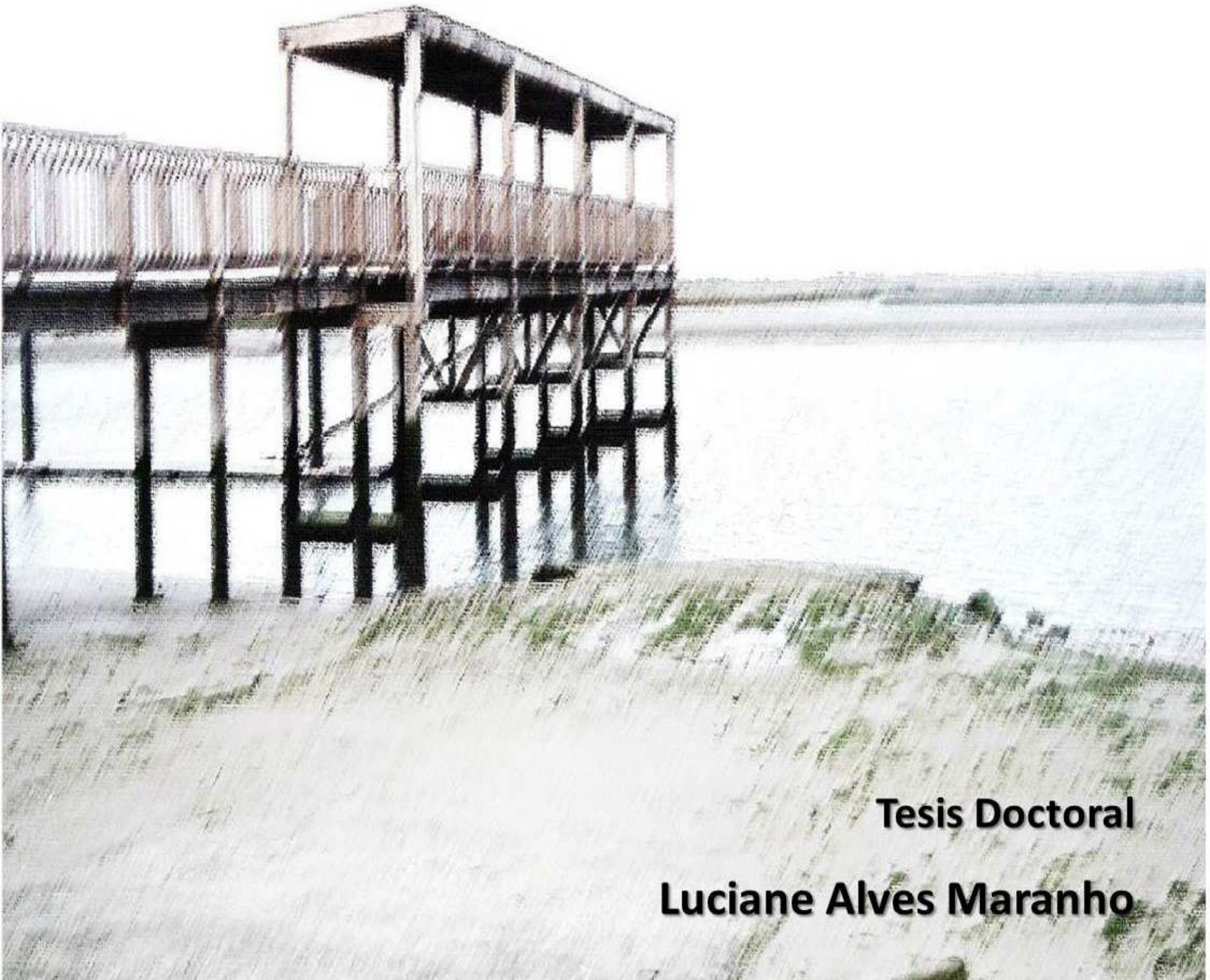
Evaluación del riesgo ambiental de sedimentos marinos afectados por la contaminación de productos farmacéuticos: estudios en laboratorio e *in situ*.



Tesis Doctoral

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UNIVERSIDAD DE CÁDIZ

FACULTAD DE CIENCIAS DEL MAR Y AMBIENTALES

EVALUACIÓN DEL RIESGO AMBIENTAL DE SEDIMENTOS MARINOS AFECTADOS POR LA CONTAMINACIÓN DE PRODUCTOS FARMACÉUTICOS: ESTUDIOS EN LABORATORIO E *IN SITU*.

Environmental Risk Assessment of Marine Sediments Affected by Contamination of Pharmaceutical Products: studies in laboratory and *in situ.*

Memoria presentada para optar al título de Doctorado Erasmus Mundus en Gestión Marina y Costera (Marine and Coastal Management)

Luciane Alves Maranho

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Dña. **M. LAURA MARTÍN DÍAZ**, Profesora Titular del Departamento de Química-Física de la Universidad de Cádiz y D. **T. ÁNGEL DEL VALLS CASILLAS**, Catedrático del Departamento de Química-Física de la Universidad de Cádiz, como sus directores,

HACEN CONSTAR,

Que esta memoria, titulada **Evaluación del riesgo ambiental de sedimentos marinos afectados por la contaminación de productos farmacéuticos: estudios en laboratorio e *in situ***, presentada por Dña. **Luciane Alves Maranho** resume su trabajo de Tesis Doctoral y reúne los requisitos legales y las condiciones de originalidad y rigor científico, por lo que autorizan su presentación y defensa para optar al grado de Doctora en Gestión Marina y Costera (Marine and Coastal Management) por la Universidad de Cádiz.

Cádiz, 02 de Octubre de 2014.

Fdo.: M. Laura Martín-Díaz

Fdo.: T. Ángel Del Valls Casillas

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El Malecón de Cádiz

Alejo Martínez

Cuando llega la bruma y ya no se ve más nada
El Malecón se escapa para la Habana
Se regalan estrellas de mares diferentes
Cuando van de paseo los continentes

Ese amor paseando la Habana vieja
Amor de lejanía que así lo deja
Los labios de quien besa, un beso vacío, que a fuerza de distancia ya sabe a olvido
Se le despierta Cádiz tan habanera, tan habanera, sorprendiendo amores que no se esperan

Cuando llega la bruma e ya no se ve más nada
El Malecón se escapa para la Habana
Se regalan estrellas de mares diferentes
Cuando van de paseo los continentes

La Bahía de Cádiz, abrazo inmenso
Cuando cierro los brazos, le dejó adentro
Y por eso se siente y de qué manera, su paso enamorado por la Alameda
Se despierta la Habana tan gaditana, tan gaditana…que imagina el perfume de sus mañanas

Cuando llega la bruma y ya no se ve más nada
El Malecón se escapa para la Habana

Cada ser tem sonhos à sua maneira.

Noite Severina

(Ney Matogrosso e Pedro Luís)

INDICE DE CONTENIDOS

LISTA DE ACRÓNIMOS Y ABREVIATURAS

Al: Alumínio

As: Arsénico

CAF: Cafeína

CBZ: Carbamazepina

Cd: Cadmio

CDNB: 1-cloro-2,4-dinitrobenceno

Cr: Cromo

Cu: Cobre

DBF: Dibenzilfluoresceína

DMSO: Dimetilsulfóxido

EBAR: Estación de bombeo de aguas residuales

EC_{50}: Concentración que causa 50% de efecto

EDAR: Estación depuradora de aguas residuales

EE2: 17α-etinilestradiol

EMA: European Medicine Agency

EqP: Equilibrio de partición

ERA: Evaluación del riesgo ambiental

EROD: Etoxi-resorufin desetilasa

FA/ PCA: Analices de factores/ Analices de componentes principales

Fe: Hierro

FX: Fluoxetina

GPX: Glutatión peroxidasa

GR: Glutatión reductasa

GSH: Glutatión reducido

GSSG: Glutatión oxidado

GST: Glutatión-S-transferasa

Hg: Mercurio

IBP: Ibuprofeno

KCl: Cloruro potásico

LAS: Sulfonato de alquilbenzeno linear

LMS: Lysosomal membrane stability

LOE: Líneas de evidencia

Log Kow: Coeficiente de partición octanol/agua

LPO: Peroxidación lipídica

MOA: Modo de acción

Mn: Manganeso

NADPH: Nicotinamida adenina dinucleótido fosfato

Ni: Níquel

NRRT: Neutral Red Retention Time

pKa: Constante de acidez

PRO: Propranolol

TOC: Carbono orgánico total

OECD: Organization for economic co-operation and development

OSPAR: Oslo and Paris (Convention)

PAH: Hidrocarburos aromáticos policíclicos

Pb: Plomo

PCB: Bifenil policlorados

PPCP: Fármacos y productos de higiene personal

ROS: Espécies reactivas de oxigeno

SDS: Sodium dodecyl sulfate

Se: Selenio

SRRI: Inhibidor selectivo de la recaptación de serotonina

TBARS: Thiobarbituric acid reactive substances

US EPA: United States Environmental Protection Agency

Zn: Zinc

CAPÍTULO 1

Introducción, objetivos y estructura de la tesis

1. Importancia de los sedimentos marinos en la evaluación de la calidad ambiental

El sedimento es un compartimento solido resultado de la erosión de la superficie terrestre, los océanos, valles, ríos o restos biológicos (organismos vivos o muertos). Debido a que es un depósito de partículas desde el punto de vista geológico, se pueden clasificar de acuerdo con el tamaño del grano, la textura y la materia constituyente. Es responsable por la protección de los ambientes costeros, tanto de los ecosistemas como de las zonas urbanas. Este sustrato es compatible con ecosistemas complejos y abriga muchas especies que son la base de la cadena trófica marina. Posee un importante papel ecológico, ya que muchas especies pelágicas tienen el comienzo de su ciclo de vida bentónico. La fauna bentónica posee un largo rango de estrategias alimenticias, que pueden clasificarse como detritívoras, filtradoras y depredadoras. La exposición a través del alimento es uno de los medios de consumo de contaminantes por la fauna bentónica. Esos contaminantes pueden ser la materia orgánica, el petróleo, pesticidas, metales y compuestos orgánicos, entre ellos los fármacos y productos de higiene personal (en inglés: pharmaceutical and personal products - PPCP).

El estudio de los sedimentos marinos es de gran importancia debido a interferencias tales como el dragado, el mal uso del suelo, la deposición de materiales de desecho muchas veces en lugares no deseados, residuos industriales y domésticos, aguas residuales, deposición de contaminantes atmosféricos, entre otros. Los sistemas marinos

están sometidos a grandes presiones antropogénicas originados de las actividades económicas, el desarrollo urbanístico y la elevada densidad de población de las zonas costeras. El manejo inadecuado de las aguas residuales, domésticas e industriales, y de los residuos sólidos puede empeorar los problemas de salud pública resultantes de la contaminación. Muchas de las especies afectadas por la contaminación de los ecosistemas costeros son de interés comercial, por lo tanto, de interés para la salud pública.

El sedimento es el primer destino de las sustancias que se introducen en los océanos. Cuando se introduce en el sistema, los contaminantes tienden a permanecer por un período corto en la columna de agua, parte disueltos en el agua y parte adsorbidos por los materiales en suspensión o asociados con sales, carbono orgánico y arcillas, precipitándose al fondo y depositándose en los sedimentos, donde entran en contacto con los organismos bentónicos. La capacidad de acumulación y la concentración de los contaminantes disponibles a la biota varían de acuerdo con las características físico-químicas del medio ambiente, tales como el pH, la salinidad y el contenido de materia orgánica (Meyer, 2002). Procesos químicos, físicos y biológicos pueden dar lugar a la liberación de contaminantes retenidos en el sedimento, tornándolos biodisponibles en la columna de agua, produciendo así posibles riesgos a la biota pelágica y bentónica.

La concentración de compuestos en el sedimento permite estimar el nivel de contaminación. Sin embargo, el impacto de los contaminantes en la biota bentónica depende de su biodisponibilidad y toxicidad para los organismos (Parsons et al., 2007). El sedimento bruto se puede considerar un componente ambiental adecuado para indicar la calidad de un ecosistema acuático, ya que es un sustrato importante para muchos organismos. Está reconocido como el principal destino de las sustancias antropogénicas introducidas en los cuerpos de agua, y tiene potencial de acumulación en niveles superiores a los observados en la columna de agua adyacente, para contaminantes como

metales y compuestos orgánicos (Adams et al., 1992). Su evaluación es crucial, ya que integra la exposición a partir del contacto directo con la fase sólida de los sedimentos, el agua intersticial y la exposición a través del alimento.

Materia orgánica, carbono orgánico total y la geoquímica del sedimento son los principales factores que controlan a adsorción de estos compuestos y que pueden afectar la biodisponibilidad a la biota. Muchas veces los coeficientes de adsorción o la evaluación de riesgo ambiental (ERA) son basados en el coeficiente de partición octanol/ agua (log K_{ow}), que reflecte la afinidad del compuesto con carbono orgánico disuelto o con lepidios. La distribución de sustancias neutras entre la fase acuosa y la fase hidrofóbica es definida como el coeficiente entre la concentración del compuesto en n-octanol y en agua. Por lo tanto, un valor de log K_{ow} < 2.5 representa que la sustancia posee un potencial de adsorción bajo y que es probable que se quede en la fase acuosa. Cuando el log K_{ow} > 4, el potencial de adsorción es alto y este compuesto probablemente estará en la fase sólida.

Equilibrio de partición (equilibrium partitioning – EqP) se define como la relación de las concentraciones de una substancia química en aceite y en agua, relacionado con la solubilidad en agua y adsorción en sedimento (Parsons et al., 2007). Los lixiviados se consideran una forma de evaluar el impacto de la transferencia de contaminantes de sedimentos a la columna de agua adyacente por proceso de resuspensión, ya sea por causas naturales o antrópicas. La evaluación de los lixiviados permite determinar la calidad ambiental del sedimento mediante el análisis de la solución obtenida mediante la mezcla de sedimento: agua (1: 4) (USEPA, 1991). Esta resuspensión puede provocar que los contaminantes antes retenidos en el sedimento pasen a estar biodisponibles en la columna de agua adyacente, lo que facilita la exposición a la biota (USEPA, 1991).

Debido a su capacidad de acumulación en el tiempo y de su importancia ecológica, la evaluación de la calidad de los sedimentos se convierte en una herramienta

básica en la gestión de los medios acuáticos. Principios generales y directrices para la efectividad de la evaluación de sedimentos se basan principalmente en las actividades de dragado. Según Nendza (2002), la evaluación de la calidad ambiental de sedimentos en las actividades de dragado es un procedimiento escalonado, siendo que primero se analizan las propiedades físicas y químicas de los materiales de dragado, incluyendo granulometría, contaminantes y nutrientes. Si la información obtenida no es suficiente para una evaluación final, se requiere la determinación de los posibles efectos ecotoxicológicos del sedimento usando bioensayos. Las características físico-químicas de los sedimentos junto con los efectos biológicos son recomendados en diversas guías, como OSPAR, Helsinki Conventions, de Canadá y Estados Unidos. Se recomienda el uso de una batería de bioensayos a fin de obtener información relevante de la exposición y los efectos de los contaminantes en los diferentes niveles ecológicos. Más información acerca de los bioensayos de toxicidad aguda y crónica frecuentemente utilizados para evaluación de la calidad ambiental están descritos en los capítulos 2 al 5.

