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"EVALUACIÓN TOXICOLÓGICA DE CILINDROSPERMOPSINA MEDIANTE MODELOS EXPERIMENTALES *IN VIVO* E *IN VITRO*: DAÑOS PRODUCIDOS POR ESTRÉS OXIDATIVO Y POTENCIAL EFECTIVIDAD DE N-ACETILCISTEÍNA PARA CONTRARRESTARLOS"

Memoria que presenta el Licenciado DANIEL GUTIÉRREZ PRAENA para optar al título de Doctor por la Universidad de Sevilla con la mención de "Doctorado Europeo"

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INFORMA:

Que la Tesis Doctoral titulada "**EVALUACIÓN TOXICOLÓGICA DE CILINDROSPERMOPSINA MEDIANTE MODELOS EXPERIMENTALES *IN VIVO* E *IN VITRO*: DAÑOS PRODUCIDOS POR ESTRÉS OXIDATIVO Y POTENCIAL EFECTIVIDAD DE N-ACETILCISTEÍNA PARA CONTRARRESTARLOS"**, presentada por el Ldo. D. DANIEL GUTIÉRREZ PRAENA para optar al grado de Doctor por la Universidad de Sevilla con la mención de "Doctorado Europeo", ha sido realizada en el Área de Toxicología de este Departamento bajo la dirección de la Dra. Ana Mª Cameán Fernández, la Dra. Ángeles Mencía Jos Gallego y la Dra. Silvia Pichardo Sánchez. 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,
Octubre de 2011.

Fdo. Ana María Cameán Fernández

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INFORMAN: Que la Tesis Doctoral titulada "**EVALUACIÓN TOXICOLÓGICA DE CILINDROSPERMOPSINA MEDIANTE MODELOS EXPERIMENTALES *IN VIVO* E *IN VITRO*: DAÑOS PRODUCIDOS POR ESTRÉS OXIDATIVO Y POTENCIAL EFECTIVIDAD DE N-ACETILCISTEÍNA PARA CONTRARRESTARLOS"**, ha sido realizada por el Ldo. D. DANIEL GUTIÉRREZ PRAENA en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar al grado de Doctor por la Universidad de Sevilla con la mención de "Doctorado Europeo".

Y para que así conste, firmamos en Sevilla,
Octubre de 2011.

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Este trabajo ha sido realizado en el Área de Toxicología del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Facultad de Farmacia de la Universidad de Sevilla y financiado por los Proyectos de Investigación AGL2009-10026, del Ministerio de Ciencia e Innovación, y P09-AGR-4672 de la Junta de Andalucía.

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"Cuando estalla la tormenta, algunas personas enmudecen de terror.

Otras, despliegan las alas cual águilas y planean"

A mi familia, compañeras

y hermano

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ADN: Ácido desoxirribonucleico

ARN: Ácido ribonucleico

ATP: Adenosín trifosfato

GST: Glutatión-S-transferasa

PSP: Paralytic Selfish Poison

MCs: Microcistinas

CAT: Catalasa

CE: Comunidad Europea

CE_{50}: Concentración Efectiva Media

CYN: Cilindrospermopsina

CYP450: Citocromo P450

DL_{50}: Dosis Letal Media

ERO: Especies reactivas de oxigeno

FAO: Food and Agriculture Organization

GI: Gastrointestinal

GPx: Glutatión peroxidasa

GR: Glutatión reductasa

GSH: Glutatión reducido

HPLC: High Performance Liquid Chromatography

IARC: International Agency on Research of Cancer

IDT: Ingesta Diaria Tolerable

i.p.: intraperitoneal

LPO: Peroxidación lipídica

NAC: N-acetilcisteína

NOAEL: Non Observed Adverse Effects Level

OATP: Organic Anion-Transporting Polypeptide

OMS: Organización Mundial de la Salud

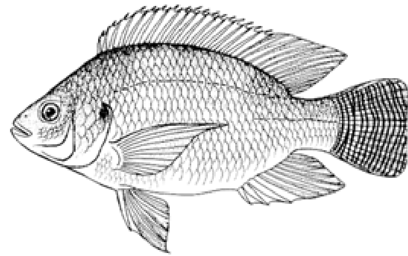
PCR: Reacción en cadena de la polimerasa

RER: Retículo Endoplásmico Rugoso

SOD: Superóxido dismutasa

µg: Microgramos / **µL:** Microlitros / **µM:** Micromolar

1. RESUMEN / SUMMARY



1. RESUMEN/SUMMARY

1.1. 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. La Cilindrospermopsina (CYN) es una de estas cianotoxinas, citotoxina que ha demostrado en roedores *in vivo* tener acción hepato y nefrotóxica, además de afectar a otros órganos, como pulmón, corazón, estómago, glándulas adrenales y sistemas vascular y linfoide. Además, se ha visto que es hepatotóxica e irritante en humanos. Se ha documentado que la CYN inhibe la síntesis de proteínas, interfiere la síntesis de glutatión reducido (GSH) y es genotóxica, aunque los estudios que se centran en ella aún son escasos.

Debido a que la principal vía de entrada de CYN en el organismo es la oral, por ingestión de aguas y alimentos contaminados, consideramos de importancia el estudio de los efectos tóxicos de CYN sobre el tracto gastrointestinal (GI) y el tejido endotelial humano, ya que la CYN se absorbe a nivel GI y se distribuye por el resto del organismo a través de la sangre. Para ello, se emplearon como modelo experimental *in vitro* la línea celular intestinal humana Caco-2, modelo enterocítico *in vitro* más usado, establecido a partir de un carcinoma de colon humano, y la línea celular endotelial humana HUVEC, establecida a partir de endotelio vascular de cordón umbilical, las cuales imitan al sistema *in vivo*, permitiendo la medida de marcadores y actividades bioquímicas de interés. Los efectos tóxicos producidos por CYN en ambas líneas celulares fueron estudiados durante 24 y 48 horas de exposición para los ensayos de citotoxicidad basal y morfológicos, y durante 24 horas de exposición para los ensayos de estrés oxidativo, ya que no existen estudios sobre estas líneas celulares que traten la generación de estrés oxidativo por parte de la CYN. Los resultados de citotoxicidad basal mostraron que la sensibilidad de las células Caco-2 y HUVEC a los efectos tóxicos de la CYN está influenciada tanto por el tiempo de exposición, como por la concentración a la que se expusieron las células, siendo la línea celular HUVEC la más

sensible de las dos. El bioindicador más sensible a la acción tóxica de la CYN en la línea Caco-2 fue la reducción de la sal de tetrazolio (MTS), mientras que en la línea celular HUVEC fue el ensayo de captación de rojo neutro (RN). En cuanto a los resultados de estrés oxidativo, se comprobó que ambas líneas celulares sufrían un incremento de las especies reactivas de oxígeno (ERO) a la vez que un descenso en los niveles de GSH y de la actividad γ-glutamilcisteina sintetasa (GCS), enzima limitante de la síntesis de GSH, a dosis de CYN bajas; a las dosis más elevadas, estos marcadores se veían incrementados y se reducía la cantidad de ERO. Las principales alteraciones morfológicas observadas en la línea celular Caco-2 fueron degeneración lipídica, daño mitocondrial y segregación nucleolar con núcleos alterados, mientras que en la línea celular HUVEC, se observaron principalmente segregación nucleolar con núcleos alterados, aumento del número de gránulos secretores, degeneración del aparato de Golgi y apoptosis Los resultados de estos experimentos han dado lugar a las siguientes publicaciones:

BIOCHEMICAL AND PATHOLOGICAL TOXIC EFFECTS INDUCED BY PURE CYLINDROSPERMOPSIN ON THE HUMAN CELL LINE CACO-2 *(Gutiérrez-Praena y col., 2011. Enviado a Water Research, en revisión)*

ALTERATIONS OBSERVED IN ENDOTHELIAL HUVEC CELL LINE EXPOSED TO PURE CYLINDROSPERMOPSIN *(Gutiérrez-Praena y col., 2011. Enviado a Archives of Toxicology)*

Siguiendo con los estudios *in vitro* y comprobados los efectos tóxicos de la CYN sobre las líneas celulares humanas, quisimos investigar el efecto que podría tener sobre la línea celular hepática del ciprínido *Poeciliopsis lucida*, PLHC-1, ya que, hasta la fecha, los estudios sobre la toxicidad de la CYN en modelos piscícolas tanto *in vitro* como *in vivo* son muy escasos, a pesar de su posible exposición al compartir hábitat. Para tratar esta cuestión, se realizaron nuevamente ensayos de citotoxicidad basal (24 y 48 horas) y de estrés oxidativo (24 horas). Los ensayos de citotoxicidad basal mostraron que la sensibilidad de las células PLHC-1 a los efectos tóxicos de la CYN era dependiente tanto de la concentración como del tiempo de exposición, siendo el bioindicador más sensible el contenido proteico total (PT). Se comprobó que la línea celular PLHC-1 era la menos sensible de las tres líneas celulares ensayadas. Con

respecto a los ensayos de estrés oxidativo, se observó un aumento de la producción de ERO así como un descenso en los niveles de GSH y de la actividad de la enzima GCS. Los resultados de este experimento dieron lugar a la siguiente publicación:

TOXICITY AND GLUTATHIONE IMPLICATION IN THE EFFECTS OBSERVED BY EXPOSURE OF THE LIVER FISH CELL LINE PLHC-1 TO PURE CYLINDROSPERMOPSIN *(Gutiérrez-Praena y col., 2011. Ecotoxicology and Environmental Safety 74, 1567-72)*

La CYN es una cianotoxina que se encuentra, en gran parte, de forma libre disuelta en el medio, por lo que un gran número de organismos acuáticos (fitoplancton, zooplancton, plantas y animales) pueden entrar en contacto con ella. Por ello, y una vez conocidos los efectos tóxicos que produce en los modelos celulares *in vitro*, consideramos de gran importancia investigar los efectos y repercusiones de dicha toxina *in vivo* sobre tilapias (*Oreochromis niloticus*), ya que es una especie de pez que crece en aguas susceptibles de contaminación por CYN, y además cada vez está cobrando mayor importancia como fuente de alimento para humanos.

Nuestro estudio se centró en investigar la inducción de estrés oxidativo como mecanismo de toxicidad asociada a CYN en función de la vía de exposición y el tiempo de sacrificio, así como los posibles daños histopatológicos que podía ocasionar. Para este fin, se intoxicaron tilapias con una dosis única de 200 μg CYN pura/kg p.c. (seleccionada en base a estudios previos) por vía oral, mediante sonda gástrica (*gavage*) y por vía intraperitoneal (i.p.). Tras 24 horas y 5 días, los peces fueron sacrificados y se analizaron diferentes actividades enzimáticas, nivel de peroxidación lipídica (LPO), oxidación de proteínas y ADN, y contenido de GSH. De esta forma, comprobamos que la CYN producía un incremento de la actividad NADPH oxidasa, de los niveles de LPO y de la oxidación de proteínas, no producía oxidación del ADN en las condiciones ensayadas, y disminuía el contenido de GSH y la actividad GCS, lo que indica la importancia de estas enzimas en la patogenicidad de la CYN. Además, se comprobó que el tiempo de sacrificio influía más en los resultados que la vía de administración de la toxina, ya que los peces sacrificados a los 5 días tras la exposición presentaban la recuperación de algunos de los biomarcadores estudiados, mientras que otros parámetros mostraban mayor afectación. Al mismo tiempo, nos planteamos el estudio

de las variaciones en la expresión génica de las enzimas Glutatión peroxidasa (GPx) y Glutatión-S-transferasa (GST), así como la abundancia relativa de GST en hígado y riñón bajo las mismas condiciones. El objetivo principal fue evaluar la alteración de los niveles de ARNm y la abundancia de proteínas de estas enzimas, ya que estos marcadores pueden proporcionar una información temprana sobre el estado del pez. Los resultados mostraron que estos parámetros se veían más afectados por la vía i.p. y en peces sacrificados tras 5 días. Por último, también se estudiaron los daños histopatológicos producidos en los diferentes órganos (hígado, riñón, intestino, corazón y branquias), comprobándose que no se producía una recuperación de las lesiones histopatológicas inducidas a los 5 días de la exposición a la toxina, lo cual demuestra la toxicidad retardada de CYN en este modelo experimental. Los resultados de este experimento dieron lugar a las siguientes publicaciones:

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 *(Gutiérrez-Praena y col., 2011. Aquatic Toxicology 105, 100-6)*

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 PEROXIDISE AND GLUTATHIONE-S-TRANSFERASE ENZYMES IN TILAPIA (OREOCHROMIS NILOTICUS) *(Gutiérrez-Praena y col., 2011. Enviado a Water Research)*

TIME-DEPENDENT HISTOPATHOLOGICAL CHANGES INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER ACUTE EXPOSURE TO PURE CYLINDROSPERMOPSIN BY ORAL AND INTRAPERITONEAL ROUTE *(Gutiérrez-Praena y col., 2011. Aceptado en Ecotoxicology and Environmental Safety)*

Tras confirmar la producción de estrés oxidativo como mecanismo de acción tóxica de la CYN tanto *in vitro* como *in vivo*, quisimos investigar el efecto protector de la N-acetilcisteína (NAC), precursor del GSH, frente al daño oxidativo e histopatológico

que pueden inducir tanto la CYN pura como liofilizados de cianobacterias productoras de CYN (+CYN) en tilapias. Los peces fueron pretratados con 0, 22 y 45 mg NAC/pez/día durante una semana, tras la cual fueron expuestos a una dosis única de, o bien 200 μg/kg pc de CYN pura o bien 200 μg/kg pc de CYN procedente de un liofilizado de *Aphanizomenon ovalisporum*, mezcladas con el alimento, y se sacrificaron a las 24 horas. Al igual que en el experimento anterior, se analizaron diferentes actividades enzimáticas, niveles de LPO, oxidación de proteínas, contenido de GSH, expresión génica de las enzimas GPx y GST y los daños histopatológicos producidos en los diferentes órganos. Los resultados mostraron un papel protector de la NAC en función de la dosis y el marcador biológico analizado. Así, sobre las actividades enzimáticas mejoraba la situación de estrés inducida por la CYN, tanto en el caso de la toxina pura como en el liofilizado de las células productoras de CYN (+CYN). Con respecto a la LPO se observa una recuperación de los valores basales y en cuanto al contenido de GSH se recuperaban los niveles tras la depleción sufrida en los grupos intoxicados con CYN sin pretratamiento. Además, estudiamos las repercusiones que la NAC podía ocasionar sobre el hígado, riñón, intestino, corazón y branquias a nivel histopatológico. Los resultados mostraron como la CYN procedente del liofilizado de *A. ovalisporum* producía daños más severos en los diferentes órganos en comparación con la CYN pura. Además, también se comprobó que la NAC ejercía un efecto protector frente a la CYN, reduciendo los daños observados. Por tanto, puede considerarse la NAC como un agente quimioprotector útil en la prevención de los efectos tóxicos en peces expuestos a CYN. Los resultados de estos experimentos han dado lugar a una solicitud de patente, y a la siguiente publicación:

PROTECTIVE ROLE OF DIERTARY N-ACETYLCYSTEINE ON THE OXIDATIVE STRESS INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN *(Gutiérrez-Praena y col., 2011. Enviado a Aquatic Toxicology)*

Por último, consideramos fundamental el inicio de las investigaciones para conocer el impacto de la CYN sobre plantas de consumo humano, ya que tras revisar la bibliografía científica, se constata la ausencia casi total de información disponible. Para ello, se comenzó con un análisis previo enfocado desde un punto de vista molecular para, de esta forma, poder identificar genes/proteínas implicados en el proceso de

producción de toxicidad. Quisimos caracterizar la expresión proteica de la planta de tomate (*Solanum lycopersicum*) expuesta a CYN pura y extractos de CYN procedentes de cultivos de *A. ovalisporum*. Se emplearon geles de dos dimensiones (2DE) para el análisis proteómico de hojas de tomate. Además, se comprobó que la toxina puede ser el principal factor responsable del estrés fisiológico observado en las plantas, aunque existen otras moléculas bioactivas sintetizadas por las células de *A. ovalisporum* que podrían contribuir a la toxicidad observada. Los resultados de este experimento han dado lugar a la siguiente publicación:

ALTERATIONS ON PROTEIN EXPRESSION INDUCED BY CYLINDROSPERMOPSIN IN TOMATO PLANTS (SOLANUM LYCOPERSICUM)
(Gutiérrez-Praena y col., 2011. Pendiente de envío)

Para finalizar, teniendo en cuenta los resultados derivados de los experimentos realizados en la presente Tesis Doctoral, queda demostrada la validez de las técnicas *in vitro* e *in vivo* empleadas para la evaluación toxicológica de la CYN, contribuyendo de esta forma a ampliar el conocimiento que actualmente podemos encontrar en la bibliografía científica con respecto a esta cianotoxina.

1.2. Summary

Cyanobacteria have acquired a big importance among the years due to their ability to produce "blooms", which can be toxic because of the production of cyanotoxins. For this reason, cyanobacteria constitute a worldwide concern in regard of environmental pollution, toxicology, health and economy, because they can affect animals and plants as well as humans. Cylindrospermopsin (CYN) is one of these cyanotoxins. This is a cytotoxin which has demonstrated to be nephrotoxic and hepatotoxic in rodents *in vivo*, and can also affect other organs such as lungs, hearth, stomach, adrenal glands, and vascular and lymphoid systems. Furthermore, it has been demonstrated that CYN is hepatotoxic and irritant for humans. It is documented CYN inhibits protein synthesis, interferes reduced glutathione synthesis (GSH) and also produce genotoxicity, although studies are still scarce.

The oral pathway is the main way of CYN exposure by ingestion of contaminated water or food. Therefore, we consider of importance the study of toxic effects in the gastrointestinal (GI) tract, and even the endothelial tissue, due to CYN is absorbed in the GI tract and is distributed to the rest of the organs through the blood. For this purpose, the human cell line Caco-2, a commonly used enterocytic model, established from a human colon adenocarcinoma, and the endothelial human cell line HUVEC, derived from human umbilical vein endothelium, were used. Both cell lines mime perfectly the *in vivo* system, allowing the measurement of different biomarkers and enzymatic activities of interest. The effects produce by CYN in both cell lines were studied after 24 and 48 hours of exposure for the basal cytotoxicity and morphologic assays, and after 24 hours of exposure for the oxidative stress studies, with no previous studies concerning to the oxidative stress generation by CYN on these cell lines. Results on basal cytotoxicity showed that the sensitivity of both Caco-2 and HUVEC cell lines to CYN is influenced by the exposure time and the toxin concentration, being the HUVEC cell line the most sensitive. The MTS tetrazolium salt reduction (MTS) was the most sensitive endpoint for the Caco-2 cell line. Meanwhile, for the HUVEC cell line was the neutral red uptake (NR). Concerning to the oxidative stress results, both cell lines experienced an increase at low CYN concentration of reactive oxygen species (ROS) and at the same time a reduction of GSH levels and the γ-gluthamylcystein syntethase (GCS) activity, the limiting enzyme in GSH synthesis. Meanwhile, at higher

concentrations, these biomarkers suffer an increase and ROS levels depletion. The more remarkable morphologic alterations on Caco-2 cells were lipidic degeneration, mitochondrial damage and nuclear segregation with altered nuclei. Meanwhile, in HUVEC cell line the main alterations were nuclear segregation with altered nuclei, increases of secretory granules, degenerated Golgi apparatus, and apoptosis. These results have leaded the following publications:

BIOCHEMICAL AND PATHOLOGICAL TOXIC EFFECTS INDUCED BY PURE CYLINDROSPERMOPSIN ON THE HUMAN CELL LINE CACO-2 *(Gutiérrez-Praena et al., 2011. Sent to Water Research, in revision)*

ALTERATIONS OBSERVED IN ENDOTHELIAL HUVEC CELL LINE EXPOSED TO PURE CYLINDROSPERMOPSIN *(Gutiérrez-Praena et al., 2011. Sent to Archives of Toxicology)*

Once evidenced the toxic effects production in the human cell lines, we studied the effect of CYN on the cyprinid *Poeciliopsis lucida* hepatic cell line, PLHC-1. Nowadays, studies *in vivo* and *in vitro* concerning CYN toxicity on fish models are scarce, even taking into account that they share the habitat. For this reason, we carried out the same basal cytotoxicity and oxidative stress assays used for the human cell lines, in order to know the behaviour of the PLHC-1 cell line when it is intoxicated with CYN. Basal cytotoxicity test showed that the sensitivity of the cell line was concentration-time dependent, being the total protein content (TP) the most sensitive biomarker. Thus, we demonstrated that the PLCH-1 cell line was the less sensitive of the three cell lines assayed. Considering the oxidative stress study, we observed an increase of ROS production correlated with a decrease in GSH levels and GCS activity reduction. The results of this experiment have led to the following publication:

TOXICITY AND GLUTATHIONE IMPLICATION IN THE EFFECTS OBSERVED BY EXPOSURE OF THE LIVER FISH CELL LINE PLHC-1 TO PURE CYLINDROSPERMOPSIN *(Gutiérrez-Praena et al., 2011. Ecotoxicology and Environmental Safety 74, 1567-72)*

CYN can be mainly found in a free form dispersed in the media, so there are a vast number of aquatic organisms (phytoplankton, zooplankton, plants and animals) that can be in contact with it. For this reason, once we knew the negative effects that CYN is able to produce *in vitro*, we considered of relevant importance to know the possible effects and repercussion of this toxin *in vivo* on tilapia fish (*Oreochromis niloticus*). This fish species was selected because it can be found in water supplies where CYN can be also found, and also is becoming an important food source for humans in many countries.

Our study consisted in investigating the oxidative stress production, such as toxicity mechanism associated to CYN, and also the histopathological damage produced by CYN in different organs. For this aim, tilapias were intoxicated with 200 μg/Kg bw of pure CYN (selected from previous studies) by *gavage* and intraperitoneal (i.p.) injection. After 24 hours and 5 days, fish were sacrificed and the different enzymatic activities, lipidic peroxidation (LPO) levels, protein and DNA oxidation, and GSH content, were evaluated. Thus, CYN was able to produce an increase in the NADPH oxidase activity, the LPO levels and the protein oxidation, does not produce DNA oxidation in the assayed conditions, and originated a decrease of GSH levels and GCS activity, highlighting the important of these enzymes in CYN pathogenicity. Furthermore, we also corroborated that the time of sacrifice was more relevant than the CYN administration pathway on the results obtained. Fish sacrificed after 5 days presented the recovery of some biomarkers, while other parameters appeared more affected. At the same time, we established the study of the gene expression of the enzymes Glutathione peroxidase (GPx) and Glutathione-S-transferase (GST), as well as the relative abundance of the enzyme GST, in liver and kidney, under the same conditions described before. The main purpose of this study was to evaluate whether the alteration of mRNA levels and protein abundance of these enzymes could be effective biomarkers to give early warning signals of the health effects on this fish species. Results showed these parameters were more affected by the i.p. way and in fish sacrificed after 5 days. Finally, histopathological damage produce in different organs (liver, kidney, intestine, heart and gills), were also evaluated, and showed no recovery of the damage after 5 days, demonstrating the delayed toxicity of CYN in this experimental model. The results of this experiment have led the following publications:

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 *(Gutiérrez-Praena et al., 2011. Aquatic Toxicology 105, 100-6)*

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 PEROXIDISE AND GLUTATHIONE-S-TRANSFERASE ENZYMES IN TILAPIA (OREOCHROMIS NILOTICUS) *(Gutiérrez-Praena et al., 2011. Sent to Water Research)*

TIME-DEPENDENT HISTOPATHOLOGICAL CHANGES INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER ACUTE EXPOSURE TO PURE CYLINDROSPERMOPSIN BY ORAL AND INTRAPERITONEAL ROUTE *(Gutiérrez-Praena et al., 2011. Accepted in Ecotoxicology and Environmental Safety)*

After confirming oxidative stress as a toxicity mechanism of CYN *in vitro* and *in vivo*, we investigated the protective role of dietary N-acetylcysteine (NAC), a GSH precursor, against the oxidative damage and the histopathological alterations that pure CYN and CYN from a lyophilized cyanobacteria culture were able to induce in tilapia. Fish received a pre-treatment with 0, 22 and 45 mg/fish/day during a week, and afterwards they were intoxicated with 200 µg/Kg bw of pure CYN or 200 µg/Kg bw of CYN from *Aphanizomenon ovalisporum* lyophilized, and mixed with the food. Fish were sacrificed after 24 hours. Similarly to the experiment before, the different enzymatic activities, LPO levels, protein oxidation, GSH content, protein expression of GPx and GST, and histopathological damage produced in different organs, were evaluated. Results showed a protective role of NAC in dependence of the dose and the biomarker analysed. Thus, the oxidative damage induced by both CYN types on the enzymatic activities was improved. Concerning to LPO and GSH levels, we observed a recovery of basal levels in both cases. Moreover, we also studied the effects of NAC on CYN-induced histopathology in liver, kidney, intestine, heart and gills. Generally, results showed that the damage induced by CYN from *A. ovalisporum* lyophilized were

more severe in comparison to those induced by pure CYN. Furthermore, NAC showed to be a useful chemoprotectant agent to prevent the toxic effects in fish exposed to CYN. The results of these experiments have led to a patent and also the following publication:

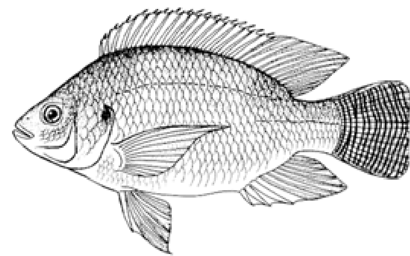
PROTECTIVE ROLE OF DIERTARY N-ACETYLCYSTEINE ON THE OXIDATIVE STRESS INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN *(Gutiérrez-Praena et al., 2011. Sent to Aquatic Toxicology)*

Finally, we considered interesting to begin the research concerning CYN impact in edible plants, as literature on this field is very scarce. We started with a previous analysis focused on a molecular point of view to identify the gens/proteins implicated in the CYN toxicity process. We characterized the protein expression of tomato plants (*Solanum lycopersicum*) exposed to pure CYN and CYN from an *A. ovalisporum* lyophilized culture. Two dimensions gels (2DE) were employed for the proteomic analysis of tomato leaves. Moreover, toxin can be the main responsible factor of the physiologic stress observed in plants, although there are other bioactive molecules synthesized by *A. ovalisporum* cells that can contribute to the toxicity of CYN. The results of this experiment have led the following publication:

ALTERATIONS ON PROTEIN EXPRESSION INDUCED BY CYLINDROSPERMOPSIN IN TOMATO PLANTS (SOLANUM LYCOPERSICUM) *(Gutiérrez-Praena et al., 2011. Waiting to be send)*

To conclude, taking into account the results derived from the experiments performed in the present Doctoral Thesis, it has been demonstrated the validity of *in vitro* and *in vivo* techniques for the toxicological evaluation of CYN, and a contribution to the knowledge of toxicological aspects of this toxin has been made.

2. INTRODUCCIÓN / INTRODUCTION



2.1. CIANOBACTERIAS Y CIANOTOXINAS

Las cianobacterias son un grupo de más de 2000 especies de organismos procariotas incluidos dentro del grupo de las eubacterias. Son conocidas como "algas verde-azuladas", aunque actualmente están clasificadas como bacterias Gram-negativas (Woese, 1987, 2002; Flores y Herrera, 2008) que se caracterizan porque tienen la capacidad de sintetizar clorofila α (Whitton y Potts, 2000). Además, estos organismos pueden adaptarse a una gran variedad de hábitats a nivel mundial (Ward y col. 1998), incluyendo zonas donde las condiciones para la vida son extremas, como pueden ser desiertos cálidos o fríos (Wynn-Williams, 2000).

Bajo determinadas condiciones ambientales (luz, temperatura, pH, etc.), eutrofización y presencia de nutrientes, principalmente nitrógeno y fósforo, estas cianobacterias pueden crecer de forma masiva dando lugar a lo que se conoce como afloramiento, floración o "bloom", los cuales adquieren una gran importancia cuando están sujetos a la producción de toxinas (cianotoxinas) como metabolitos secundarios (Duy y col., 2000). Dichas cianotoxinas pueden suponer un grave problema ambiental, ecotoxicológico, sanitario, y también económico, debido a que afectan tanto a animales y plantas como a seres humanos. Estas floraciones son más frecuentes en regiones tropicales y subtropicales (Hawkins et al., 1985; Hayman 1992) pero también se han detectado casos en otras regiones (Albay y col., 2005; Jurczak y col., 2005; Karlsson y col., 2005) y pueden ocurrir tanto en aguas salobres como dulces, de forma que se considera que presentan un distribución ubicua (Chorus y Bartram, 1999).

La aparición de floraciones de cianobacterias puede darse en cualquier época del año, si bien la mayoría de los estudios de seguimiento de estas floraciones a nivel mundial concluyen que las condiciones más favorables se dan a finales de verano y otoño. Su duración también depende de las condiciones climáticas: en zonas templadas las floraciones de cianobacterias son más frecuentes a final de verano y principios de otoño, con una duración de 2-4 meses, mientras que en regiones mediterráneas o con climas subtropicales las floraciones comienzan antes y persisten durante más tiempo. Así, en Francia, lo más común es que presenten una duración en el tiempo de unos 4 meses, mientras que en España, Portugal, Sudáfrica, Japón y el sur de Australia pueden durar 6 meses o más. En años en los que las precipitaciones son escasas, estas

floraciones pueden persistir incluso a lo largo de todo un año en zonas tropicales o subtropicales de China, Brasil y Australia (Chorus y Bartram, 1999).

Se ha estimado que aproximadamente el 50% de las floraciones de cianobacterias de aguas continentales son tóxicas (Hallegraeff, 1992). Este hecho, unido a la capacidad que presentan las cianotoxinas para producir daños en los seres humanos, ha provocado que la presencia de floraciones en el agua sea considerada como un importante problema de calidad de las aguas en todo el mundo, siendo necesario esclarecer los mecanismos de toxicidad de estas toxinas y la forma de contrarrestar sus efectos.

2.2. CLASIFICACIÓN DE LAS CIANOBACTERIAS Y CIANOTOXINAS

2.2.1. CIANOBACTERIAS

Atendiendo a su morfología, se pueden encontrar cianobacterias unicelulares que pueden presentarse tanto aisladas como agregadas en colonias con forma irregular, y que presentan formas ovoides, esféricas o cilíndricas. Además, también existen cianobacterias multicelulares con morfología filamentosa. En función de estas características podemos encontrar cinco subgrupos:

- Subgrupo I: cianobacterias unicelulares con forma esférica, cilíndrica u ovalada, que se reproducen por fisión binaria o por gemación.
- Subgrupo II: cianobacterias unicelulares que pueden formar colonias y que se dividen por fisión múltiple.
- Subgrupo III: cianobacterias filamentosas sin heterocistos que se dividen en un solo plano.
- Subgrupo IV: cianobacterias filamentosas con heterocistos que se dividen en un solo plano.
- Subgrupo V: cianobacterias filamentosas con heterocistos que se dividen en múltiples planos.

2.2.2. CIANOTOXINAS

Las cianotoxinas son un grupo diverso de sustancias naturales producidas por diversas especies de cianobacterias. Debido a su procedencia acuática, la mayoría de las toxinas son más dañinas para los animales terrestres que para los animales acuáticos (Briand y col., 2003), ya que estos últimos suelen presentar adaptaciones que les permiten vivir en presencia de las toxinas, convirtiéndose con ello en organismos bioacumuladores.

Tradicionalmente, estas cianotoxinas se clasifican en función de los efectos tóxicos que producen (Tabla 1) (de Figuereido y col., 2004; Teneva y col., 2005):

- **Dermatotoxinas**: lipopolisacáridos, lyngbyatoxina-a, aplisiatoxinas.
- **Neurotoxinas**: anatoxina-a, homoanatoxina-a, anatoxina-a(s), saxitoxinas.
- **Hepatotoxinas**: microcistinas, nodularina, cilindrospermopsina.

Grupo de toxinas	**Agente Productor (género)**	**Mecanismos y Efectos**	**Biotransformación**
Anatoxinas	*Anabaena*	Inhibición de la actividad acetilcolinesterasa	Citocromo P-450 Glutatión-S-Transferasa (GST)
Anatoxin-a(s)	*Anabaena* *Aphanizomenon* *Cylindrospermopsis* *Planktothrix* *Oscillatoria* *Microcystis*	Unión irreversible a los receptores nicotínicos de acetilcolina	Citocromo P-450 GST
Cilindrospermopsinas	*Cylindrospermopsis* *Aphanizomenon* *Umezakia* *Raphidiopsis* *Anabaena* *Lyngbya*	Inhibición síntesis de proteínas Daño citogenético en el ADN	Citocromo P-450
Lipopolisacáridos	*Anabaena* *Anacystis* *Microcystis* *Nodularia*	Irritante potencial, afectando a los tejidos expuestos	Vía alternativa de desacetilación lisosomal
Microcistinas	*Microcystis* *Anabaena* *Plankthotrix* *Nostoc* *Anabaenopsis* *Hapalosiphon*	Inhibición de las fosfatasas de proteínas (PP1 y PP2A) Estrés oxidativo	GST

Nodularinas	*Nodularia*	Inhibición de las fosfatasas de proteínas (PP1 y PP2A)	GST
Saxitoxinas	Dinoflagelados (*Protogonyaulax, Alexandrium, Gymnodinium, Pyrodinium*) Cianobacterias (*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis)*	Unión y bloqueo de canales de sodio en células nerviosas	GST

Tabla 1. Diferentes toxinas de cianobacterias, principales productores, principales efectos tóxicos y biotransformación. Modificado de Prieto (2007).

Las **dermatotoxinas** están producidas por diferentes especies de cianobacterias marinas, principalmente aquellas que pertenecen a los géneros *Lyngbya* y *Schizothrix*. Estas toxinas causan dermatitis severa por contacto e irritación de los ojos y tracto respiratorio (Arthur y col., 2006). Los lipopolisacáridos endotóxicos, producidos por especies de *Anabaena, Anacystis, Microcystis y Nodularia,* tienen acción sobre el sistema inmunitario y también afectan a los sistemas de destoxicación de diferentes organismos.

Las **neurotoxinas** presentan estructura alcaloidea y bloquean la neurotransmisión, llegando a causar la muerte por rápida parálisis respiratoria (Sivonen, 1998). Se pueden encontrar diferentes tipos:

- Anatoxina-a y homoanatoxina-a: están producidas por diferentes especies de los géneros *Anabaena, Aphanizomenon, Cylindrospermopsis*, *Planktothrix, Oscillatoria* y *Microcystis*, entre otras. Presentan estructura análoga a los agonistas nicotínicos, por lo que pueden unirse a los receptores nicotínicos de acetilcolina y provocar una sobreexcitación de las células musculares (McElhiney y Lawton, 2005). La anatoxina-a(s) es un éster de fosfato de la N-hidroxiguanina cíclica (Sivonen y Jones, 1999) que inhibe irreversiblemente la enzima acetilcolinesterasa, por lo que interfiere en la contracción muscular, causando fatiga y fallo muscular (Carmichael, 1994).
- Saxitoxina o toxina paralizante (Paralytic shellfish poison, PSP): son neurotoxinas, producidas tanto por dinoflagelados como por cianobacterias, con estructura de alcaloides, que bloquean selectivamente

los canales de sodio dependientes de voltaje, por lo que impiden la propagación del impulso nervioso (Kao, 1993).

Dentro de las **hepatotoxinas** se pueden encontrar diferentes tipos:

- Microcistinas (MCs) y nodularina: son péptidos cíclicos que pueden ser transportados activamente por los OATP o polipéptidos transportadores de aniones orgánicos (Fischer y col., 2005), los cuales se encargan principalmente de transportar sales biliares, y es por ello por lo que uno de los principales órganos diana es el hígado. Además, son inhibidores de las fosfatasas de proteínas 1 y 2A y potentes promotores de tumores en el hígado (Sivonen y Jones, 1999; Humpage y col., 2000a).
- Cilindrospermopsina (CYN): es un alcaloide tricíclico derivado de la guanidina unido a un grupo hidroximetiluracilo (Ohtani y col., 1992) (Fig.1), con un peso molecular de 415 Daltons y una elevada solubilidad en agua (Sivonen y Jones, 1999; Shaw y col., 2000), debido esto último a la carga negativa que presenta en el grupo sulfato y a la carga positiva que presenta en el grupo guanidinio (zwiterión). Además, es estable bajo diferentes condiciones de luz, temperatura y pH (Chiswell y col., 1999).

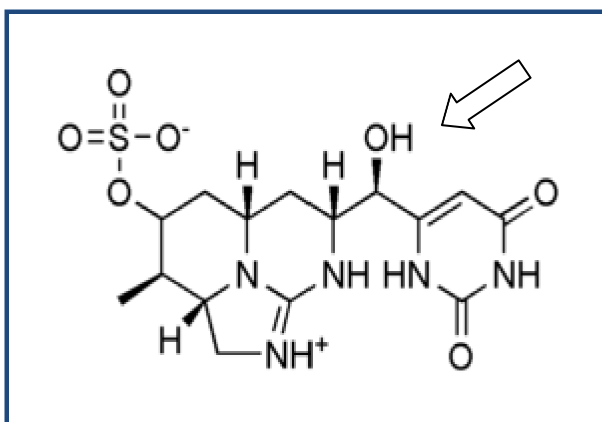


Figura 1. Estructura química de la cilindrospermopsina.

Existe un epímero de la CYN, denominado 7-epiCYN (Banker y col., 2000) (Fig.2) y otra variante de CYN denominada 7-deoxiCYN (Norris y col., 1999; Li y col., 2001) (Fig.3), que se encuentra presente junto a ésta en los reservorios de agua contaminados. La existencia de estas variantes de la molécula conllevó al estudio de la relación entre la toxicidad y la química de los compuestos. Runnegar y col. (1994)

consideraron que el grupo sulfato presente en posición 12 de la CYN podría ser necesario para el transporte hacia el interior de las células de los órganos diana. Sukenik y col. (2001) postularon que el anillo de pirimidina era esencial para la toxicidad. Por otro lado, la presencia o no del grupo hidroxilo en posición 7 de la CYN juega un papel importante en su toxicidad, reduciéndose ésta en más de 50 veces si la posición se encontraba modificada.

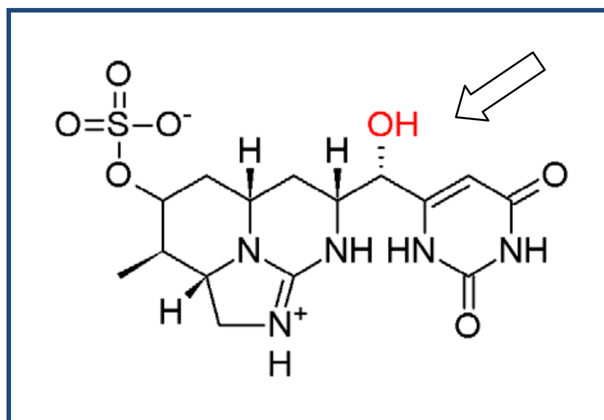


Figura 2. Estructura química del epímero 7-epicilindrospermopsina.

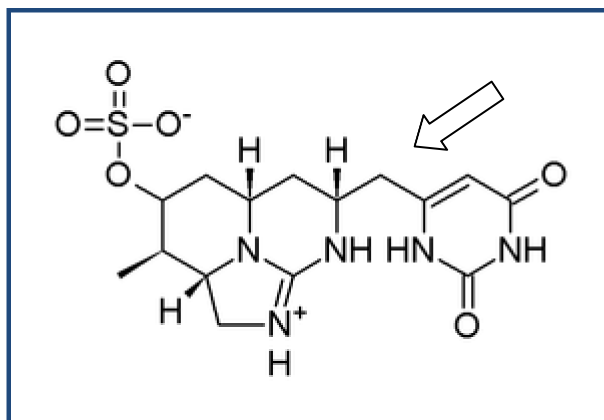


Figura 3. Estructura química de la 7-deoxicilindrospermopsina.

La CYN se aisló e identificó tras un brote de hepatoenteritis que afectó a la población australiana de Palm Island, donde se determinó que el agente causante era una cianobacteria denominada *Cylindrospermopsis raciborskii* (Bourke y col., 1983). La molécula fue definida químicamente por Ohtani y col. (1992) y posteriormente se purificó a partir de extractos de *Cylindrospermosis raciborskii* (Moore y col., 1993).

Existen distintas especies de cianobacterias capaces de sintetizar CYN, entre las que encontramos: *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum* (Shaw y col., 1999), *Umezakia natans* (Harada y col., 1994), *Anabaena bergii* (Stüken y col., 2006), *Raphidiopsis curvata* (Li y col., 2001), *Aphanizomenon flos-aquae* (Preussel y col., 2006), *Lyngbya wollei* (Seifert y col., 2007) y *Anabaena lapponica* (Spoof y col., 2006), aunque es *C. raciborskii* la principal especie productora.

Entre la diversidad de cianotoxinas existentes, en este trabajo nos hemos centrado en la CYN, ya que la presencia de la misma, así como de sus diferentes especies productoras es cada vez más ubicua, existiendo, sin embargo, pocos estudios al respecto, a pesar de los riesgos que representa tanto para la salud humana como animal (Bourke y col., 1983; Moestrup, 1996; Chorus & Bartram, 1999).

2.3. DISTRIBUCIÓN GEOGRÁFICA DE CILINDROSPERMOPSINA

Inicialmente, el crecimiento de cianobacterias productoras de CYN se limitaba a las zonas tropicales y subtropicales, pero hoy día se encuentran distribuidas por cualquier zona del mundo, encontrándose en lagos, ríos, lagunas, presas, etc. Actualmente existen datos de la presencia de cepas productoras de CYN en Asia (Chonudomkul y col., 2004), África (Fathaili y col., 2010), América del Norte (Chapman y Schelske, 1997; Richey y col., 2001; Kling, 2009), América Central (Berry y Lind, 2010), América del Sur (Molica y col., 2002), Europa (Saker y col., 2003; Quesada y col., 2006; Alster y col., 2009; Barone y col., 2009) y Oceanía (McGregor y Fabbro, 2000; Stirling y Quilliam, 2001); es decir, hasta el momento se ha encontrado en todos los continentes excepto la Antártida (Fig.4).

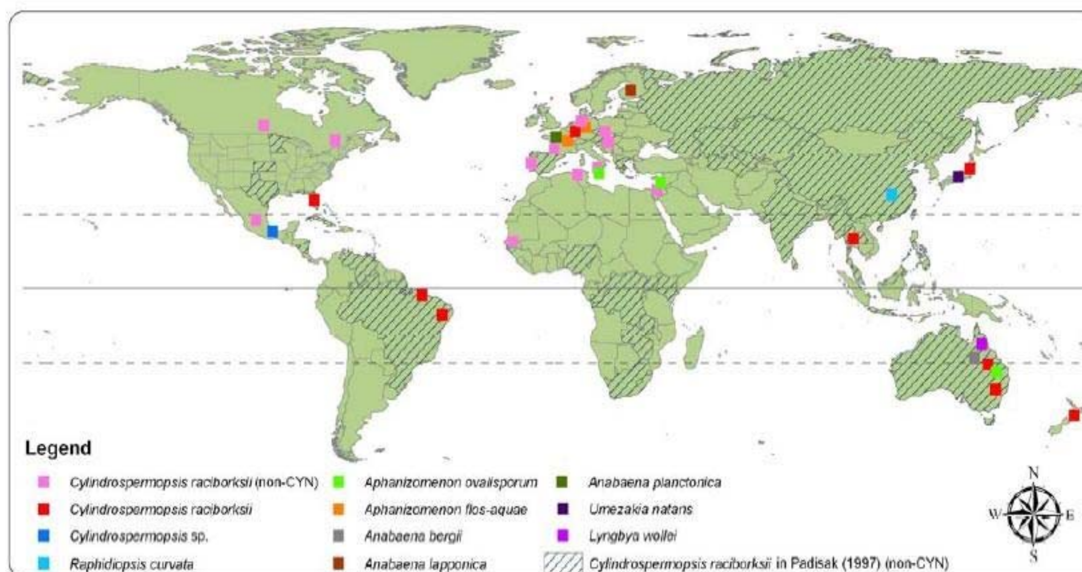


Figura 4. Distribución mundial de blooms de algas productoras de CYN o derivados de CYN (datos orientativos). Tomado de Kinnear (2010).

Por otro lado, se cree que existen muchas zonas donde aún la CYN no ha sido detectada debido a que muchos de los organismos productores no forman capas visibles en la superficie del agua y que, por tanto, su distribución puede ser mucho más amplia en comparación a la que actualmente se conoce (Kling y col., 2009).

2.4. EXPOSICIÓN A LAS CIANOTOXINAS

Los registros más frecuentes de intoxicaciones por cianotoxinas están relacionados con animales, domésticos o silvestres, que bebieron agua que contenían cianobacterias tóxicas (Francis, 1978; Moestrup, 1996), los cuales datan, incluso, del Pleistoceno (Braun y Pfeiffer, 2002). Los registros de intoxicaciones humanas a lo largo de los años han presentado diferente gravedad (Carmichael, 1981; Falconer, 1996; Falconer & Humpage, 1996). Los casos más graves se refieren al "Síndrome de Caruaru", en el que se detectó el desarrollo de fallos hepáticos severos en 100 pacientes, de los que murieron 50, de una clínica de diálisis del Norte de Brasil (Pernambuco) debido a que el agua estaba contaminada con MCs y CYN (Chorus & Bartram, 1999).

En este aspecto, cabe destacar la bioacumulación de las cianotoxinas en los organismos vivos, de forma que éstas alcanzan mayores concentraciones en los tejidos

de éstos que las que hay presentes en el medio ambiente. La bioacumulación alcanza mayores concentraciones conforme se sube de nivel trófico en la cadena alimentaria, por lo que los humanos pueden convertirse en los mayores reservorios de cianotoxinas. Además, se puede producir el fenómeno conocido como biomagnificación, que se da cuando interaccionan diferentes niveles tróficos. Entre los alimentos en los que la bioacumulación es frecuente se encuentran el pescado, los crustáceos y otras especies acuáticas con interés comercial y de recreo (Saker y Eaglesham, 1999).

En la actualidad, existen numerosos estudios concernientes a la bioacumulación y biomagnificación de otras cianotoxinas, como las MCs, mientras que los estudios centrados en la CYN aún son escasos. Se ha comprobado que las MCs pueden acumularse sobre vegetales destinados al consumo humano, como son lechuga (Codd y col., 1999; Crush y col., 2008), patatas y judías (McElhiney y col., 2001), brócoli y semillas de mostaza (Järvenpää y col., 2007), arroz (Prieto y col., en prensa), viéndose de esta forma el riesgo que conlleva para los humanos el riego de los campos de cultivo con aguas contaminadas por cianobacterias. Además, las MCs también se acumulan en una gran variedad de animales acuáticos como crustáceos, bivalvos y peces (Eriksson y col., 1989; Watanabe y col., 1997). En peces se han realizado numerosos estudios, tanto en ambientes naturales como en laboratorio, en los que se ha demostrado la inducción de efectos tóxicos de las MCs (Chen y Xie, 2005; Jos y col., 2005; Prieto y col., 2006). Vistos los efectos y la variedad de seres vivos que se ven afectados por las MCs, es de interés el estudio de los potenciales procesos de bioacumulación y biomagnificación de CYN, tanto a nivel medioambiental como de la salud humana.

2.4.1. Vías de exposición a Cilindrospermopsina

Según el Programa Internacional de Seguridad Química de la Organización Mundial de la Salud (OMS) de 1995, para que un producto sea considerado un riesgo para la salud humana, no sólo debe presentar ciertas características químicas y/o biológicas, cinéticas y dinámicas, sino que también se deben tener en cuenta el desconocimiento de sus principales vías de exposición y la posibilidad de entrar en contacto con el hombre, como es el caso de las cianobacterias.

Existen dos formas posibles de encontrar a estas cianotoxinas: dentro de las propias células productoras, o libres en el medio acuoso debido a la excreción al exterior por parte de las células o a la ruptura de éstas (Zimba y col., 2001; Li y col., 2003). Las intoxicaciones por estas toxinas pueden ocurrir de diferentes formas: por contacto dérmico, inhalación, consumo de agua contaminada, ingestión de alimentos contaminados, etc. (Codd y col., 1999).

En diferentes muestreos realizados en reservorios de agua distribuidos a lo largo del mundo, se han determinado diferentes niveles de CYN, alcanzando concentraciones muy elevadas capaces de producir daños tanto en animales y plantas como en seres humanos. En Florida se han detectado niveles de hasta 97,1 μg/L de CYN en aguas tratadas (Burns y col., 2002). En Australia se han detectado concentraciones que comprenden desde 1,17 μg/L hasta 800 μg/L de CYN en estudios realizados sobre distintas masas de agua (Chiswell y col., 1999; Saker y Eaglesham, 1999; Shaw y col., 1999; McGregor y Fabbro, 2000; Shaw y col., 2000; Hoeger y col., 2004). En Europa se han detectado niveles de CYN en diferentes países como Francia, donde se encontraron concentraciones de hasta 1,95 μg/L (Brient y col., 2009), Italia, detectándose concentraciones extracelulares de CYN de hasta 126 μg/L (Messineo y col., 2010) y Alemania, donde se encontraron niveles de CYN libre en el medio de 0,08 – 12,1 μg/L en un estudio realizado sobre 21 lagos (Rücker y col., 2007). En España, Quesada y col. (2006) determinaron concentraciones de CYN de hasta 9,4 μg/L en un lago de uso recreativo, superándose de esta forma el valor guía para la CYN propuesto por Humpage y Falconer (2003) de 1 μg/L.

En la siguiente tabla (Tabla 2) se recoge un resumen de las concentraciones de CYN (μg/L) encontradas en distintos reservorios de aguas naturales a nivel mundial:

País de Muestreo	Concentración de CYN (μg/L)	Referencia
EE.UU. (Florida)	8,1 – 97,1	Burns y col., 2002
Australia	589	Saker y Eaglesham, 1999
	4 - 120	Shaw y col., 1999
	10 - 92	Chiswell y col., 1999
	0 - 80	McGregor y Fabbro, 2000
	800	Shaw y col., 2000
	1,17	Hoeger y col., 2004

Francia	1,95	Brient y col., 2009
Italia	0 - 15	Manti y col., 2005
	18,4	Bogialli y col., 2006
	2,6 - 126	Messineo y col., 2010
Alemania	0,8 – 12,1	Rücker y col., 2007
España	1,5 – 9,4	Quesada y col., 2006

Tabla 2. Concentraciones de CYN encontradas en distintos reservorios de agua distribuidos a lo largo de todo el mundo. Tomada de Rücker y col. (2007) y Hedman y col. (2008).

En la evaluación del riesgo asociado con la exposición de cianotoxinas en humanos se deben tener en cuenta diferentes situaciones (Dietrich y Hoeger, 2005):

- Consumo de agua contaminada.
- Bioacumulación de cianotoxinas en plantas y animales.
- Exposición involuntaria mediante el consumo de suplementos de algas contaminadas con cianotoxinas.
- Contacto dérmico, nasal u oral en aguas de recreo.

El grado con el que las personas se exponen a las cianotoxinas depende en gran medida de diferentes factores como son: las condiciones climáticas, los hábitos alimentarios poblacionales, las fuentes de agua potable, el tratamiento del agua potable y la prosperidad económica de la población (Willett y col., 2004).

2.4.1.1. Consumo de agua contaminada

La mayoría de las poblaciones en países industrializados dependen de surtidores públicos para el consumo de agua potable, lo cual hace que uno de los mayores riesgos de intoxicación por cianotoxinas para los seres humanos sea a través del consumo de agua potable.

En algunos países (Argentina, Finlandia, Israel, etc.), el control de las aguas contaminadas con toxinas o células de cianobacterias no es obligatorio, llegándose a detectar hasta 9000 cel/mL incluso después del tratamiento de las aguas de bebida (Hoeger y col., 2004; 2005). Además, cabe destacar que una gran parte de la población mundial no tiene acceso a ningún tipo de agua tratada.

La primera vez que se tiene constancia de una intoxicación exclusivamente debida a CYN en la población humana por el consumo de agua fue en Palm Island (Australia), donde se registraron 148 casos de hepatoenteritis acompañado de daño tubular renal (Byth, 1980; Hawkins y col., 1985). Tras la correspondiente investigación, el motivo de dicha intoxicación se atribuyó a una floración de *C. raciborskii* en el embalse de abastecimiento de agua potable de la población.

Actualmente, aún no existe un valor guía establecido para la CYN, si bien la OMS se encuentra en aras de establecer uno (OMS, 2011). Humpage y Falconer (2003) recomiendan un valor guía de 1 μg/L de CYN en agua de bebida. En Europa, la presencia de las toxinas de cianobacterias no está claramente regulada, aunque la Directiva Europea 2000/60/CE que caracteriza los contaminantes prioritarios del agua, ha destacado las cianobacterias productoras de toxinas como potenciales contaminantes peligrosos.

Para las aguas de recreo en las que se producen floraciones de cianobacterias, la OMS ha establecido tres niveles de alerta dependiendo del riesgo de los efectos adversos para la salud humana (OMS, 2003), los cuales están basados en la densidad de cianobacterias presente en el agua. Los niveles de alerta son los siguientes:

- Baja probabilidad de efectos adversos sobre la salud: 20.000 células/mL.
- Probabilidad moderada de efectos adversos sobre la salud: 100.000 células/mL.
- Riesgo elevado de efectos adversos sobre la salud: presencia de una capa superficial espesa de cianobacterias.

La Directiva Europea 2006/7/CE relativa a la gestión de la calidad de las aguas de baño y por la que se deroga la Directiva 76/160/CEE, contempla la propensión a la proliferación de cianobacterias dentro de los perfiles de las aguas de baño.

2.4.1.2 Bioacumulación de Cilindrospermopsina en vegetales y animales

La bioacumulación, como se describió anteriormente, es el proceso de acumulación de sustancias químicas en organismos vivos, de forma que éstas alcanzan

mayores concentraciones en los tejidos de los organismos que las que hay presentes en el medio ambiente que los rodea.

Con respecto a organismos vegetales, el primer estudio que se realizó al respecto fue sobre lentejas de agua, las cuales se expusieron a concentraciones de CYN en el rango de 0-120 µg /L (Kinnear y col., 2009), viéndose que la mayor parte de la CYN se acumulaba en las paredes celulares a bajas concentraciones. En trabajos realizados con hydrilla (*Hydrilla verticillata*) se mostró que no existía bioconcentración en plantas expuestas a CYN, incluso a concentraciones de exposición de hasta 400 µg/L (White y col., 2005), mientras que sobre lentejas de agua (*Lemna punctata*) se demostró bioacumulación de CYN al emplear concentraciones de 570 µg/L (Seifert, 2007). También se han realizado estudios sobre plantas de arroz (*Oriza sativa*), expuestas a un extracto de CYN y a una mezcla de extractos de *A. ovalisporum* y *Microcystis aeruginosa*, productores de CYN y MCs, respectivamente. En ambos casos se vieron afectadas las actividades Glutatión transferasa (GST) y Glutatión peroxidasa (GPx). Además, también se comprobó que la adición simultanea de los dos extractos resultaba en la producción de efectos sinérgicos sobre las plantas (Prieto y col., 2011). Debido a la escasez de datos concernientes al efecto de la CYN sobre las plantas, es de especial interés la realización de más estudios que ayuden a esclarecer este aspecto.

Los estudios de bioacumulación de CYN sobre animales aún son escasos. El primer estudio fue llevado a cabo sobre *Cherax quadricarinatus* y *Melanotaenia eachamensis* presentes en un estanque con una concentración de CYN de 589 µg/L (Saker y Eaglesham, 1999), viéndose que la acumulación de CYN era distinta en función de los diferentes órganos y tejidos. En *Cherax quadricarinatus* la toxina se detectó tanto en músculo (900 µg/Kg tejido liofilizado) como en el hepatopáncreas (4.300 µg/Kg de tejido liofilizado). Ensayos realizados en el laboratorio confirmaron este tipo de bioacumulación tras catorce días de exposición, con valores en hepatopáncreas y tejido abdominal de 1000 y 200 µg de toxina/Kg de tejido liofilizado, respectivamente. En *Melanotaenia eachamensis*, la toxina fue recuperada con un máximo de 1.200 µg/Kg tejido liofilizado.

Se ha observado en estudios recientes sobre *Pomacea patula catemacensis* que, al ser la CYN una molécula muy soluble en agua, la bioacumulación se debe producir

en el intestino o hígado, ya que son las primeras zonas de contacto y presentan transporte activo por parte de los hepatocitos, respectivamente (Berry y Lind, 2010). En estudios realizados sobre *Anodonta cygnea*, los niveles más altos de CYN se encontraron en la hemolinfa (68,1%), seguido de las vísceras (23,3%), el pie y la gónada (7,7%), y el manto (0,9%) (Saker y col., 2004). También se demostró que la CYN y su epímero deoxiCYN, podían concentrarse en las vísceras de *Corbiculina* (Seifert y col., 2007), indicando la posible afinidad de la CYN por los tejidos sanguíneo y linfático, a diferencia de otras cianotoxinas, como las MCs, que se concentran habitualmente en el hepatopáncreas y otras vísceras (Meriluoto y col., 2008*)*. White y col. (2006) demostraron la bioacumulación de CYN en *Melanoides tuberculata*. En estudios realizados por el mismo grupo sobre *Bufo marinus* la toxina se acumuló en los tejidos, pero no en cantidades suficientes como para demostrar su bioacumulación (White y col., 2007).

La acumulación de CYN en los diferentes animales acuáticos presenta una gran variabilidad, siendo ésta mucho mayor en los organismos estructuralmente más sencillos (gasterópodos, bivalvos y crustáceos) que en los organismos más complejos (anfibios y peces), mientras que si nos referimos al grado de afectación de estos organismos, se da el orden inverso, es decir, los animales superiores se afectan en mayor medida que los animales inferiores (Kinnear y col., 2009). Se ha detectado que las especies herbívoras parecen ser las más tolerantes a la CYN, lo cual sugiere que los efectos tóxicos producidos estén relacionados con la mayor o menor capacidad de los organismos para acumular la toxina (Smith y col., 2008).

Con respecto a la biomagnificación, que es el proceso de bioacumulación de la toxina a lo largo de la cadena trófica, debido a que la CYN es una molécula muy soluble en agua, no se considera probable que este proceso ocurra, si bien la toxina puede llegar a los organismos superiores a través de cadena trófica, sin ser necesariamente acumulada por los organismos basales (Berry y Lind, 2010). Esto es de gran importancia debido a que la CYN puede depositarse en alimentos que posteriormente van a ser consumidos por los humanos (Fig. 5).

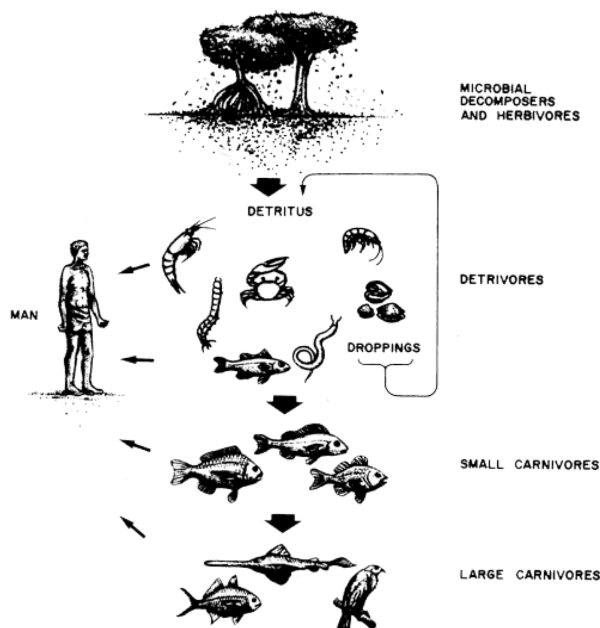


Figura 5. Esquema de una cadena trófica acuática, donde puede apreciarse cómo el ser humano puede convertirse en el mayor reservorio de cilindrospermopsina al ser consumidor de la mayoría de los organismos que intervienen en la cadena. Tomado de FAO (1998).

A partir de los pocos datos sobre la toxicidad de CYN que existen en la bibliografía, se ha establecido una ingesta diaria tolerable (IDT) provisional para esta toxina de 0,03 µg/Kg/día, la cual se ha obtenido a partir de un valor de un NOAEL (Non Observed Adverse Effects Level), derivado de ensayos en ratones, de 30 µg/Kg/día al cual se le aplica un factor de incertidumbre de 1000 (Humpage y Falconer, 2003).

2.4.1.3 Exposición involuntaria a través de suplementos de algas contaminados con cianotoxinas

En la actualidad, cada vez tiene más auge el empleo de cianobacterias como suplementos alimenticios e incluso, en algunos casos, como alimento de animales y seres humanos (Gilroy y col., 2000; Chamorro y col., 2000; Moreno y col., 2006). En distintas regiones del mundo ya se empleaban las "algas verdes-azuladas" (*Spirulina spp.* y *Nostoc spp.*) como fuente de alimento (Carmichael y col., 2000; Jensen y col., 2001). Estos suplementos suelen consumirse por sus supuestos efectos beneficiosos para la salud, ya que parece ser que aumentan el estado de alerta y la energía, mejora el estado de ánimo y ayuda en la pérdida de peso (Jensen y col., 2001). Hoy en día, los suplementos de "algas verdes-azuladas" representan una importante fuente de ingresos

para el sector económico de los países industrializados. El problema de su empleo radica en la coexistencia de estas especies nutritivas con otras especies de cianobacterias productoras de toxinas (Schaeffer y col., 1999; Saker y col., 2005). Estudios realizados para la detección de CYN no han podido confirmar la presencia de ésta en diferentes suplementos alimenticios (Liu y Scott, 2011).

2.4.1.4. Contacto dérmico, nasal u oral en aguas de recreo

Como se vio anteriormente, las épocas de mayor riesgo para la presencia de una floración de cianobacterias son la primavera, verano y principios de otoño, coincidiendo con el periodo en que un elevado número de personas utilizan los embalses, lagos o ríos con fines recreativos. Las exposiciones a aguas contaminadas con cianobacterias mientras se realizan actividades recreacionales han producido desde gastroenteritis e irritaciones cutáneas, hasta hepatitis y neumonía (Bell y Codd, 1994), estando implicada tanto la vía de exposición oral como la dérmica (Pilotto y col., 1997; Chorus y col., 2000). Cabe destacar que la exposición a la CYN por vía dérmica es de gran importancia, ya que se encuentra en su mayor parte de forma extracelular, disuelta en el medio.

2.5. TOXICIDAD DE LA CILINDROSPERMOPSINA

2.5.1. Transporte de Cilindrospermopsina

La forma en que la CYN entra en los organismos no es del todo conocida. Se piensa que el bajo peso molecular que presenta (415 Da) hace que la difusión pasiva sea un mecanismo factible en la absorción de la toxina, si bien este proceso sería lento y progresivo, ya que es dependiente del gradiente de concentración (Chong y col., 2002; Runnegar y col., 2002). No obstante, se ha visto en algunos estudios que el grupo sulfato de la CYN no es necesario para la entrada de la molécula en las células y que su naturaleza hidrofílica hace que sea poco probable que atraviese la pared celular (Froscio y col., 2009).

Se ha observado en diferentes estudios que organismos que ingieren CYN, como *Daphnia magna*, presentan daños en la pared intestinal (Nogueira y col., 2006) lo cual es de gran importancia, ya que el epitelio dañado ofrece una mayor superficie de absorción, facilitando con ello la captación de la CYN por parte de las células intestinales. Otro posible mecanismo de transporte de la CYN al hígado es mediante el sistema de transporte de las sales biliares, a las cuales se una la CYN y de esta forma llega al hígado (Chong y col., 2002).

A todos estos mecanismos de transporte también hay que añadir el hecho de que la absorción de CYN por parte de las células es mayor cuando existen otros componentes celulares procedentes del agente productor de la toxina (Seifert y col., 2007).

2.5.2. Efectos tóxicos producidos por Cilindrospermopsina

Dentro del marco medioambiental, la CYN tiene la capacidad de afectar a un amplio rango de plantas y animales acuáticos, lo que, unido a la bioacumulación que se produce y a la transferencia dentro de la cadena trófica, hace que el daño producido por la CYN se pueda extender a otras especies de plantas y animales terrestres. Existen distintos factores que influyen en la toxicidad de *C. raciborskii* y de la toxina CYN (Fig. 6).

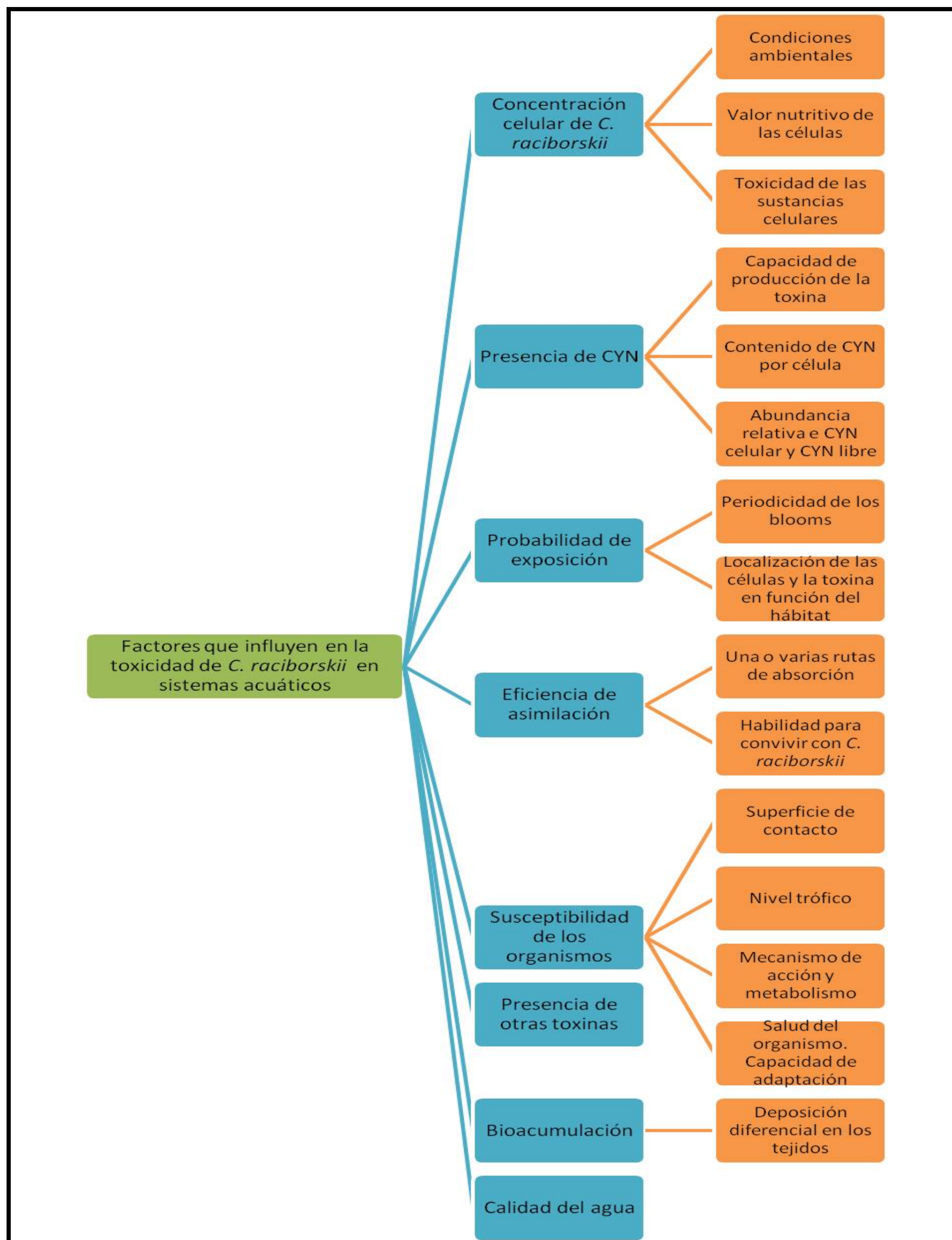


Figura 6. Cuadro resumen de los factores que influyen en la toxicidad de *C. raciborskii* y de CYN en los ecosistemas acuáticos. Adaptado de Kinnear (2010).

Se han llevado a cabo estudios sobre diferentes especies de animales con diferentes concentraciones de extractos de CYN y CYN pura, siendo estos estudios bastante escasos en peces.

En experimentos realizados sobre el crustáceo *Artemia salina* se observó, tras exposición a CYN pura, una respuesta dependiente de la dosis en la mortalidad de este organismo, disminuyendo los valores de la DL_{50} desde las 24 h a las 72 h (Metcalf y

col., 2002). Por otro lado, poblaciones naturales de *Daphnia magna* sometidas a floraciones de *C. raciborskii* experimentaron una alta mortalidad, reducción de su crecimiento corporal, así como efectos negativos en la fecundidad por la presencia de metabolitos tóxicos en células de *Cilindrospermopsis spp.* (Nogueira y col., 2004*).*

En estudios de los efectos tóxicos de CYN producidos en embriones de pez cebra (*Danio rerio)*, se observó que ésta era tóxica sólo cuando era inyectada directamente en los embriones, pero no cuando éstos se encontraban en un medio acuoso contaminado. Por el contrario, la inmersión directa de los embriones en todos los extractos de cianobacterias resultó en una mortalidad mayor y reproducible, con presencia de disfunciones en el desarrollo de los embriones, sugiriendo con ello que la CYN produce la muerte y que otros metabolitos presentes en *C. raciborskii* y *A. ovalisporum* parecen inhibir el desarrollo (Berry y col., 2009).

Con respecto al efecto de CYN sobre vertebrados terrestres, extractos liofilizados de una floración de *C. raciborskii* en una presa australiana, fueron letales para ratones a las 24 h tras inyección intraperitoneal (i.p.). En este mismo lugar, tres vacas y diez terneros afectados por haber bebido agua contaminada, mostraron signos de gran debilidad antes de su muerte. En las necropsias se detectaron derrame abdominal, mesenterios hiperémicos e inflamación y palidez hepática (Briand y col., 2003). El estudio histopatológico del hígado realizado a un becerro mostró signos similares a los efectos conocidos de CYN en estudios realizados sobre ratones, es decir, un área extensa de degeneración y necrosis hepática (Falconer y col., 1999; Seawright y col., 1999).

En cuanto a los seres humanos, los primeros efectos clínicos conocidos producidos por CYN se observaron tras la intoxicación masiva ocurrida en la localidad australiana de Palm Island, donde se observó que los afectados sufrían una inusual hepatoenteritis, que cursaba inicialmente con hepatomegalia, constipación, vómitos y dolor de cabeza, seguido todo ello de hemorragias y pérdida de proteínas, electrolitos y glucosa a través de la orina, con signos variables de deshidratación (Bourke y col., 1983; Griffiths y Saker, 2003).

Por norma general, los efectos de CYN se caracterizan por una toxicidad retardada que afecta de forma sistémica a los individuos, aunque principalmente afecta al hígado y riñón (Kinnear, 2010). La toxicidad está mediada por inhibición irreversible de la síntesis de proteínas (Froscio y col., 2001; Looper y col., 2005), así como por genotoxicidad por fragmentación del ADN, la cual fue demostrada en modelos *in vitro* (Humpage y col., 2000a; Humpage y col., 2005; Bazin y col., 2010a), e *in vivo* (Shen y col., 2002). Además, se cree que la CYN sufre una activación metabólica en el hígado, aumentando con ello su toxicidad (Runnegar y col., 1995).

Una intoxicación aguda por CYN conlleva una acumulación de lípidos en el hígado seguida de necrosis hepatocelular, la cual puede ser debida a la formación de radicales libres, probablemente debido a la disminución de la síntesis de glutatión reducido (GSH), la cual se ha observado en ensayos con ratones (Norris y col., 2002) y en hepatocitos de rata (Runnegar y col., 1995). Por otro lado, en el riñón de ratones Swiss indujo destrucción de los túbulos proximales (Falconer y col., 1999), mientras que sobre otros tejidos como pulmones, corazón, estómago, glándulas adrenales, sistema vascular y sistema linfático, se pueden producir efectos citotóxicos y trombóticos. En el timo y el bazo se produce una necrosis selectiva de linfocitos, seguida de atrofia de ambos (Kinnear, 2010). También se ha demostrado que la CYN inhibe la síntesis del nucleótido pirimidina y altera la distribución del colesterol en ratones (Reisner y col., 2004).

Por otro lado, también hay que tener en cuenta las diferencias en intensidad de los efectos tóxicos que se producen cuando se emplean la toxina pura o extractos de cianobacterias productoras de CYN. En estudios realizados sobre diferentes especies de organismos de distintos niveles tróficos, se ha visto que a concentraciones inferiores a 100 µg/L apenas se producen efectos adversos (Seifert y col. 2007). Asimismo, la exposición a extractos de *C. raciborskii* resultó en una elevada toxicidad subletal, lo cual sugiere, como ya se mencionó anteriormente, que los extractos de células pueden contener uno o más compuestos bioactivos, además de la CYN, que incrementan el riesgo de producción de efectos tóxicos (Kinnear, 2010). Estos mismos resultados de toxicidad se han visto en estudios sobre ratones (Falconer y col., 1999). Debido a esto, es importante diferenciar entre los efectos tóxicos producidos por la CYN *per se,* de

aquellos que son producidos por los compuestos bioactivos presentes en los extractos celulares.

