilindrospermopsina Número de solicitud de Patente: P201201151. 2012b.

Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal 1984; 219: 1-14.

Harada K, Ohtani I, Iwamoto K, Suzuki M, Watanabe MF, Watanabe M, Terao K. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 1994; 32: 73-84.

Harpaz S, Becker K, Blum R. The effect of dietary L-carnitine supplementation on cold tolerance and growth of ornamental cichlid fish (*Pelvicachromis pulcher*)—preliminary results. Journal of Thermal Biology 1999; 24: 57-62.

Harpaz S. Carnitine and its attributed functions in fish culture and nutrition—a review. Aquaculture 2005; 249: 3-21.

Hawkins PR, Runnegar MTC, Jackson ARB, Falconer IR. Severe hepatotoxicity caused by the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raja isolated from a domestic water supply reservoir. Applied and Environmental Microbiology 1985; 50: 1292-1295.

Hawkins PR, Chandrasena NR, Jones GJ, Humpage AR, Falconer IR. Isolation and toxicity of *Cylindrospermposis raciborskii* from an ornamental lake. Toxicon 1997; 35: 341-346.

Hiller S, Krock B, Cembella A, Luckas B. Rapid detection of cyanobacterial toxins in precursor ion mode by liquid chromatography tandem mass spectrometry. Journal of Mass Spectrometry 2007; 42: 1238-1250.

Hilton JW, Hodson PV, Slinger SJ. The requirement and toxicity of selenium in rainbow trout (*Salmo gairdneri*). The Journal of Nutrition 1980; 110: 2527-2535.

http:www.iarc.fr/

Huang S, Weng YM, Huang CH. Lipid peroxidation in sarcoplasmic reticulum and muscle of tilapia is inhibited by dietary vitamin E supplementation. Journal of Food Biochemistry 2004; 28: 101-111.

Huang C, Huang S. Effect of dietary vitamin E on growth, tissue lipid peroxidation, and liver glutathione level of juvenile hybrid tilapia, *Oreochromis niloticus* x *O. aureaus*, fed oxidized oil. Aquaculture 2004; 237:381-389.

Humpage AR, Fenech M, Thomas P, Falconer IR. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutation Research 2000; 472:155-161.

Humpage AR, Falconer IR. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environmental Toxicology 2003; 18: 94-103.

Humpage A. Toxin types, toxicokinetics and toxicodynamics. En: Hudnel, H.K. (Ed.), Cyanobacterial harmful algal blooms state of the science and research needs. Springer Press, New York 2008; 619: 383-415.

Jayaprakas V, Sambhu C, Sunil Kumar S. Effect of dietary L-carnitine on growth and reproductive performance of male *Oreochromis mossambicus* (Peters). Fishery Technology 1996; 33: 84-90.

Jones DP, Kagan VE, Aust SD, Reed DJ, Omaye ST. Impact of nutrients on cellular lipid peroxidation and antioxidant defense system. Fundamental and Applied Toxicology 1995; 26: 1-7.

Jos A, Pichardo S, Prieto AI, Repetto G, Vázquez CM, Moreno IM, Cameán AM. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. Aquatic Toxicology 2005; 72: 261-271.

Kardinaal WEA, Visser PM. Dynamics of cyanobacterial toxins. Sources of variability in microcystis concentrations. En: Harmful Cyanobacteria (Huisman J, Marthijs CP y Visser PM, Eds). Springer, Dordrecht, The Netherlands. 2005; 41-64.

Kinnear S. Cylindrospermopsin: a decade of progress on bioaccumulation research. Marine Drugs 2010; 8: 542-564.

Kiss T, Vehovszky A, Hiripi L, Kovacs A, Voros L. Membrane effects of toxins isolated from a cyanobacterium. *Cylindrospermopsis raciborskii*, on identified molluscan neurones. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 2002; 131: 167-176.

Li R, Carmichael WW, Brittain S, Eaglesham GK, Shaw GR, Liu Y, Watanabe MM. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata*. Journal of Phycology 2001; 37: 1121-1126.

Li X, Chung I, Kim J, Lee J. Sub-chronic oral toxicity of microcystin in common carp (*Cyprinus carpio L.*) exposed to *Microcystis* under laboratory conditions. Toxicon 2004; 44: 821-827.

Li L, Xie P, Chen J. In vivo studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the phytoplanktivorous bighead carp i.p.-injected with extracted microcystins. Toxicon 2005a; 46: 533-545.

Li X, Chung I, Kim J, Lee J. Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and has no effect on lipid peroxidation. Comparative Biochemistry and Physiology - Part C 2005b; 141: 292-296.

Li L, Xie P, Li S, Qiu T, Guo L. Sequential ultrastructural and biochemical changes induced in vivo by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitris*. Comparative Biochemistry and Physiology - Part C 2007; 146: 357-367.

Lin Y, Shiau S. The effects of dietary selenium on the oxidative stress of grouper, *Epinephelus malabaricus*, fed high copper. Aquaculture 2007; 267: 38-43.

Magalhaes VF, Marinho MM, Domingos P, Oliveira AC, Costa SM, Azevedo LO, Azevedo SMFO. Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). Toxicon 2003; 42: 289-295.

Miller LL, Wang F, Palace VP, Hontela A. Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. Aquatic Toxicology 2007; 83: 263-271.

Mohamed ZA, Carmichael WW, Hussein AA. Estimation of microcystins in the freshwater fish *Oreochromis niluticus* in an Egyptian fish farm containing a *Microcystis* bloom. Environmental Toxicology 2003; 18: 137-141.

Mohamed ZA, Hussein AA. Depuration of microcystins in tilapia fish exposed to natural populations of toxic cyanobacteria: a laboratory study. Ecotoxicology and Environmental Safety 2006; 63: 424-429.

Moreno IM, Maraver J, Aguete EC, Leao M, Gago-Martínez A, Cameán AM. Decomposition of microcystin-LR, microcystin-RR, and microcystin-YR in water samples submitted to in vitro dissolution tests. Journal of Agricultural and Food Chemistry 2004; 52: 5933-5938.

Moreno I, Pichardo S, Jos A, Gómez-Amores L, Mate A, Vázquez CM, Cameán AM. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 2005; 45: 395-402.

Neumann C, Bain P, Shaw G. The in vitro toxicology of deoxycylindrospermopsin. En: Australian Research Network for Algal Toxin Workshop. Moreton Bay Research Station, Australia 2005; 9-11.

Niki E. Antioxidant compounds. En: Davies KJA, ed. Oxidative damage and repair: Chemical, biological and medical aspects. Elmsford, NY: Pergamon Press 1991; 57-64.

Norris RL, Eaglesham GK, Pierens G, Shaw GR, Smith MJ. Chiswell RK, Seawright AA, Moore MR. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii***.** Environmental Toxicology 1999; 14: 163-165.

Norris RLG, Seawright AA, Shaw GR, Senogles P, Eaglesham GK, Smith MJ, Chiswell RK, Moore MR. Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 2002; 40: 471-476.

Ohlendorf HM. Selenium. En Noninfectious diseases of wildlife, eds. Fairbrother A, Locke LN and Hoff GL. Ames: Iowa State University Press 1996; 128-140.

Ohtani I, Moore RE, Runnegar M. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. American Chemical Society 1992; 114: 7941-7942.

Orun I, Ates B, Selamoglu Z, Yazlak H, Ozturk E, Yilmaz I. Effects of various sodium selenite concentrations on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss*). Fresenius Environmental Bulletin 2005; 14: 18-22.

Ozório ROA. Dietary L-carnitine supplementation to cultivated fish: a minireview. Current Nutrition & Food Science. 2009; 5: 40-48.

Padisák J. *Cylindrospermopsis raciborskii* (Woloszynka) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Archiv für Hydrobiologie 1997; 107: 563-593.

Park HD, Watanabe MF. Toxic *Microcystis* in eutrophic lakes. En: Watanabe MF, Harada K, Carmichael WW, Fujiki H (Eds.), Toxic Microcystis. CRC Press, Boca Raton, 1995; 57-77.

Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Marine Drugs 2010; 8: 1650–1680.

Peña-Llopis S, Ferrando MD, Peña JB. Fish tolerase to organophosphate-induced oxidative stress is dependen ton the glutathione metabolism and enhanced by *N*-acetylcysteine. Aquatic Toxicology 2003; 65: 337-360.

Pinho GLL, da Rosa CM, Maciel FE, Bianchini A, Yunes JS, Proença LA, Monserrat JM. Antioxidant responses after microcystin exposure in gills of an estuarine crab species pre-treated with vitamin E. Ecotoxicology and Environmental Safety 2005; 61: 361-365.

Pizzolon L. Importancia de las Cianobacterias como Factor de Toxicidad en las Aguas Continentales. Interciencia 1996; 21: 239-245.

Prieto AI, Jos A, Pichardo S, Moreno I, Cameán AM. Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (*Oreochromis* sp.). Aquatic Toxicology 2006; 77: 314-321.

Prieto AI, Pichardo S, Jos A, Moreno I, Cameán AM. Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. Aquatic Toxicology 2007; 84: 337-345.

Prieto AI, Pichardo S, Jos A, Moreno I, Cameán AM. Protective role of vitamin E on the Microcystin-induced oxidative stress in tilapia fish (*Oreochromis niloticus*). Environmental Toxicology and Chemistry. 2008; 27: 1152-1159.

Prieto AI, Jos A, Pichardo S, Moreno I, de Sotomayor MA, Moyano R, Blanco A, Cameán AM. Time-dependent protective efficacy of Trolox (vitamin E analog) against microcystin-induced toxicity in tilapia (*Oreochromis niloticus*). Environmental Toxicology 2009; 24(6): 563-79.

Puerto M, Prieto AI, Pichardo S, Moreno I, Jos A, Moyano R, Cameán AM. Effects of dietary *N*-acetylcysteine (NAC) on the oxidative stress induced in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Environmental Toxicology and Chemistry 2009; 28: 1679-1686.

Puerto M, Prieto AI, Jos A, Moreno I, Moyano R, Blanco A, Cameán AM. Dietary N-acetylcysteine (NAC) prevents histopathological changes in tilapias (*Oreochromis niloticus)* exposed to a microcystin-producing cyanobacterial water bloom. Aquaculture 2010; 306: 35-48.

Puerto M, Gutiérrez-Praena D, Prieto AI, Pichardo S, Jos A, Miguel-Carrasco JL, Vázquez CM, Cameán AM. Subchronic effects of cyanobacterial cells on the transcription of antioxidant enzyme genes in tilapia (*Oreochromis* sp.). Ecotoxicology 2011a; 20:479-490.

Puerto M, Jos MA, Pichardo S, Gutiérrez-Praena D, Cameán AM. Acute effects of pure Cylindrospermopsin on the activity and transcription of antioxidant enzymes in Tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 2011b; 20: 1852-1860.

Puerto M, Jos A, Pichardo S, Moyano R, Blanco A, Cameán A. Acute exposure to pure Cylindrospermopsin results in oxidative stress and pathological alterations in tilapia (*Oreochromis niloticus*). Environmental Toxicology 2012. Feb 13. doi: 10.1002/tox.21764

Qiu T, Xie P, Ke Z, Li L, Guo L. In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake. Toxicon 2007; 50: 365-376.

Quesada A. Cianobacterias en Aguas de Consumo y Recreo: Un Problema de Todos. En: Ponencia realizada por el Centro de Estudio y Experimentación de obras públicas. CEDEX, Madrid, 2006.

Rebouche CJ. Ascorbic acid and carnitine biosynthesis. American Journal of Clinical Nutrition 1991; 54: 1147S-1152S.

Rudolf E, Radocha J, Cervinka M, Cerman J. Combined effect of sodium selenite and campthotecin on cervical carcinoma cells. Neoplasma 2004; 51: 127-135.

Runnegar MT, Kong SM, Zhong YZ, Ge JL, Lu SC. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical and Biophysical Research Communications. 1994; 201: 235-241.

Runnegar M, Berdt N, Kong SM, Lee EYC, Zhang L. In vivo and in vitro binding of microcystin to protein phosphatases 1 and 2A. Biochemical and Biophysical Research Communications 1995a; 216: 162-169.

Runnegar M., Kong SM, Zhong YZ, Lu SC. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical Pharmacology 1995b; 49: 219-225.

Runnegar MT, Xie C, Snider BB, Wallace GA, Weinreb SM, Kuhlenkamp J. In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicological Sciences 2002; 67: 81-87.

Savanian A. Phospholipase A2: a secondary membrane antioxidant. En: Savanian A, ed. Lipid peroxidation in biological systems. Champaign, IL: American Oil Chemists' Society 1988; 84-99.

Schreiber S, Becker K, Bresler V, Fishelson L. Dietary L-carnitine protects the gills and skin of guppies (*Poecilia reticulata*) against anionic xenobiotics. Comparative Biochemestry and Physiology 1997; 117: 99-102.

Seawright AA, Nolan CC, Shaw GR, Chiswell RK, Norris RL, Moore MR, Smith MJ. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). Environmental Toxicology 1999; 14: 135-142.

Seifert M, McGregor G, Eaglesham G, Wickramasinghe W, Shaw G. First evidence for the production of cylindrospermopsin and deoxycylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei*. Harmful Algae 2007; 6: 73-80.

Sevgiler Y, Piner P, Durmaz H, Üner N. Effects of *N*-acetylcysteine on oxidative responses in the liver of fenthion exposed *Cyprinus carpio.* Pesticide Biochemistry and Physiology 2007; 87: 248-254.

Shiau S, Lin Y. Vitamin requirements of tilapia: A review. *Proceedings*, VIII Simposium Internacional de Nutrición Acuícola, Monterrey, México, 2006; 129-138.

Sivonen K, Jones G. Cyanobacterial toxins. En: Chorus I, Bartram J. (Eds.), Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. E and FN Spon, London, 1999; 41-111.

Spallholz JE, Reid T, Boylan LM, Shriver,B. Selenium free radical chemistry: applications for pharmaceuticals. En: Proceedings of the Sixth International Symposium on Selenium and Tellurium (STDA), Scottsdale, AZ, 1998; 47-56.

Tencalla F, Dietrich D. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). Toxicon 1996; 35: 583-595.

Teneva I, Dzhambazov B, Koleva L, Mladenov R, Schirmer K. Toxic potential of five freshwater Phormidium species (Cyanoprokaryota). Toxicon 2005; 45:711-725.

Terao K, Ohmori S, Igarashi K, Ohtani I, Watanabe MF, Harada KI, Ito E, Watanabe M. Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 1994; 32: 833-843.

Thorarinsson R, Landolt ML, Elliott DG, Pascho RJ, Hardy RW. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in Chinook salmon (*Oncorhyndhus tshawytscha*). Aquaculture 1994; 121: 343-358.

Tocher DR, Mourente G, Van der Eecken A, Evjemo JO, Díaz E, Bell JG, Geurden I, Lavens P, Olsen Y. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile

turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition 2002; 8: 195-207.

Tocher DR, Mourente G, Van der Eecken A, Evjemo JO, Díaz E, Wille M, Bell J, Olsen Y. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International 2003; 11: 195-216.

Torreele E, Van der Sluizen A, Verreth J. The effect of dietary L-carnitine on the growth performance in fingerlings of the African catfish *Clarias gariepinus* in relation to dietary lipid. British Journal of Nutrition 1993; 69: 289-299.

Towner RA, Sturgeon SA, Hore KE. Assessment of in vivo oxidative lipid metabolism following acute microcystin- LR-induced hepatotoxicity in rats. Free Radical Research 2002; 36 (1): 63-71.

Wiegand C, Pflugmacher S, Oberemm A, Meems N, Beattie KA, Steinberg CEW, Codd GA. Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). Environmental Toxicology 1999; 14: 89-95.

Wilhelm Filho D. Fish antioxidant defenses. A comparative approach. Brazilian Journal of Medical and Biological Research 1996; 29: 1735-1742.

Xie L, Yokoyama A, Nakamura K, Park H. Accumulation of microcystins in various organs of the freshwater snail *Sinotaia histrica* and three fishes in a temperate lake, the eutrophic Lake Suwa, Japan. Toxicon 2007; 49: 646-652.

Zimba PV, Khoo L, Gaunt P, Carmichael WW, Brittain S. Confirmation of catfish mortality from *Microcystis* toxins. Journal of Fish Diseases 2001; 24: 41-47.

Zurawell, R. W., Chen, H., Burke, J. M., & Prepas, E. E. Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health - Part B: Critical Reviews 2005; 8: 1-37.

PATENTE / PATENT

Ana Mª Cameán Fernández, Carmen M Vázquez, **Remedios Guzmán Guillén**, Ana Isabel Prieto Ortega, Isabel Moreno Navarro, Ángeles Mencía Jos Gallego, Silvia Pichardo Sánchez, María Puerto Rodríguez, Daniel Gutiérrez Praena, Mª Rosario Moyano Salvago, Alfonso Blanco Rodríguez

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(71) Solicitantes:

UNIVERSIDAD DE SEVILLA (50.0%)
OTRI-Pabellón de Brasil, Paseo de las Delicias s/n
41013 Sevilla ES y
UNIVERSIDAD DE CÓRDOBA (50.0%)

(72) Inventor/es:

CAMEAN FERNÁNDEZ, Ana María;
VÁZQUEZ CUETO, Carmen María;
GUZMÁN GUILLÉN, Remedios;
PRIETO ORTEGA, Ana Isabel;
MORENO NAVARRO, Isabel;
JOS GALLEGO, Ángeles Mencia;
PICHARDO SÁNCHEZ, Silvia;
PUERTO RODRÍGUEZ, María;
GUTIÉRREZ PRAENA, Daniel;
MOYANO SALVAGO, M. Rosario y
BLANCO RODRÍGUEZ, Alfonso

(74) Agente/Representante:

GONZÁLEZ CARVAJAL, Ramón

(54) Título: **Uso de L-Carnitina para proteger a los peces de la intoxicación por cilindrospermopsina**

(57) Resumen:

La presente invención se refiere al uso de una composición que comprende L-Camitina (LC) para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a Cilindrospermopsina (CYN). También se refiere al uso de la citada composición en la recuperación de las alteraciones histopatológicas producidas en los tejidos de la lista que comprende hígado, riñón, corazón, branquias y/o tracto gastrointestinal. Además, dicha composición se utiliza para la fabricación de un alimento funcional, un complemento vitamínico, o un complemento nutricional.

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DESCRIPCIÓN

Uso de L-Carnitina para proteger a los peces de la intoxicación por Cilindrospermopsina

La presente invención se refiere al uso de una composición que comprende L-Carnitina (LC) para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a Cilindrospermopsina (CYN). También se refiere al uso de la citada composición en la recuperación de las alteraciones histopatológicas producidas en los tejidos de la lista que comprende hígado, riñón, corazón, branquias y/o tracto gastrointestinal. Además, dicha composición se utiliza para la fabricación de un alimento funcional, un complemento vitamínico, o un complemento nutricional.

ESTADO DE LA TÉCNICA ANTERIOR

La L-carnitina (L-3-hidroxi-4-N,N,N-trimetilaminobutirato) (LC) es un derivado aminoacídico sintetizado a partir de los aminoácidos esenciales lisina y metionina con la ayuda de la vitamina C y otros compuestos secundarios producidos por el cuerpo (Harpaz S, Aquaculture 249: 3-21, 2005) que está presente en la mayoría de las especies animales y en muchos microorganismos y plantas. La LC se encuentra ampliamente distribuido por todo el organismo, aunque se presenta en mayores cantidades en el corazón y en el músculo esquelético (Rebouche C. J. FASEB J. 6: 3379-3386,1992).

La función principal de este derivado aminoacídico consiste en actuar como cofactor en el transporte de ácidos grasos al interior de la mitocondria, donde se produce la β-oxidación de los mismos, para la obtención de energía metabólica (Bremer J. Physiol. Rev. 63: 1420-1480, 1983), desempeñando un papel crucial en el metabolismo de los ácidos grasos.

El 75% de la cantidad de LC requerida por el organismo proviene de la dieta. El resto se sintetiza endógenamente en el hígado y en menor cantidad en el riñón y cerebro, a partir de los aminoácidos lisina y metionina, como indicamos con anterioridad (Tanphaichitr V. y Broquist H. P. J. Biol. Chem. 248: 2176-2181, 1973).

Aunque no se conocen con exactitud los mecanismos de acción de la LC, su uso beneficioso en algunas patologías cardiovasculares (Ferrari R *et al.*, Ann. N.Y. Acad. Sci 033: 79-91, 2004; Kendler B.S. J. Cardiovasc. Nurs. 21: 9-16, 2006) se cree debido a sus propiedades antioxidantes y antiinflamatorias (Savica V. *et al.*, Semin. Nephrol. 24: 464-468, 2004; Laviano A. *et al.*, Curr. Opin. Clin. Nutr. Metab. Care 9: 442-448, 2006).

Son múltiples los usos descritos para el uso de la LC sola o en combinación con otras sustancias. Así, distintos trabajos y patentes de investigación la describen para el tratamiento de astenias y anorexias (FR 4465), en combinación con citrato de lisina tribásico y de vitamina C; como complemento nutricional en sujetos convalecientes, personas de edad, deportistas, mujeres embarazadas o cualquier persona asténica que presente perturbaciones funcionales musculares (ES 2 043 062); alteraciones cardiacas asociada a la hipertensión arterial (P200901543); trastornos cerebrales centrales y periféricos, cardiacos, vasculopáticos, y trastornos del aprendizaje o relacionados con la edad, en combinación con el coenzima Q_{10} (ES 2 269355 T3); prevención de disfunciones hepáticas y biliares frente a lesiones inducidas por agentes hepatotóxicos exógenos y endógenos por su intenso efecto antioxidante y su eficaz mejora de la circulación periférica y función cardiaca, en combinación con un extracto de *Silybum marianum* (ES 2 225 576 T3); prevención de la fibrosis renal (P201100709) y tratamiento de todas las formas de nefropatías mediante una composición de L-acetil carnitina y L-propionil carnitina (ES 2 254 206 T3); prevención ó tratamiento de alteraciones de órganos que realizan función metabólica intensa (hígado, riñón, sistema cardiovascular y cerebro) como hepatosis, nefropatías y lesiones cardiovasculares o cerebrales provocadas por sustancias tóxicas, en combinación con glutation (ES 2 232 443 T3); la efectividad terapéutica de acetil L-carnitina en neuropatías periféricas (ES 2 039 262), y en el tratamiento de pacientes aquejados de metabolismo cerebral deficiente tal y como se produce en la demencia senil y pre-senil y enfermedad de Alzheimer (4.346.107), promoción de la regeneración del tejido nervioso, inhibición de la degeneración neuronal y trastornos cerebrales producidos por el envejecimiento y el uso de fármacos neurotóxicos, para mejorar el proceso de aprendizaje y memoria y para el tratamiento del coma y shock (ES 2 078 019 T3; ES 2 043 608; ES 2 207

287 T3); trastornos del aprendizaje en niños que sufren de déficit de atención/hiperactividad (ADHD), en combinación con la sal interna de acetil-L-carnitina y una huperzina (ES 2 237 591 T3). Además, por su mecanismo de acción, es útil para trastornos del metabolismo de los lípidos y formas alérgicas y para la activación de las defensas inmunes, en combinación con polisacáridos (ES 2 222 388 T3); propionil L-carnitina es útil para la prevención y/o tratamiento de trastornos debidos a un metabolismo lipídico anormal, tales como la hipercolesterolemia, aterosclerosis, hiperlipidemias y obesidad, en combinación con quitosana (ES 2 197 890 T3). Ha demostrado utilidad frente a la citotoxicidad inducida por agentes inmunosupresores, en combinación con glicina (ES 2 269 356 T3); enfermedades oculares como el glaucoma (ES 2 040 106); isovaleril L-carnitina ha probado ser útil para la prevención y curación de osteoporosis (ES 2 247 005 T3); enfermedades de la piel como ictiosis, psoriasis y dermatosis inducidas por una queratinización defectuosa como caspa, acné e hiperkeratosis palmar y plantar, en combinación con ácido glicólico (ES 2 088 655 T3); infecciones micóticas producidas por *Cryptococcus neoformans*, *Candida albicans* y *Aspergillus* sp. (ES 2 101 998 T3) y son útiles para el tratamiento de infecciones intestinales, gastritis tipo B y úlceras duodenales humanas producidas por *Campylobacter* sp. y *Helicobacter* sp. en combinación con alcoholes alifáticos de cadena larga (ES 2 093 395 T3).

Concretamente en peces, la LC y derivados se ha empleado con diferentes propósitos: promotor del crecimiento, prestando protección frente a concentraciones tóxicas de amoniaco y xenobióticos, aliviar el estrés inducido por temperaturas extremas,aumentando la reproducción etc., que han sido revisados en la literatura científica (Harpaz S, Aquaculture 249: 3-21, 2005), con resultados a veces contradictorios. Se ha demostrado que L-carnitina, como aditivo alimentario, mejora el crecimiento (Becker *et al.*, Aquaculture 174:313-332, 1999; Yang *et al.*, Aquaculture 342:48-55, 2012), la función reproductora (CH19950003453 19951207), el desarrolllo y la supervivencia de peces y almejas (JP19800024343 19800227) y la resistencia al estrés generado por frío en cíclidos (Harpaz *et al.*, J. Therm. Biol. 24:57-62, 1999; CH19980000400 19980219). Además se ha demostrado que ejerce efecto protector frente a la penetración de xenobióticos aniónicos en guppies (Schreiber *et al.*, Comp. Biochem. Physiol. 117C:99-102, 1997). No existiendo, hasta el momento, nada

publicado de LC con respecto a Cilindrospermopsina (CYN) objeto de esta invención y por tanto, siendo novedoso aportar el uso de la LC con respecto al tratamiento, prevención y/o recuperación de efectos inducidos por CYN en peces.

Por otro lado, la CYN es una toxina producida por cianobacterias tóxicas presentes en aguas superficiales, pertenecientes al menos a seis géneros, siendo las especies identificadas en los momentos actuales *Cylindrospermopsis raciborskii*, *Anabaena bergii, Aphanizomenon ovalisporun, Aphanizomenon flos-aquae, Umezakia natans, y Raphidiopsis curvata,* entre otras. CYN se aisló por primer vez de un cultivo de *Cylindrospermopsis raciborskii*, obtenido de los reservorios de agua de bebida que surtían a la población de Palm Island, Queensland, Australia (Ohtani I *et al.,* J. Am. Chem. Soc. 144:7941-7942, 1992), Se ha comprobado su acumulación en peces y crustáceos, afectando a la calidad y seguridad de este tipo de alimentos y suponiendo un riesgo potencial para el consumidor. En comparación con los mamíferos, los estudios sobre efectos tóxicos de CYN en peces son muy escasos, destacando que puede afectar no sólo al hígado sino también al riñón, corazón, branquias, y tracto gastrointestinal. Las cianobacterias constituyen parte de la dieta de diversos ciprinídos y cíclidos, como es el caso, por ejemplo, de las Tilapias (*Orechromis, sp.*). La Tilapia *(Oreochromis sp.*) es uno de los pescados que más rápidamente se ha introducido en acuicultura, por la facilidad que presenta su manejo, gran capacidad de adaptación a condiciones adversas y fácil reproducción; sus distintas variedades son filtradoras y consumidoras de cianobacterias y en Europa se está despertando un gran interés por su cultivo.

Como mecanismo de acción tóxica más aceptado, la CYN está considerada un citotoxina general que bloquea la síntesis de proteínas en células eucariotas de mamíferos y plantas (Terao K. *et al*,, Toxicon 32:833-843, 1994; Runnegar M.T. *et al.* Biochem. Pharmacol. 49:219–225, 1995) y disminuye los contenidos de Glutation (GSH). La disminución de GSH no parece conllevar a un incremento del estrés oxidativo en la célula, sugiriéndose que no es un mecanismo primario de la toxicidad de CYN. Sin embargo, recientemente, se ha comprobado la participación directa del

estrés oxidativo en la patogénesis de CYN en peces (Gutierrez-Praena D. *et al.*, Aquat. Tox. 105:100-106, 2011; Puerto M. *et al.*, Ecotoxicology 20: 479-490, 2011), detectándose un aumento en la producción de especies reactivas de oxígeno (ROS), lipoperoxidación (LPO), oxidación de proteínas y de ADN así como cambios en la actividad de diversas enzimas antioxidantes en peces. Los escasos estudios toxicológicos realizados hasta la actualidad han conducido al establecimiento de una Ingesta Diaria Tolerable (IDT) provisional de 0,03 µg/Kg/día de CYN, y la propuesta de un valor guía provisional de 1 µg/L de CYN en aguas de bebida.

Actualmente no existe un tratamiento antidótico específico en casos de intoxicación por CYN y sus epímeros procedentes de cianobacterias. Hasta la fecha, tan sólo el uso de la N-acetilcisteína ha demostrado actividad protectora en peces frente a la intoxicación por CYN (Gutierrez-Praena D. *et al.*, Aquat. Toxicol. 31: 1-8, 2012; P201101162, Cameán Fernández A. *et al.*, 2011). Teniendo en cuenta la ubicuidad de esta toxina, se hace necesario recuperar peces que presenten alteraciones histopatológicas con diferentes niveles de afección, que pueden impedir el ciclo de vida normal de las especies afectadas.

EXPLICACIÓN DE LA INVENCIÓN

La presente invención se refiere al uso de una composición que comprende LC para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

En tilapias (*Oreochromis sp.*) expuestas a dosis únicas y repetidas de CYN se inducen estrés oxidativo y alteraciones patológicas. En concreto, se ha comprobado aumento de los niveles de peroxidación lipídica (LPO) y de oxidación de ADN, disminución de los niveles de GSH, en diferentes órganos (hígado, riñón) de peces expuestos a 400 µg CYN/kg pez de forma aguda por vía oral. Así mismo, CYN induce múltiples alteraciones histopatológicas en órganos diversos: hígado, riñón, tracto gastrointestinal, corazón y branquias.

La LC administrada en esta invención se muestra efectiva manteniendo el estado de salud del pez, previniendo daños causados por la toxina y/o mejorando los efectos tóxicos inducidos por CYN en diversos órganos de tilapias intoxicadas.

Además, el uso de LC como aditivo alimentario no sólo mejora los niveles de GSH en hígado y riñón, sino que por su propia actividad antioxidante es capaz de disminuir la lipoperoxidación (LPO) (hígado, riñón), la oxidación de ADN (hígado, riñón) inducida por CYN, y prevenir y recuperar las lesiones histopatológicas inducidas en múltiples órganos como hígado, riñón, corazón, tracto gastrointestinal y branquias.

En este sentido, un primer aspecto de la presente invención se refiere al uso de una composición que comprende LC para la elaboración de un medicamento útil en el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

La composición de la presente invención comprende, al menos, LC. El medicamento está compuesto, al menos, por la composición anterior. La LC, sus sales, derivados farmacéuticamente aceptables o sus profármacos, se formulan en una composición farmacéutica apropiada, en la cantidad terapéuticamente efectiva, junto con uno o más vehículos, adyuvantes o excipientes farmacéuticamente aceptables. El medicamento se emplea para el tratamiento de los efectos tóxicos en peces expuestos a CYN.

Por un "derivado farmacéuticamente aceptable" se entiende cualquier sal, farmacéuticamente aceptable o cualquier otro compuesto que después de su administración, es capaz de proporcionar (directa o indirectamente) LC.

Un "vehículo farmacéuticamente aceptable" se refiere a aquellas sustancias, o combinación de sustancias, conocidas en el sector farmacéutico, utilizadas en la elaboración de formas farmacéuticas de administración e incluye sólidos o líquidos, disolventes, tensioactivos, etc.

El término "tratamiento" tal como se entiende en la presente invención supone combatir los efectos tóxicos para estabilizar el estado de toxicidad de los

individuos. El medicamento se emplea también para la prevención de los efectos tóxicos ocasionados a los peces expuestos a CYN. El término "prevención" tal como se entiende en la presente invención consiste en evitar la aparición de efectos tóxicos en peces expuestos a CYN. En este caso, previamente a la intoxicación por CYN, los peces están protegidos por un aumento de las defensas antioxidantes producido por la acción de la LC. El medicamento también se emplea para la recuperación de los efectos tóxicos ocasionados en peces expuestos a CYN.

El término "efectos tóxicos" tal como se entiende en la presente invención hace referencia a la consecuencia derivada de la exposición del pez a la CYN, es decir, la aparición de diversos efectos adversos, como por ejemplo, un daño celular que ocasiona un daño en los tejidos biológicos, lo que a su vez puede provocar un cambio en las funciones fisiológicas y en el metabolismo celular.

La CYN es una toxina de naturaleza alcaloide, en cuya estructura interviene un grupo tricíclico guanidinio unido a hidroximetiluracilo. Es producida por al menos seis géneros de cianobacterias, que se encuentran ampliamente distribuida en aguas tropicales y subtropicales. Pueden existir dos posibles epímeros de forma natural, cilindrospermopsina (CYN) y 7-epicilindrospermopsina, ambos tóxicos; la completa pérdida del grupo uracilo elimina la toxicidad de CYN. En reservas naturales de agua se ha descrito otra variante, la 7-desoxicilindrospermopsina, cuya toxicidad apenas está establecida, siendo menos tóxica en ratón que CYN.

Un segundo aspecto de la presente invención es el uso de una composición que comprende L-Carnitina para la elaboración de un medicamento útil en la recuperación de efectos tóxicos en peces expuestos a CYN. El término "recuperación" hace referencia a la desaparición de los efectos tóxicos causados por la intoxicación con CYN. Esta recuperación supone la reversión total de los daños causados en los tejidos del pez, recuperando de esta forma las funciones normales de los órganos afectados.

En una realización preferida de la presente invención, los efectos tóxicos son alteraciones histopatológicas. El término "alteraciones histopatológicas" tal como se entiende en la presente invención son daños producidos en los tejidos biológicos del pez. Estos daños son detectados por medio del análisis a nivel

microscópico de las estructuras patológicas de las diferentes muestras obtenidas, sin excluir otras técnicas de detección.

Una realización aún más preferida de la invención, es el uso donde las alteraciones histopatológicas son producidas en al menos uno de los tejidos de la lista que comprende hígado, riñón, corazón, branquias o tracto gastrointestinal. Tal como se ha mencionado anteriormente, la CYN puede acumularse en el tejido hepático y también puede llegar a otros órganos utilizando la sangre como medio de dispersión, de esta forma, la toxina puede causar efectos tóxicos y/o alteraciones histopatológicas en los citados órganos. La recuperación de los tejidos afectados por las alteraciones histopatológicas es un aspecto destacable de la presente invención ya que puede suponer la curación de los peces cultivados, peces seleccionados por diversas características para la cría, peces de especies en peligro de extinción o cualquier otro tipo de pez que presente alteraciones histopatológicas en un grado reversible.

En otra realización más preferida de la presente invención, la LC se administra en una cantidad diaria de entre 400 y 880 mg por Kg de peso del pez. Esta administración se lleva a cabo durante al menos veintiún días. Preferiblemente la cantidad diaria incorporada a los peces es a partir de 400 mg por Kg de peso.

Esta composición, se puede administrar de distintas formas, entre ellas, pero sin limitarse, intraperitonealmente, oralmente, bucalmente, intramuscularmente o de forma subcutánea. Más preferiblemente se administra de forma oral o intraperitoneal. En otra realización más preferida la composición se presenta en una forma adaptada a la administración oral o intraperitoneal.

Preferiblemente los peces intoxicados están expuestos a más de 400 µg CYN/ kg de pez por vía oral en exposición única (intoxicación aguda).

Otra realización preferida de la presente invención, comprende el uso de la composición anteriormente descrita que además incluye excipientes farmacológicamente aceptables.

El término "excipiente" hace referencia a una sustancia que ayuda a la absorción de la sustancia activa (en la presente invención, LC), estabiliza dicha sustancia activa o ayuda a la preparación del medicamento en el sentido de darle consistencia o aportar sabores que lo hagan más agradable. Así pues, los excipientes podrían tener la función de mantener los ingredientes unidos como por ejemplo almidones, azúcares o celulosas, función de endulzar, función de colorante, función de protección del medicamento como por ejemplo para aislarlo del aire y/o la humedad, función de relleno de una pastilla, cápsula o cualquier otra forma de presentación como por ejemplo el fosfato de calcio dibásico, función desintegradora para facilitar la disolución de los componentes y su absorción en el intestino sin excluir otro tipo de excipientes no mencionados en este párrafo.