1.2. Productos farmacéuticos y el medio ambiente

Durante las últimas décadas, el impacto de la contaminación química se ha centrado casi exclusivamente en los contaminantes convencionales "prioritarios", como los metales, hidrocarburos aromáticos policíclicos (PAHs), bifenil policlorados (PCBs) y pesticidas, y en especial aquellos que causan toxicidad aguda, son cancerígenos y persistentes en el medio ambiente (Daughton y Ternes, 1999). Los fármacos son considerados un grupo de compuestos emergentes, e incluyen diversas clases de contaminantes que varían en propiedades físico-químicas y farmacológicas, modos de acción, destino en el ambiente, degradación y cantidad consumida por la población. Por

estas razones, el estudio del comportamiento y posibles efectos de estos compuestos en el medio ambiente es complejo.

La presencia de los productos farmacéuticos en el medio ambiente ha sido ampliamente estudiada en los últimos años. Sin embargo, la mayoría de los estudios se centran en las medidas de la concentración de estos compuestos en el agua (Farré et al., 2001, Andreozzi et al., 2002, Ternes et al., 2002, Metcalfe et al., 2003, Hernando et al., 2006). Los productos farmacéuticos se encuentran en bajas concentraciones ambientales en aguas superficiales ($\mu g \cdot L^{-1}$- $ng \cdot L^{-1}$), pero son constantemente liberados en el medio ambiente por vertidos domésticos, lo que contribuye a su persistencia en el ambiente en general y en el marino en particular. Las concentraciones de fármacos en sedimento marino son de $ng \cdot g^{-1}$, a menudo mayor que las detectadas en las aguas superficiales (Ternes et al., 2002, Löffler y Ternes, 2003, Hernando et al., 2006, Da Silva et al., 2011, Pintado-Herrera et al., 2013), lo que indica que los sedimentos pueden actuar a largo plazo como sumidero y/ o fuente de dichos compuestos a los sistemas acuáticos (Gilroy et al., 2012).

Uno de los factores que determina la adsorción y acumulación de los productos farmacéuticos en el medio es la polaridad. Muchos fármacos son bases o ácidos débiles, que pueden ser ionizables, influenciados por el pKa (constante de acidez) o por el pH del medio. Esto está relacionado a la adsorción de los fármacos en los organismos y sedimentos. Por ejemplo, cuando más alto sea el pKa de un fármaco, más ácido se considera. Por lo tanto, un fármaco ácido expuesto a un ambiente como el marino que posee un pH 8.0 (básico), será más ionizado que la exposición de un fármaco básico. El caso contrario también es válido. Una vez ionizado, este compuesto puede dar lugar a una serie de sub-compuestos y/o ser adsorbidos a las partículas en suspensión en la columna de agua y depositados en los sedimentos. Solubilidad, materia orgánica, carbono orgánico

total (TOC) y % de partículas finas en el sedimento están entre los otros factores que diferencian los ambientes en relación a la polución y biodisponibilidad de los productos farmacéuticos, que pueden ocasionar posibles efectos adversos a la biota. La biodisponibilidad también varía entre organismos debido a su comportamiento, tipo de alimentación, composición corporal, hábitos de vida en el sedimento, entre otros factores.

Entre los productos farmacéuticos detectados en el medio ambiente, se han encontrado concentraciones de carbamazepina (CBZ), ibuprofeno (IBP), fluoxetina (FX), 17α-etinilestradiol (EE2), propranolol (PRO) y cafeína (CAF). Estos seis compuestos son altamente consumidos en todo el mundo y fueron clasificados en la lista de los 300 medicamentos más consumidos en 2005 en los Estados Unidos (http://www.rxlist.com/script/main/art.asp?articlekey=79510).

CBZ es un fármaco establecido para el control de la epilepsia, neuralgia del trigémino y el trastorno bipolar (Quinn et al., 2005). Se metaboliza predominantemente en CBZ 10, 11-epóxido y otros derivados en los seres humanos (Van Rooyen et al., 2002). Esta droga se ha detectado con frecuencia en las aguas superficiales, las aguas subterráneas y ocasionalmente en los sedimentos. Es eliminada parcialmente en las plantas de tratamiento de aguas residuales (> 10%) (Scheytt et al., 2005, Hernando et al., 2006). Sin embargo, la presencia de CBZ fue observada en los efluentes de las estaciones depuradoras de aguas residuales (EDAR; en inglés: wastewater treatment plant – WWTP) como resultado de su baja biodegradabilidad (Ternes, 1998, Andreozzi et al., 2002, Fang et al., 2012) que alcanzan concentraciones de 53.6 – 455 $ng \cdot L^{-1}$ à 1325 $ng \cdot L^{-1}$ en los efluentes de EDAR en España (Da Silva et al., 2011). Este compuesto es considerado muy toxico (EC_{50} < 1 $mg \cdot L^{-1}$) para bacterias, algas y para la mayoría de especies de invertebrados. Sin embargo, no presenta toxicidad para la mayoría de los peces expuestos (Hernando et al., 2006).

IBP es un medicamento de venta sin receta, aplicado a efectos antipiréticos, antiinflamatorios y analgésicos. Es fácilmente biodegradable en condiciones aeróbicas, siendo eliminado alrededor de 90% en las EDAR (Ternes, 1998, Scheytt et al., 2005, Hernando et al., 2006). Su liberación en el medio ambiente es en gran parte de una forma modificada, ya sea hidrolizada o conjugada, transformado en productos hidroxilo y carboxilo. No obstante, IBP se encuentra frecuentemente en los efluentes de EDAR de muchos países (Fang et al., 2011). IBP fue detectado en concentraciones más altas que otros productos farmacéuticos en los efluentes de EDAR en España, con la concentración que varía desde 75 – 1430 $ng \cdot L^{-1}$ (Da Silva et al., 2011) hasta 2134 $ng \cdot L^{-1}$ (Hernando et al., 2006). Este compuesto puede actuar a través de acciones inespecíficas por la narcosis no polar, y ser tóxico para microalgas y crustáceos de agua dulce *Daphnia* (Cleuvers, 2003).

FX es un fármaco inhibidor selectivo de la recaptación de serotonina (ISRS; en inglés: selective serotonin reuptake inhibitor – SSRI) prescrita para el tratamiento de la depresión, el trastorno bipolar y la bulimia. Se metaboliza a norfluoxetina, su metabolito activo (Brooks et al., 2003). Por lo tanto, FX se excreta principalmente con menos de 10% de compuesto inalterado en la orina (Hiemke y Härtter, 2000). Estados Unidos es uno de los principales consumidores de este compuesto. No obstante, FX fue previamente detectada en los efluentes de EDAR en los Estados Unidos a una concentración de 560 ± 250 $ng \cdot L^{-1}$ (Benotti y Brownawell, 2007). Guler y Ford (2010) observaran cambios de comportamiento (geotaxia y fototaxia) en anfípodos expuestos a bajas concentraciones de FX.

EE2 es un medicamento de esteroides y la hormona sintética más común usada en las píldoras anticonceptivas (Beausse et al., 2004). Los estrógenos se excretan en la orina con glucurónidos y sulfatos conjugados en los seres humanos

(http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5991). Este compuesto tiene relativamente baja polaridad y la adsorción en los sedimentos parece ser un proceso acumulativo (Beausse et al., 2004). La eficiencia de eliminación de las EDAR está por encima de 90% para este compuesto (Beausse et al., 2004). Sin embargo, las aguas residuales domésticas sin tratamiento adecuado mostraron altas concentraciones de EE2 (4390 $ng \cdot L^{-1}$) en el río Atibaia en Brasil (Montagner y Jardim, 2011). La eliminación farmacéutica depende de las características del tratamiento y la eficiencia de la EDAR. Schlenk (2008) publicó una revisión sobre feminización de peces debido a la exposición a esteroides como el EE2.

PRO es un agente bloqueante no selectivo del receptor β-adrenérgico (en inglés: non-selective β-blocker) ampliamente prescrito para el tratamiento de la angina de pecho, la hipertensión, la migraña y la ansiedad (Stanley et al., 2006). Se metaboliza en el hígado y se excreta en la orina: 20% como ácido naftoxilático, hasta un 25% como glucurónido propranolol y sólo un 0,5% de fármaco inalterado (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi? cid = 4.946 y loc = ec_rcs # x94). PRO posee la polaridad relativamente alta. No obstante, se ha detectado en los efluentes de EDAR la concentración de 260 $ng \cdot L^{-1}$ en los Estados Unidos (Kostich et al., 2014). PRO puede afectar negativamente la biota acuática, con alteraciones en la fisiología (Oskarsson et al., 2012) y estrés oxidativo (Franzellitti et al., 2011).

CAF puede ser el compuesto neuroactivo más ampliamente consumido en el mundo, ya que es encontrado en concentraciones significativas en los efluentes de las EDAR. CAF es considerado un buen marcador de la contaminación fecal humana (Potera, 2012). Es fácilmente biodegradable, sin embargo, altas concentraciones de CAF (15200 ± 4.400 $ng \cdot L^{-1}$) se observaron en los efluentes de las EDAR en los Estados Unidos (Kostich et al., 2014). CAF posé un potencial teratogénico que puede resultar en consecuencias de

mal desarrollo, como malformaciones en humanos y otros animales (Nehly and Debry, 1994).

Requisitos regulatorios actuales para la evaluación del riesgo ambiental (ERA) de sustancias químicas "emergentes", y su inclusión dentro del marco regulador han sido discutidos en los Estados Unidos, Canadá, Japón, Australia, Suiza y la Unión Europea (Straub y Hutchinson, 2012). Sin embargo, la Unión Europea (Directiva 93/67/CEE - Comisión de las Comunidades Europeas, 1996) clasifica los productos químicos según su valor de EC_{50} ($mg \cdot L^{-1}$). Esta directiva no es aplicable a compuestos emergentes, aunque se encuentran en concentraciones de $\mu g \cdot L^{-1}$- $ng \cdot L^{-1}$ en el medio ambiente. Conceptos regulatorios para ERA se basan generalmente en un conjunto de estudios ecotoxicológicos a corto plazo en tres o cuatro especies diferentes, comportamientos ambientales y la aplicación de los factores de evaluación de calidad para corregir las incertidumbres inherentes (Hernando et al., 2006). La "Guía sobre la evaluación del riesgo medioambiental de medicamentos de uso humano", publicado por la Agencia Europea de Medicamentos (en inglés: European Medicine Agency – EMA) (EMA/CHMP/SWP/4447/00, EMA/CHMP/SWP/44609/2010) relata la importancia de determinar los efectos letales y subletales en la evaluación del riesgo de los productos farmacéuticos de consumo humano. La guía describe un procedimiento escalonado compuesto por dos fases. La Fase I consiste en ensayos incluyendo persistencia, bioacumulación y toxicidad. La Fase II es una evaluación completa de los riesgos sobre la base de datos de destino ambiental y de efectos. Esta guía es recomendada para ambientes de agua dulce. Las estrategias de evaluación de los riesgos ambientales de los sedimentos marinos afectados por la contaminación de los productos farmacéuticos deben ser aún aclaradas, ya que los fármacos (incluso en bajas concentraciones) pueden causar efectos adversos a la biota. Sin embargo, los procedimientos para la realización de ERA de los

productos farmacéuticos en el sedimento marino deben ser mejor desarrollados y detallados, ya que los existentes y aplicados en distintas guías se basan en la evaluación de sustancias químicas convencionales, como metales (Ferrari et al., 2004). La inclusión de ensayos de toxicidad aguda y crónica que respondan más específicamente à fármacos deben ser tenidos en cuenta.

Comparativamente a los datos químicos, poco se ha publicado respecto a la ecotoxicología acuática de los productos farmacéuticos, especialmente datos relevantes a la exposición, como los posibles efectos adversos y la bioacumulación de fármacos en los organismos acuáticos. Los ensayos iniciales conocidos como "screening", para determinar si es necesaria una evaluación de riesgo más detallada, deben incluir concentraciones ambientales. La incorporación de ensayos no estándar en el proceso debe ser explorado. Dado que, los productos farmacéuticos se encuentran en bajas concentraciones en el medio ambiente y no se conoce bien su mecanismo de acción en la biota, el presente trabajo podría considerarse un primer paso en la evaluación de la idoneidad de las pruebas de toxicidad aguda y respuestas subletales en la evaluación de la calidad ambiental de sedimentos marinos afectados por los compuestos emergentes.

1.3. Hipótesis y objetivos de la Tesis

El objetivo general de esta tesis es evaluar la calidad ambiental de sedimentos marinos afectados por vertidos de aguas residuales analizando la validez de diferentes especies bioindicadoras y ensayos de toxicidad en laboratorio y campo. Para ello, la tesis fue dividida en dos partes: la evaluación de la calidad ambiental de sedimentos dopados con fármacos y de sedimentos directamente afectados por vertidos de estaciones de depuración (EDAR) o de bombeo (EBAR) de aguas residuales situadas en la Bahía de Cádiz (SW, España). Se han llevado a cabo diferentes tipos de ensayos de toxicidad bajo

condiciones de laboratorio e *in situ*. El objetivo general se desarrolla en los siguientes objetivos concretos:

1. Evaluar la toxicidad aguda de sedimentos marinos dopados con productos farmacéuticos, mediante la simulación en el laboratorio y el estudio de la viabilidad de las respuestas y las especies bioindicadoras utilizadas.
2. Determinar la toxicidad crónica (detoxificación, estrés oxidativo, neurotoxicidad, genotoxicidad) de productos farmacéuticos presentes en sedimentos marinos mediante ensayos de dopaje en el laboratorio. Para ello se analiza la viabilidad de las respuestas y las especies bioindicadoras utilizadas.
3. Examinar la distribución de contaminantes en sedimentos marinos de la Bahía de Cádiz, afectados por estaciones de depuración (EDAR) y bombeo (EBAR) de aguas residuales.
4. Monitorizar la calidad ambiental de sedimentos marinos afectados por vertidos de aguas residuales en la Bahía de Cádiz mediante estudios de toxicidad aguada y crónica en laboratorio e *in situ*.
5. Evaluar el efecto de la estacionalidad en la distribución de contaminantes en sedimentos marinos y su relación con la calidad ambiental de éstos.