La DL_{50} de la CYN, ya sea pura o procedente de extractos, obtenida a partir de estudios realizados sobre ratones, también depende del tiempo de exposición, siendo menor (más tóxica) cuanto mayor tiempo haya transcurrido tras la exposición, quedando demostrada la toxicidad retardada de CYN, al menos en dicho modelo experimental. Asimismo, también depende de la vía de administración, tal y como se recoge en la tabla 3.

CYN	Vía	DL_{50} (mg/Kg)	Tiempo de Observación	Referencia
Pura	i.p.	2,1 0,2	24 h 5-6 días	Ohtani y col. (1992)
Pura	i.p.	0,17	7 días	Duy y col. (2000)
Extracto	i.p.	50-110 20-65	24 h 7 días	Shaw y col. (1999)
Pura	oral	4,4-6,9	2-6 días	Seawright y col. (1999)

Tabla 3. DL_{50} de CYN (pura y extracto) calculada en ratones a diferentes tiempos de exposición y por diferentes vías.

Tras revisar la literatura científica existente con respecto a la toxicidad de la CYN, es patente la gran escasez de estudios realizados sobre peces y otras especies acuáticas, las cuales son de gran interés en primer lugar, porque son los organismos que pueden entrar con contacto con la cianotoxina de forma más temprana ya que ésta se libera en el mismo medio en el que viven, y por otro lado, debido a su consumo potencial por los humanos.

Nuestro equipo de investigación ha puesto de manifiesto que tilapias expuestas por vía oral a CYN experimentan un aumento marcado de LPO y de la enzima GST (Puerto y col., 2011b) así como alteraciones histopatológicas en diferentes órganos, y un aumento de la oxidación de proteínas y una disminución de los niveles de GSH en hígado y riñón (Puerto y col., enviado). No obstante, aún no se conoce la influencia que van a tener en su toxicidad variables clave, como la vía de exposición o el tiempo de sacrificio tras la misma, por lo que resulta de interés el abordaje de su estudio sobre estos modelos experimentales acuáticos.

Por otra parte, se han estudiado los efectos tóxicos que produce CYN sobre modelos *in vitro,* viéndose que produce inhibición del crecimiento celular o citotoxicidad tanto en líneas celulares (Bain y col., 2007; Neumann y col., 2007; Froscio y col., 2009a, 2009b; Bazin y col., 2010a) como en cultivos primarios (Froscio y col., 2003; Fastner y col., 2003; Young y col., 2008). En este sentido, la línea celular Caco-2 es uno de los modelos *in vitro* más utilizados. Fastner y col. (2003) emplearon las líneas celulares Caco-2 y HepG2 para evaluar la toxicidad que producían diferentes cultivos de *C. raciborskii*. En otro estudio, se comparó la sensibilidad de diferentes líneas celulares (HepG2, Caco-2, BE-2, MNA y HDF) frente a CYN y 7-deoxiCYN, resultando ser la línea celular Caco-2 la más sensible en los ensayos de citotoxicidad realizados, considerando el ensayo de reducción de la sal de tetrazolio MTS (Neumann y col., 2007). En otro estudio de citotoxicidad producida por CYN en el que se emplearon siete líneas celulares, se comprobó que la línea celular menos afectada era la Caco-2, mientras que la más sensible fue la línea celular hepática C3A, lo cual pone de manifiesto el papel que juega la activación metabólica en los daños producidos por la CYN (Froscio y col., 2009a). Además, se ha comprobado en diferentes estudios, que los efectos inducidos por CYN son dependientes tanto del tiempo de exposición como de la concentración de la toxina a la que fueron expuestos los diferentes modelos experimentales (Chong y col., 2002; Bain y col., 2007; Froscio y col., 2009a). En estudios realizados con otras cianotoxinas como las MCs, sobre las líneas celulares Caco-2, humana, y PLHC-1 y RTG-2, ambas procedentes de pez, se han presentado resultados similares a los obtenidos por estos autores (Pichardo y col., 2005, 2007; Puerto y col., 2009b, 2010b). Actualmente, los estudios de citotoxicidad de CYN sobre líneas celulares de pez son escasos, por lo que es de interés el conocer de qué forma afectaría a estas líneas celulares.

2.5.3. Mecanismos de acción toxica de la Cilindrospermopsina

2.5.3.1. Inhibición de la síntesis de proteínas

En estudios realizados por Terao y col. (1994), se investigaron los efectos morfológicos y bioquímicos producidos en ratones por una dosis de 200 μg/Kg de CYN aislada de un cultivo de *Umezakia natans* y sacrificados tras 72 h. Se observó que la CYN era un potente inhibidor de la síntesis proteica. El órgano más afectado fue el

hígado, en el que se observaron una serie de cambios, los cuales estaban relacionados con la evolución de los ratones con respecto al tiempo de exposición:

- Desprendimiento de los ribosomas del retículo endoplasmático rugoso (RER) y acumulación de éstos en el citoplasma de los hepatocitos. Por lo general, este proceso iba acompañado de condensación y reducción del tamaño del nucléolo.
- Tras 24 h, la cantidad de citocromo P450 (CYP450) se veía reducida de forma considerable en los microsomas hepáticos. Algunos autores consideran que la proliferación del retículo endoplasmático liso se debe a la peroxidación lipídica (LPO) causada por el CYP450.
- Acumulación de grasa en el centro de los lóbulos hepáticos, inducida posiblemente por los radicales libres generados en la lesión inducida.
- Necrosis hepática grave, que aparece al final del proceso antes del sacrificio.

Posteriormente se ha comprobado que los efectos sobre la síntesis de proteínas en hepatocitos primarios son un indicador temprano de la exposición a CYN y aparecen incluso a concentraciones subtóxicas (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 eliminando la toxina del medio. La explicación de este hecho puede ser una unión covalente con el sitio de unión ribosómico ó que la CYN quede atrapada dentro de la célula, ejerciendo de forma permanente su acción inhibitoria. Por otro lado, la adición de CYN a un lisado de reticulocitos, dio lugar al cese inmediato de la síntesis de proteinas, indicando que la CYN interfiere en el paso de elongación de las proteínas (Froscio, 2002). Además, Runnegar y col. (2002) demostraron que las alteraciones en el anillo de guanidina, mediante el empleo de análogos sintéticos de CYN, también potenciaban la acción inhibitoria de ésta en lisados reticulocitos de conejo.

Estudios recientes han demostrado que la CYN no estaría unida al propio ribosoma, sino a una de las proteínas solubles asociadas al sistema de traducción eucariótica a través de un enlace no covalente (Froscio y col., 2008), y de esta forma ejerce su efecto inhibidor sobre la síntesis de proteínas.

2.5.3.2. Inhibición de la síntesis de glutatión

En estudios realizados en cultivos de hepatocitos de ratas intoxicadas con CYN, Runnegar y col. (1994) comprobaron que los niveles de GSH se veían reducidos, al igual que el número de proteínas. 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, a la vez que sugirieron la implicación del CYP450 en la toxicidad de la CYN por activación metabólica (Runnegar y col., 1995). 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. A diferencia de otras cianotoxinas como las MCs, se ha comprobado la implicación de la metabolización de CYN en la toxicidad de la molécula (Humpage y col., 2005). La inhibición de la producción de GSH podría potenciar la citotoxicidad y genotoxicidad a través de la producción de especies reactivas de oxígeno (ERO).

2.5.3.3. Inducción de estrés oxidativo

Con respecto al estrés oxidativo, existen estudios con resultados contradictorios sobre la implicación de la producción de estrés oxidativo como mecanismo de toxicidad de la CYN. Humpage y col. (2005) estudiaron el efecto que producía CYN sobre hepatocitos primarios de ratones, demostrando que era citotóxica a 0,1 µM tras 18 horas de exposición; sin embargo, el marcador de LPO no experimentó alteración cuando los hepatocitos se expusieron a 5 µM de CYN tras el mismo tiempo de exposición. Por otro lado, se ha informado de un aumento del nivel de LPO en hepatocitos primarios de *Hoplias malabaricus* cuando se expusieron a concentraciones de 100µM de CYN (Silva y col. 2010) y en hepatocitos primarios de *Prochilodus lineatus* expuestos a concentraciones de 10 µg/L de CYN purificada (Liebel y col., 2011). En estudios llevados a cabo en nuestro laboratorio con concentraciones de 200 y 400 µg de CYN pura/Kg de peso corporal sobre diferentes órganos de *Oreochromis niloticus*, se observó como los niveles de LPO sufrían un aumento en ambos órganos. Además, también se vieron afectadas la actividad, la expresión génica y la abundancia relativa de los marcadores enzimáticos GST y GPx (Puerto y col., 2011b). Aun así, las investigaciones

siguen siendo escasas, por lo que es necesario llevar a cabo más estudios para poder determinar si la CYN induce daño oxidativo.

2.5.3.4. Genotoxicidad de Cilindrospermopsina

En comparación con otras cianotoxinas, los estudios acerca de los mecanismos de toxicidad de la CYN son bastantes escasos (Hawkin y col.1997; Falconer y col., 1999; Seawright y col., 1999), pero entre los mecanismos encontrados cabe destacar su potencial genotóxico.

Humpage y col. (2000b) emplearon el ensayo de micronúcleos para detectar lesiones en los cromosomas. La utilidad de esta prueba se mejoró incorporando la detección de centrómeros para diferenciar entre la pérdida del cromosoma entero y la de fragmentos cromosómicos (Fenech, 1989; Segura y col., 1997). La pérdida del cromosoma entero se observó desde las concentraciones de 1µM, y la rotura de fragmentos a partir de 6 µM. Es posible especular, de forma análoga a la citotoxicidad aguda, que la pérdida del cromosoma entero puede ser debido a la interrupción de la formación del cinetocoro por la inhibición de síntesis de proteínas, mientras que la pérdida de un fragmento puede deberse a efectos directos sobre el ADN después de la activación metabólica de la toxina (Humpage y col., 2000b). Por otro lado, en células con un metabolismo deficiente (CHO K1), y por tanto con baja activación metabólica de la CYN, no se observó la producción de efectos genotóxicos mediante el ensayo cometa (Fessard y Bernard, 2003).

Para entender el papel del CYP450 en la genotoxicidad de la CYN mediada por su activación metabólica, se estudiaron las respuestas de hepatocitos primarios de ratones en presencia y ausencia de inhibidores del CYP450 (Humpage y col., 2005). La CYN demostró ser citotóxica a concentraciones superiores a 0,1 µM. Sin embargo, a concentraciones de 0,05 µM ya existía un aumento significativo en la longitud de la cola del cometa, representativo de daño en el ADN. Al comparar los resultados en presencia de los inhibidores del CYP450 (omeprazol y SKF525A), se observó que no se producía la inducción de genotoxicidad. Por este motivo, los autores concluyen que es probable que los metabolitos derivados del CYP450 sean los responsables tanto de la citotoxicidad aguda como de la genotoxicidad inducida por CYN. Recientemente, Bazin

y col. (2010a) han confirmado *in vitro* la implicación de la activación metabólica de CYN en la mediación de su toxicidad y sugieren que la CYN es un pro-genotóxico.

Los estudios de genotoxicidad de CYN *in vivo* son escasos. Se ha demostrado que la CYN y/o sus metabolitos activos se unen a las proteínas y al ADN, formando aductos en el hígado (Shaw y col., 2000; Norris y col., 2001). En un estudio realizado por Shen y col. (2002) se ha observado un aumento en la fragmentación del ADN en el hígado de los ratones tratados con 200 µg/Kg de CYN tras inyección i.p. Recientemente, Bazin y col. (2010b), trataron ratones machos a concentraciones de 50, 100 y 200 µg/Kg de CYN por vía i.p. y con 1, 2 y 4 mg/Kg por sonda gástrica. Tras 24 h de la intoxicación, se realizaron un ensayo cometa en diferentes muestras de hígado, sangre, médula ósea, riñón, intestino y colon, y un ensayo de micronúcleos en muestras de médula ósea y colon. El examen histológico mostró focos de muerte celular en hígado y riñón de los ratones que recibieron las dos dosis más altas de CYN en ambas vías de administración.

A pesar de todas las evidencias de la genotoxicidad de la CYN, la Agencia Internacional en Investigaciones sobre el Cáncer (IARC) aún no ha clasificado la CYN por su carcinogenicidad.

2.6. REPARACIÓN DEL DAÑO OCASIONADO POR LAS CIANOBACTERIAS

Los daños producidos por las cianobacterias no sólo tienen consecuencias sobre la salud humana y animal, sino también económicas, ya que pueden afectar a la producción en piscifactorías, donde pueden desarrollarse blooms tóxicos que afectan a los peces. Por eso es interesante identificar sustancias que permitan prevenir o recuperar los efectos tóxicos que se producen, originados por ejemplo, por las especies reactivas de oxígeno (ERO), las cuales pueden ser eliminadas del organismo o inactivadas por distintos mecanismos (Fig. 7):

- Sistemas enzimáticos: catalasa (CAT), superóxido dismutasa (SOD), GPx, glutatión reductasa (GR) y glucosa-6-fosfato deshidrogenasa.
- Sistemas no enzimáticos: GSH, β-caroteno, vitaminas C y E, etc.

De esta forma, los radicales superóxido (O^-_2) presentes en los tejidos serán transformados hasta peróxido de hidrógeno (H_2O_2) mediante una reacción catalizada por la enzima SOD. El H_2O_2 puede ser reducido hasta dos moléculas de agua (H_2O) por diferentes reacciones en las que intervienen las enzimas CAT y GPx. Ésta última, requiere para ejercer su acción de dos moléculas de GSH, las cuales se transforman en una molécula de glutatión oxidado (GSSG) en el transcurso de la reacción. La enzima encargada de recuperar estas moléculas de GSH consumidas es la GR mediante el gasto de una molécula de NADPH, gracias a la enzima glucosa-6-fosfato deshidrogenasa (Fig. 7).

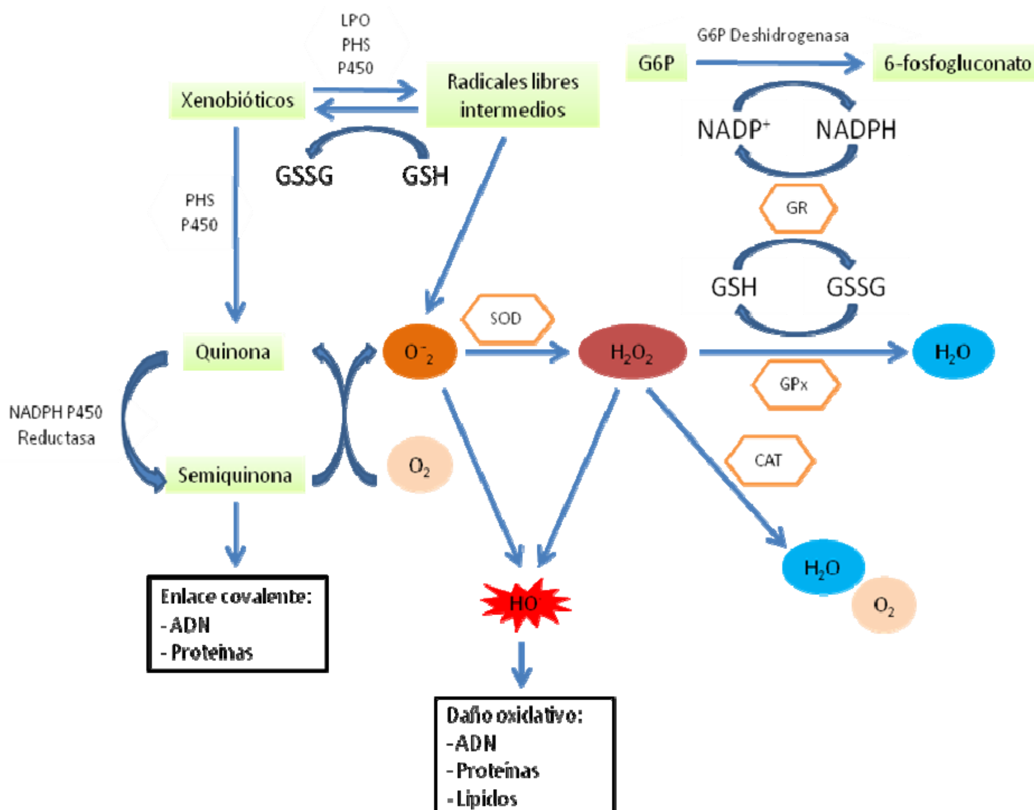


Figura 7. Principales sistemas antioxidantes celulares enzimáticos y no enzimáticos.

Como es bien sabido, en la biotransformación de los xenobióticos, las principales reacciones son catalizadas por CYP450 oxidasa y GST, que introducen grupos reactivos o polares en los xenobióticos, y los conjugan con el GSH, respectivamente. 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 ϕ), 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).

Diferentes estudios realizados en peces intoxicados con cianotoxinas (Konishi y col., 2005; Wang y col., 2006; Li y col., 2008) mostraron que el CYP450 se mantenía sin cambios tras la intoxicación, apoyando la participación de la GST y la importancia de la fase II en la destoxicación de cianotoxinas a través de la conjugación. Además, ya pronosticaban la influencia de la GPx en la prevención del estrés oxidativo. En otro estudio, la expresión de los genes de GPx y GR experimentaron un descenso de la transcripción, mientras que aumentaron los niveles de CAT y SOD (Sun y col., 2008). Por tanto, estudios centrados en alteraciones de los niveles de ARNm de las enzimas implicadas en la prevención del estrés oxidativo producido, y la abundancia relativa de proteínas de dichas enzimas, pueden ser eficaces para la prevención de los efectos de la CYN en los sistemas estudiados.

2.6.1. Sustancias que favorecen la reparación de los efectos tóxicos inducidos por cianobacterias

Los componentes no enzimáticos del sistema de defensa antioxidante juegan un papel importante en los procesos de eliminación de las ERO dentro del organismo. A continuación se muestran algunas características de las principales sustancias con capacidad antioxidante conocida, como la N-acetilcisteína y el GSH.

2.6.1.1. Glutatión (GSH)

El GSH (Fig. 8) se produce de forma natural en las células animales, estando presente en nuestra dieta diaria en alimentos tales como frutas y vegetales frescos o congelados, pescados, carnes y nueces. El GSH, junto con el selenio (Se), tiene la función de regenerar la vitamina C empleada en los procesos de eliminación de

sustancias oxidantes. Asimismo, el GSH junto con el Se y la vitamina C regeneran la vitamina E (Kelly y col., 1998). Esta relación sinérgica ayuda en el mantenimiento del sistema de defensa antioxidante celular. El mantenimiento de niveles adecuados de GSH junto con el de otros antioxidantes naturales, es crítico para tener un sistema de defensa activo contra la generación de radicales libres.

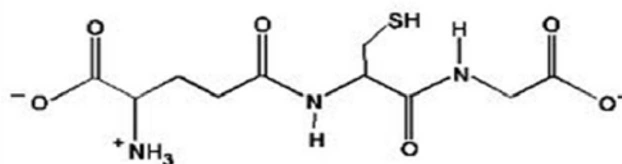


Figura 8. Estructura química del GSH.

Así, el GSH colabora en procesos fisiológicos tales como:

- Mantenimiento del sistema inmune, particularmente en la activación de los linfocitos y plaquetas.
- Reducción de la proliferación de células con tendencia a la malignidad, colaborando en la defensa de diferentes enfermedades causadas por la presencia de radicales libres (procesos inflamatorios, Alzheimer, Parkinson, cáncer, etc.).
- Revierte la capacidad de metástasis aumentando la respuesta de las células a la quimioterapia (Wu y col., 2004).

2.6.1.2. N-Acetilcisteína

La N-Acetilcisteína (NAC) es un potente antioxidante tiólico que, además de ser precursor del GSH, mantiene o incluso aumenta los niveles intracelulares del mismo. Su estructura química es la siguiente (Fig. 9):

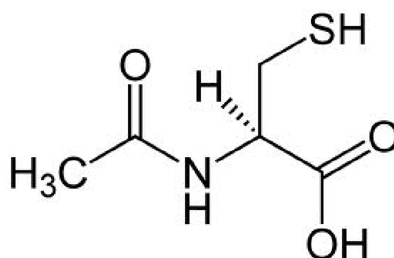


Figura 9. Estructura química de la NAC

La NAC colabora en procesos fisiológicos tales como (Fig. 10):

- Precursor de la síntesis de GSH, así como estimulador de las enzimas citosólicas involucradas en la regeneración del mismo.
- Puede actuar por reacción directa mediante su grupo tiol en la reducción de especies reactivas del oxígeno.
- Puede prevenir la muerte celular programada en células neuronales.

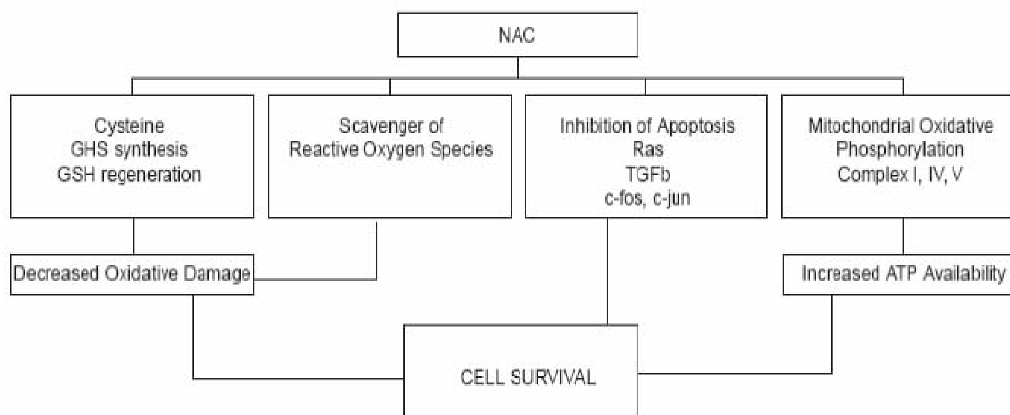


Figura 10. Actividad terapéutica de la NAC. Tomada de Banaclocha (2001).

La NAC protege a la célula de diferentes ERO como el ácido hipocloroso, radicales hidroxilo (HO^-) y H_2O_2 (Aruoma y col., 1989). Sin embargo, existen algunas controversias sobre las propiedades protectoras de la NAC, ya que varios investigadores han demostrado que presenta efectos tóxicos (Khawli y Reid, 1994). Nuestro grupo de investigación ha demostrado que la NAC ejerce un efecto protector frente al estrés oxidativo y los daños histopatológicos producidos por las cianotoxinas MCs en peces, y que la producción de efectos tóxicos depende de la dosis de NAC que se administre (Puerto y col., 2009a, 2010a), exhibiendo NAC una cierta toxicidad *per se*, a las dosis más elevadas ensayadas.

2.6.1.3. Otras sustancias antioxidantes

Además de GSH y NAC, existen otras sustancias antioxidantes como son la vitamina E, selenio y sulforanos.

La **vitamina E** se encuentra en muchos alimentos de origen vegetal, sobre todo en los de hoja verde, semillas, germen de trigo y levadura de cerveza, aunque también puede encontrarse en alimentos de origen animal, como la yema de huevo. Este compuesto, junto con su análogo sintético Trolox, podrían ser considerados aptos en la prevención y el tratamiento de animales expuestos a cianotoxinas (Gehringer y col., 2003; Prieto y col., 2008, 2009). Gehringer y col. (2003) demostraron que la vitamina E ejercía un efecto protector en hígado de ratones expuestos a extractos de MC-LR. En estudios realizados en nuestro laboratorio sobre tilapias, se demostró que la vitamina E (700 mg/kg de dieta) tenía efectos protectores, en particular en relación con la LPO y la actividad CAT (Prieto y col., 2008). En este mismo contexto, también se investigó la eficacia del Trolox (análogo de la vitamina E) en el daño oxidativo de tilapias con respecto al tiempo, viéndose que ejercía un efecto protector con respecto a los parámetros de LPO, oxidación de proteínas, antioxidantes enzimáticos y no enzimáticos, y daños histopatológicos (Prieto y col., 2009).

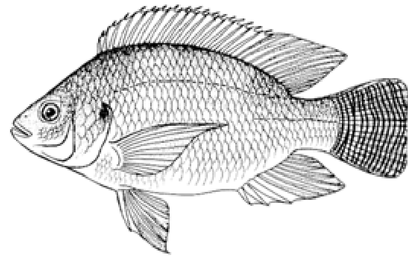
También es importante destacar como sustancia antioxidante el **selenio** (Se), el cual es un oligoelemento que se encuentra de forma natural en la tierra, agua y algunos alimentos como carnes, pescados, cereales y algunos vegetales. Es esencial ya que interviene en varias vías metabólicas formando parte del sistema enzimático de GPx, ampliamente distribuida en todos los tejidos. Estudios realizados en nuestro laboratorio comprobaron la influencia del Se en el estrés oxidativo y en las lesiones histopatológicas en tejidos de peces intoxicados con MCs. Los resultados mostraron protección dosis dependiente, viéndose mejorados diferentes parámetros de estrés oxidativo a diferentes dosis de Se. También se revierten los cambios histopatológicos inducidos por MCs en diferentes órganos (Atencio y col., 2009).

Recientemente se ha demostrado que el **sulforafano** (SFN) protege del daño inducido por la MC-LR en células HepG2, BRL-3A y células NIH 3 T3 (Gan y col., 2010). Este compuesto es un isotiocianato natural que procede de crucíferas como los brotes de brócoli. Se cree que el principal mecanismo por el cual el SFN protege a las células es a través de un factor de transcripción de GSH, elevando los niveles intracelulares de éste (Dinkova-Kostova y col., 2002). Investigaciones realizadas *in vitro* con MCs indican que el pretratamiento de las células con 10 mM de SFN durante

12 horas protege de manera significativa a las células frente al daño oxidativo (Gan y col., 2010).

Dado que los estudios con CYN son más recientes que los de MCs, aún no se ha probado el efecto protector de los antioxidantes en la intoxicación con esta cianotoxina, puesto que tampoco está completamente dilucidado el papel del estrés oxidativo en la toxicidad de dicha toxina.

3. JUSTIFICACIÓN Y OBJETIVOS / SIGNIFICANCE AND PURPOSES



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. Además, hay que tener en cuenta que presenta una distribución ubicua y que se están encontrando concentraciones ambientales capaces de producir daños tanto en el hombre como en los animales. Esta falta de información es particularmente destacable en peces, a pesar de compartir hábitat y por tanto tener un alto grado de probabilidad de exposición. Igualmente, los estudios que hayan abordado la toxicidad de CYN en modelos experimentales piscícolas *in vitro* son escasos, por lo que se planteó el estudio de su acción tóxica y la producción de estrés oxidativo en la línea celular PLHC-1 del ciprínido *Poeciliopsis lucida*.

Por otro lado, los estudios en vegetales son asimismo prácticamente inexistentes, por lo que se consideró necesario el abordaje 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 riesgo para la salud humana.

Adicionalmente, y desde el punto de vista de la seguridad alimentaria, los estudios de las consecuencias de la ingesta de CYN a través de la cadena trófica, y posterior repercusión en la salud humana debido a las consecuencias derivadas del consumo de la toxina, son escasos. Teniendo en cuenta la creciente atención y preocupación que este tema está recibiendo en la actualidad, consideramos necesario investigar los efectos que CYN es potencialmente capaz de inducir en el aparato digestivo, ya que es la principal vía de entrada, usando como modelo experimental la línea celular Caco-2, procedente de un adenocarcinoma de colon humano. Así mismo, también se considera al endotelio vascular como una posible diana para la CYN, ya que en el proceso de distribución de ésta a través de la sangre al resto de los tejidos entra en contacto con el endotelio. Por ello se ha seleccionado la línea celular de endotelio vascular de cordón umbilical, HUVEC.

Otras cuestiones que se abordan en esta Tesis consisten en investigar *in vivo* las modificaciones inducidas por CYN en las actividades de diversas enzimas implicadas en el estrés oxidativo, a nivel molecular y bioquímico, así como estudios

histopatológicos, para esclarecer, de esta forma, el impacto de esta cianotoxina en peces, considerando distintas vías y tiempos de sacrificio. También se ha considerado de interés probar la efectividad de la N-Acetilcisteína como protector de las lesiones inducidas por CYN y de esta forma confirmar la naturaleza oxidativa del daño.

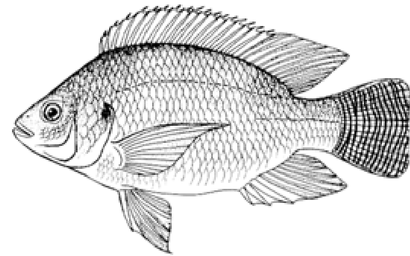
Por todo ello, los objetivos propuestos en la presente Tesis fueron:

1. Estudiar la citotoxicidad producida por CYN empleando como modelos *in vitro* la línea celular Caco-2, derivada de un adenocarcinoma de colon humano, y la línea celular HUVEC, derivada de endotelio vascular, así como las diferentes respuestas de estrés oxidativo y los cambios morfológicos que pueda producir la CYN, valorando, de esta forma, su idoneidad como método rápido de detección de riesgos para el consumidor.
2. Comprobar la producción de citotoxicidad basal y de estrés oxidativo en la línea celular hepática de peces *Poeciliopsis lucida,* PLHC-1, por exposición a CYN.
3. Investigar la inducción de estrés oxidativo *in vivo* como mecanismo de toxicidad de CYN en tilapias (*Oreochromis niloticus*) expuestas a CYN pura, determinando para ello los niveles de peroxidación lipídica y la actividad y expresión génica y proteica de enzimas antioxidantes, evaluándose además la influencia de la vía de exposición (oral e intraperitoneal) y el tiempo de sacrificio (24 horas y 5 días). Del mismo modo, determinar los posibles cambios histopatológicos que se produzcan bajo las mismas condiciones.
4. Analizar *in vivo* en tilapias (*Oreochromis niloticus*) expuestas por vía oral tanto a células de cianobacterias productoras de CYN como a CYN pura, el efecto protector de diferentes dosis de N-acetilcisteína para su potencial aplicación como suplemento en la dieta, con la finalidad de revertir los cambios inducidos sobre el estrés oxidativo y a nivel histopatológico.
5. Investigar la inducción de estrés oxidativo *in vivo* en plantas (*Solanum lycopersicum*) como mecanismo de acción tóxica de la CYN mediante la determinación de la expresión proteica de diferentes enzimas involucradas en este mecanismo a través de técnicas de proteómica.

Esta Tesis Doctoral está estructurada como una recopilación de diversos artículos de investigación, resultantes de las investigaciones científicas llevadas a cabo. 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). Los estudios histopatológicos se han realizado en el Área de Toxicología de la Universidad de Córdoba, bajo la dirección de la Dra. Rosario Moyano. Además, se integran las investigaciones llevadas a cabo recientemente 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 y las conclusiones se redactan tanto en castellano como en inglés para optar a la mención de "Doctorado Europeo".

4. RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION



CAPÍTULO 1 / CHAPTER 1

Daniel Gutiérrez-Praena, Silvia Pichardo, Ángeles Jos, F. Javier Moreno, Ana M. Cameán

BIOCHEMICAL AND PATHOLOGICAL TOXIC EFFECTS INDUCED BY PURE CYLINDROSPERMOPSIN ON THE HUMAN CELL LINE CACO-2

Enviado a *Water Research*, **2011.**

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Manuscript Number: WR18263R1

Title: Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2

Article Type: Special Issue: Cyanobacteria

Keywords: cylindrospermopsin; cytotoxicity; morphology; oxidative stress; Caco-2 cells

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Order of Authors: Daniel Gutierrez-Praena, PhD student; Silvia Pichardo; Angeles Jos, PhD; F. Javier Moreno, PhD; Ana M Camean, PhD

Abstract: Cylindrospermopsin (CYN), a cyanotoxin produced by several freshwater cyanobacteria, causes human intoxications and animal mortalities. The present study focuses on the cytotoxic effects of CYN on Caco-2 cells at 24 and 48 hours. The basal cytotoxicity endpoints studied were total protein content (TP), neutral red uptake (NR) and reduction of the tetrazolium salt (MTS). The effect of non-cytotoxic concentrations of CYN on the generation of intracellular reactive oxygen species (ROS), γ-glutamylcysteine synthetase (GCS) activity and glutathione (GSH) content was also studied and the morphological alterations in the Caco-2 cells subsequent to CYN exposure were recorded.
The most sensitive endpoint—the reduction of MTS—showed that the viability of Caco-2 cells after exposure to the highest concentration assayed (40 µg/mL CYN) was reduced by about 90%. Intracellular ROS production increased only when exposed to a concentration of 1.25 µg/mL CYN, while GSH content and GCS activity increased when exposed to 2.5 µg/mL CYN. The main insights provided by the present study are the ultrastructural alterations, which reveal lipid degeneration, mitochondrial damage and nucleolar segregation with altered nuclei. Therefore, it has been demonstrated that CYN can induce toxic effects in Caco-2 cells in a time-concentration dependent manner. Moreover, unlike the cytotoxic and biochemical alterations, which were only evident at higher concentrations, morphological damage was noticeable even at the lowest concentration used.

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13th October, 2011

Dear Editor,

We are very grateful for giving us the opportunity of sending a revised version of our manuscript entitled **"Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2"** for its publication in "Water Research" in the special issue on "Cyanobacteria".

We have rewritten the paper following all the comments made by the reviewers. Moreover, the English writing has been revised by an English speaker expert in research articles (John Bates from the Universitat Rovira i Virgili, john.bates@urv.net).

I am at your disposal for any questions you may have.

Sincerely,

Silvia Pichardo

Revision Notes
Click here to download Revision Notes: Comments to reviewers.doc

COMMENTS FROM THE EDITOR AND/OR REVIEWERS

Major revision is required for this manuscript to improve the quality of the writing. Particular attention should be paid to the structure of the discussion to ensure it is clear, concise and focussed. The discussion should also emphasise the novel aspects of this work compared with previous research. After major revision the manuscript will be re-considered for publication in the special issue.

Comments: Thank you very much for giving us the opportunity of sending a revised version of our manuscript. We have rewritten the paper in order to emphasize the novel findings observed in our research concerning the ultrastructural changes. The grammar as well as the English writing has been revised by an English speaker expert in research articles (John Bates from the Universitat Rovira i Virgili, john.bates@urv.net). Moreover, the discussion section has been shortened 92 lines instead of 155 lines, and it has been rewritten in order to focus on the data obtained in this work.

Reviewer #1: Water Research Manuscript No: WR18263
Title: Biochemical and pathalogical toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2. The authors describe the toxic effects of Cylindrospermopsin on the human cell line Caco-2. The paper is well written in most parts and adequately references the literature on cylindrospermospin.
A lot of work has been carried out investigating cylindrospermopsin toxicity in various cell lines, including Caco-2 cells lines. This is referenced by the authors. One concern about the paper is that the cytotoxicity endpoints that are used in the paper (MTS, ROS, GSH), have all been carried out previously in mammalian cells and have not generated new information about cylindrospermopsin. The authors state that "despite that use of Caco-2 cells in toxicity studies, no morphological examination has been performed". The authors are correct in that morphological studies using electron microscopy in a CYN treated cell line has not previously been published. However a number of CYN toxicity papers do report on morphological observations via light microscopy. If the ultrastructral changes following CYN exposure are the novel aspect of the paper, then emphasis should be placed on this and what is expected to be able to be achieved by carrying out this work. As currently written the paper focuses on the cytotoxicity work in the Caco-2 cells, similar to other papers already published. The electron microscopy is added on at the end. The paper would be greatly enhanced by reworking the content and emphasis of the paper.

Comments: Thank you very much for your thorough revision of our manuscript, we think that its quality has been improved thanks to your corrections and suggestions.

You are right about the fact that some authors have already carried out cytotoxicity studies in mammalian cells exposed to cylindrospermopsin. However, most of these studies have used CYN from cyanobacterial cultures to assess toxicity instead of pure toxin. Provided purified and non-purified CYN have been reported to have different toxic effects (Falconer et al. 1999; Kinnear, 2010), it is important to study the toxicity induced by them individually. This has been introduced in the text (page 6, line 122).

In addition, some authors have already studied the inhibition of GSH as one of the toxic mechanisms of CYN (Runnegar et al. 1994, 1995). But only Humpage et al (2005) have studied some oxidative stress endpoints, such as the lipid peroxidation marker,

malondialdehyde (MDA), glutathione reductase, levels of GSH, etc.; although they were not able to establish a relationship between ROS formation and cytotoxicity.

Considering all these matters, we find that the cytotoxicity and oxidative stress studies performed in our study are of interest. However, we agree with you that the novel findings observed in our research should be more focussed. Therefore, the paper has been rewritten in order to emphasize the ultrastructural changes observed, explaining the advantage of this morphological study. Particularly, the ultrastructural changes have been introduced in the aim of the work and have been profusely discussed. We have maintained the order of the results, because the cytotoxicity studies are needed to establish the exposure concentrations used in the morphological studies.

Specific comments:
Introduction
Page 5, line 78. Reword the sentence " this means a higher risk to the population since exposure to CYN is more likely to happen...'
Suggest that the sentence should be qualified to indicate that " the presence of free CYN in the drinking water poses a risk to the human health if not adequately removed at the treatment plant" Note that CYN degradation occurs upon the addition of chlorine.
Comments: Thanks for your comment. The sentence has been reworded to avoid misunderstanding.

Page 5, Line 83, remove "In fact".
Comments: Thanks, it has been removed.

Page 5, Line 85, Full stop after CYN. Then "the toxin has been strongly implicated...."
Comments: Thanks, full stop has been added.

Page 5, line 87, add the year (1979) for the Palm Island Mystery disease.
Comments: It has been added.

Page 7, line 128. The authors state that "However, morphological studies in permanent cell lines exposed to CYN are very scarce".
A number of the papers on CYN toxicity, including those on micronuclei induction (Bazin et al, 2010; Humpage et al, 2000), and the Newmann et al (2007) have reported on morphological characteristics of following CYN exposure although not necessarily provided a lot of figures.
The authors do not indicate why looking at cell morphology would improve our understanding of CYN toxicology apart to say that the morphological studies have not been carried out in Caco-2 cells before. The paper would be greatly strengthened if the study was investigating a hypothesis.
Comments: The sentence above mentioned has been erased to avoid misunderstandings.

In addition, we have introduced here the following paragraph: “most of these studies have used CYN from cyanobacterial cultures CYN to assess toxicity instead of pure toxin. Provided purified and non-purified CYN have been reported to have different toxic effects (Falconer et al. 1999; Kinnear, 2010), it is important to study the toxicity induced by them individually.”

Moreover, following the reviewer's suggestion, we have also explained the aim of our work concerning to the morphological studies performed. As it has been evidenced in our work, the morphological study is more sensitive than the cytotoxicity and oxidative stress endpoints. Therefore, alterations can be observed at lower concentration and time. Moreover, the ultrastructural visualization allows exploring the intracellular targets.

Page 9, line 194. Replace "independence with independent
Comments: It has been replaced.

Discussion
This section is too long in its current form. The discussion section needs to be more focussed on new outcomes generated by the paper.
Comments: The discussion concerning the cytotoxicity study has been severely shortened to 32 lines instead of 77. Moreover, we have rewritten some parts of this section in order to emphasize the ultrastructural changes observed (page 17, line 417).

P16, Line 373. Change the wording of "CYN is a cytotoxic toxin as it has been proved...."
Toxin after cytotoxic is a bit redundant. Suggestions " general cytotoxin" or non-specific toxin"
Remove "proved" suggest 'as has been shown in several different human cell lines"
Comments: We have made all the changes suggested by the reviewer. Thanks again for your comment.

P16 line, 387. The authors state that "Moreover, previous studies carried out in our laboratory in caco-2 cells exposed to different congeners of microcystins also confirmed this finding' when referring to the fact that effects at 48hrs were seen at lower concs than could be seen at 24hrs. This statement does not seem relevant since both CYN and MCYST are different toxins.
Comments: This sentence has been removed following your suggestion, as well as all the comparisons with previous studies carried out with microcystins performed thought the text.

Reviewer #2: The topic and results of this study are very interesting, and shed new light on the toxic mode of action of cylindrospermopsin. Unfortunately, the manuscript is of poor quality. There are numerous grammatical errors, several paragraphs in the introduction and discussion are repetitive, and the discussion is not very focused on the data, or concise.

The language of the entire manuscript needs to be edited thoroughly, and the discussion needs to be focused.

Comments: Thank you very much for your revision of the manuscript. English writing has been revised by an English speaker expert in research articles (John Bates from the Universitat Rovira i Virgili, john.bates@urv.net).

Moreover, following your comments and those from the other reviewer, the discussion section has been shortened and rewritten in order to focus on the data obtained in this work. The discussion of the results obtained in the cytotoxic and oxidative stress studies

have been shortened to 32 lines instead of 77 lines. In addition, some paragraphs have been erased in order to avoid repetitions, for example, in the introduction, page 7 lines 128-135, which is also mentioned in the discussion section.

Other changes:

* Conclusion section has been shortened.
* In the reference list we have erased Puerto et al. 2010b (therefore there is only one Puerto et al. 2010), and we have introduced Kinnear 2010.

*Manuscript
Click here to download Manuscript: Gutierrez-Praena_Water Research_revised version without changes.doc Click here to view linked References

1 **Biochemical and pathological toxic effects induced by the cyanotoxin**
2 **Cylindrospermopsin on the human cell line Caco-2**

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4 Cameán[1]
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20

21 **Abbreviations**

22 PBS: phostate buffered saline

23 CYN: cylindrospermopsin

24 DAD: Array Detector

25 DCF: dichlorofluorescein

26 DCFH-DA: 2’,7’-dichlorofluorescein diacetate

27 DeoxyCYN: deoxycylindrospermopsin

28 EC_{50}: mean effective concentration

29 GCS: γ-glutamylcysteine synthetase

30 GSH: glutathione

31 HPLC: high performance liquid chromatography

32 IC_{50}: half maximal inhibitory concentration

33 mBCl: monochlorobimane

34 MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

35 tetrazolium salt)

36 NR: neutral red

37 ROS: reactive oxygen species

38 SD: standard deviation

39 TP: total protein

40

41 **Abstract**

42 Cylindrospermopsin (CYN), a cyanotoxin produced by several freshwater
43 cyanobacteria, causes human intoxications and animal mortalities. The present study
44 focuses on the cytotoxic effects of CYN on Caco-2 cells at 24 and 48 hours. The basal
45 cytotoxicity endpoints studied were total protein content (TP), neutral red uptake (NR)
46 and reduction of the tetrazolium salt (MTS). The effect of non-cytotoxic concentrations
47 of CYN on the generation of intracellular reactive oxygen species (ROS), γ-
48 glutamylcysteine synthetase (GCS) activity and glutathione (GSH) content was also
49 studied and the morphological alterations in the Caco-2 cells subsequent to CYN
50 exposure were recorded.
51 The most sensitive endpoint—the reduction of MTS—showed that the viability of Caco-
52 2 cells after exposure to the highest concentration assayed (40 µg/mL CYN) was
53 reduced by about 90%. Intracellular ROS production increased only when exposed to a
54 concentration of 1.25 µg/mL CYN, while GSH content and GCS activity increased when
55 exposed to 2.5 µg/mL CYN. The main insights provided by the present study are the
56 ultrastructural alterations, which reveal lipid degeneration, mitochondrial damage and
57 nucleolar segregation with altered nuclei. Therefore, it has been demonstrated that
58 CYN can induce toxic effects in Caco-2 cells in a time-concentration dependent
59 manner. Moreover, unlike the cytotoxic and biochemical alterations, which were only
60 evident at higher concentrations, morphological damage was noticeable even at the
61 lowest concentration used.

62

63 **Keywords**: cylindrospermopsin; cytotoxicity; morphology; oxidative stress; Caco-2
64 cells.

65

1. Introduction

Cylindrospermopsin (CYN) is a cyanotoxin produced by such cyanobacterial species as *Cylindrospermopsis raciborskii* (Ohtani et al. 1992), *Umezakia natans* (Terao et al. 1994), *Aphanizomenon ovalisporum* (Banker et al. 1997), *Raphidiopsis curvata* (Li et al. 2001), *Anabaena bergii*, *Aphanizomenon flos-aquae*, and *Anabaena lapponica* (Bazin et al., 2010). It is a tricyclic alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al. 1992). It is zwitterionic, highly water soluble, and stable to extreme temperatures and pH (Chiswell et al. 1999). Other cyanobacterial toxins, such as microcystins, are contained largely within the cell; however, extracellular CYN can be as much as 90% of total CYN (Chiswell et al. 1999; Rücker et al. 2007). This means a higher risk because people are more likely to be exposed to CYN if it is not appropriately removed at the treatment plant.

This cyanotoxin has appeared in a number of important drinking-water sources throughout the world. Consequently, it is increasingly being recognized as a potential threat to drinking-water safety (Kuiper-Goodman et al. 1999). The major route of human exposure to CYN is oral, mainly through drinking water contaminated with CYN. The presence of CYN in water has been strongly implicated in human poisoning in Australia and Brazil (Hawkins et al. 1985; Carmichael et al. 2001). In North Queensland, Australia, in 1979 a human poisoning incident resulted in the hospitalization of a number of individuals who developed symptoms of hepatic and renal injury (Hawkins et al. 1985). CYN was also implicated, in combination with microcystins, in the Brazilian dialysis clinic tragedy in 1996 (Caruaru, Brazil) involving the death of over 50 patients (Carmichael et al. 2001).

Inhibition of protein synthesis is a well-known mode of action of CYN that has been observed in both *in vivo* and *in vitro* systems (e.g. cultured cells) in which CYN inhibits

93 messenger RNA translation (Terao et al. 1994; Froscio et al. 2001, 2003). Inhibition of
94 protein synthesis is considered to be an early indicator of exposure to CYN which
95 occurs at subtoxic concentrations and well before the onset of toxicity at higher
96 concentrations (Froscio et al. 2003). Moreover, disruption of cell growth or cytotoxicity
97 has also been reported in a range of different mammalian cell lines including hepatic-,
98 renal-, gastric- and intestinal-derived cells (Fessard and Bernard, 2003; Bain et al.
99 2007; Neumann et al. 2007; Froscio et al. 2009a,b). CYN been shown to be able to
100 enter a variety of cell types and cause toxic effects (Froscio et al. 2009a). It has also
101 been shown that cytotoxicity is dependent on a cytochrome p-450 metabolism and that
102 CYN exposure can lead to genotoxic effects (Runnegar et al. 1994; Humpage et al.
103 2000; Froscio et al. 2001, 2003).
104 As has been mentioned above, various *in vitro* models have been used to show that
105 the toxin produces multiple cytopathic effects. It is clear that cell culture-based toxicity
106 tests are of interest, because they can screen samples for a biochemical response
107 characteristic of a particular toxin class while retaining the ability to detect more general
108 cytotoxicity endpoints (Froscio et al. 2009a). In this regard, the Caco-2 cell line is a
109 commonly used enterocytic model, established from a human colon carcinoma. It
110 undergoes a process of spontaneous differentiation in culture that leads to the
111 formation of a monolayer of cells which express several morphological and functional
112 characteristics of the mature enterocyte (Sambuy et al. 2005). Several experiments
113 have already been conducted in this cell line and have shown that it is an accurate
114 experimental model for assessing the toxic effects of CYN (Fastner et al. 2003;
115 Neumann et al. 2007; Froscio et al. 2009a; Bazin et al. 2010). Fastner et al. (2003)
116 used the human cell line Caco-2, together with HepG2 cells, to assess the toxicity of
117 seven isolates of *Cylindrospermopsin raciborskii* non-containing CYN. Caco-2 cells
118 have also been used, along with four other cell lines, to assess the toxicity of the
119 cyanobacterial metabolite deoxycylindrospermopsin (deoxyCYN), and it has been

120 demonstrated that Caco-2 are the most sensitive cell line to deoxyCYN and CYN
121 (Neumann et al. 2007). Similarly, Caco-2 cells have also been used as one of the
122 seven cell lines to perform a cytotoxicity screening of CYN, and proved to be the least
123 sensitive model (Froscio et al. 2009a). Bazin et al (2010) demonstrated that CYN is
124 genotoxic not only in the Caco-2 cell line used as a human enterocytic model, but also
125 in HepaRG cells, which indicates that metabolism is involved. However, no
126 ultrastructural studies have been conducted so far in permanent cell lines exposed to
127 CYN. In addition, most of the studies mentioned above used CYN from cyanobacterial
128 cultures to assess toxicity instead of pure toxin. Since purified and non-purified CYN
129 have been reported to have different toxic effects (Falconer et al. 1999; Kinnear, 2010),
130 it is important to study the toxicity they induce individually. However, no ultrastructural
131 study has been conducted so far in permanent cell lines exposed to CYN. In addition,
132 most of these studies have used CYN from cyanobacterial cultures CYN to assess
133 toxicity instead of pure toxin. Provided purified and non-purified CYN have been
134 reported to have different toxic effects (Falconer et al. 1999; Kinnear, 2010), it is
135 important to study the toxicity induced by them individually.
136
137 Although tissue damage and alteration have been reported in cell models, studies on
138 the ultrastructural alterations induced by CYN are still limited. Moreover, although
139 Caco-2 cells have been used in toxicity studies, no morphological examinations have
140 been performed after CYN exposure. Likewise, in spite of extensive recent research to
141 elucidate the toxic mechanism of CYN, much still remains to be clarified with respect to
142 the toxicity of this cyanotoxin.
143
144 In the present paper, we aimed to study the ultrastructural morphological alteration in
145 the permanent human cell line, Caco-2 resulting from exposure to CYN together with
146 the cytotoxic and oxidative effects. First, we intended to confirm that short-term

147 exposure to pure CYN might induce a cytotoxic response by studying the alteration of
148 three endpoints: total protein content, neutral red uptake and metabolization of MTS (3-
149 (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
150 salt). Second, we studied the alteration in the oxidative status and the involvement of
151 glutathione in the toxic mechanism of CYN. Finally, we used light and electron
152 microscopy to analyze the morphological alterations induced by the cyanotoxin. The
153 ultrastructural alterations would make it possible to explore the intracellular targets of
154 the toxin. Moreover, due to its high sensitivity, the morphological study can be used as
155 an early indicator of the damage induced in the cell.
156
157 **2. Materials and Methods**
158 *2.1. Supplies and Chemicals*
159 Culture medium, fetal bovine serum and cell culture reagents were obtained from
160 BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-
161 Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was
162 obtained from BioRad (Spain).
163
164 *2.2. Model systems*
165 Caco-2 cell line derived from human colon carcinoma (ATCC® HTB-37) was
166 maintained at 37º C in an atmosphere containing 5% CO_2 at 95% relative humidity
167 (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's medium
168 supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 50 µg/ml
169 gentamicin, 1.25 µg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were
170 grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25%
171 trypsin. They were counted in an improved Neubauer haemocytometer and viability
172 was determined by exclusion of Trypan Blue. Caco-2 cells were plated at density of 7.5
173 x 10^4 cells/ml to perform the experiments.

174

175 *2.3. Toxin test solutions*

176 The cyanotoxin cylindrospermopsin standard (purity > 95%) was supplied by Alexis
177 Corporation (Lausen, Switzerland). Cyanotoxin content was confirmed by a high
178 performance liquid chromatography (HPLC) system (Varian 9012) equipped with a
179 Varian ProStar 330 Diode Array Detector (DAD) (Varian Technologies, Oxford, UK).
180 Stock solution of CYN (400 µg/mL) was prepared in 250 µL of sterilized milliQ water,
181 considering that CYN is a highly water-soluble molecule (Sivonen and Jones 1999),
182 and maintained at 4°C.

183

184 *2.4. Cytotoxicity assay*

185 From the stock solution, serial dilutions in medium were prepared 0.3, 0.6, 1.2, 2.5, 5,
186 10, 20 and 40 µg/mL CYN. Culture medium without toxin was used as control group.
187 After replacing the previous medium, the exposure solutions were added to the
188 systems, and incubated at 37ºC for 24 and 48 hours. The basal cytotoxicity endpoints
189 were protein content (TP), supravital dye neutral red cellular uptake (NR), and
190 tetrazolium salt reduction (MTS).

191

192 Total protein content (TP) is a very useful endpoint to assess cytotoxicity, since it gives
193 data about cell damage independent of the toxic mechanism involved (Pichardo et al.
194 2005). TP was quantified in situ, according to the procedure given by Bradford (1976),
195 using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which
196 exposure originally took place, in order to determine the total cell number present in the
197 culture. The culture medium was replaced by 200 µL NaOH to dissolve the proteins
198 and after 2h incubation at 37ºC, 180 µL was replaced by the same volume of a 22%
199 Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature,
200 absorbance was read at 620 nm.

201

202 NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up
203 by viable lysosomes. This assay was performed according to Borenfreund and Puerner
204 (1984). Briefly, neutral red (NR) in medium is absorbed and concentrated in lysosomes
205 of cells. NR uptake is proportional to the concentration of the NR solution and the
206 numbers of viable liver cells. The NR can be extracted from lysosomes for quantitative
207 measurement of cells viability and cytotoxicity of xenobiotics (Zhang et al. 1990).
208 Culture medium was replaced by 100 µL modified medium without serum containing 10
209 mg/mL NR. The microplate with the NR-containing medium was returned to the
210 incubator for another 3 h to allow the uptake of NR into the lysosomes of viable cells.
211 Thereafter, the medium was removed and cells were fixed for 1 min with a
212 formaldehyde-$CaCl_2$ solution. By adding 0.2 mL of acetic acid-ethanol solution to the
213 wells, the NR absorbed by the cells was extracted, brought into solution and quantified
214 at 540 nm.

215

216 MTS reduction is carried out by dehydrogenases enzymes present in mitochondria.
217 This endpoint a good marker of the damage induced in this organelle. MTS reduction
218 was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-
219 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
220 salt) tetrazolium compound added to the medium is bioreduced by cells into a coloured
221 formazan product soluble in culture medium and is directly measured
222 spectrophotometrically at 490 nm after 3 hours of incubation in the dark.

223

224 *2.5. Oxidative stress assays*

225 Concentrations used in these assays were calculated based on the cytotoxicity study
226 previously performed. The most sensitive endpoint was MTS reduction; therefore, the
227 mean effective concentration (EC_{50}) value obtained for this endpoint at 24 h, 2.5 µg/mL

228 CYN, was chosen as the higher exposure concentration for the oxidative stress studies
229 in Caco-2 cell line, along with the fractions $EC_{50}/2$ and $EC_{50}/4$, being the toxin
230 concentrations used 0, 0.625, 1.25 and 2.5 µg/mL CYN.
231 After replacing the previous medium, the exposure solutions were added to the cells,
232 and incubated at 37ºC for 24 hours. Culture medium without toxin was used as control
233 group. The oxidative stress endpoints measured were reactive oxidative species (ROS)
234 content, glutathione (GSH) levels, and γ-glutamylcysteine synthetase (GCS) activity.
235
236 The production of ROS was assessed in 96 well microplates using the
237 dichlorofluorescein (DCF) assay. The probe 2',7'-dichlorofluorescein diacetate (DCFH-
238 DA) (Molecular probes, Invitrogen) readily diffuses through the cell membrane and is
239 hydrolyzed by intracellular esterases to non-fluorescent compound (DCFH), which is
240 rapidly oxidized in the presence of ROS to the highly fluorescent DCF. Specifically,
241 cells were incubated with 200 µl 20 µM DCFH-DA in culture medium at 37ºC for 30
242 min, and then washed with phostate buffered saline (PBS) and resuspended in 200 µl
243 of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was
244 monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm.
245 ROS production was expressed as fluorescence arbitrary units (Puerto et al. 2010).
246
247 GSH content in cells was evaluated by reaction with the fluorescent probe
248 monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos et al. 2009). This
249 molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme
250 GST. After the cell exposure to CYN, medium was discarded and cells were incubated
251 at 37°C for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with
252 PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the
253 excitation/emission wavelengths of 380/460. Results were expressed as arbitrary units.
254

255 In order to measure γ-glutamylcysteine synthetase (EC 6.3.2.2) activity (GCS), one of
256 the enzymes involved in GSH synthesis, cells were seeded in a 5 mL plate and
257 incubated at 37°C for 48h. Afterwards, they were exposed to different concentrations of
258 CYN and incubated at 37ºC for 24h. After exposure, cells were collected and
259 resuspended in PBS, 20 µL of sample was added to the mixture reaction, and
260 absorbance was read at 340 nm (Seelig and Meister, 1985). Results were expressed
261 as nkat/mg protein.

262

263 *2.6. Morphology*

264 Cells were exposed to two different concentrations of CYN, 0.625 and 2.5 µg/mL,
265 during 24 and 48 hours of exposure. Afterwards, cultured cells were fixed directly in the
266 culture flasks in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at
267 4°C. They were all postfixed in 1% osmium tetroxide for 60 min at 4°C. Samples were
268 dehydrated in ethanol at progressively higher concentrations and embedded in Epon
269 (epoxy embedding medium). Toluidine blue-stained semi-thin sections (0.5 µm thick)
270 used as controls were viewed in a Leitz (Aristoplan) light microscope. Thin sections
271 (60-80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with
272 uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron
273 microscope.

274

275 *2.7. Calculations and statistical analysis*

276 All experiments were performed three times and in duplicate per concentration. Data
277 for the concentration-dependent cytotoxicity relationships of all experiments were
278 presented as the arithmetic mean percentage ± standard deviation (SD) in relation to
279 control. Statistical analysis was carried out using analysis of variance (ANOVA),
280 followed by Dunnett's multiple comparison tests. Differences were considered
281 significant from $P<0.05$.

282 EC_{50} values (mean effective concentration, concentration that modified each biomarker
283 by 50%, positive or negative, in comparison with appropriate untreated controls) were
284 derived by linear regression in the concentration-response curves.
285

286 **3. Results**

287 *3.1. Results of the cytotoxicity assays*

288 After 24 h exposure of Caco-2 cells to lower concentrations of CYN, no significant
289 changes were observed in total protein content (Fig 1a). However, at higher
290 concentrations (40 µg/mL) protein content was significantly reduced by up to 50%.
291 Exposure of 48 h to CYN resulted in a concentration-dependent reduction in protein
292 content, which was significant above a concentration of 2.5 µg/mL CYN. The EC_{50}
293 values, 36.5±2.1 and 2.0±0.5µg/ml for 24h and 48h of exposure, respectively, show
294 that the toxic effects were more evident in the longer contact.
295

296 Similarly, when Caco-2 cells were exposed to CYN for 24 h, no significant alterations
297 were recorded in neutral red uptake at the lowest concentration assayed (0.3 µg/mL).
298 At the highest concentrations, however, decreases of up to 45% were observed. After
299 48 h of exposure, NR uptake was reduced in a concentration-dependent manner, and
300 was significantly different from the control at 5 µg/mL CYN (Fig 1b). Similarly, EC_{50}
301 values for NR uptake varied between 19.0±1.3 µg/ml for 24h and 10.0±1.7 µg/ml for 48
302 h of exposure.
303

304 A progressive concentration-dependent reduction in MTS metabolism was observed at
305 both exposure times, although it was more pronounced after 48 h. All concentrations
306 led to significant changes in MTS reduction compared to the control except for the first
307 concentration at 24 h (Fig 1c). The EC_{50} values also revealed greater damage in the
308 cells after a longer exposure time (2.5±0.4 µg/ml for 24 h and 0.6±0.2 µg/ml for 48 h).

309

310 *3.2. Results of the oxidative stress assays*

311 ROS content was significantly increased only at a concentration of 1.25 μg/mL
312 CYN,(six times higher than the control group). However, at the highest concentration
313 assayed (2.5 μg/mL) no differences with the control group were discernible (Fig 2a).
314 GCS activity was only significantly increased at 2.5 μg/mL (four times higher than the
315 control group) (Fig 2b). Similarly, GSH content was significantly increased when Caco-
316 2 cells were exposed to 2.5 μg/mL (twice as high as the control group) (Fig2c).

317

318 *3.3. Results of the morphology study*

319 *3.3.1. Light microscope observation*

320 In the control group, Caco-2 cells are very heterogeneous in shape and size, with
321 irregular cell borders (Fig 3a,d). The presence of euchromatic nuclei and big nucleoli
322 are indicative of transcriptionally active cells (Fig 3a). In the cell culture,
323 undifferentiated cells coexist with cells that have a certain degree of differentiation.
324 These latter cells show secretory granules and intracytoplasmic vacuoles. In a
325 considerable percentage of multinuclear cells, micronuclei can also be seen.
326 After exposure to 0.625 μg/mL CYN, no significant changes were observed. However,
327 the vacuoles increased in number, and were big and intracellular. Some of them were
328 exocyted, which resulted in irregular cell borders (Fig 3b). Similar effects were
329 observed after exposure to 2.5 μg/mL CYN after both exposure times (Fig 3c,f).

330

331 *3.3.1. Electron microscope observation*

332 When the control group of Caco-2 cells was observed under the electron microscope,
333 their shape and size were highly heterogeneous at both 24 and 48 hours. In the same
334 culture, cells in different stages of the differentiation process coexisted and secretory
335 granules accumulated in the cytoplasm (Fig 4a). Moreover, cells that were undergoing

336 a degeneration process were shown to contain autophagosomes and
337 heterophagosomes, some of which were discharged outside the cell. Well-developed
338 Golgi apparatuses were also visible.
339
340 After 24 hours of exposure to 0.625µg/mL, Caco-2 cells underwent several alterations
341 with respect to the control. Nucleolar segregation was observed, which indicates
342 alterations in the ribosomal DNA transcription process. In the altered nuclei, the fibrillar
343 component was seen to have split from the granular component (Fig 4b) and
344 micronuclei were present (Fig 4b). The appearance of vacuoles containing lipids
345 showed that lipid degeneration was taking place (Fig 4c) and endosomes were formed
346 by vesicles containing electrodense material (Fig 4d). Mitochondria were also
347 degenerating, covered by endoplasmic reticulum cistern caused by the formation of
348 phagosomes (Fig 4d, insert). After 48 hours of exposure to this low concentration, the
349 damage was similar. Of particular note were alterations in some cellular components
350 such as nucleoli and mitochondria, and organelle-poor cytoplasm.
351
352 When Caco-2 cells were exposed to 2.5 µg/mL CYN for 24 hours, considerable
353 damage was observed. Most of the cells were profusely vacuolated and contained
354 hydrolytic enzymes. Significant alterations were evident in mitochondria, which led to a
355 high number of phagosomes (Fig 4d, insert). Moreover, the Golgi apparatus was
356 disorganized, the endomembrane system displayed alterations, and nucleolar
357 degeneration was evident. Nuclei contained disorganized nucleoli and disorganized
358 chromatin, all of which was more evident after 48 hours of exposure. Damage to nuclei
359 (Fig 5b) and segregated nucleoli (Fig 5c) was also considerable, and the cell cytoplasm
360 contained degraded components (e.g. Golgi apparatus). Mitochondrial injury was
361 greatest in cells exposed to the highest concentration at the longest exposure time

362 assayed. Finally, myelin-like membrane figures and secondary lysosomes were
363 present, appearing as multivesicular bodies and heterophagosomes (Fig 5d).
364

365 **4. Discussion**

366 CYN is a general cytotoxin as has been shown in several human cell lines (Chong et
367 al. 2002, Bain et al. 2007, Froscio et al. 2009a,b, Bazin et al. 2010) and primary
368 cultures (Froscio et al. 2003, Fastner et al. 2003, Young et al. 2008). In Caco-2 cells
369 cytotoxic as well as genotoxic effects have already been reported (Fastner et al. 2003,
370 Neumann et al. 2007, Froscio et al. 2009a, Bazin et al. 2010), but very few studies
371 have focused on morphology and the involvement of oxidative stress as a possible
372 toxic mechanism. The results of the present cytotoxicity study in Caco-2 cells exposed
373 to CYN revealed a concentration- and time-dependent decrease in all the endpoints
374 assayed, which has also been reported by other authors of similar studies performed in
375 different cell lines exposed to CYN (Chong et al. 2002; Bain et al. 2007; Neumann et al.
376 2007; Froscio et al. 2009a). Of all the cytotoxic endpoints studied in the present paper,
377 MTS metabolism assay was the most sensitive. Neumann et al. (2007) compared the
378 toxic effects induced by CYN and deoxyCYN, an analog of CYN, in four different cell
379 lines, and found that Caco-2 cells were among the most sensitive to cyanotoxin
380 exposure according to the MTS assay, even in comparison with the hepatoma cell line
381 HepG2. Bain et al (2007) reported similar reductions but at longer exposure times, up
382 to 72 hours.
383

384 As well as cytotoxic effects, alterations were also detected in the oxidative endpoints.
385 So far the relationship between CYN exposure and oxidative stress induction on
386 human cell lines has not been established. The reduction in ROS content observed in
387 our study could be related to the increase in the GSH content observed at 2.5 µg/mL,
388 since it is well known that GSH is involved in ROS quenching (Pflugmacher et al.

389 1998). The GSH content increases together with GCS activity at 2.5 µg/mL which may
390 be explained by the fact that this enzyme in the synthesis of GSH. Therefore, when
391 Caco-2 cells are exposed to high concentrations of CYN, they try to cope with the
392 assault by increasing the production of GSH in order to scavenge the damage caused
393 by the toxin. In contrast, some reports have confirmed that one of the toxic
394 mechanisms of CYN is the inhibition of GSH synthesis both *in vitro* (Runnegar et al.
395 1995, Gutierrez-Praena et al. 2011) and *in vivo* (Norris et al. 2002). Humpage et al.
396 (2005) stated that the relationship between ROS formation and cytotoxicity was not
397 clear in mouse hepatocytes exposed to CYN. However, the present study shows that
398 ROS enhancement is related to GSH synthesis.
399
400 Morphological alteration described in several tissues in *in vivo* experiments (Terao et
401 al. 1994; Seawright et al. 1999; Falconer et al. 1999; Bernard et al. 2003) have
402 indicated that as well as hepatocytes, enterocytes are also targets for CYN toxicity
403 especially by the oral route, and that exposure to low doses of CYN may alter the
404 intestinal tissue. In fact, the effects of CYN on the intestine have already been studied
405 using *in vivo* experimental models. Congestion and edema of the small intestine were
406 shown in mice orally exposed to CYN, accompanied by slight diarrhea (Seawright et al.
407 1999). Inflammation in the intestines has also been reported in mice intraperitoneally
408 dosed with *C. raciborski* extract (Saker et al. 2003),and studies on *Daphnia magna* fed
409 with cyanobacterial cultures containing and not containing CYN have observed a very
410 advanced stage of intracellular disorganization with lipid droplets in midgut and
411 digestive diverticula (Nogueira et al. 2006). However, although the morphological
412 alterations reported in rodents *in vivo* are considerable, on the alterations in cell lines
413 exposed to CYN are less abundant. Most of the alterations observed in the present
414 study are well correlated with previous morphological studies conducted in several *in*
415 *vitro* experimental models. CYN has induced alterations in the cell structure of Chinese

416 hamster ovary cells (CHO-K1) (Fessard and Bernard 2003; Gacsi et al. 2009).
417 Microscopic examinations have indicated that when the neuroblastoma cell lines BE2
418 and MNA were treated with CYN and deoxyCYN they underwent significant
419 morphological changes, while HepG2 cells at 2.5 µg/mL CYN showed increases in the
420 number of floating cells (Neumann et al. 2007).

421

422 The present study describes for the first time the ultrastructural alterations in the
423 colonic cell line Caco-2 subsequent to CYN exposure.. This description has made it
424 possible to identify such intracellular targets as nuclei, mitochondria and the Golgi
425 apparatus. Our work has shown that the morphological study is more sensitive than the
426 cytotoxicity and oxidative stress studies, and can therefore be used as an early
427 indicator of the damage induced in the cell. The formation of multivesicular bodies and
428 the extensive alteration to mitochondria has resulted in the appearance of numerous
429 phagosomes. Terao et al (1994) described numerous autophagic vacuoles in the
430 cytoplasm of hepatocytes observed after 48 hours of CYN exposure. In the present
431 study, mitochondrial alteration is greater in cells exposed to the highest concentration
432 and for the longest time. This effect was also found in the cytotoxic study performed in
433 the present paper, with MTS metabolism being the most sensitive endpoint.MTS is
434 reduced by mitochondrial dehydrogenases, so this endpoint can provide information
435 about the damage induced in this organelle (Puerto et al. 2009). In addition to the
436 damage induced in the mitochondria, the present study has also shown a considerable
437 vacuolization of the cytoplasm. This vacuolization has been observed by other authors
438 *in vitro* (Gacsi et al. 2009) and in hepatic and renal cells *in vivo* (Seawright et al. 1999,
439 Falconer et al. 1999). Likewise, other authors have also reported the presence of
440 micronuclei. Humpage et al (2000), for example, observed a significant increase in
441 centromere-positive micronuclei in the WIL2-NS lymphoblastoid cell-line exposed to 1-
442 10 µg/mL CYN (Humpage et al., 2000). Bazin et al (2010) also revealed that CYN

443 could alter chromosomes by inducing significant increases in the frequency of
444 micronuclei in both differentiated and undifferentiated human intestinal Caco-2 cells
445 (0.5-2 µg/mL CYN).
446 One of the main alterations induced by CYN observed in this study is the accumulation
447 of fat droplets, which has also been found in the liver of rodents (Terao et al. 1994,
448 Seawright et al. 1999). The increase in ROS recorded at sub-cytotoxic concentrations
449 could be the responsible for this morphological alteration (Terao et al. 1994). Similarly,
450 the impairment observed in our study on nuclear alteration was also described by
451 Maire et al. (2010), who reported morphological cell transformation in SHE cells
452 exposed to CYN with nuclear enlargement. And Terao et al. (1994) reported that the
453 nuclei of the hepatocytes became dense, round and reduced in size. The nucleolar
454 segregation observed could indicate alterations in the ribosomal DNA transcription
455 process, which could be the cause of the lipid degeneration (Cristea and Esposti 2004,
456 Deretic and Klionsky 2008). Therefore, more studies are needed to assess the
457 influence of nuclear damage and/or ROS formation on the appearance of the fat
458 droplets.
459
460 **5. Conclusions**
461 CYN is able to induce toxic effects on the biochemical and physiological level in Caco-2
462 cells in a time-concentration dependent manner, establishing a correlation between the
463 cytotoxic effects observed, the morphological alterations, and the contribution of the
464 oxidative stress in the pathogenicity of this cyanotoxin.
465
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470

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619

620

621 **Figure captions**

622 Figure 1. Protein content, TP (a); Neutral red uptake, NR (b); and reduction of
623 tetrazolium salt, MTS (c) of Caco-2 cells after 24h and 48h of exposure to 0-40 µg/mL
624 CYN. All values are expressed as mean ± s.d. * significantly different from control ($p \leq$
625 0.05).

626 Figure 2. ROS content (a); GCS activity (b); and GSH content (c) in Caco-2 cells after
627 24h of exposure to 0.625, 1.25, and 2.5 µg/mL CYN. All values are expressed as mean
628 ± s.d. * significantly different from control ($p \leq 0.05$).

629 Figure 3. Morphology of Caco-2 cells after 24 h (a, b, c) and 48 h (d, e, f) of treatment
630 observed by light microscopy. Bars= 20 µm. Unexposed control cultures (a, d) and
631 Caco-2 cells exposed to 0.625 µg/mL CYN (b, e) and 2.5 µg/mL CYN (c, f). Secretory
632 granules (stars), intracytoplasmic vacuoles (arrows) and irregular cell borders (circles)
633 are observed in Caco-2 cells.

634 Figure 4. Ultrastructural changes of Caco-2 cells after 24 h of exposure to CYN
635 observed by electron microscopy. Bars (a, d)= 2 µm. Bars (b, c)= 5µm. Bar (d, insert)=
636 1µm. Unexposed control cultures (a) and Caco-2 cells exposed to 0.625 µg/mL CYN
637 (b) and 2.5 µg/mL CYN (c, d). (a) Unexposed cells with secretory granules (white
638 stars). (b) Altered nuclei with fibrillar component splitting from the granular in exposed
639 cells (white arrow) and presence of micronuclei (black arrow). (c) Vacuoles containing
640 lipids (black arrow head). (d) Endosomes containing electrodense material (black
641 stars), and degenerated mitochondria covered by endoplasmic reticulum cistern
642 caused by the formation of phagosomes (white arrow head).

643 Figure 5. Ultrastructural changes of Caco-2 cells after 48 h of exposure to CYN
644 observed by electron microscopy. Bars (a, b, d)= 2 µm. Bar (c)= 5 µm. Unexposed
645 control cultures (a) and exposed 2.5 µg/mL CYN (b, c, d). (b) Damage in nuclei (white
646 arrow). (c) Segregated nucleoli (black arrow). (d) Presence of myelin-like membrane

647 figures and secondary lysosomes (white arrow head), appearing as multivesicular
648 bodies (black arrow head) and heterophagosomes (white star).