El término "excipiente farmacológicamente aceptable" hace referencia a que el excipiente esté permitido y evaluado de modo que no cause daño a los organismos a los que se administra.

En una realización más preferida de la invención, la composición comprende además otra sustancia activa.

En cada caso la composición se adaptará al tipo de administración utilizada, por ello, la composición de la presente invención se puede presentar bajo la forma de soluciones o cualquier otra forma de administración clínicamente permitida y en una cantidad terapéuticamente eficaz.

Otras realizaciones preferidas son el uso para la fabricación de un alimento funcional, el uso para la fabricación de un complemento vitamínico y otra más es el uso para la fabricación de un complemento nutricional.

La LC puede formar parte de un alimento funcional, complemento vitamínico, complemento nutricional o cualquiera de sus combinaciones. Tal como se entiende en la presente invención, un alimento funcional cumple una función específica como puede ser la de mejorar la salud de los peces. Para ello al alimento funcional se le puede agregar un complemento vitamínico y/o complemento nutricional. El alimento funcional, los complementos descritos o

cualquiera de sus combinaciones pueden administrarse junto con un pienso, formar parte de la composición del pienso o pueden administrarse de forma independiente.

En una realización preferida, de la presente invención, los peces son cultivados.

Se entiende por "peces cultivados" aquellos peces criados en piscifactorías, charcas o cualquier contenedor de agua de cualquier tamaño que permita la cría de peces y/o el engorde. Los peces cultivados pueden ser, sin limitar, peces destinados a la alimentación o a la cría de peces ornamentales.

En otra realización preferida, de la presente invención, los peces pertenecen al género *Oreochromis sp.*

Los peces pertenecientes a este género se conocen como Tilapias. Las Tilapias crecen en aguas cálidas dulces o saladas y tienen pocas exigencias respiratorias, rápido crecimiento y facilidad para la puesta. Los peces se pueden seleccionar, sin limitarse, a la lista que comprende *O. amphimelas, O. andersonii, O. angolensis, O. aureus, O. chungruruensis , O. esculentus, O. hunteri, O. ismailiaensis, O. jipe, O. karomo, O. karongae, O. korogwe, O. lepidurus, O. leucostictus, O. lidole, O. macrochir, O. malagarasi, O. mortimeri, O. mossambicus, O. mweruensis, O. niloticus* (Nile tilapia), *O. Pantani, O. pangani girigan, O. pangani pantani, O. placidus, O. placidus placidus, O. placidus ruvumae, O. rukwaensis, O. saka, O. salinicola, O. schwebischi, O. shiranus, O. shiranus chilwae, O. shiranus shiranus, O. spilurus, O. spilurus niger, O. spilurus percivali, O. spilurus spilurus, O. squamipinnis, O. tanganicae, O. upembae, O. urolepis, O. urolepis hornorum, O. urolepis urolepis u O. variabilis.* Más preferiblemente los peces pertenecen a la especie *O. niloticus* (Nile tilapia).

A lo largo de la descripción y las reivindicaciones la palabra "comprende" y sus variantes no pretenden excluir otras características técnicas, aditivos, componentes o pasos. Para los expertos en la materia, otros objetos, ventajas y características de la invención se desprenderán en parte de la descripción y en parte de la práctica de la invención. Las siguientes figuras y

ejemplos se proporcionan a modo de ilustración, y no se pretende que sean limitativos de la presente invención.

DESCRIPCION DE LAS FIGURAS

FIG. 1. Muestra el efecto protector de diferentes concentraciones de LC sobre la LPO en hígado y riñón de tilapias expuestas a 400 µg CYN/kg pez.

Medidas de LPO en hígado y Medidas de LPO en riñón.

Donde: el eje Y representa los valores de LPO (peroxidación lipídica) cuantificados como sustancias de degradación de la peroxidación de los lípidos que reaccionan con el ácido tiobarbitúrico (*Thiobarbituric Acid Reactive Substances*, TBARS) expresados en nmol de malonildialdehído (MDA)/g de tejido ± error estándar (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (a) comparación de los grupos tratados con CYN y LC con respecto a sus respectivos grupos control y (b) comparación del grupo tratado con CYN y LC (400 ó 880 mg LC/Kg pez/día) con su respectivo grupo no tratado con LC.

FIG. 2. Muestra el efecto protector de diferentes concentraciones de LC sobre la oxidación de ADN en hígado y riñón de tilapias expuestas a 400 µg CYN/kg pez

Medidas de oxidación de ADN (sitios AP; 100.000 pb).

Donde: el eje Y representa los valores de los sitios apurínicos/ apirimidínicos por cada 100.000 pares de bases cuantificados como medida de la oxidación de ADN expresados como media ± error estándar (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (a)

comparación de los grupos tratados con CYN y LC con respecto a sus respectivos grupos control y (b) comparación del grupo tratado con CYN y LC (400 ó 880 mg LC/Kg pez/día) con su respectivo grupo no tratado con LC.

FIG 3. Muestra el efecto protector de diferentes concentraciones de LC sobre el cociente GSH/GSSG en hígado y riñón de tilapias expuestas a 400 µg CYN/kg pez

Medidas del cociente Glutatión reducido/glutatión oxidado (GSH/GSSG)
Donde: el eje Y representa los valores de GSH/GSSG (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (a) comparación de los grupos tratados con CYN y LC con respecto a sus respectivos grupos control y (b) comparación del grupo tratado con CYN y LC (400 ó 880 mg LC/Kg pez/día) con su respectivo grupo no tratado con LC.

FIG. 4. Muestra los cambios histopatológicos en hígado de tilapias expuestas a CYN y su recuperación por LC.

A, C, E, G, I, K, M, Ñ, P: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, F, H, J, L, N, O, Q: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 5. Cambios histopatológicos en riñón de tilapias expuestas a CYN y su recuperación por LC.

A, C, E, G, I, K, M, Ñ, P: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, F, H, J, L, N, O, Q: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 6. Cambios histopatológicos en corazón de tilapias expuestas a CYN y su recuperación por LC.

A, C, E, G, I, K, M, Ñ, P: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, F, H, J, L, N, O, Q: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 7. Cambios histopatológicos en intestino de tilapias expuestas a CYN y su recuperación por LC.

A, C, E, G, I, K, M, Ñ, P: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, F, H, J, L, N, O, Q: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 8: Cambios histopatológicos en branquias de tilapias expuestas a CYN y su recuperación por LC observados con el microscopio óptico y electrónico de barrido.

A, B, C, D, I, K, M, Ñ, P: Observaciones al microscopio óptico, tinción con Hematoxilina-eosina. Las barras miden 100 µm.
B, D, F, H, J, L, N, O, Q: Observaciones ultraestructurales al microscopio electrónico de barrido (SEM: scanning electron microscope). Las barras miden 10 µm.

MODO DE REALIZACIÓN DE LA INVENCIÓN

A continuación se ilustrará la invención mediante unos ensayos realizados por los inventores que describen el uso de LC para tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

EJEMPLO 1

La invención se llevó a cabo empleando un total de 72 peces macho de *Oreochromis niloticus* (Nile tilapia), de peso medio 55,2 ± 6,77 g, y longitud de 12 ± 2 cm, obtenidos en una piscifactoría, y transferidos en acuarios (96 L) con sistema de filtración de agua y aireación adecuados, y ciclos de 12/12 h

luz/oscuridad. Los peces fueron alimentados con comida comercial (Dibaq, Segovia, España), en una cantidad de 0,3 g/día. Los peces se aclimataron durante 15 días antes del experimento. Se utilizaron 9 grupos experimentales con 8 animales en cada uno. Los peces fueron intoxicados con la toxina CYN pura (pureza > 95%, Alexis Corporation, Lausen, Suiza) ó mediante la administración de células liofilizadas de un cultivo de *Aphanizomenon ovalisporum* LEGE X-001 productor de CYN, en ambos casos por vía oral (mezclado con la comida comercial para peces). La administración de LC se realizó también a través del piensc, empleando dos niveles de dosis, 400 y 880 mg LC/Kg pez/día. Cada grupo fue introducido en un acuario independiente:

Acuario 1: peces control, alimentados solo con pienso normal durante 21 días

Acuario 2: peces alimentados con pienso durante 21 días e intoxicados con CYN (dosis única de 400 µg CYN/kg pez) procedente de un estandar puro.

Acuario 3: peces alimentados con pienso durante 21 días, intoxicados con CYN (dosis única de 400 µg CYN/kg pez) procedente de un cultivo liofilizado de *Aphanizomenon ovalisporum* productor de la misma.

Acuarios 4 y 5: Peces alimentados con pienso + LC, a las dosis de 20 y 45 mg LC/pez/día (equivalentes a 400 y 880 mg LC/Kg pez/día) durante 21 días.

Acuarios 6 y 7: Peces con pienso+ LC (400 y 880 mg LC/Kg pez/día) durante 21 días, e intoxicados con una dosis única de 400 µg CYN/kg pez.procedente de estándar puro.

Acuarios 8 y 9: Peces con pienso+ LC (400 y 880 mg LC/Kg pez/día) durante 21 días, e intoxicados con una dosis única de 400 µg CYN/kg pez.procedente de un cultivo liofilizado de *Aphanizomenon ovalisporum* productor de la misma.

Al final del experimento los peces fueron sacrificados, anestesiándolos con hielo. Se procedió a la extracción de los órganos, y se prepararon sus extractos para las determinaciones de biomarcadores enzimáticos, según Gutiérrez-Praena *et al.*(Aquat. Toxicol. 105: 100-106, 2011). Concretamente la medida de la lipoperoxidación lipídica (LPO) se realizó midiendo el malonildialdehído o

sustancias de degradación de la peroxidación de los lípidos que reaccionan con el ácido tiobarbitúrico; la oxidación de ADN mediante la determinación de los sitios apurínicos/ apirimidínicos por cada 100.000 pares de bases y se determinó además el cociente glutatión reducido/glutatión oxidado (GSH/GSSG) en hígado y riñón. Los estudios histológicos por microscopia óptica y electrónica en los distintos órganos se llevaron a cabo según Atencio *et al.* (Toxicol. Pathol. 36:449-458, 2008), incluyendo en el caso de las branquias microscopia óptica y electrónica de barrido (SEM).

Para los estudios de significación estadística entre grupos, se empleó el análisis de la varianza (ANOVA) y posteriormente el ensayo de Tukey, con una significación estadística $p<0,05$.

Los resultados más significativos fueron los siguientes:

1) En la FIG. 1 se observa como la toxina CYN (procedente de células liofilizadas de un cultivo de *Aphanizomenon ovalisporum* o de estándar puro) incrementa la lipoperoxidación (LPO) en hígado (2,1 veces y 1,7 respectivamente), y en riñón (1,4 veces y 1,5 respectivamente) frente al control, y los efectos protectores de LC se demuestran con las dos dosis ensayadas.

2) En la FIG. 2 se observa como CYN procedente de células liofilizadas de un cultivo de *Aphanizomenon ovalisporum* aumenta la oxidación de ADN en hígado y riñon (1,3 veces y 1,4 respectivamente) en relación al control, y los efectos protectores de LC se demuestran con las dos dosis ensayadas.

3) La FIG. 3 muestra la disminución de los cocientes GSH/GSSG en hígado y riñón de los peces intoxicados con CYN, y la aplicación de ambas dosis de LC mejoró este parámetro.

EJEMPLO 2. LC mejoró las alteraciones histopatológicas inducidas por CYN en hígado.

El estudio histopatológico del hígado de los peces pertenecientes a los lotes tratados con CYN puso en evidencia un proceso degenerativo que consistió en una desorganización del parénquima hepático con presencia de glucógeno en el citoplasma del hepatocito y ciertas gotas de grasa (FIG. 4 G, H, I, J), frente a una ausencia de lesiones del grupo control (FIG. 4 A, B) caracterizada por

cordones hepáticos normales, con hepatocitos con una morfología poliédrica normal.

En los dos lotes de peces tratados exclusivamente con LC se observó que, con ambas dosis (400 y 880 mg LC/Kg pez/día) no existían lesiones hepáticas y presentan una morfología aparentemente normal (FIG. 4 C, D, E, F). En los lotes de peces a los que junto a la toxina CYN se les administró 400 mg LC/kg pez/día presentan un parénquima con una morfología aparentemente normal con ausencia de grasa y glucógeno granular en el caso de la CYN procedente de liofilizado (FIG. 4 K, L) y un cierto contenido de glucógeno pero sin llegar a ser un proceso patológico en el caso de CYN pura (FIG. 4 M, N).

El estudio de los peces del lote a los que junto con CYN (procedente de liofilizado o pura) se les administró una dosis de 880 mg LC/Kg pez/día mostró un parénquima hepático aparentemente normal con recuperación total de las lesiones provocadas por la toxina, tanto al microscopio óptico, como al electrónico (FIG. 4 Ñ, O, P, Q).

A, B: Hígado de los peces control. **A**. Cordones hepáticos normales, hepatocito con una morfología aparentemente normal. **B**. Detalle de hepatocito aparentemente normal, con organoides citoplasmáticos, retículos y mitocondrias.
C, D: Tilapias tratadas con LC (400 µg/kg pez/día). **C.** Parénquima con una morfología aparentemente normal, con los hepatocitos dispuestos en cordones y zona pancreática aparentemente normal. **D**. Detalle de hepatocito aparentemente normal con organoides citoplasmáticos y ausencia de grasa y glucógeno granular.
E, F: Tilapias tratadas con LC (880 mg LC/Kg pez/día). **E.** Parénquima con una morfología aparentemente normal, con los hepatocitos dispuestos en cordones y zona pancreática aparentemente normal. **F**. Detalle de hepatocito aparentemente normal con organoides citoplasmáticos y ausencia de grasa y glucógeno granular.
G, H: Hígado de tilapias expuestas a CYN procedente de liofilizado (400 µg CYN/Kg pez). **G**. Parénquima hepático desorganizado, hepatocitos con presencia de glucógeno (círculos). **H.** El hepatocito presenta gran contenido de glucógeno en el citoplasma (círculo) y gotas lipídicas (flecha).

I, J: Hígado de peces expuestos a CYN pura (400 µg LC/Kg pez). **I.** Parénquima hepático desorganizado, hepatocitos con presencia glucógeno (circulo) y de vesículas de grasa (flecha). **J.** El hepatocito presenta con un citoplasma con escasos organoides y el resto del citoplasma repleto de grasa (circulo) y cierta presencia de glucógeno granular (flecha).

K, L: Hígado de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (400 mg LC/Kg pez/día). **K.** Parénquima con una morfología aparentemente normal, con los hepatocitos dispuestos en cordones aparentemente normales. **L.** Detalle de hepatocito aparentemente normal con organoides citoplasmáticos y ausencia de grasa y glucógeno granular.

M, N: Hígado de peces expuestos a CYN pura y tratadas con LC (400 mg LC/Kg pez/día). **M.** Cordones hepáticos normales, con cierta morfología poliédrica con núcleo central y citoplasma claro con escaso contenido en glucógeno (círculo). **N.** Detalle de hepatocito con cierto contenido en glucógeno (círculo).

Ñ, O: Hígado de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (880 mg LC/Kg pez/día). **Ñ.** Parénquima hepático aparentemente normal. **O.** Detalle de hepatocito aparentemente normal.

P, Q: Hígado de peces expuestos a CYN pura y tratadas con LC (880 mg LC/Kg pez/día). **P.** Cordones hepáticos normales, pero con cierta morfología poliédrica con núcleo central y citoplasma sin contenido de glucógeno. **Q.** Detalle de hepatocito aparentemente normal.

EJEMPLO 3. LC mejoró las alteraciones histopatológicas inducidas por CYN en riñón.

Morfológicamente los riñones de los peces de los lotes tratados con CYN (procedente de liofilizado o pura) mostraron una glomerulopatía, observándose al microscopio óptico una atrofia glomerular y dilatación de la capsula de Bowman y al microscopio electrónico se observa esta glomerulopatía con tumefacción e incluso pérdida de las microvellosidades de los túbulos contorneados proximales (FIG. 5 G, H, I, J). Los riñones de los peces del lote control presentaron una estructura aparentemente normal (FIG. 5 A, B).

El estudio de los peces de los lotes tratados solamente con las dosis de LC (400 y 880 mg LC/Kg pez/día) no mostró ninguna lesión renal (FIG. 5 C, D, E, F).

En los peces a los que se les administró CYN (procedente de liofilizado o pura) junto con 400 ó 880 mg LC/Kg pez/día se observó estructuralmente y ultraestructuralmente una morfología del parénquima renal totalmente normal (FIG. 5 K, L, M, N, Ñ, O, P, Q).

A, B: Riñón de los peces control.

C, D: Tilapias tratadas con LC (400 µg/kg pez/día). **C.** Glomérulos y túbulos aparentemente normales. **D**. Glomérulo con podocito aparentemente normal.

E, F: Tilapias tratadas con LC (880 mg LC/Kg pez/día). **E.** Glomérulos y túbulos aparentemente normales. **F**. Túbulo contorneado proximal aparentemente normal.

G, H: Riñón de tilapias expuestas a CYN procedente de liofilizado (400 µg CYN/Kg pez). **G**. Glomerulopatía y atrofia glomerular (circulo), tubulonefrosis y dilatación de la capsula de Bowman (flecha) y ligera tubulonefrosis (estrella). **H**. Microvellosidades de los túbulos contorneados proximales tumefactos y pérdidas de estas (circulo).

I, J: Riñón de peces expuestos a CYN pura (400 µg LC/Kg pez). **I**. Glomerulopatía (circulo), atrofia glomerular (estrella), ditalación de la capsula de Bowman y tubulonefrosis (flecha). **J**. Tubulonefrosis de túbulos contorneados proximal con células engrosadas e hialinizadas (flechas).

K, L: Riñón de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (400 mg LC/Kg pez/día). **K**. Glomérulos y túbulos aparentemente normales. **L**. Membrana basal aparentemente normal.

M, N: Riñón de peces expuestos a CYN pura y tratadas con LC (400 mg LC/Kg pez/día). **M.** Glomérulos y túbulos aparentemente normales. **N**. Túbulo contorneado próximal aparentemente normal, con abundantes microvellosidades.

Ñ, O: Riñón de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (880 mg LC/Kg pez/día). **Ñ**. Glomérulos y túbulos aparentemente normales. **O**. Túbulo contorneado próximas aparentemente normal.

P, Q: Riñón de peces expuestos a CYN pura y tratadas con LC (880 mg LC/Kg pez/día). **P.** Glomérulos y túbulos aparentemente normales. **Q**. Detalle de túbulo contorneado distal aparentemente normal y con abundantes mitocondrias.

EJEMPLO 4. LC mejoró las alteraciones histopatológicas inducidas por CYN en corazón.

El estudio histopatológico realizado sobre peces tratados con CYN (procedente de liofilizado o pura) mostraron al microscopio óptico procesos de miofibrolisis, con pérdida de miofibrillas, presencia de edemas y hemorragias (FIG 6. G,I). Al microscopio electrónico se observa una pérdida de las miofibrillas (FIG. 6 H, J). El corazón de los peces del lote control presentó una estructura aparentemente normal (FIG. 6 A, B).

Los peces tratados solo con LC (400 y 880 mg LC/Kg pez/día) presentan una morfología de las fibras cardiacas similares a las del grupo control (FIG. 6 C, D, E, F). ,
No se observaron lesiones en los tratados con ambas dosis de LC (400 ó 880 mg LC/Kg pez/día) y expuestos a CYN (FIG. 6 K, L, M, N, Ñ, O, P, Q).

A, B: Corazón de los peces control.
C, D: Tilapias tratadas con LC (400 µg/kg pez/día). **C.** Fibras musculares aparentemente normales. **D**. Detalle de miofibrillas aparentemente normales, con bandas perfectamente dispuestas y normales.
E, F: Tilapias tratadas con LC (880 mg LC/Kg pez/día). **E.** Fibras musculares aparentemente normales. **F**. Detalle de miofibrillas con material contráctil intacto, con bandas perfectamente dispuestas y normales
G, H: Corazón de tilapias expuestas a CYN procedente de liofilizado (400 µg CYN/Kg pez). **G**. Miofibrolisis, pérdida de miofibrillas (flecha), ciertos edemas (estrella) y hemorragias (círculo). **H**. Pérdida y desintegración de las miofibrillas (circulo)
I, J: Corazón de peces expuestos a CYN pura (400 µg LC/Kg pez). **I**. Miofibrolisis con pérdida de miofibrillas (círculos), abundantes edemas (estrella) y ciertas hemorragias (flecha). **J**. Pérdida y desintegración de las miofibrillas (circulo).
K, L: Corazón de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (400 mg LC/Kg pez/día). **K** Fibras musculares

aparentemente normales pero ciertas hemorragias (círculo). **L**. Detalle de miofibrillas normales sin pérdida de material contráctil.

M, N: Corazón de peces expuestos a CYN pura y tratadas con LC (400 mg LC/Kg pez/día). **M.** Fibras musculares aparentemente normales. **N**. Detalle de miofibrillas normales sin pérdida de material contráctil.

Ñ, O: Corazón de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (880 mg LC/Kg pez/día). **Ñ** Fibras musculares aparentemente normales. **O**. Detalle de miofibrillas aparentemente normales.

P, Q: Corazón de peces expuestos a CYN pura y tratadas con LC (880 mg LC/Kg pez/día). **P.** Fibras musculares aparentemente normales. **Q**. Detalle de miofibrillas aparentemente normales.

EJEMPLO 5. LC mejoró las alteraciones histopatológicas inducidas por CYN en intestino.

A nivel de intestino en los peces tratados con CYN (procedente de liofilizado o pura) se observaron procesos de enteritis con necrosis de enterocitos al microscopio óptico y pérdida manifiesta de microvellosidades al microscopio electrónico (FIG. 7 G, H, I, J),en comparación con el grupo control (FIG. 7 A, B). Tras la administración de las dos dosis de LC (400 y 880 mg LC/Kg pez/día) solo se observó una actividad manifiesta de las células caliciformes, sin interés patológico (FIG. 7 C, D, E, F), al igual que en los grupos de peces a los que se les administró LC junto con CYN (FIG. 7 K, L, M, N, Ñ, O, P, Q), mostrando así ambas dosis de LC un efecto protector frente a la toxina.

A, B: Intestino de los peces control. **A**. Vellosidades aparentemente normales con enterocitos aparentemente normales. **B**. Enterocitos con abundantes microvellosidades aparentemente normales (círculo).

C, D: Tilapias tratadas con LC (400 µg/kg pez/día). **C**. Vellosidades aparentemente normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). **D**. Enterocitos con abundantes células caliciformes aparentemente normales (flechas).

E, F: Tilapias tratadas con LC (880 mg LC/Kg pez/día). **E**. Vellosidades aparentemente normales con enterocitos aparentemente normales y

abundantes células caliciformes (flecha). **F**. Enterocitos con abundantes microvellosidades (circulo).

G, H: Intestino de tilapias expuestas a CYN procedente de liofilizado (400 µg CYN/Kg pez). **G**. Detalle de vellosidades intestinales con enterocitos necrosados (circulo) **H**. Detalle de enterocito con pérdida parcial de las microvellosidades (circulo).

I, J: Intestino de peces expuestos a CYN pura (400 µg LC/Kg pez). **I**. Detalle de vellosidades intestinales con enterocitos necrosados (circulo) **J**. Detalle de enterocito con pérdida parcial de las microvellosidades (circulo).

K, L: Intestino de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (400 mg LC/Kg pez/día). **K**. Vellosidades aparentemente normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). **L**. Enterocitos aparentemente normales con abundantes microvellosidades (circulo).

M, N: Intestino de peces expuestos a CYN pura y tratadas con LC (400 mg LC/Kg pez/día). **M**. Vellosidades aparentemente normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). **N**. Enterocitos con abundantes microvellosidades (circulo).

Ñ, O: Intestino de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (880 mg LC/Kg pez/día). **Ñ**. Vellosidades aparentemente normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). **O**. Enterocitos aparentemente normales con abundantes microvellosidades (circulo).

P, Q: Intestino de peces expuestas a CYN pura y tratadas con LC (880 mg LC/Kg pez/día). **P**. Vellosidades aparentemente normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). **Q**. Enterocitos con abundantes microvellosidades (circulo).

EJEMPLO 6. LC mejoró las alteraciones histopatológicas inducidas por CYN en branquias.

Al microscopio óptico las branquias de los peces tratados con CYN pura presentaron, a nivel de las laminillas,, procesos de hiperemia y hemorragia (FIG. 8 I, J), siendo estas lesiones más manifiestas en los peces tratados con CYN procedente de liofilizado (FIG. 8 G, H). Al microscopio electrónico de barrido

pueden observarse infiltrados inflamatorios, erosión y tumefacción de la superficie lamelar (FIG. 8 G, H, I, J).

Estas lesiones no fueron observadas en el lote control (FIG. 8 A, B), ni en los peces tratados con LC (400 ó 880 mg LC/Kg pez/día) (FIG. 8 C, D, E, F), ni en los grupos de a los que se les administró LC junto con CYN (FIG. 8 K, L, M, N, Ñ, O, P, Q), que presentaron una morfología aparentemente normal. Se muestra así el efecto protector de ambas dosis de LC frente a CYN.

A, B: Branquias de los peces control.
C,D: Tilapias tratadas con LC (400 µg/kg pez/día). Estructura aparentemente normal.
E,F: Tilapias tratadas con LC (880 mg LC/Kg pez/día). Estructura aparentemente normal.
G, H: Branquias de tilapias expuestas a CYN procedente de liofilizado (400 µg CYN/Kg pez). **G.** Detalle de filamento branquial con presencia procesos de hiperemia (flecha) y hemorragias en laminillas secundarias (circulo). **H.** Arco branquial con superficie erosionada y tumefacta (círculo).
I, J: Branquias de peces expuestos a CYN pura (400 µg LC/Kg pez). **I.** Detalle de filamento branquial con hiperemia en laminillas secundarias (flecha) y hemorragias (círculo). **J.** Arco branquial con pérdida de continuidad, erosionado e infiltrado celular (circulo).
K, L: Branquias de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (400 mg LC/Kg pez/día). **K.** Filamento branquial aparentemente normal. **L.** Arco branquial aparentemente normal.
M, N: Branquias de peces expuestos a CYN pura y tratadas con LC (400 mg LC/Kg pez/día). **M.** Filamento branquial aparentemente normal. **N.** Arco branquial aparentemente normal.
Ñ, O: Branquias de tilapias expuestas a CYN procedente de liofilizado y tratadas con LC (880 mg LC/Kg pez/día). **Ñ.** Filamento branquial aparentemente normal. **O.** Arco branquial aparentemente normal.
P, Q: Branquias de peces expuestos a CYN pura y tratadas con LC (880 mg LC/Kg pez/día). **P.** Filamento branquial aparentemente normal. **Q.** Arco branquial aparentemente normal.

REIVINDICACIONES

1. Uso de una composición que comprende L-Carnitina para la elaboración de un medicamento útil en el tratamiento y/o prevención de efectos tóxicos en peces expuestos a cilindrospermopsina.

2. Uso de una composición que comprende L-Carnitina, según reivindicación 1, para la elaboración de un medicamento útil en la recuperación de efectos tóxicos en peces expuestos a cilindrospermopsina.

3. Uso según cualquiera de las reivindicaciones 1 ó 2, donde los efectos tóxicos son alteraciones histopatológicas.

4. Uso según la reivindicación 3, donde las alteraciones histopatológicas son producidas en al menos uno de los tejidos de la lista que comprende hígado, riñón, corazón, branquias o tracto gastrointestinal.

5. Uso según cualquiera de las reivindicaciones 1 a 4, donde la L-Carnitina se administra en una cantidad diaria de entre 400 y 880 mg LC/Kg pez/día.

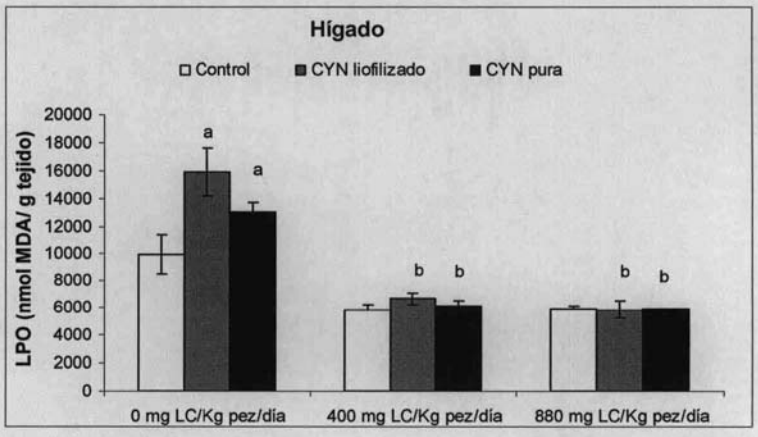
6. Uso según la reivindicación 5, donde la composición se presenta en una forma adaptada a la administración oral o intraperitoneal.

7. Uso según cualquiera de las reivindicaciones 1 a 6, donde la composición incluye excipientes farmacológicamente aceptables.

8. Uso según cualquiera de las reivindicaciones 1 a 7, donde la composición comprende además otra sustancia activa.

9. Uso según cualquiera de las reivindicaciones 1 a 8 para la fabricación de un alimento funcional.

10. Uso según cualquiera de las reivindicaciones 1 a 9 para la fabricación de un complemento vitamínico.


Hígado
Control
CYN liofilizado
CYN pura
LPO (nmol MDA/ g tejido)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
a
b
b
b
b
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

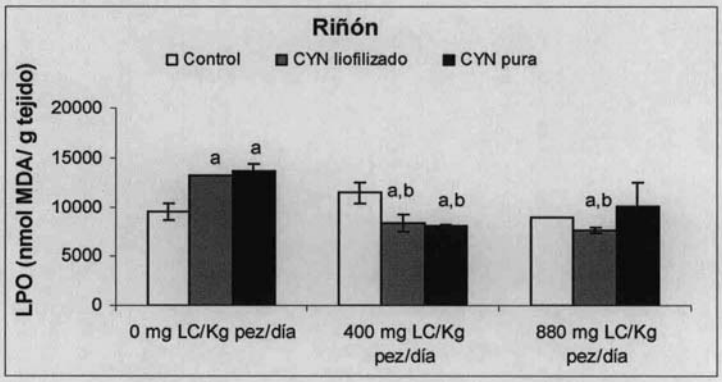
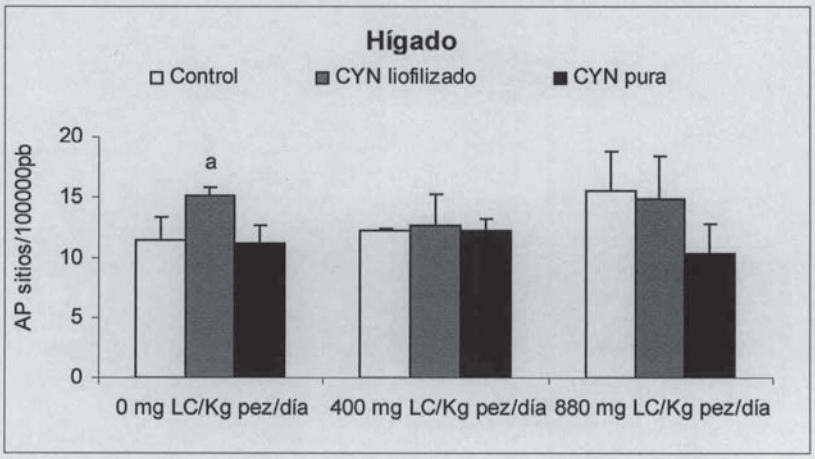

Riñón
Control
CYN liofilizado
CYN pura
LPO (nmol MDA/ g tejido)
20000
15000
10000
5000
0
a
a
a,b
a,b
a,b
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

FIG. 1


Hígado
Control
CYN liofilizado
CYN pura
AP sitios/100000pb
20
15
10
5
0
a
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

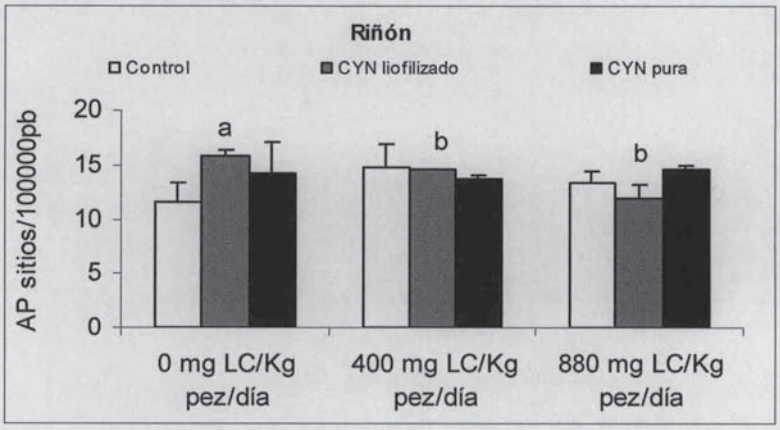
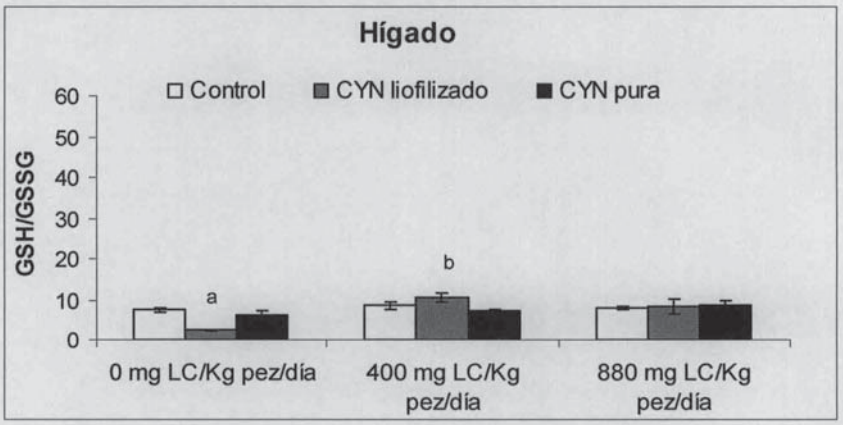

Riñón
Control
CYN liofilizado
CYN pura
AP sitios/100000pb
20
15
10
5
0
a
b
b
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

FIG. 2


Hígado
Control
CYN liofilizado
CYN pura
GSH/GSSG
60
50
40
30
20
10
0
a
b
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