1.4. Estructura de la tesis

Esta Tesis está estructurada en 6 capítulos. El primer capítulo incluye una breve introducción sobre la importancia de los sedimentos marinos en la evaluación de la calidad ambiental, sobre los productos farmacéuticos y el medio ambiente. En este capítulo se describe la hipótesis y los objetivos generales de la Tesis. El segundo capítulo comprende una breve introducción sobre la evaluación de la calidad ambiental de productos farmacéuticos en sedimento marino: ensayos agudos. En este capítulo se

incluye el artículo I sobre la evaluación de la toxicidad aguda en sedimento dopado con productos farmacéuticos. En este segundo capítulo se estima la idoneidad del uso de ensayos de toxicidad agudos para la evaluación de la calidad ambiental de sedimentos marinos afectados por la presencia de productos farmacéuticos. El tercer capítulo recopila la evaluación del riesgo ambiental de productos farmacéuticos en sedimento marino mediante la simulación en laboratorio. Se estimaron respuestas subletales a la exposición a este tipo de compuestos. Dicho capítulo incluye 3 artículos (II, III y IV). El artículo II resume la evaluación de efectos subletales determinados en anfípodos *Ampelisca brevicornis* expuestos a sedimento dopado con distintos productos farmacéuticos. Los artículos III y IV de este capítulo recopilan la descripción sobre las respuestas bioquímicas en poliquetos *Hediste diversicolor* expuestos a sedimento dopado con productos farmacéuticos. Así se analizaron no sólo la idoneidad del uso de estas especies bioindicadoras, sino también respuestas a nivel de metabolismos de detoxificación, estrés oxidativo, neurotoxicidad y genotoxicidad. Seguidamente, en el cuarto capítulo es describe la monitorización y estacionalidad de la calidad ambiental de sedimentos afectados por vertidos de efluentes de plantas de tratamiento de agua residual en la Bahía de Cádiz. Se utilizó la metodología validada en los capítulos anteriores y se evaluó la estacionalidad de las respuestas. Este capítulo se compone de 3 artículos (V, VI, VII y VIII). El artículo V es sobre ensayos agudos en laboratorio para evaluar la calidad de sedimento afectado por vertidos de aguas residuales en la Bahía de Cádiz en las dos estaciones del año, invierno y verano. El artículo VI describe las respuestas bioquímicas y celulares (NRRT) determinadas en almejas expuestas en laboratorio a muestras de sedimento afectados por vertidos de la Bahía de Cádiz. Las respuestas de los biomarcadores relacionados a gametogénesis y gastos de energía están redactadas en el artículo VII. El quinto capítulo consiste en la evaluación de la calidad de sedimentos

afectados por vertidos de la Bahía de Cádiz: respuestas sub-letales de toxicidad *in situ*. El artículo VIII explica las respuestas de los biomarcadores específicos y no específicos para fármacos, y respuestas celulares (NRRT) determinados en almejas *Ruditapes philippinarum* expuestas por 14 días en jaulas colocadas en campo durante el invierno y verano de 2011. El sexto capítulo finaliza con las conclusiones obtenidas en relación a los objetivos propuestos de esta tesis.

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CAPÍTULO 2

Evaluación de la calidad ambiental de sedimentos marinos afectados por productos farmacéuticos: ensayos de toxicidad aguda mediante simulación de concentraciones en laboratorio.

Los residuos de productos farmacéuticos y de higiene personal (en inglés: pharmaceutical and personal care products - PPPC) son considerados un problema ambiental emergente. Esta cuestión es motivo de creciente preocupación científica (Kummerer et al., 2001, Heberer, 2002), representada por el incremento en el número de artículos científicos sobre las concentraciones de los productos farmacéuticos en muestras ambientales. Estudios sobre posibles efectos adversos en la biota aumentaron en los últimos años, centrados principalmente en la toxicidad aguda de los productos farmacéuticos (Figura 1a). Los principales estudios son sobre crecimiento, reproducción y mortalidad (Figura 1b). Sin embargo, pocos grupos de investigación han desarrollado estudios destinados a comprender más profundamente la biodisponibilidad de estos compuestos y posibles efectos adversos sobre las comunidades bentónicas.

Las legislaciones europea y norte-americana incluyen guías para evaluar el riesgo ambiental provocado por la exposición a sustancias farmacéuticas. En estas directivas se aconseja la evolución del riesgo como parte del proceso de aprobación de nuevas sustancias. Todavía, es de especial importancia la determinación de la emisión de compuestos farmacéuticos al medio ambiente y la evaluación ecotoxicológica de sus posibles efectos adversos, principalmente relacionados con ambientes marinos y sedimento.

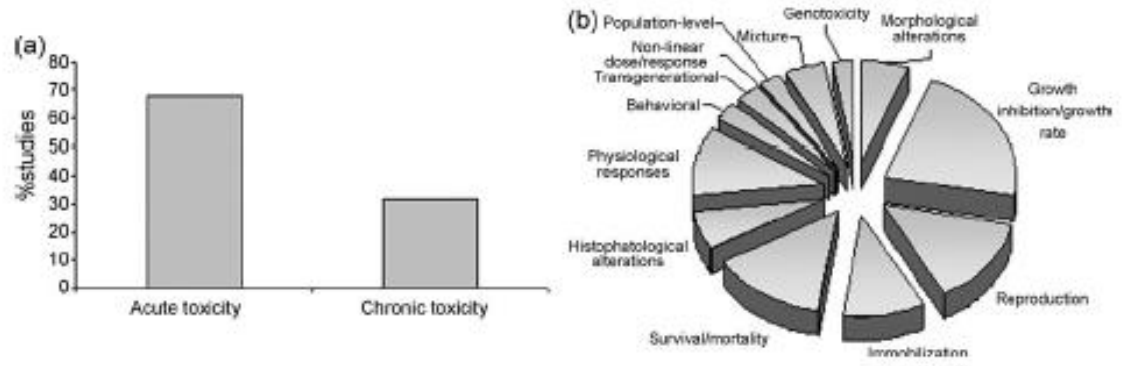

Figura 1. (a) Estudios ecotoxicológicos agudos y crónicos. (b) Los principales resultados utilizados en estudios ecotoxicológicos, expresados en porcentaje relativo (datos recogidos de 94 artículos publicados entre 1996 y 2009). Fuente: Santos et al., 2010.

En este capítulo se incluye el artículo I, en el cual se describen bioensayos de toxicidad aguda para determinar los posibles efectos adversos a la biota acuática ocasionados por fármacos asociados al sedimento marino. Los fármacos estudiados representan una amplia gama de medicamentos previamente detectados en el medio ambiente, entre ellos fármacos pertenecientes a clases terapéuticas predominantes y vendidas en todo el mundo, como carbamazepina (CBZ), ibuprofeno (IBP), fluoxetina (FX), 17α-etinilestradiol (EE2), propranolol (PRO) y cafeína (CAF). A pesar de la baja porcentaje de estudios sobre efectos adversos relacionados a productos farmacéuticos, los fármacos escogidos en el presente estudio están entre los más estudiados en los últimos años (Figura 2). CAF no está presente en la Figura 2, pero también fue incluido por ser considerado un marcador de vertidos domésticos, ser altamente consumido en todo el mundo, poseer propiedades estimuladoras y teratogénicas, y ser detectado en altas concentraciones en el medio ambiente (Potera, 2012).

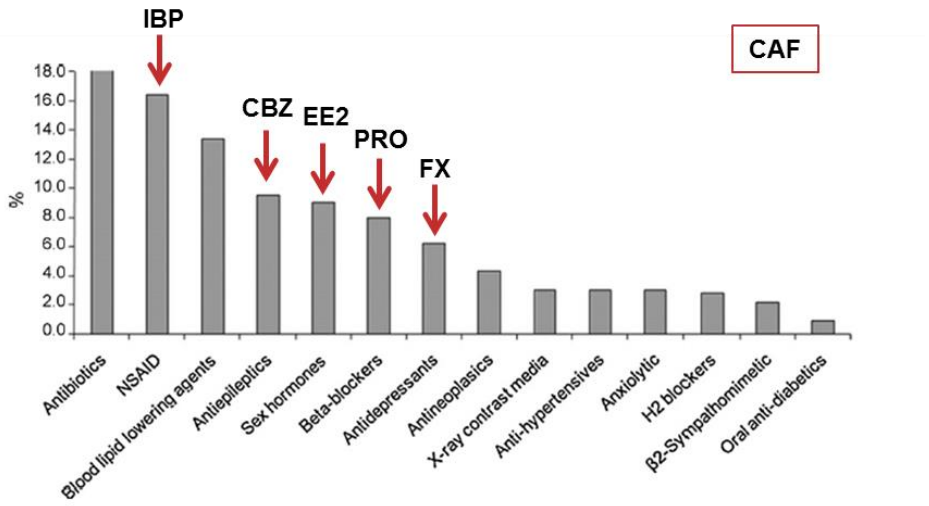

Figura 2. Porcentaje de estudios publicados sobre distintas clases terapéuticas, expresada en porcentaje relativo, descrito en 183 artículos publicados entre 1996 y 2009. Fuente: Santos et al., 2010.

Para los bioensayos, sedimento marino fue dopado con distintos fármacos. El sedimento referencia fue muestreado en el Río San Pedro (Cádiz, España) (Figura 3). El lugar de muestreo fue citado en estudios previos como un lugar de referencia y con buena calidad ambiental (Pérez et al., 2004, Solé et al., 2009, Basallote et al., 2012, De Orte et al., 2013). Una vez en el laboratorio, el sedimento fue tamizado, y secado a 70^{o}C (Atkinson y Arey, 2003), ya que la OECD (2000) recomienda sedimento seco para el proceso de dopaje. El mismo volumen de agua perdido en este proceso fue añadido al sedimento antes de doparlo. Se obtuvo agua de mar de la " Planta Experimental de Cultivos Marinos" de la Universidad de Cádiz. Esta agua de mar se viene utilizando para el cultivo y mantenimiento de organismos (peces, moluscos y plancton) desde 2002.

Las soluciones con fármacos fueran diluidas en DMSO (0.001% v/v) y agua de mar. La concentración de DMSO utilizada fue previamente considerada como no tóxica para la biota (Quinn et al., 2008 a, b, Eades y Waring, 2010, Aguirre-Martínez et al., 2013 a, b). Sedimentos dopados con fármacos fueran preparados en frascos de cristal de 3 litros, se mezclaron mediante homogeneización usando una espátula de teflón, y

herméticamente cerrados para la mezcla de sedimento y fármaco con la ayuda de rotadores (inglés: bottle roller), a 60 rpm durante 30 minutos. Pasados los 30 minutos, los botes fueran llevados a la camera a 4^{o}C y en oscuridad por 7 días, para asegurar el equilibrio entre el compuesto, agua y sedimento (Francis et al., 1984, Löffler et al., 2005). La metodología aplicada para el procedimiento de dopaje fue una adaptación de los protocolos de la ASTM (2000), USEPA (2001) y OECD (2004) (Figura 3).

Figura 3. Procedimiento de dopaje de sedimento marino con productos farmacéuticos.

Pasados los 7 días, se dio inicio a los ensayos de toxicidad aguda con sedimento bruto. Un porcentaje de sedimento bruto fue separado para la preparación de los lixiados. Esta parte liquida del sedimento simula la resuspensión y la re-disponibilidad de los contaminantes a la columna de agua. Lixiviados se obtuvieron mediante la mezcla de sedimento: agua (proporción 1: 4) puestos en frascos de cristal herméticamente cerrados, y mezclados con la ayuda de los rotadores a 60 rpm durante 30 min. Después de la decantación, se utilizó el agua sobrenadante para los bioensayos. La metodología aplicada fue una adaptación del protocolo de la USEPA (1998 a, b) (Figure 4).

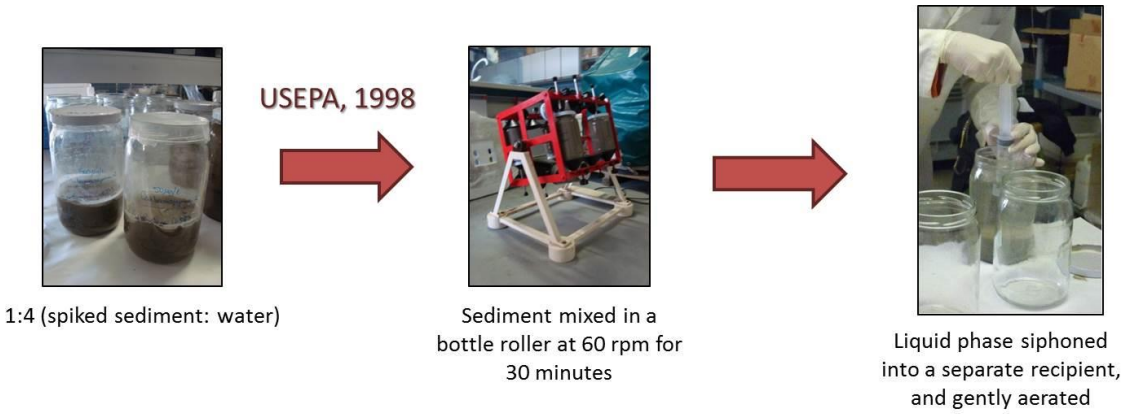

Figura 4. Preparación de las muestras de lixiviado a partir de muestras de sedimento bruto dopados con fármacos.

Una vez en el sedimento, el xenobiótico puede causar efectos agudos como mortalidad, alteraciones en la reproducción, comportamiento, entre otros. Por lo tanto, ensayos agudos hacen posible la priorización del uso de los recursos, ya que permiten distinguir los sedimentos potencialmente tóxicos de los no-tóxicos, y valorar cuales serán el objeto de un estudio más profundo en las fases siguientes de la investigación. Generalmente, son ensayos sencillos, sensibles, de bajo coste, fáciles de reproducir y que ofrecen respuestas de fácil interpretación en un corto período de tiempo.

En este capítulo se incluye el artículo I. En este artículo se describen bioensayos de toxicidad aguda de sedimento bruto llevado a cabo con las especies de bacteria *Vibrio fischeri* (Figura 5) y crustáceos anfípodos *Ampelisca brevicornis* (Figura 6). También se llevaran a cabo bioensayos de toxicidad aguda de lixiviado con las especies de bacteria *Vibrio fischeri* (Figura 5), erizos de mar *Paracentrotus lividus* (Figura 7), y microalgas *Isochrysis galbana* y *Tetraselmis chuii* (Figura 8). Cada una de las especies utilizadas en los ensayos agudos de esta Tesis se relaciona con el ambiente de distintas maneras ya que poseen hábitos de vida y de alimentación distintos. *V. fischeri* es una bacteria heterótrofa pelágica, por lo tanto, puede verse afectada en mayor medida por el estado del medio acuoso. *P. lividus* es un erizo de mar, organismo bentónico que habita en fondos duros.

La fase de reproducción puede verse afectada por la contaminación del agua, incluyendo contaminación por la resuspensión de sedimento. *A. brevicornis* es un organismo bentónico que vive enterrado en sedimentos de muy diversa textura, pero también son nadadores. Se alimentan de materia orgánica en descomposición. Siendo así, los anfípodos pueden verse afectados por la contaminación del agua y sedimento. Las microalgas bentónicas *I. galbana* y *T. chuii* son muy abundantes en el ecosistema marino, y sirven de alimento para diversos organismos que viven tanto en la columna de agua cuanto en el sedimento.