Figure 1
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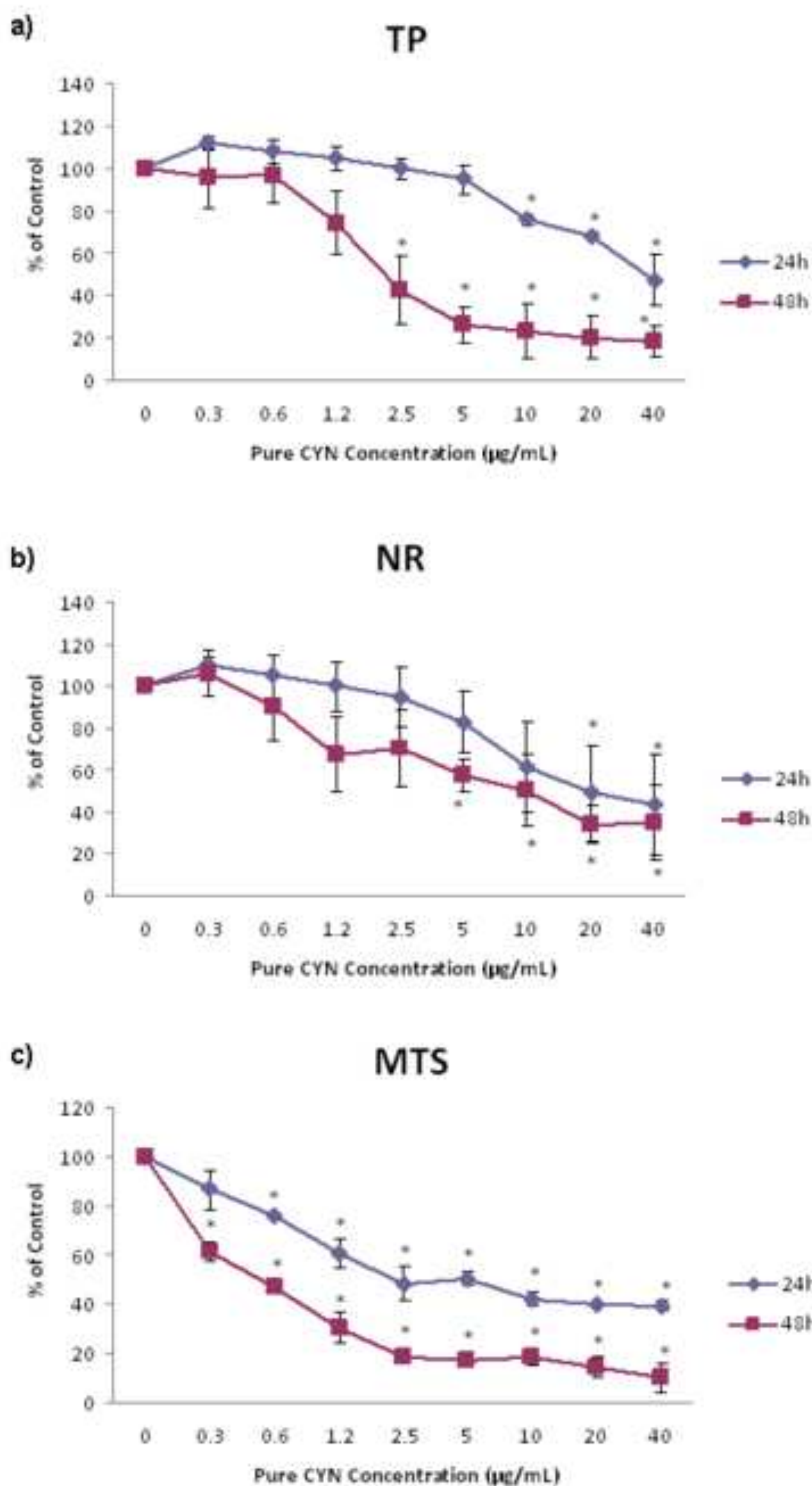

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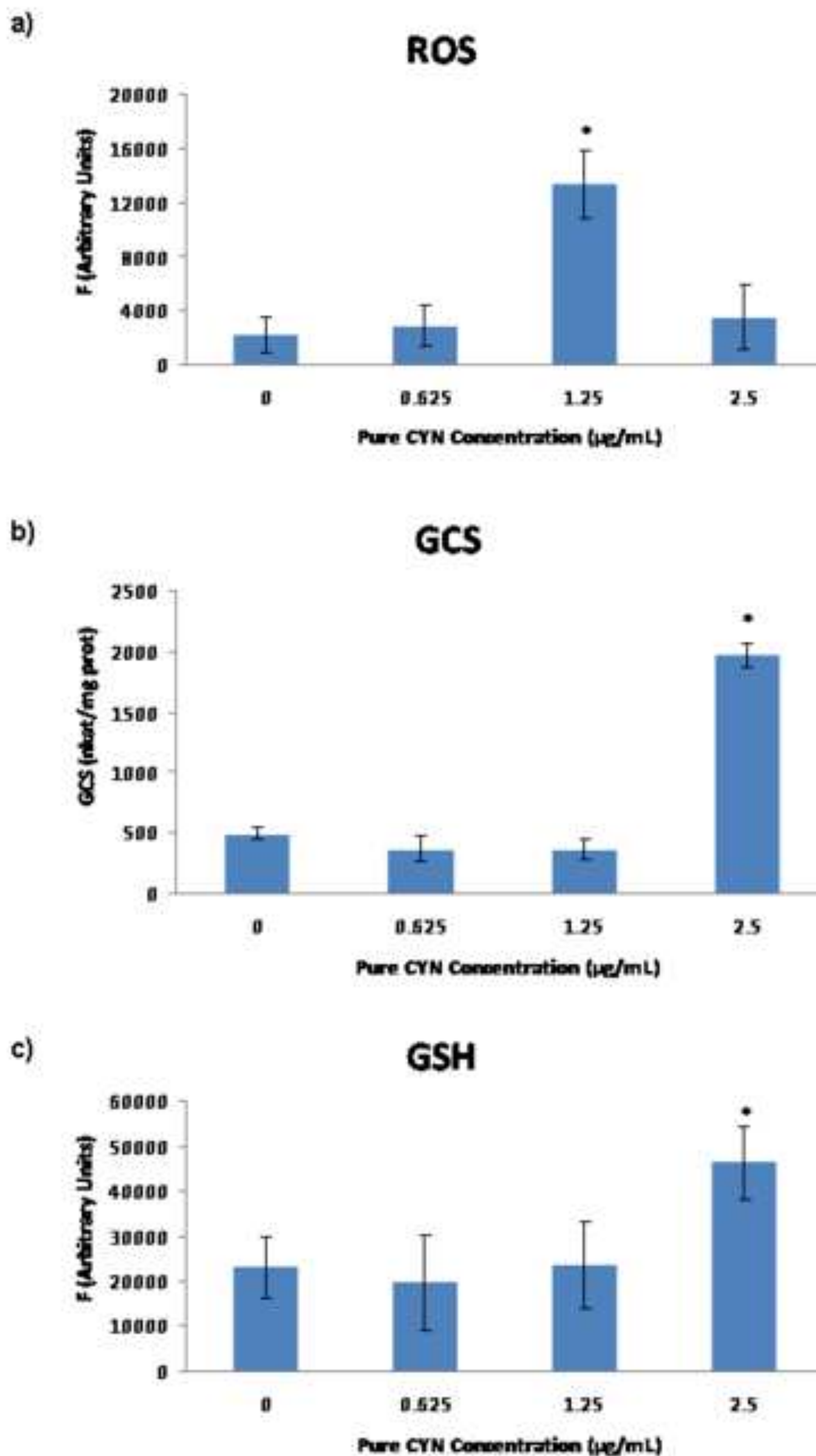

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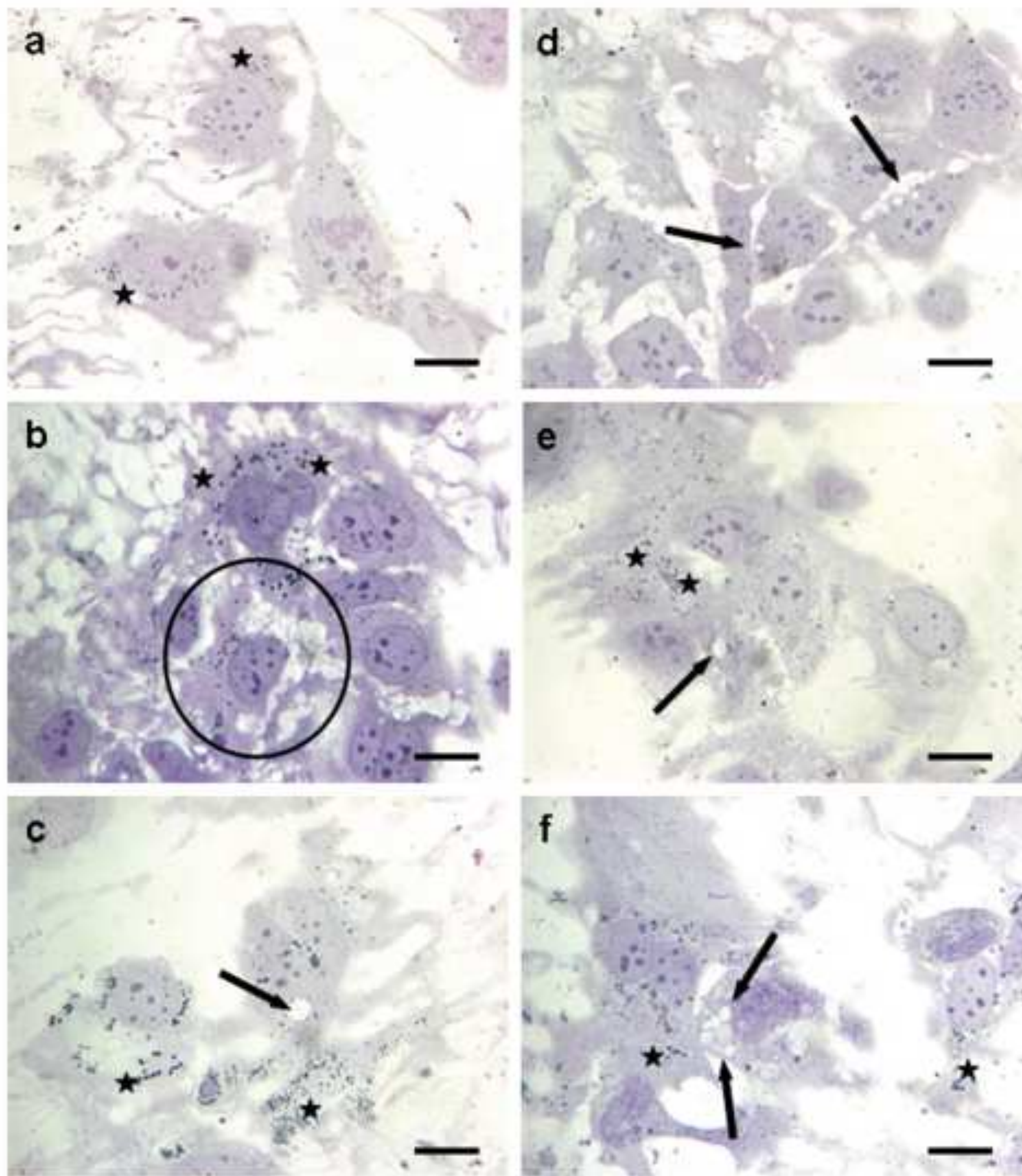

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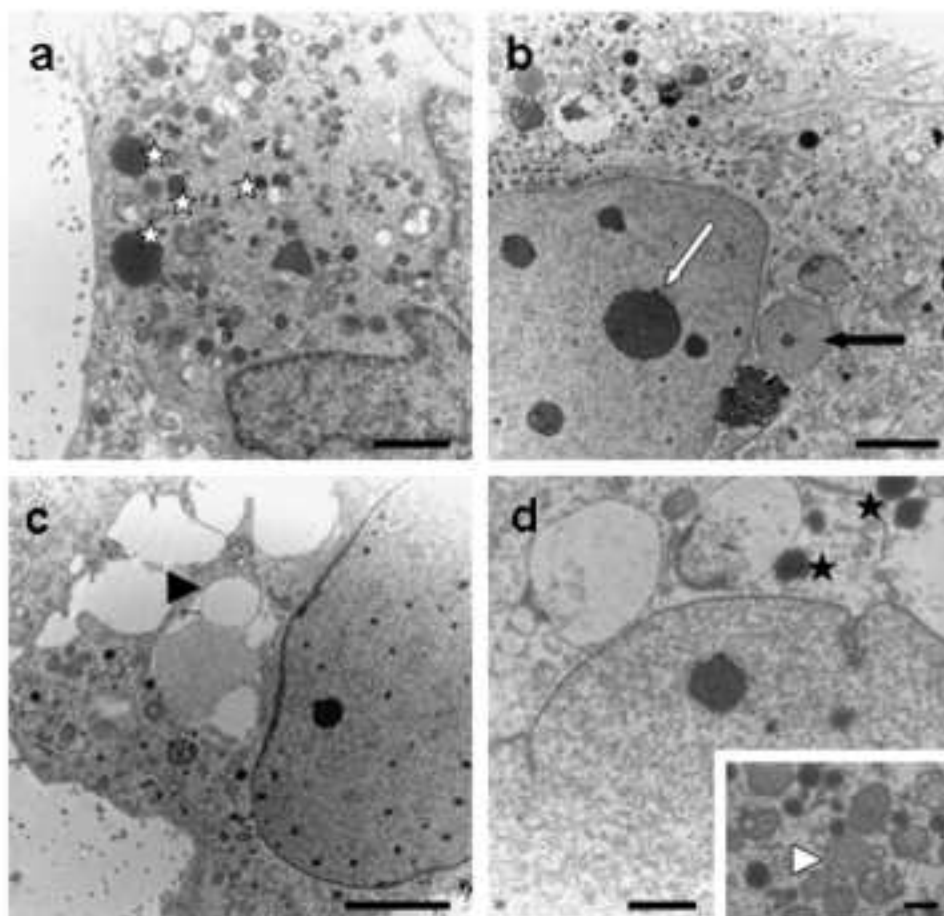

Figure 5

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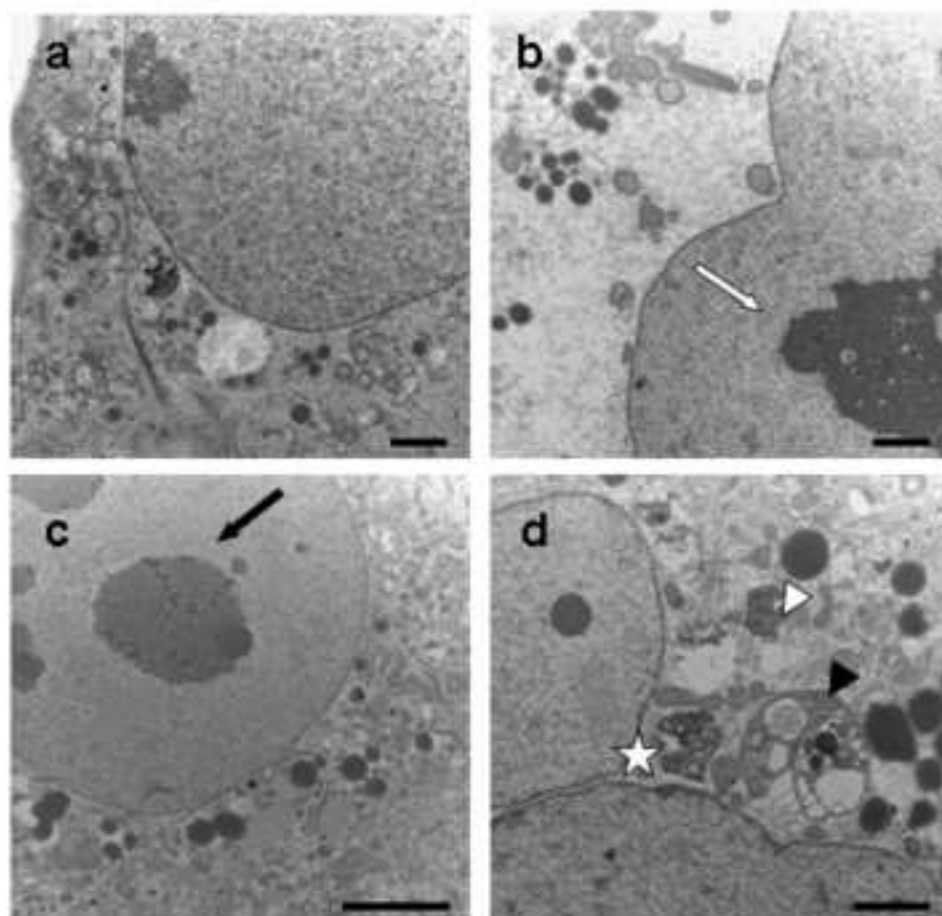

CAPÍTULO 2 / CHAPTER 2

Daniel Gutiérrez-Praena, Silvia Pichardo, Ángeles Jos, F. Javier Moreno, Ana M. Cameán

ALTERATIONS OBSERVED IN THE ENDOTHELIAL HUVEC CELL LINE EXPOSED TO PURE CYLINDROSPERMOPSIN

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Abstract:	The cyanobacterial toxin cylindrospermopsin (CYN) is receiving great interest due to its increasing presence in body waters, which has lead to recognize it as a potential threat to drinking water safety. CYN is a potent inhibitor of protein and glutathione synthesis. The present work deals for the first time the study of the effects of CYN in endothelial cells. The basal cytotoxicity endpoints studied at 24 and 48 hours were total protein content (PC), neutral red (NR) uptake and the tretazolium salt, MTS, reduction. Moreover, the effect of subcytotoxic concentrations of CYN on the generation of intracellular reactive oxygen species (ROS), the activity of γ-glutamylcysteine synthetase (GCS) and glutathione (GSH) content have been investigated. In addition, the morphology alterations of HUVEC cells subsequent to CYN exposure were recorded. The cytotoxicity endpoints revealed a decrease in the cellular viability in a time and concentration-dependent way. The most sensitive cytotoxicity endpoint was NR uptake assay, with reductions in the cell viability of 95% at 48 h of exposure to 40 µg/mL CYN. Intracellular ROS production was only increased at the lowest concentration assayed, while GCS activity and GSH content underwent concentration-dependent enhancements. The most remarkable morphological alterations observed were: nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases in the presence of granules and apoptosis.
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Alterations observed in the endothelial HUVEC cell line exposed to pure Cylindrospermopsin

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Abstract

The cyanobacterial toxin cylindrospermopsin (CYN) is receiving great interest due to its increasing presence in body waters, which has lead to recognize it as a potential threat to drinking water safety. CYN is a potent inhibitor of protein and glutathione synthesis. The present work deals for the first time the study of the effects of CYN in endothelial cells. The basal cytotoxicity endpoints studied at 24 and 48 hours were total protein content (PC), neutral red (NR) uptake and the tretazolium salt, MTS, reduction. Moreover, the effect of subcytotoxic concentrations of CYN on the generation of intracellular reactive oxygen species (ROS), the activity of γ-glutamylcysteine synthetase (GCS) and glutathione (GSH) content have been investigated. In addition, the morphology alterations of HUVEC cells subsequent to CYN exposure were recorded.

The cytotoxicity endpoints revealed a decrease in the cellular viability in a time and concentration-dependent way. The most sensitive cytotoxicity endpoint was NR uptake assay, with reductions in the cell viability of 95% at 48 h of exposure to 40 µg/mL CYN. Intracellular ROS production was only increased at the lowest concentration assayed, while GCS activity and GSH content underwent concentration-dependent enhancements. The most remarkable morphological alterations observed were: nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases in the presence of granules and apoptosis.

Keywords: cylindrospermopsin; cytotoxicity; oxidative stress; HUVEC, morphology.

1. Introduction

The cyanobacterial toxin cylindrospermopsin (CYN) can be produced by several freshwater cyanobacterial species including *Cylindrospermopsis raciborskii* (Ohtani et al. 1992), *Umezakia natans* (Harada et al. 1994), *Aphanizomenon ovalisporum* (Banker et al. 2000), *Raphidiopsis curvata* (Li et al. 2001), *Anabaena bergii* (Stüken et al. 2006), *Aphanizomenon flos-aquae* (Preussel et al. 2006), and *Anabaena lapponica* (Spoof et al. 2006). CYN is a tricyclic alkaloid that consists on a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al. 1992). This cyanotoxin causes human intoxications and animal mortalities (Kinnear et al. 2010). Consequently, CYN is receiving great interest due to its increasing presence in body waters, which has lead to recognize it as a potential threat to drinking water safety (Kuiper-Goodman et al. 1999).

It has been evidenced that CYN is able to inhibit protein synthesis *in vivo* as well as *in vitro* models (Terao et al. 1994; Froscio et al. 2003), which is considered as a primary toxic mechanism. Moreover, it has been also shown a cytochrome p-450 metabolism dependent cytotoxicity, and genotoxic effects subsequent to CYN exposure (Runnegar et al. 1994; Humpage et al. 2005; Straser et al., 2011). In addition, CYN inhibits reduced glutathione synthesis (Runnegar et al. 1994) which could increase the cytotoxicity and the genotoxicity induced by CYN because of the reactive oxygen species (ROS) generation, causing oxidative stress. In this sense, studies about oxidative stress induction by CYN as a possible toxic mechanism are scarce. Our group has found that CYN induces oxidative stress in the fish cell line PLHC-1 (Gutierrez-Praena et al. 2011a) and also in tilapia (*Oreochromis niloticus*) (Puerto et al. 2011; Gutierrez-Praena et al. 2011b).

The major route of human exposure to CYN is the oral one, mainly through drinking water contaminated with CYN, being strongly implicated in human poisoning events in Australia and Brazil (Hawkins et al. 1985; Carmichael et al. 2001). Despite the lack of data concerning the toxicokinetics of this cyanotoxin, it has been reported that its intestinal absorption needs active transport systems, making use of the bile acid transport system (Chong et al. 2002). However, due to the small size of the molecule, a limited passive diffusion through biological membranes occurs (Runnegar et al. 2002). Alternatively CYN may exploit one of the many facilitated transport mechanisms used to move solutes in the cell (Froscio et al. 2009b). In any case, once CYN has gone into the vascular system, it is distributed within the blood reaching the target organs as instance, liver and kidney. During this distribution, the vascular endothelium is in contact with the cyanotoxin and therefore, toxic effects in these cells could be expected.

Endothelial cells are involved in exchange of metabolites between blood and the tissues, in blood homeostasis and wound healing and also provide a non-thrombogenic surface (Henderson 1991), they play an important role in mediating both normal physiology and patho-physiology in human body. Endothelial cells could be target of numerous xenobiotics, as instance, the cyanotoxin CYN. To our knowledge, no study has been conducted so far in order to clarify the effects induced in this tissue by CYN.

The aim of the present study was to investigate, for the first time, the biochemical and morphological alterations in the human umbilical vein endothelial cell line, HUVEC. First, we intended to study the cytotoxic response that short-term exposure to CYN might induce by studying the alteration of three endpoints: total protein content, neutral red uptake and MTS reduction. Moreover, the alteration in the oxidative status and the implication of glutathione in the toxic mechanism of CYN has been studied. Finally,

morphological alterations induced by the cyanotoxin were observed by light and electron microscopy.

2. Materials and Methods

2.1. Supplies and Chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from LGC Standards (Spain), Gibco (Spain) and Sigma-Aldrich (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Model systems

HUVEC cells derived from a human vascular endothelium (ATCC No. CRL-1730) were maintained at 37º C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting in F-12K (LGC Standards, Spain) supplemented with fetal bovine serum, heparin and endothelial cell growth supplement. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by exclusion of Trypan Blue. HUVEC cells were plated at density of 4.5 x 10^5 cells/ml to perform the experiments.

2.3. Toxin test solutions

The cyanotoxin cylindrospermopsin standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Cyanotoxin content was confirmed by a high performance liquid chromatography (HPLC) system (Varian 9012) equipped with a Varian ProStar 330 Diode Array Detector (DAD) (Varian Technologies, Oxford, UK). Stock solution of CYN (400 µg/mL) was prepared in 250 µL of sterilized milliQ water,

128 considering that CYN is a highly water-soluble molecule (Sivonen and Jones 1999),
129 and maintained at -4°C.
130
131 *2.4. Cytotoxicity assays*
132 HUVEC cells were seeded in 96-well tissue-culture plates for the basal cytotoxicity
133 tests and incubated at 37ºC for 24h prior to exposure. From the stock solution of CYN,
134 serial dilutions in medium were prepared 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40 µg/mL
135 CYN. Culture medium without toxin was used as control group. After replacing the
136 previous medium, the exposure solutions were added to the systems, and incubated at
137 37ºC for 24 and 48 hours. The basal cytotoxicity endpoints were protein content (PC),
138 supravital dye neutral red cellular uptake (NR), and tetrazolium salt reduction (MTS).
139
140 Total protein content (PC) was quantified *in situ*, according to the procedure given by
141 Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue
142 culture plates in which exposure originally took place, in order to determine the total
143 cell number present in the culture. The culture medium was replaced by 200 µL NaOH
144 to dissolve the proteins and after 2h incubation at 37ºC, 180 µL was replaced by the
145 same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min
146 incubation at room temperature, absorbance was read at 620 nm.
147
148 NR uptake was performed according to Borenfreund and Puerner (1984). Briefly,
149 neutral red (NR) in medium is absorbed and concentrated in lysosomes of cells. NR
150 uptake is proportional to the concentration of the NR solution and the numbers of viable
151 cells. The NR can be extracted from lysosomes for quantitative measurement of cells
152 viability and cytotoxicity of xenobiotics (Zhang et al. 1990). Culture medium was
153 replaced by 100 µL modified EMEM without serum containing 10 mg/mL NR. The
154 microplate with the NR-containing medium was returned to the incubator for another 3

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

h to allow the uptake of NR into the lysosomes of viable, intact cells. Thereafter, the medium was removed and cells were fixed for 1 min with a formaldehyde-$CaCl_2$ solution. By adding 0.2 mL of acetic acid-ethanol solution to the wells, the NR absorbed by the cells was extracted, brought into solution and quantified at 540 nm.

MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) tetrazolium compound added to the medium is bioreduced by cells into a coloured formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 hours of incubation in the dark.

2.5. Oxidative stress assays

Concentrations used in these assays were calculated based on the cytotoxicity study previously performed. The most sensitive endpoint was NR uptake, therefore the mean effective concentration (EC_{50}) value obtained for this endpoint at 24 h, 1.5 µg/mL CYN, was chosen as the highest exposure concentration for the oxidative stress studies, along with the fractions $EC_{50}/2$ and $EC_{50}/4$, being the toxin concentrations used for the assays 0, 0.375, 0.75 and 1.5 µg/mL CYN.

After replacing the previous medium, the exposure solutions were added to the cells, and incubated at 37ºC for 24 hours. Culture medium without toxin was used as control group. The oxidative stress endpoints measured were reactive oxidative species (ROS) content, glutathione (GSH) levels, and γ-glutamylcysteine synthetase (GCS) activity.

The production of ROS was assessed in 96 well microplates using the dichlorofluorescein (DCF) assay. The probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Invitrogen) readily diffuses through the cell membrane and is

182 hydrolyzed by intracellular esterases to non-fluorescent compound (DCFH), which is
183 rapidly oxidized in the presence of ROS to the highly fluorescent DCF. Specifically,
184 cells were incubated with 200 µl 20 µM DCFH-DA in culture medium at 37ºC for 30
185 min, and then washed with PBS and resuspended in 200 µl of PBS. The formation of
186 the fluorescence oxidized derivative of DCF-DA was monitored at emission wavelength
187 of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as
188 fluorescence arbitrary units (Puerto et al. 2010b).

189

190 GSH content in cells was evaluated by reaction with the fluorescent probe
191 monochlorobimane (mBCl, Molecular probes, Invitrogen) (Jos et al. 2009). This
192 molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme
193 GST. After the cell exposure to CYN, medium was discarded and cells were incubated
194 at 37°C for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with
195 PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the
196 excitation/emission wavelengths of 380/460. Results were expressed as arbitrary units.

197

198 In order to measure γ-glutamylcysteine synthetase (EC 6.3.2.2) activity (GCS), the
199 limiting enzyme in GSH synthesis, cells were seeded in a 5 mL plate and incubated at
200 37°C for 48h. Afterwards, they were exposed to different concentrations of CYN and
201 incubated at 37ºC for 24h. After exposure, cells were collected and resuspended in
202 PBS, 20 µL of sample was added to the mixture reaction, and absorbance was read at
203 340 nm (Seelig and Meister, 1985). Results were expressed as nkat/mg protein.

204

205 *2.6. Morphology*

206 Cells were exposed to two different concentrations of CYN, 0.375 and 1.5 µg/mL,
207 during 24 and 48 hours of exposure. Afterwards, cultured cells were fixed directly in the
208 culture flasks in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at

209 4°C. They were all postfixed in 1% osmium tetroxide for 60 min at 4°C. Samples were
210 dehydrated in ethanol at progressively higher concentrations and embedded in Epon
211 (epoxy embedding medium). Toluidine blue-stained semi-thin sections (0.5 μm thick)
212 used as controls were viewed in a Leitz (Aristoplan) light microscope. Thin sections
213 (60-80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with
214 uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron
215 microscope.

216

217 *2.7. Calculations and statistical analysis*

218 All experiments were performed three times and in duplicate per concentration. Data
219 for the concentration-dependent cytotoxicity relationships of all experiments were
220 presented as the arithmetic mean percentage ± standard deviation (SD) in relation to
221 control. Statistical analysis was carried out using analysis of variance (ANOVA),
222 followed by Dunnett's multiple comparison tests. Differences were considered
223 significant from $P<0.05$.

224 EC_{50} values (mean effective concentration, concentration that modified each biomarker
225 by 50%, positive or negative, in comparison with appropriate untreated controls) were
226 derived by linear regression in the concentration-response curves.

227

228 **3. Results**

229 *3.1. Results of the cytotoxicity assays*

230 HUVEC cells exposed to CYN underwent a concentration and time-dependent
231 decrease in protein content (PC). Significant reductions in cell viability are shown at
232 concentrations of 1.25 μg/mL CYN and above in both exposure times. A remaining cell
233 viability of 28% and 10% was recorded after 24h and 48h of exposure to the highest
234 concentration used (40μg/mL), respectively (Fig. 1a). In addition, considering the EC_{50}

values, toxic effects were more evident in the longest exposure, being 8.5±1.2 for 24h and 1.5±0.6 µg/ml for 48h exposure.

NR uptake assay also indicates a reduction in the cellular viability, showing greater affectation than the PC assay. These decreases are significantly different from the control group at the concentration 1.25 µg/mL and above at 24 h, but when cells were exposed to CYN during two days the cell viability was significantly reduced from the first concentration assayed (Fig. 1b). The greater affectation observed in this endpoint is also visible in the low rate of cell viability observed at 40 µg/mL CYN exposure, which were 20% and 3% after 24h and 48h, respectively. Moreover, lower EC_{50} values are shown for NR uptake, in comparison to those obtained for PC assay, varying between 1.5±0.9 µg/ml for 24h and 0.8±0.5 µg/ml for 48h.

Similarly, MTS reduction performed by cells exposed to CYN also decreased in a concentration and time dependent way, showing significant changes respect to the control group from 1.25 µg/mL in both exposure times (Fig. 1c). At the highest concentration assayed, 47% cell viability was observed at 24h and only 13% after two days of contact with CYN. The EC_{50} values also evidenced a greater effect in the cells after a longer exposure time (15.5±2.1 µg/ml for 24h and 1.5±0.3 µg/ml for 24h).

3.2. Results of the oxidative stress assays

When HUVEC cells were exposed to 0.375 µg/mL CYN, ROS content was significantly enhanced, while at higher concentrations it decreased to the levels of the control group (Fig. 2a). By contrast, GCS activity was increased at the highest concentrations assayed, 0.75 and 1.5 µg/mL, with enhancements of 2.25 and 3.5-folds, respectively, compared to the control group (Fig. 2b). Moreover, GSH content underwent

261 concentration-dependent enhancements, with a 3-fold increase at the highest
262 concentration used in comparison with the control group (Fig. 2c).
263
264 *3.3. Results of the morphology study*
265 *3.3.1. Light microscope observation*
266 HUVEC cells cultured in monolayer were observed, under light microscope, as
267 elongated cells that exhibit extensive cytoplasmic projections. The nucleus is large with
268 a central position and big nucleoli. Elongated mitochondria and small dense granules
269 are visible in the cytoplasm (Fig 3a). When cells are exposed to 0.375 μg/mL CYN
270 during 24 h, no significant changes are shown, with active cells in cellular division (Fig.
271 3b). After 48 hours of exposure lamellipodia and cytoplasmic projections, are observed;
272 increasing the size of the cell. In addition, there was an enhancement in the number of
273 granules in the cytoplasm (Fig. 3c). The accumulation of granules in the cytoplasm
274 becomes more visible after 24 h exposure to 1.5 μg/mL. The nucleoli are compact,
275 showing the entry into quiescence (Fig 3d). When cells are exposed to the highest
276 concentration assayed of CYN during 48 h, a large increase of cytoplasmic granules
277 and irregular nuclei showing segregated nucleoli and micronuclei are observed.
278
279 *3.3.1. Electronic microscope observation*
280 The ultrastructural morphology of HUVEC cells after 24 h of exposure to CYN were
281 observed by electron microscopy. The most visible ultrastructural features of
282 unexposed cells are euchromatic nucleus with irregular border and reticulated
283 nucleolus. Moreover, free ribosomes are shown in the cytoplasm and abundant actin
284 filaments, as well as small rough endoplasmic reticulum with dilated cavity and dense
285 elongated mitochondria with scarce transversal crests. Plasmatic membrane is
286 characterized for an intense vesicular activity, with most of the vesicles covered by
287 clathrin (Fig 4a). No significant changes were observed after 24 h to 0.375 μg/mL CYN,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

288 except for the increase in the presence of granules, which are observed as dense
289 material in the interior and clear outer halo, frequently associated with dictyosomes (Fig
290 4b,c). However, exposure to a higher concentration of CYN induced alteration in
291 HUVEC cells, highlighting the presence of abundant heterophagosomes and irregular
292 granules near the nucleus (Fig 4d). The presence of granules in the cells increases
293 along with the time of exposure and CYN concentration. Moreover, in the projection of
294 the cytoplasm the microfilaments are highly visible, which force the alignment of
295 mitochondria (Fig 4e). Moreover, degenerated Golgi apparatus in apoptotic process is
296 shown (Fig 4f).

297

298 When HUVEC cells were exposed to 1.5 µg/mL CYN during 48 hours, greater injure
299 was observed. Most of the cells showed pleomorphic nuclei and apoptotic with
300 tendency to segregation (Fig 5a,b). Moreover, autophagosome with irregular
301 morphology and heterogeneous content as well as small dense mitochondria are also
302 visible (Fig 5c). After 24 h of exposure to CYN, great endocytic activity, with vesicles
303 showing endocytic covering together with nuclei showing segregated nucleoli, and
304 granules near the nucleus were observed (Fig 5d,e). In addition, elongated
305 mitochondria and dense irregular granules around lipids have been also observed (Fig
306 5f).

307

308 **4. Discussion**

309 CYN was first classified as hepatotoxic due to the toxic effects observed in this tissue
310 (Terao et al. 1994). However, damage in other organs that could be in contact with
311 CYN such as kidney, thymus, heart, spleen, and gastrointestional tract has been also
312 described (Terao et al. 1994; Seawright et al. 1999; Falconer et al. 1999; Norris et al.
313 2001; Bernard et al. 2003). Nowadays, CYN is considered a cytotoxin because it can

affect many tissues (Funari and Testai 2008). In this concern, no study has been performed so far in endothelial cells.

In the present study, the endothelial cells HUVEC exposed to CYN underwent a concentration and time-dependent decrease in protein content, which is a very useful endpoint informing about cell damage in independence of the toxic mechanism involved (Pichardo et al. 2005). Moreover, in the case of CYN, this decrease is particularly interesting since protein synthesis inhibition has been pointed out as one of the metabolite independent toxic mechanism of this cyanotoxin (Froscio et al. 2003 Humpage et al. 2005). In previous studies carried out in our laboratory in the hepatic fish cell line, PLHC-1, the protein content assay was found to be the most sensitive endpoint, suggesting that the protein synthesis inhibiting activity of CYN could be its primary mechanism of action (Gutierrez-Praena et al. 2011a). Nevertheless, in the present study the neutral red uptake was the most sensitive endpoint, with EC_{50} 6- and -10 fold higher than those obtained in the protein content and MTS reduction assays, respectively. Considering the MTS reduction assay, a good marker of the damage induced in mitochondria, significant changes were observed from 1.25 µg/mL in both exposure times compared to the control group. However, no alteration in the mitochondrial morphology was shown in the ultrastructural observation after exposure to 1.5 µg/mL CYN at any exposure time. Moreover, the EC_{50} values obtained for protein content and MTS reduction assays were similar, evidencing a relationship between both endpoints since cells exposed to a concentration of 8,5-15,5 µg/mL CYN for 24 h and 1,5 µg/mL CYN for 48 h are still viable but cannot replicate due to the disruption to protein synthesis (Froscio et al. 2009b).

All the studied cytotoxicity endpoints showed more marked alterations at lower concentrations at 48 hours of exposure in comparison with the exposure to 24 hours. A

341 concentration- and time-dependent decrease has also been reported by other authors
342 in similar studies performed in different cell lines exposed to CYN (Chong et al. 2002;
343 Bain et al. 2007; Neumann et al. 2007; Young et al. 2008; Froscio et al. 2009a; Bazin
344 et al. 2010b, Straser et al. 2011). Chong et al. (2002) reported reductions in the
345 tetrazolium salt MTT when human cell line KB and rat primary hepatocytes were
346 exposed to CYN, being higher after 72 h in comparison to 24 and 48 h exposures. Also
347 a time-dependent response was observed on human granulosa cells exposed to CYN,
348 showing no effects in the MTT assay after 2-6 h exposures but significant decrease
349 from 0.5 μg/ml CYN after 48 and 72 h, and at 1 μg/ml CYN after 24 h exposure (Young
350 et al. 2008). Concentration and time-dependent decreases in MTS viability assay were
351 also reported by Bain et al. (2007) in human dermal fibroblasts cells, HDF; human
352 hepatic cells, HepG2; and human intestinal cells, Caco-2 exposed up to 5 μg/mL CYN,
353 being more evident in fibroblasts. Similarly, Straser et al. (2011) observed significant
354 decreases in the MTT viability assay on HepG2 cells only at the highest concentrations
355 used 1 and 5 μg/mL CYN.
356
357 In the oxidative stress study, the ROS content was only enhanced significantly at the
358 lowest concentration used 0.375μg/mL; however, it decreased to the control values at
359 higher concentrations. This recovery of the normal ROS content can be related to the
360 concentration-dependent increase in the GSH observed, given that it is well known that
361 GSH is involved in ROS quenching (Pflugmacher et al. 1998). Moreover, the GSH
362 content increases in parallel with the GCS activity, enzyme involved in GSH synthesis.
363 Therefore, when HUVEC cells are exposed to higher concentrations of CYN, cells try to
364 face this assault by increasing the production of GSH in order to scavenge the damage
365 caused by the toxin. Similar results were observed in our laboratory in Caco-2 cells
366 exposed to CYN, with enhancements of ROS at 1.25 μg/mL CYN; however, at 2.5
367 μg/mL CYN the decrease of ROS content occur together with the increase in GCS

activity and GSH levels (Gutierrez-Praena et al. 2011c). By contrast, other studies have confirmed the inhibition of GSH synthesis as one of CYN toxic mechanism both *in vitro* (Runnegar et al. 1995, Gutierrez-Praena et al. 2011a) and *in vivo* (Norris et al. 2002).

In this study, no significant cytotoxic or morphology changes were observed when cells were exposed to 0.375 μg/mL CYN during 24 h. However, increases in the ROS and GSH contents were detected. These findings suggest that the primary damage induced by CYN on HUVEC cells is related with the production of ROS and the oxidative situation that is faced by an increase in GSH synthesis. Other authors, however, have reported that this primary damage is due to the inhibition of protein synthesis (Terao et al. 1994), being the *in vitro* inhibition of protein synthesis independent of the transport into the cells (Runnegar et al. 2002). Nevertheless, in our study, significant alterations in protein content were only observed in exposure to higher concentration, being significantly decreased from 1.25 μg/mL CYN. Hence, the induction of GSH synthesis on HUVEC cells produced by CYN at sub-toxic concentrations should be more intensely studied in order to confirm the primary mechanism of toxicity of the cyanotoxin on endothelial cells.

Concerning to the morphological studies, the most remarkable morphological alterations observed were nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases in the presence of granules and apoptosis. Terao et al. (1994) described that nucleoli in the nuclei of the hepatocytes became dense and rounded and reduced in size. These findings have been also corroborated *in vitro* in several cell lines (Fessard and Bernard, 2003; Gacsi et al. 2009; Maire et al. 2010) and *in vivo* in liver of rodents exposed to CYN (Terao et al. 1994; Seawright et al. 1999). Moreover, Terao et al. (1994) also reported altered Golgi apparatus after 24 h of exposure to CYN, and numerous autophagic vacuoles in the cytoplasm after 48 h. Finally, cell

death, both necrosis and apoptosis, has been observed subsequent to CYN exposure. Necrosis has been induced in several organs of mice intraperitoneally and orally exposed to CYN (Terao et al. 1994; Falconer et al. 1999; Seawright et al. 1999; Bernard et al. 2003; Saker et al. 2003). Besides, apoptotic cells were observed in Chinese hamster ovary cells, CHO-K1, exposed to CYN (Fessard and Bernard 2003; Gacsi et al. 2009). In addition, in the present work, exposure to 0.375 µg/mL CYN induced increases in ROS content after 24 h and alterations in the nuclei and nucleoili after 48 h exposure. Moreover, other authors have also reported the relationship between oxidative stress and genotoxicity. In this sense, Bain et al. (2007) suggested that CYN induces stress responses in human cell lines that results in the activation of the p53 transcription factor, which is involved in the DNA damage.

5. Conclusion

The present work has demonstrated for the first time that CYN is able to induce toxic effects in endothelial cells in a time-concentration dependent manner. Cytotoxic effects have been observed on HUVEC cells exposed to CYN, with NR uptake as the most sensitive endpoint. Moreover, the results obtained in the oxidative study suggest that the oxidative stress may play a role in the toxic effects induced by CYN. Concerning the morphological studies, the main alterations shown were nucleolar segregation with altered nuclei, degenerated Golgi apparatus, increases in the presence of granules and apoptosis. However, more studies are needed to establish a correlation between the cytotoxic effects observed, the morphological alterations, and the contribution of the oxidative stress in the pathogenicity of this cyanotoxin.

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Figure captions

Figure 1. Protein content, PC (a); Neutral red uptake, NR (b); and reduction of tetrazolium salt, MTS (c) of HUVEC cells after 24h and 48h of exposure to 0-40 µg/mL CYN. All values are expressed as mean ± s.d. * significantly different from control ($p \leq 0.05$).

Figure 2. ROS content (a); GCS activity (b); and GSH content (c) in HUVEC cells after 24h of exposure to 0.375, 0.75, and 1.5 µg/mL CYN. All values are expressed as mean ± s.d. * significantly different from control ($p \leq 0.05$).

Figure 3. Morphology of HUVEC cells after 24 h (a, b, d) and 48 h (c, e, f) of treatment observed by optical microscopy. Bars= 50 µm. Unexposed control culture (a) and exposed to 0.375 µg/mL CYN (b, c) and 1.5 µg/mL CYN (d, e, f). Secretory granules (stars), lamellipodia (arrow) and segregated nucleoli (black arrow head) are observed in HUVEC cells.

Figure 4. Ultrastructural changes of HUVEC cells after 24 h of exposure to CYN observed by electron microscopy. Unexposed control cultures (a) and HUVEC cells exposed to 0.375 µg/mL CYN (b,c) and 1.5 µg/mL CYN (d, e, f). (a) Unexposed cells with euchromatic nucleus, reticulated nucleolus (white arrow), free ribosomes in the cytplasm (black star), small rough endoplasmic reticulum (black arrows) and elongated mitochondria (black arrow heads). Bar= 5 µm. (b) Detail of a reticulated nucleolus (black arrow) and dictyosomes (big white arrow) Bar= 5 µm. (c) Presence of secretory granules near the nucleus (white arrow head). Bar= 2 µm. (d) Detail of a projection of the cytoplasm with visible microfilaments, aligned mitochondria (black arrow heads) and granules with dense material in the interior and clear outer halo (white arrow

head). Bar= 2 μm. (e) Presence of irregular granules (white arrow heads) and heterophagosomes (black arrow) near the nucleus. Bar= 5 μm. (f) Detail of a degenerated Golgi apparatus in apoptotic process. Bar= 0.5 μm.

Figure 5. Ultrastructural changes of HUVEC cells after 48 h of exposure to CYN observed by electron microscopy. HUVEC cells exposed to 0.375 μg/mL CYN (a,b,c) and 1.5 μg/mL CYN (d, e, f). (a) After 48 h of exposure, cells shown pleomorphic nuclei. Bar=5 μm. (a, insert): Detail of an apoptotic nucleus. Bar=0.7 μm (b) Detail of a reticulated nucleolus (black arrow) and nucleoli with tendency to segregation (white arrow). Bar=5 μm. (c) Presence of autophagosome with irregular morphology and heterogeneous content (black star); small dense mitochondria are also visible (white stars). Bar=5 μm. (d) Great endocytic activity, with vesicles showing endocytic covering (black arrow head). Bar=0.5 μm. (e) Nuclei showing segregated nucleoli (white arrow head), and granules near the nucleus (big white arrow). Bar=5 μm. (f) Presence of elongated mitochondria and granules. Bar=2 μm. (f, insert): Detail of dense irregular granules around lipids. Bar=2.5 μm.

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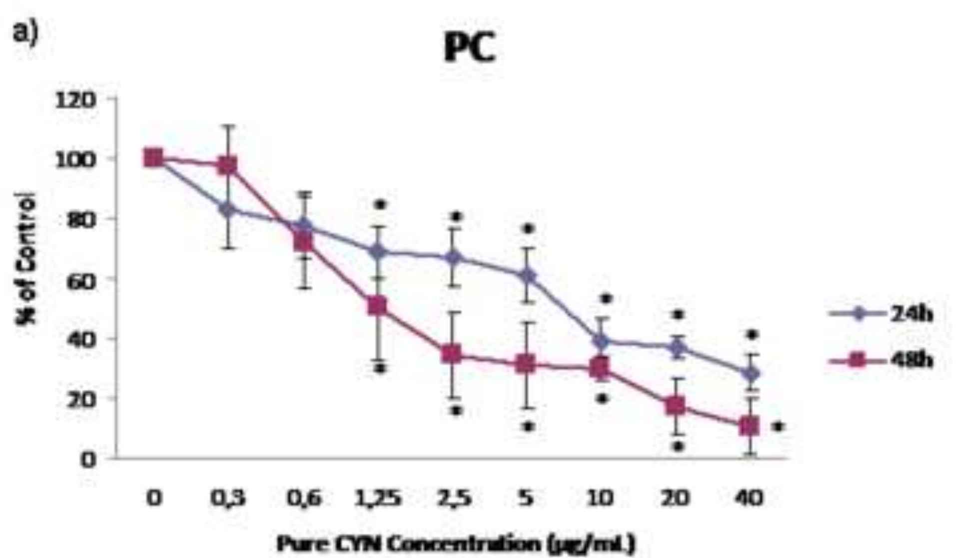

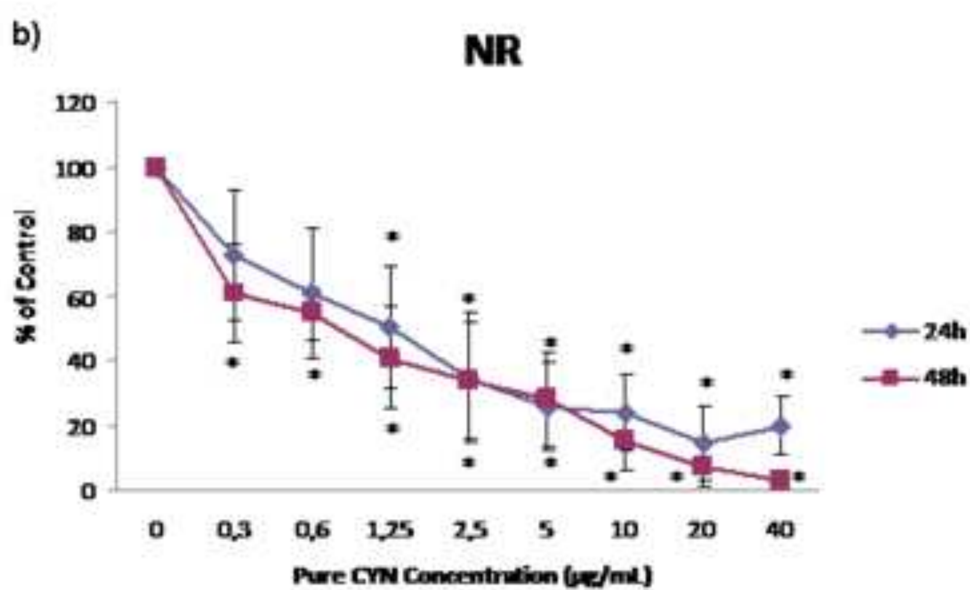

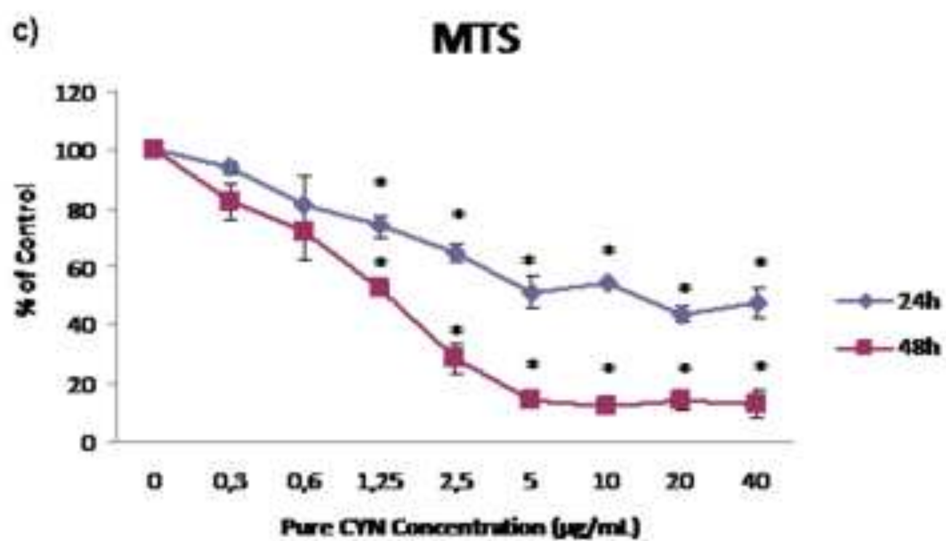

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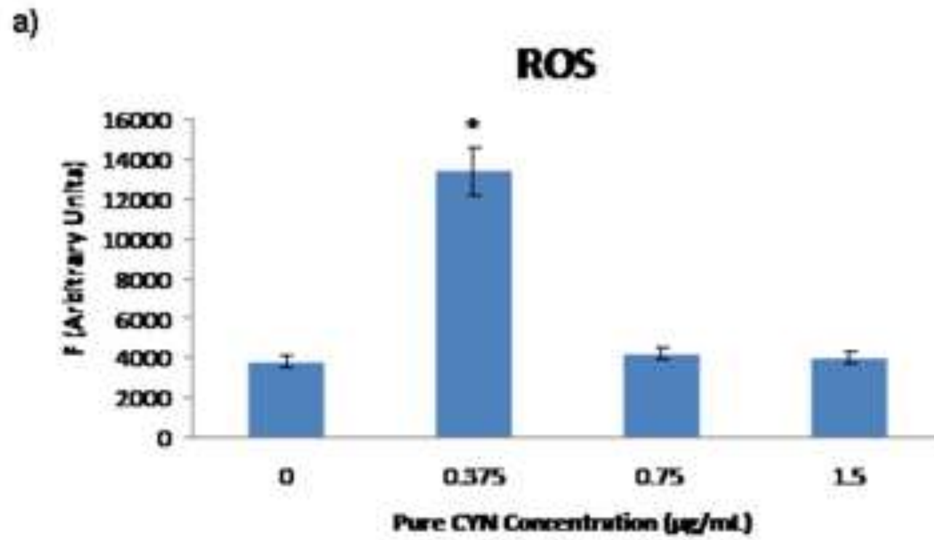

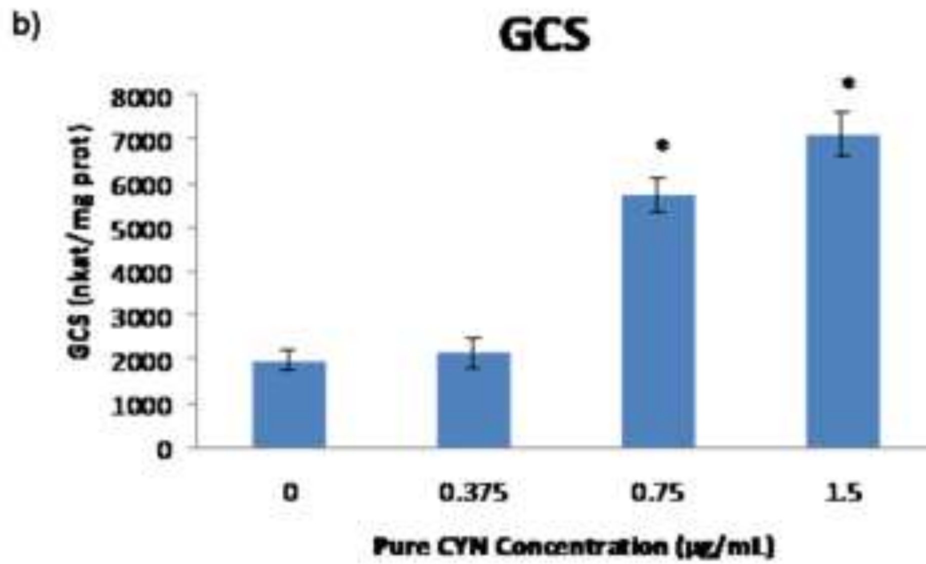

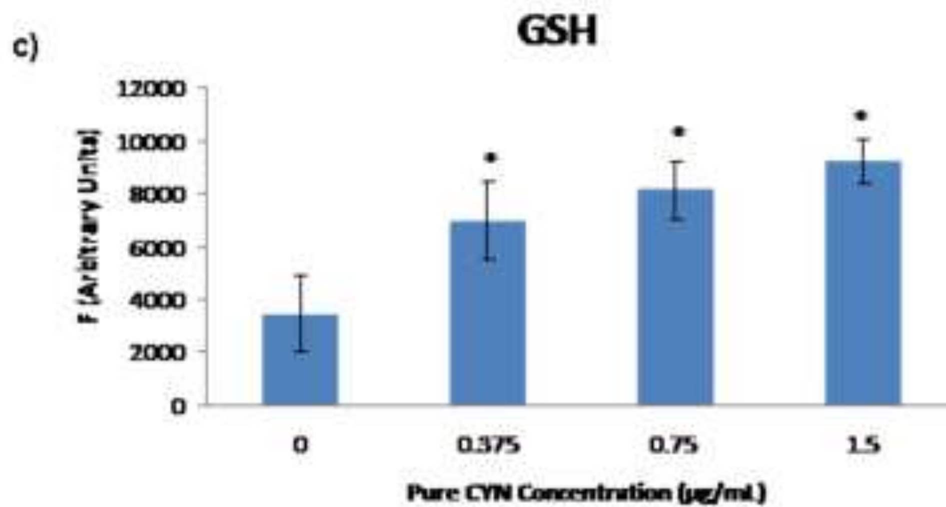

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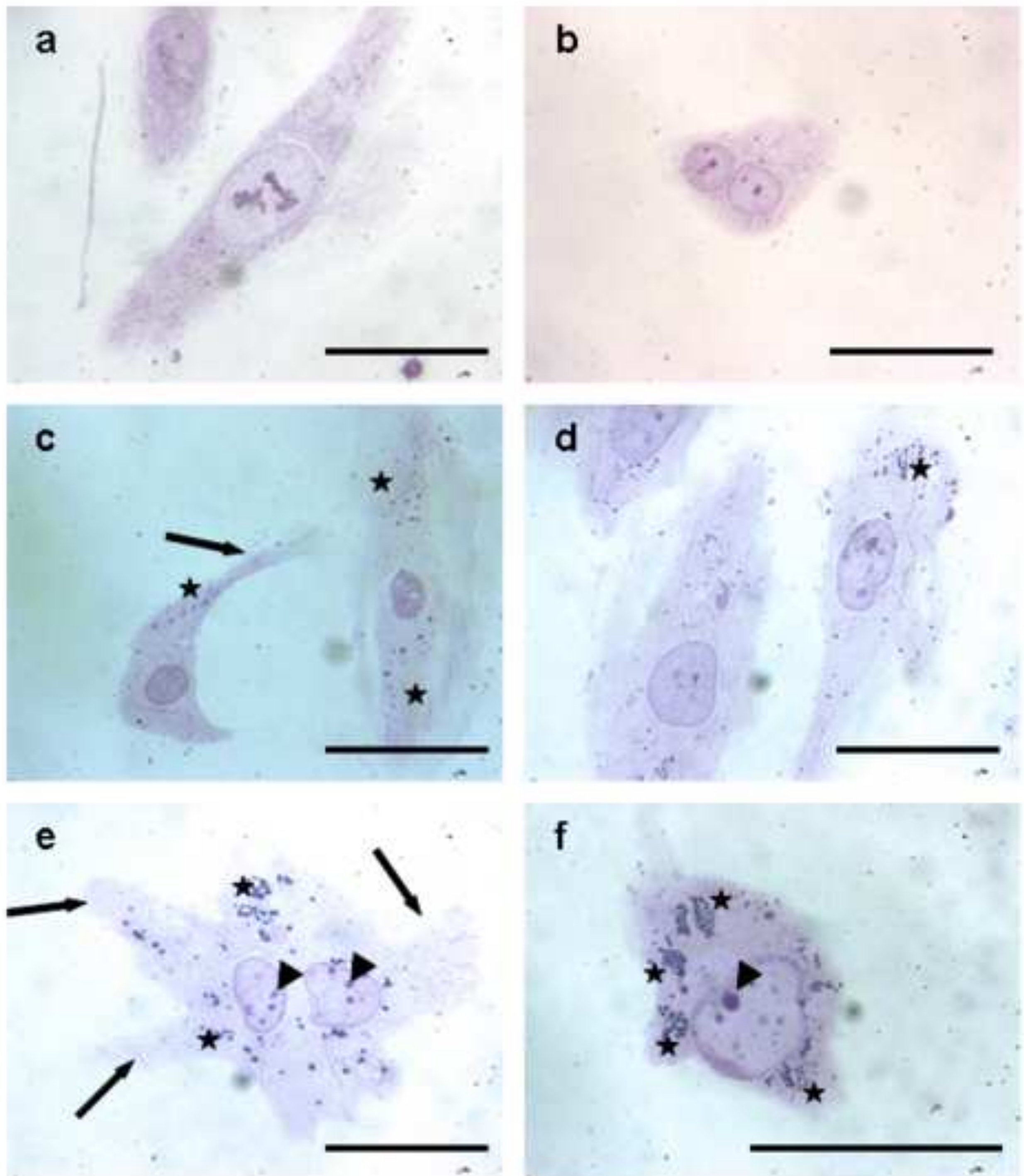

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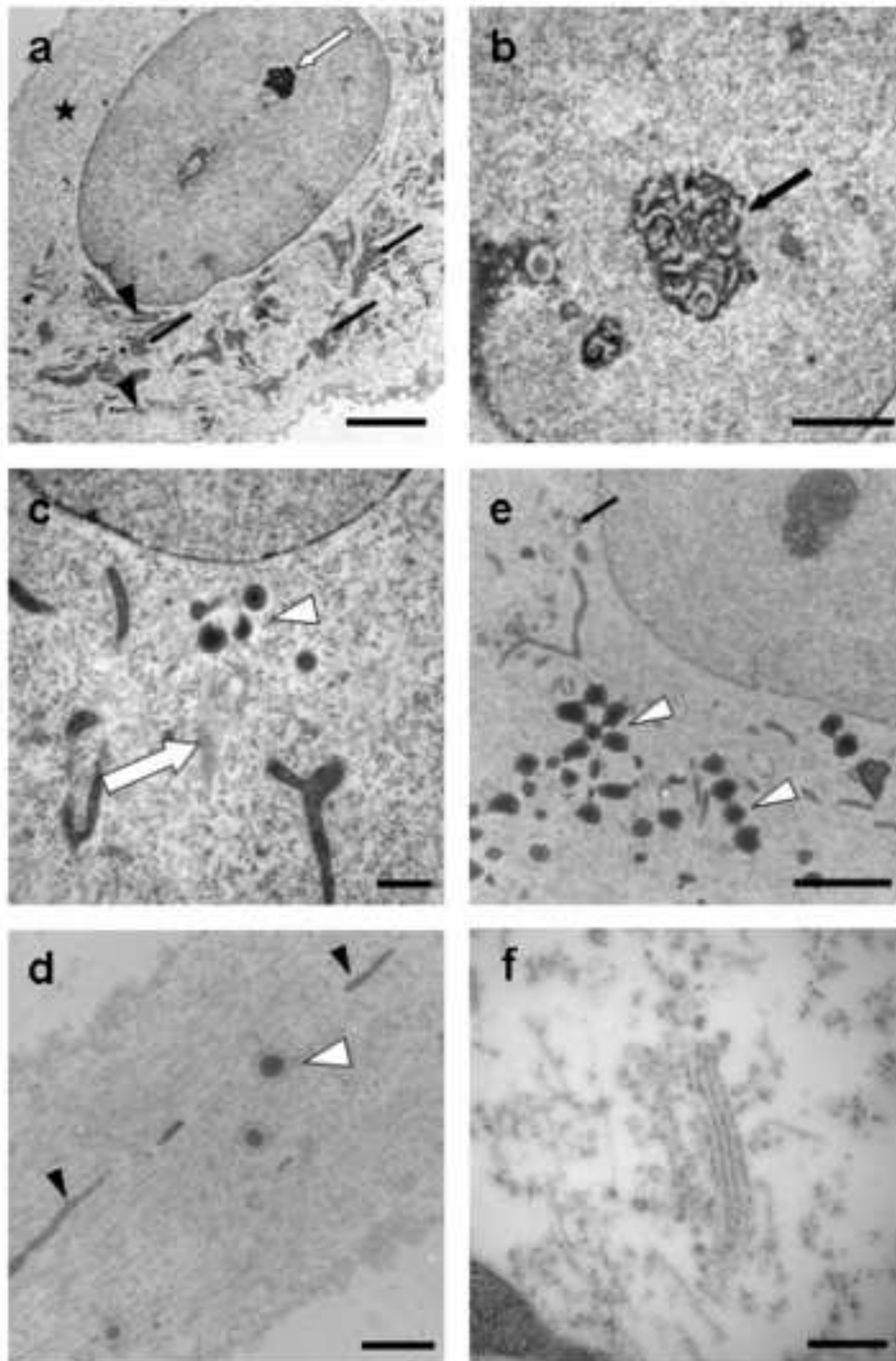

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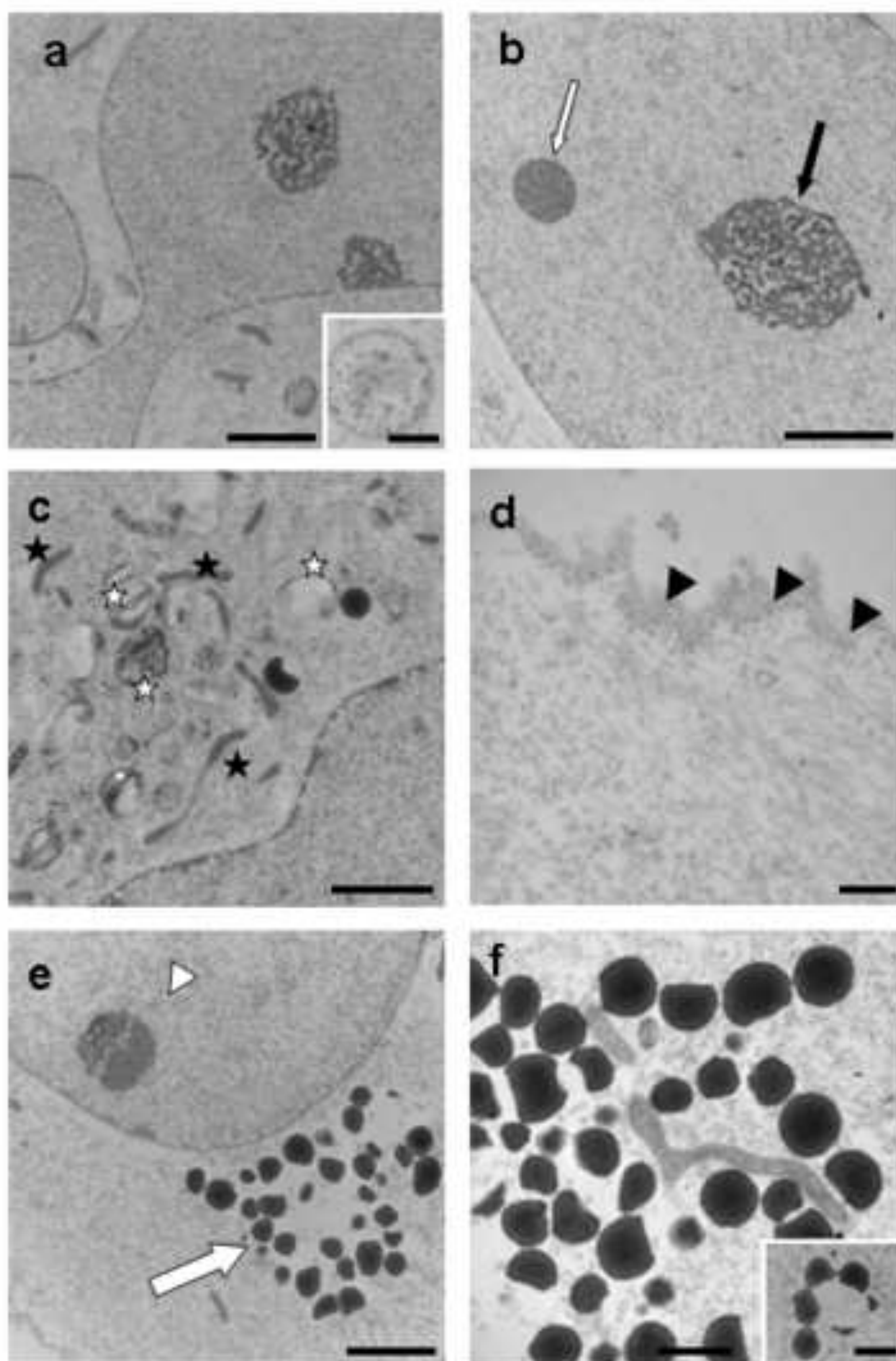

CAPÍTULO 3 / CHAPTER 3

Daniel Gutiérrez-Praena, Silvia Pichardo, Ángeles Jos, Ana M. Cameán

TOXICITY AND GLUTATHIONE IMPLICATION IN THE EFFECTS OBSERVED BY EXPOSURE OF THE LIVER FISH CELL LINE PLHC-1 TO PURE CYLINDROSPERMOPSIN

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Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin

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ABSTRACT

Cylindrospermopsin (CYN), a cyanotoxin produced by several freshwater cyanobacteria species, has been reported to cause human and animal intoxications. CYN is a potent inhibitor of protein and glutathione synthesis. In order to study these effects, various *in vitro* models have been used, which are representative of the organs targeted by the toxin. However, studies concerning CYN toxicity to fish species, both *in vivo* and *in vitro*, are still very scarce.

To our knowledge, this is the first work dealing with the effects of CYN in a fish cell line. In the present work, we tried to test the hypothesis that CYN could be hepatotoxic to fish causing cell damage and oxidative stress, which may lead to pathogenicity. To deal this purpose, PLCH-1 cells, derived from fish liver, were exposed to concentrations that ranged from 0.3 to 40 μg/mL CYN during 24 and 48 h for the cytotoxicity study, and 2, 4 and 8 μg/mL CYN for the oxidative stress assays. The basal cytotoxicity endpoints studied were protein content, neutral red uptake and the tetrazolium salt, MTS, reduction. The biomarkers used for the oxidative stress study were reactive oxygen species (ROS) content, reduced glutathione content and γ-glutamylcysteine synthetase activity.

The cytotoxicity endpoints revealed a decrease in the cellular viability in a time and concentration-dependent way. Moreover, when cells were exposed to pure CYN, an increase in the ROS content was observed, being more marked at the higher concentrations used. Finally, the present work shows alterations in GSH content and synthesis due to CYN. Moreover, a relationship between cytotoxic effects and ROS production has been evidenced. The results obtained confirm the alteration on fish liver cells, which should be considered relevant to what it may happen in real scenarios since fish are frequently in contact with this cyanotoxin.

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1. Introduction

Cyanobacterial toxins have become recognized as a potential hazard in drinking water worldwide (Carmichael et al., 2001). Among them, the cyanotoxin cylindrospermopsin (CYN) is a potent toxic alkaloid, which is produced by several cyanobacterial species. It was first isolated from *Cylindrospermopsis raciborskii* from tropical waters (Hawkins et al., 1985; Chiswell et al., 1997), although it is also produced by other species such as *Umezakia natans*, *Aphanizomenon ovalisporum*, *Anabaena bergii*, *Raphidiopsis curvata*, *Aphanizomenon flos-aquae* and *Anabaena lapponica* (Bazin et al., 2010). These organisms can flourish, in a wide range of habitats, raising an environmental concern, since they frequently occur in drinking water reservoirs (Kuiper-Goodman et al., 1999). The massive proliferation of these organisms is largely due to eutrophication. Compared to other cyanotoxins, CYN is mainly produced as an extracellular toxin; appearing consequently in a large proportion in environmental samples (van Apeldoorn et al., 2007). Although no formal guideline exists for CYN levels, Humpage and Falconer (2003) have proposed a drinking water limit of 1 μg/L total CYN. Thus, CYN can pose serious threats to human and environmental health via contamination of drinking water, recreational exposure to waterborne toxins and possible accumulation of toxins in the food-web (Kinnear, 2010).

The toxic mechanism of CYN has not been fully elucidated. It is well known that CYN inhibits directly protein synthesis powerfully, which has been reported using both *in vivo* and *in vitro* experimental models (Terao et al., 1994; Froscio et al., 2003). However, at high concentrations its acute toxicity appears to be mediated by cytochrome P-450 (CYP450) (Froscio et al., 2003). It has been suggested that the toxicity at low concentrations is

Abbreviations: CYN, cylindrospermopsin; CYP450, cytochrome P-450; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; EMEM, Eagle′s minimal essential medium; GSH, glutathione; GCS, γ-glutamylcysteine synthetase; mBCl, monochlorobimane; NR, neutral red; PBS, phosphate buffered saline; ROS, reactive oxygen species; SD, standard deviation; PC, protein content

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primarily mediated via protein synthesis inhibition, but at higher concentrations a more rapidly toxic process dominates, which is metabolism dependent (Humpage et al., 2005). Moreover, other effects such as reduction of glutathione synthesis and genotoxicity have been described (Froscio et al., 2003; Humpage et al., 2005). It has been reported that levels of glutathione (GSH) are reduced in affected cells due to the inhibition of glutathione synthesis (Runnegar et al., 1994, 1995). Although it has been suggested that this reduction does not contribute significantly to CYN acute toxicity *in vivo* (Norris et al., 2002), it seems clear that the absence of GSH to reduce high levels of reactive oxygen species (ROS) may play an important role in other toxic mechanisms, for instance, genotoxicity (Humpage et al., 2005).

Regarding CYN toxicity, both *in vivo* and *in vitro* studies have been performed trying to elucidate the effects of this cyanotoxin. *In vivo* studies in mice suggest that the liver is a major target organ; in fact CYN has been traditionally classified as hepatotoxic (de Figueiredo et al., 2004). However, heart, thymus, spleen, lungs, kidneys and ovaries can also be affected (Young et al., 2008). Chong et al. (2002) demonstrated that the uptake of CYN into hepatocytes may involve more than one mechanism.

Cell culture-based toxicity tests are of interest, having the potential to screen samples for a biochemical response characteristic to toxin class, while retaining the ability to detect more general cytotoxicity endpoints (Froscio et al., 2009). The toxicity of the cyanobacterial toxin CYN can be detected via cell-based assays as it is known to produce damage in a range of different cell types (Froscio et al., 2009). Freshly isolated hepatocytes were used as a sensitive *in vitro* model for CYN toxicity (Runnegar et al., 1994, 1995, 2002; Froscio et al., 2003; Humpage et al., 2005) with concentrations in the low micromolar (≥ 1 μM) range producing disruption of the cell membrane within 18–24 h. While the sensitivity of primary hepatocytes is thought to be due to CYP450 activation of CYN to toxic metabolites (Runnegar et al., 1995; Froscio et al., 2003; Humpage et al., 2005), CYN was also shown to exert effects in cell lines considered to be less metabolically competent. In this sense, the cell line chosen in the present work, PLHC-1 hepatic fish cell line, has a hepatocellular origin and CYN has been reported to be hepatoxic. This permanent cell line also would have metabolic activity response and uptake capabilities, very important in the pathogenicity of CYN (Humpage et al., 2005). PLHC-1 cells have shown to have an inducible CYP450 activity that can also be inhibited (Babich et al., 1991). Moreover, PHLC-1 cells can be used in an *in vitro* system to screen environmentally relevant stressors using a combined stress protein and cytotoxicity assay (Ryan and Hightower, 1994). Besides, retention of hepatocyte properties has made this cell line ideal for *in vitro* toxicology assays. In fact it has been well characterized by environmental toxicologists. It has been reported that the *in vitro* cytotoxicity of some chemicals is positively correlated with the acute toxicity *in vivo*, and therefore cytotoxicity assays may serve as an alternative for acute fish toxicity testing (Fent, 2001). In addition, very little is known about the toxic effects induced by the cyanotoxin CYN on fish. In fact, its effects of CYN on fish cell lines have not been previously examined. In this sense, the PLHC-1 cell line origin, a hepatocellular carcinoma of the cyprinid fish *Poeciliopsis lucida*, makes this experimental model ideal to study toxic effects induced by cyanobacteria, not only because they can be in contact through water, but also because cyanobacteria are an important dietary component of cichlids and cyprinids fish (Zurawell et al., 2005; Prieto et al., 2007).