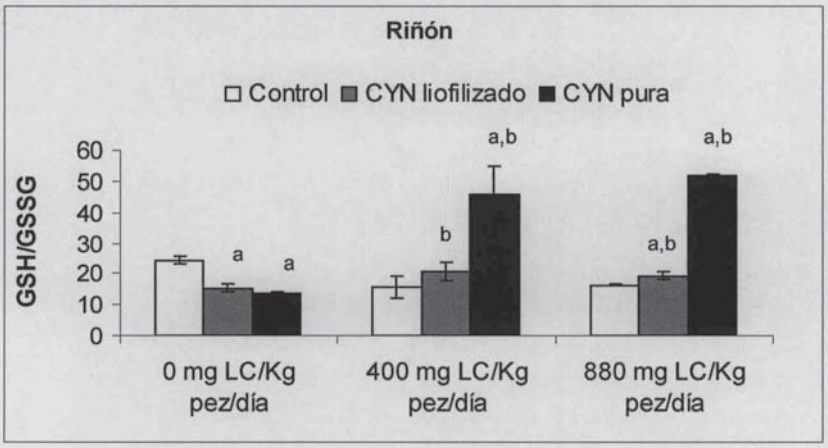
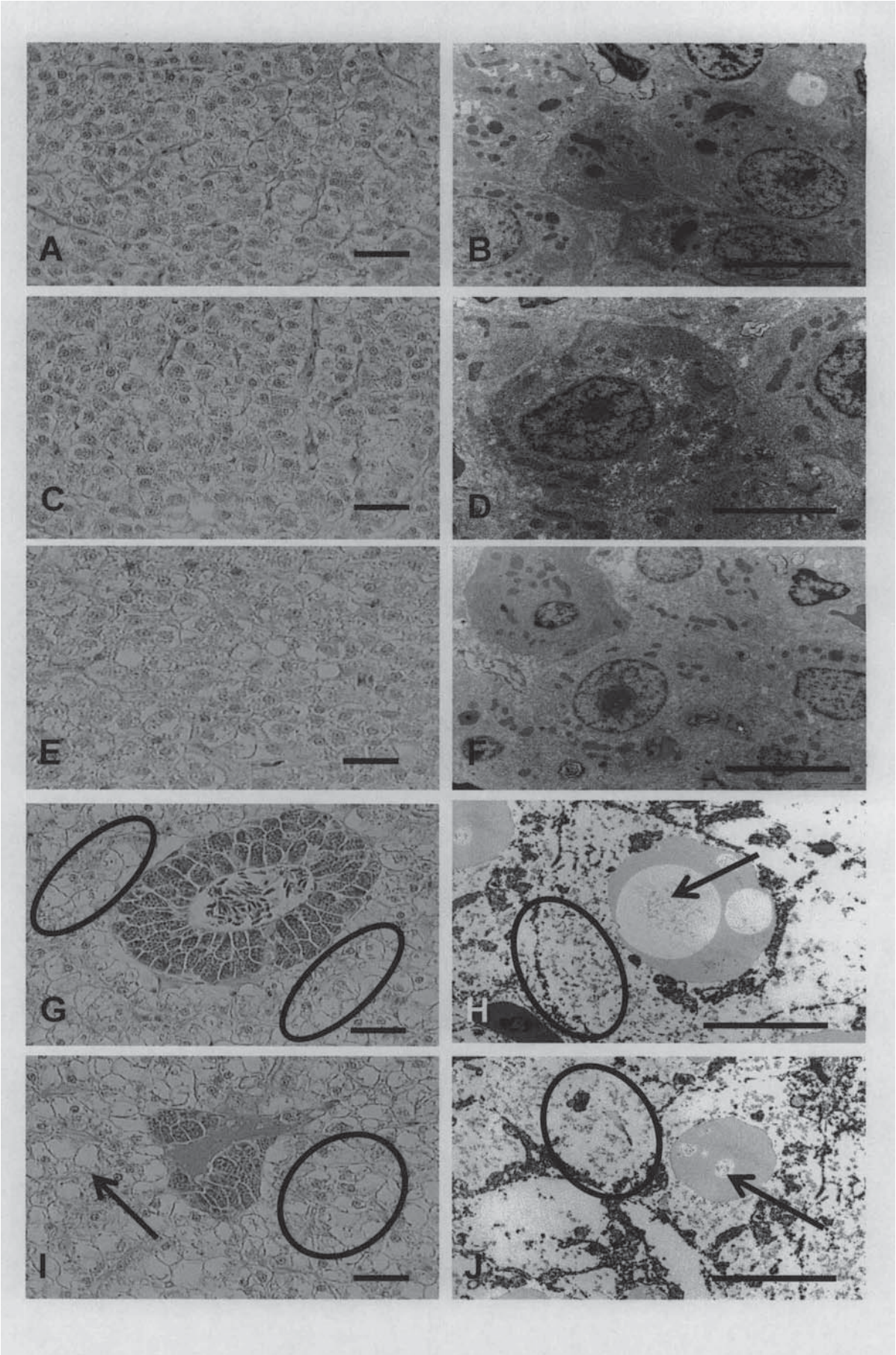

Riñón
Control
CYN liofilizado
CYN pura
GSH/GSSG
60
50
40
30
20
10
0
a
a
b
a,b
a,b
a,b
0 mg LC/Kg pez/día
400 mg LC/Kg pez/día
880 mg LC/Kg pez/día

FIG. 3


A
B
C
D
E
F
G
H
I
J

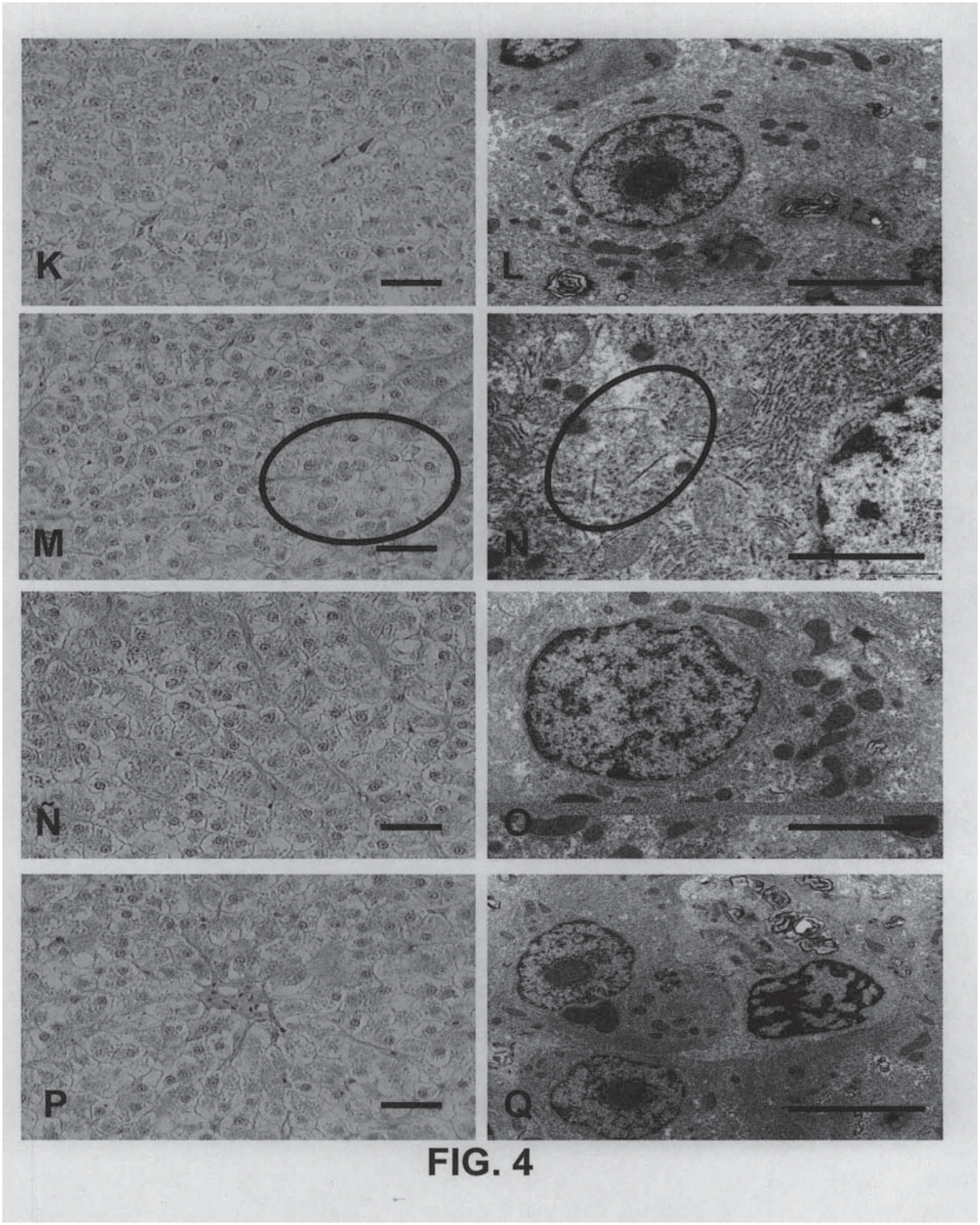
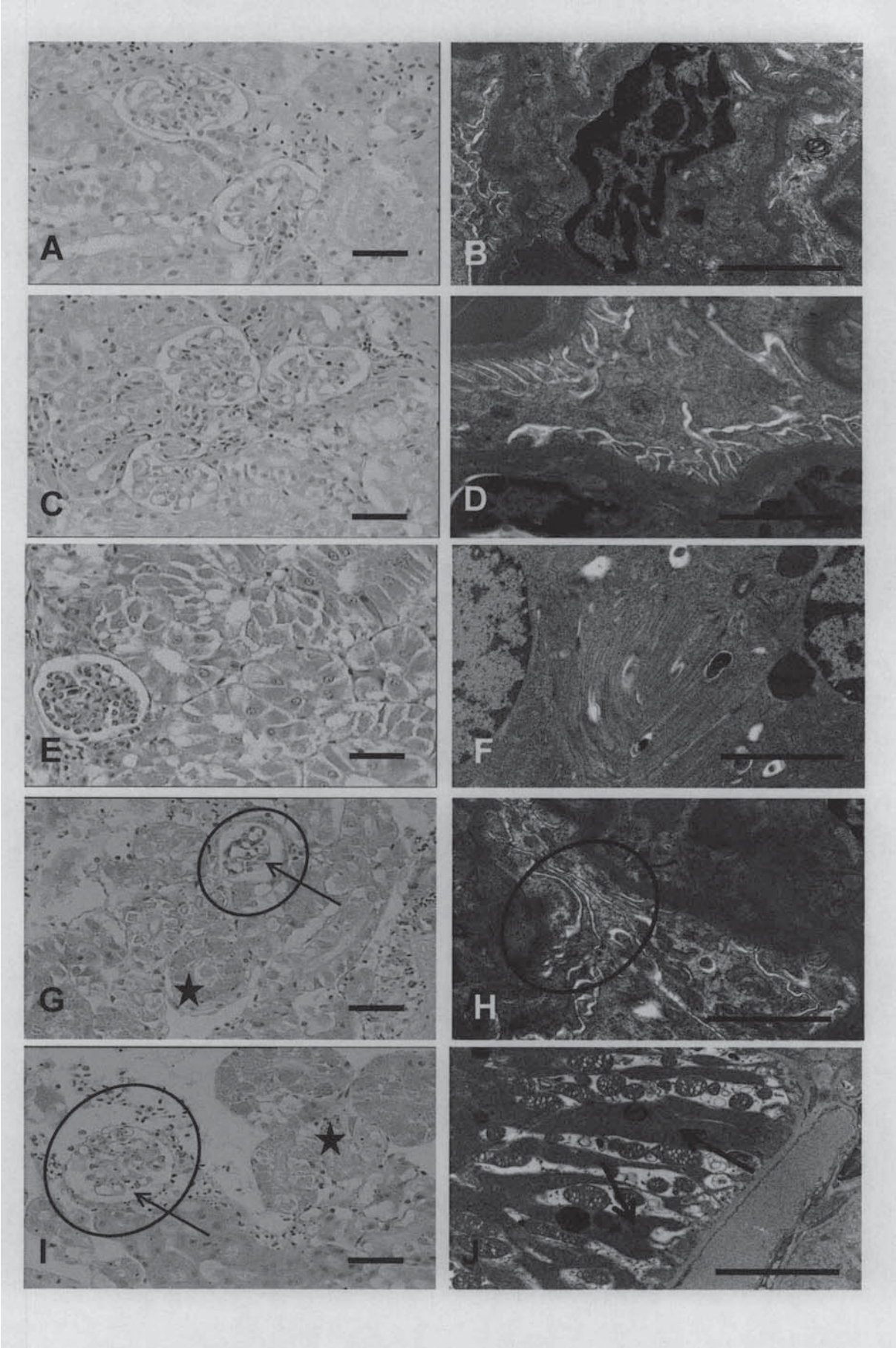


FIG. 4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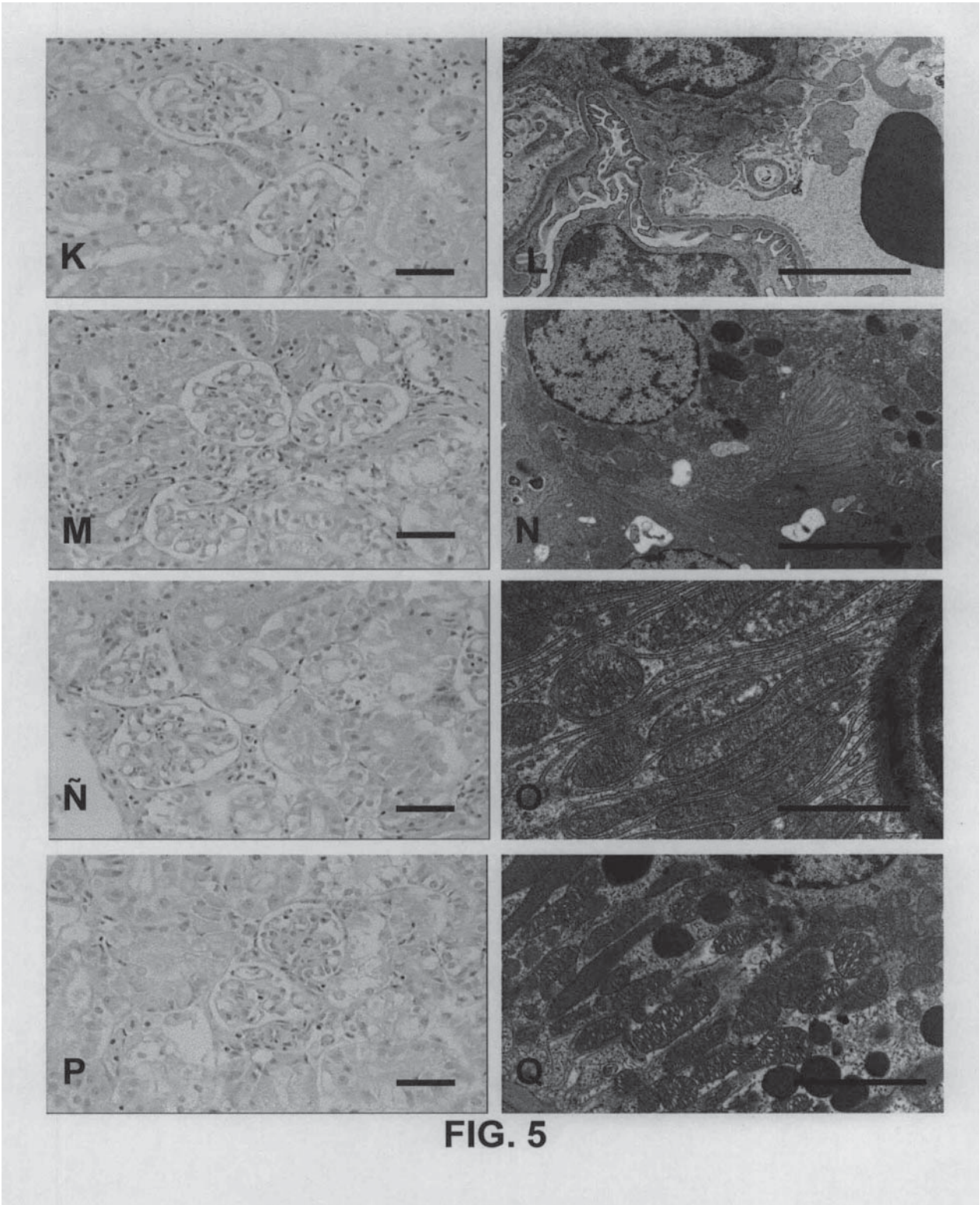
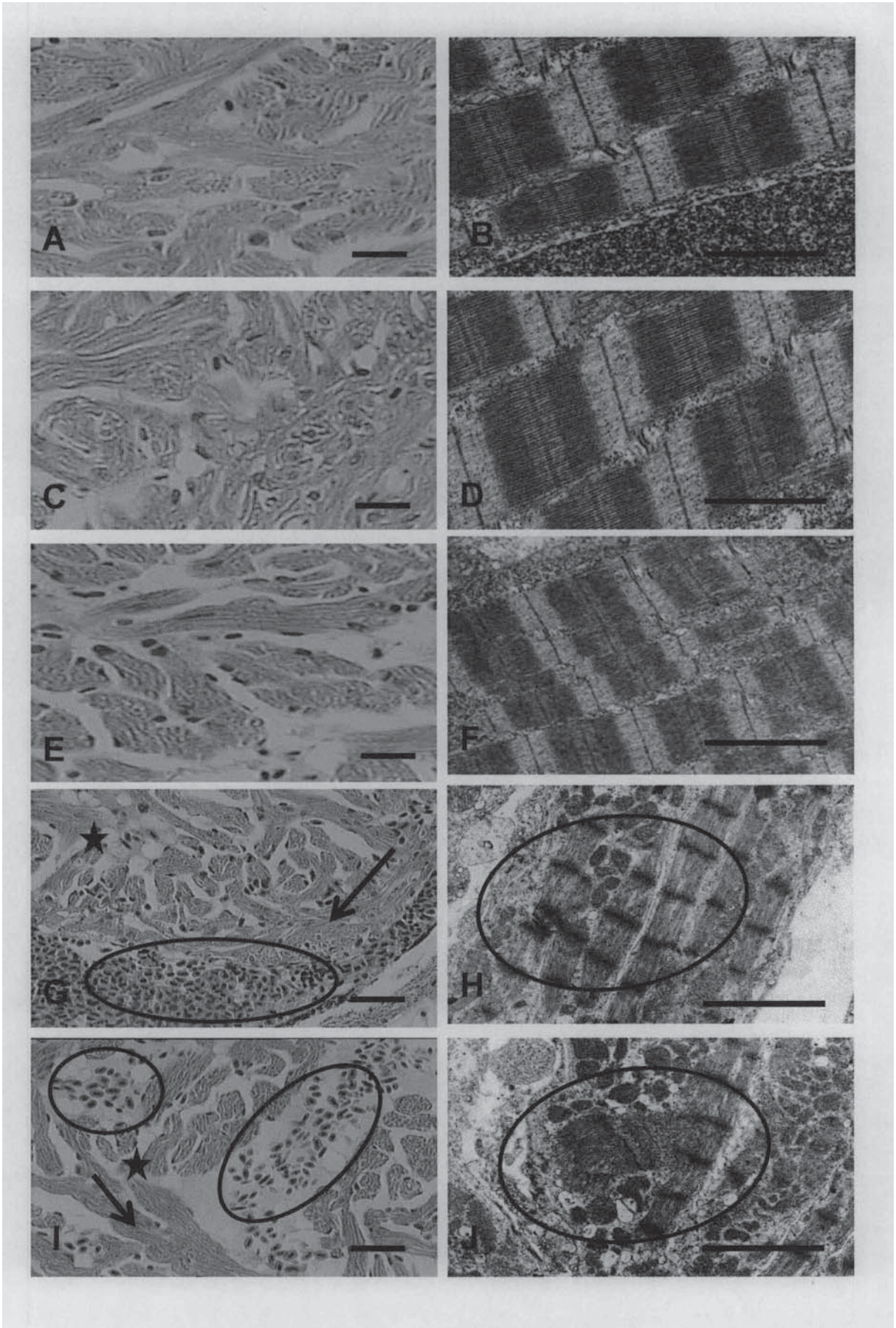


FIG. 5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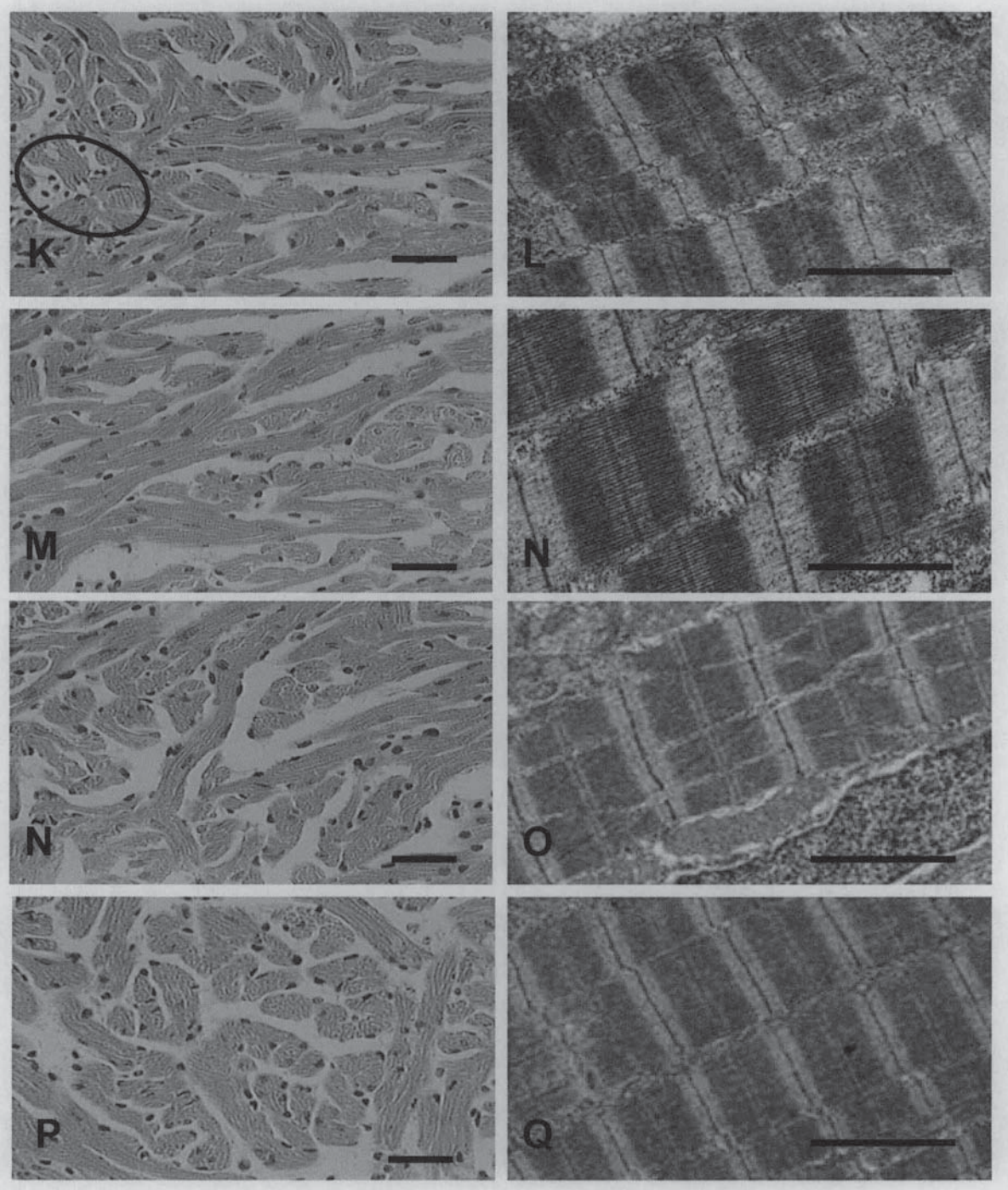
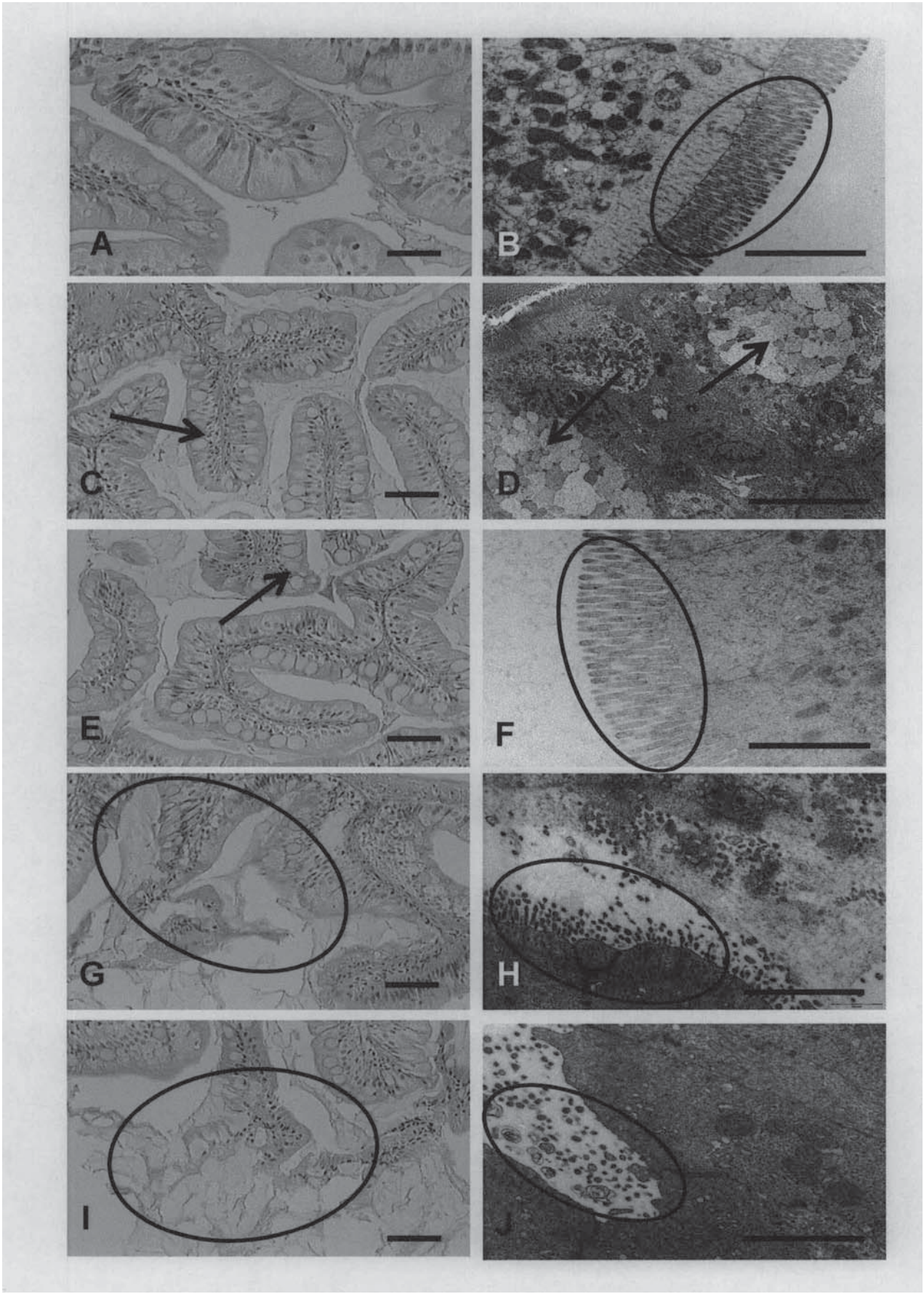


FIG. 6


A
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H
I
J

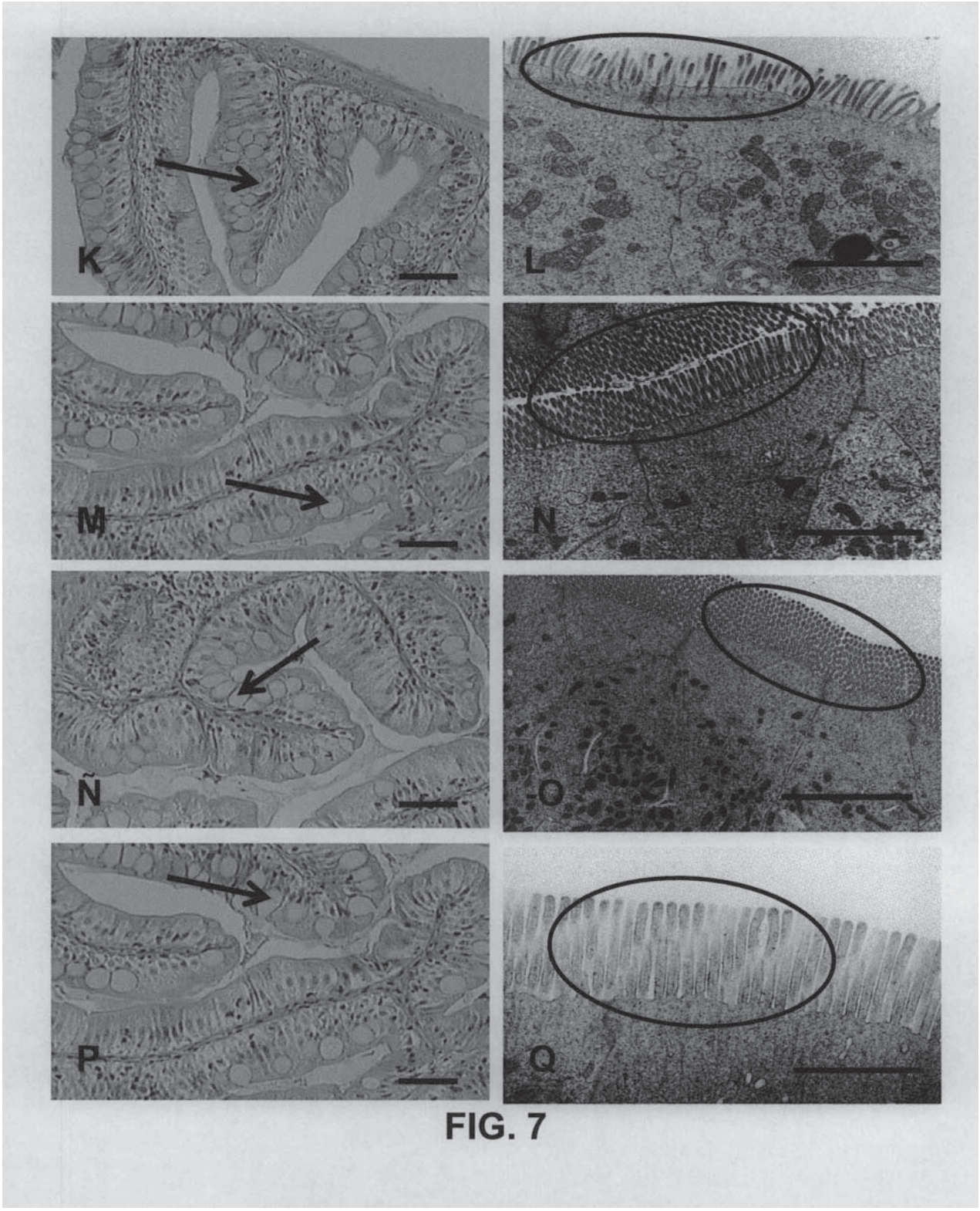
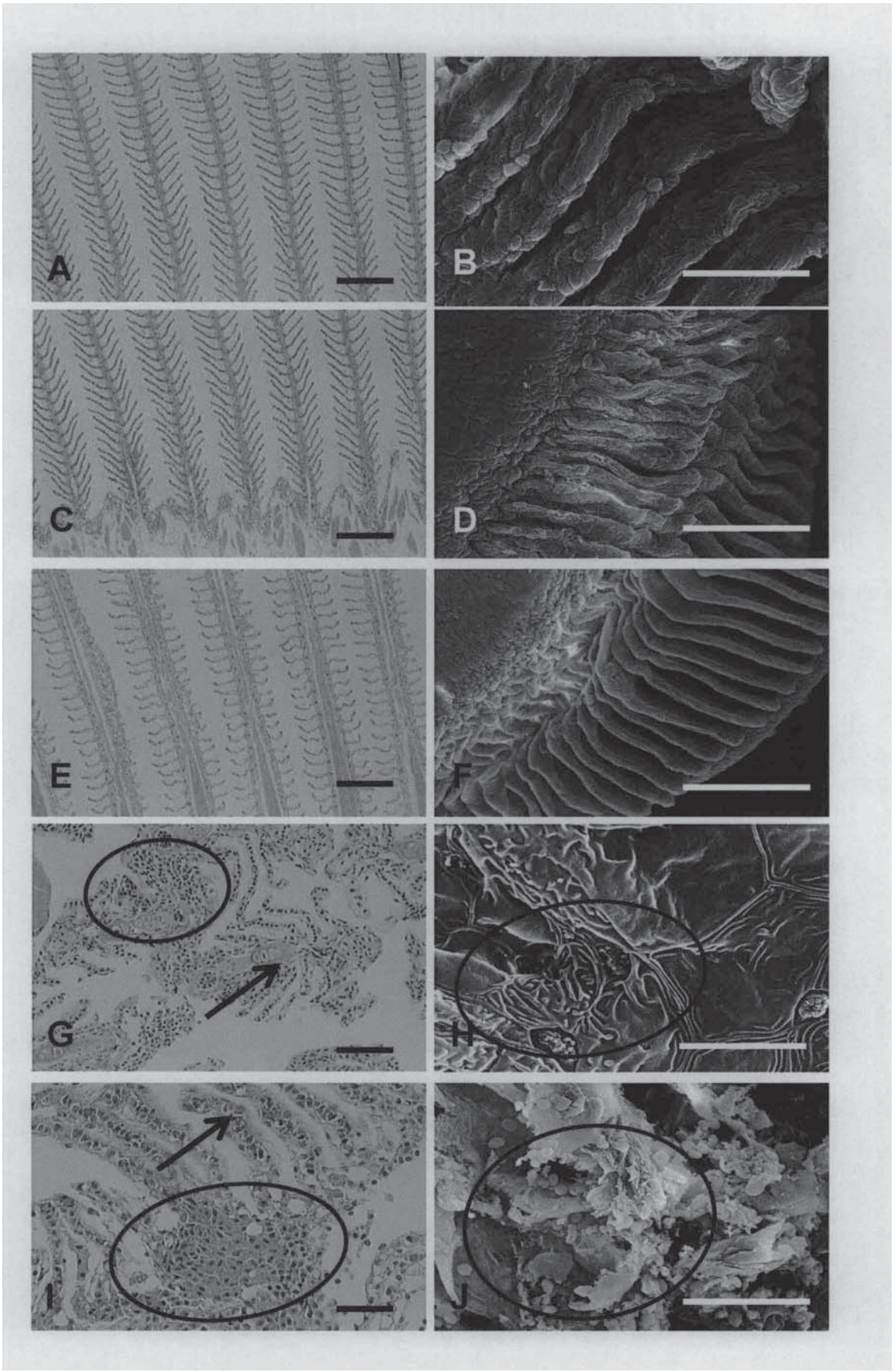


FIG. 7


A
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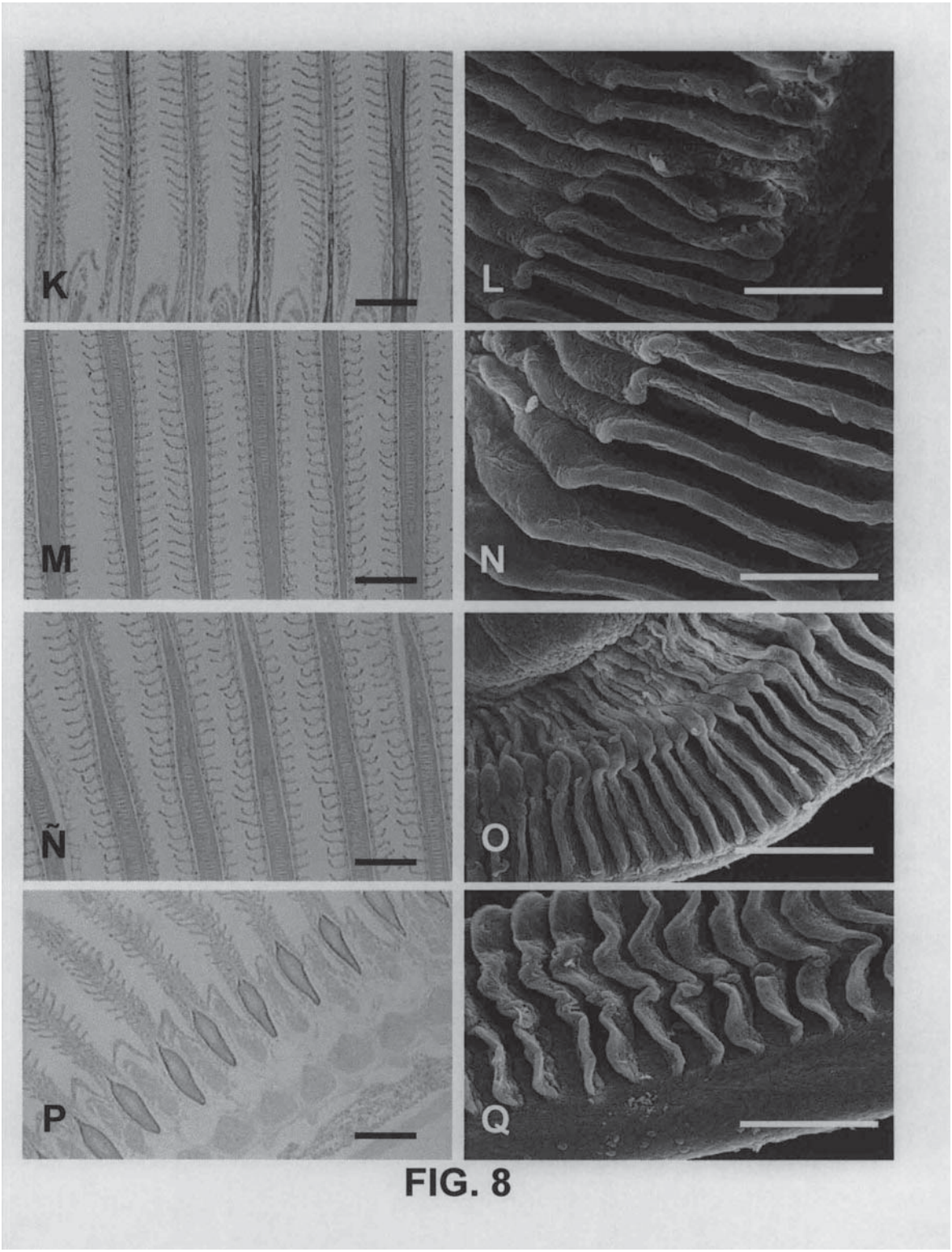

FIG. 8

OFICINA ESPAÑOLA
DE PATENTES Y MARCAS

ESPAÑA

(21) N.º solicitud: 201201151

(22) Fecha de presentación de la solicitud: 08.11.2012

(32) Fecha de prioridad:

INFORME SOBRE EL ESTADO DE LA TECNICA

(51) **Int. Cl. :** Ver Hoja Adicional

DOCUMENTOS RELEVANTES

Categoría	(56) **Documentos citados**	**Reivindicaciones afectadas**
A	GUTIÉRREZ-PRAENA DANIEL et al. Protective role of dietary N-acetylcysteine on the oxidative stress induced by cylindrospermopsin in tilapia (*Oreochromis niloticus*). Environmental toxicology and chemistry, 2012. Vol. 31 (7). ISSN 1552-8618 (Electronic) Doi: doi:10.1002/etc.1838 pubmed:22511408, páginas 1548-1555.	1-13
A	SCHREIBER S. et al. Dietary L-Carnitine Protects the Gills and Skin of Guppies (*Poecilia reticulata*) Against Anionic Xenobiotics. COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY. PART C: COMPARATIVE PHARMACOLOGY TOXICOLOGY, 19970501 ELSEVIER SCIENCE, OXFORD, GB, 1997. Vol. 117 (1) ISSN 0742-8413 Doi: doi:10.1016/S0742-8413(96)00230-7, páginas: 99-102.	1-13
A	WO 9105554 A1 (UNIV GEORGIA RES FOUND et al.) 02.05.1991, todo el documento.	1-13
A	US 2001043983 A1 (HAMILTON NATHAN D.) 22.11.2001, párrafos 10,16,22,24.	1-13
A	HARPAZ S. et al. L-Carnitine and its attributed functions in fish culture and nutrition-a review. Aquaculture, 2005, Vol. 249 (1-4) ISSN 0044-8486, páginas: 3-21.	1-13

Categoría de los documentos citados
X: de particular relevancia
Y: de particular relevancia combinado con otro/s de la misma categoría
A: refleja el estado de la técnica
O: referido a divulgación no escrita
P: publicado entre la fecha de prioridad y la de presentación de la solicitud
E: documento anterior, pero publicado después de la fecha de presentación de la solicitud

El presente informe ha sido realizado

☒ para todas las reivindicaciones ☐ para las reivindicaciones nº:

Fecha de realización del informe	**Examinador**	**Página**
17.02.2014	A. I. Polo Díez	1/4

CLASIFICACIÓN OBJETO DE LA SOLICITUD

A61K31/205 (2006.01)
A23K1/18 (2006.01)
A23K1/16 (2006.01)
A61P39/00 (2006.01)

Documentación mínima buscada (sistema de clasificación seguido de los símbolos de clasificación)

A61K, A23K, A61P

Bases de datos electrónicas consultadas durante la búsqueda (nombre de la base de datos y, si es posible, términos de búsqueda utilizados)

INVENES, EPODOC, WPI, BDTXTE, MEDLINE, BIOSIS, FSTA, HCAPLUS

Fecha de Realización de la Opinión Escrita: 17.02.2014

Declaración

Novedad (Art. 6.1 LP 11/1986)	Reivindicaciones	1-13	**SI**
	Reivindicaciones		**NO**
Actividad inventiva (Art. 8.1 LP11/1986)	Reivindicaciones	1-13	**SI**
	Reivindicaciones		**NO**

Se considera que la solicitud cumple con el requisito de aplicación industrial. Este requisito fue evaluado durante la fase de examen formal y técnico de la solicitud (Artículo 31.2 Ley 11/1986).