Diversas guías de evaluación de calidad de sedimentos incluyen el uso de diferentes testes de toxicidad, incluyendo los aplicados en el presente estudio (CEDEX, 1994, USACE, 1998, USEPA, 1998 a, b, GIPME, 2000, SEDNET, 2003). Siete bioensayos sobre condiciones controladas de laboratorio, se utilizaron para determinar la toxicidad de seis fármacos dopados en sedimento marino:

I - Inhibición de la bioluminiscencia de la bacteria *Vibrio fischeri* a través de Microtox®, utilizando diferentes técnicas para evaluar la toxicidad de lixiviado (Basic Test) y sedimento bruto (Solid Phase Test).

Figura 5. Aparato de Microtox® modelo 500. Reactivos para Microtox® Solid Phase Test (SPT)

II - La tasa de mortalidad de anfípodos *Ampelisca brevicornis* expuesto a muestras de sedimento bruto durante 10 días.

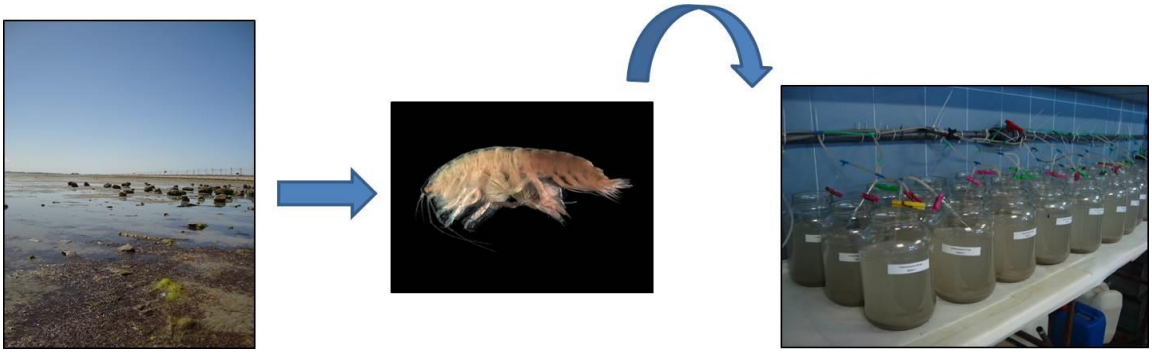

Figura 6. Anfípodos fueran colectados en una zona considerada limpia en Cádiz. Una vez en el laboratorio, los organismos fueran aclimatados y en seguida expuesto a los sedimentos dopados con fármacos.

III - Índice de huevos no-fertilizados y desarrollo embrionario anormal de larvas de erizos de mar *Paracentrotus lividus* expuestos durante 48 horas a lixiviados.

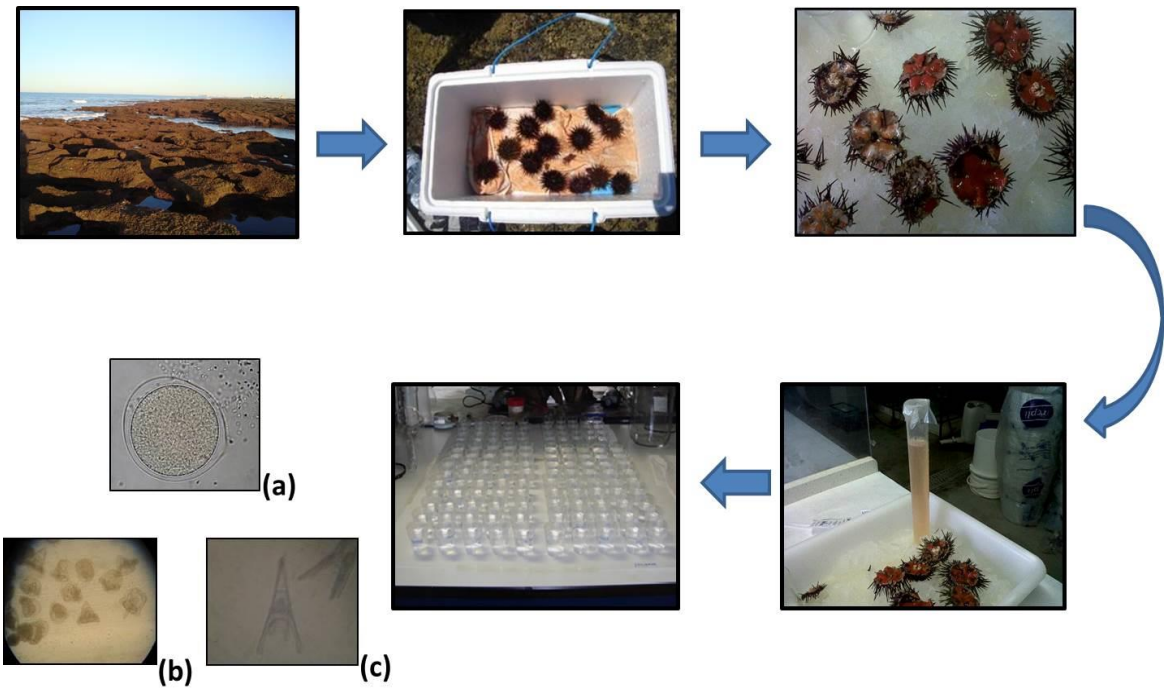

Figura 7. Erizos fueran colectados en una zona considerada limpia en Cádiz (Torre Gorda, San Fernando). Una vez en laboratorio, fueran extraídos esperma y óvulos, y se dio inicio al bioensayo de fertilización, en que el objetivo era determinar el porcentaje de huevos fecundados (a). En seguida, se hizo la fecundación *in vitro* para el bioensayo de desarrollo embriolarval. Después de 48 horas, las larvas fueran identificadas como anormales (b) y normales *pluteus* (c).

IV. Tasa de crecimiento de dos especies de microalgas (*Isochrysis galbana* y *Tetraselmis chuii*) expuestas durante 96 horas a muestras de lixiviados provenientes de sedimento dopado con fármacos.

Figura 8. Bioensayo de crecimiento de microalgas, medidos por absorbancia. La microalga color naranja es la *Isochrysis galbana*, y la de color verde es la *Tetraselmis chuii*.

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Water, Air and Soil Pollution

Under review

Suitability of standardized acute toxicity tests for marine sediment assessment: pharmaceutical contamination.

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ABSTRACT

Pharmaceutical products are found in low concentrations constantly released into the environment, which contribute to their persistence. In this study, a battery of recognized short-term bioassays was applied to evaluate the risk assessment of sediment samples spiked with pharmaceutical products: carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol, propranolol and caffeine, including the environmental concentrations. Two phases of the sediment (elutriate and whole sediment) were evaluated through the determination of seven endpoints: bioluminescence inhibition of the bacteria *Vibrio fischeri*, mortality rate of the amphipods *Ampelisca brevicornis*, spermiotoxicity and embryotoxicity of the sea-urchin *Paracentrotus lividus* and microalgae growth rate of *Isochrysis galbana* and *Tetraselmis chuii*. Lethal and sublethal responses were analysed after short-term exposure. Results refuted the fact that Microtox® Basic Test, amphipods and microalgae bioassays concerning acute toxicity were sufficient for the quality assessment of pharmaceutical-spiked sediments and the protection of the aquatic environment. Between the bioassays used in the present study, sea-urchins tests were the most sensitive endpoints, which embryotoxicity was more sensitive to evaluate the contamination by pharmaceutical products than the spermiotoxicity. Sea-urchin bioassays are recommendable for the sediment quality assessment affected by pharmaceutical products contamination.

Keywords: marine sediment, benthic organisms, short-term toxicity, pharmaceuticals, environmental concentrations.

1. INTRODUCTION

During the last decades, the impact of chemical pollution has focused on the conventional "priority" pollutants, and especially those acutely toxic/ carcinogenic displaying persistence in the environment (Daughton and Ternes 1999). Contaminants can accumulate in the sediment, where they stay in contact with benthic organisms (DelValls et al. 2002). Chemical, physical and biological factors can result in the release of contaminants retained in the sediment, re-making them available to the water column, thus producing potential risks to the aquatic biota. Environmental risk of sediment resuspended contaminants can be determined through the short-term tests applied to elutriate samples.

Pharmaceutical and personal care products (PPCPs) are organic compounds considered as emergent contaminants recently identified as potentially toxic for aquatic organisms (Quinn et al. 2008 a, b; Martín-Díaz et al. 2009 a, b; Aguirre-Martínez et al. 2013 a, b). Such compounds are continually infused into the environment via wastewater treatment plants (WWTPs) and wet weather runoff which make them "persistent" compounds. Carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethynylestradiol (EE2), propranolol (PRO) and caffeine (CAF) represent different drug classes among the most frequently detected pharmaceutical compounds in the environment (Daughton and Ternes 1999; Heberer 2002; Beausse 2004; Díaz-Cruz and Barceló 2004; Hernando et al. 2006).

Since the pharmaceutical products have a specific mode of action (MOA) in target organisms, there are concerns about the potential adverse effects on non-target species, including those caused by low concentrations observed in the environment (Fent et al. 2006; Kim et al. 2007) (Figure 1). The number of studies about possible adverse effects due to the exposure of invertebrates to pharmaceutical compounds have been increased

(De Lange et al. 2006; Quinn et al. 2008 a, b; Damásio et al. 2011; Oskarsson et al. 2012; Franzellitti et al. 2013, 2014; Aguirre-Martínez et al. 2013 a, b). Although, little attention is given to the organisms exposure to pharmaceuticals spiked in sediment samples (Gómez-Oliván et al. 2012; Gilroy et al. 2012; Mendéz et al. 2013).

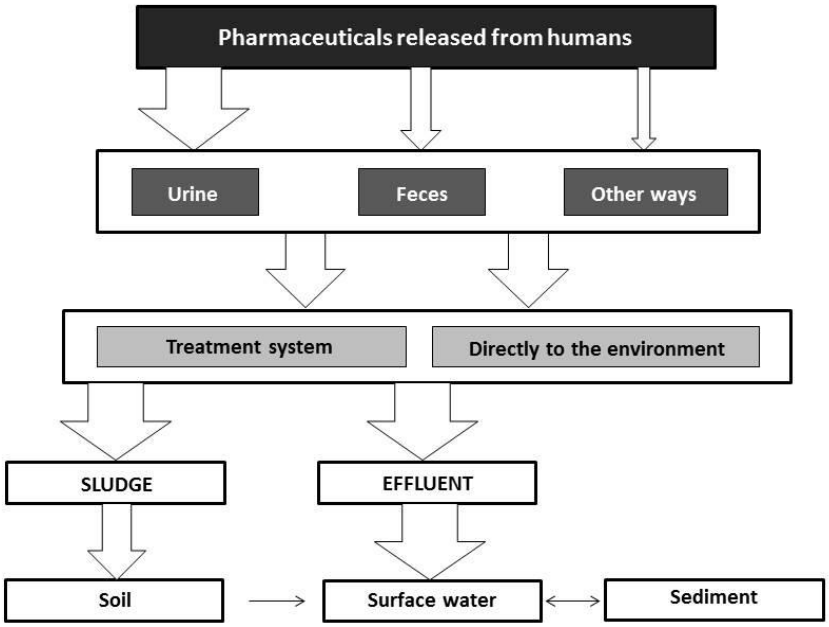


Figure 1. Pathway of pharmaceutical products into the environment (adapted from Halling-Sørensen et al., 1998).

The present work could be considered a first step to evaluate the suitability of short-term toxicity tests for the environmental quality assessment of marine sediments affected by pharmaceutical compounds. Standardized toxicity tests are recommended to screen the potential hazards of aquatic contaminants and to develop environmental quality criteria (Brooks et al. 2003). Guidelines of sediment quality assessment include the use of different toxicity tests as amphipods mortality, Microtox®, sea-urchin embryolarval development and microalgae rate (CEDEX 1994; USACE 1998; USEPA 1998; GIPME 2000; SEDNET 2003). Therefore, seven standardized well-documented sediment toxicity tests were performed to assess potential effects of pharmaceutical products on marine

biota. Six pharmaceutical products were spiked in the marine sediment samples: CBZ, IBP, FX, EE2, PRO and CAF. Lethal and sublethal endpoints were determined in marine aquatic organisms belonging to different trophic levels. Two sediment phases were analyzed: solid (whole sediment) and liquid (elutriate) phases. The cause-effect relationship among pharmaceutical products and lethal and sublethal endpoints was discussed.

2. MATERIAL AND METHODS

2.1. Chemicals

Briefly, an overview of physical-chemical characteristics, structures, production, metabolism and removal via WWTPs of CBZ, IBP, FX, EE2, PRO and CAF was shown in the Maranho et al. (2014). All reagents used in the present study were purchased from Sigma-Aldrich® (Madrid, Spain).

The reference sediment used in the spiking procedure was collected at Río San Pedro (SW, Spain) (36°31′53″ N; 6°12′48″ W), an intertidal creek area part of the Natural Park "Bahía de Cádiz". This place was considered reference area by previous studies (Pérez et al. 2004; Solé et al. 2009; Basallote et al. 2012; De Orte et al. 2013). The topmost 10 cm layer of the sediments were sampled and sieved through 2 mm mesh in order to remove any associated macrofauna and larger sediment granules. Sediment samples were dried at 70^{o}C in a way to reduce possible interferences (Atkinson and Arey 2003). The same volume of water lost in the dry sediment procedure was added to the sediment sample before the spiking procedure. Overlying seawater was obtained from "Experimental Marine Aquaculture Plant – Cultivos Marinos" at the University of Cádiz.

Physical-chemical characterization of the reference sediment was performed. Grain size (USGS 2000), organic matter (OM) (USEPA 2002a) and total organic carbon (TOC) (USEPA 2002a) of reference sediment were summarized in the Maranho et al. (2014).

2.2. Sediment spiking procedures

Reference sediment samples were spiked with increasing amounts of the test compounds in order to obtain theoretical concentrations of 100x, 10x, 1x, 0.1x and 0.01x. Theoretical concentration of 1x corresponded to the average of environmental concentrations determined in sediment (Ternes et al. 2002; Hernando et al. 2006; Schultz et al. 2010; Pintado-Herrera et al. 2013; Zhou and Broodbank 2014). Concentrations of six pharmaceutical products (environmental concentrations were underlined) were: CBZ, IBP and PRO (500 $ng \cdot g^{-1}$, 50 $ng \cdot g^{-1}$, 5 $ng \cdot g^{-1}$, 0.5 $ng \cdot g^{-1}$, 0.05 $ng \cdot g^{-1}$), FX and EE2 (100 $ng \cdot g^{-1}$, 10 $ng \cdot g^{-1}$, 1 $ng \cdot g^{-1}$, 0.1 $ng \cdot g^{-1}$, 0.01 $ng \cdot g^{-1}$) and CAF (1500 $ng \cdot g^{-1}$, 150 $ng \cdot g^{-1}$, 15 $ng \cdot g^{-1}$, 1.5 $ng \cdot g^{-1}$, 0.15 $ng \cdot g^{-1}$). Pharmaceutical stock solutions were prepared with the solvent DMSO 0.001% (v/v) (Aguirre-Martínez et al. 2013 a, b; Eades and Waring 2010; Quinn et al. 2008 a, b). Two controls were run in parallel with the bioassays: reference sediment (control) and reference sediment spiked with DMSO 0.001% (v/v) (solvent control).