Considering the background mentioned above, the aim of the present work was to study the cytotoxicity of CYN on fish cells, as well as investigating the role of GSH in CYN toxicity mechanism, including the production of oxidative stress. Moreover, the hypothesis to be tested was if PLHC-1 cells could be an appropriate experimental model to assess the toxicity induced by CYN, considering the importance of studying the effects of CYN on fish, and in order to reduce the number of fish used in bioassays.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Model systems

PLHC-1 cells derived from a hepatocellular carcinoma of the topminnow *P. lucida* (ATCC No.CRL-2406) were maintained at 30° C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's Minimum Essential Medium (EMEM) supplemented with fetal bovine serum, sodium pyruvate, gentamicine, non-essential amino acids, L-glutamine and fungizone.

2.3. Toxin test solutions

The cyanotoxin cylindrospermopsin standard (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Cyanotoxin content was confirmed by a high performance liquid chromatography (HPLC) system (Varian 9012) equipped with a Varian ProStar 330 Diode Array Detector (DAD) (Varian Technologies, Oxford, UK), using a gradient program with water and methanol as solvent, both containing 0.05% of triflouracetic acid. A stock solution of CYN (400 μg/mL) was prepared in 250 μL of sterilized milliQ water, considering that CYN is a highly water-soluble molecule (Sivonen and Jones 1999), and is maintained at -4 °C.

2.4. Cytotoxicity assay

PLHC-1 cells were diluted to 5×10^5 cells/mL, and 200 μL were placed in each well of the 96-well tissue-culture plates for the basal cytotoxicity tests and incubated at 30 °C for 24 h prior to exposure. From the stock solution, serial dilutions in medium were prepared: 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40 μg/mL CYN. Culture medium without toxin was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 30 °C for 24 and 48 h. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR) and tetrazolium salt reduction (MTS).

Protein content (PC) was quantified *in situ*, according to the procedure given by Bradford (1976) .The culture medium was replaced by 200 μL NaOH to dissolve the proteins and after 2 h incubation at 37 °C, 180 μL was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature absorbance was read at 620 nm

Neutral Red (NR) uptake was performed according to Borenfreund and Puerner (1984). Culture medium was replaced by 100 μL modified EMEM without serum containing 10 mg/mL NR. The microplate with the NR-containing medium was returned to the incubator for another 3 h to allow the uptake of NR into the lysosomes of viable, intact cells. Thereafter, the medium was removed and cells were fixed for 1 min with 1% formaldehyde–1% $CaCl_2$ solution. By adding 0.2 mL of acetic acid–ethanol–water (1:49:50) solution to the wells, the NR absorbed by the cells was extracted and brought into solution and quantified at 540 nm

MTS reduction was measured in intact cells according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) tetrazolium compound added to the medium is bioreduced by cells into a colored formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 h of incubation in the dark. All cytotoxicity endpoints were expressed as percentage of the control.

2.5. Oxidative stress assays

Assays concentrations were calculated based on the cytotoxicity study previously performed. The most sensitive endpoint was protein content (PC), therefore the mean effective concentration (EC_{50}) value obtained for this endpoint at 24 h, 8 μg/mL CYN, was chosen as the highest exposure concentration for the oxidative stress studies in the fish cell line, along with the fractions $EC_{50}/2$ and $EC_{50}/4$, being the toxin concentrations used 0, 2, 4 and 8 μg/mL CYN. Culture medium without toxin was used as control group. After replacing the previous

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medium, the exposure solutions were added to the cells, and incubated at 30 °C for 24 h. The oxidative stress endpoints measured were reactive oxygen species (ROS) content, reduced glutathione (GSH) levels and γ-glutamylcysteine synthetase (GCS) activity.

The production of ROS was assessed in 96-well microplates using the dichlorofluorescein (DCF) assay. Cells were incubated with 200 µl 20 µM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in culture medium at 30 °C for 30 min, and then washed with phosphate buffered saline (PBS) and resuspended in 200 µl of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was monitored at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. ROS production was expressed as fluorescence arbitrary units (Puerto et al., 2010).

GSH content in cells was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl) (Puerto et al., 2010). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme GST. After the cell exposure to CYN, medium was discarded and cells were incubated at 30 °C for 20 min in the presence of 40 µM mBCl. Later on, cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the excitation/emission wavelengths of 380/460. Results were expressed as arbitrary units.

In order to measure γ-glutamylcysteine synthetase (GCS), one of the responsible enzymes in GSH production, cells were seeded in a 5 mL plate and incubated at 30 °C for 48 h. Later on, they were exposed to different concentrations of CYN and incubated at 30 °C for 24 h. After exposure, cells were collected and resuspended in PBS, 20 µL of sample was added to the mixture reaction and absorbance was read at 340 nm (Seelig and Meister, 1985). Results were expressed as nkat/mg protein.

2.6. Calculations and statistical analysis

All experiments were performed three times and in duplicate per concentration. Data for the concentration-dependent cytotoxicity relationships of all experiments were presented as the arithmetic mean percentage ± standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered significant when $P \leq 0.05$.

EC_{50} values (mean effective concentration, that is concentration that modified each biomarker by 50%, positive or negative, in comparison with appropriate untreated controls) were calculated using Sigma Stat and Sigma Plot software (Jandel Scientific, San Rafael, CA, USA).

3. Results

PLHC-1 cells exposed to CYN experienced a concentration and time-dependent decrease in protein content. Significant reductions in cell viability are shown from 2.5 µg/mL pure CYN after 24 h and from the first concentration assayed at 48 h, with remaining cell viability of 5% at 24 h and 4% after 48 h of exposure to the highest concentration used (Fig. 1). Moreover, considering the EC_{50} values, toxic effects were more evident in the longer exposure, being 8.0 ± 2.1 µg/ml for 24 h and 2.6 ± 0.7 µg/ml for 48 h exposure.

NR uptake assay also indicates a reduction in the cellular viability. However, in comparison to the TP content assay, the decrease is significantly different from the control only at higher concentrations, from 5 µg/ml, at both exposures times (Fig. 2). Moreover, at 40 µg/mL pure CYN cell viability was below 20% at both exposures times: 13% and 2% after 24 h and 48 h, respectively. Moreover, higher EC_{50} values are shown for NR uptake varying between 8.6 ± 1.9 µg/ml for 24 h and 7.1 ± 0.8 µg/ml for 48 h.

MTS metabolism performed by cells exposed to pure CYN also decreased in a concentration and time dependent way, showing significant changes with respect to the control from 10 µg/mL pure CYN for 24 h and from 2.5 µg/mL pure CYN for 48 h (Fig. 3). At the highest concentration assayed, 26% viability was observed at 24 h and only 4% after 48 h of exposure. The EC_{50} values also evidenced a greater effect in the cells after a longer exposure time (2.2 ± 0.3 µg/ml for 48 h and 16.12 ± 2.3 µg/ml for 24 h).

Regarding the ROS content, an increase was observed from the lower concentration assayed (2 µg/ml) being more remarkable at the highest concentration (8 µg/mL) with enhancements of 2.5-folds of the ROS content in comparison with the control group (Fig. 4). The GSH content in cells exposed to 2 µg/ml increased significantly (Fig. 5), although at higher concentrations

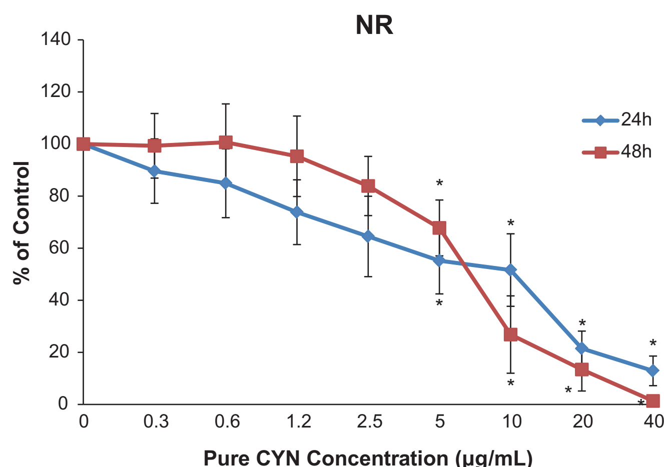

Fig. 2. Neutral red (NR) uptake of PLHC-1 cells after 24 and 48 h of exposure to 0–40 µg/mL pure CYN. All values are expressed as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

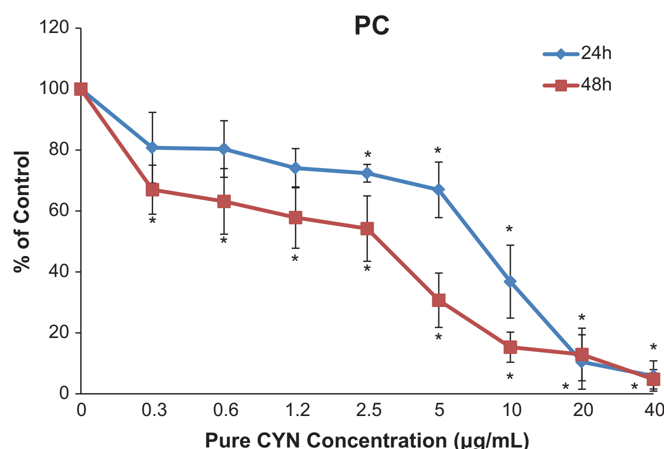

Fig. 1. Protein content (PC) of PLHC-1 cells after 24 and 48 h of exposure to 0–40 µg/mL pure CYN. All values are expressed as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

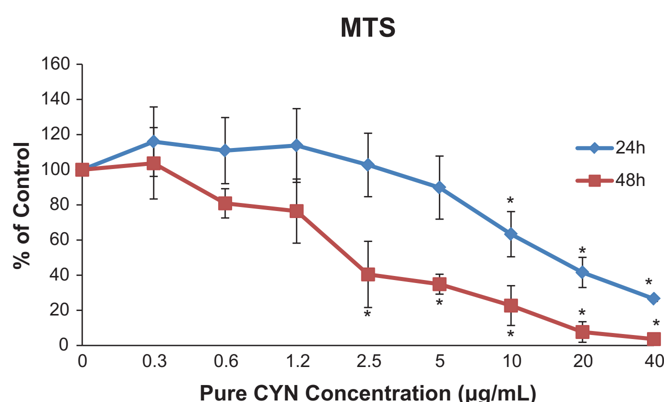

Fig. 3. Reduction of tetrazolium salt (MTS) of PLHC-1 cells after 24 and 48 h of exposure to 0–40 µg/mL pure CYN. All values are expressed as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

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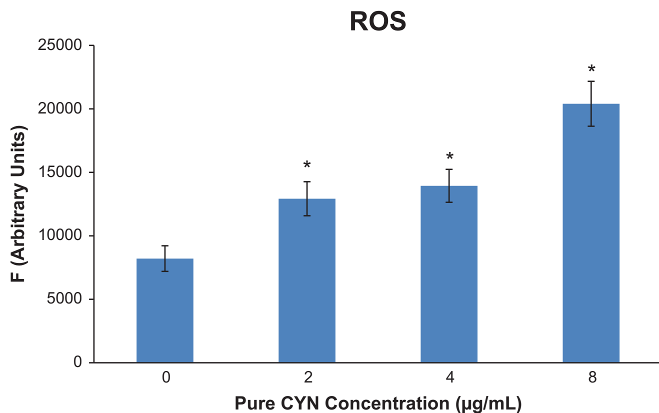

Fig. 4. ROS content in PLHC-1 cells after 24 h of exposure to 0, 2, 4 and 8 μg/mL pure CYN. All values are expressed as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

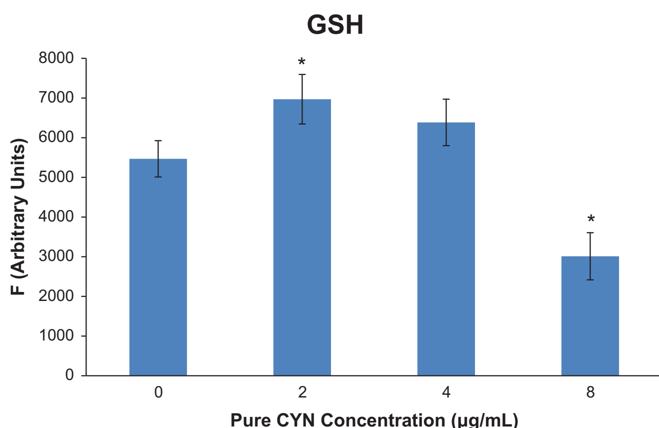

Fig. 5. GSH content in PLHC-1 cells after 10 min of exposure to 0, 2, 4 and 8 μg/mL pure CYN. All values are expressed as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

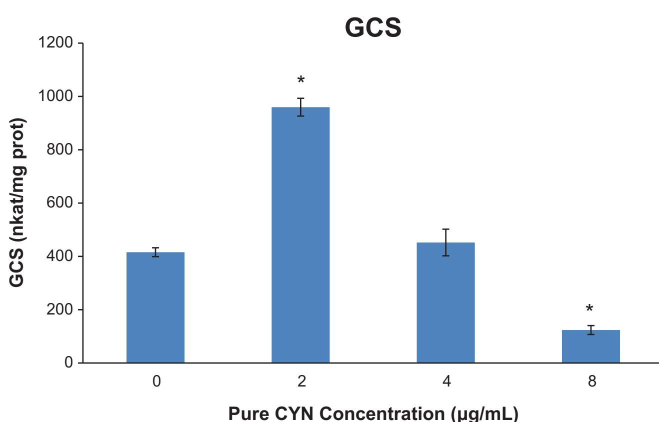

Fig. 6. GCS activity in PLHC-1 cells after 24 h of exposure to 0, 2, 4 and 8 μg/mL pure CYN. All values are expressed as nkat/protein and as mean ± s.d. *Significantly different from control ($p \leq 0.05$).

the GSH content was reduced 2-folds compared to the control group. Similarly, the GCS activity was enhanced by the exposure to 2 μg/ml of CYN, decreasing in a concentration dependent manner from that point with a 3-folds reduction at the highest concentration used in comparison with the control group (Fig. 6).

4. Discussion

Despite considerable research, much remains to be clarified with respect to the toxicity of CYN, especially on fish, as studies concerning its effects are still very scarce. The only work performed so far on fish has reported that CYN was toxic to zebrafish embryos when injected directly into them, but no effect was observed by direct immersion at doses up to 50 μg/ml (Berry et al., 2009). However, no work has been performed on isolated fish cells lines so far. To our knowledge this is the first work using a fish cell line as an experimental model to study CYN's toxic effects and the implications for GSH in its toxicity mechanism.

In the present work, cytotoxic effects have been studied. Protein content and NR assay results showed a decrease in the cellular viability in a time-concentration-dependent manner. In the same way, the MTS assay revealed that there is a reduction in the dehydrogenase activity when concentration and time increase. These results are in agreement with those obtained by Chong et al. (2002), who reported reductions in the tetrazolium salt MTT, when human cell line KB was exposed to CYN. These decreases were significantly different from the control at the higher concentration used (3.2 μg/ml) after 24 and 48 h of exposure, meanwhile at 72 h a further decrease was observed. Similarly, Chong et al. (2002) also reported a dose- and time-dependent toxicity for CYN in rat primary hepatocytes. Also a time-dependent response was observed on human granulosa cells exposed to CYN, showing no effects in the MTT assay after 2–6 h exposure to 0–1 μg/ml CYN (Young et al., 2008). However, exposure to CYN during a longer period of time (24, 48 and 72 h) resulted in a concentration-dependent cell viability reduction on human granulosa cells, reporting a significant decrease from 0.5 μg/ml CYN after 48 and 72 h to 1 μg/ml CYN after 24 h exposure (Young et al., 2008).

The cytotoxic effects induced by CYN exposure compared with those caused by other cyanobacterial toxins, such as microcystins (MCs), on the fish cell lines PLHC-1 and RTG-2 exposed to pure MC-LR, -RR and -YR have shown similar results (Pichardo et al., 2005, 2007). According to the protein content and MTS assay, CYN was reported to be more toxic than MC-LR (EC_{50} of 132 μg/ml and 47 μg/ml, respectively) and MC-RR (EC_{50} of 79 and > 100 μg/ml, respectively). Meanwhile considering the NR uptake CYN has similar toxic potency to MC-LR (EC_{50} 12 μg/ml), with MC-RR being less toxic (> 100 μg/ml). Chong et al. (2002) also reported cell viability decreases measured by the MTT assay in both CYN and MC-LR exposures on rat primary hepatocytes, showing that CYN was less toxic than MC-LR with 72 h LC_{50} of 40 and 8 ng/ml, respectively. However, other authors have pointed out that CYN exhibits a more pronounced toxic effect compared to MC-LR in CHO-K1 cells (Gácsi et al., 2009).

Different permanent cells lines have been used to test CYN toxicity. As instance, Froscio et al. (2009) performed a cytotoxicity screening test in seven cell lines representative of the organs targeted by this cyanotoxin (liver, kidney and intestine) after exposure for 24 h and 7 days, showing a time dependent effect of the toxin. Their study revealed a greater sensitivity of the hepatic cell lines with 24 h IC_{50} for CYN cytotoxicity (MTT assay) around 1.5 μM for both cell lines, C3A and Hep-G2. This greater sensitivity of rat primary hepatocytes and hepatic cell lines in comparison with colonic and fibroblast cell line has been also reported by many other authors (Fastner et al., 2003; Bain et al., 2007; Bazin et al., 2010) in exposure to pure CYN and *C. raciborskii* extracts. The differences observed in the effects induced by CYN in different mammalian cell lines may be attributed to the CYP450 profile expressed by each cell type, and consequently the production of toxic metabolites (Young et al., 2008). Moreover, the origin of the cell may have also had an influence on the extent of the

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effects induced by the toxin, with hepatic cells being the more sensitive (Froscio et al., 2009), probably due to the liver's higher metabolic activity compared to other organs. Consequently, considering *in vitro* experiments, the metabolic activity of the cell line used should be taken into account. In this sense, PLHC-1 constitutes a suitable experimental model to assess CYN toxic effects as it has been demonstrated in the present work.

Regarding the results obtained for the protein content assay in the present work, it seems predictable that among the cytotoxicity endpoints this might be the most sensitive, as the inhibition of protein synthesis is a well-known toxic mechanism for CYN (Terao et al., 1994). In comparison to the other cytotoxicity assays, significant decreases were evident from the lower concentration used at 48 h exposure. However, this reduction could be due not only to an inhibition in the protein synthesis, but also to the cellular disruption induced by CYN on the cells (Gácsi et al., 2009). Nevertheless, this finding suggests that the protein synthesis inhibition activity of CYN could be one of the mechanisms of action.

Concerning CYN's toxicity mechanism, it has been reported that it may inhibit not only the synthesis of protein but also that of GSH synthesis. In the present work, an increase in the GSH content was observed when cells were exposed to the lower concentration used. This finding can be explained because the enzyme responsible for this production, GCS, increased significantly at the same concentration of the toxin (2 μg/mL). Therefore, when PLHC-1 cells are exposed to low concentrations of CYN, cells try to face this assault by increasing the production of GSH in order to minimize the damage cause by the toxin. Nevertheless, a great reduction in GSH content was observed in exposure to higher concentrations, and this depletion was significant at 8 μg/mL. The decrease in levels of GSH in cells could result from decreased synthesis or increased utilization of this molecule to fight against oxidative situation. In the present work, we find evidence that GSH content is linked to the activity of the enzyme GCS since its activity also decreased following a similar pattern to that of GSH content reduction. Therefore we can conclude that the depletion of GSH may be due to an inhibition of its synthesis. These findings are in agreement with Runnegar et al. (1994, 1995) who also showed reduced GSH levels in hepatocytes incubated with CYN. In addition, GSH levels were also depleted significantly by 5 μM CYN exposure after 10 h exposure (Humpage et al., 2005). This finding has been also corroborated *in vivo* (Norris et al., 2002). Moreover, Runnegar et al. (1994) also pointed out that the fall in GSH caused by CYN was due to the inhibition of GSH synthesis rather than increased consumption, because in the latter case they proved that the rate of fall in GSH was not accelerated by the CYN pretreatment.

The reduction in GSH levels could lead to an increase in oxidative stress in the cell, and this could contribute to other damages such as genotoxicity. Hence, the study of oxidative stress biomarkers altered by CYN is of interest. In the present work the content of ROS has been measured, evidencing a concentration-dependent increase. In contrast, Humpage et al. (2005) reported no change in the lipid peroxidation product malondialdehyde in mouse hepatocytes exposed to 5 μM CYN for 12 h, concluding that ROS were not involved in CYN cytotoxicity. However, in the present work a relationship between cytotoxic effects and ROS production seems to be clear.

5. Conclusion

We can conclude that CYN induces cytotoxic effects on PLHC-1 cells, evidencing a relationship between this effects and ROS production. The protein content assay was found to be the most sensitive endpoint, suggesting that the protein synthesis inhibiting activity of CYN could be its primary mechanism of action. Moreover, it has been demonstrated that the depletion of GSH is due to an inhibition of its synthesis, because of the significant reductions in the activity of the enzyme GCS observed, suggesting a possible toxic mode of action of CYN. Moreover, PLCH-1 has been demonstrated to be a suitable experimental model to test CYN toxic effects on fish considering the lack of knowledge in this field and the environmental implication of the aforementioned cyanotoxin. Accordingly, this fish cell line could be useful in assessing toxic effects in real scenarios where CYN may be present.

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CAPÍTULO 4 / CHAPTER 4

Daniel Gutiérrez-Praena, Silvia Pichardo, Ángeles Jos, Ana M. Cameán

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

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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

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ABSTRACT

Cylindrospermopsin (CYN) is a toxin produced by various cyanobacterial species that are increasingly being found in freshwater systems. Although CYN can have toxic effects in humans, domestic animals and wildlife, it has been subject to very little investigation (particularly in fish). It has been reported to deplete the cellular glutathione content but the role of oxidative stress in the pathogenicity of CYN in fish is unknown. For this reason tilapia fish were exposed to 200 μg/kg pure CYN through two different exposure routes-gavage and intraperitoneal injection-and sacrificed after 24 h and 5 days. The results showed an increase in NADPH oxidase activity (a biomarker of reactive oxygen species formation), lipid peroxidation (LPO) and protein oxidation; no changes in DNA oxidation; and a reduction in glutathione levels (GSH) and γ-glutamylcysteine synthetase (GCS) activity, the limiting enzyme in glutathione synthesis. The time of sacrifice had a bigger influence on the results than the exposure route because after 5 days some of the biomarkers assayed had recovered their pre-intoxication levels, which was not the case after 24 h.

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1. Introduction

Cylindrospermopsin (CYN) is a naturally occurring toxin produced by particular strains of *Cylindrospermopsis raciborskii* and other freshwater cyanobacterial species, including *Umezakia natans*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Anabaena bergii*, *Anabaena lapponica*, *Lygnbya wollei* and *Raphidiopsis curvata* (Fastner et al., 2003; Pearson et al., 2010). Its chemical structure was elucidated by Ohtani et al. (1992). It consists of a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992), and has a molecular formula of $C_{15}H_{21}N_5O_7S$ and a molecular weight of 415.43 (Lewis, 2000). The presence of this cyanotoxin in freshwater systems is increasingly being documented in the scientific literature (Saker et al., 2004; Quesada et al., 2006; Brient et al., 2009; Fathaili et al., 2010; Messineo et al., 2010) probably because climate change favors the emergence of cyanobacterial blooms (Paerl and Huisman, 2008).

CYN is a highly biologically active alkaloid that interferes with several metabolic pathways. The liver is widely regarded as its main target but it also has general cytotoxic (Runnegar et al., 1994, 1995, 2002) and neurotoxic (Kiss et al., 2002) effects and it is considered to be a potential carcinogen (Humpage et al., 2000). Various toxic mechanisms have been suggested to explain its pathogenicity. It has proved to be a potent inhibitor of protein synthesis in an *in vitro* rabbit reticulocyte globin synthesis assay (Terao et al., 1994) and to deplete mouse hepatic glutathione *in vivo* (Norris et al., 2002), although in this latter case the effect was not of sufficient magnitude to represent the primary mechanism of CYN toxicity.

Different aquatic animals accumulate CYN in different ways. Kinnear et al. (2009) noted an emerging pattern whereby lower-level organisms accumulate greater concentrations of CYN toxin than other, more biologically complex, animals. 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. Thus, fish and other organisms with highly advanced toxin-metabolism systems are at greater risk of secondary CYN toxicity (Pearson et al., 2010). It has also been demonstrated that CYN is metabolized via the cytochrome P450 pathway, which results in compounds with increased toxicity (Norris et al., 2002).

CYN has the potential to impact a wide variety of aquatic and semi-aquatic plant and animal species. Furthermore, because it makes bioaccumulation and trophic transfer possible, toxicity may also be exerted on first, second and higher-order consumers in aquatic food webs. This could extend the damage CYN makes to the environment to include terrestrial organisms (Kinnear, 2010).

Research into CYN has been performed with purified toxin, extracted toxin or cyanobacterial cells containing the toxin, and differences in toxicity have been revealed. Seifert et al. (2007)

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demonstrated that significant adverse effects were rarely recorded for exposure to CYN concentrations below 100 μg/L of pure toxin. This was true for a range of species including a floating macrophyte, green algae, and a range of aquatic invertebrates from various trophic levels. On the other hand, exposure to *C. raciborskii* extracts resulted in greater sub-lethal toxicities. 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, so increasing the risk of toxic effects (Kinnear, 2010). This has also been observed by other authors in toxicity studies in mice (Falconer et al., 1999). Therefore, it is important to differentiate between the toxic effects induced by CYN per se from those induced by other bioactive substances from unpurified cyanobacterial materials. This observation was first made in other cyanobacterial toxins, such as microcystins (MCs) (Falconer, 2007).

Toxic effects induced by pure CYN on fish have scarcely been reported in the scientific literature, although aquatic organisms are probably exposed when a cyanobacterial bloom occurs. In tilapia exposed by gavage to 200 and 400 μg/kg bw pure CYN and sacrificed after 24 h, Puerto et al. (in press-a) observed increased lipid peroxidation (LPO) and alterations in the activity and gene expression of glutathione peroxidase (GPx) and glutathione-S-transferase (GST). This suggests that oxidative stress induction could play a role in CYN pathogenicity. Moreover, a histopathological study revealed dose-dependent alterations in liver, kidney, heart, intestines and gills (Puerto et al., in press-b). Other cyanobacterial toxins, such as MCs, have been more extensively investigated in piscine models, and it has been shown that after a single exposure the oxidative stress biomarkers studied varied in fish sacrificed after 24 h with respect to those sacrificed after 72 h (Prieto et al., 2007). CYN toxicity also depends on the time of sacrifice. Thus, the median lethal dose (LD_{50}) in mice after intraperitoneal (i.p.) injection of pure CYN is 2.1 mg/kg bw after 24 h, but it is 10-fold lower (LD_{50} = 0.2 mg/kg bw) when the observation period is prolonged to 120–144 h (Ohtani et al., 1992).

In this context the aim of this study was to investigate the influence of the time of sacrifice and the exposure route on the induction of oxidative stress as a toxicity mechanism associated to CYN pathogenicity in fish. For this purpose Tilapia (*Oreochromis niloticus*) were acutely exposed by gavage and i.p. injection to 200 μg/kg bw pure CYN. The animals were sacrificed after either 24 h or 5 days. The biomarkers assayed included NADPH oxidase activity, a biomarker of O_2^- production, lipid peroxidation (LPO), DNA oxidation, protein oxidation, GSH levels and γ-glutamylcysteine synthetase (GCS), the limiting enzyme for GSH synthesis, activity.

2. Material and methods

2.1. Chemicals

The cyanotoxin cylindrospermopsin standard (purity ≥95%) was supplied by Alexis Corporation (Lausen, Switzerland). Chemicals for the different assays were provided by Sigma–Aldrich (Madrid, Spain) and VWR International Eurolab (Seville, Spain). The protein assay reagent was obtained from BioRad Laboratories (Hercules, USA).

2.2. Experimental setup and acclimation of fish

Studies were conducted using male *O. niloticus* (Nile tilapia, Perciformes: Cichlidae) with mean weight 53.4 ± 5.2 g. Fish were obtained from Valenciana de Acuicultura SA (Valencia) and transferred to the laboratory where they were held in aquariums (8 individuals/aquarium) with 96 L of fresh water. Exposure to chlorine was minimized by filling the tanks at least 3 days before fish were introduced. The aquariums were equipped with a continuous system of water filtration and aeration (Eheim Liberty 150 with Bio-Espumador cartridges as skimmers 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 ± 0.2, conductivity 292 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 days before the beginning of the experiment.

2.3. Experimental exposure

The experiment was carried out using 8 aquaria with eight fish in each (n = 8). Fish in aquaria 1 and 2 were exposed by gavage to a single dose of 200 μg/kg in 0.5 mL of 0.9% (w/v) NaCl solution and were sacrificed after 24 h (aquarium 1) and 5 days (aquarium 2). Fish in aquaria 3 and 4 received only the vehicle solution (0.5 mL of 0.9%, w/v, NaCl), were sacrificed after 24 h (aquarium 3) and 5 days (aquarium 4), and were used as controls. Similarly, two other groups of fish (n = 8 per treatment) were exposed by i.p. injection to 200 μg/kg in 0.5 mL of 0.9% (w/v) NaCl solution and were sacrificed after 24 h (aquarium 5) and 5 days (aquarium 6). The corresponding control groups were injected with the vehicle only and were sacrificed after 24 h (aquarium 7) and 5 days (aquarium 8). The dose of pure CYN used in the experiments (200 μg/kg) was selected in accordance with a previous study performed in our lab that showed toxic effects in the same fish species exposed by gavage (Puerto et al., in press-a, in press-b).

2.4. Preparation of postmitochondrial supernatant (PMS)

At the end of the experiment, all fish were killed by transection of the spinal cord. Organs were quickly removed, weighed, rinsed with ice-cold saline, frozen in liquid nitrogen and kept at −85 °C until use. Enzyme extracts from tissues 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 LPO, protein oxidation, GCS activity and GSH content measurements. A different homogenization was performed to determine NADPH oxidase activity

2.5. 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).

2.6. NADPH oxidase determination

Chemiluminescence with lucigenin (Sigma–Aldrich, Madrid, Spain) was used to measure O_2^- production as previously described by Ohara et al., 1993 and Fortuño et al., 2005 with modifications. Samples were homogenized in phosphate buffer with protease inhibitor and centrifuged at 2000 × g for 10 min. A total of 10 μL sample, 5 μL NADPH, phosphate buffer and lucigenin were added to the cuvette to make a final volume of 300 μL. Luminescence was

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measured every 30 s for 4 min in a luminometer (Stratec Biomedical Systems AG, Germany). A buffer blank was subtracted from each reading. Measurements were expressed as relative light units (RLUs) per second.

2.7. Lipid peroxidation

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman, 1990). Malondialdehyde (MDA) was formed as an end lipid peroxidation product which reacted with TBA reagent under acidic conditions to generate a pink coloured product. Briefly, 0.5 mL of the homogenized tissue, obtained previously, and treated with 25 μl of butylhydroxytoluene 1% (v/v) in acetic acid, was mixed with 0.2 mL of sodium lauryl sulphate (8%), 1 mL of acetic acid (20%, v/v) 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-Tetraethoxypropan (TEP) was used as a standard.

Values were expressed as nmol thiobarbituric acid reactive substances (TBARs/g tissue).

2.8. Protein oxidation

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 protein, using the extinction coefficient 22,000/M/cm.

2.9. 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.

2.10. GSH levels

The GSH content was determined in liver homogenates using a commercial kit (Bioxytech GSH/GSSH-412; Oxis Research, Foster City, CA, USA) adapted to fish tissues (Atencio et al., 2008). Results are expressed as nmol GSH/mg protein.

2.11. GCS activity

GCS (EC 6.3.2.2.) is the rate limiting enzyme in GSH synthesis. Its activity was determined in liver tissue using the method described by Seelig and Meister, 1985 adapted to fish samples. The formation of ADP was monitored spectrophotometrically by coupled assay with pyruvate kinase and lactate dehydrogenase. The reaction mixture (final volume 1 mL) contained Tris–HCl buffer (100 mM, pH 8.2), sodium L-glutamate (10 mM), L-α-aminobutyrate (10 mM), $MgCl_2$ (20 mM), Na_2ATP (5 mM), sodium phosphoenolpyruvate (2 mM), KCl (150 mM), NADH (0.2 mM), pyruvate kinase (5 units), lactate dehydrogenase (10 units) and the enzyme sample. The rate of decrease in absorbance at 340 nm was monitored at 37 °C. The results were expressed as mU/mg protein.

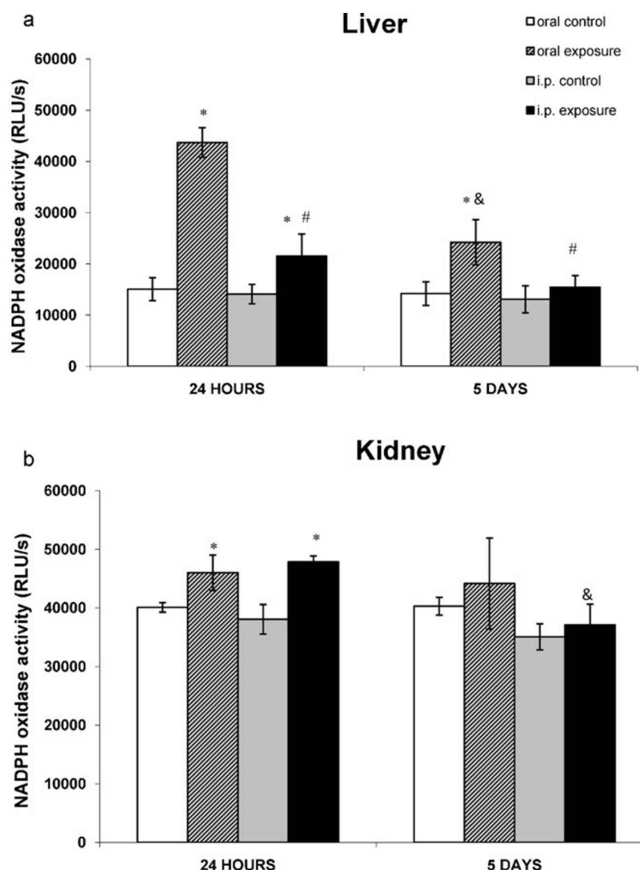

Fig. 1. NADPH oxidase activity (relative light units per second, RLU/s) in liver (a) and kidney (b) of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$). The significance levels observed are $^{*}p < 0.05$ in comparison to control group values; $^{\#}p < 0.05$ when fish were sacrificed at the same time and exposed by different routes; and $^{\&}p < 0.05$ when fish were exposed by the same route and sacrificed at different times.

2.12. Statistics

Data are expressed as mean ± standard deviation of eight animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA). When differences were significant, Tukey's test was used to compare the individual treatments. Statistical significance was inferred at $p < 0.05$.

3. Results

3.1. Effects of pure CYN on NADPH activity

When fish were exposed to pure CYN by gavage and i.p. injection and sacrificed 24 h later, NADPH oxidase activity was higher than the control levels in liver (Fig. 1). This significant increase was higher with the oral exposure (2.9-fold) than with the i.p. (1.5-fold). In fish sacrificed 5 days after the single exposure, the enzyme activity levels were lower than in those fish sacrificed after 24 h, and the fish exposed by gavage still showed a 1.6-fold increase with respect to the basal levels. In kidney, a 1.2-fold increase in activity was observed only in fish sacrificed after 24 h subject to either exposure route. In fish exposed by intraperitoneal injection there were significant differences in the results obtained at 24 h and 5 days.

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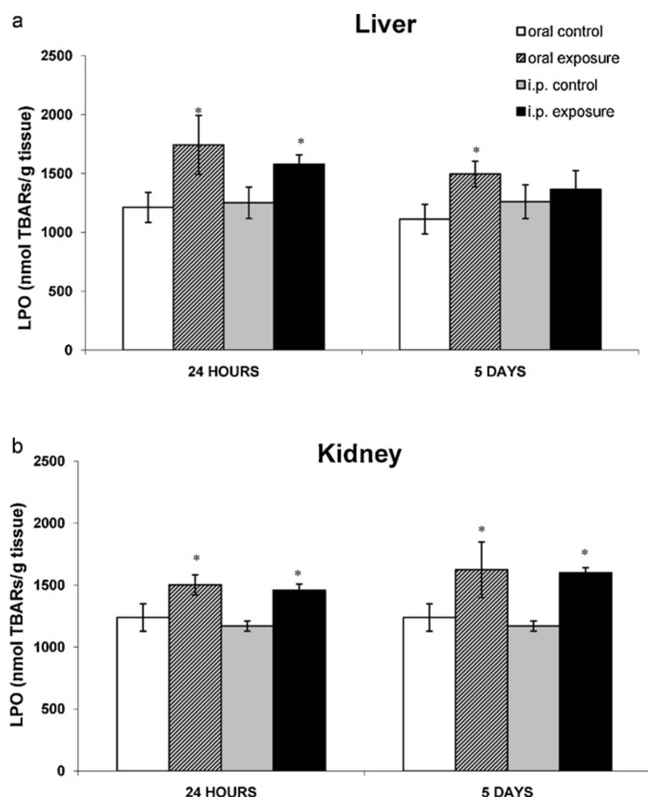

Fig. 2. Lipid peroxidation level (LPO) in liver (a) and kidney (b) of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$). LPO levels are expressed as nmol TBARs/g tissue. The significance levels observed are $^{*}p < 0.05$ in comparison to control group values.

3.2. Effects of pure CYN on lipid peroxidation

Lipid peroxidation products increased in the liver and kidney of fish orally exposed to pure CYN and sacrificed after 24 h (1.4-fold and 1.2-fold, respectively) and 5 days (1.2-fold and 1.3-fold, respectively) (Fig. 2). In those fish that had undergone i.p. exposure the same pattern was observed and no statistical differences were observed with respect to the control only in the liver of fish sacrificed after 5 days. Neither were any differences detected in fish exposed in the same way and sacrificed at both times, or in fish sacrificed at the same time and exposed in a different way.

3.3. Effects of pure CYN on protein oxidation

In liver tissue, protein oxidation only increased in fish sacrificed after 5 days (1.5-fold for both exposure routes) (Fig. 3). In kidney no changes were observed in fish exposed orally whereas those injected intraperitoneally showed a significant increase at both sacrifice times (1.3-fold and 1.5-fold, respectively). Results in kidney showed that the route of exposure influences this biomarker.

3.4. Effects of pure CYN on DNA oxidation

No significant changes in DNA oxidation were observed in the liver of fish in any of the experimental groups (Fig. 4).

3.5. Effects of pure CYN on GSH content

Fish exposed to pure CYN by gavage and sacrificed after 24 h experienced a significant GSH depletion (1.3-fold) in the liver. Those sacrificed after 5 days, however, showed no statistical differences with respect to the control group (Fig. 5). The fish that had been injected intraperitoneally also experienced a significant depletion at 24 h (1.2-fold) and a recovery of the GSH basal levels when fish were sacrificed 5 days after exposure to the toxin. Statistical differences were also observed between fish subject to either exposure route and sacrificed at the same time, and between fish subject to the same exposure route and sacrificed at different times.

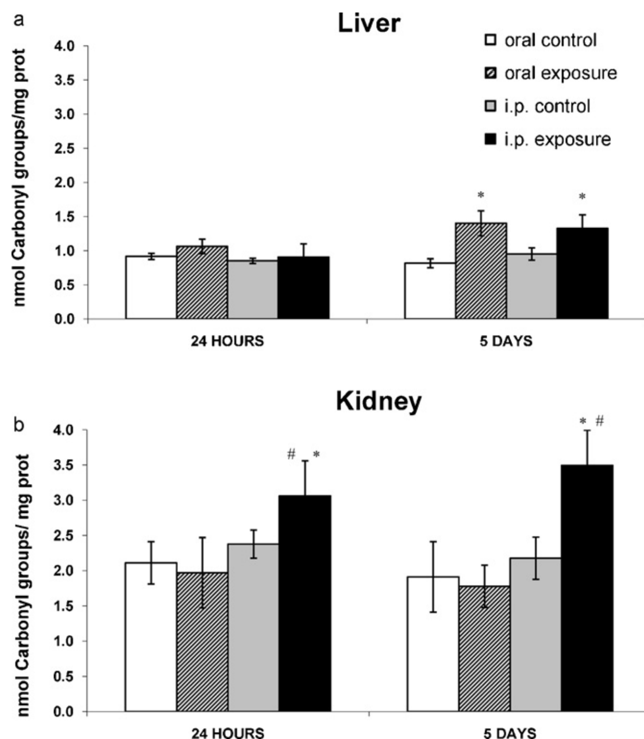

Fig. 3. Protein oxidation values (nmol carbonyl/mg protein) in liver (a) and kidney (b) of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$). The significance levels observed are $^{*}p < 0.05$ in comparison to control group values, and $^{\#}p < 0.05$ when fish were sacrificed at the same time and exposed by different routes.

3.6. Effects of pure CYN on GCS activity

GCS activity in the liver of fish showed the same pattern in both exposure types. A reduction in enzyme activity was observed in fish sacrificed at 24 h after both oral and i.p. exposures (1.2-fold and 1.7-fold decrease, respectively) (Fig. 6). Five days after the exposure, no statistical differences with respect to the control groups were observed. However, there was a statistically significant difference

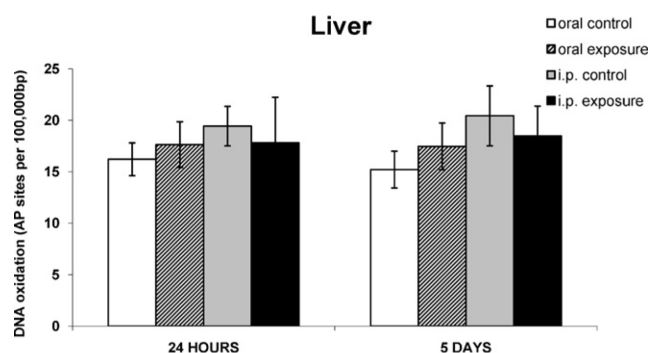

Fig. 4. DNA oxidation (AP sites per 100,000 bp) in the liver of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$).

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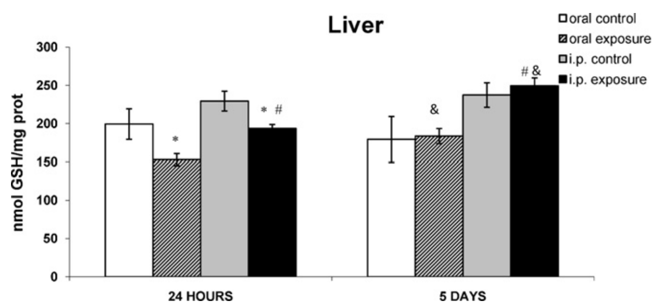

Fig. 5. Glutathione (GSH) levels (nmol GSH/mg protein) in the liver of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$). The significance levels observed are $^{*}p < 0.05$ in comparison to control group values; $^{\#}p < 0.05$ when fish were sacrificed at the same time and exposed by different routes; and $^{\&}p < 0.05$ when fish were exposed by the same route and sacrificed at different times.

between these fish and those sacrificed 24 h after exposure. Of the two exposure routes, i.p. injection is observed to have a considerably greater effect. The enzyme activity in fish sacrificed 24 h and 5 days after exposure was 1.7-fold and 1.5-fold lower, respectively, in fish exposed in this way.

4. Discussion

Very few reports are available on the toxic effects of CYN on fish even though this toxin is increasingly being found in freshwater ecosystems. To our knowledge this is the first article that explores the influence of the exposure route and the time of sacrifice on the effects of pure CYN on oxidative stress biomarkers using *O. niloticus* as the experimental model.

The toxic mechanisms involved in CYN pathogenicity have not been fully elucidated. It is known that CYN inhibits protein synthesis (Terao et al., 1994), but the protein content of liver and kidney homogenates was not observed to decrease significantly in this study (data not shown). Oxidative stress is a toxic mechanism that has been associated with the pathogenicity of other cyanobacterial toxins, such as MCs, in fish (Jos et al., 2005; Prieto et al., 2006; Cazenave et al., 2006). Whether CYN can induce oxidative damage or not has been subject to very little examination, and the reports that do exist are controversial: Silva et al., 2010 state that it can while Humpage et al., 2005 disagree. We observed increased NADPH oxidase activity, a biomarker of ROS formation, in liver and kidney, and particularly in fish sacrificed after 24 h. Similarly, Puerto et al., in press-b observed an increase in this biomarker in fish exposed by gavage to different CYN doses. The fact that NADPH oxidase activity was observed to be lower in fish sacrificed after 5 days than after 24 h could be due to the initial recovery of the oxidative status of the fish. Little is known about the detoxification of CYN. It has been reported that when this cyanotoxin is metabolized by CytP450 (Norris et al., 2002), more toxic unknown metabolites are produced. The ways in which these compounds are detoxified and the time the process takes still have to be elucidated.

In the present study, the increase deduced for ROS formation correlated with an increase in LPO products. Again, a greater toxic effect was observed in fish sacrificed 24 h after exposure. Puerto et al., in press-a also observed an increase in this biomarker in tilapia exposed to pure CYN, and Gutiérrez-Praena et al. (in press) in the PLHC-1 cell line derived from *Poeciliopsis lucida* liver. Prieto et al., 2007 evaluated this biomarker in fish exposed to a single dose of MCs and sacrificed after 24 and 72 h, and also found an increase with a similar pattern in liver. We suggest that the LPO values recover in liver because in our study they were lower 5 days after intoxication than after 24 h and no changes were observed after i.p. exposure. In contrast to our results, Humpage et al., 2005 did not observe an increase in malondialdehyde in rat isolated hepatocytes exposed to 5 μM CYN for 12 h.

Protein oxidation increased more in kidney than in liver. Indeed, other authors consider the kidney to be the main target of this toxin (Humpage and Falconer, 2003). In liver, protein oxidation was only altered in fish sacrificed after 5 days. Although the effects were higher in kidney, changes were only significant for i.p. exposure, which indicates that this route has a greater toxic effect on this biomarker. Atencio et al. (2008) found no changes in tench exposed to acute doses of *Microcystis* and sacrificed after 96 h.

No changes in DNA oxidation were observed. Some reports have demonstrated the genotoxicity of CYN but have not suggested an oxidative stress mediated mechanism for DNA damage (Humpage et al., 2005). DNA fragmentation (Shen et al., 2002) and modification (Shaw et al., 2000) have been observed in the livers of treated mice, while in human lymphoblastoid cells, CYN has been shown to induce the formation of centromere-negative micronuclei, indicating double-stranded DNA breakage (Humpage et al., 2000).

We observed a decrease in GSH levels in the liver of fish exposed by gavage and i.p. injection, and sacrificed after 24 h. Norris et al., 2002 also demonstrated a CYN induced depletion of mouse hepatic GSH *in vivo*, although they did not consider the effect to be of sufficient magnitude to be the primary mechanism of CYN toxicity. This cyanotoxin also decreased glutathione levels and the

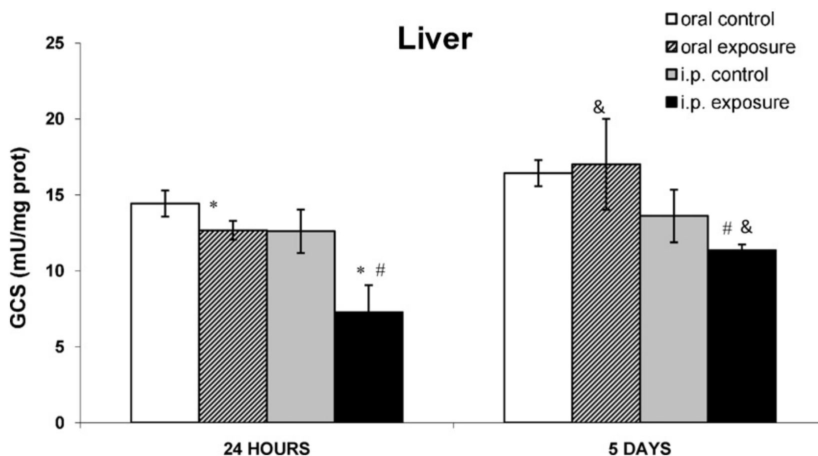

Fig. 6. γ-glutamylcysteine synthetase (GCS) activity (mU/mg protein) in the liver of fish exposed by gavage and intraperitoneal injection to 200 μg/kg bw CYN and sacrificed after 24 h and 5 days. The values are expressed as mean ± SD ($n = 8$). The significance levels observed are $^{*}p < 0.05$ in comparison to control group values; $^{\#}p < 0.05$ when fish were sacrificed at the same time and exposed by different routes; and $^{\&}p < 0.05$ when fish were exposed by the same route and sacrificed at different times.

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synthesis of glutathione and protein in cultured rat hepatocytes (Runnegar et al., 1994, 1995, 2002). In fish exposed to a single dose and sacrificed after 5 days, GSH levels recovered as no changes were observed with respect to the control. This could be due to the action of detoxification and recovery mechanisms, which were able to counteract the alteration in this biomarker in this period of time.

The GSH depletion observed agreed with the inhibition of GCS activity, the limiting enzyme in GSH synthesis, and both biomarkers followed the same temporal pattern. Runnegar et al., 1995 reported that the inhibition of GSH synthesis was the predominant mechanism for the reduction in GSH, and effectively ruled out other mechanisms such as increased consumption, increased formation of oxidized GSH, increased GSH efflux, hidden forms of GSH, decreased precursor availability and decreased cellular ATP. In fish that had been exposed by gavage and sacrificed after 5 days, the activity was no different from that of the control group, and the decrease observed in fish sacrificed after 24 h was no longer apparent. In fish exposed by i.p. injection, however, activity partially recovered in comparison to 24 h data, but did not reach control levels. Amado et al., 2011 observed similar effects-a decrease in the GSH concentration and the activity of the enzyme involved in its synthesis-in carp exposed to MCs.

Overall, the toxic effects of CYN were evident 24 h and 5 days after the exposure. Some biomarkers tended to recover their basal levels over time (NADPH oxidase activity, LPO, GSH and GCS activity in liver) while others showed bigger alterations (protein oxidation and LPO in kidney). CYN has been reported to have a late and progressive acute toxicity (Funari and Testai, 2008). This has been explained by the existence of two routes of toxic action: a rapid route probably through a CYP450 oxidation product of the toxin (Runnegar et al., 1995; Froscio et al., 2001) and a slower mechanism through the well-documented inhibition of protein synthesis, which does not require toxin metabolism (Froscio et al., 2001). The present study has not shown a decrease in the protein content of the tissue homogenates, and this may explain why the results obtained do not indicate greater toxicity over time. The two exposure routes also led to different results but there are no clear differences between them in terms of the severity of the toxic effects. Oral exposure led to higher alterations in NADPH oxidase activity and LPO in liver, and i.p. exposure resulted in higher toxic effects in LPO and protein oxidation in kidney and GCS activity in liver. Other authors have also observed that the nature, location, and time course of histological damage were similar for oral and intraperitoneal administration of *C. raciborskii* extracts in mice, with maximum damage being observed 2–3 days after treatment (Falconer et al., 1999). Likewise, Hawkins et al., 1985 and Seawright et al., 1999 found no significant pathological differences between preparations of CYN (suspension of the freeze dried culture or purified compound) or between oral and intraperitoneal administration (Norris et al., 2002).

In conclusion, pure CYN induces oxidative stress in tilapia fish exposed by gavage and i.p. injection as revealed by alterations in NADPH oxidase activity, LPO levels, protein oxidation, GSH levels and GCS activity. These effects are influenced by the exposure route and the time of sacrifice.

Acknowledgements

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CAPÍTULO 5 / CHAPTER 5

Daniel Gutiérrez-Praena, Silvia Pichardo, Ángeles Jos, Ana M. Cameán

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).

Enviado a Water Research, **2011.**

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Manuscript Draft

Manuscript Number:

Title: 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)

Article Type: Special Issue: Cyanobacteria

Keywords: Cylindrospermopsin; cyanotoxin; glutathione transferase; glutathione peroxidase; fish

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Order of Authors: Daniel Gutiérrez-Praena; A. Jos; Silvia Pichardo; María Puerto; Ana M Cameán

Abstract: Cylindrospermopsin is a cyanobacterial toxin frequently implicated in cyanobaterial blooms that is approaching an almost cosmopolitan distribution pattern. Moreover, the predominantly extracellular availability of this cyanotoxin makes it particularly likely to be taken up by a variety of aquatic organisms including fish. Recently, Cylindrospermopsin has shown to alter the activity and gene expression of some of the glutathione related enzymes in tilapias (Oreochromis niloticus), but little is known about the influence of the route of exposure and the time of sacrifice after a single exposure to Cylindrospermopsin on these biomarkers. With this aim, tilapias were exposed by gavage or by intraperitoneal injection to a single dose of 200 µg/kg bw of pure Cylindrospermopsin and after 24 hours or 5 days they were sacrificed. The activity and relative mRNA expression by real-time PCR of antioxidant enzymes glutathione peroxidase and soluble glutathione-S-transferases (sGST) and the sGST protein abundance by Western blot analysis were evaluated in liver and kidney. Results showed differential responses in dependence on the variables considered with a higher toxicity with the intraperitoneal exposure and with 5 days as time of sacrifice in general terms.

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Cover Letter, For Editor only

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October, 26 2011

Department of Biochemical Engineering
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Netherlands,

Dear Prof. van Loosdrecht,

We would be very grateful if you consider the manuscript entitled "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*)" for its publication in the special issue of Cyanobacteria in "**Water Research**".

The authors of the article were: Daniel Gutiérrez-Praena, Angeles Jos*, Silvia Pichardo, María Puerto, Ana M Cameán.

Studies on the toxic effects of cylindrospermopsin on fish are scarce, moreover those dealing with molecular biomarkers. In the present work we investigate the influence of the exposure way and the time of sacrificed after a single exposure in tilapia

I am looking forward to receiving a positive answer from you.

Sincerely,

Angeles Jos

Highlights

- CYN induce oxidative stress in fish
- CYN alters the activity and gene expression of GST and GPx enzymes
- Time of sacrifice and exposure way influence the results
- The higher effects are induced after 5 days of an intraperitoneal exposure

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1 Influence of the exposure way and the time of sacrifice on the effects induced by a
2 single dose of pure cylindrospermopsin on the activity and transcription of glutathione
3 peroxidase and glutathione-S-transferase enzymes in Tilapia (*Oreochromis niloticus*)
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14

15 **Abstract**

16

17 Cylindrospermopsin is a cyanobacterial toxin frequently implicated in cyanobaterial
18 blooms that is approaching an almost cosmopolitan distribution pattern. Moreover, the
19 predominantly extracellular availability of this cyanotoxin makes it particularly likely to
20 be taken up by a variety of aquatic organisms including fish. Recently,
21 Cylindrospermopsin has shown to alter the activity and gene expression of some of the
22 glutathione related enzymes in tilapias (*Oreochromis niloticus*), but little is known about
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24 to Cylindrospermopsin on these biomarkers. With this aim, tilapias were exposed by
25 gavage or by intraperitoneal injection to a single dose of 200 μg/kg bw of pure
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28 peroxidase and soluble glutathione-S-transferases (sGST) and the sGST protein
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30 showed differential responses in dependence on the variables considered with a higher
31 toxicity with the intraperitoneal exposure and with 5 days as time of sacrifice in general
32 terms.

33

34

35 **Keywords:** Cylindrospermopsin, cyanotoxin, glutathione transferase, glutathione
36 peroxidase, fish

37

38

39 **Abbreviations**

40

41 cDNA: complementary desoxirribonucleic acid

42 CDNB: 1-chloro-2,4-dinitrobenzene

43 CYN: Cylindrospermopsin

44 CytP450: cytochrome P450

45 GPx: glutathione peroxidase

46 GR: glutathione reductase

47 GSH: glutathione

48 GST: glutathione S-transferase

49 i.p.: intraperitoneal

50 LD_{50}: Letal Dose 50

51 LPO: lipid peroxidation

52 MCs: microcystins

53 mRNA: messenger ribonucleic acid

54 PMSF: phenylmethylsulfonylfluoride

55 RNA: ribonucleic acid

56 RT-PCR: real time polymerase chain reaction

57 sGST: soluble glutathione S-transferase

58

59

60

61 **1. Introduction**
62
63 Cylindrospermopsin (CYN) is a cyanotoxin that has been frequently implicated
64 in cyanobaterial blooms (Fessard and Bernard, 2003). A cyanobacterial bloom consists
65 on a massive growth of these bacteria on the water and they are caused by a complex
66 interaction of high concentrations of nutrients, sunlight, warm temperature, turbidity,
67 pH, conductivity, salinity, carbon availability and slow-flowing or stagnant water (van
68 Apeldoorn et al., 2007). This toxin is now approaching an almost cosmopolitan
69 distribution pattern and CYN producers are recorded from habitats including lakes,
70 reservoirs, rivers, ponds and dams (Kinnear, 2010).
71
72 Due to their habitat, aquatic organisms are exposed to both, the toxic
73 cyanobacterial cells and to the released toxins. Moreover, there are reports regarding
74 to the accumulation of CYN in redclaw crayfish (*Cherax quadricarinatusin*) in field and
75 laboratory trials and also in rainbow fish (*Melanotaenia eachamensis*) under natural
76 conditions (Saker and Eaglesham, 1999). But little is still known about the toxicity of
77 CYN on fish, in comparison to other cyanobacterial toxins more extensively studied
78 such as microcystins (MCs). From a general point of view CYN has shown to be a
79 protein synthesis inhibitor (Terao et al., 1994; Runnegar et al., 1995) and even to
80 induce genotoxicity (Humpage et al., 2000). It is classified as a cytotoxin (Kinnear,
81 2010) or a hepatotoxin (Ohtani et al., 1992), although other organs can also be affected
82 (Chong et al., 2002). It has been also reported that CYN affects glutathione (GSH)
83 synthesis in rat hepatocytes *in vitro* (Runnegar et al., 1994; 1995). *In vivo*, Norris et al.,
84 (2002) demonstrated that GSH depletion is unlikely to be of primary importance in
85 hepatic toxicity of CYN in mouse. As GSH serves as a substrate for Glutathione S-
86 transferase (GST) and Glutathione peroxidase (GPx) the response of these enzymes
87 to a CYN exposure is of interest.
88
89 LD_{50} data of CYN on fish is not known to the extent of our knowledge but its
90 toxicity has been tested on mice through intraperitoneal (i.p.) injection (Ohtani et al.,
91 1992; Harada et al., 1994; Terao et al., 1994; Falconer et al., 1999) and the oral route
92 (Falconer et al., 1999; Seawright et al., 1999). The 24h LD_{50} (via i.p. injection) was
93 found to be 2.1 mg/kg. With a lower dose of CYN (0.2 mg/kg) the mice died 5-6 days
94 after injection (Ohtani et al., 1992). The 5-day oral LD_{50} was shown to be approximately
95 6 mg/kg (Ohtani et al., 1992; Chong et al., 2002). The exposure route has therefore an
96 important role on the toxicity of CYN as it has been demonstrated in fish (Gutiérrez-
97 Praena et al., 2011a). Moreover, the time of sacrifice after a single CYN exposure can

98 also influence the changes observed in the toxicity biomarkers assayed. Thus, Prieto et
99 al. (2007) found time-dependent oxidative stress responses in tilapia (*Oreochromis*
100 *niloticus*) exposed to a single dose of MCs and sacrificed in 24 and 72h. These
101 differences are in partly due to distribution and elimination processes and the activation
102 of the defence mechanisms in the organism. In this regard, MCs are known to be
103 detoxified by GSH conjugation (Pflugmacher et al., 1998) but CYN is metabolized
104 through the cytochrome P450 (CytP450) pathway leading to more toxic compounds
105 (Norris et al., 2002).
106
107 Other important issue to consider is the differential toxicity between pure CYN
108 and CYN extracted or contained in cyanobacterial material (cultures, blooms). Thus,
109 Falconer et al. (1999) performed a toxicity study to mice after i.p.administration of four
110 different batches of *Cylindrospermopsis raciborskii* cell extracts and observed that the
111 variability in the *in vivo* toxicity and the histological damage did not correlate with, nor
112 was explained by, the known CYN content of the lysates employed. The implication
113 drawn from these data was that more than one toxin was likely to be present in that
114 organism. Previous research in our lab has shown that tilapia exposed by gavage to
115 200 and 400 μg/kg bw of pure CYN and sacrificed after 24h had alterations in different
116 biomarkers such as lipid peroxidation (LPO), activity and gene expression of the
117 enzymes GPx and GST, NADPH oxidase activity and GSH content (Puerto et al.,
118 2011a; 2011b). Moreover, an histopathological study revealed dose-dependent effects
119 in liver, kidney, heart, intestines and gills.
120
121 In the present study the influence of the exposure way and the time of sacrifice
122 of the fish after an acute dose of pure CYN were investigated, in order to know possible
123 differences in the activities and gene expression of some of the GSH related enzymes.
124 For this purpose, Tilapia were acutely exposed to 200 μg/kg bw pure CYN by gavage
125 and by i.p. injection and after 24h and 5 days they were sacrificed. The biomarkers
126 assayed included activity and gene transcription of GPx and soluble glutathione-S-
127 transferase (sGST) and protein abundance of sGST.
128
129
130 **2. Material and Methods**
131
132 *2.1 Chemicals*
133 The cyanotoxin Cylindrospermopsin standard (purity $\geq$ 95%) was supplied by
134 Alexis Corporation (Lausen, Switzerland). Chemicals for the different assays were

135 provided by Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab (Seville,
136 Spain). Protein assay reagent was obtained from BioRad Laboratories (Hercules,
137 USA).
138
139 *2.2. Experimental setup and acclimation of fish*
140 Studies were conducted using male *Oreochromis niloticus* (Nile tilapia,
141 Perciformes: Cichlidae) with mean weight 50.2 ± 5.8 g. Fish were obtained from
142 Valenciana de Acuicultura S.A. (Valencia) and transferred to the laboratory where they
143 were held in aquaria (8 individuals/aquarium) with 96 L of fresh water. Exposure to
144 chlorine was minimized by filling the tanks at least 3 days before fish were introduced.
145 The aquariums were equipped with continuous system of water filtration and aeration
146 (Eheim Liberty 150 with Bio-Espumador cartridges as skimmers and the temperature
147 was kept constant (21 ± 2°C).Dissolved oxygen values were maintained between 6.5
148 and 7.5 mg/L. Mean values for additional parameters of water quality were: pH 7.6±
149 0.2, conductivity 292 µS/cm, Ca^{2+} 0.60 mM/L and Mg^{2+} 0.3 mM/L. Fish were fed with
150 commercial fish food (ciprinidos, 2 mm, Dibaq, Segovia, Spain) and were acclimatized
151 for 15 days before the beginning of the experiment.
152
153 *2.3. Experimental exposure*
154 The experiment was carried out using 8 aquaria with eight fish in each (n=8).
155 Fish in aquaria 1 and 2 were exposed by gavage to a single dose of 200 µg/kg in
156 0.5mL of 0.9% (w/v) NaCl solution and were sacrifice after 24h (aquarium 1) and 5
157 days (aquarium 2). Fish in aquaria 3 and 4 received only the vehicle solution (0.5mL of
158 0.9%, w/v, NaCl), were sacrificed after 24h (aquarium 3) and 5 days (aquarium 4), and
159 were used as controls. Similarly, other two groups of fish (n=8 per treatment) were
160 exposed by i.p. injection to 200 µg/kg in 0.5 mL of 0.9% (w/v) NaCl solution and were
161 sacrificed after 24h (aquarium 5) and 5 days (aquarium 6). The corresponding control
162 groups were injected with the vehicle only and were sacrificed after 24h (aquarium 7)
163 and 5 days (aquarium 8). The dose of pure CYN used in the experiments (200 µg/kg)
164 was selected in accordance with a previous study performed in our lab that showed
165 toxic effects in the same fish species exposed by gavage (Puerto et al., 2011a, 2011b).
166
167
168 *2.4. Preparation of postmitochondrial supernatant*
169 At the end of the experiment, all fish were anesthetized in ice water and killed
170 by transection of the spinal cord. Liver and kidney were quickly removed, weighed,
171 rinsed with ice-cold saline, frozen in liquid nitrogen and kept at -85°C until use. Enzyme

172 extracts from each tissue were prepared from each individual (not pooled) according to
173 the method described by Puerto et al., 2009. Briefly, tissues were homogenized using
174 0.1 M potassium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol, 1 mM
175 ethylenediaminetetra-acetic acid, and 1.4 mM dithioerythritol. After removal of cell
176 debris (10 min at 13,000 *g*), the membrane fraction was separated by centrifugation at
177 105,000 *g* for 60 min. The remaining supernatant, defined as the soluble (cytosolic)
178 fraction, was used for GPx and sGST activity measurements.
179
180 *2.5. Protein estimation*
181 Protein contents in the samples were estimated by the method of Bradford
182 (1976) using bovine γ-globulin as standard. Briefly, 5 µL of the diluted samples were
183 mixed with 95 µL H_2O and 5 mL Coomassie Brilliant blue dye (Biorad Laboratories,
184 Hercules, USA) and the absorbance was read at 595 nm in the spectrophotometer
185 (Cary100, Varian, Madrid, Spain).
186
187 *2.6. Glutathione peroxidase and glutatione-S-transferase activities*
188 Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed following the
189 rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase
190 (GR). The assay mixture contained 600µL buffer with 50mM potassium phosphate+
191 1mM EDTA+1mM NaN3, (pH 7.5); 100µL 0.2mM GSH, 100 µL 0.1 mM NADPH, 8 µL
192 GR and homogenized tissue (20 µL). After 5 min of preincubation (20–25 ^{o}C), the
193 reaction was initiated by the addition of 100 µL 0.25 mM H_2O_2.The specific activity was
194 determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1} (Lawrence and Burk,
195 1976).
196 Glutathione *S*-transferase activity (sGST; EC 2.5.1.18) was measured in the liver and
197 renal homogenates according to the method described by Habig et al. (1974), by
198 monitoring at 340 nm the formation of a conjugate between 16 mM GSH and 16 mM 1-
199 chloro-2,4-dinitrobenzene (CDNB). Assays were performed in a reaction mixture
200 containing 1.75 mL of phosphate buffer, 100 µL CDNB, 100 µL GSH and 50 µL tissue
201 homogenate.
202 Both enzymatic activities were expressed in nkat/mg protein.
203
204 *2.7. RNA preparation and reverse transcription*
205 Total RNA was extracted and purified using the RNeasy Mini Kit ™ (Cat. Nº
206 74104, Qiagen, Madrid, Spain) according to the manufacturer instructions, as
207 previously described by Puerto et al. (2010). The RNA integrity was assessed by
208 agarose gel electrophoresis. RNA quality was assessed as the 260/280 nm

209 absorbance ratio using an Eppendorf biophotometer (Netheler-Hinz GmbH, Hamburg,
210 Germany). The RNA was then stored at -80 ºC before further processing. Reverse
211 transcription (RT) was performed using random hexamers primers, 4 µg of total RNA
212 and the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Madrid,
213 Spain) according to the manufacturer's instructions.
214
215 *2.8. Real-time polymerase chain reaction (RT-PCR)*
216 A semi quantitative RT-PCR protocol was developed to measure the mRNA
217 levels of GPx, sGST and beta-actin as an external control in liver and kidney of tilapia.
218 The cDNA obtained was diluted in miliQ sterile water and used for the amplification by
219 PCR. The forward and reverse primers used in this study are shown in Table 1. All
220 mRNA sequences were obtained from GenBank (DQ355022, EU234530, EF206801,
221 for GPx, sGST and beta-actin, respectively). PCR primers for GPx, sGST and beta-
222 actine were obtained from Sigma-Aldrich (Spain). Each specific gene product was
223 amplified by RT-PCR using the ABI Prism 7000 sequence detector (Applied
224 Biosystems, Foster City, USA) according to the following parameters: 50 ºC for 2 min,
225 95 ºC for 10 min, 95 ºC for 15 s (40 cycles) and 60 ºC for 1 min (40 cycles).
226 Amplification data were collected by the sequence detector and analyzed with
227 sequence detection software supplied by the manufacturer. For each assay, a standard
228 curve was constructed using increasing amounts of cDNA. In all cases, the slope of the
229 curves indicated adequate PCR conditions (slopes of 3.3–3.6). The RNA concentration
230 in each sample was determined from the threshold cycle (Ct) values and calculated
231 with the sequence detection software supplied by the manufacturer (7000 SDS
232 Software, Applied Biosystems). The quantitative fold changes in mRNA expression
233 were determined relative to beta-actine mRNA levels in each corresponding group and
234 calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).
235
236 *2.9. Western blot analysis for sGST*
237 Liver tissues were prepared for measurement of sGST protein abundance by
238 Western blot analysis. Homogenates (10% w/v) were prepared in 50 mM phosphate
239 buffer, pH 7, containing 0.01 mM EDTA, and 1 mM phenylmethylsulfonylfluoride
240 (PMSF) at 0–4ºC with a polytron homogenizer (Heidolph RZR2102 Control, Sigma,
241 Madrid, Spain). The homogenate was centrifuged at 2000 *g* for 10 min at 4 ºC, and the
242 supernatant was then used for determination of total protein concentration (Bradford,
243 1976). Liver tissue protein preparations were solubilized in Laemmli sample buffer and
244 resolved by 8% SDS-PAGE (Laemmli, 1970). Proteins were electrotransferred onto
245 nitrocellulose membranes and immunoblotted as described by Mate et al. (2006) and

246 Barfull et al. (2002) using monoclonal anti-GST antibodies (SIGMA, Madrid, Spain),
247 diluted at 1:3000. The anti-GST antibody was detected by the enhanced
248 chemiluminiscence (ECL) method according to the supplier's protocol and using a
249 peroxidase-conjugated antimouse IgG as a secondary antibody (1:2000 dilution).
250

251 *2.10. Statistics*

252 Data are expressed as mean ± standard deviation of eight animals per group.
253 Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad
254 InStat software (GraphPad Software Inc., La Jolla, USA). When significant differences
255 were indicated Tukey test was used for comparing the individual treatments. Statistical
256 significance was inferred at $P<0.05$.
257

258 **3. Results**

259

260 *3.1. Glutathione peroxidase and glutathione-S-transferase activities*

261 No significant changes in GPx activity were appreciable in liver of fish in any of
262 the exposures ways to CYN assayed at any time (Fig. 1a). In kidney, a similar
263 response was observed with no significant alterations respect to the control groups
264 (Fig. 1b).
265

266 Hepatic sGST activity increased 1.6-fold in fish orally exposed to CYN and
267 sacrificed 24h later, but the enzyme showed its basal activity when fish were sacrificed
268 5 days after the exposure. Moreover, fish exposed by i.p. injection experienced an
269 increase in this enzymatic activity at both times with the highest levels (2.6-fold) in fish
270 sacrificed 5 days after the exposure (Fig. 2a). Kidney, on the other hand, did not
271 showed any change in this enzymatic activity in fish exposed by gavage, but in those
272 fish i.p. injected a significant increase, 1.4 and 2.4 fold, was observed at both times of
273 sacrifice, respectively (Fig. 2b).
274
275

276 *3.2. Glutathione peroxidase and glutathione-S-transferase gene expression*

277

278 Significant alterations were observed in the relative gene expression of the
279 enzyme GPx in liver of fish exposed to pure CYN. The oral exposure resulted in a
280 significant 2-fold and 7-fold increase in fish sacrificed 24h and 5 days after the
281 exposure, respectively. The i.p. exposure only increased significantly in fish sacrificed
282 after 5 days. Moreover, statistical differences were evident between fish groups

exposed in a different way and sacrificed at the same time and also between fish groups exposed in the same way and sacrificed at different times (Fig 3a).

In the kidney, the oral exposure resulted in a 1.3-fold increase when fish were sacrificed after 24h and in a 9-fold decrease after 5 days. Fish exposed by i.p. injection experienced a decrease that was significant (3.5-fold) when they were sacrificed after 5 days (Fig 3b).

Regarding to sGST, in liver of fish orally exposed an increase was clear only when the sacrifice was 5 days after the exposure (5.4-fold). The i.p. injection, however, resulted in a high increase (>10-fold) at both times of sacrifice. The influence of the exposure route and the time of sacrificed was evident (Fig. 4a). In the kidney, the oral exposure increased the sGST gene expression 1.5-fold in fish sacrificed after 24h, whereas no significant changes were observed in fish sacrificed after 5 days. The i.p. exposure resulted also in an increase (>1.6-fold) at both times of sacrifice. Thus, statistical differences were observed between fish groups sacrificed after 5 days and exposed in different ways (Fig. 4b).

3.3. Glutathione S-transferase protein expression (Western blotting)

Liver experienced a significant increase in the protein expression of sGST when fish were exposed by the oral route (1.4 and 2-fold in fish sacrificed after 24h and 5 days, respectively) (Fig. 5a). However, with the i.p. exposure changes were only induced in fish sacrificed after 24h (1.4- fold increase) (Fig. 5b). In the kidney, no changes were observed in the GST expression of fish orally exposed and sacrificed at the two periods considered (Fig. 5c). The i.p. exposure, finally, only experienced a 1.4-fold increase in fish sacrificed after 5 days (Fig. 5d). Therefore, a differential response in regard to the organ, the type of exposure and the time of sacrifice was evident.