Base de la Opinión.-

La presente opinión se ha realizado sobre la base de la solicitud de patente tal y como se publica.

1. Documentos considerados.-

A continuación se relacionan los documentos pertenecientes al estado de la técnica tomados en consideración para la realización de esta opinión.

Documento	Número Publicación o Identificación	Fecha Publicación
D01	GUTIÉRREZ-PRAENA DANIEL et al.	2012
D02	SCHREIBER S et al.	1997
D03	WO 9105554 A1	02.05.1991
D04	US 2001043983 A1	22.11.2001
D05	HARPAZ et al.	2005

2. Declaración motivada según los artículos 29.6 y 29.7 del Reglamento de ejecución de la Ley 11/1986, de 20 de marzo, de Patentes sobre la novedad y la actividad inventiva; citas y explicaciones en apoyo de esta declaración

La invención se refiere, según la primera y segunda reivindicación, al uso de composiciones que comprenden L-carnitina para la elaboración de medicamentos útiles para el tratamiento y/o prevención y/o recuperación de los efectos tóxicos en peces expuestos a cilindrospermopsina (CYN)

Las reivindicaciones dependientes 3 a 13 aportan detalles sobre el uso de las reivindicaciones 1 y 2.

El documento D1 propone la utilización de la N-acetilcisteína (NAC) en la dieta para proteger a la tilapia de los efectos tóxicos que produce la exposición a las cilindroespermopsinas. La NAC es un precursor del glutatión, además de estimular las enzimas implicadas en el ciclo del GSH (Glutation-reductasa, gamma-glutamilcisteina sintetasa, glutatión-S-transferasa). Por todo ello, su ingestión contrarresta la inhibición de la síntesis de Glutation y el estrés oxidativo que produce la intoxicación con CYN.

El documento D2 demuestra el efecto protector de la L-carnitina frente a xenobióticos aniónico en peces de la especie *Poecilia reticulata*. Las membranas y capas epiteliales de las branquias y la piel de los peces a los que se les había administrado L-carnitina estaban protegidas frente a la entrada de xenobióticos aniónicos pero no de xenobióticos catiónicos (figura 1)

El documento D3 describe los efectos de alimentar un tipo de peces con un suplemento de carnitina. La L-carnitina, molécula implicada en la producción de energía metabólica, demostró ser útil para aumentar el crecimiento y para conferir resistencia frente a la toxicidad del amonio (ejemplo 3; reivindicación 9)

El documento D4 utiliza la L-carnitina junto con ácido lipóico como suplemento para animales de edad avanzada (entre los que se citan los peces) con objeto de aumentar su metabolismo mitocondrial (párrafo 16)

El documento D5 es una revisión sobre la función de la L-carnitina en peces.

Novedad y actividad inventiva (art. 6.2 y 8.2 de la L.P.)

Ninguno de los documentos citados en el estado de la técnica divulga ni sugiere el uso del L-carnitina para tratar o prevenir los efectos tóxicos en los peces expuestos a la toxina cilindrospermopsina.

El único compuesto que ha sido utilizado con éxito para tratar o prevenir los efectos tóxicos de la CYN en peces ha sido la NAC (documento D1), compuesto que no tiene similitud estructural con la L-carnitina, ni está involucrado en los mismos mecanismos fisiológicos.

Por otro lado, aunque la L-carnitina ha sido utilizado como suplemento alimenticio de los peces con muchas finalidades (ver documentos D2-D5): aumento de crecimiento, protección de ciertos xenobióticos, etc., no se deduce de manera evidente de estos documentos que la L-carnitina vaya a proteger a los peces de todo tipo de tóxicos y, en particular, de la intoxicación con CYN.

Por lo tanto, se considera que las reivindicaciones 1 a 13 tienen características que no se encuentran ni resultan evidentes del estado de la técnica, y por tanto cumplen el requisito de novedad y de actividad inventiva.

PATENTE / PATENT

Ana Mª Cameán Fernández, **Remedios Guzmán Guillén**, Ana Isabel Prieto Ortega, Isabel Moreno Navarro, Ángeles Mencía Jos Gallego, Silvia Pichardo Sánchez, María Puerto Rodríguez, Daniel Gutiérrez Praena, Sara Maisanaba Hernández, Mª Rosario Moyano Salvago, Alfonso Blanco Rodríguez

USO DE LA VITAMINA E PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA

2014

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Nº SOLICITUD: P201400428

FECHA Y HORA DE ENTRADA EN OEPM:

FECHA Y HORA DE ENTRADA EN LUGAR DISTINTO A LA OEPM: 22-05-14 12'05

LUGAR DE PRESENTACIÓN: SEVILLA CODIGO

INSTANCIA DE SOLICITUD

...ENTO DE PAGO DE TASAS
...50 de la Ley Orgánica
...Ley de Reformas Urgentes)

OFICINA ESPAÑOLA DE PATENTES Y MARCAS
30 MAYO 2014
ENTRADA

1. IDENTIFICACIÓN DE LA SOLICITUD

(1) MODALIDAD:

☒ **PATENTE DE INVENCIÓN** ☐ **MODELO DE UTILIDAD**

(2) TIPO DE SOLICITUD:	(3) EXPEDIENTE PRINCIPAL O DE ORIGEN:
☐ **ADICIÓN A LA PATENTE** ☐ **SOLICITUD DIVISIONAL** ☐ **CAMBIO DE MODALIDAD** ☐ **TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA** ☐ **ENTRADA EN FASE NACIONAL DE SOLICITUD PCT**	**MODALIDAD:** **Nº SOLICITUD:** **FECHA PRESENTACIÓN:**

2. TÍTULO DE LA INVENCIÓN (4)

USO DE VITAMINA E PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA

RECEPCIÓN
JUNTA DE ANDALUCIA
Agencia de Innovación y Desarrollo de Andalucía
22.05.2014
Registro General: 184 Sevilla
Hora: 12.05

3. IDENTIFICACIÓN DEL SOLICITANTE

(5) APELLIDOS Y NOMBRE /DENOMINACIÓN SOCIAL	NIF/PASAPORTE
UNIVERSIDAD DE SEVILLA	Q4118001I

DIRECCIÓN POSTAL	CÓDIGO POSTAL Y LOCALIDAD	PROVINCIA	PAÍS RESIDENCIA	CODIGO PAÍS RESIDENCIA
Pabellón de Brasil - Paseo de las Delicias s/n	41013 Sevilla	Sevilla	España	ES

PAÍS DE NACIONALIDAD	CÓDIGO PAIS NACIONALIDAD	CNAE (6)	PYME (7)
España	ES		

DIRECCIÓN CORREO ELECTRÓNICO	Nº TELÉFONO FIJO	Nº TELÉFONO MÓVIL	(8) INDICACIÓN DEL MEDIO DE NOTIFICACIÓN PREFERENTE
qdelgado@us.es	954488116		☐ CORREO POSTAL ☒ CORREO ELECTRÓNICO

PORCENTAJE DE TITULARIDAD (9): 100 %	NOTA: DE NO ESPECIFICARSE DICHO PORCENTAJE, LA OEPM PRESUMIRÁ IGUALES LAS CUOTAS DE LOS SOLICITANTES.
EL SOLICITANTE TAMBIÉN (10) ☐ SI ES INVENTOR:	☒ NO — MODO DE OBTENCIÓN DEL DERECHO: (11) ☒ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar):_____
(12) EL SOLICITANTE ES UNA UNIVERSIDAD PÚBLICA ESPAÑOLA	☒ SI

4. OTROS SOLICITANTES Y/O INVENTORES (13)

☒ LOS DEMAS SOLICITANTES Y/O INVENTORES SE INDICAN EN HOJA COMPLEMENTARIA

Ejemplar para el expediente

5. IDENTIFICACIÓN DEL REPRESENTANTE

(14) REPRESENTACIÓN		(15) Nº PODER GENERAL
☐ EL SOLICITANTE NO ESTÁ REPRESENTADO	EL SOLICITANTE ESTÁ REPRESENTADO POR: ☐ AGENTE DE LA PROPIEDAD INDUSTRIAL ☐ OTRO REPRESENTANTE	

(16) ACTUACIÓN POR MEDIO DE AGENTE	NOMBRE	CÓDIGO DE AGENTE

(17) ACTUACIÓN POR MEDIO DE OTRO REPRESENTANTE	NOMBRE	DIRECCIÓN POSTAL	N.I.F.

DIRECCIÓN CORREO ELECTRÓNICO	Nº TELÉFONO	(18) INDICACIÓN DEL MEDIO DE NOTIFICACIÓN PREFERENTE
		☐ CORREO POSTAL ☐ CORREO ELECTRÓNICO

6. OTROS DATOS

(19) REIVINDICACIÓN DE PRIORIDAD	PAIS ORIGEN	CODIGO PAÍS	FECHA	NÚMERO
☐ SI ☐ NO				

(20) EXPOSICIONES OFICIALES	NOMBRE	FECHA	LUGAR
☐ SI ☐ NO			

(21) EFECTUADO DEPÓSITO DE MATERIAL BIOLÓGICO	AUTORIDAD DE DEPÓSITO	CODIGO PAÍS	FECHA	NÚMERO
☐ SI ☐ NO				

LISTAS DE SECUENCIAS DE AMINOÁCIDOS Y ÁCIDOS NUCLEICOS

La descripción contiene un listado de secuencias biológicas en concordancia con la norma ST.25 OMPI ☐ SI ☐ NO

Se adjunta un soporte de datos legible por ordenador que incluye el listado de secuencias biológicas en concordancia con la norma ST.25 OMPI ☐ SI ☐ NO

El solicitante declara por medio de esta instancia, que la información registrada en el soporte de datos legible por ordenador es idéntica a la contenida en el listado de secuencias biológicas incluido en la descripción de la versión escrita de esta solicitud ☐

(22) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE TASAS PREVISTO EN EL ART. 162 DE LA LEY 11/1986 DE PATENTES	☐ SI

7. ÍNDICE DE DOCUMENTOS QUE SE ACOMPAÑAN / FECHA Y FIRMA (23)

- ☒ DESCRIPCIÓN. Nº PÁGINAS:
- ☒ Nº DE REIVINDICACIONES:
- ☒ DIBUJOS. Nº PÁGINAS:
- ☐ LISTA DE SECUENCIAS. Nº PÁGINAS:
- ☐ SOPORTE LEGIBLE POR ORDENADOR DE LISTA DE SECUENCIAS
- ☒ RESUMEN
- ☐ FIGURA A PUBLICAR EN BOPI Nº: ___
- ☐ DOCUMENTO DE PRIORIDAD
- ☐ TRADUCCIÓN DEL DOCUMENTO DE PRIORIDAD
- ☐ DOCUMENTO DE REPRESENTACIÓN
- ☐ JUSTIFICANTE DEL PAGO DE TASA DE SOLICITUD
- ☒ HOJA DE INFORMACIÓN COMPLEMENTARIA
- ☐ PRUEBAS DE LOS DIBUJOS
- ☐ SOLICITUD CAP
- ☐ OTROS:

FIRMA DEL SOLICITANTE O REPRESENTANTE

Fdo.: Ramón González Carvajal

FIRMA DEL FUNCIONARIO

Ejemplar para el expediente

ADVERTENCIA: POR DISPOSICIÓN LEGAL LOS DATOS CONTENIDOS EN ESTA SOLICITUD PODRÁN SER PUBLICADOS EN EL BOLETÍN OFICIAL DE LA PROPIEDAD INDUSTRIAL E INSCRITOS EN EL REGISTRO DE PATENTES DE LA OEPM, SIENDO AMBAS BASES DE DATOS DE CARÁCTER PÚBLICO Y ACCESIBLES VÍA REDES MUNDIALES DE INFORMÁTICA

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española de Patentes y Marcas

Nº SOLICITUD: P201400428
FECHA Y HORA DE ENTRADA EN OEPM:
FECHA Y HORA DE ENTRADA EN LUGAR DISTINTO A LA OEPM: SEVILLA 22-05-14 12'05

HOJA DE INFORMACIÓN COMPLEMENTARIA

1. IDENTIFICACIÓN DE LA SOLICITUD

(1) MODALIDAD:

☒ PATENTE DE INVENCIÓN ☐ MODELO DE UTILIDAD

2. IDENTIFICACIÓN DE LOS SOLICITANTES (2)

(3) APELLIDOS Y NOMBRE/ DENOMINACIÓN SOCIAL/ INFORMACIÓN RELATIVA A UNIVERSIDAD PÚBLICA ESPAÑOLA/FIRMA	PAÍS NAC.	CÓD. NAC.	NIF/ PASAPORTE	(4) ESTA PERSONA TAMBIÉN ES INVENTOR:	(5) MODO DE OBTENCIÓN DEL DERECHO:
UNIVERSIDAD DE CÓRDOBA ESTE SOLICITANTE ES UNIVERSIDAD PÚBLICA: ☒ SI FIRMA:	ES		Q1418001B	☐ SI ☒ NO	☒ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar): PORCENTAJE DE TITULARIDAD: 20 %
ESTE SOLICITANTE ES UNIVERSIDAD PÚBLICA: ☐ SI FIRMA:				☐ SI ☐ NO	☐ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar): PORCENTAJE DE TITULARIDAD: %
ESTE SOLICITANTE ES UNIVERSIDAD PÚBLICA: ☐ SI FIRMA:				☐ SI ☐ NO	☐ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar): PORCENTAJE DE TITULARIDAD: %
ESTE SOLICITANTE ES UNIVERSIDAD PÚBLICA: ☐ SI FIRMA:				☐ SI ☐ NO	☐ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar): PORCENTAJE DE TITULARIDAD: %
ESTE SOLICITANTE ES UNIVERSIDAD PÚBLICA: ☐ SI FIRMA:				☐ SI ☐ NO	☐ INVENCIÓN LABORAL ☐ CONTRATO ☐ SUCESIÓN ☐ OTROS (Especificar): PORCENTAJE DE TITULARIDAD: %

NOTA: DE NO ESPECIFICARSE EL PORCENTAJE DE TITULARIDAD, LA OEPM PRESUMIRÁ IGUALES LAS CUOTAS DE LOS SOLICITANTES.

Ejemplar para el expediente

3. IDENTIFICACIÓN DE LOS INVENTORES (Incluir sólo aquellos inventores que no figuren como solicitantes) (6)

APELLIDOS Y NOMBRE	PAÍS NACIONALIDAD	CODIGO NAC.	NIF/PASAPORTE
CAMEÁN FERNÁNDEZ, ANA MARÍA	ESPAÑOLA	ES	28669922-P
REMEDIOS GUZMÁN, GUILLÉN	ESPAÑOLA	ES	48824434-B
PRIETO ORTEGA, ANA ISABEL	ESPAÑOLA	ES	53272253-K
MORENO NAVARRO, ISABEL	ESPAÑOLA	ES	28748803-E
JOS GALLEGO, ÁNGELES MENCÍA	ESPAÑOLA	ES	44958744-T

4. OTROS DATOS

(7) EXPOSICIONES OFICIALES

NOMBRE	FECHA	LUGAR

(8) REIVINDICACIÓN DE PRIORIDAD

PAÍS ORIGEN	CÓDIGO PAÍS	FECHA	NÚMERO

Ejemplar para el expediente

3. IDENTIFICACIÓN DE LOS INVENTORES (Incluir sólo aquellos inventores que no figuren como solicitantes) (6)

APELLIDOS Y NOMBRE	PAÍS NACIONALIDAD	CODIGO NAC.	NIF/PASAPORTE
PICHARDO SÁNCHEZ, SILVIA	ESPAÑOLA	ES	28783594-Z
PUERTO RODRÍGUEZ, MARÍA	ESPAÑOLA	ES	31725821-N
GUTIÉRREZ PRAENA, DANIEL	ESPAÑOLA	ES	28821142-A
MAISANABA HERNÁNDEZ, SARA	ESPAÑOLA	ES	30230099A
MOYANO SALVAGO, MARIA ROSARIO	ESPAÑOLA	ES	30449826-B

4. OTROS DATOS

(7) EXPOSICIONES OFICIALES

NOMBRE	FECHA	LUGAR

(8) REIVINDICACIÓN DE PRIORIDAD

PAÍS ORIGEN	CÓDIGO PAÍS	FECHA	NÚMERO

Ejemplar para el expediente

3. IDENTIFICACIÓN DE LOS INVENTORES (Incluir sólo aquellos inventores que no figuren como solicitantes) (6)

APELLIDOS Y NOMBRE	PAÍS NACIONALIDAD	CODIGO NAC.	NIF/PASAPORTE
BLANCO RODRÍGUEZ, ALFONSO	ESPAÑOLA	ES	29987559-K

4. OTROS DATOS

(7) EXPOSICIONES OFICIALES

NOMBRE	FECHA	LUGAR

(8) REIVINDICACIÓN DE PRIORIDAD

PAÍS ORIGEN	CÓDIGO PAÍS	FECHA	NÚMERO

Ejemplar para el expediente

Uso de vitamina E para proteger a los peces de la intoxicación por Cilindrospermopsina

La presente invención se refiere al uso de una composición que comprende vitamina E (vit E) para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a Cilindrospermopsina (CYN). También se refiere al uso de la citada composición en la recuperación de las alteraciones histopatológicas producidas en los tejidos de la lista que comprende hígado, riñón, corazón, tracto gastrointestinal, branquias y/o cerebro. Además, dicha composición se utiliza para la fabricación de un alimento funcional, un complemento vitamínico, o un complemento nutricional.

III. ESTADO DE LA TÉCNICA
Este apartado presenta el estado del arte hasta la fecha y permite definir la novedad de la invención que se solicita registrar. Por esa razón debemos describir todo aquello que conocemos, los procedimientos o técnicas utilizadas hasta la fecha, los productos que hay en el mercado para..., los dispositivos que se utilizan en ..., etc. Se debe hacer una relación de todos los documentos, bibliográficos o de patentes que reflejen el estado de la técnica anterior y que se conozcan sobre el tema. En el caso de publicaciones se deberá citar entre otros el autor, revista, fecha e incluso página o líneas; en el caso de patentes, número, fecha de publicación y titular de la patente. Deben estar citados, en la medida de lo posible, todos los documentos que reflejen el estado de la técnica anterior
(extensión máxima recomendada DOS páginas)

ESTADO DE LA TÉCNICA ANTERIOR

La vitamina E (vit E) es un nutriente esencial sintetizado en la membrana de los cloroplastos por las plantas (Soll *et al*., Arch. Biochem. Biophys. 238: 290-299, 1985). Estructuralmente comprende una familia de compuestos poliprenoides formados por un anillo aromático con un grupo hidroxilo y una cadena polipronoide saturada, presentando en estado natural ocho isoformas (α, β, γ, δ- tocoferol y α, β, γ, δ –tocotrienol) y siendo α-Tocoferol la que ha mostrado una mayor actividad biológica en mamíferos (Peuthert and Pllugmacher, Toxicon 56: 411-417, 2010; Atkinson, Encyclopedia of biological chemistry (Second edition) pp 545-550, 2013; Traber, Encyclopedia of human nutrition (Third edition) pp 383-89, 2013). La vitamina E se encuentra ampliamente distribuida por todo el organismo estando presente en todas las células de los tejidos corporales (Pekmezci, Chapter eight in Vitamins & Hormones 86: 179-215, 2011).

Por su capacidad para eliminar radicales peroxilo, la función principal de la vit E es la de antioxidante formando parte de la primera línea de defensa frente a la peroxidación lipídica a nivel celular (Pekmezci, Chapter eight in Vitamins & Hormones 86: 179-215, 2011) contribuyendo a mantener la

integridad de las membranas y la bioactividad celular (Traber and Atkinson, Free Rad. Biol. Med., 43: 4-15, 2007). La vitamina E presenta efectos inmunomoduladores y juega un papel importante enfermedad isquémica cardiaca limitando la progresión de la aterosclerosis y en la carcinogénesis por sus propiedades antioxidantes frente al cáncer (Pekmezci, Chapter eight in Vitamins & Hormones 86: 179-215, 2011). Asimismo se ha demostrado que la vit E protege, de forma dosis dependiente, el ADN de leucocitos mononucleares frente a oxidantes reactivos generados por neutrófilos activados (Staden, Mut. Res., 288: 257-262, 1993).

Son múltiples los usos descritos de la vit E principalmente en combinación con otras sustancias. Así, distintos trabajos y patentes de investigación la describen para prevención y tratamiento de distintas patologías incluyéndola en formulaciones de vacunas antigripales para ancianos debido a su efecto positivo sobre la respuesta inmunitaria (ES 2 420 829 T3). A nivel cardiovascular se describe su uso en asociación con fenofibrato como medicación anti-ateroma, presentando sinergismo en la función protectora antioxidante de las lipoproteinas de baja densidad (LDL) del plasma (ES 2 148 694 T3); en productos farmaceúticos o alimenticios en mezcla sinergética con licopeno para la prevención de la oxidación de lipoproteínas de baja densidad (LDL) (ES 2 218 833 T3); en formulaciones para administración oral que ejerce un efecto reconstituyente sobre el sistema cardiovascular (ES 2 308 550 T3); en preparados para la prevención y tratamiento de dislipemias que incluyen nuevos ligandos de receptores activados por proliferador de peroxisomas (PPAR) que no causan retención de líquidos, edema o insuficiencia cardiaca (ES 2 385936 T3); como agentes bifuncionales que poseen actividad antioxidante y antiarrítmica utilizados en el tratamiento de lesiones por isquemia-reperfusión, así como en enfermedades referidas a radicales libres y/o arritmias (ES 2 231 514 T3). Además, estudios epidemiológicos realizados en mujeres de mediana edad sugieren que el uso de suplementos de vit E se asocia con un menor riesgo de enfermedad coronaria (Stampfer, N. Engl. J. Med. 328: 1444-1449, 1993). Otros usos de la vit E se han descrito en formulaciones farmacéuticas que contienen productos activos antiinflamatorios (ES 2 215 101 T3); en composición antiulcerosa combinado con el ácido ascórbico (ES 2 046 229 T3); en solución o emulsión acuosa de ubiquinona, análogo o derivado, de uso oftálmico para reducir los efectos de estrés oxidativo a nivel del ojo y como solubilizante efectivo para la propia ubiquinona (ES 2 385037 T3); en procedimientos de encapsulación de ácidos grasos poliinsaturados (ES 2 357 965 T3) o en forma de derivado en composición farmacéutica con ciclosporina A y un emulsionante (ES 2 148 345 T3). Existen distintas

patentes de investigación que recogen su uso en formulaciones cosméticas (ES 2382975 T3) como son composiciones cicatrizantes mejoradas para el tratamiento de una lesión causada por el acné (ES 2 176 343 T3) y composiciones para el tratamiento de uñas junto con aceite de soja o girasol (US 3887702 A). La vitamina E en combinación con la vitamina A, vitamina C y zinc forma parte de preparados para acelerar el brote del cabello (DE 19757921), disminuir su caída y/o favorecer su repigmentación (ES 2 282 213 T3, FR 760358) y junto con extractos de plantas, vitamina B6 y biotina en composición revitalizadora del cabello (ES 2 159 580 T3) y se ha descrito su uso en composiciones bucales (ES 2 043 856). También se ha demostrado su utilidad en combinación con otras sustancias en composiciones nutricionales. Concretamente, junto a beta-caroteno, vitamina C, selenio y otros compuestos ha mostrado utilidad para la prevención y tratamiento de caquexia y anorexia por cáncer (ES 2 210 523 T3); junto a extracto de Panax ginseng, vitaminas y minerales en composición para la activación del sistema inmune (ES 2 343 119 T3); en preparados para la alimentación parenteral u oral de pacientes con función inmunológica debilitada (ES 2 187 595 T3); como suplemento dietético formando parte de un Kit nutricional infantil para regular el ciclo de sueño-vigilia (ES 2 259 885 A1) e incluso en la preparación de salsas culinarias y preparados alimenticios como alimento funcional graso rico en vitaminas (ES 2 264 866 B1). Se ha introducido el uso de la vit E en la práctica deportiva para mejorar la eficiencia energética de los atletas y los índices de rendimiento físico (ES 2 382115 T3) o junto con oligoelementos, vitaminas dermoprotectoras y otros antioxidantes en composiciones para aliviar el estrés del calor restaurando el equilibrio electrolítico debido a la sudoración en ausencia de ejercicio físico (ES 2 178 257 T3).

Concretamente en peces, se ha mostrado un requerimiento diario de vit E en distintas especies como el salmón Atlántico (120 mg / kg de dieta) (Hamre y Lie, 1995., Comp. Biochem. Physiol., 111 A:547-554, 1995) o la carpa común (200 a 300 mg / kg de dieta) (Watanabe *et al*., Bull. Jpn. Soc. Sci. Fish. 43: 935-946, 1977; Wilson *et al*., J. Nutr. 114: 2053-2058, 1984), describiéndose su uso en composiciones lipídicas marinas para alimentar organismos acuáticos (ES 2 320 851 T3). Además, se ha demostrado una mejora del sistema inmunitario y de la resistencia a enfermedades en la carpa herbívora (CN 102940157 A 20130227). Un aumento de vit E en la dieta de peces (de 300 a 1500 mg vit E / kg dieta) puede reducir la tasa de oxidación de lípidos en filetes de pescado, reducir la formación de sabores desagradables (Chaiyeapechara *et al.*, Aquaculture 219: 715-735, 2003) y mejorar la calidad del filete de especies como rodaballo (Ruff *et al*., Aquac. Nutr. 9: 91-103, 2003), así como aumentar la acumulación de vitaminas lipofílicas en pescados y mariscos (JP 2001275578 A 20011009). Se ha

demostrado que una disminución de vit E en la dieta de rodaballo (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) y dorada (*Sparus aurata* L.) conlleva a actividades más altas de las enzimas antioxidantes en hígado y a niveles más altos de peróxidos lípidicos (Tocher *et al*. Aquac. Nutr. 8:195-207, 2002). Además se ha mostrado que un aumento del nivel de ácido graso poliinsaturado (PUFA) en la dieta de salmón Atlántico o carpa común provoca un aumento de las necesidades de vit E (Watanabe *et al*, Bull. Jpn. Soc. Sci. Fish. 43: 935-946, 1981; Schwarz *et al*, Aquaculture 69: 57-67, 1988; Hamre and Lie, Aquaculture Res. 26: 175-184, 1995). Concretamente, en tilapias (*Oreochromis* sp) alimentadas con aceite oxidado se ha demostrado que un suplemento de vit E impidió el aumento de peroxidación lipídica (LPO) y aumentó el nivel de GSH en hígado (Huang and Huang, Aquaculture 237: 381-389, 2004). Se ha demostrado que la vit E mejora el efecto protector de la taurina frente a las mareas rojas en peces y crustáceos (JP S60110250 A 19850615). A pesar del papel protector que tiene la vit E, se conoce muy poco acerca de la modulación de la defensa antioxidante que ejerce en organismos acuáticos expuestos a cianotoxinas. Hasta el momento, solo se ha demostrado el efecto protector del pretratamiento con N-aceticisteína (NAC) (P200803360 de Cameán Fernández A, *et al.* 2011) y Selenio (Se) (P200803359 de Cameán Fernández A, *et al.* 2011) en tilapias (*Oreochromis niloticus*) expuestas a Microcistinas (MCs) y que la vit E modula las respuestas antioxidantes en cangrejos de estuario (*Chasmagnathus granulatus*) y en tilapias expuestas a MCs (Pinho *et al*., Ecotoxicol. Environ. Saf. 61: 361-365, 2005; Prieto *et al*., Environ Toxicol. Chem. 27: 1152-1159, 2007; Prieto *et al*., Environ. Toxicol. 54: 563-579, 2008). No existiendo, por lo tanto, nada publicado de vit E con respecto a Cilindrospermopsina (CYN) objeto de esta invención y siendo novedoso aportar el uso de la vit E con respecto al tratamiento, prevención y/o recuperación de efectos inducidos por CYN en peces.

Por otro lado, la CYN es una toxina producida por cianobacterias tóxicas presentes en aguas superficiales, pertenecientes a trece especies de cianobacterias capaces de sintetizar CYN (*Anabaena lapponica, Anabaena planctonica, Anabaeana bergii, Aphanizomenon ovalisporum, Aphanizomenon flos-aquae, Aphanizomenon flos-aquae var klebahnii, Aphanizomenon gracile, Cylindrospermopsis rasiborskii, Lyngbya wollei, Raphidiopsis mediterránea, Rapidiopsis curvata, Umezakia natans, Oscillatoria* sp. PCC 6506*)* que se reparten en cuatro de los cinco continentes (Moreira *et al*., J. Applied Microbiol. 114: 605-620, 2013). CYN se aisló por primera vez de un cultivo de *Cylindrospermopsis raciborskii*, obtenido de los reservorios de agua de bebida que surtían a la población de Palm Island, Queensland, Australia (Ohtani I *et al.,* J. Am. Chem. Soc. 144:7941-7942, 1992). Se ha comprobado su acumulación en peces y

crustáceos, afectando a la calidad y seguridad de este tipo de alimentos y suponiendo un riesgo potencial para el consumidor. En comparación con los mamíferos, los estudios sobre efectos tóxicos de CYN en peces son muy escasos, destacando que puede afectar no sólo al hígado sino también al riñón, corazón, branquias, y tracto gastrointestinal. Las cianobacterias constituyen parte de la dieta de diversos ciprinídos y cíclidos, como es el caso, por ejemplo, de las Tilapias (*Orechromis, sp*.). La Tilapia *(Oreochromis sp*.) es uno de los pescados que más rápidamente se ha introducido en acuicultura, por la facilidad que presenta su manejo, gran capacidad de adaptación a condiciones adversas y fácil reproducción; sus distintas variedades son filtradoras y consumidoras de cianobacterias y en Europa se está despertando un gran interés por su cultivo.

Como mecanismo de acción tóxica más aceptado, la CYN está considerada un citotoxina general que produce bloqueo de la síntesis de proteínas en células eucariotas de mamíferos y plantas (Terao *et al*., Toxicon 32: 833-843, 1994; Froscio *et al*., Toxicon 51: 191–198, 2008), genotoxicidad por fragmentación del ADN (Bazin *et al*., Environ. Molec. Mutag. 51: 251-259, 2009; Zegura *et al*., Toxicon 58: 471–479, 2011; Zegura *et al*., Mutat. Res. 727: 16–41, 2011) y que disminuye los contenidos de Glutation (GSH) debido a una disminución de su síntesis a través de la formación de metabolitos vía CYP450 (Runnegar *et al*., Biochem. Pharmacol. 49: 219–225, 1995) más que a un aumento de su consumo por fenómenos de conjugación a través de la enzima Glutation s transferasa (GST). La disminución de GSH no parece conllevar a un incremento del estrés oxidativo en la célula (Humpage *et al*., J. Toxicol. Environ. Health Part A 68: 739–753, 2005) por lo que se sugirió que no era un mecanismo primario de toxicidad de CYN. Sin embargo, recientemente, se ha comprobado la participación directa del estrés oxidativo en la patogénesis de CYN en peces expuestos de forma aguda (Gutierrez-Praena *et al*., Aquat. Tox. 105:100-106, 2011; Puerto *et al*., Ecotoxicology, 20:1852-1860, 2011) y subcrónica (Guzmán-Guillén *et al*., Chemosphere, 90:1184-1194, 2013) detectándose un aumento en la producción de especies reactivas de oxígeno (ROS), lipoperoxidación (LPO), oxidación de proteínas y de ADN así como cambios en la actividad de diversas enzimas antioxidantes en peces. Los escasos estudios toxicológicos realizados hasta la actualidad han conducido al establecimiento de una Ingesta Diaria Tolerable (IDT) provisional de 0,03 µg/Kg/día de CYN, y la propuesta de un valor guía provisional de 1 µg/L de CYN en aguas de bebida. Actualmente no existe un tratamiento antidótico específico en casos de intoxicación por CYN y sus epímeros procedentes de cianobacterias. Hasta la fecha, tan sólo el uso de la N-acetilcisteína

(Gutierrez-Praena D. *et al.*, Aquat. Toxicol. 31: 1-8, 2012; P201101162, Cameán Fernández A. *et al.,* 2011) y L-carnitina (Guzmán-Guillén *et al*., 132-133: 141-150, Aquat. Toxicol., 2013; solicitud de patente Nº P201201151, Cameán Fernández A, *et al.,* 2012) han demostrado actividad protectora en peces frente a la intoxicación por CYN.