The methodology applied for the spiking procedure was an adaptation of ASTM (2000), USEPA (2001) and OECD (2004) protocols. Pharmaceutical-spiked sediments were initially prepared in 3-L glass beakers, mixed by homogenization using a Teflon spatula, and subsequently placed at 60 rpm for 30 min in airtight glass flasks on a rotator mixer. Pharmaceutical-spiked sediments were incubated for 7 days ($4^{o}C$ in the dark) (Francis et al. 1984; Löffler et al. 2005). After the equilibration period, sediment samples

spiked with the highest pharmaceutical concentrations were diluted with the reference sediment to obtain the concentrations proposed for the bioassays.

Sediment elutriates were obtained by rotator mixer of sediment: water (proportion 1:4) at 60 rpm for 30 min in airtight glass flasks (USEPA 1998). After the decantation, the overlying water was used for the bioassays. The highest concentrations of the six selected pharmaceuticals were measured in the spiked sediments (Jelic et al. 2009). Measured concentrations of target compounds in spiked sediments and the MRM transitions are shown in Maranho et al. (2014). No degradation products were assumed, since the half-lives of CBZ (Lam et al. 2004; Loofler et al. 2005), IBP (Beausse et al. 2004), FX (Lam et al. 2004), EE2 (Beausse 2004; Mes et al. 2005), PRO (Andreozzi et al. 2002) and CAF (Bradley et al. 2007) were higher than 10-days, that was the maximum exposure period applied in the present study.

2.3. Toxicity tests

The bioassays applied are a benchmark for contaminated marine sediments and dredged material characterization, using different species depending of the localization. Whole sediment toxicity was assessed by the bioluminescence inhibition of the bacteria *Vibrio fischeri* (Microtox® Solid Phase Test - SPT) and mortality rate of amphipods *Ampelisca brevicornis*. Short-term toxicity of elutriate samples were evaluated by the bioluminescence inhibition of the bacteria *V. fischeri* (Microtox® Basic Test), fecundation success (spermiotoxicity) and abnormal larval development (embryotoxicity) assays with the sea-urchin *Paracentrotus lividus*, and growth rate of the marine microalgae's *Isochrysis galbana* and *Tetraselmis chuii*. Physical chemical parameters (oxygen saturation, pH, photoperiod and salinity) were monitored during the experiments.

2.3.1. Amphipods mortality

Individuals of *A. brevicornis* were obtained from a reference area at the Bay of Cádiz (SW, Spain) (36°29′16″ N; 6°15′52″ W) (Casado-Martínez et al. 2007; Ramos-Gómez et al. 2009). The sediment toxicity test was performed in 3-L glass beakers containing the proportion 1:4 of sediment and water. Thirty amphipods per beaker were exposed for 10 days in triplicate (ASTM 1993). After the exposure period, the percentage of mortality was checked.

2.3.2. Microtox® bioluminescence inhibition

Bioluminescence inhibition of *V. fischeri* assays were performed with whole sediment (Microtox® Solid Phase Test - SPT) and elutriate (Microtox® Basic Test) samples. Absorbance values were recorded at 5, 15 and 30 min. Regression statistics of concentration (log C) on the gamma parameter were used to estimate the correlation of toxic effect (IC_{50}).

2.3.3. Sea-urchin spermiotoxicity and embryotoxicity

Adults of *Paracentrotus lividus* were collected from a reference area at Cádiz (SW, Spain) (36°27′6″ N; 6°15′1″ W). This species was used as bioindicator by previous studies (Casado-Martínez et al. 2007; Beiras et al. 2012; Carballeira et al. 2012).

Spermiotoxicity assay was based on USEPA protocol (2002 b). Gametes were obtained by direct extraction. Briefly, a standard sperm solution was added to the vials containing elutriate samples of each pharmaceutical product concentration. Five replicates were used per sample. The incubation period was 60 min at room temperature (20 ± 2^{o}C), and then eggs were added to the vials. After 30 min (time for the egg fertilization), samples were fixed with formaldehyde. The percentage of unfertilized eggs was

determined. Embryotoxicity assay was conducted following the methodology described by Fernández and Beiras (2001) and USEPA (2002 b). Once the fecundation was successfully completed, embryos (density of 20–30 embryos per mL) were introduced in the vials containing elutriates samples. Five replicates were used per sample. After 48h of exposure at 20°C in the dark, samples were fixed with formaldehyde. Results were expressed as the percentage of abnormal larvae development occurrence.

2.3.4. Microalgae growth rate

Inoculums of *I. galbana* and *T. chuii* were obtained from "Experimental Marine Aquaculture Plant – Cultivos Marinos" at the University of Cádiz. Microalgae´s were cultivated under aseptic conditions in a nutritive medium composed by synthetic seawater (USEPA 2002 a) and received a supply of nutrients and vitamins according to the f/2 nutritive medium (Guillard and Ryther 1962). Experimental protocol followed the method 1003.0 (absorbance) for measuring the toxicity of effluents and receiving waters (USEPA 2002 a).

Microalgae´s bioassay´s were performed in sterilized vials of borosilicate glass under continuous illumination (cold white light of 11000 lux) and controlled temperature ($20 \pm 2^{o}C$). Each test vial received elutriates samples and concentrated algae inoculums. Thirty minutes after the inoculation of the microalgae to the replicates and every 24h, biomass concentrations of the treatments were measured in terms of optical density (USEPA 2002 a) at wavelength of 690 nm (Nannocolor® PT-3 Macherey-Nagel). Microalgae´s were exposed during 96h. Areas under the growth curves were calculated and the growth or inhibition percentage was obtained. Response was calculated through the equation [(Ns - Nc)/ Nc] x100, where Ns, is the cell number in the sample and Nc is

the cell number in the control (Mucha et al. 2003). Quadratic curve was fitted in a way to calculate the EC_{50}.

2.4. Data analysis

Significant differences between short-term responses and pharmaceutical-spiked sediments were determined by one-way ANOVA. Post-hoc examination was carried out using Dunnett´s test. Significance level was set up at $p < 0.05$. Data was log transformed prior to analysis to improve normality and variance homoscedasticity. Significant correlations between pharmaceutical concentrations in sediments and short-term responses were examined by Spearman´s correlation analysis. Significance level was set up at $p < 0.01$ and $p < 0.05$. Results were analysed using SPSS/PC + statistical package. Minimum of quadratic functions was applied to adjust the curves for *I. galbana* and *T. chuii* utilizing the R-program **(**R version 3.1.1**)**.

3. RESULTS

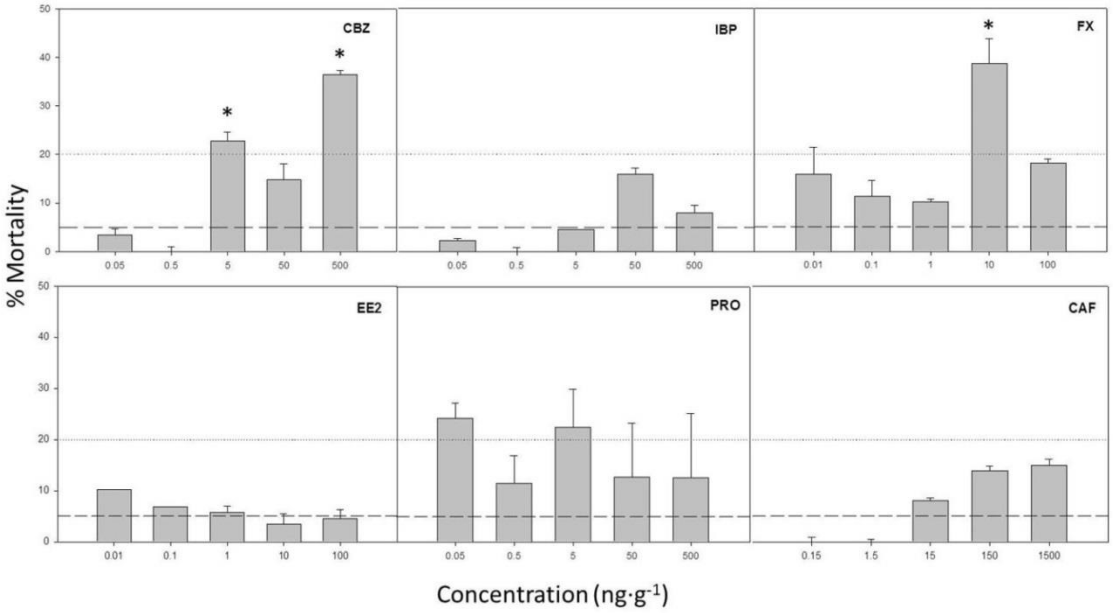
All bioassays were performed in acclimatized room (20 ± 2^{o}C). The averages of pH and salinity were determined for the amphipods (pH: 7.60 ± 0.33; salinity: 36.94 ± 1.04) and for elutriate (pH: 7.85 ± 0.72; salinity: 37.95 ± 0.83) bioassays. Oxygen saturation was always above 80%. No significant differences were observed between control and solvent control responses.

3.1. Observed short-term toxicity of whole pharmaceutical spiked-sediment

There was no significant difference between control and solvent control. Amphipods mortality (Figure 2) was significantly higher than the control ($p < 0.05$) for sediment spiked with CBZ (500 $ng \cdot g^{-1}$, 5 $ng \cdot g^{-1}$) and FX (10 $ng \cdot g^{-1}$). Some agencies

classified as toxic sample when the amphipod mortality is 20% higher than observed in the control sediment (USEPA 1998; Environment Canada 2000; Casado-Martínez et al. 2006 c). According to this classification, PRO (5 $ng \cdot g^{-1}$ and 0.05 $ng \cdot g^{-1}$) were considered toxic for *A. brevicornis*.

Figure 2. Mortality rate of amphipods *A. brevicornis* exposed to six pharmaceutical compounds spiked in marine sediment samples.



* = significance difference set up at $p < 0.05$. Long dash line (---) represents the mortality of the solvent control. Dot line (⋯) represents 20% mortality higher than control sediment (USEPA, 1998, Environment Canada, 2000, Casado-Martínez *et al.*, 2006 c).

For the Microtox® SPT assay, the highest concentration of each pharmaceutical product was tested to evaluate the bioluminescence inhibition of *V. fischeri*: CBZ 500 $ng \cdot g^{-1}$, IBP 500 $ng \cdot g^{-1}$, PRO 500 $ng \cdot g^{-1}$, FX 100 $ng \cdot g^{-1}$, EE2 100 $ng \cdot g^{-1}$ and CAF 1500 $ng \cdot g^{-1}$. Control and solvent control were not toxic for *V. fischeri*. After 30 min of exposure, IC_{50} of the control sediment was the highest observed in this study (5967 $mg \cdot kg^{-1}$, 95% confidence range: 2317 to 5649 $mg \cdot kg^{-1}$), followed by the solvent control (4589 $mg \cdot kg^{-1}$, 95% confidence range: 3952 to 6920 $mg \cdot kg^{-1}$). Microtox® SPT is

assumed by different international agencies to evaluate sediment quality. The Canadian Standards (Environment Canada 2002) considered the limit of IC_{50} = 1000 mg·kg^{-1}. According to the Spanish Standards (CEDEX 1994; Casado-Martínez et al. 2006 a; Morales-Caselles et al. 2007), this limit is 750 mg·kg^{-1} (dry weight). Good sediment quality status of the control and solvent control was confirmed.

Microtox® SPT results were shown in the Table 4. Concerning the IC_{50} values after 30 min of exposure, FX (IC_{50} = 36.1 ng·g^{-1}) was the most toxic pharmaceutical product tested, followed by EE2 (IC_{50} = 39.4 ng·g^{-1}), CBZ (IC_{50} = 95.6 ng·g^{-1}), IBP (IC_{50} = 100.6 ng·g^{-1}), PRO (IC_{50} = 163.9 ng·g^{-1}) and CAF (IC_{50} = 507.6 ng·g^{-1}).

Table 4. Bioluminescence inhibition of the bacteria *V. fischeri* (IC_{50}) exposed to marine sediment samples spiked with carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethinylestradiol (EE2), propranolol (PRO) and caffeine (CAF). Results are expressed in ng·g^{-1}.

Microtox® Solid Phase Test (IC_{50})			
	5 min	**15 min**	**30 min**
CBZ	412.8	205	95.6
IBP	291.1	217.8	100.6
FX	54.4	67	36.1
EE2	57.8	52.9	39.4
PRO	272.8	206.1	163.9
CAF	735	646.5	507.6

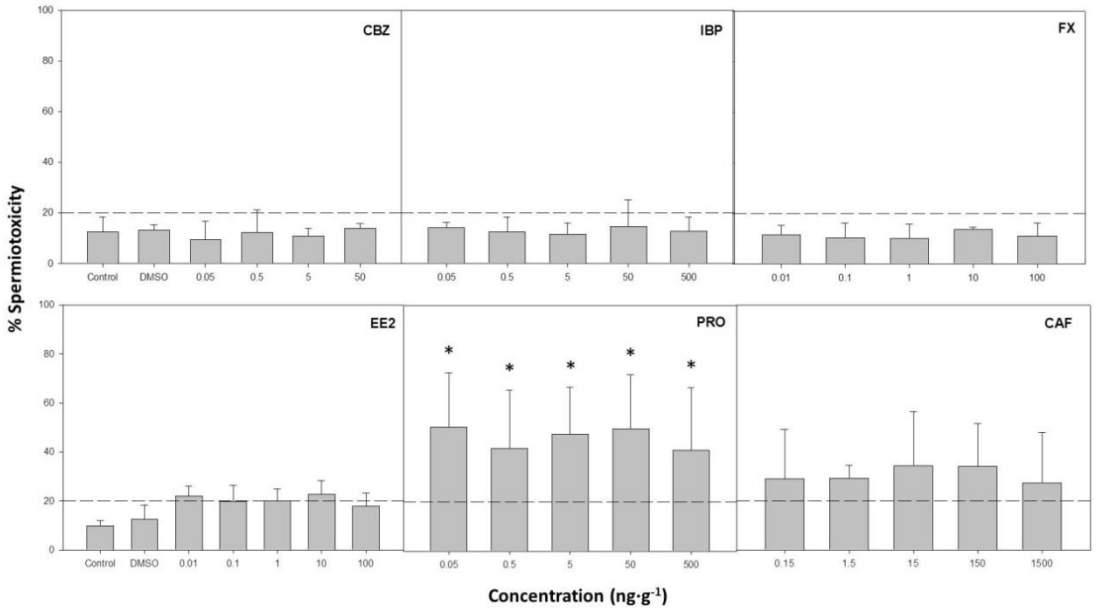
3.2. Observed short-term toxicity of elutriates

For the Microtox® bioassay (Basic Test), hormesis was observed in all elutriate samples tested.