4. Discussion

This study demonstrated that type of exposure to CYN (oral or i.p.) and the time of sacrifice of tilapias (24h or 5 days) have an influence on the effects observed. GPx activity did not change with any treatment, nor in the liver, neither in the kidney. Puerto et al. (2011b) also did not found changes in this biomarker in tilapias exposed in the same way. GST activity, on the other hand, experienced in liver and increase in fish orally exposed and sacrificed after 24h whereas fish sacrificed 5 days after the single

320 exposure showed similar levels to the control group. The initial increase was also
321 observed by Puerto et al. (2011b). The absence of alterations in fish sacrificed after 5
322 days could suggest a recovery of the enzyme. These results are in accordance with a
323 previous study that showed a reduction of GSH levels, that could be linked to the
324 higher GST activity observed in this study and with a reduction of gamma-
325 glutamylcysteine synthetase activity, a enzyme involved in the GSH synthesis, in fish
326 sacrificed 24h after the exposure (Gutiérrez-Praena et al., 2011a). No one of these
327 three biomarkers showed significant alterations in fish orally exposed and sacrificed
328 after 5 days. The i.p. exposure, however, showed a different pattern of effects. In this
329 case, both in liver and kidney there was an increase in the GST activity in fish
330 sacrificed in 24h, and after 5 days of the exposure this increase was even higher. This
331 could suggest a higher toxicity of this type of exposure.
332
333 Although GPx activity did not change, the genetic expression of the enzyme
334 was modified in both organs. This could suggest a post-transcriptional regulation of the
335 enzyme or the combined effect of different GPx genes, as various forms of GPx are
336 found in vertebrates (Vernet et al., 1999; Birringer et al., 2002; Wang et al., 2006). Fish
337 orally exposed to CYN and sacrificed in 24h showed an increase in liver and kidney
338 that was in agreement with a previous study by Puerto et al. (2011b). Zegura et al.
339 (2011) also found an increase in GPx expression in human lymphocytes exposed to
340 0.5 μg/ml CYN for 24h. Fish sacrificed 5 days after the exposure experienced a high
341 increase in the liver and a decrease in the kidney. This could be due because the
342 higher defensive capacity of the liver. Moreover, also with the i.p. exposure the more
343 toxic effects were observed in the kidney. Thus, no changes were observed in any
344 organ after 24h of the i.p. exposure, and after 5 days an adaptative response (an
345 increase) was observed in liver whereas in kidney the genetic expression was
346 decreased. Therefore, in this parameter the major toxic effects were found in the
347 kidney in fish sacrificed 5 days after the exposure. These results confirm that although
348 CYN was primary considered a hepatotoxin (Norris et al., 2002; Berry et al., 2009) it
349 can also affect other organs such as kidney in this particular case. Actually, Humpage
350 and Falconer (2003) considered that kidney appeared to be the more sensitive organ to
351 this toxin in mice.
352
353 The sGST gene expression observed was different in dependence on the
354 organ, the type of exposure and the time of the sacrifice. Again, the i.p. exposure
355 showed the higher effects. With this type of exposure a correspondence between the
356 genetic expression and the enzymatic activity was found, with an increase in all cases.

357 The oral exposure only induced effects on the mRNA levels of sGST in the liver of fish
358 sacrificed after 5 days and in the kidney of fish sacrificed 24h after the exposure. The
359 differences observed with the enzymatic activity could be explained by the existence of
360 different isoforms as GSTs are a multiple gene family of dimeric enzymes. Moreover,
361 between the gen expression of a particular enzyme and its final activity there are
362 different molecular processes that can be influenced and could explain the differences
363 observed. In the present study, when the toxic treatment has induced a change in the
364 sGST activity or its gene expression, this has been always an increase. This response
365 could indicate the action of the defensive systems of the fish to detoxify endogenous
366 compounds such as peroxidised lipids (Leaver and George, 1998), as well as
367 breakdown of xenobiotics. Actually, fish exposed in a similar way showed an increase
368 in lipid peroxidation products in all cases (Gutiérrez-Praena et al., 2011a). Also, sGST
369 catalyze the conjugation of GSH to electrophilic centers on a wide variety of substrates.
370 However, it has been reported that CYN is metabolized by CytP450 (Norris et al.,
371 2002) to more toxic metabolites, but the way they are detoxified is still not kwon.
372
373 Other cyanobacterial toxins such as MCs have shown to induce changes in the
374 genetic expression of GPx and GST on fish (Fu and Xie, 2006; Wang et al., 2006;
375 Puerto et al., 2010) but the effects of CYN on these parameters has been still scarcely
376 investigated (Puerto et al., 2011a) and this if the first study that explore the differential
377 responses induced by the type of exposure and the time of sacrifice.
378
379 The relative abundance of sGST proteins did not correlate in all cases with the
380 activity and the gene expression of the enzyme suggesting that there is a regulation at
381 transcriptional and translational level.
382 CYN induced no changes or an increase in the sGST relative protein abundance.
383 However, CYN is known to inhibit protein synthesis (Terao et al., 1994). Also Puerto et
384 al. (2011b) did not show a decrease in this biomarker in fish exposed in a similar way.
385 Maybe the dose used was not high enough to induce this effect.
386
387 Regarding to the dose employed in this study, 200 μg CYN/kg fish, this is 10 μg
388 CYN/fish, it could be considered environmentally relevant. CYN levels in freshwaters
389 are very variable and for example 8.1-97.1 μg/L has been reported in Northern America
390 (Burns et al., 2002), 0-15 μg/L in Italy (Manti et al., 2005), and 4-120 μg/L (Shaw et al.,
391 1999) or even 800 μg/L (Shaw et al., 2000) in Australia. Moreover, the predominantly
392 extracellular availability of this cyanotoxin makes it particularly likely to be taken up by

393 a variety of aquatic organisms (Kinnear, 2010) and therefore, acute and subchronic
394 exposure of fish in contaminated waters can occur.
395
396 Other cyanobacterial toxins have shown to induce differential time-dependent
397 responses. Thus, Prieto et al., (2007), observed a recovery of the oxidative damage
398 72h after a single exposure to 120 μg/fish MC-LR. A recovery of toxic histopathological
399 effects caused by MCs was reported by Ernst et al. (2006) to occur at 24–48 h in
400 European whitefish (*C. lavaretus*). Li et al. (2005) observed recovery of hepatocytes in
401 bighead carp 48 h postinjection. Malbrouck et al. (2004) found recovery of the liver
402 structure and regeneration of hepatocytes 96 h post-injection in goldfish *Carasius*
403 *auratus* as well as reconstruction of the tissue structure 21 days post-injection (of 125
404 mg/kg body weight of MC-LR). However, after CYN exposure, a general recovery of
405 the different biomarkers was not observed, even more toxic effects were evident in
406 some cases (i.e. GST activity in liver and kidney after i.p. exposure, GPx gene
407 expression with the same kind of exposure, etc.). Gutiérrez-Praena et al. (2011a) found
408 both kind of responses (lower or higher toxicity) with the time of sacrifice, but an
409 histopathological study in fish exposed in the same way revealed more severe damage
410 after 5 days in comparison to 24 h (Gutiérrez-Praena et al., 2011b). These differences
411 observed between cyanobacterial toxins could be due to their different toxicokinetics
412 and metabolization as MCs are detoxified through GSH conjugation (Pflugmacher et
413 al., 1998) and CYN is bioactivated by CytP450 (Norris et al., 2002). Moreover, Kinnear
414 (2010) reported that CYN has a delayed toxicity involving multiple organ systems,
415 principally the liver and kidney. In regard to the type of exposure, in general the
416 biomarkers selected showed higher alterations after an i.p. exposure. However, other
417 authors observed that the nature, location, and time course of histological damage
418 were similar for oral and intraperitoneal administration of *C. raciborskii* extracts in mice
419 (Falconer et al., 1999).
420
421 Overall, both variables studied, the type of exposure and the time of sacrifice,
422 play a role in the toxic effects induced by CYN. These effects are evident not only at
423 biochemical level (enzymatic activity) but also at molecular level (gene and protein
424 expression). This study can contribute to a deeper insight into the toxic mechanisms
425 involved in CYN pathogenicity and to understand the consequences that the fish
426 exposure to CYN could have.
427
428

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430

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435

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437

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581 **Figure captions**

582

583 **Figure 1.** Glutathione peroxidase (GPx) activity (nkat/mg protein) in liver (a) and kidney
584 (b) of fish exposed by gavage and intraperitoneally to 200 µg/kg bw CYN and sacrificed
585 after 24h and 5 days. The values are expressed as mean ± sd (n=8).

586

587 **Figure 2.** Glutathione-S-transferase (GST) activity (nkat/mg protein) in liver (a) and
588 kidney (b) of fish exposed by gavage and intraperitoneally to 200 µg/kg bw CYN and
589 sacrificed after 24h and 5 days. The values are expressed as mean ± sd (n=8). The
590 significance levels observed are *$p < 0.05$ in comparison to control group values; #
591 $p<0.05$ when fish sacrificed at the same time and exposed in a different way are
592 compared; and & $p<0.05$ when fish exposed in the same way and sacrificed at different
593 times are compared.

594

595 **Figure 3.** Relative gene expression of glutathione peroxidase (GPx) in liver (a) and
596 kidney (b) of fish exposed by gavage and intraperitoneally to 200 µg/kg bw CYN and
597 sacrificed after 24h and 5 days. The values are expressed as mean ± sd (n=8). The
598 significance levels observed are *$p < 0.05$ in comparison to control group values; #
599 $p<0.05$ when fish sacrificed at the same time and exposed in a different way are
600 compared; and & $p<0.05$ when fish exposed in the same way and sacrificed at different
601 times are compared.

602

603 **Figure 4.** Relative gene expression of soluble glutathione-S-transferase (sGST) in liver
604 (a) and kidney (b) of fish exposed by gavage and intraperitoneally to 200 µg/kg bw
605 CYN and sacrificed after 24h and 5 days. The values are expressed as mean ± sd
606 (n=8). The significance levels observed are *$p < 0.05$ in comparison to control group
607 values; # $p<0.05$ when fish sacrificed at the same time and exposed in a different way
608 are compared; and & $p<0.05$ when fish exposed in the same way and sacrificed at
609 different times are compared.

610

611 **Figure 5.** Relative abundance of glutathione-S-transferase (sGST) protein in liver of
612 fish exposed by gavage (a) and and intraperitoneally (b), and in kidney of fish exposed
613 by gavage (c) and intraperitoneally (d) to 200 µg/kg bw CYN and sacrificed after 24h
614 and 5 days. The values are expressed as mean ± sd (n=8). Results are expressed as
615 relative abundance %. The significance levels observed are *$p < 0.05$ in comparison to
616 control group values; # $p<0.05$ when fish sacrificed at the same time and exposed in a

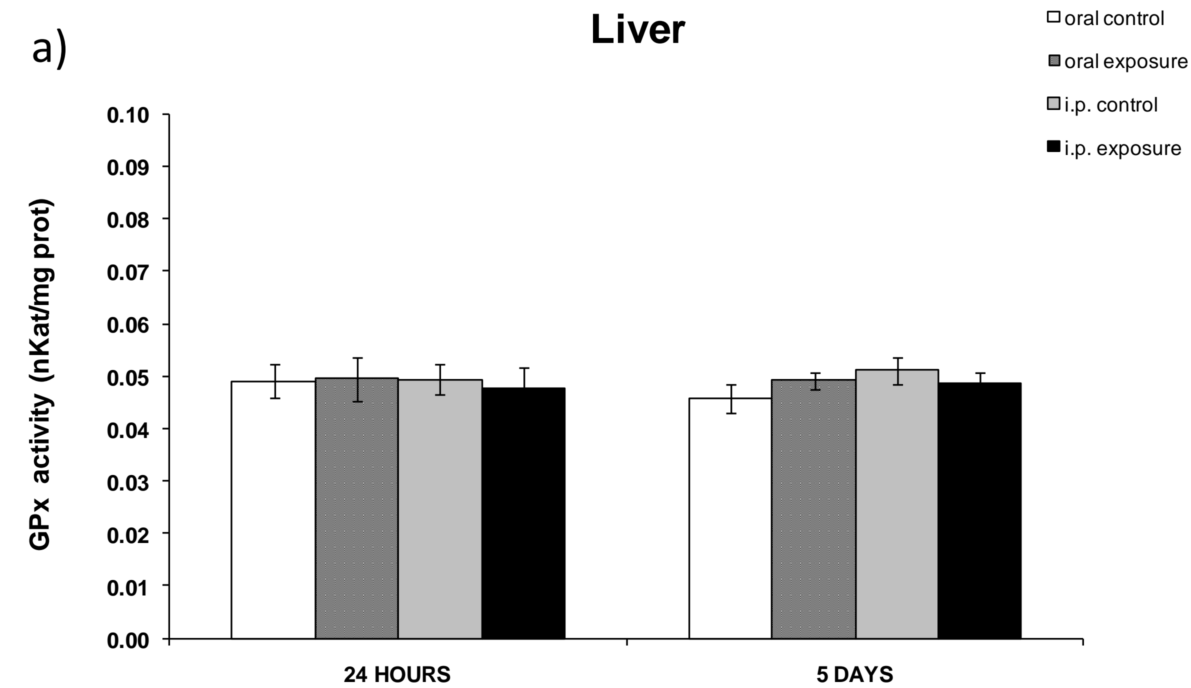
617 different way are compared; and & $p<0.05$ when fish exposed in the same way and
618 sacrificed at different times are compared.
619

Table 1
Click here to download Table: Table 1.doc

Table 1. Nucleotides sequences (5’-3’) of PCR primers of beta-actine, GPx and sGST.

Gene	Fordward primer	Reverse primer
beta-actine	CAATGAGAGGTTCCGTTGC	AGGATTCCATACCAAGGAAGG
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA

Figure 1



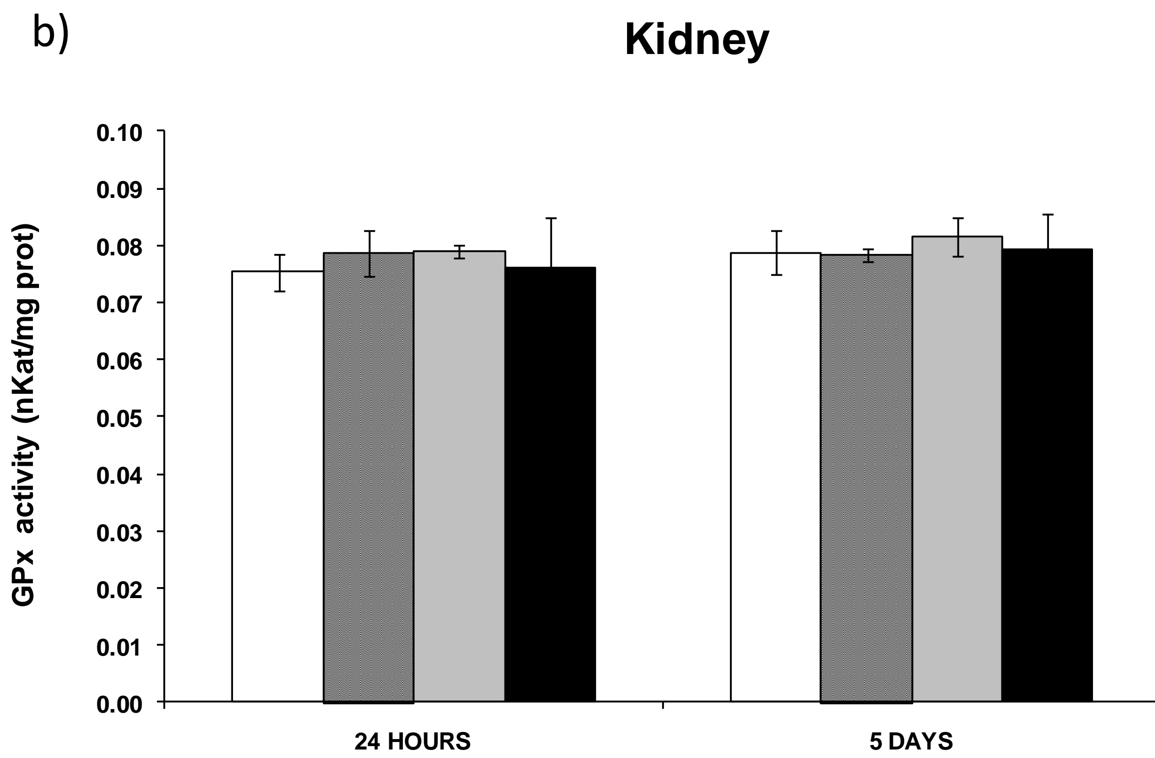
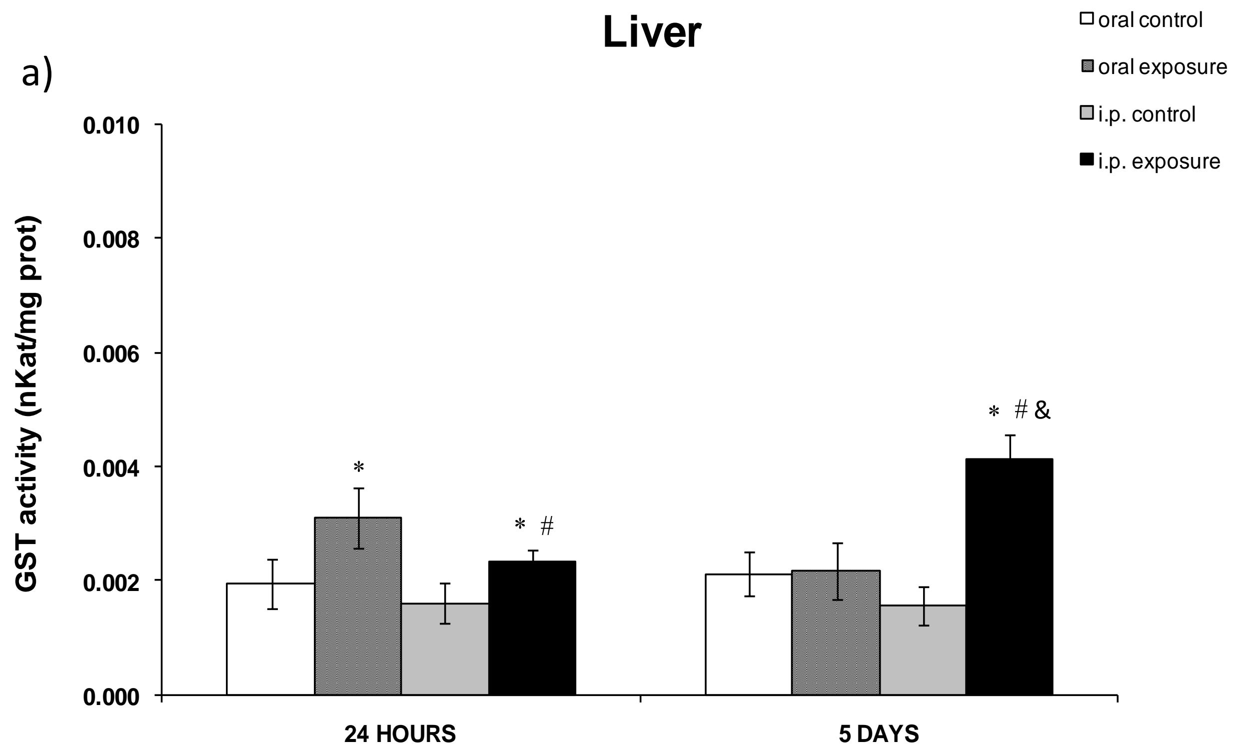


Figure 2



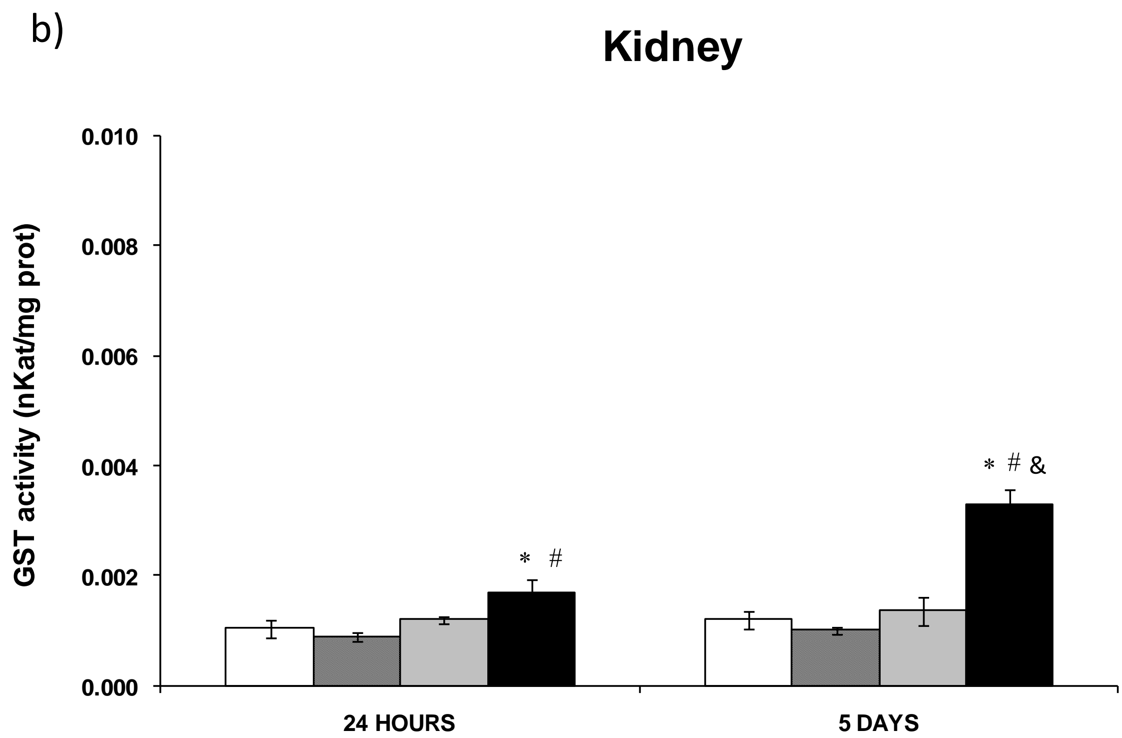
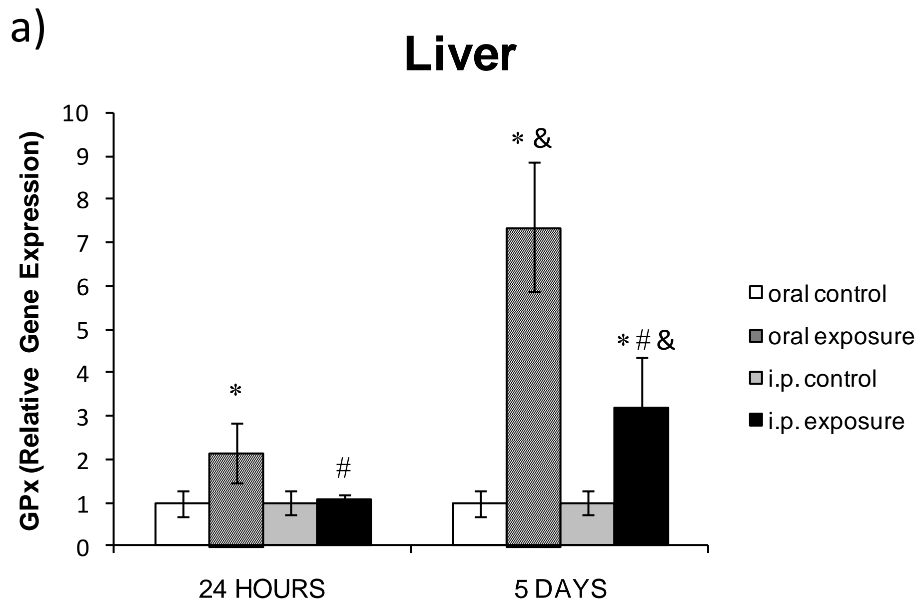


Figure 3

a)



b)

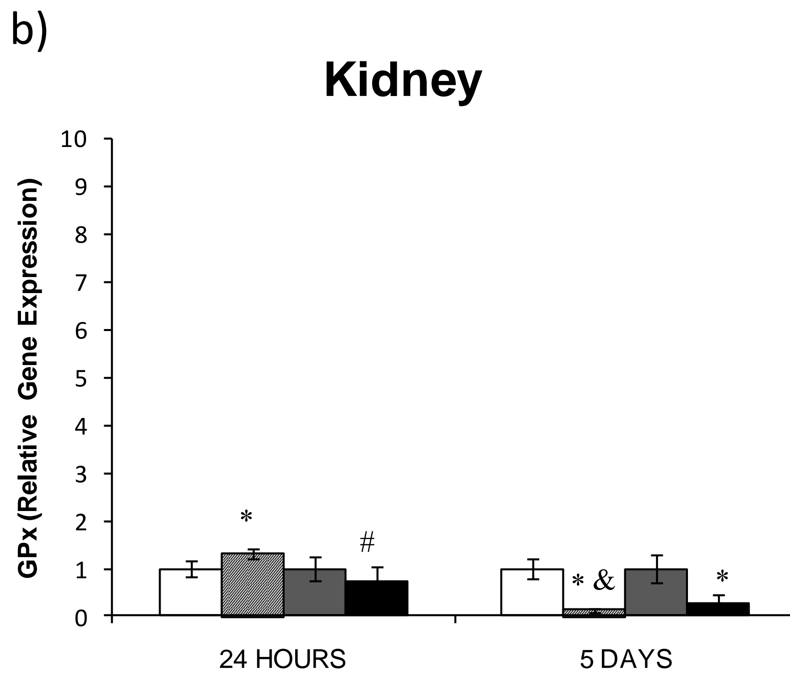


Figure 4

a)

Liver

oral control
oral exposure
i.p. control
i.p. exposure

GST (Relative Gene Expression)

0 2 4 6 8 10 12 14

24 HOURS
5 DAYS

* #
* &
* # &

b)

Kidney

GST (Relative Gene Expression)

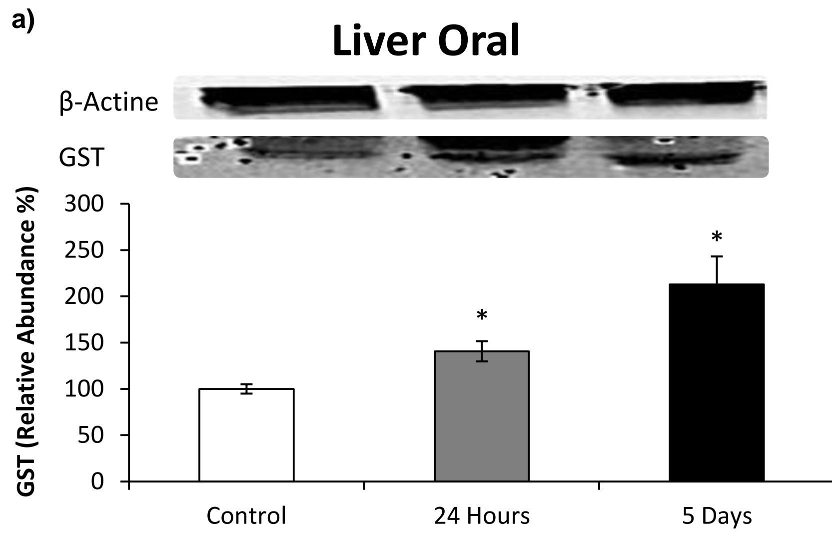
0 2 4 6 8 10 12 14

24 HOURS
5 DAYS

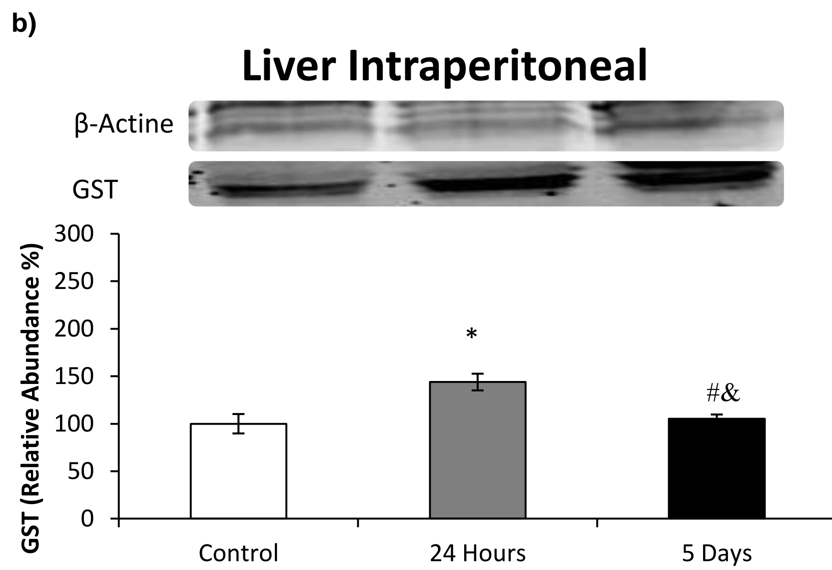
*
*
&
* #

Figure 5

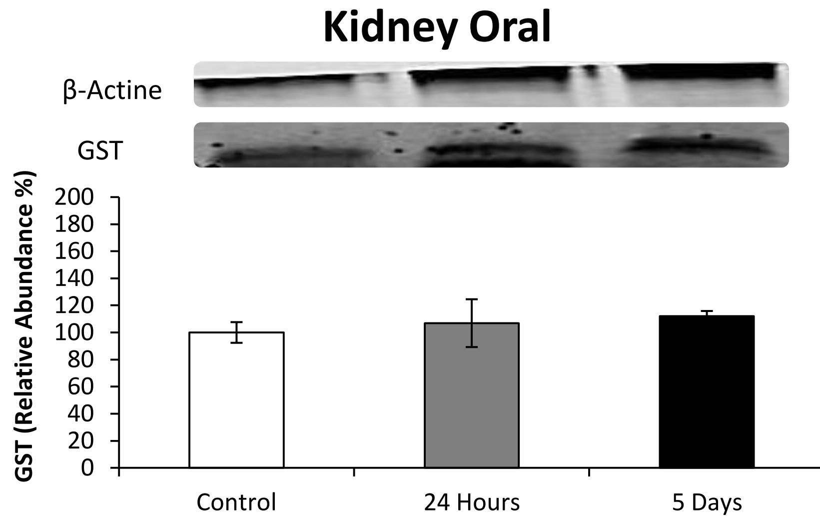
a)



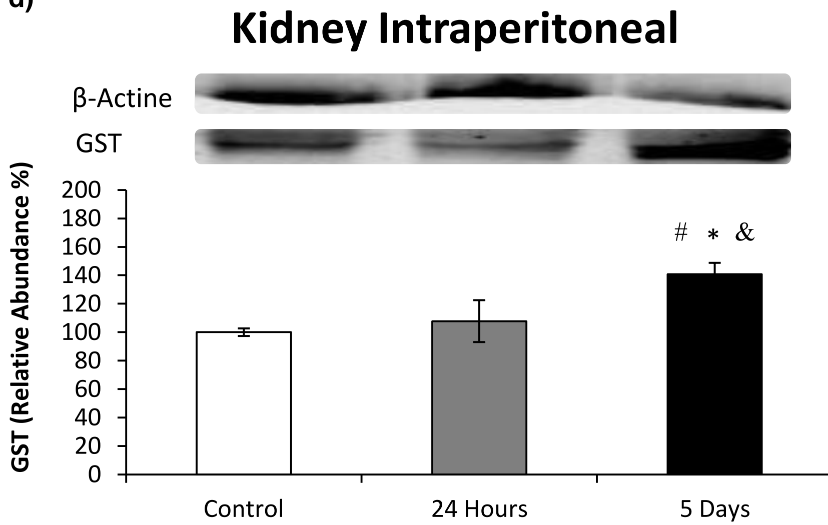
b)



c)



d)



CAPÍTULO 6 / CHAPTER 6

Daniel Gutiérrez-Praena, Ángeles Jos, Silvia Pichardo, Rosario Moyano, Alfonso Blanco, José G. Monterde, Ana M. Cameán

TIME-DEPENDENT HISTOPATHOLOGICAL CHANGES INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) AFTER ACUTE EXPOSURE TO PURE CYLINDROSPERMOPSIN BY ORAL AND INTRAPERITONEAL ROUTE.

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Time-dependent histopathological changes induced in Tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral and intraperitoneal route

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ABSTRACT

Although fish and aquatic organisms can be in contact with the cyanotoxin cylindrospermopsin (CYN), toxicological studies are practically nonexistent. CYN has a late and progressive acute toxicity in rodents, but no data have been reported in fish. In this work, tilapia (*Oreochromis niloticus*) were exposed for the first time to an acute dose of CYN (200 μg/kg fish) by intraperitoneal (i.p.) injection, and the effects were compared with the oral route (gavage). In both cases, fish were sacrificed after 24 h or 5 days of the toxin administration. CYN induced multiorganic damage, being the liver and kidney the main targets of toxicity. The histological findings were more pronounced after i.p. administration (in the liver, kidney, heart, gills) with the exception of the gastrointestinal tract. The time of sacrifice influenced the degree of histological damage in all organs studied, and was more severe after 5 d in comparison to 24 h. Moreover, CYN induced an increase in the average nuclear diameter of hepatocytes in the liver, and decreased cross sections of proximal and distal convoluted tubules in the kidney. The changes in these parameters were also more severe by i.p. route, and with the time of sacrifice, supporting the histopathological results obtained in these organs. Thus, both parameters could be useful for quantifying the extent of the damage in fish after CYN exposure.

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1. Introduction

The cyanobacterial toxin cylindrospermopsin (CYN) occurs in a number of important drinking-water sources in the world and is being increasingly recognized as a potential threat to drinking water safety (Chorus and Bartram, 1999; Humpage et al., 2005). Blooms of CYN-producing cyanobacteria have been reported in rivers, freshwater lakes and reservoirs (Watanabe, 1987; Baker and Humpage, 1994; Banker et al., 1997; Humpage and Falconer, 2003; Fastner et al., 2003; Saker et al., 2003). Today, the distribution of CYN-producing cyanobacteria is now known to extend into temperate regions, and emphasizes that the potential for human health risk from this toxic organism, and consequently to CYN, is not limited to the tropics, but it is also relevant to temperate climates (Falconer and Humpage, 2006).

The acute toxicity of CYN has been well established in rodents, based on diverse intraperitoneal (i.p.) and oral exposure studies providing a picture of general organ damage of extracts or purified toxin (Hawkins et al., 1985; Ohtani et al., 1992; Falconer et al., 1999; Seawright et al., 1999; Humpage and Falconer, 2003). The toxin primarily accumulates, and exerts toxic effects, in the liver and in the kidney (Funari and Testai, 2008), although the toxin causes damage in several organs, including the lungs, heart, stomach, adrenal glands, the vascular system, and the lymphatic system (Hawkins et al., 1985; Falconer and Humpage, 2006).

In rodents, CYN has a late and progressive acute toxicity. It has been shown in mice after ip injection of pure CYN, that the LD_{50} decreases between 24 hours (h) and 5 days (d) , with the 5–6 day i.p. LD_{50} of 200 μg/kg body weight in comparison to the 24-h LD_{50} of 2100 μg/kg bw (approximately one-tenth of the 24 h) (Ohtani et al., 1992). No data on the oral toxicity of pure CYN are available, but studies with aqueous extracts of *C. raciborskii* provide preliminary indications (Chorus and Bartram, 1999; van Apeldoorn et al., 2007). After administering to mice a single oral dose of an aqueous extract of freeze-dried *Cilindrospermopsis* cells, a LD_{50} in the range of 4.4–6.9 mg/kg toxin equivalent was determined, with death occurring 2–6 days after treatment (Seawright et al., 1999). According to these results, the

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oral toxicity appears to be over tenfold lower than i.p. toxicity (Chorus and Bartram, 1999).

The mechanisms of CYN toxicity are under investigation (Falconer and Humpage, 2006), and its mode of action has been associated with inhibition of protein synthesis (Terao et al., 1994; Runnegar et al., 1995). Data from primary hepatocytes show two routes of toxic action, a rapid route probably through toxicity of a CYP450 oxidation product of the toxin (Runnegar et al., 1995; Froscio et al., 2001), and a slower mechanism through the well-documented inhibition of protein synthesis, which does not require toxin metabolism. Although oxidative stress, as measured by the lipid peroxidation (LPO) rate, was primarily excluded as toxicity mechanism (Froscio, 2002), more recent investigations indicated that it could have a role (Silva et al., 2010; Puerto et al., in press; Gutiérrez-Praena et al., 2011a).

In relation to the effects on aquatic organisms, blooms of *C. raciborskii* produced high mortality, reduced body growth and negative effects on fecundity in natural *Daphnia* populations (Nogueira et al., 2004). In spite of their likely exposure to CYN, and although its 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), the toxicological studies on CYN in fish are practically nonexistent (Berry et al., 2009), and LD_{50} data has been not reported. Recently, we have demonstrated that acute exposure by oral route (gavage) to 200 and 400 μg pure CYN/kg bw fish induced dose-dependent histopathological effects, in the liver, kidney, heart, intestines, and gills of Tilapia (*Oreochromis* sp.) (Puerto et al., unpublished results). Nevertheless, the time course of these effects remained unknown. Tilapia has been chosen as an adequate experimental model to study the effects of toxins from cyanobacterial cells, such as MCs (Jos et al., 2005; Molina et al., 2005) because it is an emerging and attractive species for aquaculture (Costa-Pierce and Rakocy, 1997).

Taking all these data into account, the aim of the present study was to evaluate the possible time-dependent effects of CYN in Tilapia fish acutely exposed to a single dose of 200 μg/kg bw fish employing two routes of administration: oral route by gavage, or by i.p. injection. The fish were euthanized 24 h or 5 d after the exposure, and histological damage in liver, kidney, heart, intestinal mucosa and gills were studied. From the results it is possible to evaluate: (1) differences in the toxic potency of CYN linked to the route of administration, and (2) if there is a slow and gradual poisoning in Tilapia induced by the toxin, similar to that found in rodents, or rather a spontaneous recovery of the damage.

2. Material and methods

2.1. Chemicals

The cyanotoxin cylindrospermopsin standard (purity ≥95%) was supplied by Alexis Corporation (Lausen, Switzerland). Chemicals for the different techniques were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain). CYN purity was confirmed by LC-DAD, using a Varian 9012 equipped with a Varian ProStar 330 Diode Array Detector. Chromatographic data were processed with a Star Chromatography Workstation (Varian Technologies). Chromatographic separation of CYN was performed according to Welker et al. (2002) on a 250 mm × 4.6 mm i.d., 5 μm, LiChrosphere C18 column purchased from Merck (Darmstadt, Germany). Standard solutions of CYN were prepared and diluted in water for their analytical determination.

2.2. Experimental setup and acclimation of fish

Studies were conducted using male *Oreochromis niloticus* (Nile tilapia, Perciformes: Cichlidae) with mean weight 55.2 ± 6.7 g and 12 ± 2 cm length. Fish were obtained from a fish hatchery (Valenciana de Acuicultura, Valencia) and transferred to the laboratory where they were held in aquaria (5 individuals/aquarium) with 96 L of tap water. Exposure to chlorine was minimized by filling the tanks at least 3 d before fish were introduced. The aquaria were also set up with continuous system of water filtration and aeration (Eheim Liberty 150 with Bio-Espumador cartridges (Bio-Espumador) as skimmers, 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 ± 0.2, conductivity 292 μS/cm, Ca^{2+} 0.60 mM/L and Mg^{2+} 0.3 mM/L. Nitrite/nitrogen and nitrate levels were not measured because of the aquaria were set up with fresh water and the experimental trial was short. 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.

2.3. Experimental exposure

The experiment was carried out using six aquaria with 5 fish in each (n=30 fish). Fish treatment groups consisted of control groups (aquaria 1–2), CYN-fish groups euthanized after 24 h of exposure (aquaria 3–4), and CYN-fish groups euthanized after 5 d of exposure (aquaria 5–6). For each group of fish, two alternative routes of administration were assayed: 1) oral route: Tilapia were exposed by gavage to a single dose of 200 μg/kg fish bw in 0.5 mL of 0.9% (w/v) NaCl solution (aquaria 3 and 5) ; in this case, fish control group only received the vehicle solution (0.5 mL of 0.9%, w/v, NaCl); 2) intraperitoneal route (i.p.): Tilapia were exposed to a single dose of 200 μg/kg fish bw in 0.5 ml of 0.9% (w/v) NaCl solution (aquaria 4 and 6); the respective control group of fish received only the vehicle solution (0.5 ml of 0.9%, w/v, NaCl). Due to the scarce toxicity data of CYN on fish, the dose was selected in accordance with those reported to produce toxic effects in rodents after gavage exposure to CYN (Humpage and Falconer, 2003), and with our previous experiments carried out in this fish species, in which this dose administered by gavage induced damage (Puerto et al., in press; Gutiérrez-Praena et al., 2011b).

After 24 h or 5 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.

2.4. 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 using an automatic processor. Sections of 3–5 μm were mounted. After they had been deparaffinized, the sections were rehydrated, stained with haematoxylin and eosin (HE), 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% 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).

2.5. 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 HE.

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® 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.

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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 were estimated. The morphometric data 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.

2.6. Statistical analysis

Data were analysed by applying bivariate comparisons considering non parametric methods. Statistical software (Statistica, version 6. Statsoft inc) was used for performing Mann–Whitney comparisons between groups. Null hypothesis was rejected by selecting a p-level <0.05.

3. Results

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

The microscopic examination of the HE-stained liver sections of fish exposed to CYN by oral route revealed a light degenerative process, with glycogen accumulation and lipid droplets (Fig. 1C, D), in comparison to control group (Fig. 1A, B), which showed a normal cord-like parenchymal structure of the liver, and an apparently normal pancreatic area along the portal vessels within the liver. These alterations were more pronounced in fish treated with CYN (oral route) and euthanized later, at 5 days. In those fish a general degeneration process in the liver, with large glycogen deposits and steatosis were observed. Moreover, moderately degranulated cells in the pancreatic tissue were also evident, and some of them suffered necrosis (Fig. 1E, F). In comparison to oral route, exposure to CYN by i.p. injection caused more considerable lesions in the liver, characterized by a general cytoplasmic glycogen accumulation and steatosis. The microscopic examination of the HE-stained section revealed hepatocytes with cytoplasmic vacuolization, glycogen deposits, and in the pancreas there were degenerative cells with scarce granules, vacuolization and some signs of necrosis (Fig. 1G). The glycogen accumulation was more evident in the ultrastructural study, in which there was a noticeable reduction of cytoplasmic organelles, and obvious mitochondrial tumefaction appeared (Fig. 1H). When fish were euthanized at 5 d, samples showed in addition to glycogen accumulation, steatosis in the cytoplasm of the hepatocytes resulting in so-called signet ring cells, with displaced nuclei because of lipid accumulation. In the pancreas areas of degeneration and focal necrosis were detected (Fig. 1I). Ultrastructurally, abundant glycogen deposits, lipid vacuoles, and scarce cytoplasmic organelles were observed (Fig. 1J).

In kidney, fish exposed to CYN by oral or i.p. routes showed a glomerulopathy with glomerular capillary atrophy, hyperemia and decreased width of the proximal and distal convoluted tubules. Globally, the pathological changes were more severe when fish were euthanized at 5 d, and also when they were intoxicated by i.p. route. At 24 h, histological changes in the renal parenchyma of fish intoxicated by oral route are mainly a membranous glomerulopathy, with atrophic glomeruli, dilated Bowman's capsule, and decreased width of the proximal and distal convoluted tubules (p,d) (Fig. 2C). Ultrastructurally, an elongation of the podocyte primary foot processes was observed (Fig. 2D). At 5 d, the atrophic glomerular degeneration process was more evident, with dilatation of the Bowman's capsule and a decrease in the width of proximal and distal convoluted tubules (Fig. 2E). Electron microscopy revealed hyperemia in the capillaries, and very elongated podocyte primary foot processes (Fig. 2F). By i.p. route, fish euthanized at 24 h showed atrophic glomeruli with hyalinization and decreased width of the proximal and distal convoluted tubules (Fig. 2G). The ultrastructural study showed that the podocyte primary foot processes were well developed (Fig. 2H). In fish euthanized after 5 d, all these histopathological changes were considerably more severe. By light microscopy, atrophic and hyalinized glomeruli were evident, and also some tubular cells with necrotic nuclei (Fig. 2I). Electronic microscopy revealed a marked hyperemia and greater increase in podocyte primary foot processes (Fig. 2J).

Heart lesions were characterized by myofibrolysis with marked edema. These processes were scarce in fish intoxicated by oral route and euthanized at 24 h, in which myofibrolysis was detected only in some cases (Fig. 3C, D). However, in fish sacrificed at 5 d, destruction of myofibrils and presence of interstitial edema were observed (Fig. 3E). Moreover, the ultrastructural study revealed a clear loss of the muscular fibers, and disorganization of their different bands (Fig. 3F). Fish treated by i.p. route of CYN showed the presence of pleomorphic fibers and abundant edema at 24 h (Fig. 3G), whereas at 5 d these processes were more evident, and large areas of myofibrolysis were observed (Fig. 3I). The electron microscopy revealed also the destruction of myofibrils, while maintaining the presence of abundant mitochondria in fish at 24 h (Fig. 3H). In fish euthanized at 5 d, there was an extensive destruction of myofibrils with abundant loss of mitochondria (Fig. 3C).

The gastrointestinal tract of fish treated with CYN by oral or i.p. routes showed a catarrhal enteritis process followed by necrotic changes with the presence of edema, hyperemia and loss of microvilli, these lesions being more intense in fish exposed by the oral route. After 24 h of CYN exposure, intestinal villi with necrotic enterocytes, and severe interstitial edema were observed (Fig. 4C). The ultrastructural study showed decreased number of microvilli and a reduction of their size (Fig. 4D). Fish euthanized after 5 d showed more necrotic enterocytes, abundant caliciform cells and microhemorrhages (Fig. 4E), with a partial loss of microvilli and cytoplasmic vacuolization (Fig. 4F). Similar lesions (necrotic enterocytes, increased caliciform cells) were also observed in fish after i.p. administration of CYN (Fig. 4G). The ultrastructural study analysis showed areas with a loss of microvilli alongside other areas with apparently normal ones (Fig. 4H). All these alterations were more pronounced 5 d after toxin exposure (Fig. 4I, J).

Morphological alterations observed in gills consisted in tumefaction processes, hyperemia, and certain inflammatory foci. Twenty-four hours after exposure to CYN by the oral route mainly hyperemia and edema in gill lamellae of fish were observed (Fig. 5C). After 5 d very hyperemic lamellae with edema and even inflammatory foci were detected (Fig. 5E). The scanning electron microscopy images showed tumefaction and desquamation in gill lamellae by 24 h (Fig. 5D), and some isolated microhemorrhages (Fig. 5F). The administration of CYN i.p. induced more tissue damage in fish, and very hyperemic gill lamellae and microhemorrhages were observed at 24 h, (Fig. 5G); even interstitial and lamellar edema at 5 d was observed (Fig. 5I). Also, the scanning electron microscopy study revealed tumefaction in lamellae with hemorrhagic contents by this route (Fig. 5H) in comparison to oral administration, and more evident microhemorrhages were observed at 5 d (Fig. 5J).

Regarding the results obtained in the morphometric study, in the liver, average hepatocyte nuclear diameters were clearly affected by CYN exposure (Fig. 6). An increase of this parameter was observed in fish exposed to pure CYN by gavage only after 5 d of administration; whereas by i.p. route, fish experienced significant increases after 24 h of exposure (1.2 fold), which were more pronounced in fish sacrificed 5 d after exposure (1.9 fold). Statistical differences were observed between fish subject to

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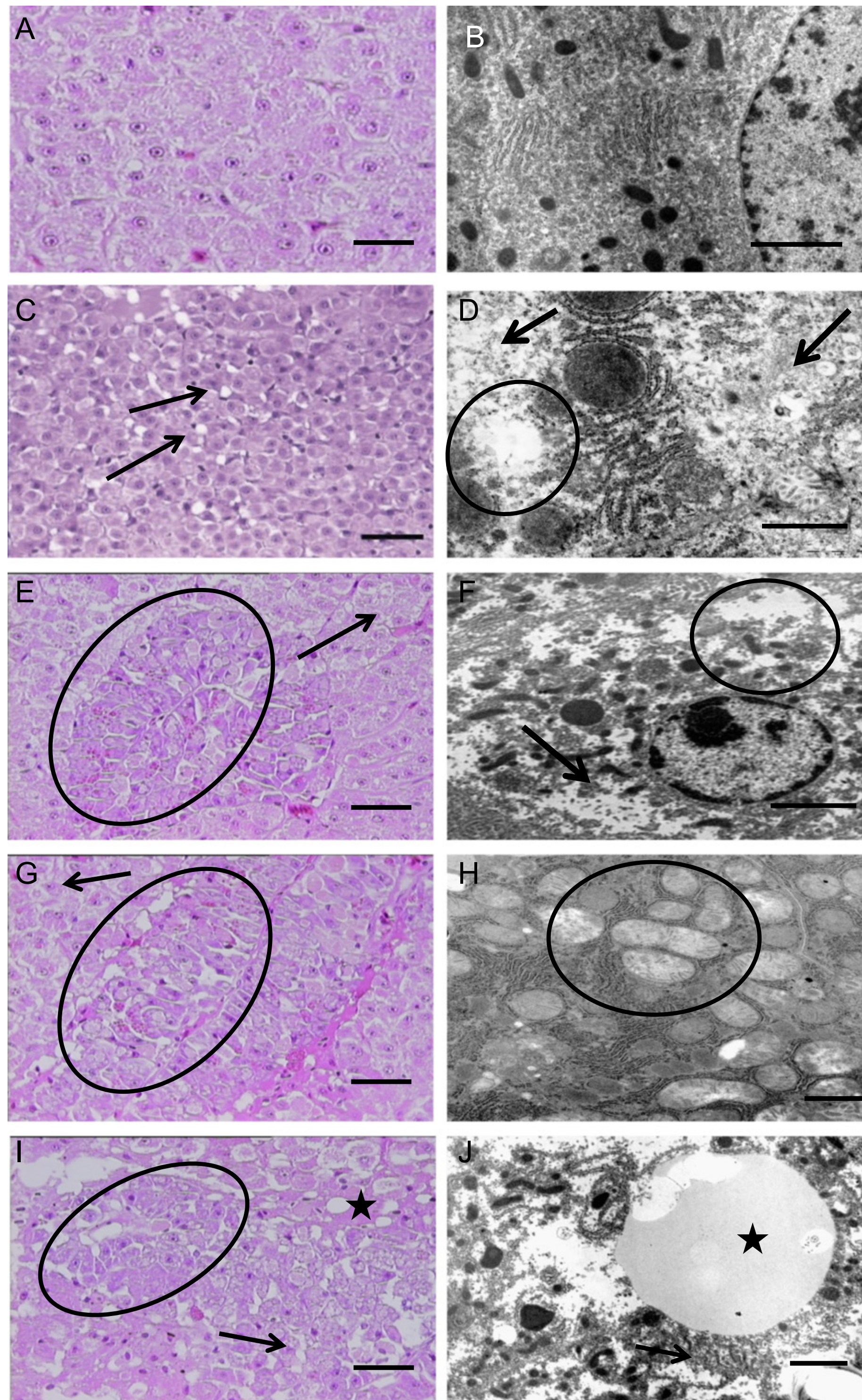

Fig. 1. Histopathological changes in liver and pancreas of Tilapia exposed to 200 μg CYN/kg b.w fish standard (purity ≥ 95%) (A, C, E, G, I): HE-stained liver section. Bars, 100 μm. (B,D,F,H,J): Ultrastructural observations. Bars, 10 μm. (A, B) Control fish: (A) normal hepatic cords, hepatocytes appeared as polyedric cells, with central nucleus and clear cytoplasm; (B) Normal hepatocyte, with cytoplasmic organelles, and mitochondria. (C,D) Tilapia exposed by oral route and euthanized at 24 h: (C) Hepatic parenchymal with cells with clear cytoplasm and lipid vesicles (arrow), and (D) hepatocytes with big deposits of glycogen (arrow) and small lipids droplets (circle). (E,F) Fish exposed by oral route and euthanized at 5 d: (E) cells with clear cytoplasm (arrow), and degenerative and necrotic pancreatic cells (circle), and (F) hepatocyte with nuclei surrounded by organelles with scarce endoplasmic reticulum, glycogen (arrow) and fat droplets (circle). (G,H) Tilapia exposed by i.p. route and euthanized at 24 h: (G) hepatocytes with clear cytoplasm (arrow), necrotic signs in the pancreatic area (circle), and (H) hepatocytes with abundant mitochondria, mitochondrial tumefaction (circle). (I,J) Tilapia exposed by i.p. route and euthanized at 5 d: (I) Cells with clear cytoplasm (arrow) and signet-ring cells (star), degenerative (circle) and necrotic (triangle) pancreatic cells, and (J) predominant glycogen deposits (arrow), and large lipid droplets (star).

either exposure route and sacrificed at the same time, and between fish subject to the same exposure route and sacrificed at different times.

In the kidney, the data concerning the average cross sections of the proximal convoluted tubules showed a significant decrease in CYN-exposed fish for either exposure route, when they were

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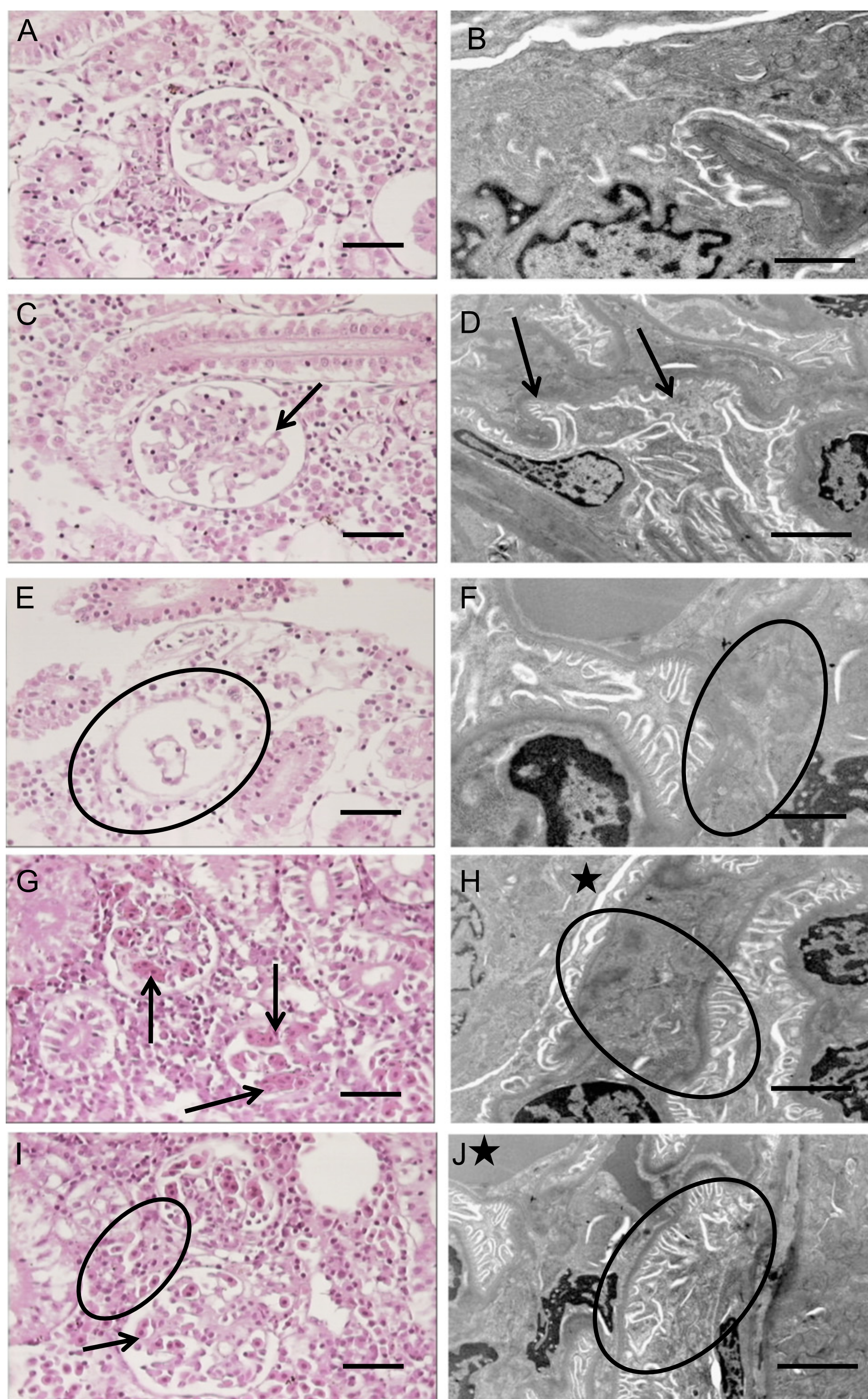

Fig. 2. Histopatohological changes in kidney of Tilapia exposed to 200 μg CYN/kg b.w fish standard (purity ≥ 95%). (A, C, E,G, I): HE-stained kidney section. Bars, 100 μm. (B,D,F,H,J): Ultrastructural observations. Bars, 10 μm. (A, B) Control fish. (C,D) Tilapia exposed by oral route and euthanized at 24 h: (C) Glomerular atrophy and dilated Bowman's capsule (arrow) and decrease the width of the proximal and distal convoluted tubules (p,d), and (D) elongated podocyte primary foot processes (arrow). (E,F) Tilapia exposed by oral route and euthanized at 5 d: (E) Detail of the renal parenchyma showing atrophic glomerulus (star) and dilated Bowman's capsule (arrow), and decrease the width of the proximal and distal convoluted tubules (p,d), (F) hyperemia in the capillaries (star) and elongation of podocyte foot processes (circle). (G,H) Tilapia exposed by i.p. route and euthanized at 24 h: (G) atrophic glomerulus and hyalinization (circle) and de decrease the width of the proximal and distal convoluted tubules (p,d) , and (H) exacerbated increase of podocyte primary foot processes (circle). (I,J) Tilapia exposed by i.p. route and euthanized at 5 d: (I) Atrophy and hyalinization of glomerulus (circle), big decrease the width of the proximal and distal convoluted tubules (p,d), and necrotic tubular cells (arrow), and (J) hyperemia (star), hyalinization (arrow) and very elongated podocyte foot processes (circle).

euthanized at 5 d (Fig. 7a), in comparison to their respective control fish. The average cross sections of distal convoluted tubules showed a significant decrease in fish administered by oral route only after 5 d of exposure. In fish exposed by i.p. route, the effects were significant at 24 h as well as after 5 d of exposure (Fig. 7b). The time of sacrifice influences the effects of CYN on the average cross sections of distal convoluted tubules in fish exposed by the same route (oral or i.p.).

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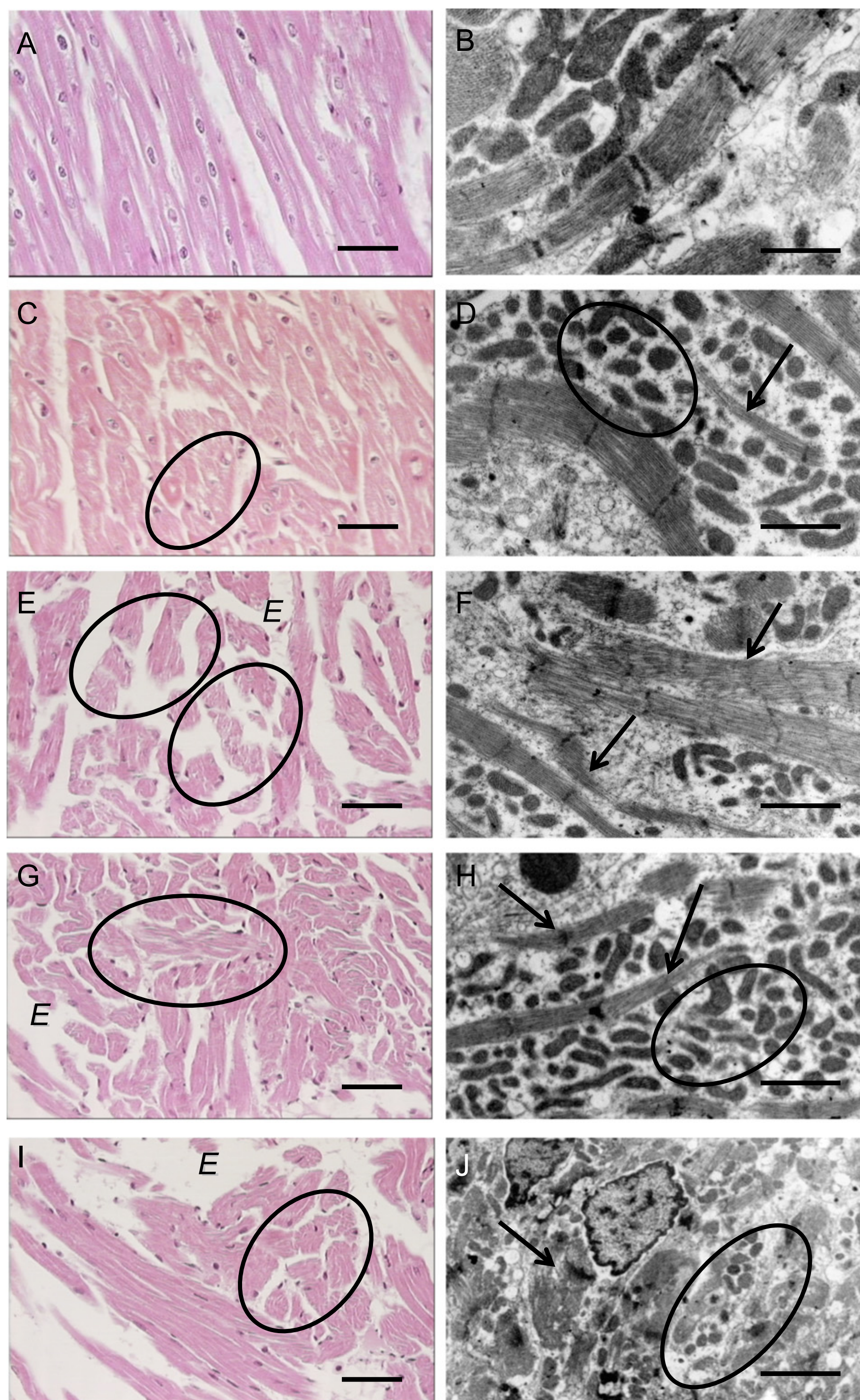

Fig. 3. Histopatohological changes in heart of Tilapia exposed to 200 μg CYN/kg b.w fish standard (purity ≥95%). (A, C, E,G, I): HE-stained heart section. Bars, 100 μm. (B,D,F,H,J): Ultrastructural observations. Bars, 10 μm. (A, B) Control fish. (C,D) Tilapia exposed by oral route and euthanized at 24 h: (C) cardiac fibers with some degree of destruction (circle), and (D) abundant mitochondria (circle) and destruction of myofibrils (arrow). (E,F) Tilapia exposed by oral route and euthanized at 5 d: (E) destruction of myofibrils (circle) with interstitial edema (*E*), and (F) destruction of myofibrils, with disorganized bands (arrow). (G,H) Tilapia exposed by i.p. route and euthanized at 24 h: (G) pleomorphic fibers with partial destruction (circle) and edema (*E*), and (H) partial destruction of myofibrils (arrow) which maintain abundant mitochondria (circle) (I,J) Tilapia exposed by i.p. route and euthanized at 5 d: (I) myofibrolysis (circle), marked edema (circle), and (J) destruction of myofibrils (arrow) with loss of mitochondria (circle).

4. Discussion

The data base on oral toxicity of pure CYN is limited by a small number of studies and insufficient reporting, according to the US Environmental Protection Agency (EPA, 2011). Moreover, there are no studies dealing with the acute oral toxicity of pure CYN in rodents. Studies with aqueous extracts of *Cylindrospermopsis* indicated that the oral toxicity appears to be over tenfold lower than i.p. toxicity (Seawright et al., 1999).

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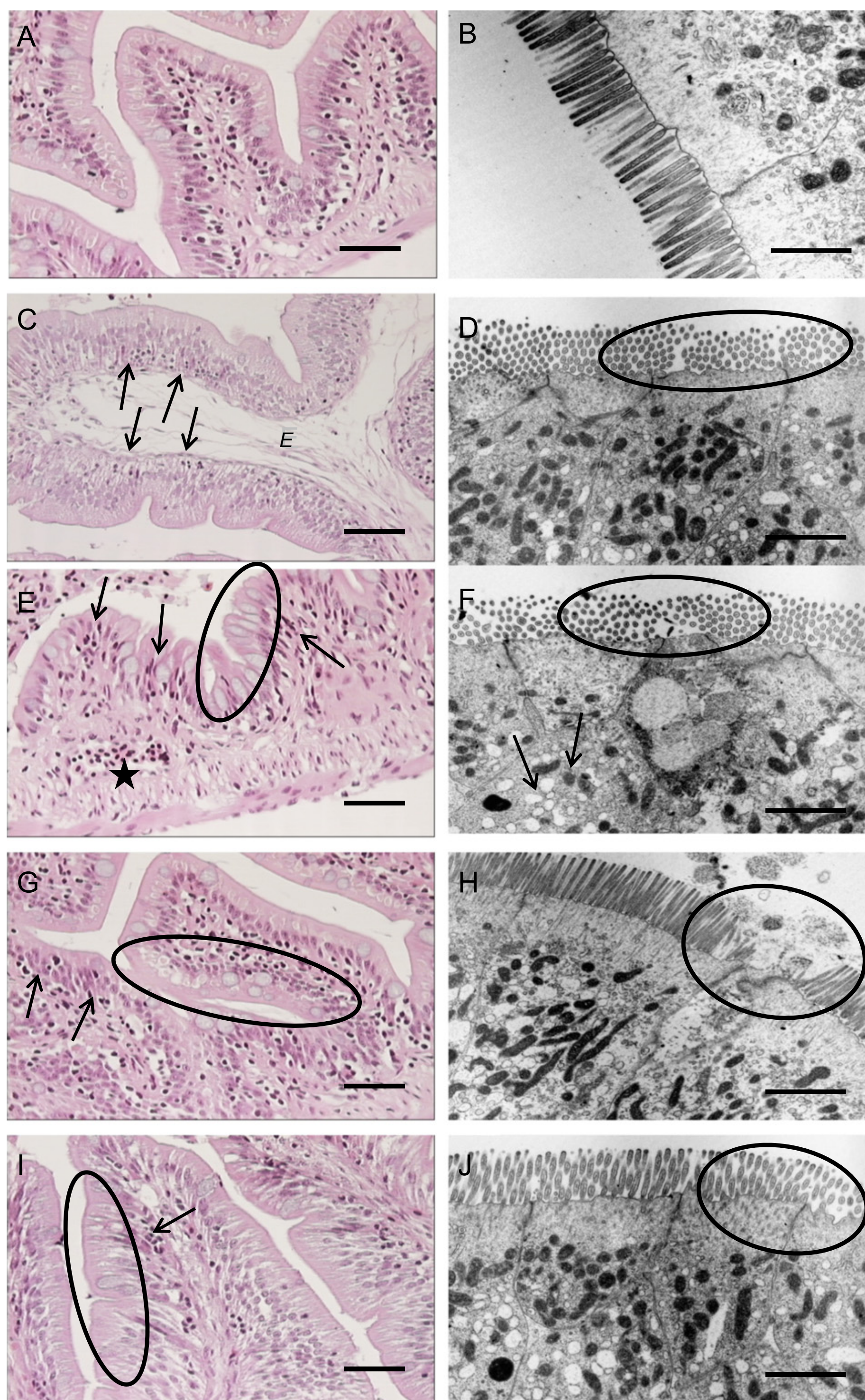

Fig. 4. Histopatohological changes in gastrointestinal tract of Tilapia exposed to 200 μg CYN/kg b.w fish standard (purity ≥ 95%). (A, C, E,G, I): HE-stained intestine section Bars, 100 μm. (B,D,F,H,J): Ultrastructural observations. Bars, 10 μm. (A, B) Control fish: (A) apparently normal intestinal villi and enterocytes, and (B) enterocytes with abundant normal microvilli.(C,D) Tilapia exposed by oral route and euthanized at 24 h: (C) intestinal villi with necrosis of enterocytes (arrow) and severe interstitial edema (E), and (D) decreased size and partial loss of microvilli (circle). (E,F) Tilapia fish exposed by oral route and euthanized at 5 d: (E) detail of intestinal villi with necrotic enterocytes (arrow), abundant caliciform cells (circle) and microhemorrhages (star), and (F) Detail of enterocyte showing a partial loss of microvilli (circle) and cytoplasmic vacuolization (arrow). (G,H) Tilapia exposed by i.p. route and euthanized at 24 h: (G) Intestinal villi showing an increase of caliciform cells (circle) and necrotic enterocytes (arrow), and (H) destruction of microvilli along with other seemingly normal areas. (I,J) Tilapia exposed by i.p. route and euthanized at 5 d: (I) necrotic enterocytes (arrow), abundant caliciform cells (circle), and (J) decreased size and loss of microvilli (circle).

In comparison to mammals, experimental data of CYN in aquatic organisms are very scarce (Nogueira et al., 2004; Berry et al., 2009). Recently, studies from our laboratory have demonstrated that CYN induced changes in oxidative stress biomarkers at biochemical and molecular levels (Puerto et al., in press; Gutiérrez-Praena et al., 2011b), and also histopathological effects in Tilapia. Among the morphological changes, disorganized parenchymal architecture in the liver, dilated Bowman's space in the

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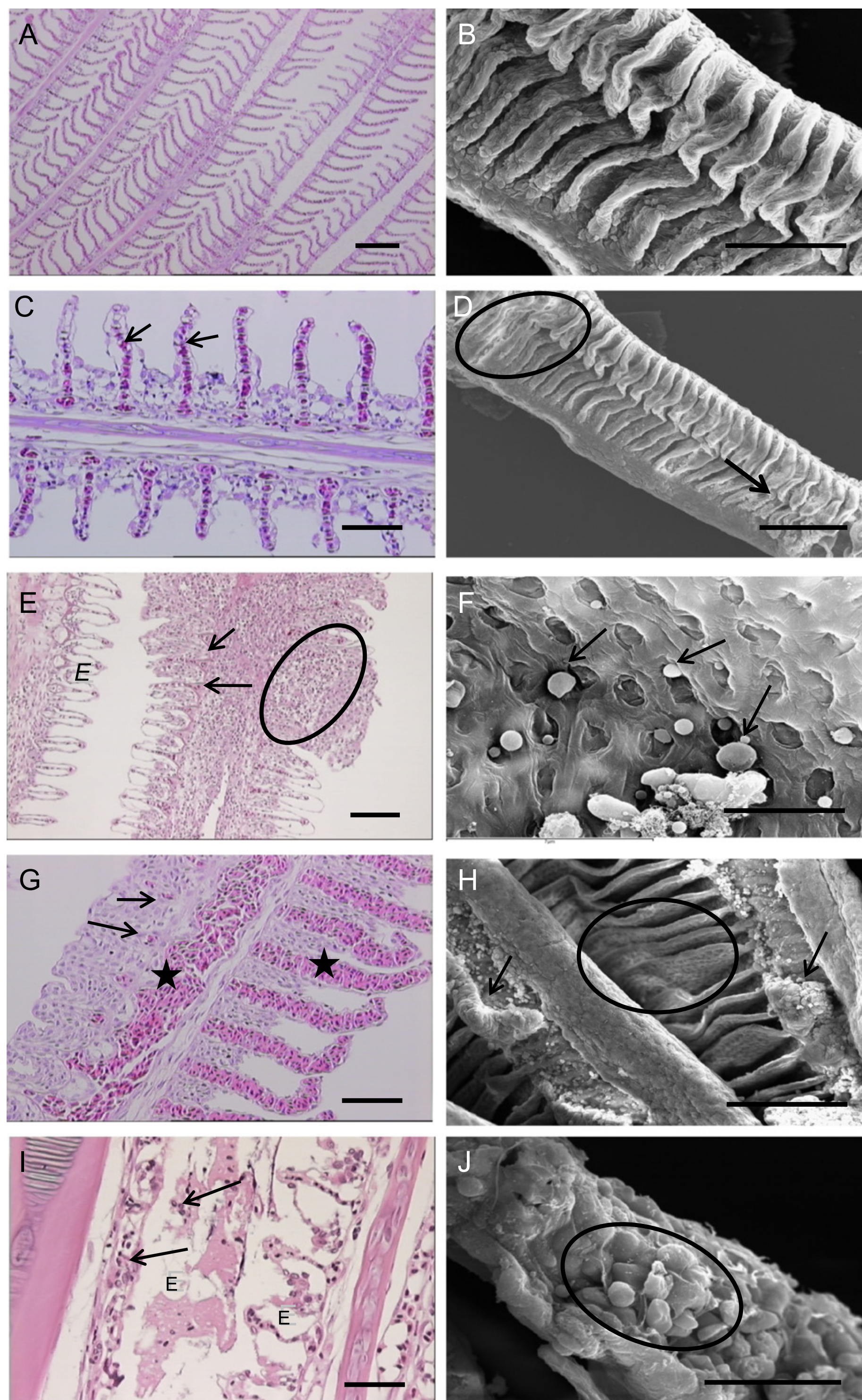

Fig. 5. Histopatohological changes in gills of tilapia exposed to CYN standard (purity ≥95%). (A, C, E): HE-stained gill section. Bars, 100 μm. (B,D,F): Ultrastructural observations. Bars, 100 μm. (A, B) Control fish. (C,D) Tilapia exposed by oral route and euthanized at 24 h: (C) hyperemia and edema in lamellae (arrow), and (D) tumefaction (circle) and some degree of desquamation (arrow) in lamellae. (E,F) Tilapia fish exposed by oral route and euthanized at 5 d: (E) very hyperemic lamellae (arrow), edema (*E*) and microhemorrhages (circle), and (F) secondary gill lamellae with some microhemorrhages (arrow). (G,H) Tilapia exposed by i.p. route and euthanized at 24 h: (G) very hyperemic lamellae (star) and microhemorrhages (arrow), and (H) tumefaction of the lamellae, which become eroded (circle) and hemorrhagic contents (arrow). (I,J) Tilapia exposed by i.p. route and euthanized at 5 d: (I) branchial filament with microhemorrhages (arrow), interstitial and lamellar edema (*E*), and (J) detail of lamellae showing very evident microhemorrhages (circle).

kidney, fibrolysis in the heart, necrotic enteritis in the intestines, and hemorrhages in the gills, were observed (Puerto et al., unpublished results).