Teniendo en cuenta la ubicuidad de esta toxina, se hace necesario recuperar peces que presenten alteraciones histopatológicas con diferentes niveles de afección, que pueden impedir el ciclo de vida normal de las especies afectadas.

EXPLICACIÓN DE LA INVENCIÓN

La presente invención se refiere al uso de una composición que comprende vit E para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

En tilapias (*Oreochromis sp*.) expuestas a dosis únicas y repetidas de CYN se inducen estrés oxidativo y alteraciones patológicas. En concreto, se ha comprobado aumento de los niveles de peroxidación lipídica (LPO), disminución de los niveles de GSH, y alteración de la actividades enzimáticas antioxidantes Glutatión-s-transferasa (GST) y Glutatión peroxidasa (GPx) en diferentes órganos (hígado, riñón) de peces expuestos a 400 µg CYN/kg pez de forma aguda por vía oral. Así mismo, CYN induce múltiples alteraciones histopatológicas en órganos diversos: hígado, riñón, corazón, tracto gastrointestinal, branquias y cerebro.

La vitamina E administrada en esta invención se muestra efectiva manteniendo el estado de salud del pez, previniendo daños causados por la toxina y/o mejorando los efectos tóxicos inducidos por CYN en diversos órganos de tilapias intoxicadas.

Además, el uso de vit E como aditivo alimentario no sólo mejora los niveles de GSH en hígado, sino que por su propia actividad antioxidante es capaz de disminuir la lipoperoxidación (LPO) (hígado, riñón), recuperar las alteraciones de las actividades enzimáticas antioxidantes Glutatión-s-transferasa (GST) (hígado, riñón) y Glutatión peroxidasa (GPx) (hígado) inducida por CYN, y prevenir y recuperar las lesiones histopatológicas inducidas en múltiples órganos como hígado, riñón, corazón, tracto gastrointestinal, branquias y cerebro.

En este sentido, un primer aspecto de la presente invención se refiere al uso de una composición que comprende vit E para la elaboración de un medicamento útil en el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

La composición de la presente invención comprende, al menos, vit E. El medicamento está compuesto, al menos, por la composición anterior. La vit E, sus sales, derivados farmacéuticamente aceptables o sus profármacos, se formulan en una composición farmacéutica apropiada, en la cantidad terapéuticamente efectiva, junto con uno o más vehículos, adyuvantes o excipientes farmacéuticamente aceptables. El medicamento se emplea para el tratamiento de los efectos tóxicos en peces expuestos a CYN.

Por un “derivado farmacéuticamente aceptable” se entiende cualquier sal, farmacéuticamente aceptable o cualquier otro compuesto que después de su administración, es capaz de proporcionar (directa o indirectamente) vit E.

Un “vehículo farmacéuticamente aceptable” se refiere a aquellas sustancias, o combinación de sustancias, conocidas en el sector farmacéutico, utilizadas en la elaboración de formas farmacéuticas de administración e incluye sólidos o líquidos, disolventes, tensioactivos, etc.

El término “tratamiento” tal como se entiende en la presente invención supone combatir los efectos tóxicos para estabilizar el estado de toxicidad de los individuos. El medicamento se emplea también para la prevención de los efectos tóxicos ocasionados a los peces expuestos a CYN. El término “prevención” tal como se entiende en la presente invención consiste en evitar la aparición de efectos tóxicos en peces expuestos a CYN. En este caso, previamente a la intoxicación por CYN, los peces están protegidos por un aumento de las defensas antioxidantes producido por la acción de la vit E. El medicamento también se emplea para la recuperación de los efectos tóxicos ocasionados en peces expuestos a CYN.

El término “efectos tóxicos” tal como se entiende en la presente invención hace referencia a la consecuencia derivada de la exposición del pez a la CYN, es decir, la aparición de diversos efectos adversos, como por ejemplo, un daño celular que ocasiona un daño en los tejidos biológicos, lo que a su vez puede provocar un cambio en las funciones fisiológicas y en el metabolismo celular.

La CYN es una toxina de naturaleza alcaloide, en cuya estructura interviene un grupo tricíclico guanidinio unido a hidroximetiluracilo. Es producida por al menos

siete géneros de cianobacterias, que se encuentran ampliamente distribuida en aguas tropicales y subtropicales. Pueden existir dos posibles epímeros de forma natural, cilindrospermopsina (CYN) y 7-epicilindrospermopsina, ambos tóxicos; la completa pérdida del grupo uracilo elimina la toxicidad de CYN. En reservas naturales de agua se ha descrito otra variante, la 7-desoxicilindrospermopsina, cuya toxicidad apenas está establecida, siendo menos tóxica en ratón que CYN.

Un segundo aspecto de la presente invención es el uso de una composición que comprende vitamina E para la elaboración de un medicamento útil en la recuperación de efectos tóxicos en peces expuestos a CYN. El término "recuperación" hace referencia a la desaparición de los efectos tóxicos causados por la intoxicación con CYN. Esta recuperación supone la reversión total de los daños causados en los tejidos del pez, recuperando de esta forma las funciones normales de los órganos afectados.

En una realización preferida de la presente invención, los efectos tóxicos son alteraciones histopatológicas. El término "alteraciones histopatológicas" tal como se entiende en la presente invención son daños producidos en los tejidos biológicos del pez. Estos daños son detectados por medio del análisis a nivel microscópico de las estructuras patológicas de las diferentes muestras obtenidas, sin excluir otras técnicas de detección.

Una realización aún más preferida de la invención, es el uso donde las alteraciones histopatológicas son producidas en al menos uno de los tejidos de la lista que comprende hígado, riñón, corazón, tracto gastrointestinal, branquias o cerebro. Tal como se ha mencionado anteriormente, la CYN puede acumularse en el tejido hepático y también puede llegar a otros órganos utilizando la sangre como medio de dispersión, de esta forma, la toxina puede causar efectos tóxicos y/o alteraciones histopatológicas en los citados órganos. La recuperación de los tejidos afectados por las alteraciones histopatológicas es un aspecto destacable de la presente invención ya que puede suponer la curación de los peces cultivados, peces seleccionados por diversas características para la cría, peces de especies en peligro de extinción o cualquier otro tipo de pez que presente alteraciones histopatológicas en un grado reversible.

En otra realización más preferida de la presente invención, la vit E se administra en una cantidad diaria de 700 mg por Kg de peso del pez. Esta administración se lleva a cabo durante al menos siete días.

Esta composición, se puede administrar de distintas formas, entre ellas, pero sin limitarse, intraperitonealmente, oralmente, bucalmente, intramuscularmente o de forma subcutánea. Más preferiblemente se administra de forma oral o intraperitoneal. En otra realización más preferida la composición se presenta en una forma adaptada a la administración oral o intraperitoneal.

Preferiblemente los peces intoxicados están expuestos a más de 400 µg CYN/ kg de pez por vía oral en exposición única (intoxicación aguda).

Otra realización preferida de la presente invención, comprende el uso de la composición anteriormente descrita que además incluye excipientes farmacológicamente aceptables.

El término “excipiente” hace referencia a una sustancia que ayuda a la absorción de la sustancia activa (en la presente invención, vit E), estabiliza dicha sustancia activa o ayuda a la preparación del medicamento en el sentido de darle consistencia o aportar sabores que lo hagan más agradable. Así pues, los excipientes podrían tener la función de mantener los ingredientes unidos como por ejemplo almidones, azúcares o celulosas, función de endulzar, función de colorante, función de protección del medicamento como por ejemplo para aislarlo del aire y/o la humedad, función de relleno de una pastilla, cápsula o cualquier otra forma de presentación como por ejemplo el fosfato de calcio dibásico, función desintegradora para facilitar la disolución de los componentes y su absorción en el intestino sin excluir otro tipo de excipientes no mencionados en este párrafo.

El término “excipiente farmacológicamente aceptable” hace referencia a que el excipiente esté permitido y evaluado de modo que no cause daño a los organismos a los que se administra.

En una realización más preferida de la invención, la composición comprende además otra sustancia activa.

En cada caso la composición se adaptará al tipo de administración utilizada, por ello, la composición de la presente invención se puede presentar bajo la forma de soluciones o cualquier otra forma de administración clínicamente permitida y en una cantidad terapéuticamente eficaz.

Otras realizaciones preferidas son el uso para la fabricación de un alimento

funcional, el uso para la fabricación de un complemento vitamínico y otra más es el uso para la fabricación de un complemento nutricional.

La vitamina E puede formar parte de un alimento funcional, complemento vitamínico, complemento nutricional o cualquiera de sus combinaciones. Tal como se entiende en la presente invención, un alimento funcional cumple una función específica como puede ser la de mejorar la salud de los peces. Para ello al alimento funcional se le puede agregar un complemento vitamínico y/o complemento nutricional. El alimento funcional, los complementos descritos o cualquiera de sus combinaciones pueden administrarse junto con un pienso, formar parte de la composición del pienso o pueden administrarse de forma independiente.

En una realización preferida, de la presente invención, los peces son cultivados.

Se entiende por “peces cultivados” aquellos peces criados en piscifactorías, charcas o cualquier contenedor de agua de cualquier tamaño que permita la cría de peces y/o el engorde. Los peces cultivados pueden ser, sin limitar, peces destinados a la alimentación o a la cría de peces ornamentales.

En otra realización preferida, de la presente invención, los peces pertenecen al género *Oreochromis sp.*

Los peces pertenecientes a este género se conocen como Tilapias. Las Tilapias crecen en aguas cálidas dulces o saladas y tienen pocas exigencias respiratorias, rápido crecimiento y facilidad para la puesta. Los peces se pueden seleccionar, sin limitarse, a la lista que comprende *O. amphimelas, O. andersonii, O. angolensis, O. aureus, O. chungruruensis , O. esculentus, O. hunteri, O. ismailiaensis, O. jipe, O. karomo, O. karongae, O. korogwe, O. lepidurus, O. leucostictus, O. lidole, O. macrochir, O. malagarasi, O. mortimeri, O. mossambicus, O. mweruensis, O. niloticus* (Nile tilapia)*, O. Pantani, O. pangani girigan, O. pangani pantani, O. placidus, O. placidus placidus, O. placidus ruvumae, O. rukwaensis, O. saka, O. salinicola, O. schwebischi, O. shiranus, O. shiranus chilwae, O. shiranus shiranus, O. spilurus, O. spilurus niger, O. spilurus percivali, O. spilurus spilurus, O. squamipinnis, O. tanganicae, O. upembae, O. urolepis, O. urolepis hornorum, O. urolepis urolepis u O. variabilis.* Más preferiblemente los peces pertenecen a la especie *O. niloticus* (Nile tilapia).

A lo largo de la descripción y las reivindicaciones la palabra "comprende" y sus variantes no pretenden excluir otras características técnicas, aditivos,

componentes o pasos. Para los expertos en la materia, otros objetos, ventajas y características de la invención se desprenderán en parte de la descripción y en parte de la práctica de la invención. Las siguientes figuras y ejemplos se proporcionan a modo de ilustración, y no se pretende que sean limitativos de la presente invención.

DESCRIPCION DE LAS FIGURAS

Figura 1: Muestra el efecto protector de la vit E sobre la LPO en hígado y riñón de tilapias expuestas a 400 µg CYN/kg pez.

Medidas de LPO en hígado y Medidas de LPO en riñón.
Donde: el eje Y representa los valores de LPO (peroxidación lipídica) cuantificados como sustancias de degradación de la peroxidación de los lípidos que reaccionan con el ácido tiobarbitúrico (*Thiobarbituric Acid Reactive Substances*, TBARS) expresados en nmol de malonildialdehído (MDA)/g de tejido ± error estándar (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (*) grupo de peces expuestos a CYN con respecto a su grupo control y (#) grupo de peces expuestos a CYN y pretratados con vit E (700 mg/Kg pez/día) versus grupo no pretratado con vit E.

Figura 2. Muestra el efecto protector de la vit E, aumentando el cociente GSH/GSSG en hígado de peces intoxicados con 400 µg CYN/kg pez.

Medidas del cociente Glutatión reducido/glutatión oxidado (GSH/GSSG).
Donde: el eje Y representa los valores de GSH/GSSG (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (*) grupo de peces expuestos a CYN con respecto a su grupo control y (#) grupo de peces expuestos a CYN y pretratados con vit E (700 mg/Kg pez/día) versus grupo no pretratado con vit E.

Figura 3. Muestra el efecto protector de la vit E, recuperando la actividad normal de la enzima antioxidante GST en hígado y riñón de peces intoxicados con 400 µg CYN/kg pez.

Medidas de la actividad enzimática antioxidante Glutatión-s-transferasa (GST).
Donde: el eje Y representa los valores de GST expresados en nkat/ mg de proteína ± error estándar (n=8). Los niveles de significación, es decir, que al comparar entre los grupos seleccionados la diferencia entre ellos sea estadísticamente significativa para lo cual el parámetro p debe ser menor de 0.05 ($p<0.05$), son los siguientes: (*) grupo de peces expuestos a CYN con respecto a su grupo control y (#) grupo de peces expuestos a CYN y pretratados con vit E (700 mg/Kg pez/día) versus grupo no pretratado con vit E.

Figura 4. Muestra el efecto protector de la vit E, recuperando la actividad normal de la enzima antioxidante GPx en hígado de peces intoxicados con 400 µg CYN/kg pez.

Medidas de la actividad enzimática antioxidante glutatión peroxidasa (GPx).
Donde: el eje Y representa los valores de GPx expresados en nkat/ mg de proteína ± error estándar (n=8). El nivel de significación ($p<0.05$) es (*) grupo de peces expuestos a CYN con respecto a su grupo control.

FIG. 5. Muestra los cambios histopatológicos en hígado de tilapias expuestas a CYN y su recuperación por vit E.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 6. Cambios histopatológicos en riñón de tilapias expuestas a CYN y su recuperación por vit E.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 7. Cambios histopatológicos en corazón de tilapias expuestas a CYN y su recuperación por vit E.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 8. Cambios histopatológicos en intestino de tilapias expuestas a CYN y su recuperación por vit E.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 9: Cambios histopatológicos en branquias de tilapias expuestas a CYN y su recuperación por vit E observados con el microscopio óptico y electrónico de barrido.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 10: Cambios histopatológicos en cerebro de tilapias expuestas a CYN y su recuperación por vit E observados con el microscopio óptico y electrónico de barrido.

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 100 µm. B, D, B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

EJEMPLOS

A continuación se ilustrará la invención mediante unos ensayos realizados por los inventores que describen el uso de vit E para tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

EJEMPLO 1

La invención se llevó a cabo empleando un total de 32 peces macho de *Oreochromis sp.* (Nile tilapia, Perciformes, Cichlidae), de peso medio 55,2± 6,7 g y longitud de 12±2 cm, obtenidos en una piscifactoría, y transferidos en acuarios (96 L) con sistema de filtración de agua y aireación adecuados, y ciclos de 12:12 h luz/oscuridad. Los peces fueron alimentados con comida comercial (Dibaq, Segovia, Spain), en una cantidad de 0,3 g/día. Los peces se aclimataron durante 15 días antes del experimento. Se realizaron 4 grupos experimentales (8 animales cada uno). Los peces fueron intoxicados con 400 µg/ kg pez de la toxina CYN procedente de un estándar puro (pureza > 95%, Alexis Corporation, Lausen, Suiza) por vía oral (mezclado junto con el pienso). La administración de vit E se realizó también a través del pienso durante un pretratamiento de 7 días, empleando dosis de 700 mg vit E/pez/día siguiendo el siguiente esquema:

Acuario 1: peces control, alimentados solo con pienso normal durante 7 días

Acuario 2: peces alimentados con pienso durante 7 días e intoxicados con CYN (dosis única de 400 µg CYN/kg pez) procedente de estándar puro.

Acuario 3: peces alimentados con pienso + vit E (700 mg/Kg pez/día) durante 7 días.

Acuario 4: peces alimentados con pienso + vit E (700 mg/Kg pez/día) durante 7 días e intoxicados con CYN (dosis única de 400 µg CYN/kg pez) procedente de estándar puro.

Al final del experimento, a las 24 h de la administración de CYN, los peces fueron sacrificados mediante anestesia con hielo, se procedió a la extracción de los órganos, y se prepararon sus extractos para las determinaciones de biomarcadores enzimáticos, según Gutiérrez-Praena *et al*. (Aquat. Toxicol. 105: 100-106, 2011). Concretamente la medida de la lipoperoxidación lipídica (LPO) se realizó midiendo el malonildialdehído o sustancias de degradación de la peroxidación de los lípidos que reaccionan con el ácido tiobarbitúrico; se determinó el cociente glutatión reducido/glutatión oxidado (GSH/GSSG) mediante un kit comercial según indicaciones (Bioxytech GSH/GSSH-412, Oxis Research, Foster City, CA, USA) y las actividades enzimáticas glutatión s transferasa (GST) y glutatión peroxidasa (GPx) (Habig *et al*,. J. Biol. Chem. 249: 7130-7139, 1974; Lawrence and Burk, Bioch. Bioph. Res. Com. 71: 952-958.1976) en hígado y riñón. Los estudios histológicos por microscopia óptica y electrónica en los distintos órganos se llevaron a cabo según Atencio *et al*. (Toxicol. Pathol. 36:449-458, 2008). Para los estudios de significación estadística entre grupos se empleo el análisis de la varianza (ANOVA) y posteriormente el ensayo de Tukey, con una significación estadística $p<0,05$.

Los resultados más significativos fueron los siguientes:

1) En la **Figura 1** se observa como CYN incrementa la LPO en hígado y en riñón (1,3 veces en ambos órganos) frente al control, y los efectos protectores de vit E volviendo este parámetro a los valores basales.

2) En la **Figura 2** se observa como CYN disminuye el cociente GSH/GSSG en hígado (2 veces) y que la aplicación de vit E mejoró este parámetro devolviendo este cociente hasta los valores control.

3) En la **Figura 3** se observa como CYN produce una disminución de la actividad enzimática GST en hígado (2.2 veces) y riñón (1.6 veces) y que la aplicación de vit E recuperó este parámetro hasta los valores control.

4) En la **Figura 4** se observa como CYN produce un incremento de la actividad enzimática GPx en hígado (1.2) y que la aplicación de vit E recuperó este parámetro hasta los valores control.

EJEMPLO 2. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en hígado.

El estudio histopatológico del hígado de los peces pertenecientes a los lotes tratados con CYN puso en evidencia un proceso degenerativo hepático evidenciado por presencia de glucógeno y pérdida de la estructura hepática (FIG. 5 E, F), frente a una ausencia de lesiones del grupo control (FIG. 5 A, B) caracterizada por cordones hepáticos normales, con hepatocitos con una morfología poliédrica normal.

En los dos lotes de peces tratados exclusivamente con vit E se observó que no existían lesiones hepáticas presentando una morfología aparentemente normal (FIG. 5 C, D). En los lotes de peces a los que junto a la toxina CYN se les administró 700 mg vit E/kg pez/día presentan un parénquima hepático con una morfología aparentemente normal con escasa presencia de glucógeno que no llega a ser un proceso patológico (FIG. 5 G, H).

A, B: Hígado de pez control. **A**: Cordones hepáticos normales, morfología poliédrica con núcleo central y citoplasma claro **B**: Detalle de hepatocito aparentemente normal, con abundantes organoides citoplasmáticos, retículos y mitocondrias.

C, D: Hígado de pez control tratado con vitamina E. **C**: Parénquima hepático aparentemente normal con hepatocitos en cordones bien estructurados **D**: Detalle de hepatocito con morfología aparentemente normal.

E, F: Hígado de pez tratado con 400 μg de CYN. **E**: Hepatocitos con citoplasma muy claro, núcleo central con membrana celular de aspecto rígido todo ello debido al gran contenido de glucógeno (círculo) **F**: Detalle del hepatocito con alto contenido de glucógeno y escasos organoides (círculos).

G, H: Hígado de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G**: Detalle de parénquima hepático con cordones hepáticos de estructura aparentemente normal **H**: Hepatocitos con núcleos centrales rodeados de escaso contenido de glucógeno (círculos).

EJEMPLO 3. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en riñón.

Morfológicamente los riñones de los peces de los lotes tratados con CYN mostraron glomerulopatía, hemorragias, tumefacción tubular, dilatación de los túbulos contorneados proximales (TCP) y desorganización de los túbulos contorneados distales (TCD) (FIG. 6 E, F). Los riñones de los peces del lote control presentaron una estructura aparentemente normal (FIG. 6 A, B).

El estudio de los peces tratados solamente con 700 mg vit E/Kg pez/día no mostró ninguna lesión renal (FIG. 6 C, D).
En los peces a los que se les administró CYN pura junto con vit E se observó estructuralmente y ultraestructuralmente una morfología del parénquima renal totalmente normal (FIG. 6 G, H).

A, B: Riñón de pez control. **A**: Parénquima renal donde su estructura es aparentemente normal. Glomérulos (círculo), túbulos contorneados proximales (TCP) y túbulos contorneados distales (TCD) aparentemente normales **B:** Detalle de podocitos aparentemente normales (Po).

C, D: Riñón de pez control tratado con vitamina E. **C**: Detalle de parénquima renal con glomérulos (círculo) y túbulos contorneados (TCP, TCD) aparentemente normales **D**: Detalle de glomérulo renal con los pedicelos (Pe) de los podocitos aparentemente normales.

E, F: Riñón de pez tratado con 400 μg de CYN. **E:** Detalle de parénquima renal con glomérulos donde existe un aumento de las células (círculo) y presencia de hemorragias (flecha). Túbulos contorneados proximales dilatados (TCP) y túbulos contorneados distales (TCD) desorganizados **F**: Glomérulo renal con aumento de la densidad electrónica de los pedicelos muy manifiesta (Pe).

G, H: Riñón de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G**: Glomérulos renales (círculo) y túbulos contorneados (TCP, TCD) aparentemente normales **H:** Detalle de glomérulo renal con pedicelos (Pe) aparentemente normales.

EJEMPLO 4. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en corazón.

El estudio histopatológico realizado sobre peces tratados con CYN mostraron al microscopio óptico presencia de edemas y microhemorragias (FIG. 7 E). Al microscopio electrónico se observa una degeneración de las miofibrillas y presencia de edema intracelular (FIG. 7 F). El corazón de los peces del lote control presentó una estructura aparentemente normal (FIG. 7 A, B).

Los peces tratados solo con vit E presentan una morfología de las fibras cardiacas aparentemente normal y similares a las del grupo control (FIG. 7 C, D).
No se observaron lesiones en los peces tratados con 700 mg vit E/Kg pez/día y expuestos a CYN, observándose miofibrillas aparentemente normales (FIG. 7 G, H).

A, B: Corazón de pez control. **A**: Detalle de fibras cardiacas aparentemente normales **B**: Miofibrillas aparentemente normales con evidencia de las bandas escaleriformes formadas por desmosomas (círculo).

C, D: Corazón de pez control tratado con vitamina E. **C**: Fibras cardiacas aparentemente normales (círculo). **D**. Detalle de miofibrillas aparentemente normales (círculo).

E, F: Corazón de pez tratado con 400 μg de CYN. **E**: Fibras cardiacas desorganizadas (círculo) con presencia de edemas (estrellas) y microhemorragias (flecha) **F**: Detalle de miofibrillas degeneradas (círculo) y presencia de edema intracelular (estrella).

G, H: Corazón de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G**: Detalle del parénquima cardiaco aparentemente normal con presencia de ligeros edemas (estrella) **H**: Detalle de miofibrillas aparentemente normales.

EJEMPLO 5. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en intestino.

A nivel de intestino en los peces tratados con CYN se observaron procesos de enteritis con enterocitos necróticos, abundantes células caliciformes y desorganización de microvellosidades (FIG. 8 E, F) en comparación con el grupo control (FIG. 8 A, B).

Tras la administración de la vit E se observaron células caliciformes y enterocitos con abundantes mitocondrias y microvellosidades aparentemente normales (FIG. 8 C, D), al igual que en los grupos de peces a los que se les administró vit E junto con CYN (FIG. 8 G, H), mostrando así la vit E un efecto protector frente a la toxina.

A, B: Intestino de pez control. **A**: Vellosidades aparentemente normales **B:** Enterocitos con microvellosidades muy desarrolladas y aparentemente normales.

C, D: Intestino de pez control tratado con vitamina E. **C**: Mucosa intestinal con vellosidades y células caliciformes aparentemente normales **D**. Enterocitos con abundantes mitocondrias (Mi) y abundantes microvellosidades (círculo).

E, F: Intestino de pez tratado con 400 μg de CYN. **E:** Mucosa intestinal con abundantes enterocitos necróticos (flechas) y abundantes células caliciformes (círculo) **F:** Detalle de enterocitos con microvellosidades desorganizadas (círculo) y presencia de abundantes células caliciformes (Cc).

G, H: Intestino de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G:** Detalle de microvellosidades intestinales aparentemente normales y escasas células caliciformes (círculo) **H:** Detalle de enterocitos con abundantes microvellosidades aparentemente normales (círculo).

EJEMPLO 6. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en branquias.

Al microscopio óptico las branquias de los peces tratados con CYN pura presentaron procesos hiperémicos en las lamelas del arco branquial con fusión de las mismas y presencia de infiltrados de células inflamatorias (FIG. 9 E). Al microscopio electrónico se observaron una desorganización de la pared de las lamelas branquiales con infiltrados inflamatorios (FIG. 9 F).

Estas lesiones no fueron observadas en el lote control (FIG. 9 A, B), ni en los peces tratados con vit E (FIG. 9 C, D), ni en los grupos de a los que se les administró vit E junto con CYN (FIG. 9 G, H), que presentaron una morfología de branquias y lamelas aparentemente normales. Se muestra así el efecto protector de la vit E frente a CYN.

A, B: Branquia de pez control. **A**: Detalle de branquias aparentemente normales **B**: Estructura de las lamelas aparente normales.

C, D: Branquias de pez control tratado con vitamina E. **C**: Detalle de branquias aparentemente normales **D**: Estructura de las lamelas aparente normales.

E, F: Branquias de pez control tratado con 400 μg de CYN. **E**: Lamelas del arco branquial con fuertes procesos hiperémicos (flechas), fusión de las lamelas (círculo) y con infiltrado de células inflamatorias (estrella) **F:** Desorganización de la pared de las lamelas branquiales con acumulo de células inflamatorias (círculo).

G, H: Branquias de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G**. Detalle de lamelas aparentemente normales. **H**. Detalle de las lamelas aparentemente normales.

EJEMPLO 7. Vit E mejoró las alteraciones histopatológicas inducidas por CYN en cerebro.

A nivel de sistema nervioso (cerebro) en los peces tratados con CYN se observa un proceso necrótico y degenerativo de las neuronas caracterizado por presencia de neuronas tumefactas con cierto grado de vacuolización, núcleo denso e irregular (FIG. 10 E, F) en comparación con el grupo control (FIG. 10 A, B) y con el grupo tratado exclusivamente con vit E (FIG. 10 C, D).
Tras la administración de la vit E junto con CYN se observaron las neuronas aparentemente normales mostrando así el efecto protector de la vit E frente a la toxina. (FIG. 10 G, H).

A, B: Cerebro de pez control. **A**. Detalle de neuronas piramidales **B**. Detalle de neurona aparentemente normal con gran cantidad de organoides citoplasmáticos.

C, D: Cerebro de pez control tratado con vitamina E. **C**. Detalle de la capa de neuronas granulares aparentemente normales (círculo) **D**. Detalle de neurona aparentemente normal.

E, F: Cerebro de pez control tratado con 400 μg de CYN. **E**. Neuronas degeneradas tumefactas muy basófilas y con cierto grado de vacuolización (círculo) **F**. Detalle de neurona con núcleo muy denso e irregular (Nu) y con todos los organoides membranosos vacuolizados (flechas).

G, H: Cerebro de pez pretratado con 700 mg de vit E y tratado con 400 μg CYN. **G**. Neuronas aparentemente normales **H**. Detalle de neurona aparentemente normal.

REIVINDICACIONES

1. Uso de una composición que comprende vitamina E para la elaboración de un medicamento útil en el tratamiento y/o prevención de efectos tóxicos en peces expuestos a cilindrospermopsina.

2. Uso de una composición que comprende vitamina E para la elaboración de un medicamento útil en la recuperación de efectos tóxicos en peces expuestos a cilindrospermopsina.

3. Uso según cualquiera de las reivindicaciones 1 ó 2, donde los efectos tóxicos son alteraciones histopatológicas.

4. Uso según la reivindicación 3, donde las alteraciones histopatológicas son producidas en al menos uno de los tejidos de la lista que comprende hígado, riñón, corazón, tracto gastrointestinal, branquias o cerebro.

5. Uso según cualquiera de las reivindicaciones 1 a 4, donde la vitamina E se administra en una cantidad diaria de 700 mg/Kg pez/día.

6. Uso según la reivindicación 5, donde la composición se presenta en una forma adaptada a la administración oral o intraperitoneal.

7. Uso según cualquiera de las reivindicaciones 1 a 6, donde la composición incluye excipientes farmacológicamente aceptables.

8. Uso según cualquiera de las reivindicaciones 1 a 7, donde la composición comprende además otra sustancia activa.

9. Uso según cualquiera de las reivindicaciones 1 a 8 para la fabricación de un alimento funcional.

10. Uso según cualquiera de las reivindicaciones 1 a 9 para la fabricación de un complemento vitamínico.

11. Uso según cualquiera de las reivindicaciones 1 a 10 para la fabricación de un complemento nutricional.

12. Uso según cualquiera de las reivindicaciones 1 a 11, donde los peces son cultivados.

13. Uso según cualquiera de las reivindicaciones 1 a 12, donde los peces pertenecen al género *Orechromis sp.*

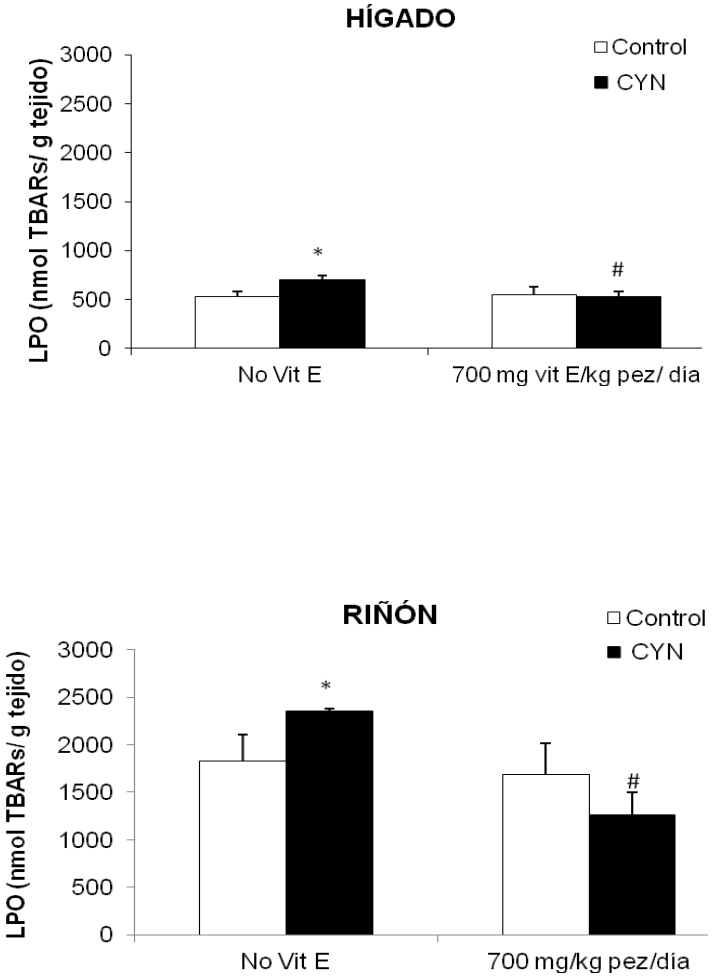
HÍGADO
Control
CYN
LPO (nmol TBARs/ g tejido)
3000
2500
2000
1500
1000
500
0
*
#
No Vit E
700 mg vit E/kg pez/ día
RIÑÓN
Control
CYN
LPO (nmol TBARs/ g tejido)
3000
2500
2000
1500
1000
500
0
*
#
No Vit E
700 mg/kg pez/día

FIG. 1

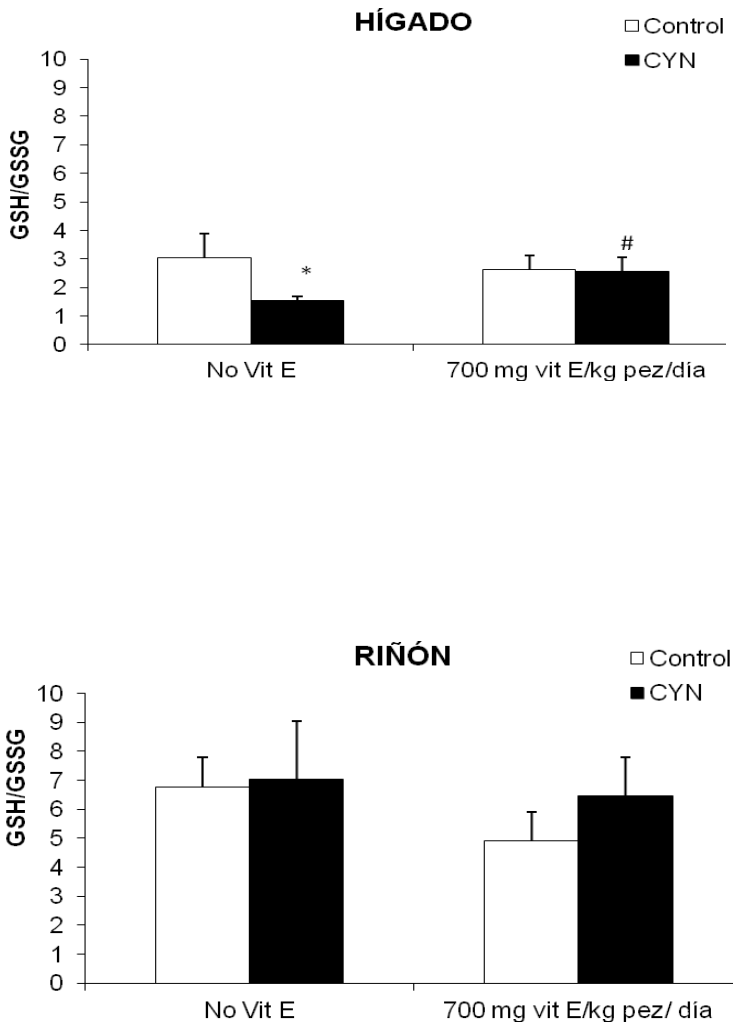
HÍGADO
Control
CYN
GSH/GSSG
10
9
8
7
6
5
4
3
2
1
0
*
#
No Vit E
700 mg vit E/kg pez/día
RIÑÓN
Control
CYN
GSH/GSSG
10
9
8
7
6
5
4
3
2
1
0
No Vit E
700 mg vit E/kg pez/ día