Spermiotoxicity data was shown in the Figure 3. Optimum sperm-to-egg ratio was determined by pre-test in the laboratory, as that which targets an optimum rate of 80% fertilization under control conditions (Environment Canada 2011). Fecundation success

was significantly lower than the control ($p < 0.05$) for PRO (all concentrations tested). EC_{50} or EC_{10} was not calculated because PRO concentrations were responsible for an average of 47.38% (± 3.94) of unfertilized eggs without variability between the different concentrations.

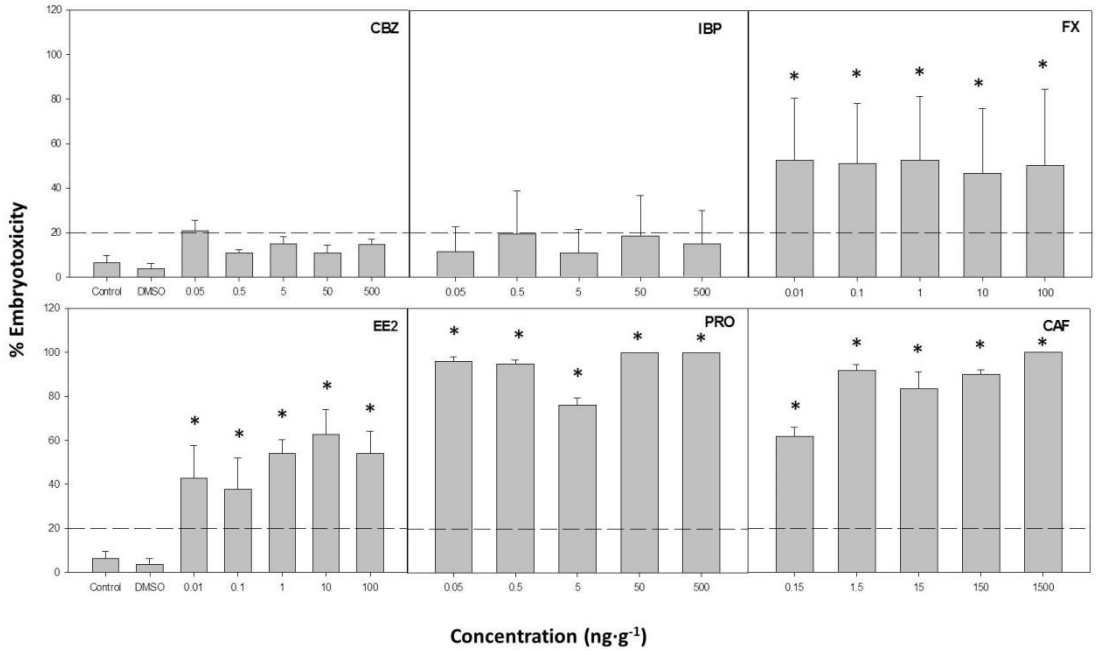
Figure 3. Absence of fecundation membrane in eggs of *P. lividus* exposed to different concentrations of pharmaceutical products (CBZ, IBP, FX, EE2, PRO and CAF).



* = significance difference set up at $p < 0.05$. Long dash line (---) represents the optimum sperm-to-egg ratio was determined by pre-test in the laboratory, as that which targets an optimum rate of 80% fertilization under control conditions (Environment Canada 2011).

Embryotoxicity was significantly higher compared with the control ($p < 0.05$) for all concentrations of FX, EE2, PRO and CAF. EC_{50} or EC_{10} were not calculate because these concentrations were responsible for an abnormal larvae development average of 44.36% (± 2.06) for FX, 43.92% (± 8.88) for EE2, 87.08% (± 8.87) for PRO and 78.96% (± 13.01) for CAF, without variability between the concentrations that could permit the calculation of EC_{50} or EC_{10}.

Figure 4. Abnormal larvae development of *P. lividus* exposed to different concentrations of pharmaceutical products (CBZ, IBP, FX, EE2, PRO and CAF).



* = significance difference set up at $p < 0.05$. The Spanish Standard limit of toxicity (20% of abnormal embryo-larval development) (DelValls et al. 2003; Casado-Martínez et al. 2006 b) is represented as a long dash line (---).

I. galbana (Figure 5) and *T. chuii* (Figure 6) microalgae's inhibitions were significantly different compared with the control ($p < 0.05$) for CBZ (50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1}, 0.05 ng·g^{-1}). *T. chuii* showed growth inhibition significantly different compared with the control ($p < 0.05$) for EE2 (1 ng·g^{-1}) and PRO (50 ng·g^{-1}). Minimum of quadratic functions was applied to adjust the curves for *I. galbana* (Figure 5) and *T. chuii* (Figure 6). Nevertheless, it was possible to calculate EC_{50} only for CBZ (21 ng·g^{-1} for *I. galbana* and 84.61 ng·g^{-1} for *T. chuii*). In fact, the maximum inhibition of *I. galbana* reached up to 50% for CBZ (50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1}, 0.05 ng·g^{-1}), IBP (5 ng·g^{-1}), FX (100 ng·g^{-1}, 1 ng·g^{-1}, 0.01 ng·g^{-1}), EE2 (all concentrations tested), PRO (500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1}) and CAF (0.15 ng·g^{-1}). Additionally, the maximum percentage of inhibition of *T. chuii* reached up to 50% for CBZ (50 ng·g^{-1}, 5 ng·g^{-1}, 0.5

ng·g^{-1}, 0.05 ng·g^{-1}), IBP (500 ng·g^{-1}, 5 ng·g^{-1}), FX (100 ng·g^{-1}, 10 ng·g^{-1}), EE2 (1 ng·g^{-1}, 0.01 ng·g^{-1}) and PRO (500 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1}, 0.05 ng·g^{-1}).

Figure 5. Growth inhibition rate of *I. galbana* exposed to different concentrations of pharmaceutical products (CBZ, IBP, FX, EE2, PRO and CAF).

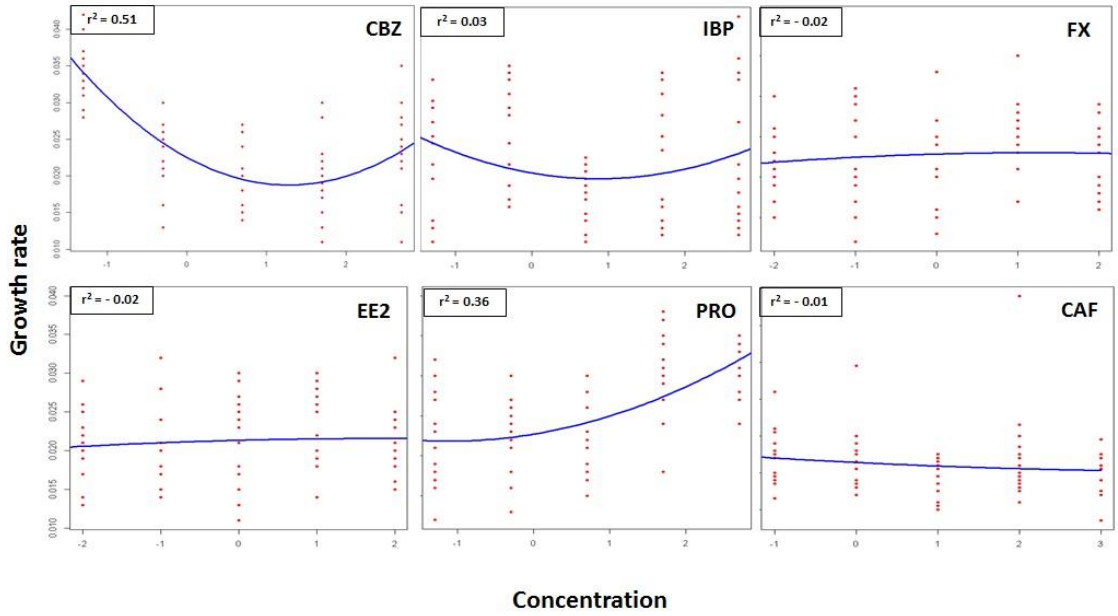
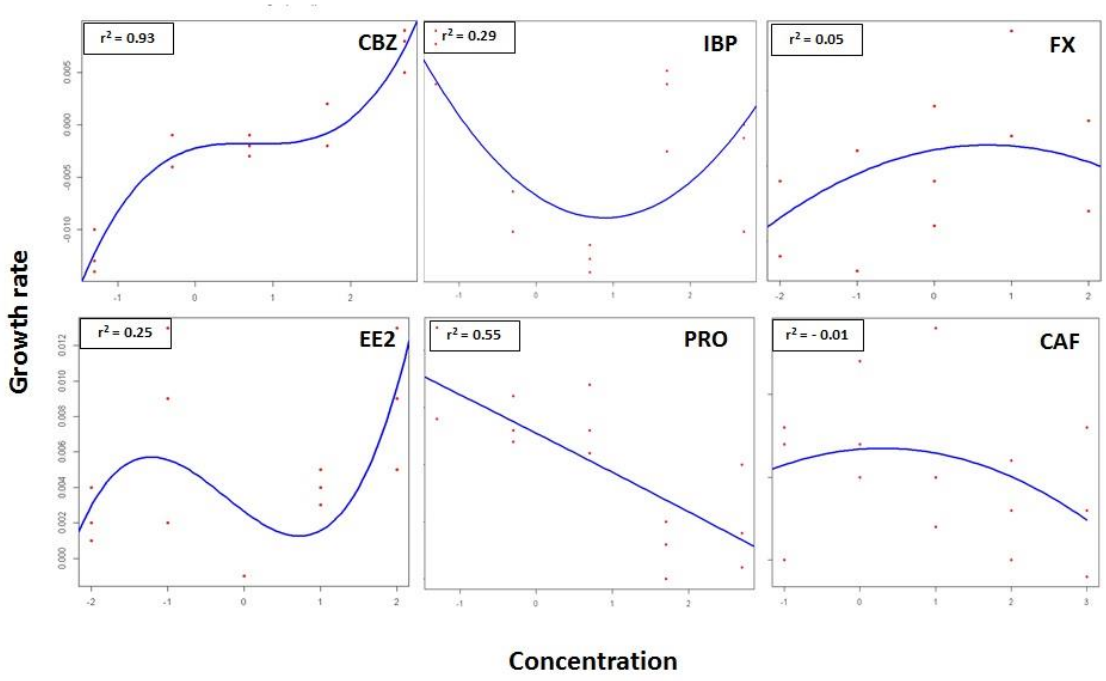


Figure 6. Growth inhibition rate of *T. chuii* exposed to different concentrations of pharmaceutical products (CBZ, IBP, FX, EE2, PRO and CAF).



3.3. Correlation between short-term responses

Correlation between concentration of pharmaceutical products and bioassays applied to different species to evaluate the short-term toxicity of pharmaceutical-spiked sediment samples was analyzed by Spearman's correlation test showed in the Table 5. Positive correlation was obtained between the concentration and sea-urchin embriotoxicity ($p < 0.05$) and spermiotoxicity ($p < 0.01$). Sea-urchin embryotoxicity and spermiotoxicity were highly positive correlated between them ($p < 0.01$). Microtox® SPT was highly negative correlated with growth rate of *I. galbana* ($p < 0.01$). *I. galbana* growth rate was positive correlated with sea-urchin spermiotoxicity ($p < 0.05$) and embryotoxicity ($p < 0.05$).

Table 5. Spearman´s correlation between acute responses of sediment samples (liquid and solid phase) and concentration of pharmaceutical products spiked in sediment samples.

	Concentration	Microtox® SPT	Amphipods mortality	Sea-urchin spermiotoxicity	Sea-urchin embryotoxicity	Growth rate *I. galbana*	Growth rate *T. chuii*
Concentration	1	.206	.293	.330*	.559**	.061	-.182
Microtox® SPT		1	-.078	.055	-.155	-.454**	-.168
Amphipods mortality			1	.229	.270	.075	.231
Sea-urchin spermiotoxicity				1	.681**	.358*	.091
Sea-urchin embriotoxicity					1	.386*	-.025
Growth rate *I. galbana*						1	.098
Growth rate *T. chuii*							1

**Significant correlation at level 0.01 (bilateral).

*Significant correlation at level 0.05 (bilateral).

4. DISCUSSION

Pharmaceuticals products are designed for a specific and targeted biochemical effect. Specific modes of action are well known in target organisms, which do not necessarily address the same dose-response in non-target organisms. Pharmaceuticals are often at least moderately lipophilic (Oetken et al. 2005), which might pose a potential risk for benthic community. Data on the environmental hazards associated with these compounds are emerging but still scarce (Molander et al. 2009). A high variability in the short-term toxicity results might be due different susceptibilities, mode of action and bioavailability of the pharmaceutical products in sediment, elutriate and water column. This fact highlights the importance of using a battery of bioassays with different organisms.

Microtox® SPT is assumed by different international agencies to evaluate environmental sediment quality. Previous study applied Microtox® to evaluate the toxicity of CBZ, diclofenac and clofibric acid (Ferrari et al. 2003, 2004). Although these compounds did not reveal high toxic impact for *V. fischeri*, they can act as toxic tracers.

Recent studies revealed the low biodegradability of the antiepileptic drug CBZ in sewage treatments (Ternes et al. 1998; Heberer 2002; Beausse 2004; Furlong et al. 2004). De Lange et al. (2006) observed reduced locomotion of the amphipod *Gammarus pulex* exposed to CBZ (1–1000 $ng \cdot L^{-1}$ for 1.5 h) which might interfere with feeding behavior. Quinn et al. (2008 a) demonstrated significantly reduction of feeding activity of *Hydra attenuata* exposed to 50 $mg \cdot L^{-1}$ of CBZ. For the amphipods *A. brevicornis*, the concentrations of 500 $ng \cdot g^{-1}$ and 5 $ng \cdot g^{-1}$ of CBZ after 10-days of exposure caused significant mortality compared with the control ($p < 0.05$). Locomotion and feeding behavior could interfere in the mortality of *A. brevicornis*, but to ensure more studies should be done in this direction.

Different pharmaceutical products can present the same toxicity level in the same organisms tested, for example IBP and FX. De Lange et al. (2006) exposed the freshwater amphipod *G. pulex* to low concentrations of FX and IBP (10 $ng \cdot L^{-1}$ – 100 $ng \cdot L^{-1}$) which resulted in significant decrease in animal´s movement, whereas at higher concentrations (1 $\mu g \cdot L^{-1}$ – 1 $mg \cdot L^{-1}$) this activity was similar to the control. In the present study, FX (10 $ng \cdot g^{-1}$) presented significant amphipods mortality when compared with the control ($p < 0.05$). Brooks et al. (2003) observed no mortality of the freshwater amphipod *H. azteca* across treatment levels of IBP (maximum of 43.2 $mg \cdot kg^{-1}$), as the present study. EE2 did not present toxicity for amphipods *A. brevicornis*. This should be better investigated with the inclusion of bioaccumulation analysis, even the capacity of the amphipod *H. azteca* to bioaccumulate EE2 was previously reported (Dussault et al. 2009).