As far as we know, this is the first study showing that single doses of CYN pure standard (200 μg CYN/kg fish bw) administered by i.p. route induced pathological changes in Tilapia. Liver and kidney were the main targets of toxicity, but histopathological changes have been observed in other organs, such as heart, intestines and gills. Moreover, the major pathological changes observed in all organs in this work were more severe in fish

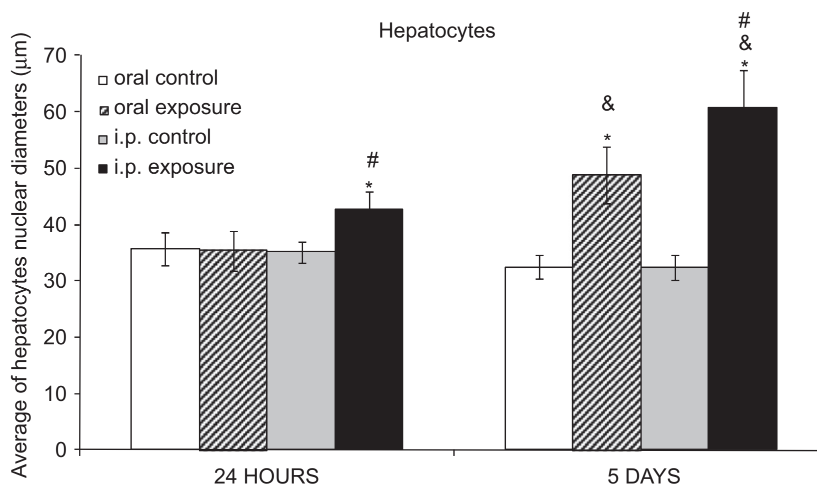

Fig. 6. Hepatocyte nuclear diameters values (µm) of fish exposed by gavage and i.p. injection to 200 µg/kg bw CYN and euthanized after 24 h and 5 d. The values are expressed as mean ± sd ($n=5$). The significance levels observed are $^{*}p<0.05$ in comparison to control group values, # $p<0.05$ when fish were sacrificed at the same time and exposed by different routes, and $p<0.05$ when fish were exposed by the same route and sacrificed at different times.

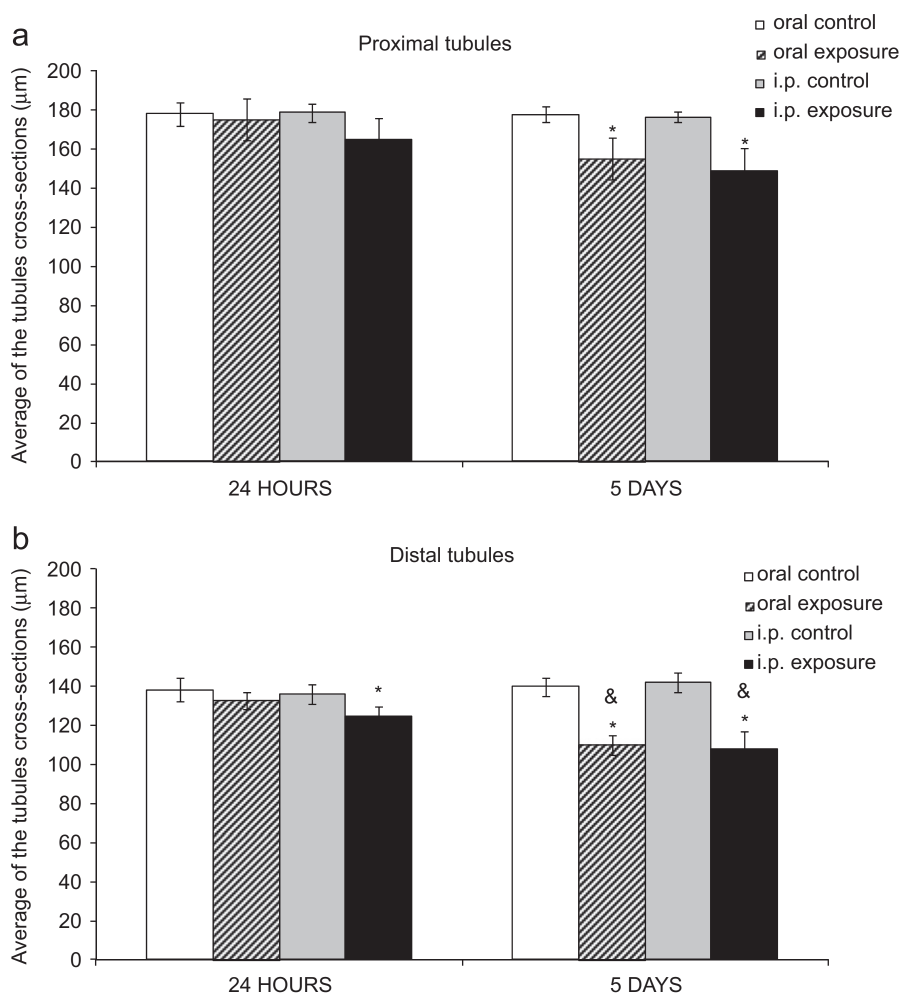

Fig. 7. Proximal (a) and Distal (b) convoluted tubules cross sections (µm) of fish exposed by gavage and i.p. injection to 200 µg/kg bw CYN and euthanized after 24 h and 5 d. The values are expressed as mean ± sd ($n=5$). The significance levels observed are $^{*}p<0.05$ in comparison to control group values, and $p<0.05$ when fish were exposed by the same route and sacrificed at different times.

euthanized after 5 d, in comparison to fish euthanized after 24 h; consequently, no recovery of histological effects induced by CYN in fish was observed. Also, the histological findings were more intense after i.p. administration of CYN, in comparison to fish exposed to CYN by oral route, with the exception of gastrointestinal effects.

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Our major histological findings in the liver of fish exposed to CYN for both routes (loss of the parenchymal architecture, general degeneration with glycogen deposits and steatosis) are in accordance with a previous study from our lab performed in Tilapia exposed by oral route (Puerto et al., unpublished results). In this work it is noticeable that a generalized degeneration in the pancreatic acinus with necrotic cells has been detected in fish exposed i.p. to CYN. Similar alterations were found in the same fish species exposed to MC-LR and MC-RR (500 μg toxin/fish) by i.p. route (Atencio et al., 2008), including the degeneration in the pancreas. The lower potency of CYN after gavage administration in comparison to i.p. injection, could be due to the lack of efficient uptake from the gastrointestinal lumen, as it has been demonstrated in other experimental models. Thus, Froscio et al. (2009) suggested that the delayed entry and low cellular potency of CYN could be explained by its limited uptake into Vero-GFP cells. 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. When toxins enter the liver via the hepatic portal vein, they are removed by a process known as presystemic hepatic elimination, which eliminated toxins during a single pass through the liver under optimal conditions, and this process minimizes their distribution to other parts of the body.

Moreover, an aggravation of the injuries has been detected with the time of sacrifice (5 d vs. 24 h), and hepatocytes showed steatosis to so-called signet ring cells. Lipid accumulation may result from the degenerative processes suffered by the hepatocytes. Vacuolization of hepatocytes and increased lipid content could be not 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 glypoprotein synthesis by CYN, as it is a well know protein synthesis inhibitor and GSH 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).

Accumulation of fat droplets in hepatocytes in the central portion of hepatic lobules was considered as the third phase of the intoxication induced by CYN in mice (Terao et al., 1994). According to the observation in vivo reported by Terao et al. (1994) the appearance of fat droplets lipids has been associated with enhancement in ROS content. In this sense, ROS increase has been observed in PLHC-1 cells exposed to subcytotoxic concentrations of CYN (Gutiérrez-Praena et al., 2011a, 2011b) and also in Tilapia exposed by gavage to this toxin (Puerto et al., unpublished results), where an increase in the NADPH oxidase activity was detected. In any case, the specific mechanism for liver toxicity is incompletely characterized, but involves inhibition of protein synthesis (Froscio et al., 2003; Terao et al., 1994). Moreover, the liver of mice treated with CYN showed membrane proliferation, fat droplet accumulation and reduced amount of total P450 in microsomes, indicating that mechanisms other than protein synthesis inhibition must also contribute to CYN toxicity. Additional support for the involvement of CYP450 in the hepatotoxicity of CYN is the finding that liver histopathology is mainly induced in the region (periacinar) where CYP450-catalyzed xenobiotic metabolism occurs (Shaw et al., 2000). In fish, it is generally not possible to differentiate liver necrosis on a zonal basis in relation to its lobular disposition, as is the case in higher animals, because of the lack of clear lobulation in fish liver which is arranged more as tubules of hepatocytes (Roberts and Rodger, 2003).

To our knowledge no morphometric studies in regard to hepatic changes in rodents or fish, after administration of CYN, have been found in the scientific literature. In this work, the increased size of the nuclear diameter of hepatocytess associated with CYN exposure for both routes, especially in the case of i.p. route and sacrificed at 5 d, support microscopic and ultrastructural findings. In kidney, the main histopathological findings (glomerulopathy with glomerular capillary atrophy, hyperemia, decreased width of the proximal and distal convoluted tubules), were more severe after 5 d of the exposure, especially by i.p. route. Fish showed atrophic and hyalinised glomeruli (light microscopy) and increase in podocyte primary foot processes. Similarly, epithelial hypertrophy, narrowing of the tubular lumen, atrophy of the glomerulus, broader Bowman's capsule, and necrosis in the epithelial cells were observed in kidney tissues of the fresh water fish *Clarias gariepinus* exposed to cypermethrin (Velmurugan et al., 2009). All these alterations indicated a reduced filtration rate leading to higher hydrostatic pressure in the glomerulus and less fluid in the tubules. Moreover, either exposure route or sacrifice time influenced the severity of the lesions detected in tubules, as it can be shown in the width of proximal and distal convoluted tubules, with decreased tubule luminal diameters, indicative of a collapse.

Results from the quantitative study revealed decreases in the cross sections of the urinary tubules of Tilapia exposed to CYN for the first time, which supported the histological findings mentioned above (epithelial hypertrophy, narrowing of the tubular lumen). These effects were always more significant with time. Du et al. (2004) reported that measurements of the luminal diameter are critical to assess changes in hydrodynamic forces and torques, and indicated that a knowledge of the diameters of the tubules is useful in determining the rate of fluid flow through the urinary tubules. Again, the higher potency of CYN after i.p. injection could be explained because the kidneys are potentially exposed to greater concentrations of toxins when they are not subject to presystemic hepatic elimination, and this may occur when CYN enters the body by i.p. injection or if water soluble CYN enters directly through branchial, dermal or conjunctival routes (Carbis et al., 1996).

In comparison to rodents, Falconer et al. (1999) reported that the i.p. administration in mice of extracts of the cyanobacterium *C. raciborkii* 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. The oral toxicity of the material was lower than i.p. toxicity by more than 25-fold, and tissue repair ensued within 5 d. By contrast, in this study no recovery of the renal effects induced by CYN was observed with time. These differences in mice and fish could be attributed to toxicokinetics differences between the species assayed, or in the nature and composition of the materials administered (pure toxin in the present study vs. extracts from a cyanobacterial strain). The proximal renal tubular damage in mice (Humpage and Falconer, 2003), 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).

Histological changes in the heart (myofibrolysis, edema) and gills (mainly tumefaction processes, hyperemia, desquamation areas) were more pronounced when fish were exposed by i.p. route, showing abundant edema and large areas of myofibrolysis in the heart, and very hyperemic gill lamellae and microhemorrhages in gills. Branchial injury was also more severe when the peritoneal route was used to administer MCs in *Cyprinus carpio* L. (Carbis et al., 1996). According to these authors, when the toxin is injected into the peritoneal cavity, it enters the lymphatic system and is directed to the heart via the posterior cardinal vein. The blood is then pumped directly to the gills for the uptake of

oxygen, and CYN would be more accessible to the gill epithelium via the peritoneal route. The occurrence of these changes in gills may be due to the direct action of CYN on the tissue, or are secondary to their effects on receptors bound to epithelial cell membranes, similarly to the pathological effects induced by MCs (Puerto et al., 2010). It is not known whether cardiac edema is directly due to cardiac function failure *per se* or consequent to indirect effects on the kidney, or branchial circulation leading to loss of fluid and ion control.

Intestines of fish exposed to CYN by oral route showed pathological changes (necrotic enterocytes, severe interstitial edema) more intense in comparison to fish intoxicated by i.p. route, and were more pronounced with the time. By the oral route, CYN can cause gastroenteritis through injury to the gut lining, and hemorrhage from blood vessel injury (Duy et al., 2000). The lesions on the gastrointestinal tract could be attributed to its intestinal absorption, which needs active transport systems although due to the small size of CYN, a limited passive diffusion through biological membranes occurs, as indicated by in vitro studies (Chong et al., 2002). Some of these pathological changes (necrotic enteritis, partial desquamation of intestinal villi, edema) were also described in Tilapia exposed i.p. to pure MCs (Atencio et al., 2008), or exposed to MC-LR from *Microcystis* cells by the oral route (Molina et al., 2005), which were more severe at 72 h than at 24 h (Prieto et al., 2009). By contrast, some authors have found recovery of histopathological effects caused by MCs with time (Malbrouck and Kestemont, 2006; Ernst et al., 2006).

Overall, all these histopathological results indicated the delayed toxicity exhibited by CYN in fish (*Oreochromis niloticus*) exposed to a single dose of pure CYN (200 μg CYN/kg fish bw) by i.p. route or by oral administration (gavage). Moreover, the severity of the pathological effects was more pronounced when CYN was administered by i.p. route in comparison to gavage, with the exception of intestines. The liver and kidney were main targets of toxicity, and results from the morphometric study indicated that the average of nuclear diameter of hepatocytes, and cross sections of proximal a distal convoluted tubules may be useful for quantifying the extent of damage or the severity of CYN toxicity in both organs, respectively. Both factors considered, exposure route and the time of sacrifice also influenced the changes on some oxidative stress biomarkers induced by CYN in Tilapia (Gutiérrez-Praena et al., 2011b). Further investigations including clarification of possible biotransformations, and toxicokinetics studies in different models, and especially in aquatic organisms, are required in order to explain the differences in toxicity show by this toxin with the time, and related to the exposure route.

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CAPÍTULO 7 / CHAPTER 7

Daniel Gutiérrez-Praena, María Puerto, Ana Isabel Prieto, Ángeles Jos, Silvia Pichardo, Vitor Vasconcelos, Ana M. Cameán

PROTECTIVE ROLE OF DIETARY N-ACETYLCYSTEINE ON THE OXIDATIVE STRESS INDUCED IN TILAPIA (OREOCHROMIS NILOTICUS) EXPOSED TO CYLINDROSPERMOPSIN

Enviado a Aquatic Toxicology, **2011.**

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Abstract: Cylindrospermopsin (CYN) is a toxin produced by various cyanobacteria species. Fish can be exposed to this cyanotoxin in natural waters and fish farms and may suffer from oxidative damage. The present study investigates the effects of dietary N-acetylcysteine (NAC), a glutathione precursor, on the oxidative stress induced by pure CYN and CYN from lyophilized cells of Aphanizomenon ovalisporum in tilapia (Oreochromis niloticus). Fish were pretreated with 0, 22 and 45 mg NAC/fish/day for a week and on day seven they received a single dose of 200 µg/Kg CYN and were euthanized after 24 hours. Oxidative biomarkers evaluated included lipid peroxidation, protein oxidation, GSH/GSSG ratio, the activity of the enzyme gamma-glutamylcysteine synthetase, and the activity and gene expression of glutathione-S-transferase and glutathione peroxidase. Results showed that CYN induced oxidative stress, as evidenced by the increase of lipid peroxidation and protein oxidation, the decrease in GSH/GSSG, and the alteration of the enzymatic activities assayed. Moreover, the exposure to cyanobacterial cells containing CYN induced higher toxic effects in comparison to pure CYN. NAC supplementation was effective in reducing the CYN induced toxicity, particularly at the highest dose employed, with a recovery of some of the biomarkers assayed to the basal levels. Therefore, NAC can be considered to be a useful chemoprotectant that reduces hepatic and renal oxidative stress in the prophylaxis and treatment of CYN-related intoxications in fish.

Cover Letter

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Dear Prof. M.J. Nikinmaa,

We would be very grateful if you consider the manuscript entitled "Protective role of dietary N-Acetylcysteine on the oxidative stress induced by Cylindrospermopsin in Tilapia (*Oreochromis niloticus*)" for its publication in "**Aquatic Toxicology**".

The authors of the article were: Daniel Gutiérrez-Praena, María Puerto, Ana Isabel Prieto, Ángeles Jos, Silvia Pichardo, Vitor Vasconcelos, and Ana M. Cameán.

Studies dealing with the toxic effects of cylindrospermopsin in fish are scarce, in spite of the occurrence of this cyanobacterial toxin is increasing in fresh water bodies. We have already reported that oxidative stress was involved in the pathogenicity of CYN on tilapia fish (Aquat. Toxicol 2011. 105, 100-106). In the present paper we demonstrated that dietary N-acetylcysteine has a protective effect on this toxic insult, therefore it can be considered a useful chemoprotectant in the prophylaxis and treatment of CYN-related intoxications in fish.

I am looking forward to receiving a positive answer from you.

Sincerely,

Angeles Jos

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1 Protective role of dietary N-Acetylcysteine on the oxidative stress induced by
2 Cylindrospermopsin in Tilapia (*Oreochromis niloticus*)
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1 **Abstract -** Cylindrospermopsin (CYN) is a toxin produced by various cyanobacteria
2 species. Fish can be exposed to this cyanotoxin in natural waters and fish farms and
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5 pure CYN and CYN from lyophilized cells of *Aphanizomenon ovalisporum* in tilapia
6 (*Oreochromis niloticus*). Fish were pretreated with 0, 22 and 45 mg NAC/fish/day for a
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10 synthetase, and the activity and gene expression of glutathione-S-transferase and
11 glutathione peroxidase. Results showed that CYN induced oxidative stress, as
12 evidenced by the increase of lipid peroxidation and protein oxidation, the decrease in
13 GSH/GSSG, and the alteration of the enzymatic activities assayed. Moreover, the
14 exposure to cyanobacterial cells containing CYN induced higher toxic effects in
15 comparison to pure CYN. NAC supplementation was effective in reducing the CYN
16 induced toxicity, particularly at the highest dose employed, with a recovery of some of
17 the biomarkers assayed to the basal levels. Therefore, NAC can be considered to be a
18 useful chemoprotectant that reduces hepatic and renal oxidative stress in the
19 prophylaxis and treatment of CYN-related intoxications in fish.
20

21 **Keywords**: Cyanobacteria, Cylindrospermopsin, Tilapia fish, Oxidative stress, N-
22 Acetylcysteine

1. INTRODUCTION

The cyanobacterial toxin cylindrospermopsin (CYN) is a potent toxic alkaloid which is produced by several cyanobacterial species, including *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Anabaena bergii*, *Anabaena lapponica*, *Lyngbya wollei* and *Raphidiopsis curvata* (Fastner et al., 2003; Pearson et al., 2010). Its chemical structure consists of a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992), and has a molecular weight of 415 Da and high water solubility (Sivonen and Jones, 1999). Furthermore, this cyanotoxin is relatively stable under different light, pH and temperature conditions (Chiswell et al., 1999). The presence of CYN in freshwater systems is raising an environmental concern, since it occurs in recreational and drinking water reservoirs (Brient et al., 2009; Fathaili et al., 2010; Messineo et al., 2010; Quesada et al., 2006; Saker et al., 2004). Its occurrence has been reported in different countries (Rücker et al., 2007) with levels ranging from not detected to 800 µg/L in subtropical Australia (Shaw et al., 2000).

It has been reported that CYN penetrates into cells by passive diffusion (Chong et al., 2002). The toxic mechanism of CYN still has not been completely elucidated. *In vivo* studies suggest the liver and the kidney are the main target organs (Humpage and Falconer, 2003; de Figueiredo et al., 2004) and it also has general cytotoxic (Runnegar et al., 1994, 1995, 2002) and neurotoxic (Kiss et al., 2002) effects. However, other organs such as kidney, heart, thymus, spleen, lungs and ovaries can also be affected by CYN (Young et al., 2008). It is well known that CYN inhibits protein synthesis in both *in vivo* and *in vitro* models (Terao et al., 1994; Froscio et al., 2003) and it is considered to produce a reduction of glutathione (GSH) synthesis and genotoxicity mediated by DNA fragmentation (Froscio et al., 2003; Humpage et al., 2005; Bazin et al., 2010). The high levels of reactive oxygen species (ROS) due to absence of reduced glutathione (GSH) may contribute to genotoxicity (Humpage et al., 2005).

CYN accumulation in different aquatic animals presents a high variability. Organisms such as gastropods, bivalves and crustaceans accumulate higher concentrations of CYN than other more evolved animals (amphibians and fish). The reverse relationship appears to be true for the susceptibility of organisms to CYN toxicity (Kinnear et al., 2009). The predominantly availability of extracellular CYN makes easy the exposure of aquatic organisms (Kinnear, 2010).

1
2 Research into CYN has been performed with purified toxin, extracted toxin or
3 cyanobacterial cells containing the toxin, but these studies are still scarce in fish.
4 Seifert et al. (2007) demonstrated that significant adverse effects were rarely recorded
5 for exposure to CYN concentrations under 100 µg/L of pure CYN for a range of aquatic
6 species from various trophic levels. On the other hand, exposure to *C. raciborskii*
7 extracts resulted in greater sub-lethal toxicity. This suggests that cell extracts are likely
8 to contain more bioactive compounds other than CYN that increase the toxic effects
9 (Kinnear, 2010). Thus, it is important to differentiate between the toxic effects induced
10 by CYN *per se* from those induced by other bioactive substances contained in cell
11 extracts.
12
13 Toxic effects induced by pure CYN on fish have been scarcely reported in the
14 scientific literature. It has been demonstrated that CYN can produce *in vitro* oxidative
15 damage in PLHC-1 cells, derived from fish liver (Gutiérrez-Praena et al., 2011a). *In*
16 *vivo*, studies carried out in tilapia (*Oreochromis niloticus*) exposed by gavage to
17 different doses of pure CYN and sacrificed after 24 h, showed an increase of lipid
18 peroxidation (LPO) and alterations in the activity and gene expression of glutathione-
19 related enzymes (Puerto et al., 2011a). Furthermore, the extent of the oxidative and
20 histopathological damage induced by pure CYN in liver, kidney, heart, intestines and
21 gills in tilapia has shown to be dependent on the exposure way and the time of sacrifice
22 (Gutiérrez-Praena et al., 2011b; 2011c). Thus, taking into account the oxidative nature
23 of CYN toxic effects on fish, the increased occurrence of CYN on freshwater systems,
24 and the commercial importance of tilapias in the aquaculture sector, it would be of
25 interest to find compounds that could prevent these effects without additional toxicity.
26
27 Literature about the protective effect of antioxidants against the toxic action of
28 cyanotoxins in aquatic organisms is scarce. Our group has previously investigated the
29 beneficial effect of some antioxidants compounds like vitamin E, selenium and N-
30 acetylcysteine (NAC) in fish exposed to another cyanobacterial toxin, microcystin (MC)
31 (Prieto et al., 2008; Atencio et al., 2009; Puerto et al., 2009, 2010). NAC, a derived
32 form of the naturally occurring amino acid L-cysteine not present in food, is a known
33 thiolic antioxidant, administered as a mucolytic agent for a variety of respiratory
34 illnesses, that can act against cellular degeneration by several mechanisms. It is a
35 precursor of GSH synthesis as a cysteine supplier and stimulates cytosolic enzyme
36 activities involved in the GSH cycle, such as glutathione reductase (GR), which

enhances the rate of GSH generation (Banaclocha, 2001). As CYN has been reported to inhibit GSH synthesis, the use of NAC as chemoprotectant could counteract this effect. Moreover, NAC also protects the cell by direct reaction with reactive oxygen species (ROS) (Aruoma et al., 1989), such as hypochlorous acid, hydroxyl radical (OH·), and hydrogen peroxide (H_2O_2), whose levels could be increased due to the GSH depletion.

In this context, the aim of the present work was to investigate the dose-dependent role of NAC pretreatment on the toxicity induced by pure CYN and CYN producing cyanobacterial cells in tilapia exposed by the oral route to explore its utility as a chemoprotective agent in intoxicated fish. The biomarkers evaluated included lipid peroxidation (LPO), protein oxidation, GSH/GSSG levels, the activity of gamma-glutamylcysteine synthetase (GCS), and the activity and gene expression of glutathione-S-transferase (GST) and glutathione peroxidase (GPx).

2. MATERIALS AND METHODS

2.1 Chemicals

The cyanotoxin cylindrospermopsin standard (purity $\geq$ 95%) was supplied by Alexis Corporation (Lausen, Switzerland). N-acetylcysteine and all other chemicals for the different assays were provided by Sigma-Aldrich (Madrid, Spain) and VWR International Eurolab (Seville, Spain). Protein assay reagent was obtained from BioRad Laboratories (Hercules, USA).

2.2 Collection of Aphanizomenon ovalisporum strain culture and determination of cyanobacterial toxins

A. ovalisporum (LEGE-X001) was cultured in the Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR, Porto, Portugal). CYN was extracted from the dried cell material using the method of Welker et al. (2002) modified in our laboratory (Guzmán-Guillén et al., 2010). The lyophilized cells (14 mg) were extracted three times with 3 mL of MilliQ water, sonicated for 15 minutes, stirred for 1 hour and sonicated for 15 min. The resulting mixture was centrifuged at 4500 r.p.m. for 10 minutes, after which the supernatant was collected and 6 mL of 0.1% trifluoroacetic acid (TFA) were added. Them it was stirred for 1 hour and allowed to stand 3 hours. The supernatant was taken for further purification / concentration.

The extract obtained was subjected to a purification procedure (Clean-up) which was adapted from Wormer et al. (2009): 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 MilliQ water. Subsequently, the sample is passed through the cartridges and eluted with 10 mL of DCM/MeOH. The sample, was concentrated by evaporation in a rotary evaporator and resuspended in 500 µL MilliQ water. The liquid chromatographic (LC) system used to analyse the toxin contents was a Varian 9012 equipped with a Varian ProStar 330 Diode Array Detector (DAD). Chromatographic data were processed with a Star Chromatography Workstation (Varian Technologies). Chromatographic separation of CYN was performed according to Welker et al. (2002) on a 250 mm x 4.6 mm i.d., 5 µm, LiChrosphere C18 column purchased from Merck (Darmstadt, Germany). 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 lyophilized cells was 7.3 µg/mg.

2.3 Experimental setup and acclimation of fish

Seventy-two male *O. niloticus* (Nile tilapia; Perciformes:Cichlidae; wt, 51 ± 7 g; length, 12 ± 3 cm) were obtained from Valenciana de Acuicultura SA (Valencia). Fish were transferred to the laboratory, where they were held in nine glass aquaria (n = 8 individuals/aquarium), with 96 L of dechlorinated tap water, a continuous system of water filtration and aeration (Eheim Liberty 150 Bio-Espumador cartridges), and a 12:12-h light:dark photoperiod. Temperature was maintained at 21 ± 2ºC 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) containing 6% lipids, 31% proteins, 37% carbohydrates, 2.5% fiber, 1.5% total phosphorus, 12% ash, 200 mg α-tocopherol/kg, 1,700 IU vitamin D3/kg feed, and 10,000 IU vitamin A/kg feed. The amount of commercial food administered per fish was 0.3 g/d. Fish were acclimatized for 15 d before the beginning of the experiments.

2.4 Exposure

The different experimental groups considered are shown in table 1. Fish were fed a mixture of the different components (fish food, pure toxin, toxic lyophilized cyanobacterial cells and NAC, depending on the treatment) that had been manually crushed in a mortar followed by sonication. 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. The pellets were placed in the tank and drifted to the bottom for the fish to take. It was visually ensured that all the pellets were eaten within an hour. The amount of commercial fish food administered per fish was 0.3 g/day.

Fish in aquaria 1-3 (n=8) were fed daily with commercial fish food for 7 days. Tilapias in NAC groups (aquaria 4 - 9) were fed in a similar way but receiving NAC supplementation (22 or 45 mg/fish/day), as shown in table 1, daily for 7 days. These NAC doses were chosen according to the previous study carried out by Puerto et al., (2009) on the same fish species intoxicated with MC. After day 7 fish from aquaria 5,6,8 and 9 received single doses of CYN as described in table 1, being aquarium 1 used as control (no CYN).

At the end of the experiment, 24h after the toxin exposure, all fish were anaesthetised in ice and killed by transection of the spinal cord. Liver and kidney 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). 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 biochemical measurements.

2.5 Lipid peroxidation, protein levels and protein carbonyl content

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman, 1990). Values were presented as nmol TBARS /g tissue.

Protein contents in the liver samples were estimated by the method of Bradford (1976) using bovine γ-globuline as standard. Results were expressed as mg protein/g tissue.

Protein carbonyl content was assayed by the method described by Levine et al. (1990) using 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 M HCl, 20% trichloro acetic acid (w/v), and 6 M guanidine hydrochloride, as it has been described by Atencio

et al., (2008). Results are expressed as nmol carbonyl/mg protein, using the extinction coefficient 22,000 M^{-1} cm^{-1}.

2.6 GSH/GSSG levels and enzymatic assays

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

Gamma-glutamylcysteine synthetase (EC 6.3.2.2.) is the limiting enzyme in GSH synthesis. It was determined using the method described by Seelig and Meister (1985) adapted to fish samples (Gutiérrez-Praena et al., 2011b). Glutathione S-transferase activity (GST, EC 2.5.1.18) was measured in the liver and renal homogenates according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase as described by Lawrence and Burk, (1976). All enzymatic activities were expressed in nkat/mg prot.

2.7 RNA preparation and reverse transcription

Total RNA was extracted and purified using the RNeasy Mini Kit ™ (Cat. Nº 74104, Qiagen, Madrid, Spain) according to the manufacturer instructions, as previously described by Puerto et al. (2010). The RNA integrity was assessed by agarose gel electrophoresis. RNA quality was assessed as the 260/280 nm absorbance ratio using an Eppendorf biophotometer (Netheler-Hinz GmbH, Hamburg, Germany). The RNA was then stored at -80 ºC before further processing. Reverse transcription (RT) was performed using random hexamers primers, 4 µg of total RNA and the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions.

2.8 Real-time polymerase chain reaction (RT-PCR)

A semi quantitative RT-PCR protocol was developed to measure the mRNA levels of sGST, GPx and beta-actin as an external control in liver and kidney of tilapia. The cDNA obtained was diluted in sterile water and used for the amplification by the PCR. The forward and reverse primers used in this study are shown in Table 2. All mRNA sequences were obtained from GenBank (EU234530, EF206801, for sGST, GPx and beta-actin, respectively). PCR primers for sGST, GPx and beta-actine were

obtained from Sigma-Aldrich (Spain). Each specific gene product was amplified by Real time PCR using the ABI Prism 7000 sequence detector (Applied Biosystems) according to the following parameters: 50 ºC for 2 min, 95 ºC for 10 min, 95 ºC for 15 s (40 cycles) and 60 ºC for 1 min (40 cycles). Amplification data were collected by the sequence detector and analyzed with sequence detection software. For each assay, a standard curve was constructed using increasing amounts of cDNA. In all cases, the slope of the curves indicated adequate PCR conditions (slopes of 3.3–3.6). The RNA concentration in each sample was determined from the threshold cycle (Ct) values and calculated with the sequence detection software supplied by the manufacturer. The quantitative fold changes in mRNA expression were determined relative to beta-actine mRNA levels in each corresponding group and calculated using the $2^{-\Delta\Delta CT}$ method.

2.9 Statistical analysis

Biochemical results are subjected to one-way analysis of variance (ANOVA) and represent mean ± SD of eight animals 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

3.1 Effects on lipid peroxidation and protein oxidation

Both toxic treatments induced a 1.5-fold increase of LPO values in the liver of exposed fish non-pretreated with NAC, with no differences regarding the type of CYN employed (pure or from cyanobacterial cells). The NAC pretreatment reduced these values, particularly with 22 mg NAC/fish/day, these groups did not show significant changes respect to their corresponding NAC-only exposed fish group (Fig. 1a). Similarly, in the kidney the toxin exposure resulted in an increase, whereas both NAC doses reduced the values to their respective control levels (Fig. 2a).

Protein oxidation did not show changes with any of the treatments in the liver (Fig. 2a), but on the contrary, the kidney experienced an increase (1.5 and 1.2-fold with pure CYN and cyanobacterial cells, respectively) that was partially reduced with 22 mg NAC/fish/day (the increase is still maintained in cyanobacterial cells exposed group) and restored to basal values with 45 mg NAC/fish/day (Fig. 2b).

1 *3.2 Effects on GSH/GSSG levels and enzymatic assays*

2 The GSH/GSSG ratio in the liver experienced a deep decrease that was more
3 accentuated with lyophilized cells (4-fold) than with pure CYN (2.6-fold) in non-
4 supplemented fish. The lowest dose of NAC although could not restore the basal
5 levels, ameliorated the toxic effect and the GSH/GSSG ratio only decreased 1.4-fold in
6 comparison to the 22 mg NAC only exposed fish group. The highest NAC dose, 45
7 mg/fish/day, prevent the toxic effect induced by pure CYN, as evidenced by the lack of
8 change on this biomarker, but it could not counteract completely the decrease due to
9 the cyanobacterial cells exposure (1.6-fold) (Fig. 3a). In the kidney, while pure CYN did
10 not modify the GSH/GSSG levels, cyanobacterial cells induced a 6.3-fold decrease,
11 value that was partially improved with the lowest dose of NAC that reduced the
12 decrease to 1.6-fold. Fish exposed to cyanobacterial cells and the highest dose of NAC
13 showed no differences respect to the NAC-only control group preventing this toxic
14 effect (Fig. 3b).
15
16 The activity of GCS in the liver was reduced with both toxic exposures, being
17 the treatment with cyanobacterial cells the one that induced a higher decrease (1.7-
18 fold). The pretreatment with both doses of NAC induced the decrease of this parameter
19 not statistically significant in comparison to their respective control groups (Fig 4a). In
20 the kidney also a decrease of this enzymatic activity was observed with both toxic
21 exposures (1.2-1.3-fold). Both NAC doses prevented the enzymatic inhibition induced
22 by pure CYN, but the lowest dose of NAC (22 mg/fish/day) increased significantly this
23 parameter (1.3-fold) when fish were exposed to cyanobacterial cells. Moreover, the
24 highest dose of NAC (45 mg/fish/day) enhanced the values of this biomarker in fish
25 without toxic treatment (1.4-fold) (Fig 4b).
26
27 Glutathione-S-transferase activity in the liver experienced an increased with
28 both toxic treatments (1.5-fold). The increase induced by pure CYN was reduced with
29 NAC in a dose-dependent manner and with the highest dose no changes respect to the
30 corresponding control group were observed. When fish were intoxicated with
31 cyanobacterial cells both NAC doses avoid the increase of this enzymatic activity and
32 no changes respect to the corresponding control groups was observed (Fig 5a). In the
33 kidney this parameter was not modified by any of the toxic treatments and the NAC
34 supplementation had no effect on fish exposed to pure CYN. However, when fish were
35 exposed to cyanobacterial cells the GST activity increased significantly (2-fold) with
36 both NAC doses.

Regarding to the GPx activity, none of the toxic exposures induced significant changes in the liver. However, intoxicated fish experienced a gradual increase of the enzymatic activity with the NAC pretreatment. Thus, an increase was evident in fish exposed to pure CYN and the highest dose of NAC (1.2-fold) and in fish exposed to cyanobacterial cells and both NAC doses (> 2-fold) (Fig. 6a). In kidney, on the other hand, neither the toxic exposure nor the NAC supplementation induced any change in this biomarker.

3.3 Glutathione-S-transferase and glutathione peroxidase gene expression

No significant change was observed in the relative gene expression of the enzyme GST in liver when tilapia fish were exposed to pure and lyophilized CYN. However, the pretreatment with 22 mg NAC/fish/day resulted in a significant enhancement (3-fold) in the expression of the enzyme in the exposure to pure CYN. This increase was less marked in liver of fish treated with the highest dose of NAC (Fig. 7a). The most remarkable change observed in kidney is the significant increase (4-fold) in the group exposed to CYN from lyophilized cyanobacteria, although the gene expression decrease to the control values when fish were also exposed to NAC. Moreover, the pretreatment with NAC induced a significant dose-dependent increase in the control groups (Fig 7b).

Regarding effect of NAC in the relative transcriptional abundance of GPx genes in liver, significant dose-dependent increases were observed in all the groups of fish exposed to CYN from lyophilized material. However, this enzyme gene expression was only enhanced in fish feed with pure CYN when they were pretreated with 22 mg NAC/fish/day. Similarly to the results observed in GST, NAC *per se* is able to increase the expression of the GPx (Fig. 8).

4. DISCUSSION

As it has been proved in this study and in previous investigations, CYN can induce oxidative stress (Gutiérrez-Praena et al., 2011b; Puerto et al., 2011a) and histopathological lesions (Gutiérrez-Praena et al., 2011c, in press; Puerto et al., 2011b, in press) on fish. Cyanobacterial blooms can occur in different freshwater systems both in natural and in man-made facilities such as aquaculture ponds. The presence of CYN

1 in these environments could have a negative impact not only in natural ecosystems but
2 also on the economy of this particular industrial sector. In this sense, finding
3 compounds that allow the fish to bear with this toxic insult is of great interest. This is
4 the first study that explores the utility of dietary NAC on the prophylaxis of CYN induced
5 damage on fish.
6
7 In natural conditions fish are exposed to cyanobacterial bloomsand these
8 organisms may contain not only CYN but also other bioactive unknown compounds.
9 Our results showed that both types of exposure (pure CYN or CYN from lyophilized cell
10 cultures) induced oxidative stress, as evidenced by the increase in LPO and protein
11 oxidation, decrease of GSH/GSSG levels and alteration of the enzymatic activities
12 assayed. But the exposure to CYN from cyanobacterial cells induced the toxic effects
13 in a major extent, particularly in GSH/GSSG ratio and GCS activity. Other authors have
14 observed the same response, with a higher toxicity induced by *C. raciborskii* extracts in
15 comparison to pure CYN in aquatic invertebrates (Seifert et al. 2007). Also, Falconer et
16 al. (1999) performed a toxicity study in mice after i.p. administration of four different
17 batches of *Cylindrospermopsis raciborskii* cell extracts and observed that the variability
18 in the *in vivo* toxicity and the histological damage did not correlate with, nor was
19 explained by, the known CYN content of the lysates employed. The implication drawn
20 from these data was that more than one toxin was likely to be present in that organism.
21 These effects are similar to those observed with other cyanobacterial toxins such as
22 MC and different authors have reported higher toxic effects of lyophilized cells in
23 comparison to pure toxins (Falconer, 2007; Vasconcelos et al., 2007; Puerto et al.,
24 2010; Pichardo and Pflugmacher, 2011).
25
26 As we have previously mentioned GSH/GSSG ratio and GCS activity are highly
27 affected by CYN exposure. The reduction in GSH levels could result from decreased
28 synthesis or increased utilization of this molecule to fight against oxidative situation, as
29 the increase in LPO and protein carbonyl groups showed. GSH is synthesized in two
30 steps by GCS and glutathione synthetase (Meister and Anderson, 1983). Glutathione
31 biosynthesis by way of the gamma-glutamyl cycle is important for maintaining GSH
32 homeostasis and normal redox status (Zhang et al., 2006). Considering that the
33 reduction in GSH content occurs together with the decrease in the activity of the
34 enzyme GCS, the depletion of GSH may be due to an inhibition of its synthesis. In fact,
35 other compounds such as chloroform has been reported to inhibit GCS activity and to
36 reduce GSH levels in rat hepatocytes (Ekström et al., 1982). Our results agree with

1 previous in vitro assays in fish hepatic cells exposed to CYN (Gutiérrez-Praena et al.,
2 2011a). These effects are in concordance with the reported toxicity mechanism of CYN
3 as inhibitor of GSH synthesis (Runnegar et al., 1994, 1995). However, Norris et al.
4 (2002) pointed out that depletion of GSH by CYN could be not a primary mechanism
5 for CYN toxicity and suggested that CYN activation by P450 had a more important role.
6
7 NAC is a precursor of GSH synthesis (Banaclocha, 2001). This agrees with our
8 results that show a recovery of the decrease in the GCS activity induced by the toxin
9 and also in GSH/GSSG levels. GCS is the limiting enzyme in the GSH synthesis so this
10 biomarker and GSH levels are prone to be influenced by a NAC supplementation, as it
11 has been corroborated in this study. Also, Sevgiler et al. (2007) pointed out that the
12 decrease observed in GSH content was the reflection of a decline in GCS activity in
13 *Cyprinus carpio* exposed to fenthion and NAC. Moreover, these authors also reported
14 reductions in GST activity. However, NAC injection improved significantly the GCS and
15 GST specific enzyme activities, which were reduced with the dichlorvos treatment in
16 the liver of *A. anguila* (Peña-Llopis et al., 2003). Puerto et al. (2009) also observed a
17 recovery of GSH/GSSG levels mediated by NAC on tilapia fish orally exposed to MC.
18 On the contrary to the results observed in the present research, there are also studies
19 that do not show a protective effect of NAC on the oxidative stress induced by
20 chemicals such as fenthion (Piner et al., 2007).
21
22 NAC, apart from acting as a GSH precursor, it can interact directly with ROS
23 (Aruoma et al., 1989), and this could explain the decrease in LPO and protein oxidation
24 observed in the pretreated fish in comparison to intoxicated only groups. Also, in
25 general (except in the gene expression of GST and GPx), NAC supplementation did
26 not affect the biomarker values in fish non-exposed to the toxin, indicating that it only
27 ameliorates or prevents the toxic effects induced by CYN.
28
29 Different results have been observed between the activity and the gene
30 expression of GST and GPx. Several authors have also evidenced alterations of the
31 antioxidant enzymes gene expression induced by different cyanotoxins (MCs and CYN)
32 (Li et al., 2008; Sun et al., 2008; Zegura et al., 2011, in press), but none of them
33 explore the relationship between the activity and gene expression. This finding could
34 be due to a post-transcriptional regulation of these enzymes (Puerto et al., 2011a).
35 Moreover, the different activity and gene expression observed in GST in liver and
36 kidney may be due to a tissue-specific expression pattern as other authors have

1 suggested (Gadagbui and James, 2000) perhaps related to their functional
2 requirements. The increase in the activity of GST observed in kidney of tilapias
3 exposed to CYN, previously found in our laboratory in fish orally and intraperitoneally
4 exposed to this toxin for 24 h (Gutiérrez-Praena et al., 2011d), could be a signal of the
5 detoxification activity of this tissue. The pretreatment with NAC induced, in general, a
6 significant dose-dependent increase in the gene expression of both enzymes, GST and
7 GPx, in the control group. Therefore, these effects induced by NAC could indicate the
8 role of this compound in the defensive systems of fish to detoxify endogenous
9 compounds such as peroxidised lipids (Banaclocha, 2001), since an increase in the
10 LPO was also found in the present work.
11
12 Regarding to the doses of NAC employed (22 and 45 mg/fish/day), both were
13 effective in preventing the oxidative stress induced by CYN, but in general the highest
14 dose induced a higher protective effect. The dose of the chemoprotectant is quite
15 important because it could produce toxic effects *per se*. Thus, Puerto et al. (2009)
16 evaluated the effect of 3 doses of NAC (20.0, 44.0, and 96.8 mg NAC/fish/d) on the
17 toxic effects induced by a single dose of MC on fish and observed that 96.8 mg
18 NAC/fish/d induced significant alteration of some enzymatic activities, such as
19 superoxide dismutase, GPx, and glutathione reductase. Some authors found that high
20 doses of NAC could be toxic via oxidative stress by increasing free radical formation in
21 endotoxin lipopolysaccharide–intoxicated rat lungs (Sprong et al., 1998). Also, it has
22 been reported that high NAC concentrations (10 mM) caused mortality in fish (Peña-
23 Llopis et al., 2003). It was suggested that the pro-oxidant activity is the result of
24 transition metal–dependent auto-oxidation yielding superoxide radical (O_2^-), H_2O_2, and
25 the reduced form of the transition metal, which may behave as a catalyst in free radical
26 reactions (Sevgiler et al., 2007). In the present study, the highest dose of NAC used
27 (45 mg/fish/day) did not produce additional toxicity. The low toxicity of NAC join to other
28 characteristics (e.g. low price, and high solubility) makes it suitable as a dietary
29 supplement for preventing cyanobacterial intoxication in fish.
30
31
32
33 **5. CONCLUSION**
34
35 Dietary NAC supplementation contributed to restore the values of oxidative
36 stress biomarkers altered by pure CYN and CYN from *A. ovalisporum* lyophilized cells

1 on tilapia. Therefore, NAC can be considered as a useful chemoprotectant in the
2 prophylaxis and treatment of CYN-related intoxications in fish.
3
4

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2

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34 1281-1292.

1 **Figure captions**

2

3 Figure 1. Lipid peroxidation (LPO) values in liver and kidney of control fish and fish
4 exposed to 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and
5 two different levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg
6 NAC/fish/day) or without it. The values are expressed as mean ± sd (n=8). Values of
7 LPO are expressed as nanomoles of thiobarbituric acid reactive susbstances (TBARS)
8 per gram of tissue. The significance levels (p<0.05) observed are: (a) in comparison to
9 their respective control group and (b) 22 or 45 mg NAC vs 0 mg NAC groups are
10 compared.

11

12 Figure 2. Protein oxidation values in liver and kidney of control fish and fish exposed to
13 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and two different
14 levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg NAC/fish/day) or
15 without it. The values are expressed as mean ± sd (n=8). Values are expressed as
16 nanomoles of carbonyl groups per milligram of protein. The significance levels (p<0.05)
17 observed are: (a) in comparison to their respective control group, (b) when 22 or 45 mg
18 NAC only vs 0 mg NAC groups are compared, and (d) when pure CYN vs lyophilized
19 CYN only exposed groups are compared.

20

21 Figure 3. Reduced glutathione/oxidised glutathione ratio (GSH/GSSG) in liver and
22 kidney of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from
23 lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC)
24 supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed
25 as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison
26 to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups
27 are compared, (c) when 22 mg NAC vs 45 mg NAC only groups are compared, and (d)
28 when pure CYN vs lyophilized CYN only exposed groups are compared.

29

30 Figure 4. Gamma glutamyl cysteine synthetase (GCS) activity (nkat/mg protein) in liver
31 and kidney of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from
32 lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC)
33 supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed
34 as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison
35 to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups

are compared, (c) when 22 mg NAC vs 45 mg NAC only groups are compared, and (d) when pure CYN vs lyophilized CYN only exposed groups are compared.

Figure 5. Glutathion-S-transferase (GST) activity (nkat/mg protein) in liver and kidney of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups are compared, and (c) when 22 mg NAC vs 45 mg NAC only groups are compared.

Figure 6. Glutathione peroxidase (GPx) activity (nkat/mg protein) in liver and kidney of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups are compared, (c) when 22 mg NAC vs 45 mg NAC only groups are compared, and (d) when pure CYN vs lyophilized CYN only exposed groups are compared.

Figure 7. Relative gen expression of glutathion-S-transferase (GST) in liver and kidney of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups are compared, (c) when 22 mg NAC vs 45 mg NAC only groups are compared and (d) when pure CYN vs lyophilized CYN only exposed groups are compared.

Figure 8. Relative gen expression of glutathione peroxidase (GPx) in liver of control fish and fish exposed to 200 µg/Kg of pure CYN or CYN from lyophilized *A. ovalisporum* cells and two different levels of N-acetylcysteine (NAC) supplementation (22 and 45 mg NAC/fish/day) or without it. The values are expressed as mean ± sd (n=8). The significance levels (p<0.05) observed are: (a) in comparison to their respective control group, (b) when 22 or 45 mg NAC only vs 0 mg NAC groups are compared, (c) when

1 22 mg NAC vs 45 mg NAC only groups are compared and (d) when pure CYN vs
2 lyophilized CYN only exposed groups are compared.

Table(s)

Table 1 – Feeding conditions (with or without NAC for 7 days) of *O. niloticus* and exposure conditions to pure CYN or *A. ovalisporum* lyophilized cells containing CYN.

Treatment Condition	1	2	3	4	5	6	7	8	9
Pure CYN	-	+	-	-	+	-	-	+	-
Lyophilized cells with CYN	-	-	+	-	-	+	-	-	+
7 day NAC 22 mg/fish	-	-	-	+	+	+	-	-	-
7 day NAC 45 mg/fish	-	-	-	-	-	-	+	+	+

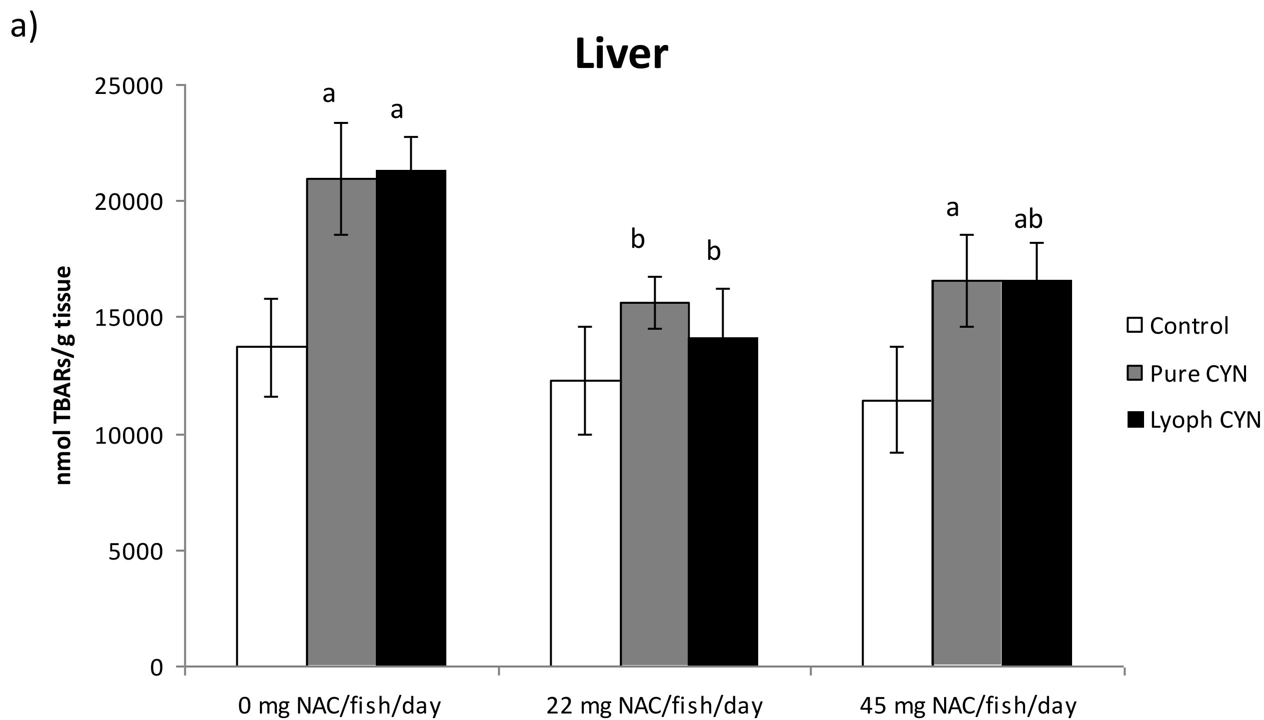
Table(s)

Table 1. Nucleotides sequences (5’-3’) of PCR primers of beta-actine, GPx and sGST.

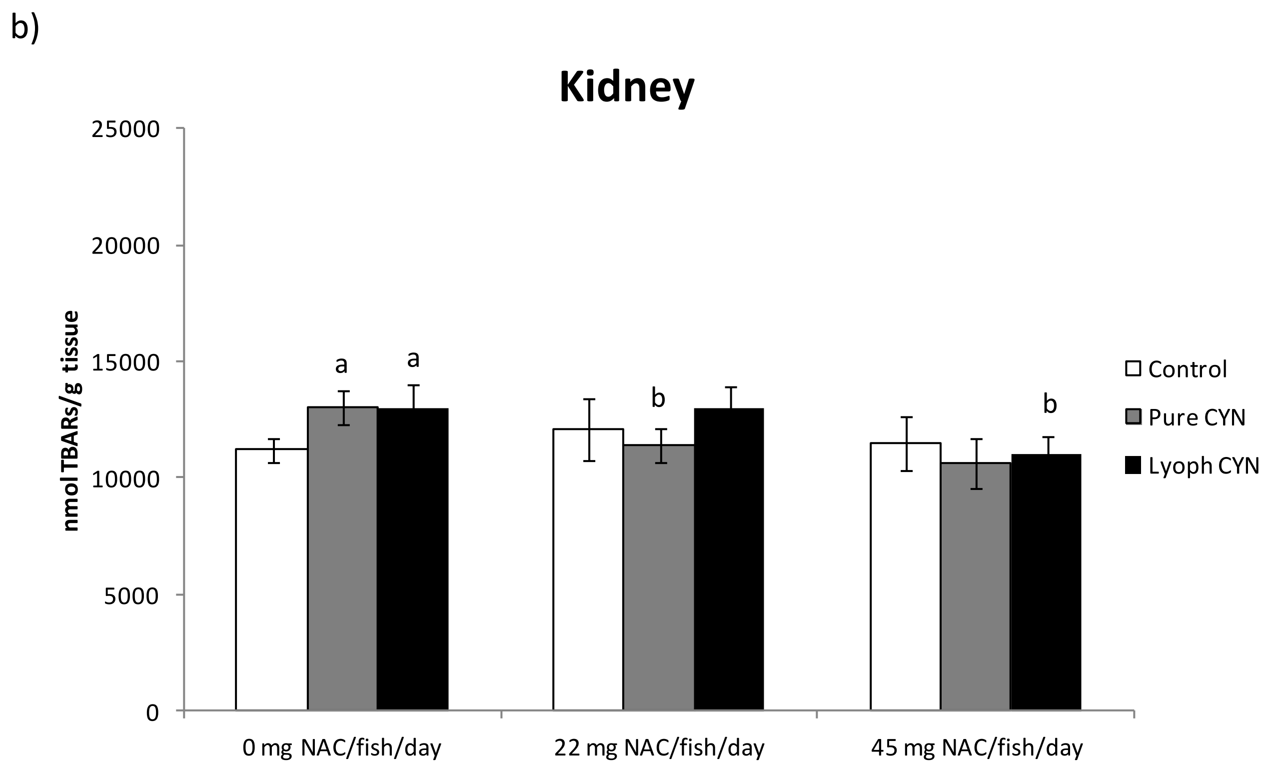
Gene	Fordward primer	Reverse primer
beta-actine	CAATGAGAGGTTCCGTTGC	AGGATTCCATACCAAGGAAGG
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA

Figure(s)

a)



b)



Figure(s)

a)

Liver

b)

Kidney

Figure(s)

a)

Liver

2.5
2.0
1.5
1.0
0.5
0.0

GSH/GSSG

bc
a
a
ab
ab
abd

□ Control
■ Pure CYN
■ Lyoph CYN

0 mg NAC/fish/day
22 mg NAC/fish/day
45 mg NAC/fish/day

b)

Kidney

16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0

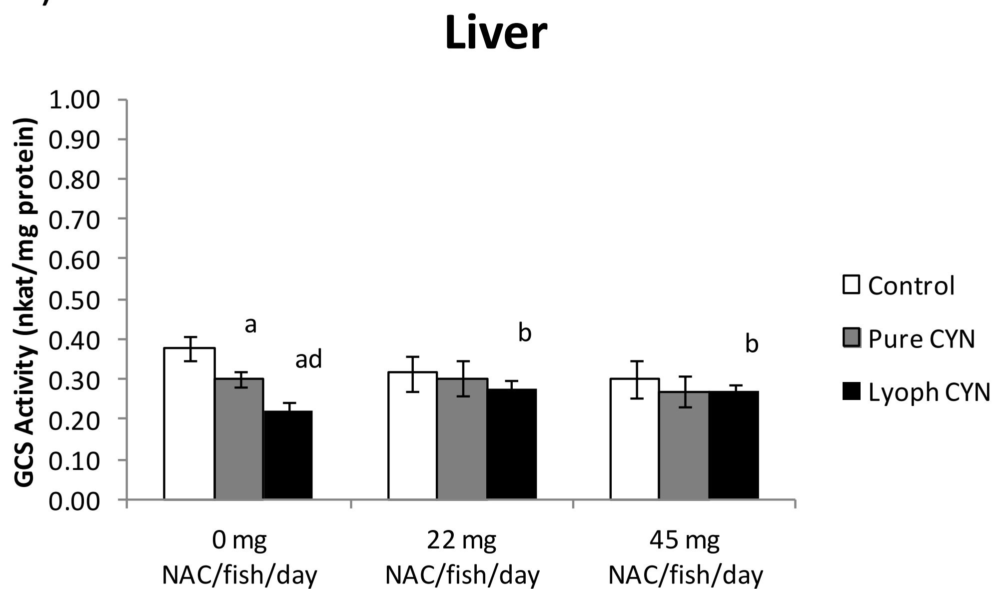
GSH/GSSG

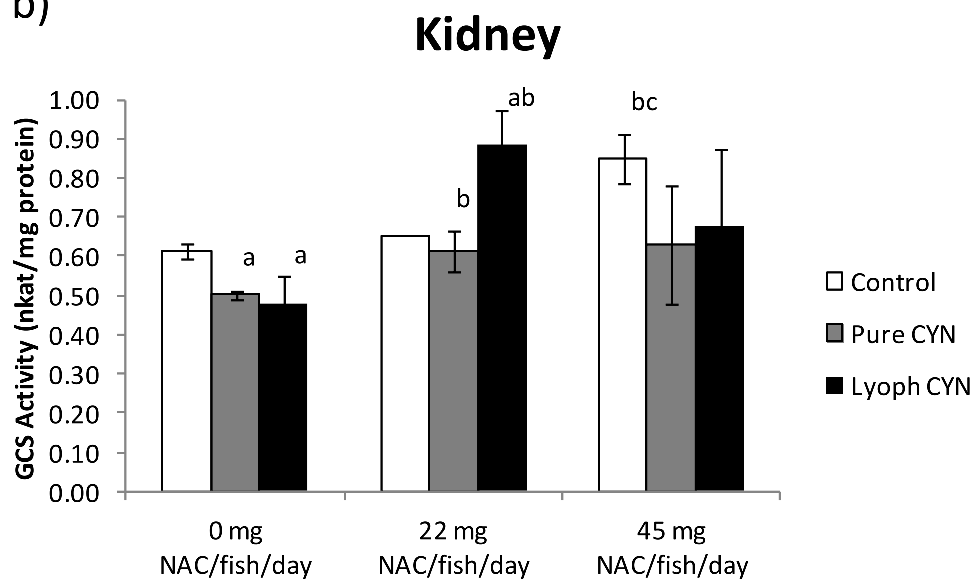
abd
b
ad

□ Control
■ Pure CYN
■ Lyoph CYN

0 mg NAC/fish/day
22 mg NAC/fish/day
45 mg NAC/fish/day

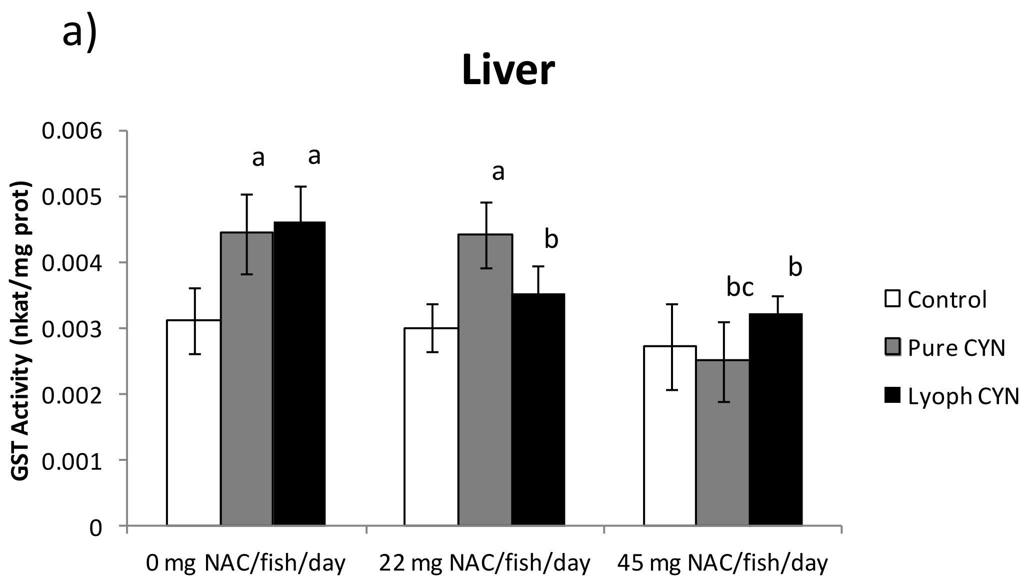
Figure(s)



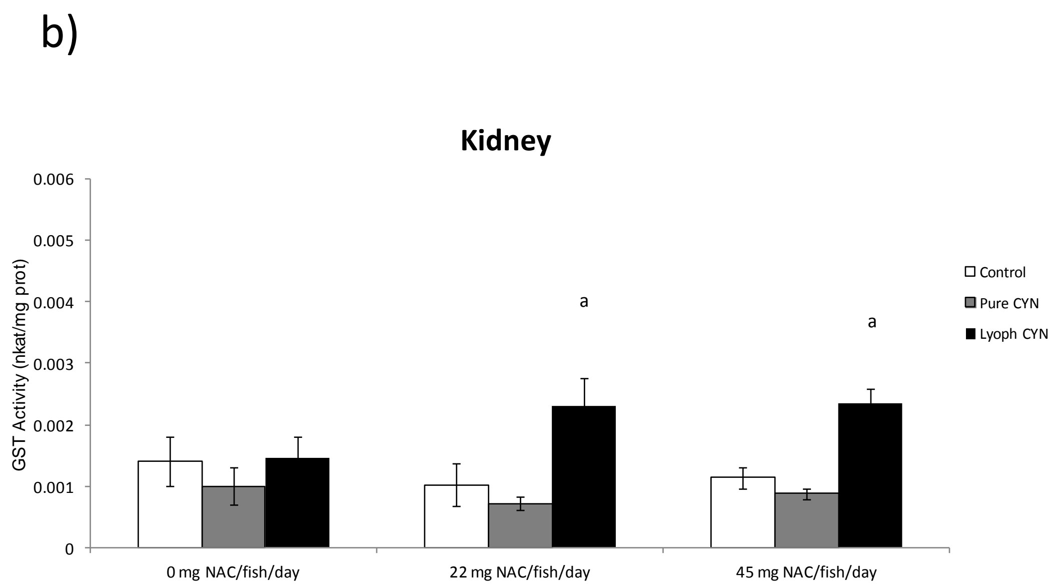


Figure(s)

a)

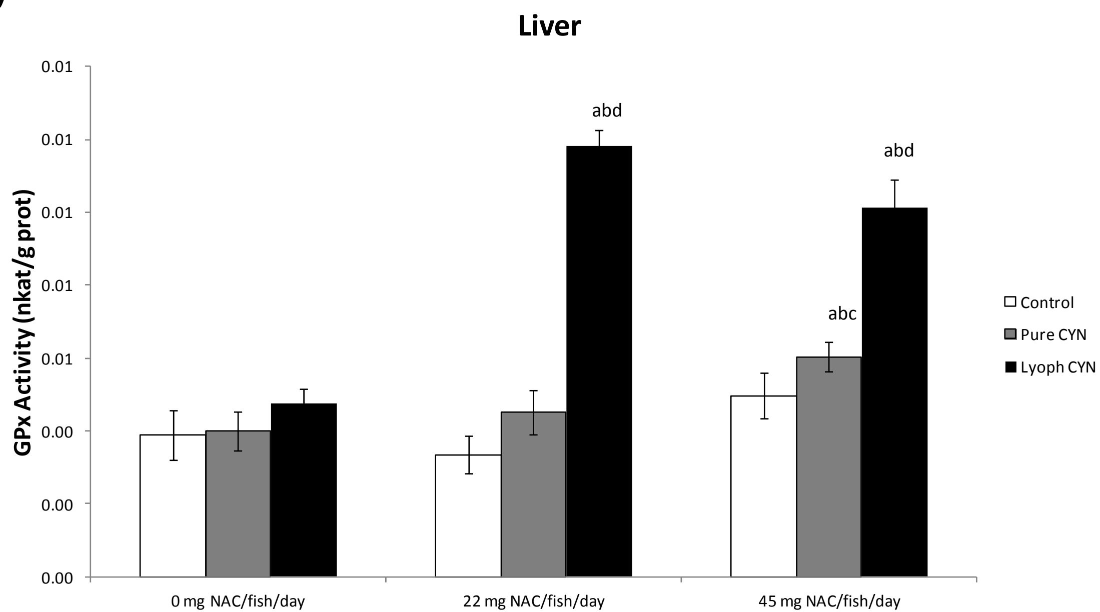


b)

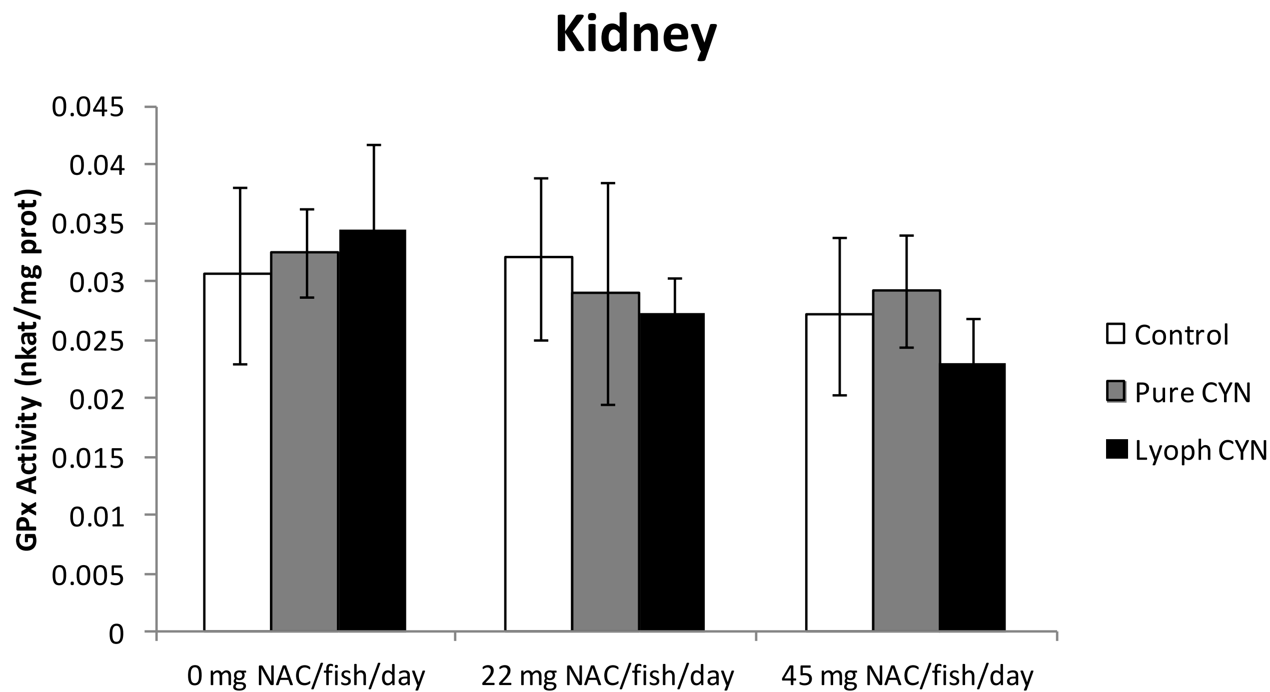


Figure(s)

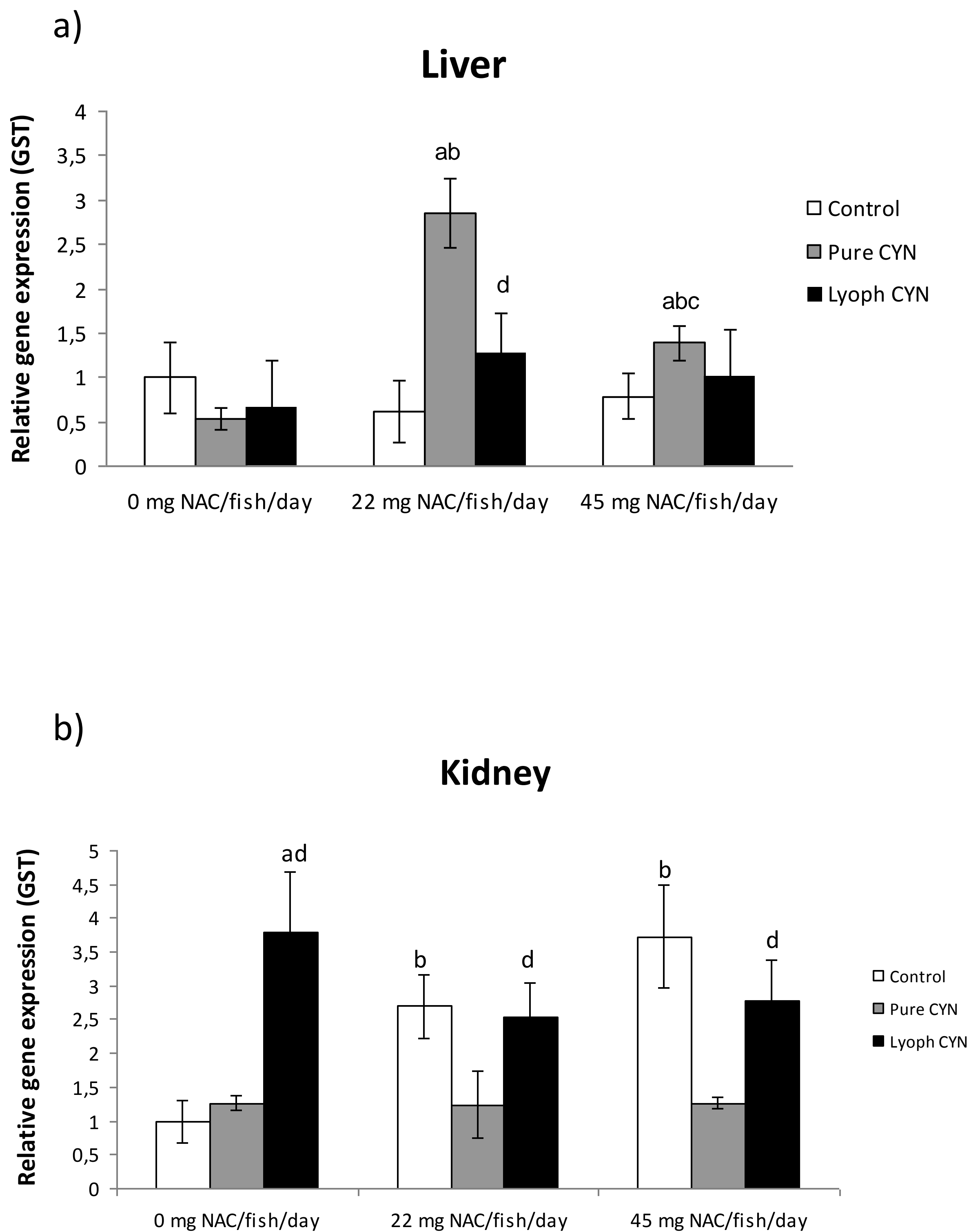
a)



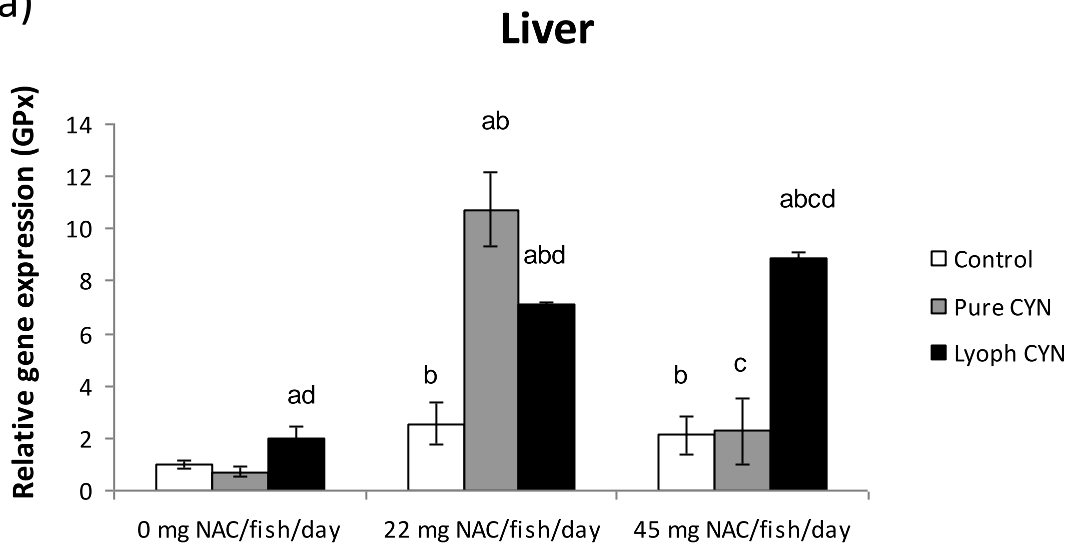
b)



Figure(s)



Figure(s)



***Highlights**

1. CYN induces oxidative stress in tilapia

2. NAC prevents the oxidative damage induced by CYN

3. Both NAC doses (22 and 45 mg/fish/day) were effective as chemoprotectant

CAPÍTULO 8 / CHAPTER 8

Daniel Gutiérrez-Praena, Alexandre Campos, Joana Azevedo, Ana M. Cameán, Vitor Vasconcelos

ALTERATIONS ON PROTEIN EXPRESSION INDUCED BY CYLINDROSPERMOPSIN IN TOMATO PLANTS (SOLANUM LYCOPERSICUM)

Pendiente de envío

1 Alterations on protein expression induced by Cylindrospermopsin in tomato plants
2 (*Solanum lycopersicum*)
3

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15
16 [a] Contributed equally to this work
17

18 **Abstract**

19

20 Toxic cyanobacteria are considered emerging world threats, being responsible for the
21 degradation of the aquatic ecosystems. *Aphanizomenon ovalisporum* produces the
22 toxin Cylindrospermopsin (CYN) being a concern in fresh water habitats. This work
23 aims to increase our knowledge on the effects of these toxic cyanobacteria in plants by
24 studying the alterations on protein expression and oxidative stress status of tomato
25 plants (*Solanum lycopersicum*) exposed to pure CYN or CYN extracted from a
26 lyophilized *A. ovalisporum* culture, dissolved on irrigation water during a period of two
27 weeks. In this work, a proteomics approach based in the two-dimensional gel
28 electrophoresis and mass spectrometry was used to study the effects of the exposure
29 in roots and leaves, and also, a determination of CYN accumulation by HPLC in roots
30 leaves and fruits after 7 and 14 days of exposure was performed.