FIG. 2

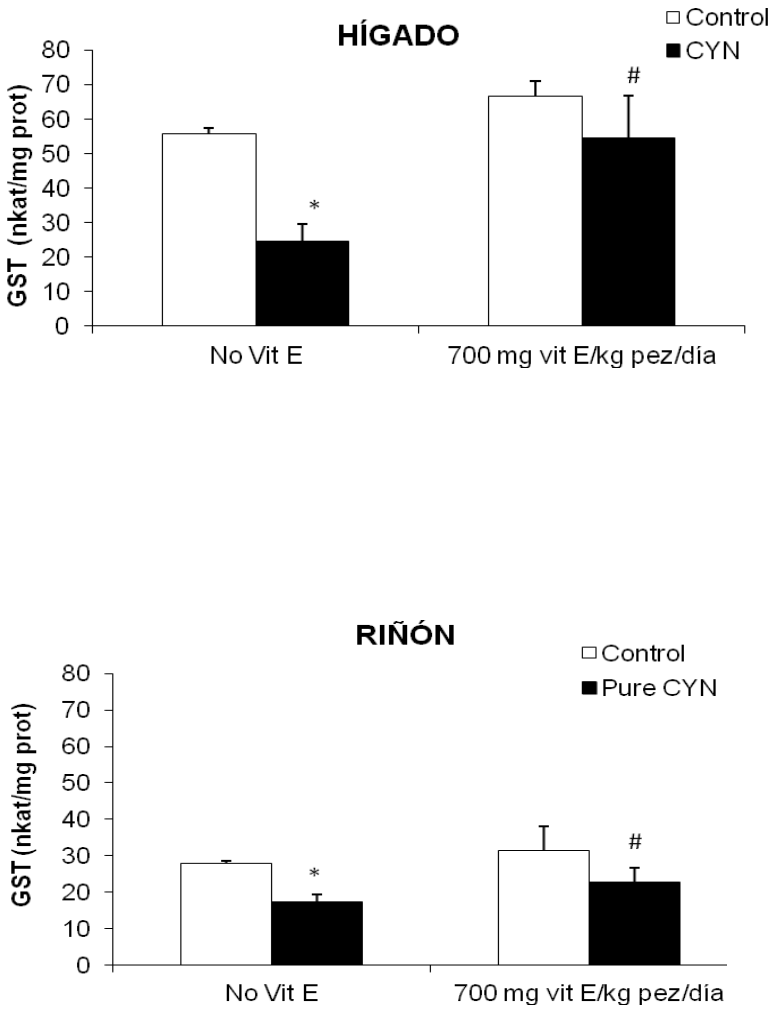
HÍGADO
Control
CYN
GST (nkat/mg prot)
80
70
60
50
40
30
20
10
0
*
#
No Vit E
700 mg vit E/kg pez/día
RIÑÓN
Control
Pure CYN
GST (nkat/mg prot)
80
70
60
50
40
30
20
10
0
*
#
No Vit E
700 mg vit E/kg pez/día

FIG. 3

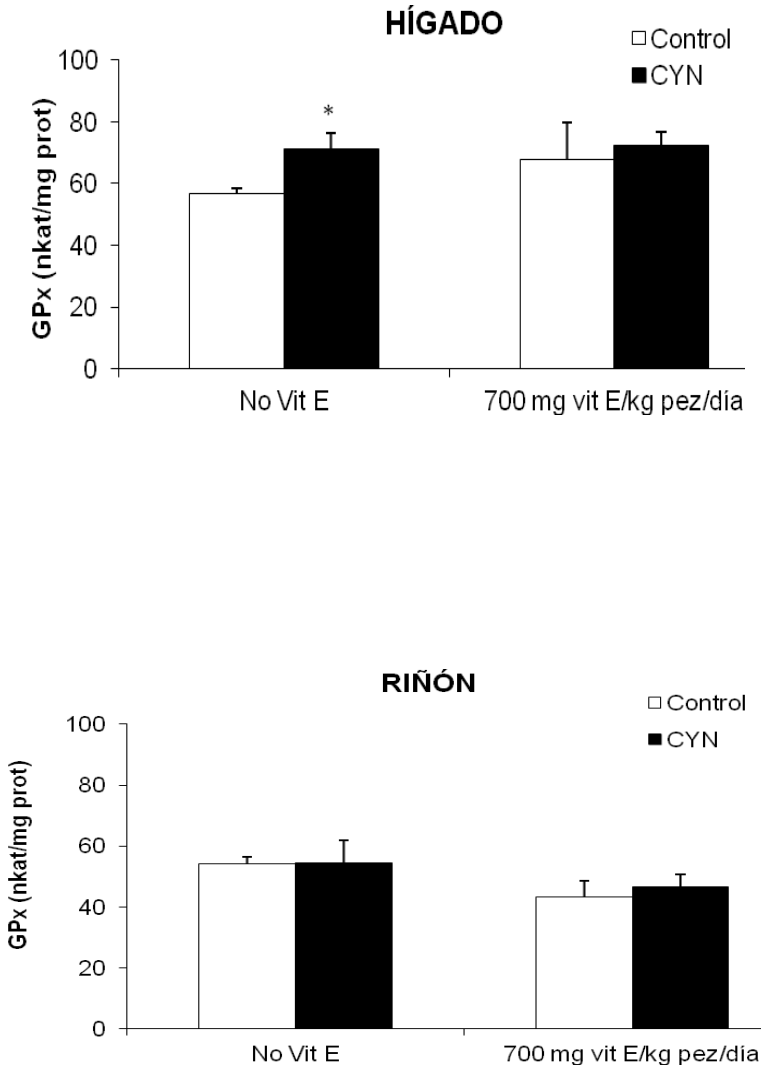
HÍGADO
Control
CYN
GPx (nkat/mg prot)
100
80
60
40
20
0
*
No Vit E
700 mg vit E/kg pez/día
RIÑÓN
Control
CYN
GPx (nkat/mg prot)
100
80
60
40
20
0
No Vit E
700 mg vit E/kg pez/día

FIG. 4

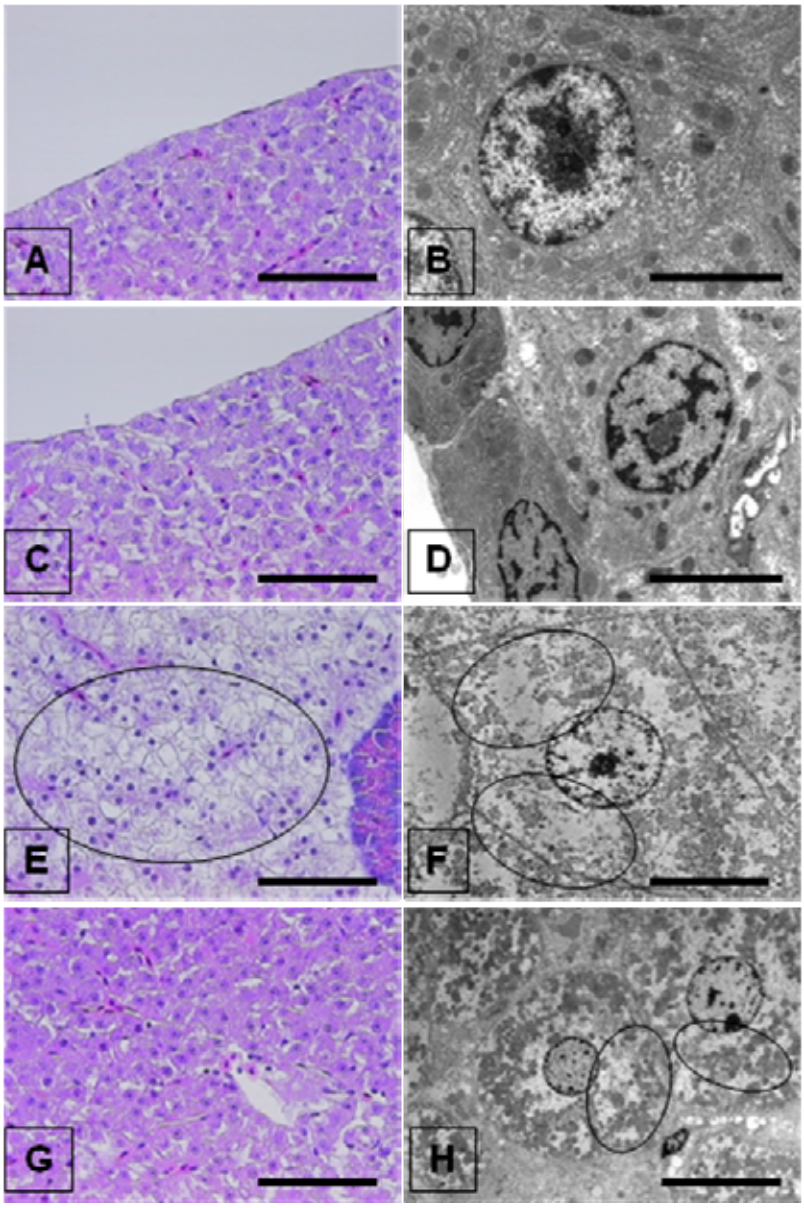

FIG. 5

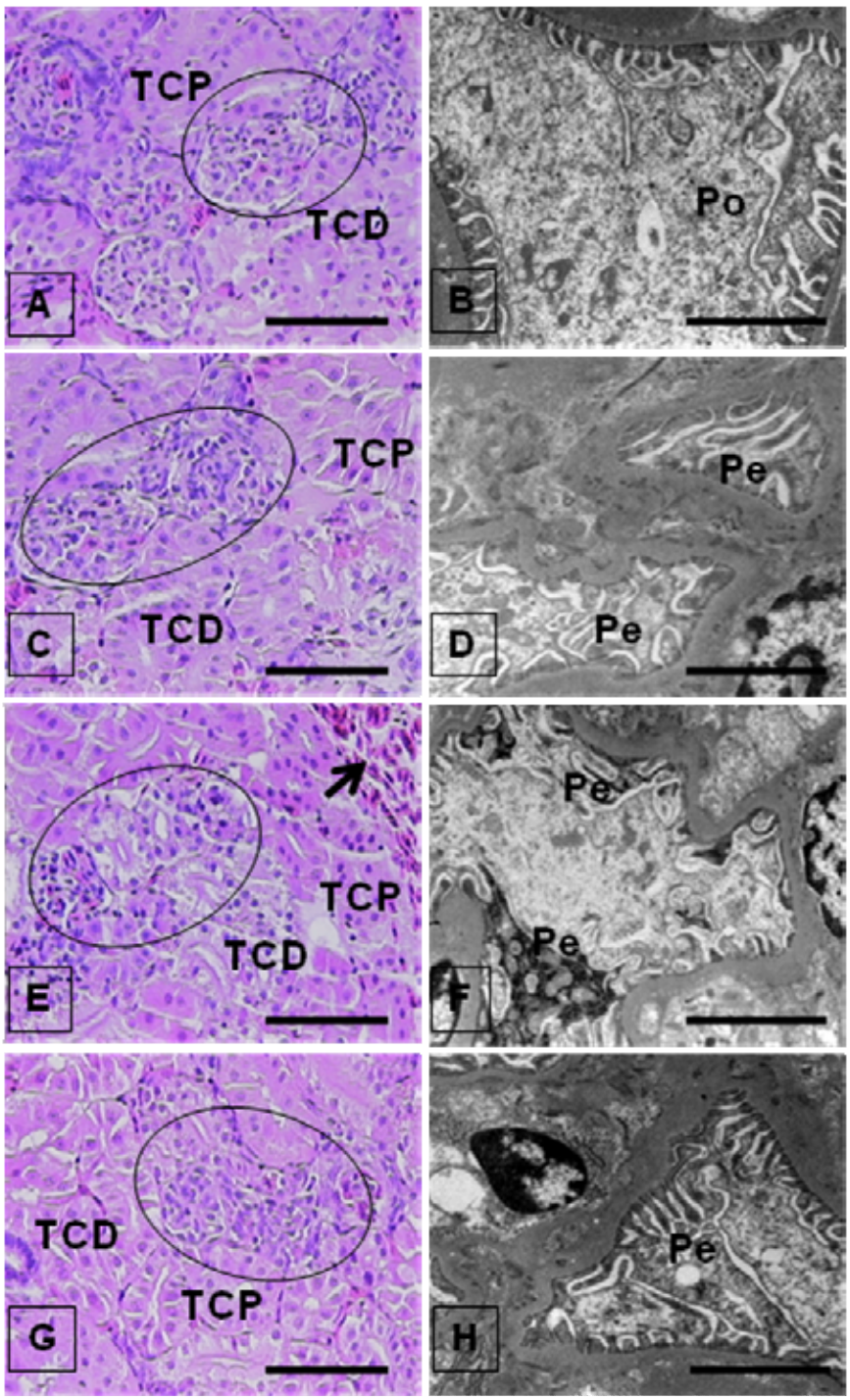

FIG. 6

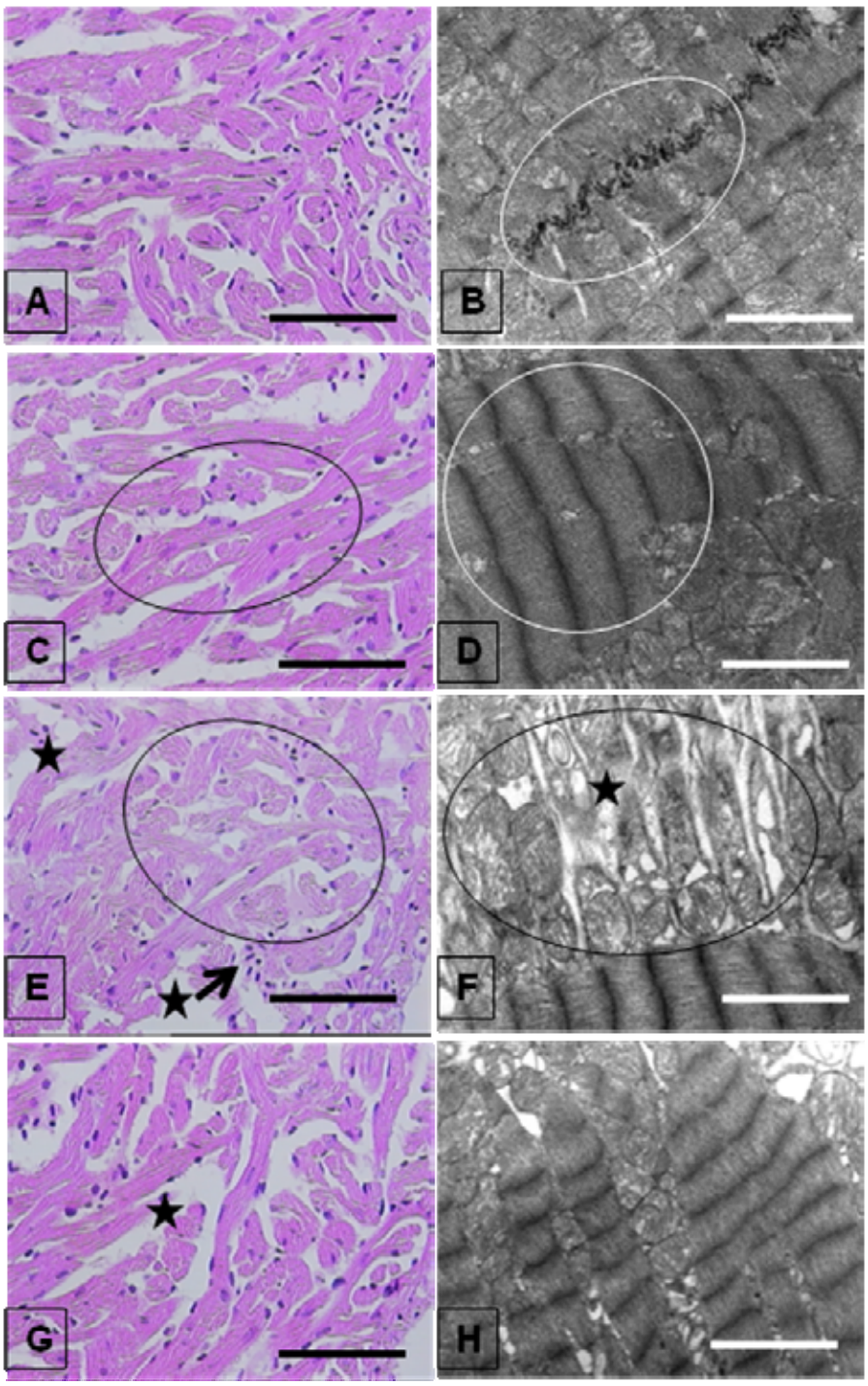

FIG. 7

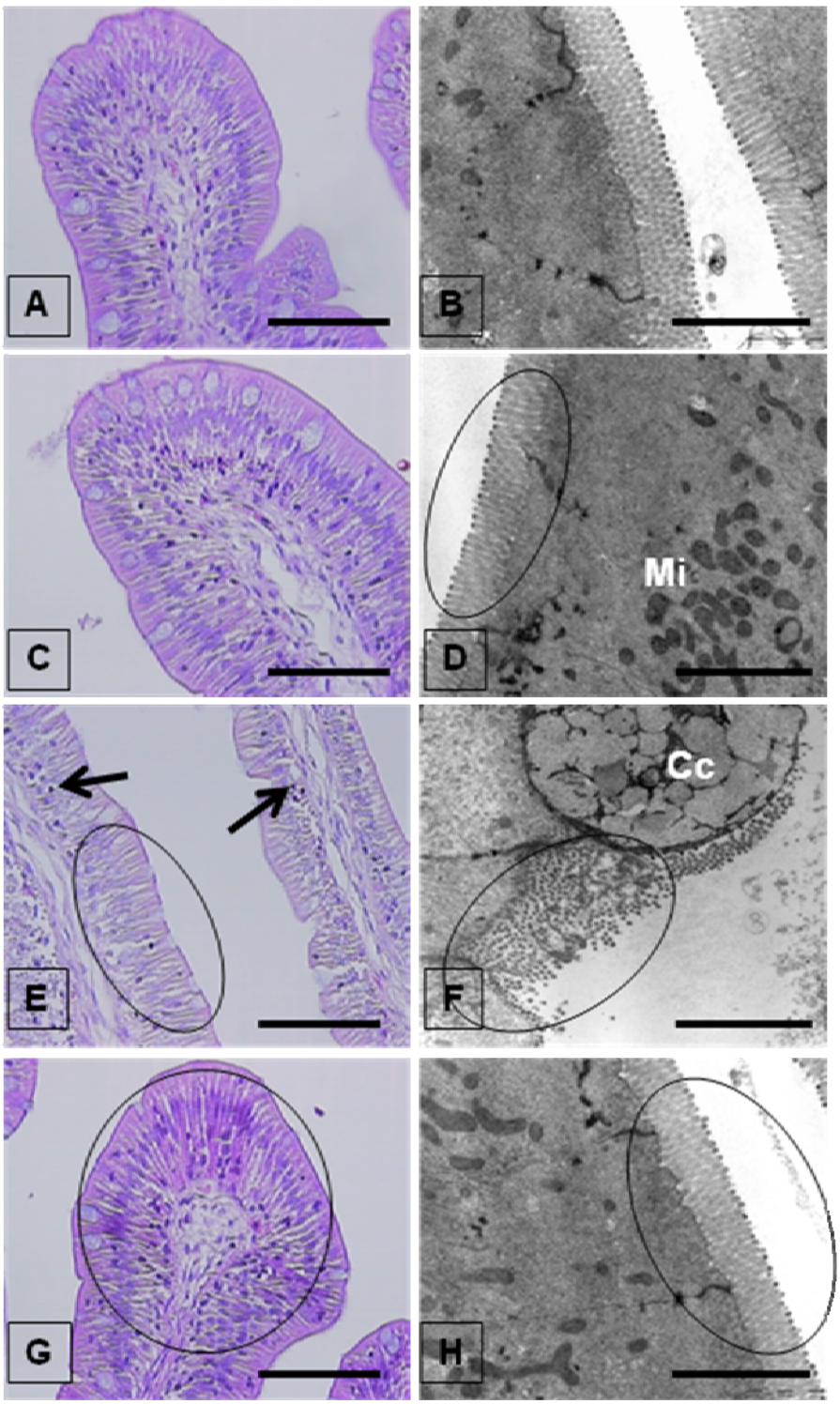

FIG. 8

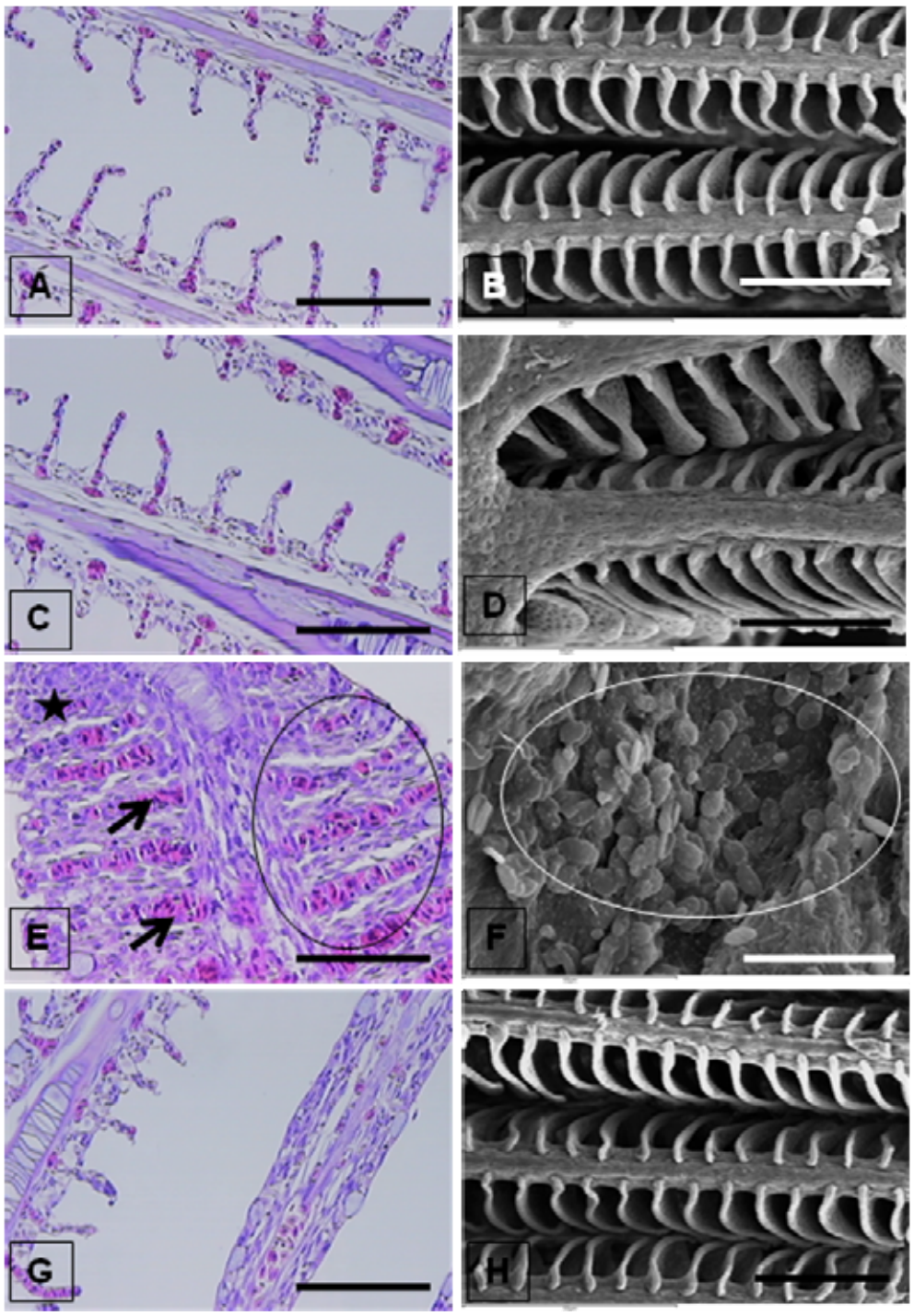

FIG. 9

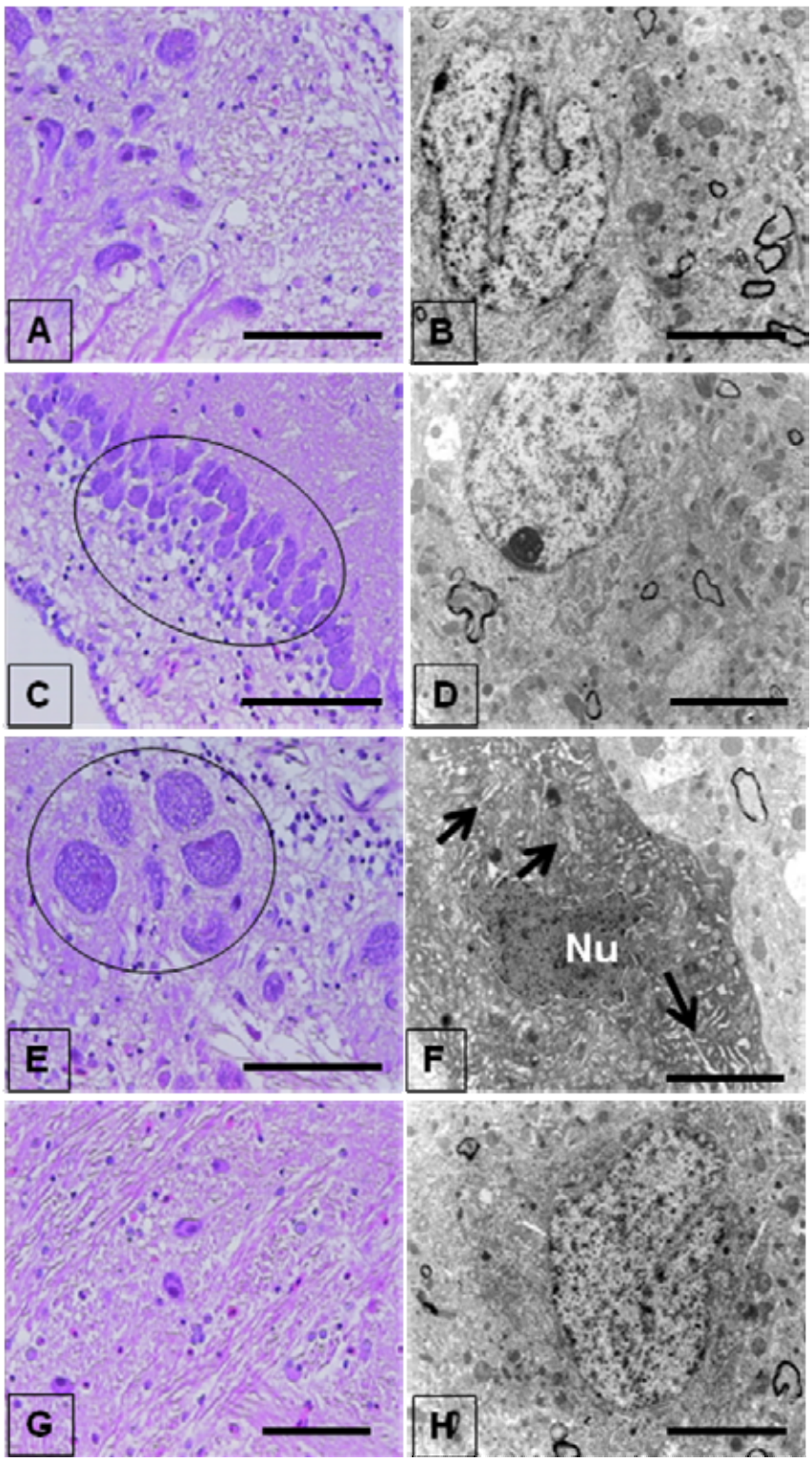

FIG. 10

VIII. REFERENCIAS / REFERENCES

Aguete, E.C., Gago-Martinez, A., Leao, J.M., Rodriguez-Vazquez, J.A., Menard, C., Lawrence, J.F. (2003). HPLC and HPCE analysis of microcystins RR, LR and YR present in cyanobacteria and water by using immunoaffinity extraction**.** Talanta 59, 697-705.

Altuntas, I., Delibas, N., Sutcu, R. (2002). The effects of organophosphate insecticide methidathion on lipid peroxidation and anti-oxidant enzymes in rat erythrocytes: Role of vitamins E and C. Hum Exp Toxicol 21, 681-685.

Anderson, L., Fabbro, L.D., Cowden, K. (2003). Assessment of Blue-Green Algal Toxins in Barramundi, Red Clay and Mussels from Awoonga Dam. Central Queensland University: Gladstone, Australia.

Ann, J., Carmichael, W.W. (1994). Use of a colorimetric protein phosphatase inhibition assay and enzyme linked inmuno sorbent assay for the study of microcystins and nodularins. Toxicon 32, 1495-1507.

Anwar, M., Nanda, N., Bhatia, A., Akhtar, R., Mahmood, S. 2013. Effect of antioxidant supplementation on digestive enzymes in radiation induced intestinal damage in rats. Int J Radiat Biol 89(12), 1061-1070.

Aráoz, R., Molgó, J., Tandeau de Marsac, N. (2010). Neurotoxic cyanobacterial toxins. Toxicon 56, 813-828.

Atencio, L., Moreno, I., Prieto, A.I., Moyano, R., Molina, A.M., Cameán, A.M. (2008a). Acute effects of microcystins MC-LR and MC-RR on acid and alkaline phosphatase and pathological changes in intraperitoneally exposed tilapia fish (*Oreochromis sp*.). Toxicol Pathol 36, 449-458.

Atencio, L., Moreno, I., Jos, A., Prieto, A.I., Moyano, R., Blanco, A., Cameán, A.M. (2009). Effects of dietary selenium on the oxidative stress and pathological changes in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Toxicon 53, 269-282.

Azevedo, S.M.F.O., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K. (2002). Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. Toxicology 181-182, 441-446.

Banaclocha, M.M. (2001). Therapeutic potential of N-acetylcysteine in age-related mitochondrial neurodegenerative diseases. Med Hypotheses 56, 472-477.

Banker, R., Carmeli, S., Hadas, O., Teltsh, B., Porat, R., Sukenik, A. (1997). Identification of cylindrospermopsin in *Aphanizomenon ovalisporum* (Cyanophyceae) isolated from Lake Kinneret, Israel. J Phycol 33, 613-616.

Banker, R., Carmeli, S., Werman, M., Telsch, B., Porat, R., Sukenik, A. (2001). Uracil moiety is required for toxicity of the cyanobacterial hepatotoxin cylindrospermopsin. J Toxicol Environ Health Sci 62, 281-288.

Banker, R., Teltsch, B., Sukenik, A., Carmeli, S. (2000). 7-Epicylindrospermopsin, a Toxic Minor Metabolite of the Cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel. J Nat Prod 63, 387-389.

Barco, M., Rivera, J., Caixach, J. (2002). Analysis of cyanobacterial hepatotoxins in water samples by microbore reversed-phase liquid chromatography-electrospry ionisation mass spectrometry. J Chromatogr A 959, 103-111.

Batcioglu, K., Karagözler, A.A., Ozturk, I.C., Genc, M., Bay, A., Ozturk, F., Aydogdu, N. (2005). Comparison of chemopreventive effects of Vitamin E plus selenium versus melatonin in 7,12-dimethylbenz(a)anthracene-induced mouse brain damage. Cancer Detect Prev 29, 54-58.

Batcioglu, K., Ozturk, I.C., Karagözler, A.A., Karatas, F. (2002). Comparison of selenium level with GSH-Px activity in the liver of mice treated 7,12-DMBA. Cell Biochem Funct 20, 115-118.

Bazin, E., Mourot, A., Humpage, A.R., Fessard, V. (2010). Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environ Mol Mutagen 51, 251–259.

Bazin, E., Huet, S., Jarry, G., Le Hégarat, L., Munday, J.S., Humpage, A.R., Fessard, V. (2012). Cytotoxic and genotoxic effects of cylindrospermopsin in mice treated by gavage or intraperitoneal injection. Environ Toxicol 27, 277-284.

Bebianno, M.J., Lopes, B., Guerra, L., Hoarau, P., Ferreira, A.M. (2007). Glutathione S-tranferases and cytochrome P450 activities in *Mytilus galloprovincialis* from the South coast of Portugal: Effect of abiotic factors. Environ Int 33, 550-558.

Belay, B., Esteban-Cruciani, N., Walsh, C.A., Kaskel, F.J. (2006). The use of levo-carnitine in children with renal disease: a review and a call for future studies. Pediatr Nephrol 21, 308-317.

Bell, S.G., Codd, G.A. (1994). Cyanobacterial toxins and human health. Rev Med Microbiol 5, 256-264.

Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L. (2009). Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53, 289-299.

Berry, J.P., Lind, O. (2010). First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (*Pomacea patula catemacensis*). Toxicon 55, 930-938.

Berry, J.P., Jaja-Chimedza, A., Dávalos-Lind, L., Lind, O. (2012). Apparent bioaccumulation of cylindrospermopsin and paralytic shellfish toxins by finfish in Lake Catemaco (Veracruz, Mexico). Food Addit Contam A: Chem Anal Control Expo Risk Assess 29, 314-321.

Beyer, D., Surányi, G., Vasas, G., Roszik, J., Erdodi, F., M-Hamvas, M., Bácsi, I., Bátori, R., Serfozo, Z., Szigeti, Z.M., Vereb, G., Demeter, Z., Gonda, S., Máthé, C. (2009). Cylindrospermopsin induces alterations of root histology and microtubule organization in common reed (*Phragmites australis*) plantlets cultured *in vitro*. Toxicon 54, 440–449.

Bilinski, E., Jonas, R.E.E. (1970). Effects of coenzyme A and carnitine on fatty acid oxidation in rainbow trout mitochondria. J Fish Res Board Can 27, 857–864.

Bláhová, L., Oravec, M., Marsalek, B., Sejnohova, L., Simek, Z., Blaha, L. (2009). The first occurrence of the cyanobacterial alkaloid toxin cylindrospermopsin in the Czech Republic as determined by inmunochemical and LC/MS methods. Toxicon 53, 519-524.

Bogialli, S., Bruno, M., Curini, R., Di Corcia, A., Fanali, C., Lagana, A. (2006). Monitoring Algal Toxins in Lake Water by Liquid Chromatography Tandem Mass Spectrometry. Environ Sci Technol 40, 2917-2923.

Bogialli, S., Bruno, M., Curini, R., Di Corcia, A., Laganá, A., Mari, B. (2005). Simple assay for analyzing five microcystins and nodularin in fish muscle tissue: hot water extraction followed by liquid chromatography-tandem mass sepectrometry. J Agr Food Chem 53, 6586-6592.

Bonilla, S., Rautio, M., Vincent, W.F. (2009). Phytoplankton and phytobenthos pigment strategies: implications for algal survival in the changing Arctic. Polar Biology 32(9), 1293-1303.

Bourke, A.T.C., Hawes, R.B., Neilson, A., Stallman, N.D. (1983). An outbreak of hepatoenteritis (the Palm Island mystery disease) possibly caused by algal intoxication, Toxicon Supplement 3, 45-48.

Boyland, E., Chasseaud, L.F. (1969). The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Adv Enzymol Relat Areas Mol Biol 32, 173-219.

Briand, J.F., Jacquet, S., Bernard, C., Humbert, J.F. (2003). Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. Vet Res 34, 361-377.

Brient, L., Lengronne, M., Bormans, M., Fastner, J. (2009). First occurrence of cylindrospermosin in freshwater in France. Environ Toxicol 24, 415-420.

Burch, M.D. (2006). Effective doses, guidelines & regulations. In Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB), State of the Science and Research Needs ed. Hudnell, H.K. 831–853. New York, USA: Springer.

Burger, J., Gaines, K.F., Boring, C.S., Snodgrass, J., Stephens, W.L.J., Gochfeld, M. (2004). Effects of cooking on Radiocesium in fish from the Savannah river, exposure differences for the public. Arch Environ Contam Toxicol 46, 231–235.

Burns, J. (2008). Toxic cyanobacteria in Florida waters. Adv Exp Med Biol. 619, 127-137.

Byth, S. (1980). Palm island mystery disease. Med J Australia 2, 40-42.

Calabrese, V., Giuffrida Stella, A.M., Calvani, M., Butterfield, D.A. (2006). Acetylcarnitine and cellular stress response: roles in nutritional redox homeostasis and regulation of longevity genes. J Nutr Biochem 17, 73-88.

Caló, L.A., Pagnin, E., Davis, P.A., Semplicini, A., Nicolai, R., Calvani, M., Pessina, A.C. (2006). Antioxidant efecto of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. Int J Cardiol 107, 54-60.

Campos, A., Vasconcelos, V.M. (2010). Molecular mechanisms of microcystin toxicity in animal cells. Int J Mol Sci 11, 268–287.

Canbaz, H., Akca, T., Tataroglu, C., Caglikulekci, M., Dirlik, M., Ayaz, L., Ustunsoy, A.B., Tasdelen, B., Aydin, S., 2007. The effects of exogenous l-carnitine on lipid peroxidation and tissue damage in an experimental warm Hepatic Ischemia- Reperfusion Injury Model. Curr Ther Res 68, 32–46.

Cappitelli, F., Abbruscato P., Foladori, P., Zanardini, E., Ranalli, G., Principi P., Villa, F., Polo, A., Sorlini, C. (2009). Detection and elimination of Cyanobacteria from frescoes: The case of St. Brizio Chapel (Orvieto Cathedral, Italy). Microbial Ecol 57, 633-639.

Carmichael, W.W. (2001). Health effects of toxin-producing cyanobacteria: the CyanoHABs**.** Hum Ecol Risk Assess 7, 1393-1407.