The β-adrenergic receptor blocking PRO ($mg \cdot L^{-1}$) significantly reduced cladoceran heart rate and metabolic activity (Dzialowski et al. 2002). PRO was reported as negatively affected the physiology of benthic amphipod *Gammarus spp* from a Baltic Sea community exposed to concentration of 1000 $\mu g \cdot L^{-1}$(Oskarsson et al. 2012). PRO and CAF were not toxic for amphipods *A. brevicornis*.

All elutriate samples tested with Microtox® Basic Test presented hormesis. In this case, the xenobiotic works as a stimulant in small concentrations, but can be inhibitory in large concentrations. The result is a biphasic (or U-shaped) concentration-response relationship (OECD 2011). Similar behavior was found by Illés et al. (2013) concerning the short-term toxicity of IBP determined in *V. fischeri*: due to considerable decrease of IBP concentration upon irradiation, the inhibition of *V. fischeri* bioluminescence initially increased and then decreased. Studies about the toxic degradation of the pharmaceutical products could bring more information about hormesis.

Main results on elutriates demonstrated the good capacity of sea-urchin bioassays to discriminate different pollution/ bioavailability levels among different pharmaceutical products. The combined use of spermiotoxicity and embryotoxicity tests proved effective in highlighting clear toxicity fingerprints to the complementarity of endpoints, as previous described by Losso and Ghirardini (2010). Dose-response was related for sea-urchin embriotoxicity and spermiotoxicity. Nevertheless, EC_{50} and EC_{10} were not calculated since the presence of the pharmaceutical products seemed to interfere in the bioassay, which suggests that even low concentrations of pharmaceutical products could be toxic enough to modify the way of life of benthic organisms.

Therefore, effects caused by CBZ were not observed on the reproduction of the worms *L. variegatus* (Oetken et al. 2005). In this study, CBZ and IBP did not affect the reproduction of the sea-urchins *P. lividus* (spermiotoxicity and embryotoxicity). For embryotoxicity, all concentrations of FX, EE2, PRO and CAF were significantly different compared with the control ($p < 0.05$). FX can increase the level of cAMP in incubated rabbit corneas (Costagliola et al. 2008). The alteration of cAMP could result in changes in the meiotic division which can cause toxicity. During observations of developing embryos of Japanese medaka exposed for 4-weeks to FX treatments, several abnormalities in the embryos were noted (Foran et al. 2004). As an endocrine disruptor, EE2 interferes with the normal physiological functions related to reproduction, justifying studies of its potential developmental toxicity in bivalves *C. gigas* embryos (Wessel et al. 2007) and sea-urchin *P. lividus* (Roepke et al. 2005, Barbaglio et al. 2005). Results were consistent with recent data reported, since the sea-urchin *P. lividus* larvae exposed to EE2 showed embryotoxicity ($p < 0.05$). The role of hormones on the physiology of this specie should be deeply investigated (Barbaglio et al. 2005). PRO and CAF inhibit adenylyl cyclase and Na^+ channels, which decreases intercellular cAMP and influences the meiotic

division. Teratogenic effect of CAF was clearly demonstrated in rodents by Nehlig and Debry (1994).

Findings indicated that CBZ caused short-term toxicity at environmental relevant concentrations for both microalgae´s assays. Otherwise, no inhibition of the microalgae *S. capricornutum* was found within 96h of exposure to CBZ (Andreozzi et al. 2002). However, in this study the growth inhibition of *I. galbana* and *T. chuii* exposed to CBZ (50 $ng \cdot g^{-1}$, 5 $ng \cdot g^{-1}$, 0.5 $ng \cdot g^{-1}$, 0.05 $ng \cdot g^{-1}$) were significant different compared with the control ($p < 0.05$). There was no effect on the microalgae´s growth rate concerning IBP and FX concentrations. Results corroborated with Brooks et al. (2003) which microalgae cell density was not significantly affected by FX associated to sediments. Brooks et al. (2003) observed cell deformities and cell sizes reduction. FX is known to have bacteriostatic effects, perhaps exerted by efflux pump inhibition which can influence the mechanism of toxicity for observed cell deformities (Munoz-Bellido et al. 2000). Such effects of pharmaceutical products on microalgaes cells warrant further investigation. Further studies should bring information about microalgaes growth rate and concentrations of EE2, PRO and CAF.

5. CONCLUSION

In summary, the present study refuted the fact that Microtox® procedures (SPT and Basic Test) and microalgae bioassays were sufficient for the protection of the aquatic environment and to provide a useful quality assessment of pharmaceutical-spiked sediments. Between the bioassays used in the present study, amphipod mortality was recommended to assess pollution by CBZ, FX and PRO. Sea-urchins tests were the most sensitive endpoints, which embryotoxicity showed more sensitive than the spermiotoxicity bioassay. We recommend a tier-testing approach, involving additional

short-term and chronic toxicity tests, and specific tests to establish the bioaccumulation associated with sediment contamination by pharmaceutical products.

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CAPÍTULO 3

Evaluación de la calidad ambiental de sedimentos marinos afectados por productos farmacéuticos: ensayos de toxicidad crónica mediante simulación de concentraciones en laboratorio.

La necesidad de comprender los impactos de las actividades humanas sobre los ecosistemas ha llevado al desarrollo de varios métodos para evaluar los efectos biológicos de la contaminación. Biomarcadores de estrés y efecto se han incorporado en los estudios ambientales para evaluar los efectos tóxicos y metabólicos de los contaminantes, por separado o en combinación, en niveles cada vez más pequeños de organización biológica.

Para que biomarcadores sean incluidos en legislaciones ambientales, es necesario presentar las bases conceptuales de su utilización y aplicación en cumplimiento de la política ambiental. Los biomarcadores son herramientas recomendadas por diversos organismos internacionales como el PNUMA, OSPAR, OECD, ICES y ICO (Cajaraville et al., 2000, Solé et al., 2009). La Agencia de Medio Ambiente Canadiense ha realizado estudios previos sobre los efectos agudos y crónicos, en laboratorio y campo, de productos farmacéuticos y vertidos de aguas residuales. La exposición a concentraciones ambientales de este tipo de compuestos puede inducir al estrés oxidativo así como daños genéticos, en los organismos expuestos en laboratorio (Gagné et al., 2006). Sin embargo, los estudios son sobre agua en su gran mayoría, y por lo tanto son necesarias investigaciones más profundas relacionadas a sedimentos, más específicas sobre la toxicidad subletal, mediante el uso de herramientas como los biomarcadores (Viarengo et al., 2007).

Debido a la continua presencia de bajas concentraciones en el medio ambiente acuático, su persistencia y el continuo aporte a través de estaciones depuradoras de aguas residuales, los fármacos tienen más probabilidades de causar efectos crónicos o sub-crónicos que efectos agudos. Los efectos tóxicos en un individuo se dan a partir de la interacción de los contaminantes con biomoléculas, proporcionando cambios estructurales y/ o funcionales esenciales a la actividad celular. Estos cambios afectan los distintos niveles tróficos representados por el "efecto dominó", desde la bioquímica celular, pasando por órganos y tejidos hasta llegar al ecosistema y biosfera. En este contexto, se han propuesto herramientas conocidas como "*early warning tools*", que permiten la evaluación de los efectos adversos, proporcionando información sobre la dinámica de acción de los contaminantes y anticipando el conocimiento de los efectos ecológicos que se pueden producir (Handy et al., 2003).

Cuando el compuesto original biodisponible o sus metabolitos se unen a macromoléculas celulares, los efectos tóxicos se manifiestan. En última instancia, lo que puede conducir a la ruptura de la membrana, daño celular y/ o efectos genotóxicos posteriormente pueden resultar en el desarrollo y progresión de enfermedades (por ejemplo, cáncer). El metabolismo es, por lo tanto, un determinante importante de la actividad y de la vida media del compuesto en el organismo (Timbrell, 1998). La primera batería de biomarcadores estudiada en esta Tesis está directamente relacionada al metabolismo, actividades antioxidantes, neurotoxicidad, estrés oxidativo y efectos adversos causados por xenobióticos en la salud (Figura 9).

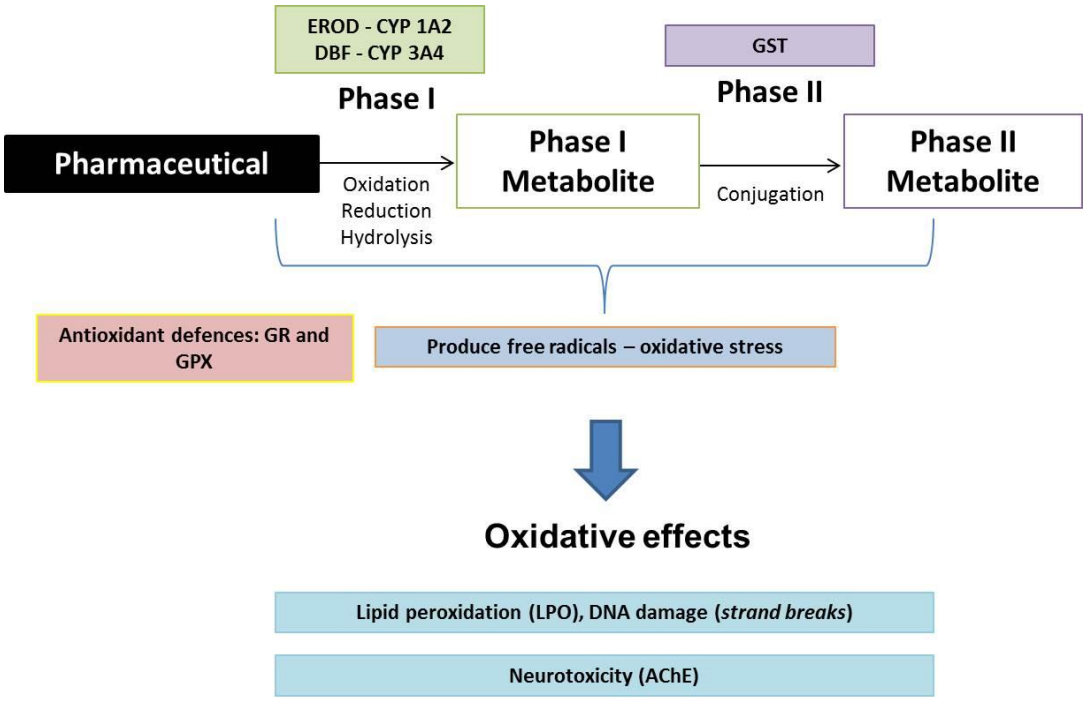

Figura 9. Biomarcadores de estrés relacionados al metabolismo y respuestas antioxidantes. Biomarcadores de efecto relacionados a peroxidación lipídica, daño genético y neurotoxicidad.

Biotransformación o metabolismo pueden ser definidos como reacciones de enzimas catalizadoras, lo que permite la conversión de un compuesto xenobiótico en una forma más soluble, para que sea excretada más fácilmente que el compuesto original.

La mayoría de los xenobióticos son catalizados por reacciones enzimáticas de la fase I del metabolismo, también conocidas como sistema de función mixta oxidasa (en inglés: mixed function oxidases - MFO). Los citocromos P450 (CYP 450) comprenden una gran familia de proteínas hemo, ubicadas en el retículo endoplásmico (Stegeman et al., 1992), donde se cataliza la oxidación de sustratos lipófilos mediante la utilización de NADPH y O_2. En la presente Tesis, dos actividades enzimáticas relacionadas con la fase I fueran determinadas: ethoxyresorufin O-deethylase (EROD) y dibenzylfluorescein dealkylase (DBF). La actividad enzimática de EROD está relacionada al CYP 450 1A1 (Gagné et al., 2007 a), implicado en reacciones de mono-oxigenación de dioxinas e hidrocarburos aromáticos policíclicos (HPA, en inglés: PAH) (Martín-Díaz et al., 2007),

y fármacos como la CBZ. La actividad enzimática de DBF está relacionada al CYP 450 3A4, implicado en el metabolismo de muchos productos farmacéuticos (Gagné et al., 2007 a). Los productos farmacéuticos pueden activar o desactivar las actividades enzimáticas de la fase I del metabolismo, como descritos en los siguientes artículos de este capítulo.

La fase II del metabolismo promueve la combinación de endógenos y xenobióticos en el tripéptido glutatión, haciéndolos solubles y más fácilmente excretados. La actividad de la enzima glutathione S-transferase (GST) es una de las actividades relacionadas a esta fase, situada preferentemente en el citosol.

En cuanto al sistema de defensa antioxidante, cambios en las actividades enzimáticas pueden reflejar un aumento en la síntesis de especies reactivas de oxígeno (O_2^-, H_2O_2) inducidos por la exposición a xenobióticos, asociadas con el estrés oxidativo, los mecanismos de defensa y la superposición de las fuerzas pro-oxidantes. La enzima glutathione peroxidase (GPX) aje en la degradación del H_2O_2 con reconocida función en la preservación del estrese oxidativo. Enzimas complementarias como la glutathione reductase (GR) producen GSH y NADPH para mantener este mecanismo de defensa antioxidante en la célula (Reed, 1986).

En cuanto a la acción de los contaminantes sobre el sistema neuronal, se ha prestado especial atención a las funciones de la enzima acetylcholinesterase (AChE), que realiza la función del neurotransmisor acetilcolina y descomponen en las uniones neuromusculares y las sinapsis, lo que impide la activación continua del nervio, siendo de vital importancia para el buen funcionamiento de los sistemas sensoriales y neuromusculares del organismo (Payne et al., 1996).

Dado que las perturbaciones que ocurren a partir de la exposición a los contaminantes se demuestran mediante cambios en diferentes procesos bioquímicos, estos

pueden resultar en el daño celular, como peroxidación lipídica (en inglés: lipid peroxidation, LPO) y daño en el ADN (en inglés: DNA damage – *strand breaks*).

Sin embargo, hay una falta de datos sobre la biota marina y estuarina expuestas a productos farmacéuticos. Daughton y Ternes (1999) sugirieron que los bioensayos o biomarcadores deberían centrarse en los mecanismos específicos de acción farmacéutica sobre la biota. La segunda clase de biomarcadores propuestos en esta Tesis son los relacionados con el modo de acción (en inglés: mode of action - MOA) de la droga en cuestión, con posibles efectos sobre la supervivencia, la función inmune y la reproducción del organismo (Gagné et al., 2007 b) (Figura 10). Este grupo está relacionado con el estado endocrino de la gametogénesis y los niveles de energía del organismo: la actividad de la monoamine oxidase (MAO), la actividad de la cyclooxygenase (COX), los niveles de vitellogenin-like proteins (Vtg), el contenido total de lípidos (en inglés: total lipids - TLP) y el transporte de electrones en la mitocondria (en inglés: mitochondrial electron transport - MET). La actividad de la MAO tiene una función de vital importancia, ya que juega un papel en la inactivación de los neurotransmisores (por ejemplo: noradrenalina, serotonina, dopamina). La actividad de la COX es una actividad enzimática responsable por la formación de importantes mediadores biológicos. Se corresponde al estado neuroendocrino y también puede ser una medida para la inmunotoxicidad. La inhibición de esta actividad puede provocar el alivio del dolor y la inflamación. Vtg-like proteins son las proteínas precursoras de la formación del huevo, por lo general en silencio en los machos, pero que puede ser inducida por la exposición a estrógenos (Gagné et al., 2011). TLP está directamente relacionada a la energía reservada en el organismo, en cuanto que el MET está relacionado con el gasto de energía, principalmente aquella relacionada a la respiración.