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47 Keywords: Solanum lycopersicum, cylindrospermopsin, Aphanizomenon ovalisporum,
48 oxidative stress, proteomics

49

Introduction

Cylindrospermopsin (CYN) is a tricyclic alkaloid produced by cyanobacterial species such as *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*. The molecule has bioactive properties and is currently considered toxic to animals and humans. Outbreaks of human poisoning and cattle mortality have been related with the proliferation of *C. raciborskii* and the accumulation of CYN (Saker et al., 1999). Therefore a major concern exists for the presence of these cyanobacterial species in aquatic ecosystems and the decrease in water quality (Saker et al., 2003). Despite the increasing investigation on CYN, the biological role of this molecule is still under debate. One hypothesis discussed by Bar-Yosef et al. (2010) is that CYN in the water column may increase the availability of organic phosphorous by inducing the extracellular alkaline phosphatases produced by other phytoplankton species. In this way, species that do not produce these enzymes, such as *A. ovalisporum*, would better control the source of organic phosphorous.

The cyanotoxin biosynthesis involves the activity of an amidinotransferase, as well as the non-ribosomal polypeptide synthetase (NRPS) and polyketide synthase (PKS) enzymes AoaA, AoaB, and AoaC genes (Schembri et al., 2001; Shalev-Alon et al., 2002; Mihali et al., 2008).

CYN target organs in mammals may include the liver, kidney, lungs, spleen, thymus and heart (Hawkins et al., 1985; Griffiths and Saker, 2003). Moreover, the cyanotoxin was reported to induce cytotoxicity and genotoxicity both *in vivo* and *in vitro* (Bain et al., 2007; Bazin et al., 2009). It is established that CYN may act through the inhibition of glutathione and protein synthesis (Terao et al., 1994; Froscio et al., 2001, 2008) but the process may be mediated by cytochrome P-450 (CYP450)-generated metabolites (Humpage et al., 2005).

Exposure and feeding experiments with CYN-producing *C. raciborskii* cells and cell extracts showed that aquatic animals such as *Melanoides tuberculata* and *Bufo marinus* tadpoles can accumulate the cyanotoxin (White et al., 2006, 2007). This feature may be involved in the decrease of relative growth rate, mortality and injuries in multiple organs of the animals (Kinnear et al., 2007; White et al., 2007). CYN concentrations up to 2.52 µg/g tissue dry weight were described in the freshwater mussel *Anodonta cygnea* when exposed to CYN-producing *C. raciborskii* (Saker et al.,

86 2004). Moreover distinct CYN concentrations were found in the different organs of the
87 animals (Saker et al., 2004).
88
89 The effects of CYN and cyanobacterial cell extracts in plants are relatively less known.
90 Nevertheless this is an important area of investigation providing essential data on the
91 putative impacts of the use of contaminated water and soils in crop production,
92 cyanotoxin accumulation and transfer through the food chain. Vasas et al. (2002) first
93 demonstrated the CYN toxicity in plants by reporting the growth inhibition of etiolated
94 *Sinapsis alba* seedlings exposed to the isolated toxin from *A. ovalisporum*. Metcalf et
95 al. (2004) reported the inhibition of tobacco (*Nicotiana tabacum*) pollen germination by
96 purified CYN at concentrations greater than 5 µg/ml. Recently, Beyer et al. (2009)
97 showed that CYN, at concentrations between 0.5 and 40 µg/ml, decreases root and
98 shoot elongation in the aquatic macrophyte *Phragmites australis*. Nevertheless, the
99 cyanotoxin led to an increase of root number and induced the formation of a callus-like
100 tissue and necrosis in root cortex. Evidences suggest that CYN induces abnormal
101 mitosis and alterations in microtubule organization in the exposed macrophytes (Beyer
102 et al., 2009). Variable responses were observed in *Hidrilla verticillata* exposed to *C.*
103 *raciborskii* whole-cell extracts, according to the cyanotoxin concentration (25, 50, 100,
104 200, and 400 µg/L) and length of exposure (Kinnear et al., 2008). In specific conditions
105 the exposure led to a significant increase in root production and decrease in the main
106 stem elongation (Kinnear et al., 2008). Decreases in chlorophyll and changes in the
107 chlorophyll *a:b* ratio pointed out that extracts might exert complex effects on
108 photosynthesis (Kinnear et al., 2008).
109
110 Studies have shown that a large proportion (over 50%) of the total pool of the
111 cyanotoxin may be present in the extracellular growth media or water phase (Norris et
112 al., 2001; Metcalf et al., 2002). Besides, CYN has a relatively low molecular weight of
113 415 Da and is highly soluble in water (Neilan et al., 1999). This situation increases the
114 risks of contamination by the direct use of water for plant irrigation and crop growth. In
115 this context, it is important to understand how plants are affected by the exposure to
116 the cyanotoxin and if this molecule is incorporated and accumulated in plant tissues.
117
118 In comparison to CYN, for Microcystins (MCs), one important group of toxins produced
119 by cyanobacteria, there is a considerable body of information on their adverse effects
120 in plants (Pflugmacher et al., 2006, 2007). It has been also pointed out that MCs could
121 be accumulated in the edible plants (Codd et al., 1999, Crush et al., 2008) being
122 therefore transferred to human beings through the food chain.

123 One putative mechanism of cyanotoxin action in plants is the induction of oxidative
124 stress. This has been suggested for MCs since the cyanotoxins are capable of
125 physiological stress in plants concomitant with the alterations in low-molecular-weight
126 antioxidants and anti-oxidative enzyme activities (Pflugmacher et al., 2007). Activities
127 of enzymes from the anti-oxidative mechanism, such as glutathione-S-transferase
128 (GST), peroxidases and glutathione reductase were subsequently measured to assess
129 the effects of MC-LR and cyanobacterial cell extracts in different plant species
130 (Pichardo and Pflugmacher, 2011). Recent studies suggest a relation between the
131 toxicity of CYN and the production of reactive oxygen species (ROS), alterations in
132 glutathione levels and activities of GST and glutathione peroxidase (GPx) in animal
133 models (Gutiérrez-Praena et al., 2011; Puerto et al., 2011). Nevertheless it is not
134 known if CYN is able to induce oxidative stress in plants.
135
136 In this work we exposed the tomato plants (*Solanum lycopersicum*) to pure CYN and
137 CYN extracted from an *A. ovalisporum* lyophilized culture, in an attempt to mimic a
138 putative situation in the natural environment in which plants are grown in fields
139 contaminated with toxic cyanobacteria blooms or irrigated with contaminated water.
140 The adverse effects on tomato plants were determined by measuring biochemical
141 parameters such as the differences in protein expression as well as the activity of
142 glutathione S-transferase (GST) and glutathione peroxidase (GPx) were registered to
143 assess the plant physiological condition. An ecological relevant concentration (100
144 µg/L) was used and the effects analyzed up to 14 days of exposure.
145
146 **2. Material and Methods**
147
148 *2.1. Biological material*
149 Tomato seeds were sterilized with ethanol (70%, v/v) for 1 min, followed by 3 washes
150 with sterile distilled water (5 min) and subsequently treated with sodium hypochlorite
151 (0.5%, w/v) for 5 min. Seeds were hydrated for 1 h in sterile distilled water and
152 germinated for 7 days in trays containing sterile full strength Hoagland solution as
153 described by Leggett and Frere (1971), at 25 °C and sun light intensity with a light/dark
154 period of 14/10 h. Plants were further grown in 4 L hydroponic cultures, in the same
155 conditions. The nutrient solution was aerated and renewed every 3 days. *A.*
156 *ovalisporum* strain (Nogueira et al., 2006) was grown as described by Saker et al.
157 (2003) in bulk axenic cultures with medium Z8, at 25 °C, 22 $\mu Em^{-2} s^{-1}$ light intensity
158 with a light/dark period of 14/10 h. After 45 days of culture, the biomass were collected
159 and concentrated by filtration from *A. ovalisporum* culture and by continuous

160 centrifugation. Finally, the concentrates were frozen at –80 °C and lyophilized (Telstar
161 cryodos 80 model).
162
163 *2.2. Cyanobacterial cell extracts and quantification of CYN*
164 *A. ovalisporum* extracts were obtained based on the methods described by Welker et
165 al. (2002). Briefly, freeze dried cells (0.7 g) were sonicated for 15 min in 5 ml distilled
166 water. The homogenate was stirred for 1 h at room temperature, centrifuged and the
167 supernatant collected. Five microliters of TFA (0.1%, v/v) was added to the
168 supernatant, stirred for 1 h, allowed to stand for 3 h and centrifuged a second time.
169 The LC system used to analyze the cyanotoxin content was a Varian 9012 equipped
170 with a Varian ProStar 330 Diode Array Detector. Chromatographic data were
171 processed with a Star Chromatography Workstation (Varian Technologies).
172 Chromatographic separation of CYN was performed according to Welker et al. (2002)
173 on a 250 mm×4.6 mm i.d., 5 μm, LiChrosphere C18 column purchased from Merck
174 (Darmstadt, Germany). CYN standard of 95% purity was supplied by Alexis (San
175 Diego, CA, USA), the limit of detection for this compound in extracts of freeze dried
176 cells is 0.92 mg/L. Standard solutions of CYN were prepared in water (100 μg/ml) and
177 diluted as required with water for their use as working solutions (0.08–5.0 μg/ml). After
178 analysis, the concentration of CYN obtained from lyophilized cells was 3.64 μg CYN/g
179 lyophilized. The extract was diluted to a final concentration of 100 μg CYN/L.
180
181 *2.3. Exposure experiments*
182 The experiment was performed in this work to assess the effects of pure CYN and a
183 CYN extracted from an *A. ovalisporum* lyophilized culture in tomato plants. One month
184 old tomato plants were exposed to 100 μg CYN/L three times a week for two weeks.
185 After plants exposure, fruits, leaves and roots were separated, the fresh weight
186 determined, and subsequently stored at −80°C until biochemical and proteomics
187 analysis. Samples were collected at days 7 and 14.
188
189 *2.4. Cyanotoxin extraction and quantification from plant tissues*
190 The dry weight of freeze dried plant tissues was determined and total CYN extracted
191 following the methods described in Section 2.2 after a process of freezing at –80°C,
192 freeze drying and subsequent sonication of plant tissues. CYN was extracted in
193 distilled water with TFA (0.1%, v/v). The total CYN were subsequently quantified using
194 the commercially available Elisa tests from Abraxis (Los Angeles, California, USA)
195 specific CYN molecule. The cyanotoxin quantifications were performed according to
196 the manufacturer's instructions. In the analysis, control samples were introduced

197 (extracts from leaves, roots and fruits of plants not exposed to cyanobacterial cell
198 extracts) to screen for false positives. The Elisa assays of controls were negative.
199 Moreover a quality control positive sample from the Elisa kit and also a sample
200 consisting in an extract (from each matrix spiked) with a known dose of these toxins
201 were analyzed in order to avoid false negatives.
202

203 *2.5. Sample preparation for 2DE*
204 Plants parts were ground in liquid nitrogen with mortar and pestle. The ground tissue
205 powder (0.1 g) was homogenized for 1 h in 250 µl of urea (8 M), thiourea (2 M), 3- [(3-
206 cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS) (4%, w/v),
207 dithiothreitol (65mM) and ampholytes, pH 4–7 (0.8%, v/v) (protein solubilization buffer,
208 SB). Proteins were precipitated with acetone and the homogenate was centrifuged at
209 16,000 g, for 20 min at 22°C. The supernatant was collected and proteins quantified
210 with the method of Bradford (1976). Protein samples were stored at −80°C.
211

212 *2.6. Two-dimensional electrophoresis (2DE)*
213 The two-dimensional electrophoresis was based in the procedure described by Puerto
214 et al. (2011). Protein samples with 400 µg of protein were diluted to 300 µl in SB buffer.
215 The protein samples were loaded in 17 cm, pH 4–7 IEF gel strips (Bio-Rad, Hercules,
216 CA, USA) and proteins separated by isoelectric focusing (IEF) in a Protean IEF Cell
217 (Bio-Rad, Hercules, CA, USA) with the following program: 16 h at 50V (strip
218 rehydration); step 1, 15 min at 250 V; step 2, 3 h voltage gradient to 10,000V (linear
219 ramp); step 3, 10,000V until achieving 60,000 V/h (linear ramp). Wet paper strips were
220 used in the electrodes to remove the excess of salts from the samples. After the first
221 dimension IEF gel strips were stored at −20°C until performing the second-dimension
222 SDS-PAGE. IEF Gel strips were equilibrated as described by Campos et al. (2009)
223 using 10 mg/ml dithiothreitol and 25 mg/ml iodoacetamide in urea (6 M), glycerol (30%,
224 v/v), SDS (2%, w/v). Subsequently IEF gel strips were placed on top of 12% (w/v)
225 acrylamide SDS-PAGE slab gels (20cm×20cm×1 cm) and proteins separated by SDS-
226 PAGE in a Protean Xi Cell (Bio-Rad, Hercules, CA, USA) at 24mA per gel. In this
227 procedure one 2DE gel was run per replicate. Gel staining and protein visualization
228 was performed as described by Neuhoff et al. (1988).
229

230 *2.7. Gel image acquisition and protein expression analysis*
231 2DE Gel images were acquired in the GS-800 Calibrated Densitometer (Bio Rad,
232 Hercules, CA, USA) and protein spots detected automatically with the PDQuest 2-D
233 Analysis Software (Bio-Rad, Hercules, CA, USA), reproducing the sensitivity

234 parameters for every gel image. Spot detection and spot matching was manually
235 revised in the software. Protein spot intensities were normalized in terms of the total
236 density in the gel image. For protein expression analysis a master gel was obtained in
237 the software with all the spots detected in the 2DE gel images. The presence/absence
238 of spots and quantitative variations in spot intensities was subsequently analyzed by
239 comparison of the intensity of each protein spot between the experimental groups. In
240 this analysis only spots that were detected in at least two replicate gels were taken in
241 consideration. The quantitative variations were statistically validated using t-Student
242 test ($P \leq 0.05$).
243
244 *2.8. Statistical analysis*
245 All experiments were performed in triplicate. Data were subjected to one-way analysis
246 of variance (ANOVA) and represented by the mean value ± SE. Differences in mean
247 values between groups were assessed by the Tukey's test and were considered
248 statistically significant at $P<0.05$ level.
249
250 **3. Results**
251
252 *3.1 Plant growth*
253 Fresh weight and dry weight of tomato roots, leaves and fruits were measured after 7
254 and 14 days of exposure to extracts of *A. ovalisporum* and pure CYN in order to assess
255 possible changes in this parameter.
256
257 *3.2. Two-dimensional gel electrophoresis of proteins*
258 Proteins from leaves and roots of *Solanum lycopersicum* were separated in well
259 defined/delimited colloidal Coomassie Blue stained spots by 2DE. Proteins were
260 separated between the pH 4 and 7 and molecular mass 15 kDa and 80 kDa in the 2DE
261 gels as shown in figure 1.
262
263 *3.3. Differential protein expression*
264 In this study the expression of individual protein spots resolved by 2DE from the tomato
265 exposed to the CYN extract from *A. ovalisporum* and pure CYN were compared with
266 the expression of the same protein spots resolved by 2DE from control plants. The
267 differential protein expression (DPE) comprised several proteins as summarized in
268 Table 1.
269
270 **4. Discussion**

271 In this work one-month-old tomato plants were exposed to CYN extracted from an *A.*
272 *ovalisporum* lyophilized culture and pure CYN aiming to study the potential effects that
273 can result from the use of contaminated water or soils in agriculture, namely in the
274 growth of tomato. Plant growth and biochemical parameters were determined from
275 roots and leaves in order to evaluate the effects caused by this exposure.
276
277 Elisa assay detected the presence of free CYN in root and leaf tissues from tomato
278 plants exposed during 7 and 14 days to *A. ovalisporum* cell extracts and pure CYN
279 suggesting an up-take of this molecule by root cells and further translocation to other
280 plant organs such as leaves. The bio-concentration factor (BCF) is an efficient way to
281 measure the bioaccumulation of xenobiotics and toxins in the organisms.
282 Bioaccumulation in a biological system occurs when the BCF values are superior to 1.
283 The parameter can be calculated dividing the CYN concentration in plant tissues by the
284 exposure concentration (Karjalainen et al., 2003). BCFs of 0.82 and 0.68 were
285 therefore estimated for CYN in the roots and leaves of tomato plants, respectively,
286 indicating that, under the particular experimental conditions used, tomato plants did not
287 accumulate the cyanotoxin CYN. The bound-CYN was not estimated in this work
288 nevertheless this form of the toxin is likely to occur together with the free-CYN in plant
289 tissues contributing to an increase of the total CYN accumulation. Further studies will
290 be required to estimate the percentage of CYN that might be bound to other molecules
291 and cellular structures, and not properly extracted by the procedures employed in this
292 work.
293
294 Bioaccumulation of CYN was studied by White et al. (2005) in the aquatic macrophyte
295 *H. verticillata*. The authors reported the presence of free CYN in the tissues of the
296 aquatic plant and an average BCF of 0.045 indicating no CYN bioaccumulation, which
297 is in agreement with our findings. Despite the lack of cyanotoxin bioaccumulation the
298 authors recorded highest tissue cyanotoxin concentrations in plants exposed to high
299 CYN concentrations (superior to100 µg/L). Interestingly the BCF reported in this work
300 for tomato is 18 times higher than the BCF reported by White et al. (2005) for the
301 aquatic macrophyte, which might suggest for differences between species regarding
302 the CYN up-take and detoxification processes. Moreover, the authors hypothesize that
303 CYN is not incorporated into the cells in high extent but is instead adsorbed in the plant
304 cell walls. This would explain the easy elimination of almost all cyanotoxin from the
305 macrophyte tissues during the depuration experiment (White et al., 2005).
306 Nevertheless, the CYN detected in the leaves of tomato plants suggests a process of
307 cyanotoxin transport through the vascular system with an uptake at the root level via

308 plasma membrane and symplastic transport, allowing the cross of the endodermis
309 barrier. Plants have been shown to up-take and partition organic forms of nitrogen (N)
310 such as amino acids, peptides and proteins (Tegeder and Rentsch, 2010). These
311 compounds may be regarded as additional N sources, especially in conditions of
312 limited availability of this element (Tegeder and Rentsch, 2010). Families of protein
313 transporters, with different specificities, have been discovered playing a crucial role in
314 these plant capabilities (Tegeder and Rentsch, 2010). Thereby it should be considered
315 the possibility of CYN accumulation in plant tissues involving similar transport systems.
316 In this sense, some authors suggest that the characteristic nature of CYN to cause root
317 stimulation at low doses (Kinnear et al., 2008; Beyer et al., 2009) suggests that the
318 transport of the cyanotoxin to the roots may be possible, because the roots are buried
319 in the sediment unlikely to come into contact with CYN suspended in the water column.
320
321 Environmental concentrations of CYN were monitored by Bogialli et al. (2006) in the
322 eutrophic Albano Lake in Italy, during a 4 month period, using liquid chromatography
323 and tandem mass spectrometry. The authors reported variations in total CYN content
324 (intra and extracellular) between 0.41 and 18.4 μg/L in the contaminated waters.
325 Variations were related with the month and the depth of sampling. Highest CYN
326 concentrations were verified in the surface waters (up to 5 m depth). More recently,
327 Gallo et al. (2009) detected extracellular CYN in the Averno Lake, south Italy, at 0.58
328 and 1.8 μg/L. These data indicate that 2.5 μg/L of CYN, considered in this work, can be
329 present in contaminated superficial waters being environmentally relevant. The effects
330 verified in the tomato plants emphasize the potential consequences of the use of such
331 waters, in plant growth and physiology. The impact to the environment, food production
332 and animal and human health attributed to the water use can be increased if we
333 consider that CYN is a relatively stable compound (Chiswell et al., 1999), persisting in
334 the water after cyanobacterial senescence (Eaglesham et al., 1999).
335
336 The effects in protein expression of tomato plant, from the exposure to A. ovalisporum
337 cells extract and pure CYN, were evaluated by comparing the 2DE protein profiles of
338 control plants with the protein profiles of plants exposed to +CYN A. ovalisporum cells
339 and pure CYN. The 2DE followed by MALDITOF/TOF analysis allowed characterizing
340 protein variations in roots and leaves of the tomato plant when exposed to the
341 cyanobacteria cells and pure toxin. The proteomics approaches followed were
342 therefore particularly important for the sensitivity in reporting the plants physiological
343 condition.
344

345 The majority of the differentially expressed proteins identified belong to the group of
346 structural proteins. Alterations in these kind of proteins have long been reported in
347 situations of cellular stress and apoptosis (Alvarez and Sztul, 1999; Bursch et al., 2000;
348 Kanlaya et al., 2009) and recently Gácsi et al. (2009) described alterations in
349 cytoskeletal structures and apoptosis in Chinese hamster ovary cells (CHO-K1)
350 mediated by CYN. At the proteomic level correlations were found between the changes
351 in the expression of the cytoskeletal proteins and stress factors in different biological
352 systems including bovine kidney and blood cells (Riedmaier et al., 2009; Zhang et al.,
353 2009), human cell lines (Ou et al., 2008), in the fish sea bream (Ibarz et al., 2010), the
354 fresh water bivalve *C. fluminea* (Martins et al., 2009) and in rabbits (Almeida et al.,
355 2010). Taking in consideration this knowledge we can infer that the changes in proteins
356 observed in this work translate to a condition of physiological stress and cellular injury
357 in tomato plants exposed to CYN.
358
359 **5. Conclusions**
360
361 In this work we investigated the effects of tomato plants exposure to CYN from the
362 cyanobacteria *A. ovalisporum* and pure CYN. Environmentally relevant concentrations
363 of CYN were considered in this work which permits to raise some concerns on the use
364 of contaminated waters for plant growth and food production. Proteomics and
365 biochemical analysis were conducted to assess the physiological condition of the
366 organisms. Alterations in the expression of cytoskeleton proteins were reported in the
367 tomato parts. Furthermore, the toxin may not be the main factor responsible for the
368 physiological stress induced in plants. The presence of other bioactive molecules
369 synthesized by *A. ovalisporum* cells should be clarified in order to be possible a more
370 rigorous evaluation of the ecological implications of +CYN *A. ovalisporum* blooms in
371 water supplies. This work demonstrates the importance of proteomics to assess the
372 biochemical changes and the physiological conditions of the organisms. Moreover it is
373 a promising approach towards the elucidation of the underlying mechanisms of toxicity
374 in plants induced by +CYN *A. ovalisporum* cells and pure CYN.
375

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377

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617 **FIGURES CAPTIONS**

618

619 **Figure 1.** Profile of protein expression from leaves of *Solanum lycopersicum* assessed
620 by two-dimensional gel electrophoresis. Proteins were separated using 17 cms IEF gel
621 strips with a pH gradient from 4 to 7. Gels were stained with colloidal Coomassie Blue.

622

623 **Figure 2.** Quantitative variation in the expression of protein spot SSP4203, assessed
624 through analysis of spot intensities in PDQuest software. Variations of this nature were
625 investigated for every spot in each experimental condition.

626

627 **TABLE CAPTIONS**

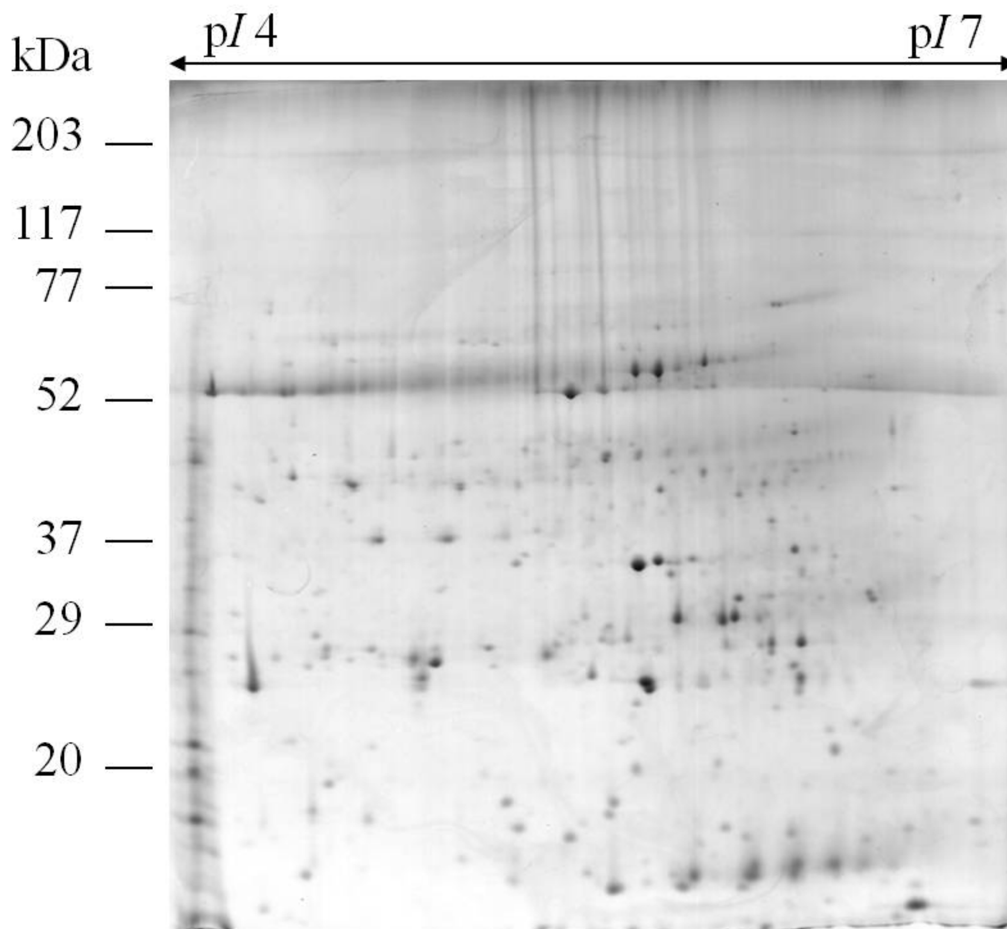
628

629 **Table 1.** Variations in protein expression of *S. lycopersicum* exposed to a CYN *A.*
630 *ovalisporum* cell extract and pure CYN. Normalized intensity and standard deviation of
631 each identified protein in each experimental group, Control plants, plants exposed to
632 CYN *A. ovalisporum* cell extract and pure CYN is presented.

633

634

635 **Figure 1.**



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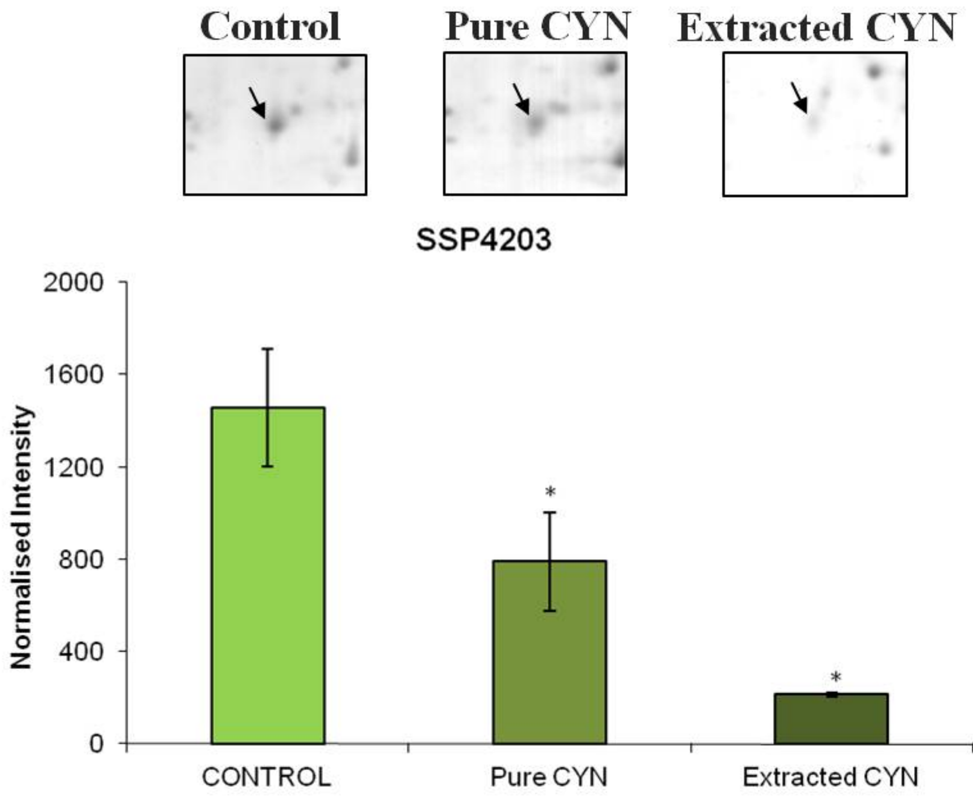
638 **Figure 2.**



639

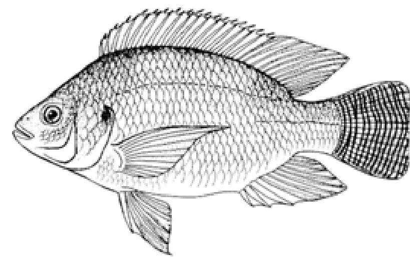
640 **Table 1.**

641

SSP (spot)	Control		A. Ovalisporum CYN extract		Pure CYN	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
1501	**3326,48**	763,10	**7082,45**	596,58	**21774,05**	3475,35
4117	**545,36**	140,16	**439,45**	45,74	**683,23**	196,28
4404	**4848,48**	220,44	**8012,15**	1856,93	**4974,05**	811,82
5125	**833,26**	88,76	**570,53**	82,23	**771,33**	168,85
5404	**2373,95**	1045,31	**2752,33**	404,86	**2427,10**	767,41
5692	**1543,30**	440,28	**1121,10**	438,68	**2553,36**	897,98
6157	**1059,03**	144,19	**858,66**	53,56	**2665,85**	66,39

642

5. DISCUSIÓN GENERAL / GENERAL DISCUSSION



Tal y como se ha comentado anteriormente, la CYN se ha 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 estas razones por las que decidimos realizar una serie de estudios que nos permitieran conocer en mayor medida el mecanismo de acción tóxica de la CYN y sus efectos tóxicos, así como la posibilidad de contrarrestar los daños producidos por ésta.

5.1. ESTUDIOS *IN VITRO*

La CYN ha sido objeto de diversos estudios *in vitro* en los que se ha puesto de manifiesto su toxicidad basal y mecanismos de acción tóxica (genotoxicidad, inhibición de la síntesis de proteínas, inhibición de la síntesis de GSH, etc.) en distintos modelos, tales como líneas celulares establecidas y cultivos primarios de mamíferos. No obstante, la implicación del estrés oxidativo en la patogenia inducida por CYN no se ha demostrado. Como paso previo, se procedió a establecer la concentración efectiva media (CE_{50}) de diferentes marcadores de citotoxicidad basal en dos líneas celulares humanas (Caco-2 y HUVEC) con el fin de seleccionar las dosis adecuadas para los estudios de estrés oxidativo y los estudios morfológicos.

Los resultados obtenidos en el ensayo de citotoxicidad mostraron que había una disminución de la viabilidad celular dependiente tanto de la concentración de CYN administrada (0-40 µg/mL) como del tiempo de exposición (24-48 horas). Resultados similares fueron obtenidos por otros grupos de investigación en los que se empleaban diferentes líneas celulares procedentes tanto de humanos como de mamíferos. Chong y col. (2002) observaron como la viabilidad de la línea celular KB, derivada de carcinoma nasofaríngeo humano, evaluada por el ensayo de MTT, se reducía conforme aumentaban tanto la concentración de CYN (0-10 µg/mL) como el tiempo de exposición (0-72 horas). Del mismo modo, Bain y col. (2007) determinaron la dependencia tanto de la concentración de CYN (0-5 µg/mL) como del tiempo de exposición (24-72 horas) mediante el ensayo de viabilidad de reducción de la sal de tetrazolio (MTS), en las líneas celulares humanas HepG2 (derivada de hepatocitos humanos), HDF (fibroblastos de humano adulto) y Caco-2. Por último, Froscio y col. (2009a) encontraron resultados

similares mediante el empleo del ensayo de viabilidad MTT realizado sobre siete líneas celulares y un cultivo primario de hepatocitos.

En cuanto a la sensibilidad a los diferentes marcadores de citotoxicidad basal estudiados en ambos modelos celulares, se comprobó que en la línea celular Caco-2 el indicador más sensible era el ensayo de MTS mientras que para la línea celular HUVEC era el de captación de rojo neutro (RN). Comparando ambas líneas celulares, se demostró una mayor sensibilidad de la línea celular HUVEC, ya que presentaba un valor de CE_{50} para la CYN (μg/mL) inferior al de Caco-2 (1,5 ± 0,9 μg/mL y 2,5 ± 0,4 μg/mL, respectivamente). En estudios similares, Neumann y col. (2007) observaron que la línea celular Caco-2 era la más sensible al evaluar el ensayo de viabilidad celular MTS, comparada con otras líneas celulares de similar procedencia. Igualmente, Bain y col. (2007) demostraron que las líneas celulares presentaban una pérdida de viabilidad causada por la CYN al realizar los ensayos de MTS. En este caso, la línea celular más sensible fue la HDF.

En base a estos resultados, se seleccionaron las concentraciones de CYN adecuadas para la realización de los estudios de estrés oxidativo a partir de la CE_{50} calculada para cada uno de los indicadores más sensibles:

Línea Celular	**CE_{50} (μg/mL)**	**$CE_{50}/2$ (μg/mL)**	**$CE_{50}/4$ (μg/mL)**
Caco-2	2,5 ± 0,4	1,25	0,625
HUVEC	1,5 ± 0,9	0,75	0,375

En los ensayos de estrés oxidativo se comprobó que ambas líneas celulares sufrían un incremento de las ERO a concentraciones inferiores a la CE_{50}, para posteriormente recuperar los valores basales, gracias al incremento de la actividad GCS, que contribuía al aumento de los niveles de GSH para combatir el daño oxidativo producido, por lo que de esta forma se puede establecer una relación entre los niveles de GSH y la actividad de la enzima limitante en su síntesis. Por el contrario, existen diferentes publicaciones que establecen la inhibición de la síntesis del GSH como un mecanismo de toxicidad de la CYN, tanto *in vivo* (Norris y col., 2002) como *in vitro* (Runnegar y col., 1994, 1995). Debido a esto, tal y como observaron Humpage y col. (2005) en hepatocitos aislados de ratón, no se puede establecer una relación directa

entre la citotoxicidad basal y el estrés oxidativo producidos por CYN, siendo necesarios más estudios que ayuden a esclarecer esta relación.

En lo concerniente a los estudios morfológicos que se realizaron sobre ambas líneas celulares, la CYN demostró producir diferentes alteraciones tales como degeneración lipídica y segregación nucleolar con alteraciones del núcleo celular, sobre la línea celular Caco-2, mientras que segregación nucleolar con alteraciones del núcleo celular, aumento de gránulos secretores, degeneración del aparato de Golgi y apoptosis fueron las alteraciones más comunes en la línea celular HUVEC. Estas alteraciones fueron ya observadas en ensayos *in vivo* e *in vitro*. Fessard y Bernard (2003) y Gacsi y col. (2009) observaron estas alteraciones sobre otras líneas celulares expuestas a CYN como la línea celular CHO-K1 (derivada de ovario de hámster chino) y las líneas celulares BE2 (derivada de neuroblastoma humano), MNA (derivada de carcinoma de hígado humano) y HepG2 (Neumman y col., 2007). Así mismo, Terao y col. (1994) encontraron alteraciones del aparato de Golgi en hígado de roedores expuestos a CYN durante 24 horas. Las alteraciones del núcleo encontradas también fueron corroboradas por estudios realizados sobre la línea celular SHE (derivada de embrión de hámster sirio) y hepatocitos por Maire y col. (2010) y Terao y col. (1994), respectivamente. Por último, la presencia de células apoptóticas también se estableció sobre la línea celular CHO-K1 (Fessard y Bernard, 2003).

Para continuar, si bien los efectos tóxicos de la CYN han sido estudiados previamente utilizando modelos *in vitro* de mamíferos, en modelos piscícolas no se han realizado hasta fechas muy recientes, a pesar de ser estos los que más probabilidad tienen de estar expuestos a la toxina. Por este motivo, se procedió en primer lugar a la determinación de la toxicidad basal en la línea celular PLHC-1 y posteriormente se realizaron los estudios de estrés oxidativo, siendo nuestro equipo el primero en investigar sobre líneas celulares piscícolas.

Los resultados de citotoxicidad basal mostraron un comportamiento similar al que se daba en las líneas celulares humanas, es decir, había una disminución de la viabilidad celular dependiente tanto del tiempo (24-48 horas) como de la concentración de CYN (0-40 μg/mL). En este caso, se comprobó que el indicador más sensible fue la determinación del contenido proteico total (PT), con una CE_{50} de 8 ± 2,1 μg/mL.

Recientemente, Liebel y col. (2011) encontraron resultados similares en cultivos primarios de hepatocitos aislados de peces intoxicados con 0,1 y 1 µg/L de CYN purificada.

En cuanto a los ensayos de estrés oxidativo, la línea celular PLHC-1 mostró un incremento de la actividad GCS y los niveles de GSH a la concentración más baja ensayada, producido por una posible respuesta de las células frente a la presencia de bajas concentraciones de CYN, para posteriormente sufrir un descenso de estos parámetros a la CE_{50} con niveles inferiores al valor basal. Este hecho puede estar relacionado con el aumento dependiente de la concentración de las ERO, alcanzando su máximo a la CE_{50}. Estos resultados concuerdan por los ya ofrecidos por Runnegar y col. (1994, 1995) y Humpage y col. (2005), quienes establecían la inhibición de la síntesis del GSH como posible mecanismo de acción toxica de la CYN. Recientemente, Silva y col. (2011) encontraron que la CYN generaba un aumento de ERO en hepatocitos de pez a concentraciones de 0,1, 1 y 10µg/L, pero en este caso, los niveles de GSH no se veían alterados con las diferentes concentraciones ensayadas. Algunos autores han descrito que al tratarse de una línea celular hepática, puede ser más sensible a los daños producidos debido a la organotropicidad que presenta la CYN (Froscio y col., 2009a; Bazin y col., 2010a). Además, hay que tener en cuenta la elevada presencia de CYP450 en este tipo de células, la cual metaboliza a la CYN dando lugar a metabolitos más tóxicos (Young y col., 2008).

Comparando estos resultados con estudios realizados sobre líneas celulares similares pero con otras cianotoxinas como las MCs, se observa que los valores de CE_{50} obtenidos a partir de los estudios de citotoxicidad basal en la línea celular Caco-2 para diferentes congéneres de MCs son superiores a los de la CYN, siendo de: 140, >200 y 114 µg/mL, para MC-LR, -RR e -YR, respectivamente (Puerto y col., 2009b, 2010b). De esta forma queda demostrado que la CYN es más tóxica que las MCs en la línea celular Caco-2, lo cual puede explicarse por la escasez de transportadores de MCs en la línea celular, ya que se han postulado diferentes mecanismos de entrada en la célula para la CYN, siendo más fácil para esta cianotoxina penetrar en las células. Además, *in vivo* se ha visto que los valores de DL_{50} tanto de CYN, comprendidos entre 0,17-6,9 mg/Kg (Ohtani y col., 1992; Shaw y col., 1999; Seawright y col., 1999; Duy y col., 2000), como los diferentes tipos de MCs, comprendidos entre 0,15-5 mg/Kg (OMS,

1998), no difieren mucho entre ellos. Del mismo modo, estudios realizados sobre las líneas celulares de pez PLHC-1 y RTG-2 con los diferentes congéneres de MCs demostraron que los valores de CE_{50} obtenidos eran muy superiores a los obtenidos con la CYN, por lo que corroboramos la mayor toxicidad de la CYN en comparación con las MCs (Pichardo y col., 2005, 2007) en las células piscícolas.

Para concluir, podemos decir que los resultados obtenidos del estudio de los efectos tóxicos de la CYN sobre las diferentes líneas celulares humanas y de pez son similares, pero la extensión de los efectos que se producen podría depender del organismo de procedencia, del tejido de procedencia de estas líneas y por tanto, de la sensibilidad de las diferentes líneas celulares. En nuestro caso vimos que las líneas celulares humanas eran más sensibles, con valores de CE_{50} inferiores a los presentados por la línea celular de pez PLHC-1; esta línea era la que mayor afectación presentaba en el estudio de los daños de estrés oxidativo, posiblemente debido a su procedencia hepática.

5.2. ESTUDIOS *IN VIVO*

5.2.1. Estudios realizados sobre tilapia (*Oreochromis niloticus*)

Nos propusimos estudiar el efecto de la CYN sobre peces, en este caso particular en tilapias (*Oreochromis niloticus*), ya que tal y como se ha comentado anteriormente, en la literatura científica no hay investigaciones al respecto. Para ello, se plantearon dos experimentos diferentes considerando diversas variables de interés, tales como: la vía de administración de la toxina (oral / i.p.); el tiempo de sacrificio tras la exposición (24 horas / 5 días); el tipo de toxina empleada (pura o procedente de cultivos de *Aphanizomenon ovalisporum* productor de CYN); y la influencia del uso de un agente quimioprotector como NAC (0 / 22 / 45 mg/Kg/día). En todos los casos la dosis de CYN administrada fue de 200 µg/Kg pez y se llevaron a cabo estudios de biomarcadores de estrés oxidativo e histopatológicos. Los órganos empleados para los estudios fueron el hígado y riñón, dos de las principales dianas de la CYN, y en los estudios histopatológicos se emplearon además, intestino, branquias y corazón.

La influencia de las distintas variables en los resultados obtenidos se presenta a continuación:

- **Vía de exposición:** se emplearon tres vías de exposición a CYN diferentes, exposición por vía i.p. y exposición oral en dos modalidades, sonda gástrica (*gavage*) y mezclada junto con el alimento.

  En todos los casos se produjo daño oxidativo, en mayor o menor medida, denotado por un incremento de LPO y oxidación de proteínas por encima de los valores basales, además de alteración de las diferentes enzimas que intervienen en el proceso de destoxicación del organismo, así como un descenso en los niveles de GSH.

  El incremento en LPO también ha sido demostrado por nuestro grupo de investigación en tilapias expuestas a diferentes dosis de CYN pura mediante sonda gástrica (Puerto y col., en prensa). Del mismo modo, este marcador también se vio afectado en tilapias expuestas a una dosis única de liofilizado de MCs mezclado con la comida (Prieto y col., 2007). Por el contrario, Humpage y col. (2005) no encontraron cambios en los niveles de MDA en hepatocitos de ratas expuestas a CYN. En cuanto a los niveles de GSH reducido, los resultados concuerdan con los obtenidos en estudios tanto *in vitro* (Runnegar y col., 1994, 1995) como *in vivo* (Norris y col., 2002), en los que se establece la inhibición de la síntesis de GSH como posible mecanismo de acción toxica de la CYN, como ya se ha mencionado anteriormente.

  En lo concerniente a la influencia de la vía de exposición en la toxicidad, vimos por primera vez en peces que, al comparar la administración oral por sonda gástrica y la inyección i.p., los efectos más severos se producían al administrar la toxina intraperitonealmente, ya que, tal y como mencionan Carbis y col. (1996) en un estudio realizado sobre peces intoxicados con MCs, puede haber una eliminación pre sistémica de la toxina al administrarla por vía oral. La única excepción fue el intestino, en el que la CYN produjo mayores daños a nivel histopatológico al ser

administrada por sonda gástrica, ya que es absorbida por el organismo en dicho órgano. Berry y col. (2009) también encontraron que la CYN producía mayores daños al ser administrada por vía i.p. en comparación a cuando se encuentra disuelta en el medio acuoso en peces de la especie *Danio rerio*. En cuanto a los estudios morfométricos, se vio como los peces intoxicados por vía i.p. se veían más afectados que aquellos expuestos por vía oral. De esta forma, se observó un aumento en el diámetro nucleolar de los hepatocitos y una disminución en el diámetro de los túbulos contorneados distal y proximal. La afectación más marcada producida en el riñón por la vía i.p. podría explicarse por el hecho de que la CYN no sufría una eliminación hepática presistémica previa.

Al comparar la administración de la CYN pura por sonda gástrica y mezclada con la comida observamos cómo no había diferencias en los niveles de LPO encontrados, mientras que la oxidación de proteínas se veía más pronunciada en el riñón de los peces en los que la CYN se administró con el alimento. En cuanto a los niveles de GSH, se vieron reducidos de forma más severa en la exposición a CYN mezclada con el alimento. De esta forma vemos que, globalmente, cuando la CYN era administrada junto con el alimento, producía un daño oxidativo mayor.

- **Tiempo de sacrificio:** se llevó a cabo un experimento en el que los peces fueron sacrificados tras 24 horas ó 5 días tras la exposición a la dosis única de CYN (por sonda gástrica o vía i.p.). Algunos de los parámetros bioquímicos evaluados se veían más afectados tras 24 horas de exposición (actividad NADPH oxidasa, LPO, GSH y actividad GCS en hígado) mientras que otros lo eran a los 5 días (LPO y oxidación de proteínas en riñón), posiblemente debido, por un lado, a la participación del CYP450 en la activación metabólica de la CYN (Froscio y col., 2001) y, por otro lado, que tras 5 días, muchos de estos parámetros se veían parcialmente recuperados por los mecanismos de destoxificación y recuperación de los órganos. Otros estudios realizados por nuestro grupo de investigación, han demostrado como en tilapias, expuestas a diferentes concentraciones de liofilizado de CYN (10-100 μg/L) disperso en el medio acuoso durante dos

tiempos de exposición (7-14 días), se afectan los diversos biomarcadores estudiados, siendo estos daños dependientes del tiempo y la concentración de CYN (Guzmán-Guillén y col., 2011). En este sentido, Prieto y col. (2007) observaron una recuperación del daño oxidativo producido en tilapia 72 horas después de una exposición única a MCs, al igual que Li y col. (2005) y Malbrouck y col. (2004) en hepatocitos de peces tras 48 y 96 horas, respectivamente, tras intoxicación con MCs.

Por otro lado, los parámetros moleculares mostraron estar más afectados a los 5 días tras la exposición a CYN, ya que la intoxicación inicial podría estimular mecanismos celulares que requiriesen más tiempo para verse reflejados en estos marcadores. Del mismo modo, Puerto y col. (2011a) encontraron resultados similares al determinar la actividad GST, la expresión génica relativa de las enzimas GPx y GST, y la abundancia relativa de GST en hígado, en tilapias intoxicadas con MCs durante 21 días. En cuanto a los resultados histopatológicos encontrados en hígado (pérdida de la arquitectura del parénquima hepático, degeneración generalizada con depósitos de glucógeno y esteatosis), riñón (glomerulopatía con atrofia capilar del glomérulo, hiperemia y disminución del grosor de los túbulos contorneados proximal y distal), corazón (miofibrolisis y edema), branquias (procesos de tumefacción, hiperemia y áreas de descamación celular) e intestino (enterocitos necróticos y edema intersticial severo), la mayor afectación se produjo a los 5 días tras la exposición. Resultados similares se obtuvieron previamente en nuestro laboratorio en tilapias intoxicadas con una dosis única de MC-LR y sacrificadas tras 24 horas (Puerto y col., 2010a). En roedores, CYN presenta una toxicidad aguda retardada, siendo la DL_{50} calculada diez veces inferior a los 5 días en comparación a la calculada tras 24 horas (Ohtani y col., 1992). En los estudios morfométricos, se comprobó cómo había un incremento del diámetro nuclear de los hepatocitos en función del tiempo al administrar la CYN. En riñón, se observó cómo había una disminución del diámetro de los túbulos contorneado y distal, siendo éste más marcado en aquellos peces sacrificados tras 5 días.

- **Tipo de CYN administrada:** en los estudios realizados se emplearon tanto CYN pura como CYN procedente de un cultivo liofilizado de *A. ovalisporum* productor de CYN (+CYN). Tras la realización de los ensayos pertinentes de determinación de los biomarcadores de estrés oxidativo se observó que, de forma general, la CYN administrada como liofilizado producía mayores daños en los biomarcadores de estrés oxidativo que la CYN pura. Esto podría explicarse por la posible presencia de otros compuestos bioactivos en el liofilizado que favorecieran la entrada en el organismo y la acción de la CYN, tal y como señalaban Seifert y col. (2007).

- **Cantidad y efectividad de NAC administrada:** se llevó a cabo un pretratamiento de una semana con NAC, utilizándose dos dosis diferentes (22 y 45 mg/pez/día) administradas junto con el alimento, y se valoró su repercusión en los efectos tóxicos inducidos tanto por CYN pura como por CYN proveniente de un cultivo celular liofilizado de *A. ovalisporum* (+CYN) administradas en la dieta. Se comprobó que la NAC ejerce un efecto protector frente al daño oxidativo producido por CYN, tanto pura como liofilizada, mostrando una mayor eficacia a la dosis mayor utilizada, Resultados similares fueron obtenidos por este mismo grupo cuando se administró a tilapias, intoxicadas con MCs, dosis de NAC semejantes a las empleadas en este estudio (Puerto y col., 2009a). De hecho, la selección de las dosis de NAC empleadas se basó en el experimento previo de Puerto et al. (2009a) en el que se observó que mientras a 20 y 44 mg/pez/día la NAC se producían efectos protectores, a 96,8 mg/pez/día la NAC exhibía efectos tóxicos *per se*. Ello era debido a la actividad pro oxidante que presenta la NAC, por lo que la selección de la dosis del quimioprotector es un factor importante a tener en cuenta. La recuperación de los parámetros de estrés oxidativo estudiados en peces pretratados con NAC podría deberse a que ésta reacciona directamente con las ERO (Aruoma y col., 1989) y además estimula a las enzimas que intervienen en la síntesis del GSH, del cual es un precursor (Banaclocha, 2001). En los estudios histopatológicos realizados se observó que la recuperación de los tejidos no era muy

diferente entre ambas dosis de NAC empleadas, destacando en algunos casos la mayor dosis empleada (corazón).

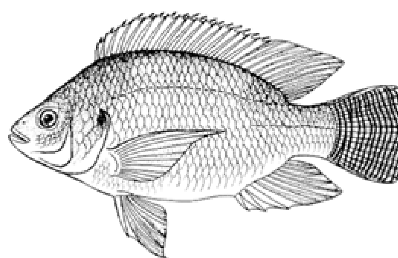
Igualmente, en los experimentos *in vivo* realizados, se observa que, por norma general, el hígado era el órgano que presentaba mayores alteraciones a nivel de producción de estrés oxidativo, si bien el riñón también se veía afectado de forma más severa en algunos parámetros. La mayor sensibilidad del hígado a los efectos de la CYN puede ser debida a la mayor cantidad de CYP450, implicada en la toxificación de la CYN. El estudio de la generación de estrés oxidativo como posible mecanismo de toxicidad de la CYN es de gran interés, siendo aún más interesante realizar estos estudios sobre diferentes organismos que pueden verse con mayor frecuencia expuestos a CYN, como son los peces que viven en aguas contaminadas, plantas irrigadas con aguas contaminadas con CYN, etc., por lo que es importante que haya más avances respecto a este asunto en el futuro.

5.2.2. Estudios realizados sobre plantas de tomate (*Solanum lycopersicum*)

Los estudios realizados sobre plantas de tomate para conocer los efectos de la CYN, pura o extracto de un liofilizado de un cultivo de *A. ovalisporum*, cuando se administra en el agua de regadío, indican que hay una mayor afectación de la planta a nivel oxidativo cuando la CYN procede de un extracto de liofilizado, aunque aún son necesarios más estudios para esclarecer este hecho y conocer cuál/cuáles son las partes de la planta más afectadas (raíz, hoja o fruto). En un estudio similar sobre plantas de arroz (*Oriza sativa*) en el que se añadieron en el agua de riego extractos de CYN y MCs, procedentes de *A. ovalisporum* y *Microcystis aeruginosa*, respectivamente, así como una mezcla de ambos extractos, se afectaron diferentes biomarcadores de estrés oxidativo, siendo estos efectos más pronunciados cuando se administraban ambas toxinas conjuntamente, lo que sugiere un mecanismo de sinergia (Prieto y col., 2011, aceptado). Mientras que la bibliografía en lo concerniente a la generación de estrés oxidativo en plantas por MCs es numerosa (Chen y col., 2004; Pflugmacher y col., 2004, 2006, Pichardo y Pflugmacher, 2011), la correspondiente a CYN es inexistente. Es por este motivo por lo que los estudios de los efectos de CYN sobre plantas de consumo humano es de interés, ya que esta 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.

6. CONCLUSIONES / CONCLUSIONS



De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se han sacado las siguientes conclusiones:

PRIMERA. En los estudios de citotoxicidad basal realizados sobre las líneas celulares Caco-2, procedente de un adenocarcinoma de colon humano, HUVEC, procedente de endotelio vascular de cordón umbilical humano, y PLHC-1, procedente de hígado de pez (*Poeciliopsis lucida*), para conocer los efectos de CYN pura, se ha comprobado que el daño producido por ésta es dependiente del tiempo de exposición y de la concentración de toxina.

SEGUNDA. En base a los datos de CE_{50} obtenidos en los biomarcadores de citotoxicidad basal, se observó una mayor sensibilidad de las líneas celulares humanas con respecto a la línea celular piscícola, siendo la línea celular HUVEC el modelo celular más sensible.

TERCERA. Una vez optimizadas las condiciones para el estudio de la capacidad de producción de estrés oxidativo por parte de la CYN sobre las tres líneas celulares, se observó en las líneas celulares humanas un máximo de producción de ERO a bajas concentraciones, que posteriormente disminuía hacia los valores basales, gracias a la acción de la enzima GCS y del GSH, no pudiendo establecerse una relación directa entre la producción de citotoxicidad basal y el daño oxidativo, y requiriéndose, por tanto, más estudios al respecto. Por el contrario, en la línea celular PLHC-1, CYN es capaz de inducir estrés oxidativo con un incremento de los valores de ERO dependiente de la concentración de toxina administrada, a la vez que daba lugar a un descenso similar en la actividad GCS y los niveles de GSH tras un intento inicial de contrarrestar el daño producido.

CUARTA. Las principales alteraciones morfológicas observadas a ambos tiempos de exposición sobre la línea celular Caco-2 fueron degeneración lipídica, segregación nucleolar con alteraciones en los núcleos celulares y daño mitocondrial, mientras que la línea celular HUVEC fueron el aumento de gránulos secretores y de la apoptosis.

QUINTA. Se ha comprobado que la producción de daños oxidativos en tilapias (*Oreochromis niloticus*) tras la administración de una dosis única de CYN pura (200

μg/Kg p.c.) depende en mayor medida del tiempo de sacrificio que de la vía de administración. Hay biomarcadores que presentan una mayor afectación a las 24 horas tras el sacrificio (actividad NADPH oxidasa, LPO, GSH y actividad GCS en hígado) y que tienden a recuperar los valores basales con el paso del tiempo, mientras que otros (oxidación de proteínas, actividad GST y LPO en riñón, y actividad GST en hígado) se muestran más alterados a los 5 días tras la exposición.

SEXTA. La expresión génica de las enzimas GPx y GST, así como la abundancia relativa de GST, han demostrado ser unos biomarcadores moleculares sensibles para la determinación de estrés oxidativo producido por CYN, viéndose más afectados, por norma general, en aquellos peces sacrificados tras 5 días y expuestos por vía i.p.

SÉPTIMA. En cuanto a las alteraciones histopatológicas producidas tras la administración de una dosis única de CYN pura en tilapias, se comprobó que los daños eran más severos en los órganos de los peces sacrificados tras 5 días y expuestos por vía i.p., a excepción de los intestinos. Además se demostró que el hígado y riñón eran los principales órganos diana de la toxina. En el hígado, las alteraciones más características observadas fueron: pérdida de la arquitectura del parénquima hepático, degeneración generalizada con depósitos de glucógeno, esteatosis y un incremento del diámetro celular de los hepatocitos. En riñón, glomerulopatía con atrofia capilar del glomérulo, hiperemia y disminución del grosor de los túbulos contorneados proximal y distal.

OCTAVA. Se ha comprobado que la CYN administrada por vía oral, a partir de un liofilizado de células de *A. ovalisporum* productor (+CYN), produce mayores daños en los órganos estudiados tanto en los biomarcadores de estrés oxidativo evaluados como en los estudios histopatológicos realizados, en comparación con la toxina pura.

NOVENA. Se ha demostrado la utilidad de administrar junto con la dieta suplementos de N-Acetilcisteína (NAC) (22-45 mg/pez/día) frente a los daños de tipo oxidativo e histopatológicos producidos en tilapias tras la administración de una dosis única de dos tipos de CYN (pura / liofilizada), ya que se recuperan los valores de los diferentes marcadores de estrés oxidativo hasta niveles basales. Por ello se puede considerar la NAC como un agente quimioprotector útil en la prevención de daños producidos por la CYN en peces.

DÉCIMA. En los estudios preliminares realizados sobre plantas de tomate (*Solanum lycopersicum*), se observaron alteraciones a nivel proteico cuando son intoxicadas con CYN y estas alteraciones dependen del origen y naturaleza de la toxina que se les administre (pura o procedente de un liofilizado de células de *A. ovalisporum* productor), así como de la zona de la planta estudiada (raíz, hoja, fruto).

The main conclusions that can be drawn on the basis of the obtained results in the present Thesis are:

FIRST. The citotoxicity studies carried out using the Caco-2 cell line from a human colon carcinoma, HUVEC, from human umbilical vein endothelium, and PLHC-1, from fish liver (*Poeciliopsis lucida*), in order to establish the effects of pure CYN, have demonstrated that this effect depends on the time of exposure and toxin concentration.

SECOND. Taking into account the results obtained from the study of citotoxicity biomarkers, a major sensitivity was observed in human cell lines compared with the fish cell line, being the HUVEC cell line the most sensitive cellular model.

THIRD. The conditions to study the CYN capacity to produce of oxidative stress in the three cell lines were optimized. The results showed a maximum production of ROS for the human cell lines at low concentrations, being these restored to control levels by the action of the enzyme GCS and GSH. It was not possible to establish a direct relation between the production of basal citotoxicity and oxidative stress, so further studies are needed. On the contrary, CYN was able to induce oxidative stress in a dose-dependent way in the PLHC-1 cell line, as well as to decrease GCS activity and GSH levels, after a first recovery process against de injury produced.

FORTH. The most remarkable morphological effects for both exposure periods in the Caco-2 cell line were lipidic degeneration, nuclear segregation with altered nuclei and mitochondrial damage, while in the HUVEC cell line the main effects were nucleolar segregation with altered nuclei, increases in the presence of secretory granules, degenerated Golgi apparatus, and apoptosis.

FIFTH. It has been demonstrated that the production of oxidative damage in tilapia (*Oreochromis niloticus*) after the administration of a single dose of pure CYN (200 µg/kg bw) depends on the time of exposure, more than on the administration route. Some biomarkers showed to be more affected after 24 hours post-sacrifice (NADPH oxidase activity, LPO, GSH and GCS activity in liver), whereas others (protein oxidation, GST activity and LPO in kidney, and GST activity in liver) resulted more affected 5 days after exposure.

SIXTH. GPx and GST gene expression, as well as GST relative abundance, have shown to be sensitive molecular biomarkers useful for the determination of oxidative stress produced by CYN, being generally more affected in fish sacrificed after 5 days and by i.p. route.

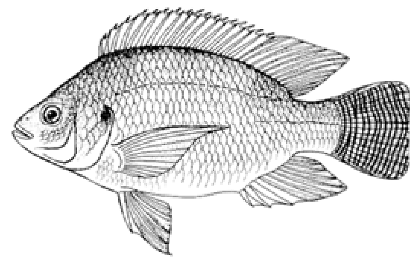
SEVENTH. The histopathological effects produced by a single dose of pure CYN in tilapia fish, showed to be more severe in the organs of fish sacrificed after 5 days and exposed by i.p. route, except the intestines. Moreover, it was demonstrated that liver and kidney were the main target organs of the toxin. The major alterations observed in the liver were: loss of the parenchyma architecture, generalized degeneration with glycogen deposits, steatosis and an increase of hepatocytes cellular diameter. In the kidney, glomerulopathy with glomerular capillary atrophy, hyperemia and decreased thickness of the proximal and distal convoluted tubules.

EIGHTH. CYN administered by oral route as a lyophilized of cells from *A. ovalisporum* CYN producer (+CYN) has shown to cause a more severe damage in both the oxidative stress biomarkers and the histopathological studies carried out.

NINTH. The usefulness of N-acetylcysteine (NAC) supplementation (22-45 mg/fish/day), within the diet, in the defense against oxidative stress and histopathological changes produced by a single dose of pure and lyophilized CYN in Tilapia (*Oreochromis niloticus*), has been demonstrated, by restoring the different oxidative stress biomarkers values to the control levels. It can be considered, therefore, as a useful chemoprotectant for prevention of the damages produced by CYN in fish.

TENTH. In preliminary studies carried out with tomatoes plants (*Solanum lycopersicum*), some alterations were observed at protein level after intoxication with CYN. These alterations depend on the origin and nature of CYN administered (pure or a lyophilized cell strain from *A. ovalisporum* CYN producer) and on the different studied plant part (root, leave, fruit).

7. OTROS MÉRITOS / OTHER MERITS



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I

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TOMO I

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INTERÉS TOXICOLÓGICO DE LA CILINDROSPERMOPSINA

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Resumen

Las toxinas producidas por cianobacterias se han convertido en un peligro para la salud humana. La cianotoxina cilindrospermopsina (CYN) es un potente alcaloide tóxico que se aisló por primera vez de *Cylindrospermopsis raciborskii*, aunque también lo producen otras cianobacterias. La proliferación masiva de estos organismos se produce en gran medida por la eutrofización de los hábitats en los que se encuentran. El creciente interés se debe a que crecen en reservorios de agua de todo el mundo, incluidos los de agua de bebida, y además existen animales usados en alimentación humana (pescados, crustáceos, moluscos, etc.) que están en contacto con esta toxina, por lo que existe un riesgo potencial de exposición humana.

La CYN es un inhibidor de la síntesis de proteinas y glutatión. Además, produce otros efectos como genotoxicidad, carcinogenicidad, citotoxicidad, etc. Para estudiar estos efectos se han empleado modelos *in vivo* e *in vitro* para, de esta forma, conocer la toxicidad que produce en el organismo así como los órganos diana de la CYN. Los primeros síntomas clínicos que se producen por intoxicación de CYN son fallos del hígado y los riñones, aunque también se sabe que afecta al bazo, corazón, pulmones, etc.

Actualmente no existe una reglamentación que regule los niveles de CYN en el agua de bebida ni en los alimentos, ni tampoco existe un método analítico validado que se pueda emplear para su determinación.

Palabras clave**:** cilindrospermopsina, seguridad alimentaria, toxicidad, inhibición síntesis de proteinas, inhibición síntesis glutatión

Abstract

Cyanobacterial toxins have been recognized as a hazard for the human health. The cyanotoxin cylindrospermopsin (CYN) is a potent toxic alkaloid first isolated from *C. raciborskii*, although it can be produced by several cyanobacterial species. The massive proliferation of these organisms is largely due to eutrophication in a wide range of ecological habitats. There is an increasing environmental interest as it frequently occurs in water reservoirs worldwide, even drinking water reservoirs, so aquatic animals used in human nutrition, like fish, crayfish, molluscs, and other animal species are prone to suffer from CYN effects as they share the same habitat. For these reasons, there is a human potential exposure.

CYN is a potent inhibitor of protein and glutathione synthesis. However, other effects such as genotoxicity, carcinogenicity, cytotoxicity, etc., have been described. In order to investigate these effects, various *in vivo* and *in vitro* models have been used, which were employed to know the toxicity produced by CYN and the target organs, respectively. The first clinical symptoms of poisoning are liver and kidney failure, and also it is demonstrated that CYN is able to produce damage on spleen, heart, lungs, and other organs in humans.

There is not a legislation that establishes legal limits of CYN concentration in drinking water and food, and neither exist a validated analytical method for CYN determination.

Keyword**s:** cylindrospermopsin, food safety, toxicity, protein synthesis inhibition, glutathione synthesis inhibition

1. CIANOBACTERIAS

Las cianobacterias son un grupo de más de 2000 especies de organismos procariotas que se caracterizan porque tienen la capacidad de sintetizar clorofila α [1]. Además, estos organismos tienen la capacidad de adaptarse a una gran variedad de hábitats por todo el mundo, incluyendo zonas donde las condiciones para la vida son extremas.

Bajo determinadas condiciones ambientales (luz, temperatura, pH, etc.) y presencia de nutrientes, estas cianobacterias pueden crecer de forma descontrolada dando lugar a lo que se conoce como afloramiento, floración o *bloom*, los cuales adquieren una gran importancia cuando están sujetos a la producción de toxinas (cianotoxinas), que suponen un grave problema económico y sanitario debido a que afectan tanto a animales como a seres humanos. Estos *blooms* son más frecuentes en regiones tropicales y subtropicales [1], pero también se han detectado casos en otras regiones [2-4]. Se ha estimado que aproximadamente el 25-75% de las floraciones son tóxicas [5].

La capacidad de las cianotoxinas para producir daños en los seres humanos ha hecho que se conviertan en un importante tema de investigación en todo el mundo, para poder esclarecer, de esta forma, sus mecanismos de toxicidad y la forma de contrarrestarlos. Además del riesgo que suponen para los humanos, las cianotoxinas también se han identificado como un peligro para el medioambiente, ya que afectan tanto a plantas como animales [1].

Existen dos formas posibles de encontrar a estas cianotoxinas: dentro de las propias células productoras o libres en el medio acuoso debido a la excreción al exterior por parte de las células o a la ruptura de las éstas [6-7]. La entrada de estas toxinas en el organismo puede ocurrir de diferentes formas: por contacto dérmico, por ingestión de alimentos contaminados o por consumo de agua contaminada.

En este aspecto, cabe destacar la bioacumulación de las cianotoxinas en los organismos, entendiendo bioacumulación como el proceso de acumulación de sustancias químicas, en este caso las cianotoxinas, en organismos

vivos, de forma que éstas alcanzan mayores concentraciones en los tejidos que las que hay presentes en el medio ambiente. La bioacumulación alcanza mayores concentraciones conforme se sube de nivel trófico en la cadena alimentaria, por lo que los humanos pueden convertirse en los mayores reservorios de cianotoxinas. Además, se puede producir el fenómeno conocido como biomagnificación, que se da cuando interaccionan diferentes niveles tróficos. Entre los alimentos en los que la bioacumulación es frecuente se encuentran el pescado, los crustáceos y otras especies acuáticas con interés comercial y de recreo [1].

2. CLASIFICACIÓN DE LAS CIANOTOXINAS

Las cianotoxinas son un grupo diverso de sustancias naturales tanto desde el punto de vista químico como toxicológico. Debido a su procedencia acuática, la mayoría de las toxinas son más dañinas para los animales terrestres que para los animales acuáticos [8]. Tradicionalmente, estas cianotoxinas se clasifican en función de los efectos tóxicos que producen (Tabla 1) [9-10].

- **Dermatotoxinas**: lipopolisacáridos, lyngbyatoxina-a, aplisiatoxinas.
- **Neurotoxinas**: anatoxina-a, homoanatoxina-a, anatoxina-a(s), saxitoxinas.
- **Hepatotoxinas**: microcistinas, nodularina, cilindrospermopsina.

Grupo de toxinas	Agente Productor (género)	Efectos	Biotransformación
Anatoxinas	*Anabaena*	Inhibición de la actividad acetilcolinesterasa	Citocromo P-450 GST
Anatoxin-a(s)	*Anabaena* *Aphanizomenon* *Cylindrospermopsis* *Planktothrix* *Oscillatoria* *Microcystis*	Unión irreversible a los receptores nicotínicos de acetilcolina	Citocromo P-450 GST
Cilindrospermopsinas	*Cylindrospermopsis Aphanizomenon* *Umezakia Raphidiopsis* *Anabaena*	Inhibición síntesis de proteínas Daño citogenético en el ADN	Citocromo P-450
Lipopolisacáridos	*Anabaena* *Anacystis* *Microcystis* *Nodularia*	Irritante potencial, afectando a los tejidos expuestos	Vía alternativa de desacetilación lisosomal
Microcistinas	*Microcystis* *Anabaena* *Plankthotrix*	Inhibición de las fosfatasas de proteína (PP1 y PP2A)	GST
Nodularinas	*Nodularia*	Inhibición de las fosfatasas de proteína (PP1 y PP2A)	GST

Saxitoxinas	Dinoflagelados (*Protogonyaulax, Alexandrium, Gymnodinium, Pyrodinium*) Cianobacterias (*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis)*	Unión y bloqueo de canales de sodio en células nerviosas	GST

Tabla 1. Diferentes toxinas de cianobacterias, principales productores, principales efectos tóxicos y biotransformación. Tomado de [11].

Las **dermatotoxinas** están producidas por diferentes especies de cianobacterias marinas, principalmente las pertenecientes a los géneros *Lyngbya spp.* y *Schizothrix spp*. Estas toxinas causan dermatitis severa por contacto e irritación de los ojos y tracto respiratorio [12]. Los lipopolisacáridos endotóxicos, producidos por especies de *Anabaena, Anacystis, Microcystis y Nodularia,* tienen acción sobre el sistema inmunitario (involucrados en shock séptico) y también afectan a los sistemas de destoxicación de diferentes organismos [1].

Las **neurotoxinas** bloquean la neurotransmisión, llegando a causar la muerte por una rápida parálisis respiratoria [13]. Presentan estructura alcaloidea, y se pueden encontrar diferentes tipos:

- Anatoxina-a y homoanatoxina-a: están producidas por diferentes especies de los géneros *Anabaena, Aphanizomenon, Cylindrospermopsis*, *Planktothrix, Oscillatoria, Microcystis*, entre otras. Presentan una estructura análoga a los agonistas nicotínicos, por lo que pueden unirse a los receptores nicotínicos de acetilcolina y provocar una sobre-estimulación de las células musculares. La anatoxina-a(s) es un éster de fosfato de la N-hidroxiguanina cíclica [1] y tiene un mecanismo de acción similar a los insecticidas organofosforados como el paratión y malatión, inhibiendo irreversiblemente la enzima acetilcolinesterasa, por lo que interfiere en la contracción muscular, causando fatiga y fallo muscular [14].
- Saxitoxina o toxina paralizante (Paralytic shellfish poison, PSP): está producida tanto por dinoflagelados como por cianobacterias, y son neurotoxinas con estructura de alcaloides que bloquean selectivamente los canales de sodio dependientes de voltaje, por lo que impiden la propagación del impulso nervioso [15].

Dentro de las **hepatotoxinas** se pueden encontrar diferentes tipos:

- Microcistinas y nodularina: son péptidos cíclicos que pueden ser transportados activamente por los polipéptidos transportadores de aniones orgánicos [16], los cuales se encargan principalmente de transportar sales biliares, y es por esto por lo que uno de los principales órganos diana es el hígado. Además, son inhibidores

de las fosfatasas de proteínas 1 y 2A (PP1 y PP2A) y potentes promotores de tumores en el hígado [1, 17].