Carmichael, W.W. (2008). A world view – one-hundred twenty-seven years of research on toxic cyanobacteria – where do we go from here? En: Hudnell, K.H. (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, Series. Adv Exp Med Biol 619, 105-120 XXIV.

Carmichael, W.W., Azevedo, S.M.F.O., An, J.S., Molica, R.J.R., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K. (2001). Human fatalities form cyanobacteria: chemical and biological evidence for cyanotoxins. Environ Health Perspect 109, 663-668.

Carmichael, W.W., Beasly, V., Bunner, D. L., Eloff, J. N., Falconer, I., Gorham, P., Harada, K.I., Krishnamurty, T., Min-Juan, Y., Moore, R. E., Rinehart, K., Runnegar, M., Skulberg, O.M., Watanabe, M. (1988). Naming cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26, 971-973.

Cazenave, J., Bistoni, M.A., Pesce, S.F., Wunderlin, D.A. 2006. Differential detoxification and antioxidant response in diverse organs of Corydoras paleatus exposed to Microcystin-RR. Aquat. Toxicol 76, 1–12.

Chen, H., Pellett, L.J., Andersen, H.J., Tappel, A.L. (1993). Protection by vitamin E, Selenium, and β-carotene against oxidative damage in rat liver slices and homogenate. Free Radical Biol Med 14, 473-482.

Chen, J., Xie, P., Zhang, D., Ke, Z., Yang, H. (2006). In situ studies on the bioaccumulation of microcystins in the phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*) stocked in Lake Taihu with dense toxic Microcystis blooms. Aquaculture 261, 1026-1038.

Chen, J., Zhang, D., Xie, P., Wang, Q., Ma, Z. (2009). Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and wáter bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic *Microcystis* blooms. Sci Total Environ 407, 3317-3322.

Chiswell, R.K., Shaw, G.R., Eaglesham, G., Smith, M.J., Norris, R.L., Seawright, A.A., Moore, M.R. (1999). Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: effect of pH, temperature, and sunlight on decomposition. Environ Toxicol 14, 155-161.

Chong, M.W.K., Wong, B.S.F., Lam, P.K.S., Shaw, G.R, Seawright, A.A. (2002). Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon 40,, 205-211.

Chorus, I. (2001). Cyanotoxin occurrence in freshwaters-a summary of survey results from different countries. En: Chorus I (Ed.) Cyanotoxins. Umweltbundesamt, Berlin, Germany 75-82.

Chorus, I., Bartram, J. (1999). Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management, World Health Organization.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. (2000). Health risk caused by freshwater cyanobacteria in recreational waters. J Toxicol Environ Health B Crit Rev 3, 323-347.

Codd, G.A., Morrison, L.F., Metcalf, J.S. (2005). Cyanobacterial toxins: risk management for health protection. Toxicol Appl Pharmacol 203, 264-272.

Codex Alimentarius Commission. (2003). Joint FAO/WHO Food Standards Programme, 4th Session, Yokohama, Japan.

Corbel, S., Mougin, C., Bouaicha, N. (2014). Cyanobacterial toxins: Modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96, 1–5.

Dai, M., Xie, P., Liang, G., Chen, J., Lei, H. (2008b). Simultaneous determination of microcystin-LR and its gluthatione conjugate in fish tissue by liquid chromatography–tandem mass spectrometry. J Chromatogr B: Analyt Technol Biomed Life Sci 862, 43-50.

Davis, T.W., Orr, P.T., Boyer, G.L., Burford, M.A. (2014). Investigating the production and release of cylindrospermopsin and deoxy-cylindrospermopsin by *Cylindrospermopsis raciborskii* over a natural growth cycle. Harmful Algae 31, 18-25.

Dawson, R.M. (1998). The toxicology of microcystins. Toxicon 36, 953-962.

Dayanandan, A., Kumar, P., Panneerselvam, C. (2001). Protective role of l-carnitine on liver and heart lipid peroxidation in atherosclerotic rats. J Nutr Biochem 12, 254–257.

De la Cruz, A.A., Hiskia, A., Kaloudis, T., Chernoff, N., Hill, D., Antoniou, M.G., He, X., Loftin, K., O'Shea, K., Zhao, C., Pelaez, M., Han, C., Lynch, T.J., Dionysiou, D.D. (2013). A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. Environ Sci Process Impacts 15, 1979-2003.

De León, L. (2002). Floraciones de cianobacterias en aguas continentales del Uruguay: causas y consecuencias. En: Perfil Ambiental de Uruguay, Domínguez, A. y R. G. Prieto (eds.), Nordan-Comunidad, Montevideo 28-37.

Dell'Aversano, C., Eaglesham, G.K., Quilliam, M.A. (2004). Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography– mass spectrometry. J Chromatogr A 1028, 155-164.

Deng, R., Chow, T.J. (2010). Review: hypolipidemic, antioxidant, and antiinflammatory activities of microalgae *Spirulina*. Cardiovasc Ther 28, 33-45.

Derin, N., Izgut-Uysal, V.N., Agac, A., Aliciguzel, Y., Demir, N. (2004). L-Carnitine protects gastric mucosa by decreasing ischemiae reperfusion induced lipid peroxidation. J Phys Pharm 55, 595-606.

Devesa, V., Vélez, D., Montoro, R. (2008). Effect of thermal treatments on arsenic species contents in food. Food Chem Toxicol 46, 1–8.

Dittmann, E., Wiegand, C. (2006). Cyanobacterial toxins – occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50, 7-17.

Domingo, J.L. (2011). Influence of cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food: a review of the published literature. Crit Rev Food Sci Nutr 51, 29-37.

Doyen, P., Bigot, A., Vasseur, P., Rodius, F., 2008. Molecular cloning and expression study of pi-class glutathione S-transferase (piGST) and selenium-dependent glutathione peroxidase (Se-GPx) transcripts in the freshwater bivalve *Dreissena polymorpha*. Comp Biochem Physiol C 147, 69–77.

Eaglesham, G.K., Norris, R.L., Shaw, G.R., Smith, M.J., Chiswell, R.K., Davis, B.C., Neville, G.R., Seawright, A.A., Moore, M.R. (1999). Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. Environ Toxicol 14, 151-154.

Eisler, R. (2000). Handbook of chemical risk assessment: Health hazards to humans, plants, and animals. Boca Raton, FL: Lewis.

El Khalloufi, F., El Ghazali, I., Saqrane, S., Oufdou, K., Vasconcelos, V., Oudra, B. (2012). Phytotoxic effects of a natural bloom extract containing microcystins on Lycopersicon esculentum. Ecotoxicol Environ Saf 79, 199–205.

Emami Naini, A., Moradi, M., Mortazavi, M., Amini Harandi, A., Hadizadeh, M., Shirani, F., Basir Ghafoori, H., Emami Naini, P. (2012). Effects of oral L-carnitine supplementation on lipid pro-file, anemia, and quality of life in chronic renal disease patients under hemodialysis: a randomized, double-blinded, placebo-controlled trial. J Nutr Metab Vol. 2012, Article ID 510483, 6 pages http://dx.doi.org/10.1155/2012/510483

Emmanouil, C., Green, R.M., Willey, F.R., Chipman, J.K. (2008). Oxidative damage in gill of *Mytilus edulis* from Merseyside, UK, and reversibility after depuration. Environ Pollut 151, 663–668.

Everson, S., Fabbro, L., Kinnear, S., Wright, P. (2011). Extreme differences in akinete, heterocyte and cylindrospermopsin concentrations with depth in a successive bloom involving *Aphanizomenon ovalisporum* (Forti) and *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju. Harmful Algae 10, 265-276.

Everson, S.R., Fabbro, L.D., Kinnear, S., Eaglesham, G., Wright, P.J. (2009). Distribution of the cyanobacterial toxins cylindrospermopsin and deoxycylindrospermopsin in a stratified lake in north-eastern New South Wales, Australia. Mar Fresh Res 60, 25-33.

Fageria, N.K., Baligar, V.C., Clark, R.B. (2002). Micronutrients in crop production. En:Donald L. Sparks. Advances in Agronomy. Burlington: Academic Press 77, 185–268.

Falconer, I.R. (1998). Algal toxins and human health, En: The handbook of environmental chemistry, J. HRUBEC, Berlin Heidelberg, Springer-Verlag, 5 Part C Quality and treatment of drinking water 2, 53–82.

Falconer, I.R., Burch, M.D., Steffensen, D.A., Choice, M., Coverdale, O.R. (1994). Toxicity of the blue-green alga (Cyanobacterium) *Microcystis aeruginosa* in drinking water to growing pigs, as an animal model for human injury and risk assesment. Environ Toxic Water 9, 31-139.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J., Hawkins, P.R. (1999). Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ Toxicol 14, 143-150.

Falconer, I.R., Humpage, A.R. (2001) Preliminary evidence for in vivo tumour initiation by oral administration of extracts of the bluegreen alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. Environ Toxicol 16, 192-195.

Falconer, I.R., Humpage, A.R. (2006). Cyanobacterial (blue-green algal) toxins in water supplies: Cylindrospermopsins. Environ Toxicol 21, 299-304.

Fastner, J., Heinze, R., Humpage, A.R., Mischke, U., Eaglesham, G.K., Chorus, I. (2003). Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates. Toxicon 42, 313-321

Fastner, J., Neumann, U., Wirsing, B., Weckesser, J., Wiedner, C., Nixdorf, B., Chorus, I. (1999). Microcystins (Hepatotoxic Heptapeptide) in German Fresh Water Bodies. Environ Toxicol 14, 13-22.

Fastner, J., Rücker, J., Stüken, A., Preußel, K., Nixdorf, B., Chorus, I., K€ohler, A. and Wiedner, C. (2007). Occurrence of the cyanobacterial toxin cylindrospermopsin in Northeast Germany. Environ Toxicol 22, 26-32.

Fatouros, I.G., Douroudos, I., Panagoutsos, S., Pasadakis, P., Nikolaidis, M.G., Chatzinikolaou, A., Sovatzidis, A., Michailidis, Y., Jamurtas, A.Z., Mandalidis, D., Taxildaris, K., Vargemezis, V. (2010). Effects of L-carnitine on oxidative stress responses in patients with renal disease. Med Sci Sports Exerc 42, 180-181.

Ferreira, M., Moradas-Ferreira, P., Reis-Henriques, M.A. (2005). Oxidative stress biomarkers in two resident species, mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*), from a polluted site in River Douro Estuary, Portugal. Aquat Toxicol 71, 39-48.

Ferreira, M., Moradas-Ferreira, P., Reis-Henriques, M.A. (2007). The effect of long-term depuration on levels of oxidative stress biomarkers in mullets (*Mugil cephalus*) chronically exposed to contaminants. Mar Environ Res 64, 181-190.

Fessard, V., Bernard, C. (2003). Cell alterations but no DNA strand breaks induced in vitro by cylindrospermopsin in CHO K1 cells. Environ Toxicol 18, 353-359.

Filipič, M., Žegura, B., Štraser, A. (2011). Genotoxicity and potential carcinogenicity of cyanobacterial toxins- a review. Mutat Res-Rev Mutat 727, 16-41.

Fischer, W.J., Dietrich, D. (2000). Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). Toxicol Appl Pharmacol 164, 73-81.

Foss, A. J., Aubel, M. T. (2013). The extraction and analysis of cylindrospermopsin from human serum and urine. Toxicon 70, 54-61.

Fraga, M., Vilariño, N., Louzao, M.C., Rodríguez, L.P., Alfonso, A., Campbell, K., Elliott, C.T., Taylor, P., Ramos, V., Vasconcelos, V., Botana, L.M. (2014). Multi-detection method for five common microalgal

toxins based on the use of microspheres coupled to a flow-cytometry system. Anal Chim Acta 850, 57-64.

Freitas, M., Azevedo, J., Carvalho, A.P., Campos, A., Vasconcelos, V. (2014). Effects of storage, processing and proteolytic digestion on microcystin-LR concentration in edible clams. Food Chem Toxicol 66, 217-223.

Freitas, R., Ramos Pinto, L., Sampaioc, M., Costac, A., Silva, M., Rodrigues, A.M., Quintino, V., Figueira, E. (2012). Effects of depuration on the element concentration in bivalves: Comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. Estuar Coast Shelf Sci 110, 43-53.

Froscio, S.M. (2002). Investigation of the mechanisms involved in cylindrospermopsin toxicity: hepatocyte culture and reticulocyte lysate studies. Ph.D. Thesis, University of Adelaide, Adelaide, Australia.

Froscio, S.M., Humpage, A.R., Burcham, P.C., Falconer, I.R. (2003). Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ Toxicol 18, 243-251.

Froscio, S.M., Humpage, A.R., Wickramasinghe, W., Shaw, G., Falconer, I.R. (2008). Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. Toxicon 51 191-198.

Froscio, S.M., Fanok, S., Humpage, A.R. (2009a). Cytotoxicity screening for the cyanobacterial toxin cylindrospermopsin. J Toxicol Environ Health A 72(5), 345-349.

Froscio, S.M., Cannon, E., Lau, H.M., Humpage, A.R. (2009b). Limited uptake of the cyanobacterial toxin cylindrospermopsin by Vero cells. Toxicon 54, 862-868

Funari, E., Testai, E. (2008). Human health risk assessment related to cyanotoxins exposure. Crit Rev Toxicol 38, 97-125.

Gagnaire, B., Cavalie, I., Camilleri, V., Adam-Guillermin, C. (2013). Effects of depleted uranium on oxidative stress, detoxification, and defence parameters of zebrafish *Danio rerio*. Arch Environ Contam Toxicol 64, 140-150.

Gago-Martinez, A., Piñeiro, N., Aguete, E.C., Vaquero, E., Nogueiras, M., Leao, J.M., Rodríguez-Vázquez, J.A., Dabek-Zlotorzynska, E. (2003). Further improvements in the application of high-performance liquid chromatography, capillary electrophoresis and capillary electrochromatography to the analysis of algal toxins in the aquatic environment**.** J Chromatogr A 992, 159-168.

Galanti, L.N., Ame, M.V., Wunderlin, D.A. (2013). Accumulation and detoxification dynamic of cyanotoxins in the freshwater shrimp *Palaemonetes argentinus*. Harmful Algae 27, 88-97.

Gallo, P., Fabbrocino, S., Cerulo, M.G., Ferranti, P., Bruno, M., Serpe, L. (2009). Determination of cylindrospermopsin in freshwaters and fish tissue by liquid chromatography couple to electrospray ion trap mass spectrometry. Rapid Commun Mass Sp 23, 3279-3284.

Gantar, M., Svircev, Z. (2008). Microalgae and cyanobacteria: food for thought. J Phycol 44, 260-268.

Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Nose, D., Ren, T. (2013). Interactive Effects of Vitamin C and E Supplementation on Growth, Fatty Acid Composition, and Lipid Peroxidation of Sea Cucumber, Apostichopus japonicus, Fed with Dietary Oxidized Fish Oil. J World Aquacult Soc 44, 536–546.

Gehringer, M.M., Govender, S., Shah, M., Downing, T.G. (2003). An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. Environ Toxicol 18(2), 142-148.

Gkeliz, S., Zaoutsos, N. (2014). Cyanotoxin occurrence and potentially toxin producing cyanobacteria in freshwaters of Greece: a multi-disciplinary approach. Toxicon 78, 1-9.

Głowacka, J., Szefel-Markowska, M., Waleron, M., Łojkowska, E., Waleron, K. (2011). Detection and identification of potentially toxic cyanobacteria in Polish water bodies. Acta Biochim Pol 58, 321-333.

Gómez-Amores, L., Mate, A., Miguel-Carrasco, J.L., Jiménez, L., Jos, A., Cameán, A.M., Revilla, E., Santa-María, C., Vázquez, C.M. (2007). L-Carnitine attenuates oxidative stress in hypertensive rats. J Nutr Biochem 18, 540-553.

González, A.G., Herrador, M.A., Asuero, A.G. (2010). Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. Talanta 82, 1995-1998.

Grosse, Y., Baan, R., Straif, K., Secretan, B., El Ghissassi, F., Cogliano, V. (2006). Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet Oncol 7, 628-629.

Grusak, M. A. (2001). Plant Macro - and Micronutrient Minerals. Encyclopedia of Life Sciences, 1-5.

Gülçin, I., (2006). Antioxidant and antiradical activities of L-carnitin. Life Sciences 78, 803–811.

Gurbuz, F., Metcalf, J.S., Karahan, A.G., Codd, G.A. (2009). Analysis of dissolved microcystins in surface water samples from Kovada Lake, Turkey. Sci Total Environ 407, 4038-4046.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Cameán, A.M. (2011a). Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotoxicol Environ Saf 74, 1567-1572.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Cameán, A.M. (2011b). Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory conditions: Influence of exposure route and time of sacrifice. Aquat Toxicol 105, 100-106.

Gutierrez-Praena, D., Pichardo, S., Jos, A., Moreno, F.J., Cameán, A.M. (2012a) Biochemical and pathological toxic effects induced by the cyanotoxin cylindrospermopsin on the human cell line Caco-2. Water Res 46, 1566-1575.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Moreno, FJ, Cameán, AM. (2012b). Alterations observed in the endotelial HUVEC cell line exposed to pure Cylindrospermosin. Chemosphere 89, 1151-1160.

Gutiérrez-Praena, D., Jos, M.A., Pichardo, S., Moyano, R., Blanco, A., Monterde, JG., Cameán, A.M. (2012c). Time-dependent histopathological changes induced in tilapia (*Oreochromis niloticus*) after acute exposure to pure Cylindrospermopsin by oral and intraperitoneal route. Ecotoxicol Environ Saf 76, 102-113.

Gutiérrez-Praena, D., Puerto, M., Prieto, A.I., Jos, A., Pichardo, S., Vasconcelos, V., Cameán, A.M. (2012d). Protective role of dietary N-acetylcysteine on the oxidative stress induced by cylindrospermopsin in tilapia (*Oreochromis niloticus*). Environ Toxicol Chem 31, 1548-55.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Moreno, I.M., Cameán, A.M. (2013a). Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: a review. Food Chem Toxicol 53, 136-152.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Puerto, M., Cameán, Ana M. (2013b). Influence of the exposure way and the time of sacrifice on the effects induced by a single dose of pure cylindrospermopsin on the activity and transcription of glutathione peroxidase and glutathione-S-transferase enzymes in Tilapia (*Oreochromis niloticus).* Chemosphere 90, 986-992.

Gutiérrez-Praena, D., Risalde, M., Pichardo, S., Jos, A., Moyano, R., Blanco, A., Vasconcelos, V., Cameán, A. (2014a). Histopathological and immunohistochemical analysis of Tilapia (*Oreochromis niloticus*) exposed to cylindrospermopsin and the effectiveness of N-Acetylcysteine to prevent its toxic effects. Toxicon 78, 18-34.

Gutiérrez-Praena, D., Campos, A., Azevedo, J., Neves, J., Freitas, M., Guzmán-Guillén, R., Vasconcelos, V. (2014b). Exposure of *Lycopersicon Esculentum* to Microcystin-LR: Effects in the Leaf Proteome and Toxin Translocation from Water to Leaves and Fruits. Toxins 6, 1837–1854.

Guzmán, R.E., Solter, P.F. (2002). Characterization of sublethal microcystin-LR exposure in mice. Vet Pathol 39, 17-26.

Haande, S., Rohrlack, T., Ballot, A.R., Berg, K., Skulberg, R., Beck, M., Wiedner, C. (2008). Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, cyanobacteria) isolates from Africa and Europe. Harmful Algae 7, 692-701.

Habdija, I., Latjner, J., Belinic, I. (1995). The contribution of gastropod biomass in macrobenthic communities of a karstic river. Int Rev Ges Hydrobiol 80, 103-110.

Halliwell, B. (2007). Biochemistry of oxidative stress. Biochem Soc Trans 35, 147-1150.

Harada, K.I., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K. (1994). Isolation of cylindrospermopsin from a cyanobacterium *Umezekia natans* and its screening method. Toxicon 32, 73-84.

Harada, K., Tsuji, K., Watanabe, M.F., Kondo, F. (1996). Stability of microcystins from cyanobacteria – III. Effect of pH and temperature. Phycologia 35, 83–88.

Harpaz, S. (2005). Carnitine and its attributed functions in fish culture and nutrition-a review. Aquaculture 249, 3–21.

Harpaz, S., Becker, K., Blum, R. (1999). The effect of dietary lcarnitine supple-mentation on cold tolerance and growth of ornamental cichlid fish (*Pelvicachromis pulcher*)-preliminary results. J Therm Biol 24, 57-62.

Hawkins, P.R., Runnegar, M.T., Jackson, A.R., Falconer, I.R. (1985). Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. Appl Environ Microb 50, 1292-1295.

Hawkins, P.R., Chandrasena, N.R, Jones, G.J., Humpage, A.R., Falconer, I.R. (1997). Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. Toxicon 35, 341-346.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R. (2005). Glutathione transferases. Annu Rev Pharmacol Toxicol 45, 51-88.

Hernández-Moreno, D., Soler, F., Míguez, M.P., Pérez-López, M. (2010). Brain acetylcholinesterase, malondialdehyde and reduced glutathione as biomarkers of continuous exposure of tench, Tinca tinca, to carbofuran or deltamethrin. Sci Total Environ 408, 4976-4983.

Herrero, A., Flores, E. (2008). The cyanobacteria: Molecular biology, genomics and evolution. Caister Academic Press. Norfolk (UK). 484 pp (ISBN: 978-1-904455-15-8).

Hoarau, P., Gnassia-Barelli, M., Roméo, M., Girard, J.P. (2001). Differential induction of glutathione S-transferases in the clam Ruditapes decussatus exposed to organic compounds. Environ Toxicol Chem 20, 523–529.

Hoarau, P., Garello, G.;, Gnassia-Barelli, M., Romeo, M., Girard, J.P. (2004). Effect of three xenobiotics compounds on glutathione S-transferase in the clam Ruditapes decussatus. Aquat Toxicol 68, 87–94.

Hoeger, S.J., Shaw, G., Hitzfeld, B.C., Dietrich, D.R. (2004). Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. Toxicon 43, 639-649.

Hori, T., Nakagawa, R., Tobiishi, K., Jida, T., Tsutsum, T., Sasaki, K., Toyoda, M. (2005). Effects of cooking on concentrations of Polychlorinated Dibenzo-p-dioxins and related compounds in fish and meat. J Agric Food Chem 53, 8820–8828.

Huang, C., Huang, S. (2004). Effect of dietary vitamin E on growth, tissue lipid peroxidation, and liver glutathione level of juvenile hybrid tilapia, *Oreochromis niloticus* x *O. aureaus*, fed oxidized oil. Aquaculture 237, 381-389.

Huang, C.H., Chang, R.J., Huang, S.L., Chen, W.L. (2003). Dietary vitamin E supplementation affects tissue lipid peroxidation of hybrid tifapia, *Oreuchromis niioticus* x *0. aureus.* Comp Biochem Physiol 134, 265-270.

Huang, S., Weng, Y.M., Huang, C.H. (2004). Lipid peroxidation in sarcoplasmic reticulum and muscle of tilapia is inhibited by dietary vitamin E supplementation. J Food Biochem 28, 101-111.

Hui, T., Wang, J. and Zhu, Y. (2001). Effect of cadmium on hepeatic GSH metabolism in *Tilapia niloticus.* J Zhejiang Unv Agri Life Sci 27, 575-578.

Humpage, A.R., Fenech, M., Thomas, P., Falconer, I.R. (2000a). Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mut Res 472, 155-161.

Humpage, A., Hardy, S., Moore, E., Froscio, S., Falconer, I. (2000b). Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J Toxicol Environ Health A 61, 155-165.

Humpage, A.R., Falconer, I.R. (2003). Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline value. Environ Toxicol 18, 94-103.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R. (2005). Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. J Toxicol Environ Health A 68, 739-753.

Ibelings, B.W., Chorus, I. (2007). Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: a review. Environ Pollut 150, 177-192.

Ito, E.; Kondo, F.; Harada, K.H. (2000). First report of the distribution of orally administered Microcystin-LR in mouse tissue using an immunostaining method. Toxicon 38, 37–48.

Järvenpää, S., Lundberg-Niinistö, C., Spoof, L., Sjövall, O., Tyystjärvi, E., Meriluoto, J. (2007). Effects of microcystins on broccoli and mustard, and analysis of accumulated toxin by liquid chromatographymass spectrometry. Toxicon 49, 865-874.

Jayaprakas, V., Sambhu, C., Sunil Kumar, S. (1996). Effect of dietary l-carnitine on growth and reproductive performance of male *Oreochromis mossambicus* (Peters). Fish Tech 33, 84-90.

Jochimsen, E.M., Carmichael, W.W., An, J., Cardo, D.M., Cookson, S.T., Holmes, C.E.M., Antunes, B.C., Liho, D.A.M., Lyra, .TM., Barreto, V.S.T., Azevedo, S.M.F.O., Jarvis, W.R. (1998). Liver Failure and Death After Exposure to Exposure to Microcystins at a Hemodyalisis Center in Brazil. N Engl J Med 338, 873-878.

Jones, D.P., Kagan, V.E., Aust, S.D., Reed, D.J., Omaye, S.T. (1995). Impact of nutrients on cellular lipid peroxidation and antioxidant defense system. Fund Appl Toxicol 26, 1-7.

Juhel, G., Davenport, J., O´Halloran, J., Culloty, S.C., O´Riordan, R.M., James, K.F., Furey, A., Allis, O. (2006). Impacts of microcystins on the feeding behaviour and energy balance of zebra mussels, *Dreissena polymorpha*: a bioenergetics approach. Aquat Toxicol 79, 391-400.

Juttner, F., Luthi, H. (2008). Topology and enhanced toxicity of bound microcystins in *Microcystis* PCC 7806. Toxicon 51, 388-397

Kaebernick, M., Neilan, B.A. (2001). Ecological and molecular investigations of cyanotoxin production. FEMS Microbiol Ecol 35, 1-9.

Kalaiselvi, T., Panneerselvam, C. (1998). Effect of L-carnitine on the status of lipid peroxidation and antioxidants in aging rats. J Nutr Biochem 9, 575-581.

Kampfenkel, K., Montagu, M.V., Inzé, D. (1995). Effects of Iron Excess on *Nicotiana plumbagnifolia* Plants' lmplications to Oxidative Stress. Plant Physiol 107, 725-735.

Kankaanpää, H., Leinio, S., Olin, M., Sjövall, O., Meriluoto, J., Lehtonen, K.K. (2007). Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68, 1210-1217.

Kavitha, P., Venkateswara Rao, J. (2009). Sub-lethal effects of profenofos on tissue-specific antioxidative responses in a Euryhyaline fish, Oreochromis mossambicus. Ecotox Environ Safe 72, 1727-1733.

Kelly, S.A., Havrilla, C.M., Brady, T.C., Abramo, K.H., Levin, E.D. (1998). Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ Health Persp 106, 375-384.

Kelly, G.S. (1998). L-carnitine: therapeutic applications of a conditionally-essential amino acid. Altern Med Rev 3, 345-360.

Kinnear, S. (2010). Cylindrospermopsin: a decade of progress on bioaccumulation research. Mar Drugs 8, 542-564.

Kinnear, S.H.W., Fabbro, L.D., Duivenvoorden, L.J., Hibberd, E.M.A. (2007a). Multipleorgan toxicity resulting frim cylindrospermopsin exposure in taspoles of the cane toad (*Bufo marinus*). Environ Toxicol 22, 550-558.

Kinnear, S.H.W., Duivenvoorden, L.J., Fabbro, L.D. (2007b). Sublethal responses in *Melanoides tuberculata* following exposure to *Cylindrospermopsis raciborskii* containing cylindrospermopsin. Harmful Algae 6, 642-650.

Kinnear, S., Fabbro, L., Duivenvoorden, L. (2008). Variable growth responses of water thyme (*Hydrilla verticillata*) to whole-cell extracts of *Cylindrospermopsis raciborskii*. Arch Environ Contam Toxicol 54, 187-194.

Kinnear, S.H.W., Duivenvoorden, L.J., Fabbro, L.D. (2009). Ecotoxicity and bioaccumulation of toxin from *Cylindrospermopsis raciborskii*: towards the development of environmental protection guidelines for contaminated water bodies. En: Lake Pollution Research Progress; Miranda FR, Bernards LM, Eds.; Nova Science Publishers, Inc.: New York, NY, USA, 81-105.

Kiss, T., Vehovszky, A., Hiripi, L., Kovacs, A. and Voros, L. (2002). Membrane effects of toxins isolated from a cyanobacterium, Cylindrospermopsis raciborskii, on identified molluscan neurones. Comp Biochem Physiol C: Toxicol Pharmacol 131, 167–176.

Kittler, K., Schreiner, M., Krumbein, A., Manzei, S., Koch, M., Rohn, S., Maul, R. (2012). Uptake of the cyanobacterial toxin cylindrospermopsin in *Brassica* vegetables. Food Chem 133, 875-879

Kobayashi, A., Fujisawa, S. (1994). Effect of L-carnitine on mitochondrial acyl CoA esters in the ischemic dog heart. J Mol Cell Cardiol 26, 499-508.

Kokocinski, M., Dziga, D., Spoof, L., Stefaniak, K., Jurczak, T., Mankiewicz-Boczek, J., Meriluoto, J. (2009). First report of the cyanobacterial toxin cylindrospermopsin in the shallow, eutrophic lakes of western Poland. Chemosphere 74, 669-675.

Kondo, F., Ito, Y., Yamada, S., Tsuji, K., Imokawa, M., Niimi, Y., Harada, K., Ueno, Y., Miyazaki, Y. (2002). Determination of microcystins in lake water using reusable immunoaffinity column**.** Toxicon 40, 893-899.

Konishi, T., Kato, K., Araki, T., Shiraki, K., Takagi, M., Tamaru, Y. (2005). A new class of glutathione S-transferase from the hepatopancreas of the red sea bream Pagrus major. J Biochem 388, 299–307.

Kubo, T., Sano, T., Hosoya, K., Tanaka, N., Kaya, K. (2005). A new simply and effective fractionation method for cylindrospermopsin analyses. Toxicon 46, 104-107.

Lance, E., Josso, C., Dietrich, D., Ernst, B., Paty, C., Senger, F., Bormans, M., Gérard, C. (2010). Histopathology and Microcystin distribution in *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacterial or dissolved microcystin-LR exposure. Aquat Toxicol 98, 211-220.

Lankoff, A., Wojcik, A., Lisowska, H., Bialczyk, J., Dziga, D. and Carmichael, W.W. (2007). No induction of structural chromosomal aberrations in cylindrospermopsin-treated CHO-K1 cells without and with metabolic activation. Toxicon 50, 1105-1115.

Lawrence, F., Menard, C. (2001). Determination of microcystins in blue-green algae, fish, and water using liquid chromatography with ultraviolet detection after sample clean-up employing immunoaffinity chromatography. J Chromatogr A 922, 111-117.

Lawton, L.A., Codd, G.A. (1991). Cyanobacterial (blue-green algal) toxins and their significance in UK and European waters. J Ind Waste Management Eval Model 5, 460-465.

Lei, H., Xie, P., Chen, J., Liang, G., Dai, M., Zhang, X. (2008). Distribution of toxins in various tissues of crucian carp intraperitoneally injected with hepatotoxic microcystins. Env Toxicol Chem 27, 1167-1174.

Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Mahakhant, A., Noparatnaraporn, N., Yongmanitchai, W., Kaya, K., Watanabe, M.M. (2001a). Isolation and identification of the cyanotoxin cylindrospermopsin and deoxy-cylindrospermopsin from a Thailand strain of *Cylindrospermopsis raciborskii* (Cyanobacteria). Toxicon 39, 973-980.

Li, R.H., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y.D., Watanabe, M.M. (2001b). First report of the cyanotoxins cylindrospermopsin and deoxy-cylindrospermopsin from *Raphidiopsis curvata* (Cyantobacteria). J Phycol 37, 1121-1126.

Liebel, S., Oliveira Ribeiro, C.A., Silva, R.C., Ramsdorf, W.A., Cestari, M.M., Magalhães, V.F., Garcia, J.R.E., Esquivel, B.M., Filipak Neto, F. (2011). Cellular responses of *Prochilodus lineatus* hepatocytes after cylindrospermopsin exposure. Toxicol In Vitro 25, 1493-1500.

Liras, V., Lindberg, M., Nyström, P., Annadotter, H., Lawton, L., Graf, B. (1998). Can ingested cyanobacteria be harmful to the signal crayfish (*Pacifastacus leniusculus*)? Freshwater Biol 39, 233-242.

Liu, H., Scott, P.M. (2011). Determination of the cyanobacterial toxin cylindrospermopsin in algal food supplements. Food Addit Contam A 28, 786-790.

Looper, R.E., Runnegar, M.T., Williams, R.M. (2005). Synthesis of the putative structure of 7-deoxycylindrospermopsin: C7 oxygenation is not required for the inhibition of protein synthesis. Angew Chem Int Ed Engl 44, 3879-3881.

Looper, R.E., Runnegar, M., Williams, R.M. (2006). Syntheses of the cylindrospermopsin alkaloids. Tetrahedron 62, 4549-4562.

Lürling, M. (2003). Effects of microcystin-free and microcystin-containing strains of the cyanobacterium *Microcystis aeruginosa* on growth of the grazer *Daphnia magna*. Environ Toxicol 18, 202-210.

Machado, J. (2014). Implicações do Uso de Água Contaminada com Microcistina na Qualidade de *Daucus Carota* (Cenoura). Universidade do Porto.

Magalhães, V.F., Marinho, M.M., Domingos, P., Oliveira, A.C., Costa, S.M., Azevedo, L.O., Azevedo, S.M.F.O. (2003). Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). Toxicon 42, 289-295.

Maire, M.A., Bazin, E., Fessard, V., Rast, C., Humpage, A.R., Vasseur, P. (2010). Morphological cell transformation of Syrian hamster embryo (SHE) cells by the cyanotoxin, cylindrospermopsin. Toxicon 55, 1317-1322.

Mankiewicz, J., Tarczynska, M., Fladmark, K.E., Doskeland, S.O., Walter, Z., Zalewski, M. (2001). Apoptotic effect of cyanobacterial extract on rat hepatocytes and human limphocytes. Environ Toxicol 16, 225-233.

Manti, G., Mattei, D., Messineo, V., Melchiorre, S., Bogialli, S., Sechi, N., Casiddu, P., Luglié, A., di Brizio, M., Bruno, M. (2005). First report of *Cylindrospermopsis raciborskii* in Italy. Harmful Algal News 28, 8-9.

Marbun, Y.R., Yen, H.K., Lin, T.F., Lin, H.L., Michinaka, A. (2012). Rapid on-site monitoring of cylindrospermopsin-producers in reservoirs using quantitative PCR. Sustain Environ Res 22, 143-151.

Martins, J., Campos, A., Osório, H., da Fonseca, R., Vasconcelos, V. M. (2014). Proteomic profiling of cytosolic glutathione transferases from three bivalve species: *Corbicula fluminea, Mytilus galloprovincialis* and *Anodonta cygnea*. Int J Mol Sci 15, 1887-1900.

Martins, J.C., Vasconcelos, V.M. (2009). Microcystin dynamics in aquatic organisms. J Toxicol Env Health B 12, 65-82.

Masten, S., Carson, B. (2000). Toxicological Summary for Cylindrospermopsin, Final Report. Rev Toxicol Lit T. Integrated Laboratory System, Research Triangle Park, NC, USA, 1-8.

Mazmouz, R., Chapuis-Hugon, F., Mann, S., Pichon, V., Mejean, A., Ploux, O. (2010). Biosynthesis of cylindrospermopsin and 7-epicylindrospermopsin in *Oscillatoria* sp. strain *PCC 6506*: identification of the *cyr* gene cluster and toxin analysis. Appl Environ Microb 76, 4943-4944.

McGregor, G.B., Fabbro, L.D. (2000). Dominance of *Cylindrospermopsis raciborskii* (Nostocales, Cyanoprokaryota) in Queensland tropical and subtropical reservoirs: implications for monitoring and management. Lake Reserv: Res Manage 5, 195-205.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., Thomas, O. (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins (Review). Environ Int 59, 303-327.

Messineo, V., Melchiorre, S., Di Corcia, A., Gallo, P., Bruno, M. (2010). Seasonal succesion of Cylindrospermopsis raciborskii and Aphanizomenon ovalisporum blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. Environ Toxicol 25, 18-27.