- Cilindrospermopsina (CYN): es un alcaloide tricíclico derivado de la guanidina unido a un grupo hidroximetiluracilo (Fig. 1), con un peso molecular de 415 Daltons y una elevada solubilidad en agua [1] debido, esto último, a la carga negativa que presenta en el grupo sulfato y a la carga positiva que presenta en el grupo guanidina (zwiterión). Además, es estable bajo diferentes condiciones de luz, temperatura y pH [1]. Además, existe un epímero, denominado 7-epiCYN (Fig. 2), que presenta una toxicidad similar a la CYN. Otra variante de la CYN es la denominada 7-deoxyCYN (Fig. 3), que se encuentra presente junto a ésta en los reservorios de agua.

La CYN se aisló e identificó tras un brote de hepatoenteritis que afectó a la población australiana de Palm Island, donde se determinó que el agente causante era una cianobacteria denominada *Cylindrospermopsis raciborskii* [1].

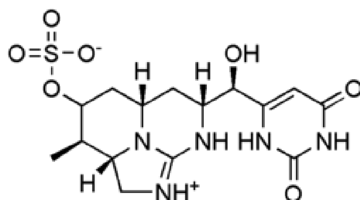

Figura 1. Estructura química de la cilindrospermopsina.

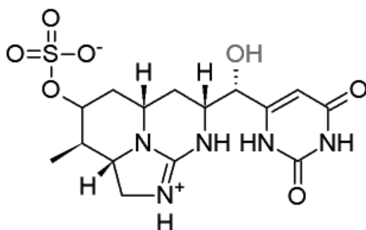

Figura 2. Estructura química del epímero 7-epicilindrospermopsina.

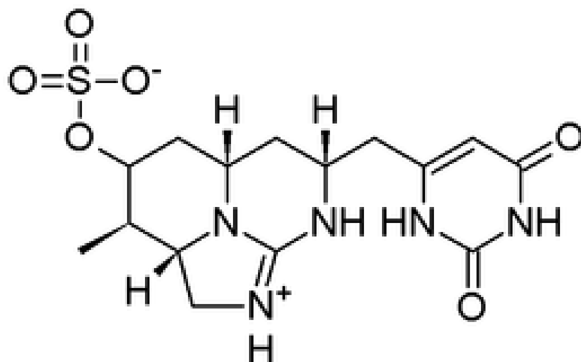

Figura 3. Estructura química de la 7-deoxicilindrospermopsina.

Existen distintas especies de cianobacterias capaces de sintetizar CYN, entre las que encontramos, además de *Cylindrospermopsis raciborskii, a Aphanizomenon ovalisporum* [18], *Umezakia natans* [1], *Anabaena bergii* [19], *Raphidiopsis curvata* [1], *Aphanizomenon flos-aquae* [1] y *Anabaena japponica* [1], aunque es *C. raciborskii* la principal especie productora.

3. DISTRIBUCIÓN DE CILINDROSPERMOPSINA

Actualmente existen datos de la presencia de CYN en Asia, África, América del Norte, América Central, América del Sur, Europa y Oceanía, es decir, de todos los continentes excepto la Antártida (Fig. 4) [1]. Aunque inicialmente el crecimiento de este tipo de cianobacterias se limitaba a las zonas tropicales y subtropicales, hoy día se encuentran distribuidas por cualquier zona del mundo, y se pueden encontrar en lagos, ríos, lagunas, presas, etc.

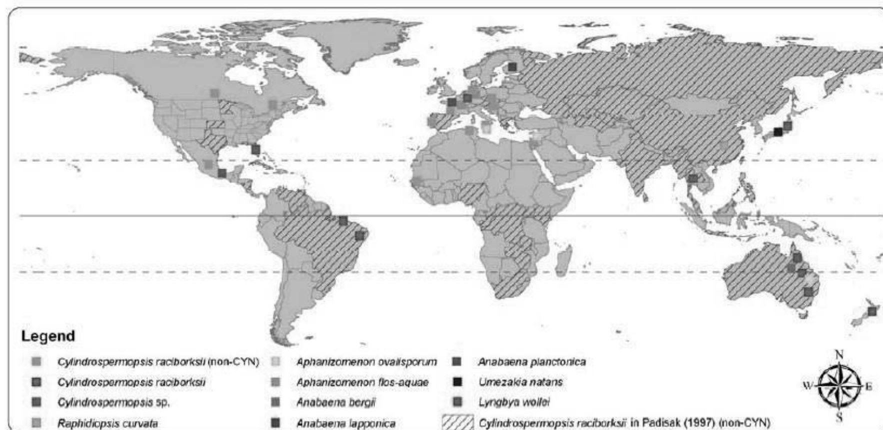

Figura 4. Distribución mundial de *blooms* de algas productoras de CYN o derivados de CYN (datos orientativos). Tomado de [1].

Por otro lado, se cree que existen muchas zonas donde aún la CYN no ha sido detectada debido a que muchos de los organismos productores no forman capas visibles en la superficie del agua y que, por tanto, su distribución puede ser mucho más amplia [1].

4. TOXICIDAD DE LA CILINDROSPERMOPSINA

La forma en que la CYN entra en el organismo para posteriormente acumularse en él no se conoce del todo bien. Se ha visto en algunos estudios que el grupo sulfato de la CYN no es necesario para la entrada de la molécula en las células, y que su naturaleza hidrofílica hace que sea poco probable que atraviese la pared celular [1]. No obstante, se piensa que el bajo peso molecular que presenta la CYN hace la difusión pasiva sea un mecanismo factible en la absorción de la toxina [1]. Se ha observado en diferentes estudios que los organismos que ingieren CYN presentan daños en la pared del intestino [1], lo cual es de gran importancia ya que el epitelio dañado ofrece una mayor superficie de absorción, por lo que se facilita la absorción de la CYN. Cuando la vía de exposición a la CYN es la vía oral, la implicación de las sales biliares en su absorción es menor que la que se ha observado en diferentes estudios sobre MC-LR. En el caso de la CYN, este proceso se ve desplazado hacia la difusión pasiva, lo cual se comprobó en estudios en los que la CYN producía citotoxicidad en la línea celular KB, la cual carece de transporte por parte de las sales biliares [1]. También

hay que añadir que la absorción de CYN es mayor cuando existen otros componentes celulares procedentes del agente productor de la toxina [1].

Los efectos clínicos conocidos que produce la CYN sobre los humanos se observaron tras la intoxicación masiva por CYN ocurrida en Palm Island (Australia), donde se observó que se producía una inusual hepatoenteritis, que cursaba inicialmente con agrandamiento del hígado, constipación, vómitos y dolor de cabeza. A esto lo seguían hemorragias y pérdida de proteínas, electrolitos y glucosa a través de la orina, con signos variables de deshidratación [20].

Por norma general, la exposición a CYN en humanos se caracteriza por una toxicidad retardada que afecta de forma sistémica a los individuos, aunque principalmente afecta a hígado y riñón [1]. La toxicidad está mediada por inhibición irreversible de la síntesis de proteinas [1, 21-22], así como por genotoxicidad por fragmentación del ADN [1]. Los efectos genotóxicos fueron demostrados en modelos *in vitro* por Humpage [23-24] mediante diferentes ensayos, e *in vivo* por Shen [25] tras la administración intraperitoneal de una dosis de CYN. Además, se cree que la CYN sufre una activación metabólica en el hígado, aumentando con ello su toxicidad, aunque la vía exacta por la que se produce este proceso aun no está dilucidada [1, 22]. Un envenenamiento agudo por CYN conlleva a una acumulación de lípidos en el hígado seguida de necrosis hepatocelular [1], la cual puede ser debida a la formación de radicales libres, lo que puede ser posible debido a la disminución de la síntesis de glutatión reducido (GSH), la cual se ha observado en ensayos con ratones [26] y en hepatocitos de rata [1, 27]. Por otro lado, en el riñón se produce destrucción de los túbulos proximales [28], mientras que sobre otros tejidos como pulmones, corazón, estómago, glándulas adrenales, sistema vascular y sistema linfático, se producen efectos citotóxicos y trombóticos [29]. En el timo y el bazo se produce una necrosis selectiva de linfocitos, seguida de atrofia de ambos [1]. También se ha visto que la CYN inhibe la síntesis del nucleótido pirimidina y que altera la distribución del colesterol en ratones [1]. Por último, también existen indicios de carcinogenicidad *in vivo* [1], pero los resultados aún son escasos.

Dentro del marco medioambiental, la CYN tiene la capacidad de afectar a un amplio rango de plantas y animales acuáticos, lo que, unido a la bioacumulación que se produce y a la transferencia dentro de la cadena trófica, hace que el daño producido por la CYN se pueda extender a otras especies de plantas y animales terrestres. Existen distintos factores que influyen en la toxicidad de *C. raciborskii* y de la CYN (Fig. 5).

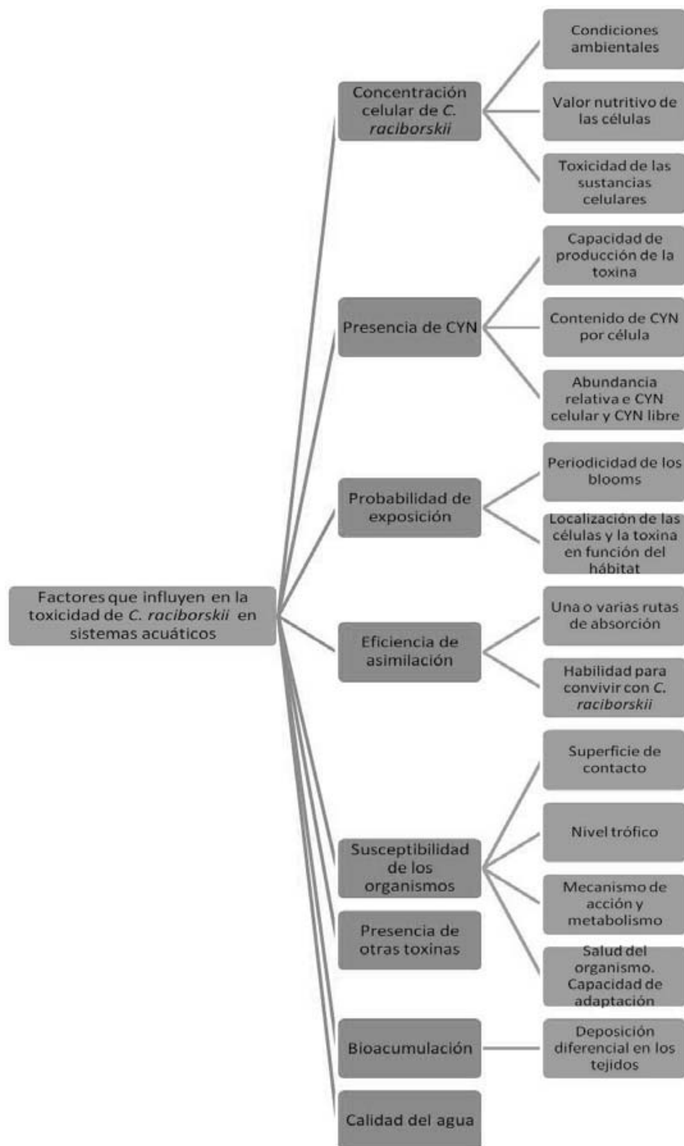

Figura 5. Cuadro resumen de los factores que influyen en la toxicidad de C. raciborskii y la CYN en los ecosistemas acuáticos. Tomado de [1].

Estudios llevados a cabo sobre diferentes especies de animales y plantas con concentraciones inferiores a 100 µg/L de CYN pura demostraron que se producían pocos efectos adversos [1]. Por otro lado, la exposición a extractos de *C. raciborskii* resultó en toxicidad subletal, lo que sugiere que las células productoras contienen más compuestos activos diferentes a la CYN que incrementan el riesgo de que se produzcan efectos tóxicos [1].

5. BIOACUMULACIÓN DE CILINDROSPERMOPSINA

La bioacumulación, como ya se describió anteriormente, es el proceso de acumulación de sustancias químicas en organismos vivos, de forma que éstas alcanzan mayores concentraciones en los tejidos que las que hay presentes en el medio ambiente que los rodea. Extractos de células enteras o cultivos vivos de *C. raciborskii* han producido daños en los tejidos y cambios en el crecimiento, comportamiento, mortalidad y reproducción en animales y plantas acuáticos [1]. Existen estudios de bioacumulación de CYN en diferentes organismos vertebrados e invertebrados como pueden ser: *Melanoides tuberculata* y caracol Tegogolos (gasterópodos); *Anodonta*, *Alathyria* y *Corbiculina* (bivalvos); cangrejo *Cherax*, *Melanotaenia* y renacuajos de *Bufo marinus* [1]. En plantas los estudios son más limitados, comprobándose que solo en la lenteja de agua *Lemna* se concentra la toxina [1].

La acumulación de CYN en los diferentes animales acuáticos presenta una gran variabilidad, siendo ésta mucho mayor en los organismos estructuralmente más sencillos (gasterópodos, bivalvos y crustáceos), que en los organismos más complejos (anfibios y peces). En lo que se refiere al grado de afectación de estos organismos, se da el orden inverso, es decir, los animales superiores se afectan en mayor medida que los animales inferiores [1]. En esta bioacumulación hay que tener también en cuenta la abundancia relativa de CYN intracelular y extracelular, ya que la intracelular se acumla en mayor cantidad que la extracelular [1] (Fig. 6).

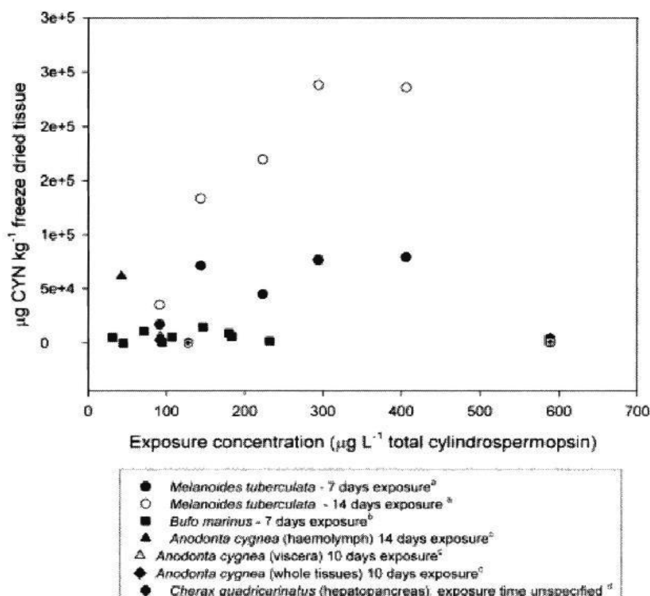

Figura 6. Concentración de CYN por kg de tejido seco encontrada en diferentes animales acuáticos en la literatura disponible sobre el tema. Tomado de [1].

Con respecto a la biomagnificación, debido a que la CYN es una molécula muy soluble en agua, no se considera probable, si bien la toxina puede llegar a los organismos superiores a través de cadena trófica, sin ser necesariamente acumulada por los organismos basales [1]. Esto es de gran importancia debido a que la CYN se puede depositar en carnes que luego van a ser consumidas por los humanos.

Otros estudios establecieron que la bioacumulación tanto de CYN como de deoxiCYN ocurre en el pez gato, *Tandanus tandanus*, pero no en otras especies de peces como son *Macquaria ambigua*, *Bidyanus bidyanus* o *M. novemaculeata* [1].

Estudios más recientes han establecido que la CYN puede bioacumularse en gran cantidad incluso cuando la concentración en el medio es pequeña, y esto se ha visto en caracoles Tegogolo, donde el factor de acumulación era muy elevado [1].

Norris [1] observó que la CYN se acumula en hígado y riñón de ratones tras 6 horas de exposición mediante inyección intraperitoneal, y que la

acumulación disminuía progresivamente su velocidad desde el quinto al séptimo día. También observó que gran parte de la toxina era eliminada con la orina y las heces.

1. 6. VÍAS DE EXPOSICIÓN A CILINDROSPERMOPSINA

La principal fuente de exposición a la CYN, es el agua de consumo humano contaminada, ya sea por el agua de bebida o por ingestión de agua en la realización de actividades de recreo acuáticas. Como ya se ha descrito anteriormente, el agua de bebida contaminada ha sido la causa de brotes de hepatoenteritis y otras afectaciones en poblaciones humanas (Australia, Brasil, etc.) [1].

Otra vía es el consumo de alimentos contaminados con CYN debido a los procesos de bioacumulación que se dan a través de la cadena trófica, aunque en comparación con otras cianotoxinas como las microcistinas, los estudios son escasos y confusos. Los principales alimentos propensos a contener esta cianotoxina son los crustáceos, bivalvos y pescados. Por otro lado, cabe pensar que si la CYN se encuentra como agente contaminante en aguas de riego, puedan verse afectadas especies de plantas cultivadas para consumo humano.

Por último, también cabe destacar la exposición a estas toxinas por vía dérmica, ya que la CYN se encuentra en su mayor parte de forma extracelular, disuelta en el medio.

A partir de los pocos datos sobre toxicidad de la CYN que hay en la bibliografía, se ha establecido una ingesta diaria tolerable (IDT) provisional de 0,03 µg/kg/día, la cual esta obtenida a partir de un valor de NOAEL (non observed adverse effect level), obtenido de ensayos en ratones, de 30 µg/kg/día [1], al cual se le aplica un factor de incertidumbre de 1000 (100 por variabilidad inter e intraespecies, y 10 por falta de datos de toxicidad crónica) [31-32]. También existe un valor guía para la presencia de CYN en agua de 1 µg/L de agua de bebida [1, 31].

7. TRATAMIENTOS DE INACTIVACIÓN DE *C. Raciborskii* Y ELIMINACIÓN DE LA CILINDROSPERMOPSINA

Debido a que cada vez los blooms de CYN son más frecuentes y a que éstos presentan una gran toxicidad, ha sido necesario investigar diferentes técnicas que sean capaces de eliminar al agente productor de CYN (*C. raciborskii*, principalmente) y de inactivar a la CYN, en cualquiera de las formas en las que se presenta en el medio. Las técnicas se han centrado en

las aguas de consumo público, debido a que la intoxicación por esta vía es la más frecuente. Para ello se han llevado a cabo diferentes experimentos donde se emplearon varios agentes oxidantes (cloro, dióxido de cloro, monocloramina, permanganato y ozono) y radiación UV que se emplean normalmente en el tratamiento de desinfección de las aguas de bebida. El objetivo es inactivar a la cianobacteria y destruir tanto la CYN intracelular como la CYN extracelular.

El empleo de cloro resultó ser muy efectivo en la oxidación de la CYN, reduciendo la vida media de la CYN a 1,7 min al emplear concentraciones de cloro de 1 mg/L. El empleo de cloro también resultó en una inactivación completa de *C. raciborskii* [33]. Otro agente que también es muy efectivo es el ozono, el cual es muy reactivo con la CYN, provocando su total eliminación del medio. Además, la misma concentración de ozono sirvió para inactivar a *C. raciborskii* [31-35]. En ambos casos no se observó un aumento de la CYN extracelular al destruir las células.

En el caso de los otros 3 agentes oxidantes (dióxido de cloro, monocloramina y permanganato) se observó que la CYN era resistente, y que no había una inactivación completa de las células de *C. raciborskii* [33-35].

Por último, al emplear radiación UV a las dosis normales para tratamiento de aguas no se observó ni destrucción de la CYN ni inactivación de las células de *C. raciborskii*, y tan solo a dosis mucho más elevadas se observaba una ligera pérdida de viabilidad por parte de las células [33].

8. MÉTODOS DE DETECCIÓN DE CILINDROSPERMOPSINA

En comparación con las microcistinas y las saxitoxinas, se han desarrollado pocos métodos para la detección de CYN.

Harada [1] desarrolló el primer método de screening para CYN usando Cromatografía Líquida de Alta Resolución (HPLC) en fase reversa acoplada a un detector de diodo array (DAD), la cual se ha empleado para determinar la CYN en distintas muestras ambientales (embalses y lagos) [1, 39] y HPLC-MS/MS [1]. También se ha empleado la técnica de HPLC acoplado a detector UV para cuantificar la CYN a partir de células liofilizadas de cianobacterias productoras, al igual que una técnica de electroforesis capilar [40] (Tabla 2).

Método	Límite de detección	Comentarios	Fuente
HPLC-UV		Uso Limitado	[36]
HPLC- PDA	1-300 ng	Interferencias de la matriz del método de extracción.	[1]
LC-MS/MS	1-600 µg/L	Procesamiento mínimo. Caracterización y cuantificación de las muestras (crustáceos).	[1]
Ratón	0,2 mg/kg	Son necesarias instalaciones especializadas y se requiere licencia. Ensayo muy largo.	[1]
Gamba	8,1 µg/mL	Ensayo largo y tedioso.	[37]

Tabla 2. Métodos de detección de CYN. Tomado de [38].

Otra técnica recogida últimamente en la literatura y que ha demostrado ser selectiva para la determinación de CYN es la cromatografía liquida de retención de iones LC/ESI-MS/MS [1].

Debido al gran porcentaje de CYN que se encuentra disuelta en el medio, ha aumentado la demanda de métodos efectivos de extracción y concentración para una correcta cuantificación de la toxina en muestras de campo [1, 37].

A pesar de haberse desarrollado estos métodos, todavía las agencias oficiales de medio ambiente no han aceptado ninguno de ellos como método estándar, aunque existen algunos países, como Australia y Nueva Zelanda, en los que los blooms son frecuentes, que tienen una cierta regulación y establecen métodos oficiales de detección de CYN. En el caso de Australia la técnica LC-MS es el método de elección para la determinación de CYN [1], al igual que en Nueva Zelanda, donde la New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters también establece la LC-MS como método de elección para la determinación de CYN.

En resumen, las investigaciones sobre el desarrollo y optimización de métodos sensibles, sencillos y rápidos que permitan una adecuada monitorización de estas toxinas, es un reto importante. Ello permitirá disponer de datos más fiables para establecer un rango de concentraciones relevantes en el medio ambiente, y evaluar el riesgo derivado de la presencia de estas toxinas en aguas y alimentos contaminados.

9. LEGISLACIÓN SOBRE CILINDROSPERMOPSINA

Los distintos estudios toxicológicos existentes hasta la actualidad han dado lugar al establecimiento de diversos límites de seguridad para CYN, como por ejemplo una IDT provisional de 0,03 µg/kg/día de CYN [1], al ser una de las toxinas procedente de cianobacterias más potentes que se conoce.

El peligro que supone esta toxina por la grave afectación del hígado que produce por su consumo en el agua de bebida ha sido la principal razón por la que se ha establecido un valor guía para la presencia de CYN de 1 µg/L en agua de bebida [1,31], comprendiendo tanto a la CYN intracelular como a la extracelular.

En Europa las toxinas de cianobacterias aún no están claramente reguladas, aunque la Directiva 2000/60/CE [41] de la Unión Europea las considera específicamente como potenciales contaminantes peligrosos.

Para aguas recreacionales con floraciones de cianobacterias, la Organización Mundial de la Salud ha establecido tres niveles de alerta de riesgos sanitarios, dependiendo del riesgo de los efectos adversos para la salud [35], que están basados en la densidad de cianobacterias. La Directiva Europea 2006/7/CE [42] relativa a la gestión de la calidad de las aguas de baño contempla, entre otros parámetros de evaluación, la propensión a la proliferación de cianobacterias dentro de los perfiles de las aguas de baño.

10. CONCLUSIONES

Como conclusiones de esta revisión sobre la CYN se pueden establecer las siguientes:

- La presencia de cianobacterias y cianotoxinas, entre ellas la CYN, en el agua constituye un problema de interés creciente.
- Las principales vías de exposición humana a la CYN son el contacto dérmico, y la ingesta de alimentos contaminados y agua de bebida.
- El principal órgano diana de la CYN es el hígado, aunque también puede afectar a otros órganos como riñón, pulmones, corazón, estómago, glándulas adrenales, sistema vascular y sistema linfático.
- Los mecanismos de acción tóxica que presenta son, entre otros, inhibición de la síntesis de proteínas, reducción de GSH, genotoxicidad, citotoxicidad y carcinogenicidad, aunque muchos de los resultados que se han obtenido hasta la fecha no son concluyentes.
- Actualmente no existen métodos analíticos de referencia que estén aceptados por la Comunidad Internacional, si bien algunos países, en los que la presencia de blooms es frecuente, sí que tienen métodos analíticos establecidos.
- Por último, la legislación actual es escasa y debe ir evolucionando conforme se vayan desarrollando las técnicas analíticas, para de esta forma establecer valores límites reales que regulen de esta

forma los niveles de CYN que llegan al consumidor a través de los alimentos y el agua de bebida.

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PATENTE

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USO DE N-ACETILCISTEÍNA PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA

2011

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MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

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- [ ] PCT: ENTRADA FASE NACIONAL

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- [ ] EL SOLICITANTE ES EL INVENTOR
- [x] EL SOLICITANTE NO ES EL INVENTOR O ÚNICO INVENTOR

(9) MODO DE OBTENCIÓN DEL DERECHO:
[x] INVENC. LABORAL [ ] CONTRATO [ ] SUCESIÓN

(10) TÍTULO DE LA INVENCIÓN:
USO DE N-ACETILCISTEINA PARA PROTEGER A LOS PECES DE LA INTOXICACIÓN POR CILINDROSPERMOPSINA

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(13) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN	CÓDIGO PAÍS	NÚMERO	FECHA

(14) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASAS PREVISTO EN EL ART. 162. LEY 11/86 DE PATENTES [ ]

(15) AGENTE /REPRESENTANTANTE: NOMBRE Y DIRECCIÓN POSTAL COMPLETA. (SI AGENTE P.I., NOMBRE Y CÓDIGO) (RELLÉNESE, ÚNICAMENTE POR PROFESIONALES)

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- [ ] LISTA DE SECUENCIAS Nº DE PÁGINAS:
- [ ] RESUMEN
- [ ] 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
- [ ] CUESTIONARIO DE PROSPECCIÓN
- [ ] OTROS:

FIRMA DEL SOLICITANTE O REPRESENTANTE

Universidad de Sevilla — Fdo. Ramón González Carvajal, Vicerrector de Transferencia Tecnológica

(VER COMUNICACIÓN)

FIRMA DEL FUNCIONARIO

NOTIFICACIÓN SOBRE LA TASA DE CONCESIÓN:
Se le notifica que esta solicitud se considerará retirada si no procede al pago de la tasa de concesión; para el pago de esta tasa dispone de tres meses a contar desde la publicación del anuncio de la concesión en el BOPI, más los diez días que establece el art. 81 del R.D. 2245/1986.

MOD. 3101i – 2 – EJEMPLAR PARA EL SOLICITANTE

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NÚMERO DE SOLICITUD

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FECHA DE PRESENTACIÓN

[x] PATENTE DE INVENCIÓN   [ ] MODELO DE UTILIDAD

(5) SOLICITANTES: APELLIDOS O DENOMINACIÓN SOCIAL	NOMBRE	NACIONALIDAD	CÓDIGO PAÍS	DNI/CIF	CNAE	PYME

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(12) EXPOSICIONES OFICIALES: LUGAR	FECHA

(13) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN	CÓDIGO PAÍS	NÚMERO	FECHA

MOD. 3102i – 2 – EJEMPLAR PARA EL SOLICITANTE

NO CUMPLIMENTAR LOS RECUADROS ENMARCADOS EN ROJO

<u>Uso de N-acetilcisteína para proteger a los peces de la intoxicación por Cilindrospermopsina</u>

La presente invención se refiere al uso de una composición que comprende N-acetilcisteína (NAC) 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 NAC, derivado acetilado del aminoácido L-cisteína, es un conocido antioxidante que contiene grupos tiol.

Las aplicaciones de la NAC se basan en que puede actuar frente al estrés oxidativo por diferentes caminos. Es un precursor de la síntesis de GSH actuando como proveedor de cisteína y estimulando la actividad de las enzimas citosólicas involucradas en el ciclo del GSH, tales como Glutatión reductasa (GR), que aumenta la tasa de regeneración de GSH. Por otro lado, *in vivo* e *in vitro* actúa directamente, a través de su grupo tiol, sobre diferentes especies reactivas de oxígeno (ROS) tales como ácido hipocloroso, radical hidroxilo y peróxido de hidrógeno (Aruoma O.I. *et al.* (1989) <u>Free Rad. Biol. Med. 6:593-597</u>).

Se ha descrito que la inyección de NAC mejora el estado redox de GSH en el hígado de anguilas (*A. anguilla*) intoxicadas con organofosforados (diclorvos), incrementando su tolerancia al estrés oxidativo y la necrosis provocadas por ingesta de pesticidas, particularmente en el caso de organofosforados o piretroides (ES2249167 A1). En el campo de las cianotoxinas, su uso queda restringido a su actividad protectora frente al estrés oxidativo y la reversión de las lesiones histopatológicas inducidas por microcistinas (MCs) en peces (WO2010061018 -A1, Caméan Fernández A, et al., 2010).

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.,* (1992) J. Am. Chem. Soc. 144:7941-7942), 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*,, (1994) Toxicon 32:833-843; Runnegar M.T. *et al.* (1995) Biochem. Pharmacol. 49:219–225) 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.* (2011) Aquat. Tox. 105:100-106; Puerto M. *et al.*, (2011) Ecotoxicology, aceptado, en prensa), detectándose un aumento en la producción de especies reactivas de oxígeno (ROS), lipoperoxidación (LPO) y oxidación de

proteínas, 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
5 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 no se conoce ningún tratamiento capaz de recuperar a los humanos,
10 mamíferos y peces intoxicados con 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 los peces afectados.

15 **DESCRIPCIÓN DE LA INVENCIÓN**

La presente invención se refiere al uso de una composición que comprende NAC para el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

20

En tilapias (*Oreochromis sp.*) expuestas a dosis únicas y repetidas de CYN se inducen estrés oxidativo y alteraciones patológicas. En concreto, se han comprobado variaciones dosis-dependiente en la actividad de diversas enzimas antioxidantes, disminución de los niveles de Glutatión (GSH),
25 aumento de los niveles de lipoperoxidación (LPO), de oxidación de proteínas y del ADN, en diferentes órganos (hígado, riñón), y múltiples alteraciones histopatológicas en órganos diversos como hígado, riñón, corazón, tracto gastrointestinal y branquias.

30 La NAC 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.

35 Además, el uso de NAC 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 proteínas en riñón inducida por CYN, y recuperar las lesiones histopatológicas inducidas en múltiples órganos como hígado, riñón, corazón, tracto gastrointestinal y branquias.

5

En este sentido, un primer aspecto de la presente invención se refiere al uso de una composición que comprende NAC para la elaboración de un medicamento útil en el tratamiento, prevención y/o recuperación de efectos tóxicos en peces expuestos a CYN.

10

NAC es el derivado N-acetil del aminoácido L-cisteína. La NAC es el precursor del glutatión. El glutatión tiene un grupo tiol (sulfidrilo) que le confiere efectos antioxidantes por medio de la reducción de radicales libres.

15 La composición de la presente invención comprende, al menos, NAC. El medicamento está compuesto, al menos, por la composición anterior. La NAC, 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
20 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
25 administración, es capaz de proporcionar (directa o indirectamente) NAC.

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
30 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
35 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 NAC. 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 N-acetilcisteína 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 NAC 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 una semana. Preferiblemente la cantidad diaria incorporada a los peces es de entre 400 y 880 mg por Kg de peso (equivalentes a 22 y 45 mg NAC/Kg/día).

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.

Los peces intoxicados están expuestos a más de 10 μg CYN/L agua por día durante 14 días (intoxicación subcrónica). Preferiblemente los peces están expuestos a más de 200 μg CYN/ kg de pez en exposición única.

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, NAC), 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 NAC 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.

5 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
10 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.*

15 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,*
20 *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.*
25 *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).

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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
35 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 NAC sobre la LPO en hígado y riñón de tilapias expuestas a a 200 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 NAC con respecto a sus respectivos grupos control y (b) comparación del grupo tratado con CYN y NAC (22 ó 45 mg NAC/pez/día) con el grupo no tratado con NAC.

FIG. 2. Muestra el efecto protector de diferentes concentraciones de NAC sobre la oxidación de proteínas en riñón de tilapias expuestas a 200 µg CYN/kg pez

Medidas de oxidación de proteínas (grupos carbonilos; mg proteína).

Donde: el eje Y representa los valores de grupos carbonilos/mg proteína cuantificados como medida de la oxidación de proteínas 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 NAC con respecto a sus respectivos grupos control y (b) comparación del grupo tratado con CYN y NAC (22 ó 45 mg NAC/pez/día) con el grupo no tratado con NAC.

5 **FIG 3. Muestra el efecto protector de diferentes concentraciones de NAC sobre el cociente GSH/GSSG en hígado de tilapias expuestas a 200 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
10 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 NAC con respecto a sus respectivos grupos control, (b) comparación del grupo tratado con CYN y
15 NAC (22 ó 45 mg NAC/pez/día) con el grupo no tratado con NAC, y (c) comparación del grupo tratado con CYN y 22 mg NAC/pez día NAC, con el grupo tratado con CYN y 45 mg NAC/pez/día.

FIG. 4. Muestra los cambios histopatológicos en hígado de tilapias
20 **expuestas a CYN y su recuperación por NAC**.

A, C, E, G, I : Tinción con Hematoxilina-eosina. Las barras miden 10 µm. B, D, F, H, J: Observaciones ultraestructurales. Las barras miden 10 µm.

25 **FIG. 5. Cambios histopatológicos en riñón de tilapias expuestas a CYN y su recuperación por NAC.**

A, C, E, G, I: Tinción con Hematoxilina-eosina. Las barras miden 10 µm. B, D, F, H, J: Observaciones ultraestructurales. Las barras miden 10 µm.
30

FIG. 6. Cambios histopatológicos en corazón de tilapias expuestas a CYN y su recuperación por NAC

A, C, E, G, I: Tinción con Hematoxilina-eosina. Las barras miden 10 µm. B, D, F, H, J: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 7. Cambios histopatológicos en intestino de tilapias expuestas a CYN y su recuperación por NAC

A, C, E, G: Tinción con Hematoxilina-eosina. Las barras miden 10 µm. B, D, F, H: Observaciones ultraestructurales. Las barras miden 10 µm.

FIG. 8: Cambios histopatológicos en branquias de tilapias expuestas a CYN y su recuperación por NAC observados con el microscopio óptico.

A, B, C, D: Tinción con Hematoxilina-eosina. Las barras miden 10 µm.

FIG. 9: Cambios histopatológicos en branquias de tilapias expuestas a CYN y su recuperación por NAC observados por microscopía electrónica.

A, C, E: Observaciones ultraestructurales al microscopio electrónico de barrido (SEM: scanning electron microscope). Las barras miden 10 µm. B, D, F: Observaciones ultraestructurales al microscopio electrónico de transmisión (TEM: transmisión 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 NAC 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 48 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 6 grupos experimentales con 8 animales en cada uno. Los peces fueron intoxicados con la toxina CYN
5 pura (pureza > 95%, Alexis Corporation, Lausen, Suiza) por vía oral (sonda nasogástrica). La administración de NAC se realizó a través del pienso, empleando dos niveles de dosis, 22 y 45 mg NAC/pez/día (equivalentes a 400 y 800 mg NAC/pez/día). Cada grupo fue introducido en un acuario independiente:

10 Acuario 1: Peces control, alimentados sólo con pienso normal durante 7 días.
Acuario 2: Peces alimentados con pienso durante 7 días, intoxicados con CYN en la dieta (dosis única de 200 µg/ kg pez).
Acuarios 3 y 5: Peces alimentados con pienso + NAC (22 y 45 mg de NAC/pez, respectivamente) durante 7 días.
15 Acuarios 4 y 6: Peces con pienso + NAC (22 y 45 mg de NAC/pez, respectivamente) durante 7 días. Los peces se intoxicaron con una dosis única de 200 µg CYN/kg pez.

Al final del experimento los peces fueron sacrificados, anestesiándolos con hielo.
20 Se procedió a la extracción de los órganos, y se prepararon sus extractos para las determinaciones de biomarcadores enzimáticos, según Gutiérrez-Praena et al., 2011 (Aquat. Toxicol. 105: 100-106). 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
25 con el ácido tiobarbitúrico; la oxidación de proteínas mediante la determinación de los grupos carbonilo, 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., 2008 (Toxicol Pathol 36:449-
30 458), incluyendo en el caso de las branquias microscopia electrónica de barrido (SEM) y microscopia electrónica de transmisión (TEM).

Para los estudios de significación estadística entre grupos, se empleó el análisis de la varianza (ANOVA) y posteriormente el ensayo de Tukey, con una
35 significación estadística $p<0,05$.

Los resultados más significativos fueron los siguientes:

1) En la FIG. 1 se observa cómo la toxina CYN incrementa la lipoperoxidación (LPO) en hígado (1,5 veces), y en riñón (1,2 veces) frente al control, y los efectos protectores de NAC se demuestran con las dos dosis ensayadas.

2) En la FIG. 2 se observa cómo CYN aumenta la oxidación de proteínas en riñon (1,5 veces) con respecto al control, y los efectos protectores parciales de la dosis mas baja de NAC (22 mg NAC/pez/dia) y totales de la dosis más elevada de 45 mg NAC/pez/día.

3) La FIG. 3 muestra la disminución de los cocientes GSH/GSSG en hígado de los peces intoxicados, y la aplicación de NAC mejoró este parámetro.

Ejemplo 2. NAC mejoró las alteraciones histopatológicas inducidas en hígado.

El estudio histopatológico del hígado de los peces pertenecientes al lote tratado con CYN puso de evidencia un proceso degenerativo que consistió en una desorganización de los hepatocitos y presencia de vesículas de grasa en el citoplasma (FIG. 4C), frente a una ausencia de lesiones del grupo control (FIG. 4A), caracterizada por cordones hepáticos normales, con hepatocitos con una morfología poliédrica normal. Al microscopio electrónico se observa que las células muestran el citoplasma repleto de grasa (FIG. 4D).

En los dos lotes de peces tratados exclusivamente con NAC se observó que, con ambas dosis (22 y 45 mg NAC/pez/dia) no existían lesiones hepáticas (FIG. 4 E, F). En los lotes de peces a los que junto a la toxina CYN se les administró 22 mg NAC/kg pez/día las lesiones inducidas por CYN prácticamente no se presentan, observándose en los hepatocitos cierta actividad metabólica, con contenido de glucógeno sin llegar a ser patológico (FIG 4G). Al microscopio electrónico se observa el detalle de un hepatocito con cierto contenido de glucógeno en el citoplasma (FIG. 4H).

El estudio de los peces del lote a los que junto con CYN se les administró una dosis de 45 mg NAC pez/día mostró una recuperación total de las lesiones provocadas por la toxina, tanto al microscopio óptico, como al electrónico (FIG. 4 I, J).

A, B: Hígado de los peces control. A. Cordones hepáticos normales, morfología poliédrica con núcleo central y citoplasma claro. B. Detalle de hepatocito aparentemente normal, con organoides citoplasmáticos, retículos y mitocondrias.

C, D: Tilapias expuestas sólo a CYN (200 µg/kg pez/día C. Parénquima hepático desorganizado, hepatocitos con presencia de vesículas de grasa (cículo). D. El hepatocito presenta un núcleo denso normal rodeado de un escaso citoplasma repleto de grasa (círculo).

E, F: Tilapias expuestas solo a NAC (dosis alta, 45 mg NAC/pez/día). E. Parénquima hepático 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 tratadas con CYN+NAC (22 mg NAC/pez/día). G. Hepatocitos en cordones con morfología aparentemente normal, morfología poliédrica con núcleo central y citoplasma claro con cierto contenido en glucógeno (círculo) H. Detalle de hepatocito con cierto contenido de glucógeno (círculo).

I, J: Hígado de peces tratados con CYN+NAC (45 mg NAC/pez/día). I. Parénquima con hepatocitos en cordones aparentemente normal. L. Detalle de hepatocito normal

Ejemplo 3. NAC mejoró las alteraciones histopatológicas inducidas en riñón.

Morfológicamente los riñones de los peces del lote tratados con CYN mostraron una glomerulopatía, observándose al microscopio óptico una atrofia glomerular y dilatación de la capsula de Bowman (FIG. 5C), y al microscopio electrónico, engrosamiento y densificación de las membranas basales y colapso de los capilares fenestrados (FIG. 5D). 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 NAC (22 y 45 mg NAC/pez/día) no mostró ninguna lesión renal (FIG. 5 E, F).

En los peces a los que se les administró CYN junto con 45 mg NAC/pez/día se observó estructuralmente y ultraestructuralmente una morfología del parénquima renal totalmente normal (FIG. 5 G, H).

5 A, B: Riñón de los peces control.
C, D: Histología de peces tratados con CYN. C. Detalle de parénquima renal con glomérulos atróficos (círculo) y dilatación de la capsula de Bowman (estrella). D. Microvellosidades de los túbulos contorneados proximales tumefactos y pérdida de dichas microvellosidades (flecha).
10 E, F: Tilapias expuestas sólo a NAC (45 mg NAC/pez/día). E. Parénquima renal con glomérulo renal aparentemente normal. F. Detalle del túbulo contorneado proximal aparentemente normal.
G, H: Histología del riñón de peces tratados con CYN+ NAC (45 mg NAC/pez/día) G. Glomérulos y túbulos con apariencia normal. H Membrana
15 basal aparentemente normal.

Ejemplo 4. NAC mejoró las alteraciones histopatológicas inducidas en corazón.

20 El estudio histopatológico realizado sobre peces tratados con CYN mostraron estructuralmente a nivel de corazón un proceso de miofibrolisis, con pérdida de miofibrillas, presencia de edemas y hemorragias. Ultraestructuralmente se apreció claramente esta miofibrolisis de la fibra cardiaca, caracterizada por una pérdida de las miofibrillas (FIG. 6 C, D). El corazón de los peces del lote control
25 presentó una estructura aparentemente normal (FIG. 6 A, B).

Los peces tratados solo con NAC (22 y 45 mg NAC/pez/dia) presentan una morfología de las fibras cardiacas similares a las del grupo control (FIG. 6 E, F).
Los procesos degenerativos en corazón que se observaron en los peces
30 tratados con CYN fueron mucho más leves en aquellos peces tratados con suplemento de NAC a la dosis más baja (22 mg NAC/pez/dia), observándose cierta pérdida de miofibrillas y ligeras hemorragias aisladas, de escaso interés (FIG. 6 G, H). No se observaron lesiones en los tratados con la dosis más elevada (45 mg NAC/pez/día) (FIG. 6 I, J).

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A, B: Corazón de peces control.

C, D: Histología del corazón de peces tratados con CYN. C. Miofibrolisis, pérdida de miofibrillas (círculo), ciertos edemas (estrella). D. Pérdida y desintegración de las miofibrillas (círculo).
E, F: Corazón de peces tratados con NAC (dosis 45 mg NAC/pez/día) E. Detalle de corazón con fibras musculares aparentemente normal. F. Estructura de las miofibrillas prácticamente normal.
G, H: Histología del corazón de peces tratados con CYN+NAC (22 mg NAC/pez/día) G. Detalle de miocardio con fibras musculares aparentemente normales pero con ciertas hemorragias (círculo). H. Detalle de miofibrillas con escasa pérdida de material contráctil (círculo).
I, J: Histología del corazón de peces tratados con CYN+NAC (45 mg NAC/pez/dia) I. Detalle de corazón con fibras musculares aparentemente normal. J. Detalle de miofibrillas aparentemente normal.

Ejemplo 5. NAC mejoró las alteraciones histopatológicas inducidas en intestino.

A nivel de intestino en los peces tratados con CYN se observaron lesiones, frente al lote control (FIG. 7 A, B), caracterizadas por un proceso de enteritis con necrosis de enterocitos, al microscopio óptico (FIG. 7C), y al microscopio electrónico, por una pérdida de microvellosidades muy manifiesta (FIG. 7D).

Tras la administración de las dos dosis de NAC (22 y 45 mg NAC/pez/día, equivalentes a 400, 880 mg/kg) solo se observó una actividad manifiesta de las células caliciformes, sin interés patológico (FIG. 7 E, F).

Los peces a los que se les administró NAC junto con la toxina CYN mostraron una protección con ambas dosis, con una estructura aparentemente normal cuando fueron observadas tanto al microscopio óptico como electrónico (FIG. 7 G, H).
A, B: Intestino de peces control. A. Vellosidades aparentemente normales con enterocitos aparentemente normales. B. Enterocitos con abundantes microvellosidades aparentemente normales y células caliciformes.
C, D: Histología de intestino de tilapias tratadas con CYN. C. Detalle de vellosidades intestinales con enterocitos necrosados (círculo) D. Alteración de los enterocitos con pérdida parcial de las microvellosidades (círculo).

E, F: Intestino de peces tratados con NAC (dosis 45 mg NAC/pez/día) E. Vellosidades aparentemente normales y abundantes células caliciformes (flecha). F. Enterocitos con abundantes microvellosidades (círculo).
G, H: Intestino de Tilapias tratadas con CYN + NAC (45 mg NAC/pez/día) G. Detalle de vellosidades intestinales normales con enterocitos aparentemente normales y abundantes células caliciformes (flecha). J. Enterocitos con abundantes microvellosidades (circulo) y células caliciformes aparentemente normales (flecha).

Ejemplo 6. NAC mejoró las alteraciones histopatológicas inducidas en branquias.

Al microscopio óptico las branquias de los peces tratados con CYN presentaron, a nivel de las laminillas primarias y secundarias, procesos de hiperemia y pérdida de las prolongaciones lamelares (FIG. 8B). Estas lesiones no fueron observadas en el lote control, que presentó una morfología aparentemente normal (FIG. 8A). En los lotes de peces tratados con CYN y ambas dosis de NAC se demuestra una recuperación de las lesiones provocadas (FIG. 8C).

Al microscopio electrónico de barrido (SEM) se observó una alteración de la morfología con pérdida de continuidad del arco branquial, y presencia de un infiltrado celular (FIG. 9C), y al microscopio de transmisión electrónica (TEM) se detectó una fuerte hiperemia de los capilares con presencia de células de infiltrado inflamatorio (FIG. 9D). Los peces del grupo control (FIG. 9 A, B) no presentaron anomalías.

Los peces tratados con CYN y ambas dosis de NAC (22 y 45 mg NAC/pez/dia, equivalentes a 400 y 800 mg/kg de NAC, respectivamente) mostraron recuperación de las lesiones provocadas por la toxina, presentándose filamentos branquiales y arcos branquiales aparentemente normales (FIG 9 E, F).

FIG. 8 (Microscopia óptica) A: Branquias de peces control.
B: Branquias de peces tratados con CYN. Detalle de filamento branquial con presencia de hiperemia en laminillas secundarias (circulo).
C: Branquias de peces tratados con NAC (45 mg NAC/pez/día). Detalle de filamento branquial aparentemente normal.

D: Histología de branquias de peces tratados con CYN+NAC (22 y 45 mg NAC/pez/día) . Detalle de filamento branquial aparentemente normal.

FIG. 9 A, B: peces control.

5 C, D: Branquias de peces tratados con CYN pura. C. Arco branquial con pérdida de continuidad, erosionado y presencia de un infiltrado celular (círculo). D. Detalle de laminilla con hiperemia de capilares (círculo).

E, F: Branquias de peces tratados con CYN+NAC (45 mg NAC/pez/día, equivalente a 800 mg NAC/kg pez). E. Filamento branquial aparentemente

10 normal. F. Arco branquial aparentemente normal.

REIVINDICACIONES

1. Uso de una composición que comprende N-acetilcisteína 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 N-acetilcisteína 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 N-acetilcisteína se administra en una cantidad diaria de entre 22 y 45 mg NAC/pez/día (equivalentes a 400 y 880 mg por Kg de peso del pez, respectivamente).

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.

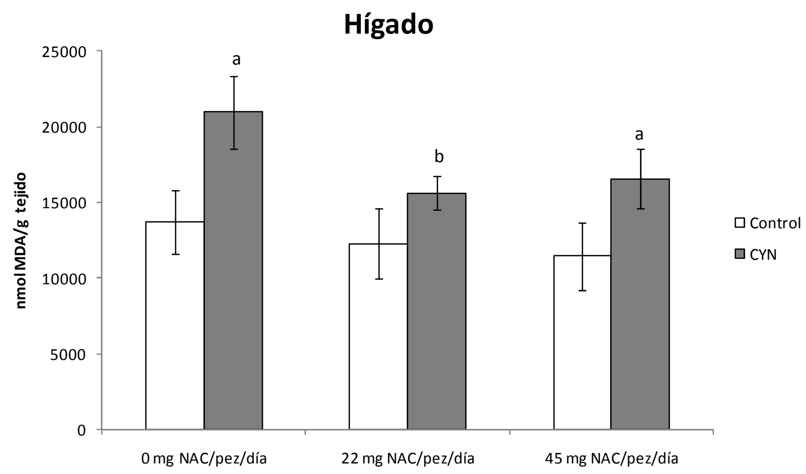
10. Uso según cualquiera de las reivindicaciones 1 a 9 para la fabricación de un complemento vitamínico.

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.

5

13. Uso según cualquiera de las reivindicaciones 1 a 12, donde los peces pertenecen al género *Orechromis sp.*



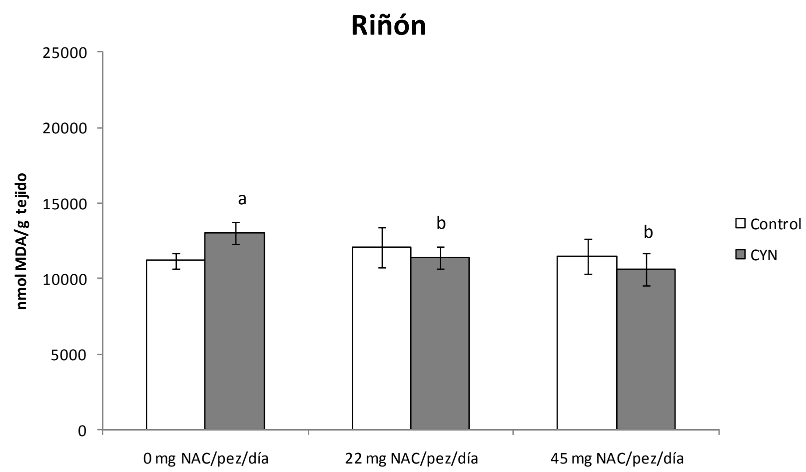


FIG. 1

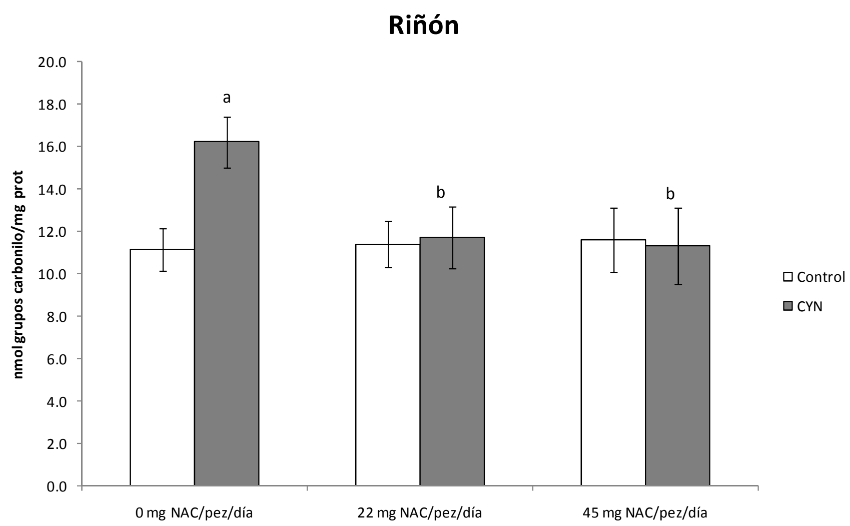


FIG. 2

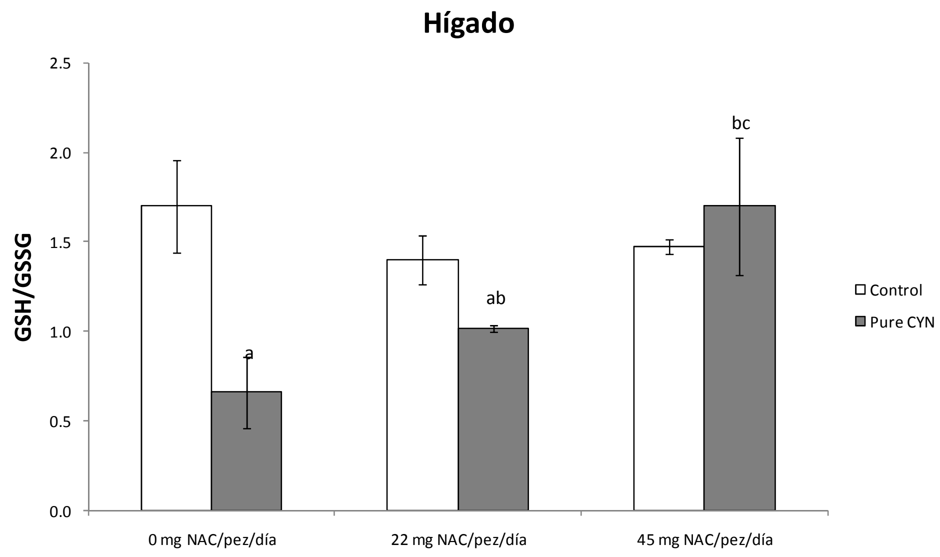


FIG. 3

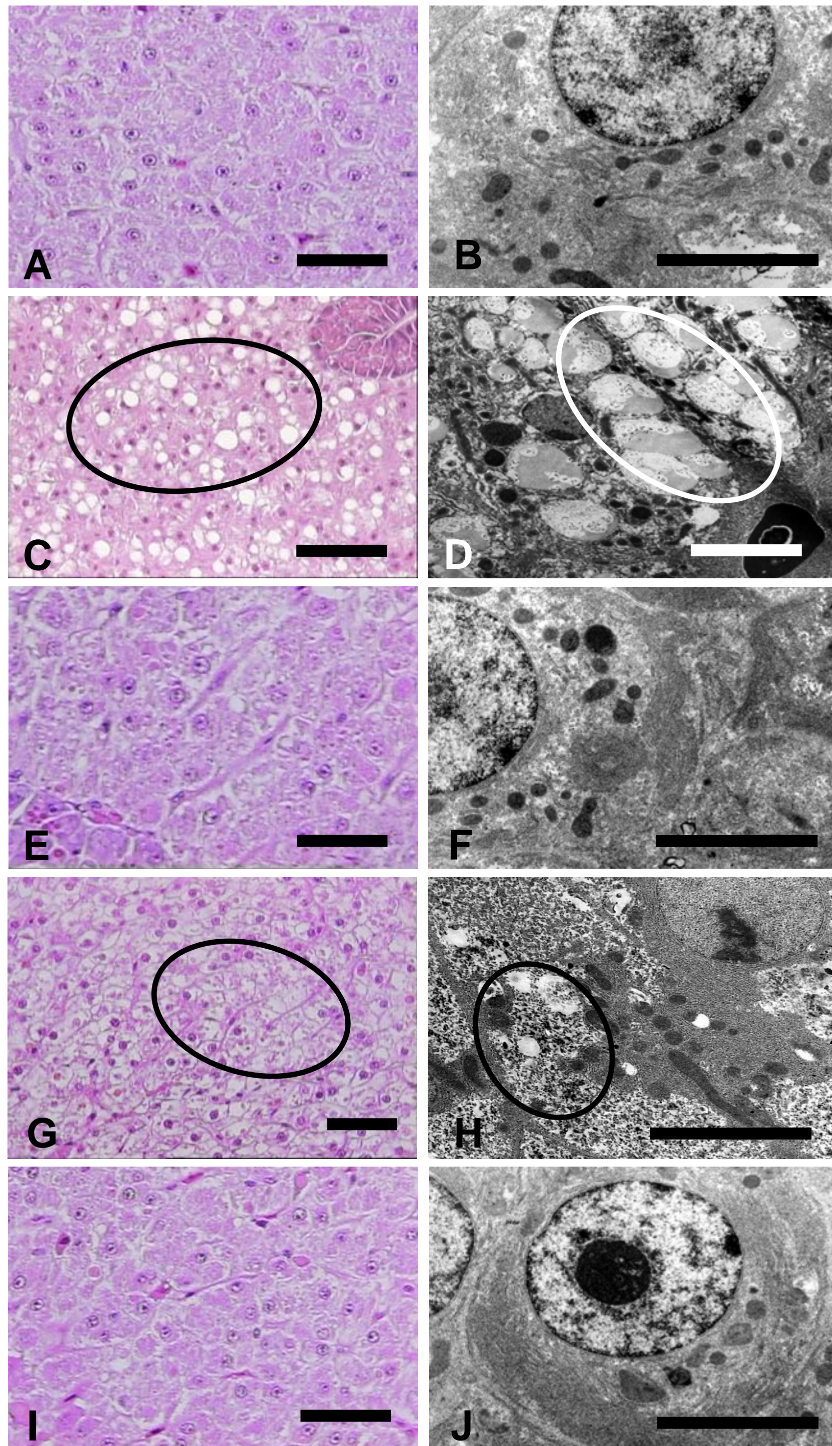


FIG. 4

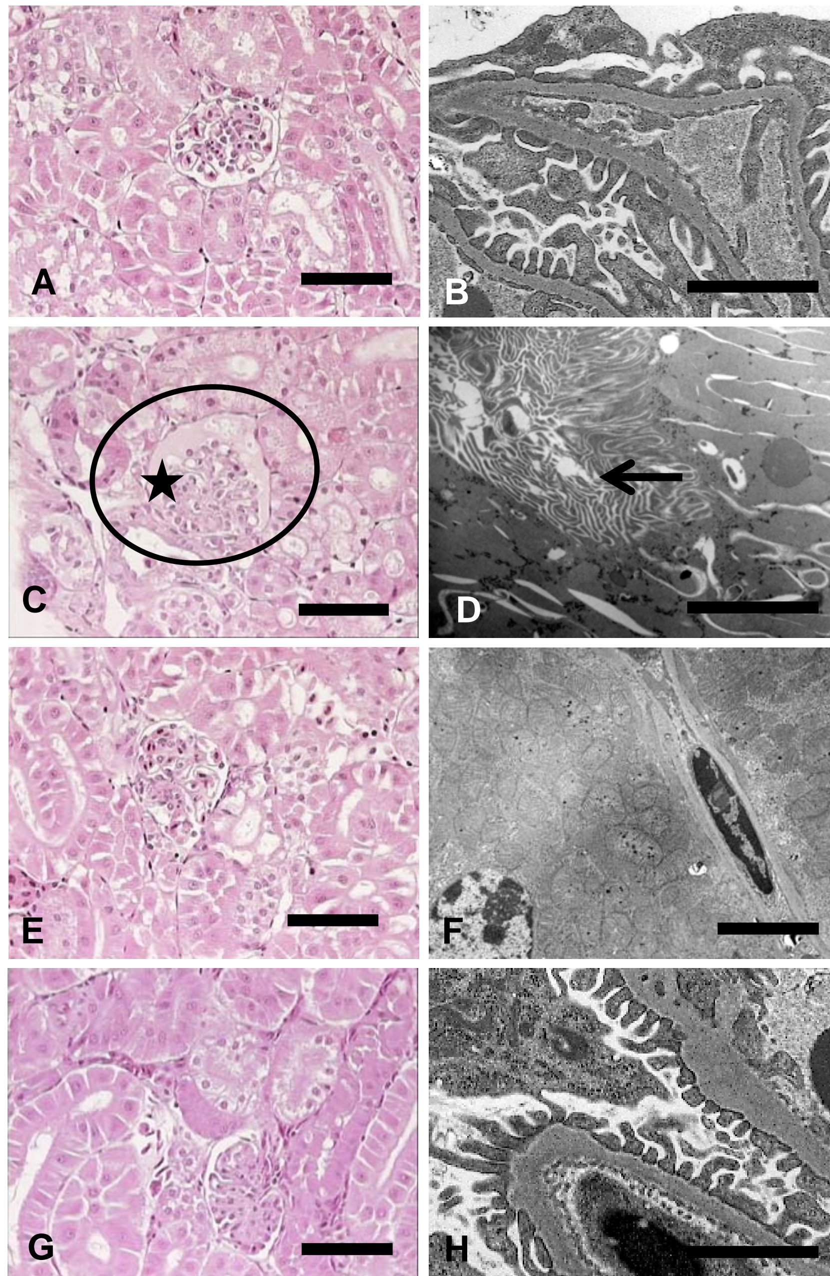


FIG. 5

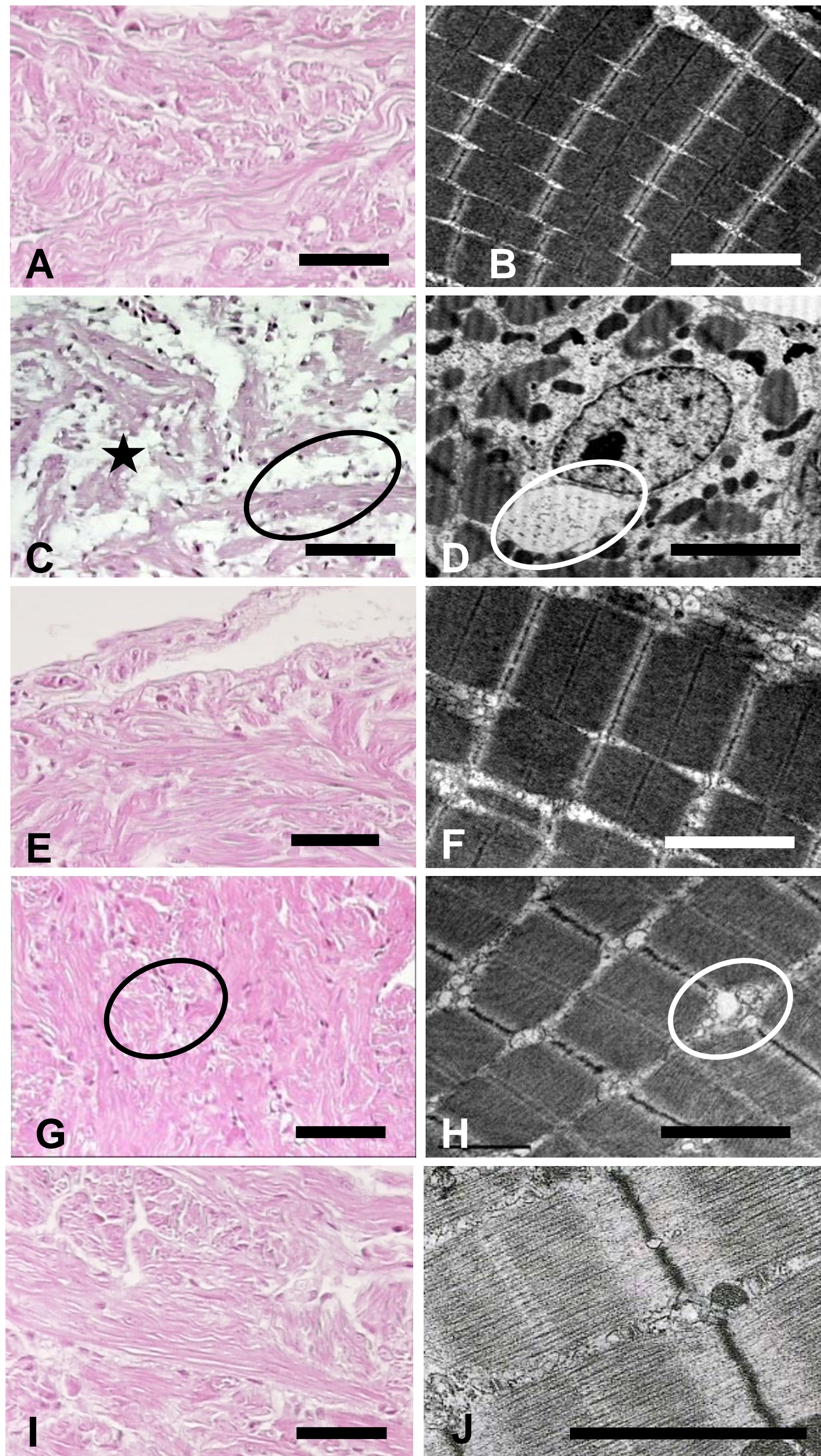


FIG. 6

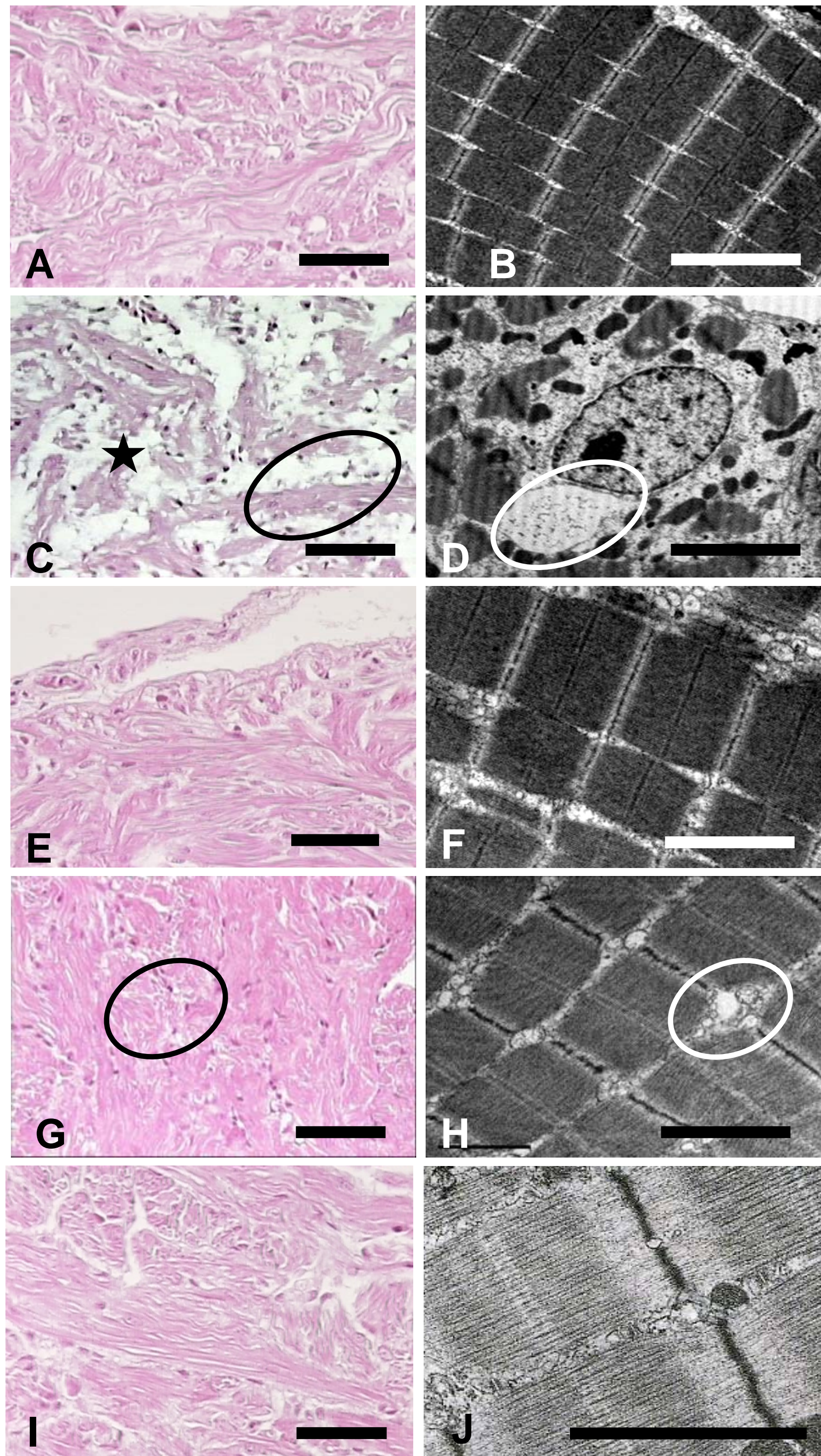


FIG. 7

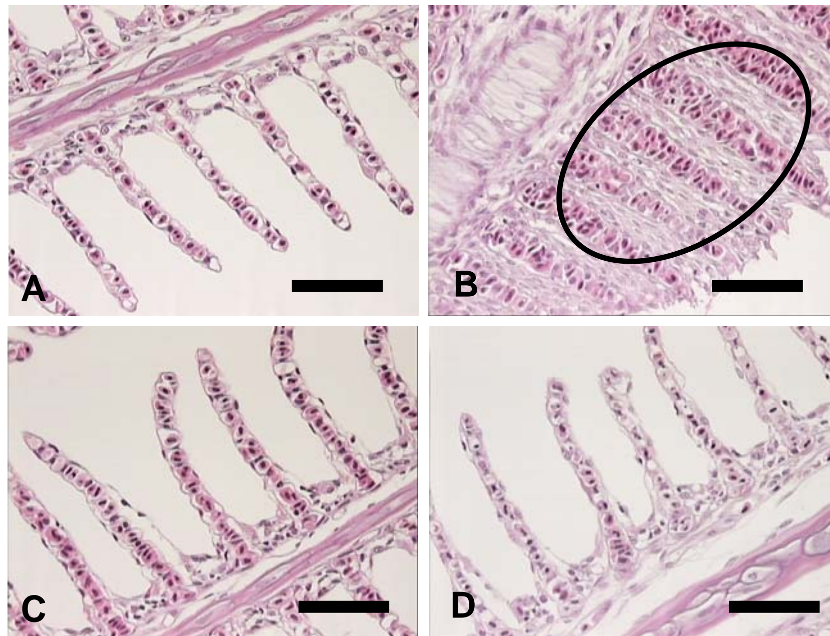


FIG. 8

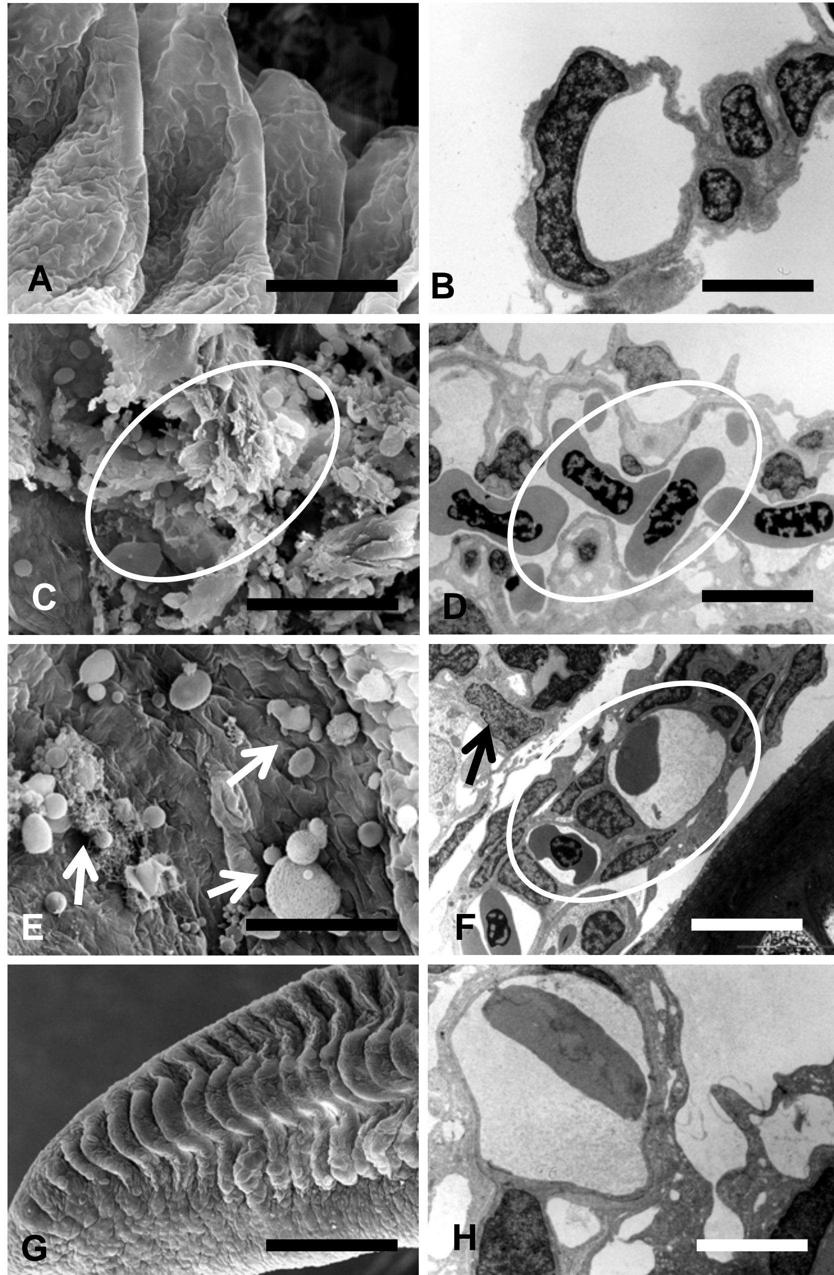


FIG. 9

RESUMEN

La presente invención se refiere al uso de una composición que comprende N-acetilcisteína (NAC) 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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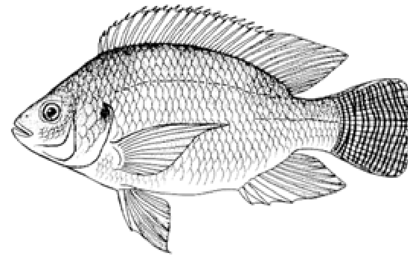

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