Metcalf, F.J.S., Codd, G.A. (2000). Microwave oven and boiling waterbath extraction of hepatotoxins from cyanobacterial cells. FEMS Microbiol Lett 184, 241-246.

Metcalf, J.S, Beattie, K.A., Pflugmacher, S., Codd, G.A. (2000). Immuno-crossreactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. FEMS Microbiol Lett 189, 155-158.

Metcalf, J.S., Beattie, K.A., Saker, M.L., Codd, G.A. (2002). Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. FEMS Microbiol Lett 216, 159-164.

Metcalf, J.S., Barakate, A., Codd, G.A. (2004). Inhibition of plant protein synthesis by the cyanobacterial hepatotoxin, cylindrospermopsin. FEMS Microbiol Lett 235, 125-129.

Miguel-Carrasco, J.L., Monserrat, M.T., Mate, A., Vázquez, C.M. (2010). Comparative effects of captopril and l-carnitine on blood pressure and antioxidant enzyme gene expression in the heart of spontaneously hypertensive rats. Eur J Pharmacol 632, 65–72.

Miller, L.L., Wang, F., Palace, V.P., Hontela, A. (2007). Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. Aquat Toxicol 83, 263-271.

Mohamed, Z.A., Hussein, A.A. (2006). Depuration of microcystins in tilapia fish exposed to natural populations of toxic cyanobacteria: a laboratory study. Ecotoxicol Environ Saf 63, 424-429.

Mohamed, Z.A. (2007). First report of toxic *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* (Cyanoprokaryota) in Egyptian fresh waters. FEMS Microbiol Ecol 59, 749-761.

Mohamed, Z.A., Al-Shehri, A.M. (2013). Assessment of cylindrospermopsin toxin in an arid Saudi lake containing dense cyanobacterial bloom. Environ Monit Assess 185, 2157-2166.

Morais, J., Augusto, M., Carvalho, A.P., Vale, M., Vasconcelos, V.M. (2008). Cyanobacteria hepatotoxins, microcystins, bioavailability in contaminated mussels exposed to different environmental conditions. Eur Food Res Technol 227, 949-952.

Moreira, C., Azevedo, J., Antunes, A., Vasconcelos, V. (2012). Cylindrospermopsin: occurrence, methods of detection and toxicology. J Appl Microbiol 114, 605-620.

Moreira, C., Martins, A., Azevedo, J., Freitas, M., Vale, M., Regueiras, A., Antunes, A. and Vasconcelos, V. (2011). Application of real-time PCR in monitoring *Cylindrospermopsis raciborskii* in a Portuguese freshwater system, abundance and toxicological evaluation. Appl Microbiol Biotechnol 92, 189-197.

Moreira, C., Ramos, V., Azevedo, J., Vasconcelos, V. (2014). Methods to detect cyanobacteria and their toxins in the environment. Appl Microbiol Biotechnol 98, 8073-8082.

Moreno, I., Repetto, G., Carballal, E., Gago, A., Cameán, A.M. (2005a). Cyanobacteria and microcystins occurrence in the Guadiana river (SW Spain). Int J Environ Anal Chem 85, 461-474.

Moreno, I., Molina, R., Jos, A., Picó, Y., Cameán, A.M. (2005b). Determination of microcystins in fish by solvent extraction and liquid chromatography. J Chromatogr A 1080, 199-203.

Moreno, I.M., Pereira, P., Franca, S., Cameán, A.M. (2004). Toxic cyanobacteria strains isolated from blooms in the Guadiana river (Southwest of Spain). Biol Res 37, 405-417.

Msagati, T.A.M., Siame, B.A., Shushu, D.D. (2006). Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. Aquat Toxicol 78, 382-397.

Mukhopadhyay M. & Sharma, A. (1991). Manganese in cell metabolism of higher plants. Bot Rev 57, 117-149.

Muthuswamy, A.D., Vedagiri, K., Ganesan, M., Chinnakannu, P. (2006). Oxidative stress-mediated macromolecular damage and dwindle in antioxidant status in aged rat brain regions: role of L-carnitine and DL-alpha-lipoic-acid. Clin Chim Acta 368, 84–92.

Namikoshi, M., Yuan, M., Sivonen, K., Carmichael, W.W., Rinehart, K.L., Rouhiainen, L., Sun, F., Brittain, S., Otsuki, A. (1998). Seven new microcystins possessing two L-glutamic acid units, isolated from *Anabaena sp*. strain 186. Chem Res Toxicol 11, 143-149.

Neumann, C., Bain, P., Shaw, G. (2005). The *in vitro* toxicology of deoxycylindrospermopsin. En: Australian Research Network for Algal Toxin Workshop, Moreton Bay Research Station, Australia, 9–11th.

Neumann, C., Bain, P., Shaw, G. (2007). Studies of the comparative *in vitro* toxicology of the cyanobacterial metabolite deoxycylindrospermopsin. J Toxicol Environ Health A 70, 1679-1686.

Niedobitek, G., Kremmer, E., Herbst, H., Whitehead, L., Dawson, C.W., Niedobitek, E., von Ostau, C., Rooney, N., Grässer, F.A., Young, L.S. (1997). Immunohistochemical detection of the Epstein-Barr Virus-encoded latent membrane protein 2A in Hodkin's disease and infectious mononucleosis. Blood 90, 1664-1672.

Nogueira, I.C., Pereira, P., Dias, E., Pflugmacher, S., Wiegand, C., Franca, S., Vasconcelos, V.M. (2004b). Accumulation of paralytic shellfish toxins (PST) from the cyanbacterium *Aphanizomenon issatschenkoi by* the cladoceran *Daphnia magna*. Toxicon 44, 773-780.

Nogueira, I.C.G., Saker, M.L., Pflugmacher, S., Wiegand, C., Vasconcelos, V.M. (2004a). Toxicity of the cyanobacterium *Cylindrospermopsis raciborskii* to *Daphnia magna*. Environ Toxicol 19, 453-459.

Norris, R.L., Eaglesham, G., Pierens, G., Shaw, G., Smith, M.J., Chiswell, R.K., Seawright, A.A., Moore, M.R. (1999). Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. Environ Toxicol 14, 163-165.

Norris, R.L.G., Seawright, A.A., Shaw, G.R., Smith, M.J., Chiswell, R.K., Moore, M.R. (2001a). Distribution of 14C Cylindrospermopsin in vivo in the mouse. Environ Toxicol 16(6), 498-505.

Norris, R.L., Eaglesham, G.K., Shaw, G.R., Senogles, P., Chiswell, R.K., Smith, M.J., Davis, B.C., Seawright, A.A. (2001b). Extraction and purification of the zwitterions cylindrospermopsin and deoxycylindrospermopsin from *Cylindrospermopsis raciborskii*. Environ Toxicol 16, 391-396.

Norris, R.L., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., et al. (2002). Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon 40, 471-476.

Nuñez, A.J. (2011). Terapia antioxidante, estrés oxidativo y productos antioxidantes: retos y oportunidades. Rev Cubana Salud Pública (online) 37, 644-660. ISSN 0864-3466.

O´Neil, J.M., Davis, T.W., Burford, M.A., Gobler, C.J. (2012). The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. Harmful Algae 14, 313-334.

Oehrle, SA., Southwell, B., Westrick, J. (2010). Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. Toxicon 55, 965-72.

Ognjanovic BI, Pavlovic SZ, Maletic SD, Zikic RV, Stajn AS, Radojicic RM, Saicic ZS, Petrovic VM (2003). Protective influence of vitamin E on antioxidant defense system in the blood of rats treated with cadmium. Physiol Res 52, 563-570.

Ohlendorf, H.M. (1996). Selenium. En: Noninfectious diseases of wildlife, eds. Fairbrother A, Locke LN and Hoff GL. Ames: Iowa State University Press, 128-140.

Ohtani, I., Moore, R.E., Runnegar, M.T.C. (1992). Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J Am Chem Soc 114, 7941-7942.

Oosterwijk, E., Ruiter, M.D., Wakka, J.C., Huiskens-van der Meij, J.W., Jonas, U., Fleuren, G.J., Zwartendijk, J., Hoedemaeker, P., Warnaar, S.O. (1986). Immunohistochemical analysis of monoclonal antibodies to renal antigens. Application in the diagnosis of renal cell carcinoma. J Am Pathol 123, 301–309.

Orr, P.T., Rasmussen, J.P., Burford, M.A., Eaglesham, G.K., Lennox, S.M. (2010). Evaluation of quantitative real-time PCR to characterize spatial and temporal variations in cyanobacteria, *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya et Subba Raju and cylindrospermopsin concentrations in three subtropical Australian reservoirs. Harmful Algae 9, 243–254.

Ozawa, K., Yokoyama, A., Ishikawa, K., Kumagai, M., Watanabe, M.F., Park, H.D. (2003). Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. Limnology 4, 131-138.

Özcan Oruç, E. (2010). Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in *Oreochromis niloticus* exposed to chlorpyrifos. Pestic Biochem Phys 96, 160-166.

Ozório, R.O.A. (2009). Dietary L-carnitine supplementation to cultivated fish: A mini-review. Curr Nutr Food Sci 5, 40-48.

Paerl, H.W., Hall, N.S., Calandrino, E.S. (2011). Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. Sci Total Env 409, 1739–1745.

Paerl, H.W., Huisman, J. (2008). Climate Blooms like it hot**.** Science 320, 57-58.

Paerl, H.W., Huisman, J. (2009). Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microb Rep 1, 27–37.

Papadimitriou, T., Kagalou, I., Stalikas, C., Pilidis, G., Leonardos, I.D. (2012). Assessment of microcystin distribution and biomagnification in tissues of aquatic food web compartments froma shallow lake and evaluation of potential risks to public health. Ecotoxicology 21, 1155-1166.

Park, H., Watanabe, M.F. (1996). Toxic Microcystis in Eutrophic Lakes. En: Watanabe MF, Harada KH, Carmichael WW, Fujiki H (Ed.) Toxic Microcystis. C.R.C. Press, Boca Raton, 57-77.

Parker, R.S. (1989). Dietary and biochemical aspects of vitamine E. Adv Food Nutr Res 33, 157-232.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B. (2010). On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar Drugs 8, 1650-1680.

Peña, V.J., Martín, I., Ruiz, S. (2005). Requerimientos nutricionales e ingestas dietéticas recomendadas. En: Gil Hernández A. (Ed) Nutrición humana en el estado de salud (Tomo III). Grupo acción médica. Madrid, España, 63-73.

Peña-Llopis, S., Ferrando, M.D., Peña, J.B. (2003). Fish tolerase to organophosphate-induced oxidative stress is dependen ton the glutathione metabolism and enhanced by N-acetylcysteine. Aquat Toxicol 65, 337-360.

Pereira, L., Fernandes, M.N., Martínez, C.B.R. (2013). Hematological and biochemical alterations in the fish Prochilodus lineatus caused by the insecticide clomazone. Environ Toxicol Pharmacol 36, 1-8.

Pereira, S., Vasconcelos, V., Antunes, A. (2011). The phosphoprotein phosphatase family of Ser/Thr phosphatases as principal targets of naturally occurring toxins. Crit Rev Toxicol 41, 83-110.

Perelló, G., Martí-Cid, R., Llobet, J.M., Domingo, J.L. (2008). Effects of various cooking processes on the concentrations of arsenic, cadmium, mercury and lead in foods. J Agric Food Chem 56, 11262–11269.

Pflugmacher, S., Jung, K., Lundvall, L., Neumann, S., Peuthert, A. (2006). Effects of cyanobacterial toxins and cyanobacterial cell-free crude extract on germination of alfalfa (*Medicago sativa*) and induction of oxidative stress. Environ Toxicol Chem. 25, 2381-2387.

Pilotto, L.S., Douglas, R.M., Burch, M.D., Cameron, S., Beers, M., Rouch, G.J., Robinson, P., Kirk, M., Cowie, C.T., Hardiman, S., Moore, C., Attewell, R.G. (1997). Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. Aust NZ J Publ Heal 21(6), 562-566.

Pinho, G.L.L., da Rosa, C.M., Maciel, F.E., Bianchini, A., Yunes, J.S., Proença, L.A., Monserrat, J.M. (2005). Antioxidant responses after microcystin exposure in gills of an estuarine crab species pre-treated with vitamin E. Ecotoxicol Environ Saf 61, 361-365.

Pizolon, L. (1996). Importancia de las Cianobacterias como Factor de Toxicidad en las aguas Continentales. Interciencia 21, 239-245.

Pouria, S., De Andrade, A., Barbosa, J., Cavalcanti, R.L., Barreto, V.T.S., Ward, C.J., Preiser, W., Poon, G.K., Neild, G.H., Codd, G.A. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. The Lancet 352, 21-26.

Pretto, A., Loro, V.L., Morsch, V.M., Moraes, B.S., Menezes, C., Clasen, B., Hoehne, L., Dressler, V. (2010). Acetylcholinesterase activity, lipid peroxidation, and bioaccumulation in silver catfish (*Rhamdia quelen*) exposed to cadmium. Arch Environ Contam Toxicol 58, 1008–1014.

Prieto, A.I., Pichardo, S., Jos, A., Moreno, I., Cameán, A.M. (2008). Protective role of vitamin E on the Microcystin-induced oxidative stress in tilapia fish (*Oreochromis niloticus*). Environ Toxicol Chem 27, 1152-1159.

Prieto, A.I., Jos, A., Pichardo, S., Moreno, I., de Sotomayor, M.A., Moyano, R., Blanco, A., Cameán, A.M. (2009). Time-dependent protective efficacy of Trolox (vitamin E analog) against microcystin-induced toxicity in tilapia (*Oreochromis niloticus*). Environ Toxicol 24, 563-579.

Prieto, A.I., Campos, A., Cameán, A.M., Vasconcelos, V.M. (2011). Effects on growth and oxidative stress status of rice plants (*Oriza sativa*) exposed to two extracts of toxin-producing cyanobacteria (*Aphanizomenon ovalisporum* and *Microcystis aeruginosa*). Ecotoxicol Environ Saf 74, 1973-1980.

Puerto, M., Prieto, A.I., Pichardo, S., Moreno, I., Jos, A., Moyano, R., Cameán, A.M. (2009). Effects of dietary N-acetylcysteine on the oxidative stress induced in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Environ Toxicol Chem / SETAC, 28, 1679-1686.

Puerto, M., Prieto, A.I., Jos, A., Moreno, I., Moyano, R., Blanco, A., Cameán, A.M. (2010). Dietary N-Acetylcysteine (NAC) prevents histopathological changes in tilapias (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. Aquaculture 306, 35-48.

Puerto, M., Jos, M.A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M. (2011). Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852-1860

Quesada, A. (2006a). Cianobacterias en Aguas de Consumo y Recreo: Un Problema de Todos. En: Ponencia realizada por el Centro de Estudio y Experimentación de obras públicas. CEDEX, Madrid.

Quesada, A., Moreno, E., Carrasco, D., Paniagua, T., Wörmer, L., De Hoyos, C., Sukenik, A. (2006b). Toxicity of *Aphanizomenon ovalisporum* (Cyanobacteria) in a Spanish water reservoir. Eur J Phycol 41, 39-45.

Rajasekar, P., Palanisamy, N., Anuradha, C.V. (2007). Increase in nitric oxide and reductions in blood pressure, protein kinase C beta II and oxidative stress by L-carnitine: a study in the fructose-fed hypertensive rat. Clin Exp Hypertens 29, 517–530.

Rasmussen, J.P., Giglio, S., Monis, P.T., Campbell, R.J., Saint, S.P. (2008). Development and field testing of a real-time PCR assay for cylindrospermopsin-producing cianobacteria. J Appl Microbiol 104, 1503-1515.

Reisner, M., Carmeli, S., Werman, M., Sukenik, A. (2004). The Cyanobacterial Toxin Cylindrospermopsin Inhibits Pyrimidine Nucleotide Synthesis and Alters Cholesterol Distribution in Mice. Toxicol Sci 82, 620-627.

Reznick, A.K., Kagen, V.E., Ransey, R., Tsuchiya, M., Serbinova, E.A., Packer, L. (1992). Antiradical effect in l-propionyl carnitine. Protection of the heart against ischemic reperfusion injury. The possible role of ion chelation. Arch Biochem Biophys 296, 349–401.

Ríos, M.V., Guzmán-Guillén, R., Prieto, A.I., Moreno, I.M., Cameán, A.M. (2013). Alteración de biomarcadores de estrés oxidativo en Tilapias (*Oreochromis niloticus*) expuestas a dosis repetidas de Cilindrospermopsina por diferentes vías de exposición. Rev Toxicol 30, 149-160.

Ríos, V., Guzmán-Guillén, R., Moreno, I.M., Prieto, A.I., Puerto, M., Jos, A., Cameán, A.M. (2014). Influence of two depuration periods on the activity and transcription of antioxidant enzymes in tilapia exposed to repeated doses of cylindrospermopsin under laboratory conditions. Toxins 6, 1062–1079.

Risalde, M.A., Molina, V., Sánchez-Cordón, P.J., Pedrera, M., Romero- Palomo, F., Bautista, M.J., Moreno, A., Gómez-Villamandos, J.C. (2013). Comparison of pathologic changes and viral antigen distribution in tissues of calves with and without pre-existing bovine viral diarrhea virus infection following challenge with bovine Herpesvirus-1. Am J Vet Res 74, 598–610.

Ernst, B., Hitzfeld, B.C., Dietrich, D.R. (2001). Presence of *Planktothrix sp*. and cyanobacterial toxins in Lake Ammersee Germany and their impact on whitefish (*Coregonus lavaretus L.*). Environ Toxicol 16, 483-488.

Roem, A.J., Kohler, C.C., Stickney, R.R. (1990). Vitamin E requieriments of blue tilapia, *Oreochromis aureus* (Steindachner) in relation to dietary lipid level. Aquaculture 87, 155-164.

Roepstorff, C., Halber, N., Hillig, T., Saha A.K., Ruderman N.B., Wojtaszewski J.F., Richter, E.A., Kiens, B. (2005). Malony-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. Am J Physiol Endocrinol Metab 288, E133-E142.

Rogers, E.H., Zehr, R.D., Gage, M.I., Humpage, A.R., Falconer, I.R., Marr, M., Chernoff, N. (2007). The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon 49, 855-864.

Rohrlack, T., Dittmann, E., Börner, T., Christoffersen, K. (2001). Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. Appl Environ Microb 67, 3523-3529.

Rücker, J., Stüken, A., Nixdorf, B., Fastner, J., Chorus, I., Wiedne, C. (2007). Concentrations of particulate and disolved cylindrospermopsin in 21 *Aphanizomenon*-dominated temperate lakes. Toxicon 50, 800-809.

Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Ge, J.L., Lu, S.C. (1994). The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Biophys Res Commun 201, 235-241.

Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Lu, S.C. (1995). Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Pharmacol 49, 219-225.

Runnegar, M.T., Xie, C., Snider, B.B., Wallace, G.A., Weinreb, S.M., Kuhlenkamp, J. (2002). In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol Sci 67, 81-87.

Rzymski, P., Poniedziałek, B. (2012). Dermatotoxins synthesized by blue-green algae (Cyanobacteria). Adv Dermatol Allergol 29, 47–50.

Saker, M.L., Eaglesham, G.K. (1999). The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. Toxicon 37, 1065-1077.

Saker, M.L., Nogueira, I.C.G., Vasconcelos, V.M., Neilan, B.A., Eaglesham, G.K., Pereira, P. (2003). First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. Ecotoxicol Environ Saf 55, 243-250.

Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M. (2004). Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. Toxicon 43, 185-94.

Saker, M.L., Jungblut, A.D., Neilan, B.A., Rawn, D.F., Vasconcelos, V.M. (2005). Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*. Toxicon 46, 555-562.

Saqrane, S., Ouahid, Y., El Ghazali, I., Oudra, B., Bouarab, L., del Campo, F.F. (2009). Physiological changes in *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lens esculenta* cultivars, caused by irrigation with water contaminated with microcystins: a laboratory experimental approach. Toxicon 53, 786-796.

Satoh, S., Takeuchi, T., Watanabe, T. (1987). Requirement of tilapia for α-tocopherol. Nippon Suisan Gakk 53, 119-164.

Sayed-Ahmed, M.M., Khattab, M.M., Gad, M.Z., Mostafa, N. (2001). l-Carnitine prevents the progression of atherosclerotic lesions in hypercholesterolaemic rabbits. Pharmacological Research 44, 235–242.

Schaeffer, D.J., Malpas, P.B., Barton, L.L. (1999). Risk assessment of microcystin in dietary *Aphanizomenon flos-aquae*. Ecotoxicol Environ Saf 44, 73-80.

Schereiber, B. (2005). Levocarnitine and dialysis: a review. Nutr Clin Pract 20, 218-243.

Schoeb, T.R., Heaton-Jones, T.G., Clemmons, R.M., Carbonneau, D.A., Woodward, A.R., Shelton, D., Poppenga, R.H. 2002. Clinical and necropsy findings associated with increasing mortality among American alligators of Lake Griffin, Florida. J. Wildlife Dis. 39, 320–337.

Schreiber, S., Becker, K., Bresler, V., Fishelson, L. (1997). Dietary L-carnitine protects the gills and skin of guppies (*Poecilia reticulata*) against anionic xenobiotics. Comp Biochem Physiol 117, 99– 102.

Seawright, A.A., Nolan, C.C., Shaw, G.R., Chiswell, R.K., Norris, R.L., Moore, M.R., Smith, M.J, (1999). The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). Environ Toxicol 14, 135-142.

Seifert, M. (2007). The ecological effects of the cyanobacterial toxin cylindrospermopsin. The University of Queensland: Brisbane, Australia.

Seifert, M., McGregor, G., Eaglesham, G., Wickramasinghe, W., Shaw, G. (2007). First evidence for the production of cylindrospermopsin and deoxycylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. Harmful Algae 6, 73-80.

Sekadende, B.C., Lyimo, T.J., Kurmayer, R. (2005). Microcystin production by cyanobacteria in the Mwanza Gulf (Lake Victoria, Tanzania). Hydrobiologia 543, 299-304.

Senogles, P., Shaw, G., Smith, M., Norris, R., Chiswell, R., Mueller, J., Sadler, R., Eaglesham, G. (2000). Degradation of the cyanobacterial toxin cylindrospermopsin from *Cylindrospermopsis raciborskii*, by chlorination. Toxicon 38, 1203-1213.

Sevgiler, Y., Piner, P., Durmaz, H., Üner, N. (2007). Effects Nacetylcysteine on oxidative responses in the liver of fenthion exposed *Cyprinus carpio*. Pestic. 35 Biochem Physiol 87, 248-254.

Sharma, N.K., Tiwari, S.P., Tripathi, K., Rai, A.K.. (2011). Sustainability and cyanobacteria (blue-green algae): facts and challenges. J Appl Phycol 23, 1059–1081.

Shaw, G.R., Seawright, A.A., Moore, M.R., Lam, P.K.S. (2000). Cylindrospermopsin, a cyanobacterial alkaloid: evaluation of its toxicologic activity. Ther Drug Monit 22, 89-92.

Shaw, G.R., Sukenik, A., Livne, A., Chiswell, R.K., Smith, M.J., Seawright, A.A., Norris, R.L., Eaglesham, G.K., Moore, M.R. (1999). Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. Environ Toxicol 14, 167-177.

Shen, X., Lam, P.K., Shaw, G.R., Wickramasinghe, W. (2002). Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. Toxicon 40, 499-501.

Shiau, S., Lin, Y. (2006). Vitamin requirements of tilapia: A review. Proceedings, VIII Simposium Internacional de Nutrición Acuícola, Monterrey, México, 129-138.

Silva, R.C., Neto, F., Oliveira, C.A., Azevedo, S.M.F.O., Magalhaes, V.F. (2010). Cylindrospermopsin effects on primary cultured hepatocytes of the neotropical fish *Hoplias malabaricus*. In: The 8th International Conference on Toxic Cyanobacteria, Istanbul, August 29th–September 4th, 188.

Sipiä, V.O, Kankaanpää, H.T., Pflugmacher, S., Flinkman, J., Furey, A., James, K.J. (2002). Bioaccumulation and Detoxication of Nodularin in Tissues of Flounder (*Platichthys flesus*), Mussels (*Mytilus edulis*, *Dreissena polymorpha*), and Clams (*Macoma balthica*) from the Northern Baltic Sea. Ecotox Environ Safe 53, 305-311.

Sivonen, K. (1996). Cyanobacterial toxins and toxin production. Phycologia 35, 12-24.

Sivonen, K., Jones, G. (1999). Cyanobacterial Toxins En: Chorus, I., Bartram, M.J. (Eds.) Toxic Cyanobacteria in Water. A guide to Their Public Health, Significance, Monitoring and Management, The World Health Organization/E and F.N. Spon, London, UK, 41-111..

Smith, J.L., Haney, J.F. (2006). Foodweb transfer, accumulation, and depuration of microcystins, a cyanobacterial toxin, in pumpkinseed sunfish (*Lepomis gibbosus*). Toxicon 48, 580-589.

Sotton, B., Domaizon, I., Anneville, O., Cattanéo, J.G. (2014). Nodularin and cylindrospermopsin: a review of their effects on fish. Rev Fish Biol Fisheries. DOI 10.1007/s11160-014-9366-6.

Stephens, F.B., Constantin-Teodosiu, D., Greenhaff, P.L. (2007). New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. J Physiol 581, 431-444.

Stewart, I., Seawright, A.A., Schluter, P.J., Shaw, G.R. (2006a). Primary irritant and delayed-contact hypersensitivity reactions to the freshwater cyanobacterium *Cylindrospermopsis raciborskii* and its associated toxin cylindrospermopsin. BMC Dermatol 6, doi: 10.1186/1471-5945-6-5.

Stewart, I., Robertson, I.M., Webb, P.M., Schluter, P.J., Shaw, G.R. (2006b). Cutaneous hypersensitivity reactions to freshwater cyanobacteria-human volunteer studies. BMC Dermatol 6, 6.

Stewart, I., Schluter, P.J., Shaw, G.R. (2006c). Cyanobacterial lipopolysaccharides and human health - a review. Environ Health 24, 5-7.

Štraser, A. Filipič, M., Zegura, B. (2011). Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line. Arch Toxicol 85, 1617-1626.

Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi, H., Wang, X. (2006). Bioaccumulation, depuration and oxidative stress in fish Carassius auratus under phenanthrene exposure. Chemosphere 63, 1319–1327.

Suzuki, Y., Kamikawa, T., Yamazaki, N. (1981). Effects of L-carnitine on ventricular arrhythmias in dogs with acute myocardial ischemia and a supplement of excess free fatty acids. Jpn Circ 45, 552-559.

Tein, I. (2003). Carnitine transport: pathophysiology and metabolism of known molecular defects. J Inherit Metab Dis 26, 147-169.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M. (1994). Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon 32, 833-843.

Thomas, A.D., Saker, M.L., Olsen, R.D. (1998). Cyanobacterium *Cylindrospermopsis raciborskii* as a probable cause of death in cattle in northern Queensland. Aust Vet J 76, 592-594.

Thorarinsson, R., Landolt, M.L., Elliott, D.G., Pascho, R.J., Hardy, R.W. (1994). Effect of dietary vitamin E and selenium on growth, survival and the prevalence of Renibacterium salmoninarum infection in Chinook salmon (*Oncorhyndhus tshawytscha*). Aquaculture 121, 343-358.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Díaz, E., Bell, J.G., Geurden, I., Lavens, P., Olsen, Y. (2002). Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquacult Nutri 8, 195-207.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Díaz, E., Wille, M., Bell, J., Olsen, Y. (2003). Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquacult Int 11, 195-216.

Torokne, A., Palovics, A., Bankine, M. (2001). Allergenic (sensitization, skin and eye irritation) effects of freshwater cyanobacteria-experimental evidence. Environ. Toxicol. 16, 512–516.

Törökné, A., Aszatols, M., Bánkinné, M., Bickel, H., Borbély, G., Carmeli, S., Codd, G.A., Fastner, J., Huang, Q., Humpage, A., Metcalf, J.S., Rábai, E., Sukenik, A., Surányi, G., Vasas, G., Weiszfeiler, V. (2004). Interlaboratory comparison trial on cylindrospermopsin measurement. Anal Biochem 332, 280-284.

Torreele, E., Van der Sluizen, A., Verreth, J. (1993). The effect of dietary L-carnitine on the growth performance in fingerlings of the African catfish *Clarias gariepinus* in relation to dietary lipid. British J Nutr 69, 289–299.

Trubetskova, I.L., Haney, J.F. (2006). Effects of differing concentrations of microcystin-producing *Microcystis aeruginosa* on growth, reproduction, survivorship and offspring of *Daphnia magna*. Arch Hydrobiol 167, 533-546.

U.S. EPA. 1991. Guidelines for Developmental Toxicity Risk Assessment. Fed. Reg. 56(234), 63798-63826.

Van Apeldoorn, M.E., Van Egmond, H.P., Speijers, G.J.A., Bakker, G.J.I. (2007). Toxins of cyanobacteria. Mol Nutr Food Res 51, 7-60

Vasas, G., Bacsi, I., Suranyi, G., Hamvas, M.M., Mathe, C., Nagy, S.A., Borbely, G. (2010). Isolation of viable cell mass from frozen *Microcystis viridis* bloom containing microcystin-RR. Phytoplankton 639, 147-151.

Vasas, G., Gáspár, A., Surányi, G., Batta, G., Gyémánt, G., M-Hamvas, M., Máthé, C., Grigorszky, I., Molnár, E., Borbély, G. (2002). Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from aphanizomenon ovalisporum, by plant test (blue-green sinapis test). Anal Biochem 302, 95–103.

Vasconcelos, V.M. (1995). Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. Aquat Toxicol 32, 227-237.

Vasconcelos, V.M., Sivonen, K., Evans, W.R., Carmichael, W.W., Namikoshi, M. (1996). Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. Water Res 30, 2377-2384.

Vincent, W.F. (2000). Cyanobacterial dominance in the polar regions. En: Cyanobacteria: Their Diversity in the Time and Space. B. Whitton and M. Potts (Eds.) Dordrecht, Kluwer Academic Publishers, 321-338.

Vincent, W.F., Mueller, D., Bonilla, S. (2004). Ecosystem on ice: the microbial ecology of Markham Ice Shelf in the High Arctic. Cryobiology 48, 103-112.

Wannemacher, R.W. (1989). Chemical stability laboratory safety of naturally occurring toxins. US Army Medical Research, Institute of infection diseases. Frot Detrick, 9–11.

Ward, D.M., Ferris, M.J., Nold, S.C., Bateson, M.M., Kopczynski, E.D., Ruff-Roberts, A.L. (1994). Species diversity in hot spring microbial mats as revealed by both molecular and enrichment culture approaches-relathionship between biodiversity and community structure. En: Microbial Mats. Structure, development and environmental significance. L.J. Stal and P. Caumette (Eds.) Berlin, Springer-Verlag. 35, 33-44.

Welker, M., Bickel, H., Fastner, J. (2002). HPLC-PDA detection of cylindrospermopsin-opportunities and limits. Water Res 36, 4659-4663.

Whalen, K.E., Morin, D., Lin, C.Y., Tjeerdema, R.S., Goldstone, J.V. y col. (2008). Proteomic identification, cDNA cloning and enzymatic activity of glutathione S-transferases from the generalist marine gastropod, *Cyphoma gibbosum*. Arch Biochem Biophys 478, 7–17.

White, S.H., Duivenvoorden, L.J., Fabbro, L.D., Eaglesham, G.K. (2006). Influence of intracellular toxin concentration on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). Toxicon 47, 497-509.

White, S.H., Duivenvoorden , L.J., Fabbro, L.D., Eaglesham, G.K. (2007). Mortality and toxin bioaccumulation in *Bufo marinus* following exposure to *Cylindrospermopsis raciborskii* cell extracts and live cultures. Environ Pollut 147, 158-167.

Whitton, B., Potts, M. (2000). The ecology of cyanobacteria. Dordrecth, Kluwer Academic Publishers, 669.

Wiedner, C., Visser, P.M., Fastner, J., Metcalf, J.S., Codd, G.A., Mur, L.R. (2003). Effects of light on the microcystin content of *Microcystis* strain *PCC 7806*. Appl Environ Microb 69, 1475-1481.

Wilhelm Filho, D. (1996). Fish antioxidant defenses. A comparative approach. Braz J Med Biol Res 29, 1735-1742.

Williams, C.D., Burns, J.W., Chapman, A.D., Pawlowicz, M., Carmichael, W.W. (2006). Assessment of cyanotoxins in Florida's surface waters and associated drinking water resources. Final Annual Report to the Florida Harmful Algal Bloom Task Force, Florida Marine Research Institute, St. Petersburg, Fla.

Wimmer, K.M., Strangman, W.K., Wright, J.L.C. (2014). 7-Deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin: Two new cylindrospermopsin analogs isolated from a Thai strain of *Cylindrospermopsis raciborskii***.** Harmful Algae 37, 203-206.

Wörmer, L., Carrasco, D., Cirés, S., Quesada, A. (2009). Advances in solid phase extraction of the cyanobacterial toxin cylindrospermopsin. Limnol Oceanogr Methods 7, 568-575.

Wu, J., Shao, S., Zhou, F., Wen, S., Chen, F., Han, X. (2014). Reproductive toxicity on female mice induced by microcystin-LR. Environ Toxicol Phar 37, 1-6.9

Yang, Y., Park, Y., Cassada, D.A., Snow, D.D., Rogers, D.G., Lee, J. (2011). *In vitro* and *in vivo* safety assessment of edible blue-green algae, *Nostoc commune* var. *sphaeroides Kützing* and *Spirulina plantensis*. Food Chem Toxicol 49, 1560-1564.

Yilmaz, M., Phlips, E.J., Szabo, N.J., Badylak, S. (2008). A comparative study of Florida strains of *Cylindrospermopsis* and *Aphanizomenon* for cylindrospermopsin production. Toxicon 51, 130-139.

Yoshida, T., Makita, Y., Tsutsumi, T., Nagata, S., Tashiro, F., Yoshida, F., Sekijima, M., Tamura, S.I., Harada, T., Maita, K., y col. (1998). Immunohistochemical localization of Microcystin-LR in the liver of mice: A study on the pathogenesis of Microcystin-LR-induced hepatotoxicity. Toxicol Pathol 26, 411–418.

Young, F.M., Micklem, J., Humpage, A.R. (2008). Effects of blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells *in vitro*. Reprod Toxicol 25, 374-80.

Young, F.M., Zebian, D., Froscio, S., Humpage, A. (2012). Cylindrospermopsin, a blue-green algal toxin, inhibited human luteinized granulose cell protein in vitro. Toxicol In Vitro 26, 656–662.

Yu, T., Xie, P., Dai, M., Liang, G. (2009). Determinations of MC-LR and [Dha7] MC-LR concentrations and physicochemical properties by liquid chromatography–tandem mass spectrometry. Bull Environ Contam Toxicol 83, 757–760.

Zagatto, P.A., Buratini, S.V., Arag˜ao, M.A., Ferrao-Filho, A.S. (2012). Neurotoxicity of two *Cylindrospermopsis raciborskii* (cyanobacteria) strains to mice, Daphnia and fish. Environ Toxicol Chem 31, 857–862.

Zambrano, S., Blanca, A.J., Ruiz-Armenta, M.V., Miguel-Carrasco, J.L., Revilla, E., Santa-María, C., Mate, A., Vázquez, M.C. (2013). The renoprotective effect of L-carnitine in hypertensive rats is mediated by modulation of oxidative stress-related gene expression. Eur J Nutr 52, 1649-1659

Žegura, B., Lah, T., Filipič, M. (2006). Alteration of intracelular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mut Res 611, 25-33.

Žegura, B., Gajski, G., Štraser, A., Garaj-Vrhovac, V. (2011). Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress. Toxicon 58, 471-479.

Zhang, D., Xie, P., Chen, J. (2010). Effects of temperature on the stability of microcystins in muscle of fish and its consequences for food safety. Bull Environ Contam Toxicol 84, 202–207.

Zhang, X., Zhou, J., Wu, Y., Zheng, X., Ma, G., Wang, Z., Jin, Y., He, J., Yan, Y. (2009). Differential Proteome Analysis of Host Cells Infected with Porcine Circovirus Type 2. J Proteome Res 8, 5111–5119.

Zhang, J., Liu, S., Yang, P., Sui, G. (2011). Rapid detection of algal toxins by microfluidic immunoassay. Lab Chip 11, 3516-3522.