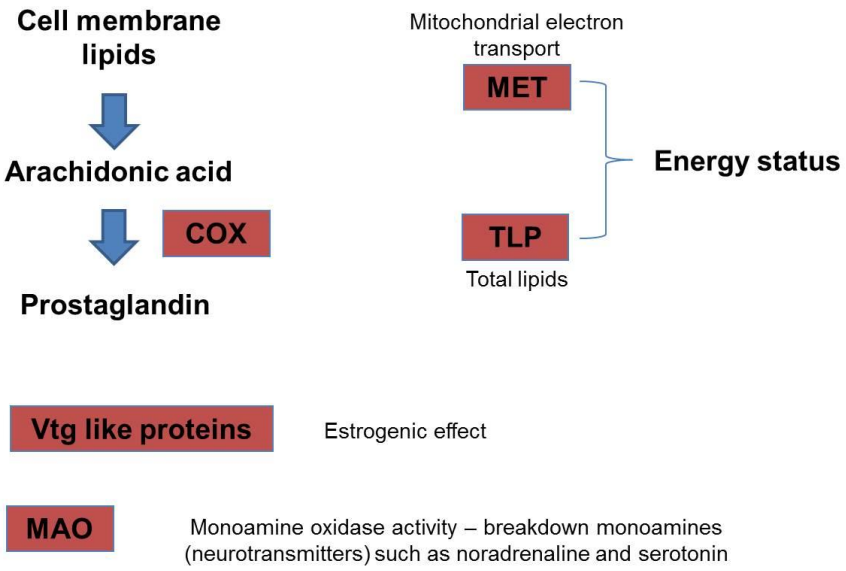

Figura 10. Biomarcadores relacionados a los posibles efectos al estado energético, sistema endócrino e inmunitario, asociados a la reproducción.

La evolución histórica de los biomarcadores está estrechamente vinculada a los avances en la medicina humana y veterinaria, pero las medidas son también posibles para invertebrados. En esta Tesis, fueran utilizadas dos especies de invertebrados bentónicos con el objetivo de identificar posibles respuestas subletales debido a la exposición de organismos marinos a distintos productos farmacéuticos dopados en el sedimento. La primera especie utilizada fue el anfípodo *Ampelisca brevicornis*. La misma metodología aplicada para el dopaje de sedimento, anteriormente detallada, fue empleada en esta fase, y el bioensayo de anfípodos se basó en las mismas premisas descritas en el capítulo 2. Una vez pasado los 10 días de exposición, los organismos fueran congelados a -80^{o}C, y posteriormente homogenizados para la determinación de los biomarcadores. Anfípodos fueran expuestos a sedimento dopado con CBZ, IBP, FX, EE2, PRO y CAF. Los biomarcadores analizados en esta especie fueran: EROD, DBF, GST, GPX, GR, AChE, LPO y daño en ADN. Los datos están detalladamente descritos en al artículo II.

El siguiente organismo utilizado en la exposición a sedimento dopado con fármacos fue el poliqueta *Hediste* (= *Nereis*) *diversicolor*. Los organismos fueran colectados en una

marisma entre San Fernando y Chiclana de la Frontera, parte del Parque Nacional de la Bahía de Cádiz. Los organismos fueran aclimatados antes de su exposición al sedimento dopado, que duró 14 días (Figura 11).

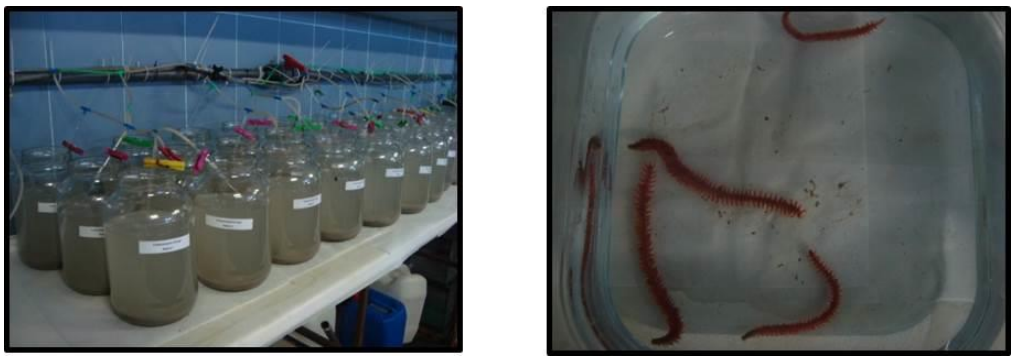

Figura 11. Bioensayo con la especie *Hediste diversicolor*.

Poliquetas fueran expuestos a sedimento dopado con CBZ, IBP, FX, EE2 y PRO. La misma metodología aplicada para el dopaje de sedimento anteriormente descrita fue empleada en esta fase. La metodología para el bioensayo fue basada en ASTM (2009) y Thain y Bifield (2001). Después del periodo de exposición, los organismos sobrevivientes pasaran una noche en agua limpia, para depuración. Los organismos fueran congelados a -80^{o}C, y posteriormente homogenizados para la determinación de los biomarcadores. El artículo III trata de las respuestas de los biomarcadores de estrés y efecto a xenobióticos en general. El artículo IV incluye análisis de los biomarcadores relacionados al modo de acción de diferentes fármacos, relacionados con reproducción y nivel energético del organismo en cuestión. Todos los biomarcadores del artículo IV nunca habían sido medidos antes en poliquetos, lo que refuerza la originalidad de este artículo.

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A Candidate Short-Term Toxicity Test Using *Ampelisca brevicornis* to Assess Sublethal Responses to Pharmaceuticals Bound to Marine Sediments

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Abstract Lethal and sublethal responses related to different phases of metabolism (phases I and II enzymatic activities), neurotoxicity (acetylcholinesterase activity), oxidative stress (lipid peroxidation and antioxidant enzyme activities), and genetic damage (DNA strand breaks) were analysed to assess the possible adverse effects of pharmaceuticals bound to marine sediments. The crustacean amphipod *Ampelisca brevicornis* was chosen as the bioindicator species. Organisms were exposed for 10 days to sediment spiked with pharmaceutical compounds frequently used and previously detected in the environment: carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethynylestradiol (EE2), propranolol (PRO), and caffeine (CAF). Short-term bioassay to evaluate amphipod mortality was recommended to assess pollution by CBZ, FX, and PRO. IBP and PRO were metabolized by phases I and II detoxification enzymatic activities. Oxidative stress was caused by PRO and CAF. Contrary to expected results, DNA damage (strand breaks) decreased after the exposure of amphipods to sediment spiked with IBP, FX, EE2, PRO, and CAF (including environmental concentrations). FX was neurotoxic to amphipods. The battery of biomarkers tested allowed the assessment of bioavailability, oxidative stress, genotoxicity, and neurotoxicity of the pharmaceuticals analysed. The results of this study suggested that pharmaceutical products at concentrations currently found in the environment might cause a wide variety of adverse effects (based on laboratory studies). The results obtained here are useful for environmental risk assessment of marine sediments contaminated by pharmaceuticals. Nevertheless, more research is needed using field-based marine sediments.

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Pharmaceuticals and personal care products (PPCPs) are discharged into aquatic environments by way of wastewater-treatment plants (WWTPs) and are preferentially adsorbed on solid environmental matrices (Beausse 2004). Sediments may therefore act as a sink as well as a long-term source of these compounds in aquatic ecosystems (Gilroy et al. 2012).

Sediment-quality guidelines from different regulatory frameworks have recommended different toxicity tests including the mortality rate of amphipods exposed to sediment for 10 days (CEDEX 1994; the United States Army Corps of Engineers (USACE) 1998; the United States Environmental Protection Agency (USEPA) 1998a, b; Global Investigation of Pollution in the Marine Environment (GIPME) 2000; European Sediment Network (SedNet) 2003). Amphipod tests have been recognized as a useful tool to evaluate marine and estuarine sediment quality (American Society for Testing and Materials (ASTM) 1991; USEPA 2001; Casado-Martínez et al. 2007; Ramos-Gómez et al. 2009). Although pharmaceuticals at

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current environmental concentrations do not cause mortality, they can produce sublethal effects on exposed aquatic organisms (Quinn et al. 2008a, b; Martín-Díaz et al. 2009; Oviedo-Gómez et al. 2010; Parolini et al. 2011; Aguirre-Martínez et al. 2013a, b). A battery of sublethal responses may provide information about the bioavailability of pharmaceuticals bound to sediments and their possible adverse effects on benthic biota.

Pharmaceuticals can affect the benthic community due to changes in metabolism. Metabolism is defined as enzyme-catalysed conversion responsible for the biotransformation of the xenobiotic in a more easily excreted compound (Lech and Vodicnik 1985). When the bioavailable parent compound or its metabolites bind to cellular macromolecules, toxic effects manifest themselves, which may lead to membrane disruption, cell damage, neurotoxicity, and/or genotoxicity. Such effects can result in the development and progression of diseases. Metabolism is therefore an important determinant of the activity and half-life of a compound in the body (Timbrell 1998). Specific redox reactivity of PPCPs forms the basis for their respective metabolism, elimination, biological effects, and toxicity. Induction of oxidative metabolism caused by different pharmaceuticals on aquatic invertebrates has been previously reported (Quinn et al. 2004; Gagné et al. 2007; Parolini et al. 2011; Aguirre-Martínez et al. 2013a, b; Franzellitti et al. 2013, 2014).

The purpose of this study was to evaluate the lethal and sublethal effects of pharmaceutical-spiked sediments on the amphipod *Ampelisca brevicornis*. Six widely used pharmaceutical products were selected: carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethynylestradiol (EE2), propranolol (PRO), and caffeine (CAF). Lethality was assessed using the short-term 10-day amphipod toxicity test, and sublethal responses were assessed using various biomarkers as follows: exposure [*i.e.*, ethoxyresorufin O-deethylase (EROD) and dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) and acetylcholinesterase (AChE)] and effect [*i.e.*, lipid peroxidation (LPO), and DNA strand breaks].

Materials and Methods

Spiking Procedure

Sediment was sampled at Río San Pedro[southwest (SW) Spain] (36°31′53″N, 6°12′48″W), an intertidal creek area part of the Natural Park Bahía de Cádiz, which has been considered as a reference site by previous studies (Pérez et al. 2004; Solé et al. 2009; Basallote et al. 2012; De Orte et al. 2013). The topmost 10-cm layer was sampled and sieved through a 2-mm mesh to remove any associated macrofauna and debris. After sieving, sediment samples were dried at 70 °C to eliminate possible interferences and contaminants (Organisation for Economic Co-operation and Development (OECD) 2000). Dry sediment was kept at 4 °C in the dark. The same volume of water lost in the dry-sediment procedure was added to the sediment sample before the spiking procedure. Overlying seawater was obtained from Experimental Marine Aquaculture Plant–Cultivos Marinos at the University of Cádiz. This water has been used for the culture and maintenance of test organisms (fish, molluscs, and plankton) since 2002.

Table 1 Physicochemical properties of sediment used for the pharmaceutical spiking procedure

Properties	Percentage (%)
Gravel	0.84
Sand	57.39
Fines	41.77
OM	8.07 ± 0.34
TOC	1.20 ± 0.09

Physicochemical characteristics of the reference sediment are listed in Table 1 (Maranho et al. 2014). For sediment grain size, an aliquot of dry sediment was analysed according to the methodology recommended by United States Geological Survey (2013). Total organic carbon (TOC) and organic matter (OM) content were determined following the methods reported by USEPA (2002).

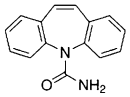
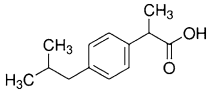
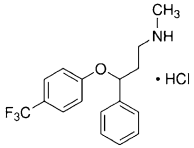
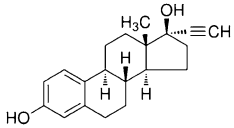
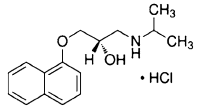
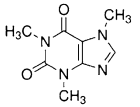
The antiepileptic drug CBZ, the antisteroidal drug IBP, the selective serotonin reuptake inhibitor (SSRI) drug FX, the oral female contraceptive EE2, the β-blocker drug PRO, and the stimulant drug CAF were spiked in the sediment samples. Such compounds are highly consumed worldwide.

All pharmaceutical products were purchased from Sigma Aldrich (Madrid, Spain). An overview of physicochemical characteristics, structures, production, metabolism, and removal by way of WWTPs is listed in Table 2 (Maranho et al. 2014).

Pharmaceutical stock solutions were prepared with solvent dimethyl sulfoxide (DMSO) 0.001 % (v/v) as recommended by Quinn et al. (2008a, b), Eades and Waring (2010), and Aguirre-Martínez et al. (2013a, b). Two controls were run in parallel with the experiments: reference sediment (control) and reference sediment spiked with DMSO 0.001 % (v/v) (solvent control). Due to the lack of significant difference between controls, only the reference sediment was considered for statistical analysis.

The spiking procedure was an adaptation of ASTM (2000), USEPA (2001), and OECD (2004) protocols, and previously described in Maranho et al. (2014). Sediment

Table 2 Overview of physicochemical characteristics, structures, production, metabolism, and removal of pharmaceuticals by way of WWTPs (CBZ, IBP, FX, EE2, PRO, and CAF)

	CBZ	IBP	FX	EE2	PRO	CAF
CAS no.	298-46-4	15687-27-1	56296-78-7	57-63-6	318-98-9	58-08-2
Therapeutic class	Anticonvulsant	Anti-inflammatory	Antidepressant	Oral contraceptive	Cardiac disorder	Stimulant
Developed formula						
Molecular weight (g mol^{-1})	236.27	206.28	345.79	296.4	295.8	194.2
Water solubility (mg L^{-1})	Insoluble[a]	1,00,000[a]	4,000[a]	4.8–11.3[f]	5,000[a]	16,000[a]
pKa	14[b]	4.52[b]	9.39[e]	10.4	9.5[h]	6.1[h]
Log kow	1.51[b]	2.4[8b]	1.22[d]	3.6–4.2[f]	1.2–3.48[i]	<0[i]
Pharmacokinetic excretion rate (%)	1–2 Unchanged[c]	1–8 Unchanged[c]	NA	≤80 Not metabolized[g]	NA	NA
Total removal by way of STP (%)	7[c]	60[j]	NA	NA	96[j]	NA

NA no data available

[a] www.sigmaaldrich.com

[b] Scheytt et al. (2005)

[c] Ternes et al. (1998)

[d] Kwon and Ambrust (2008)

[e] Vasskog et al. (2006)

[f] Nagpal and Meays (2009)

[g] De Mes et al. (2005)

[h] Yu and Chu (2009)

[i] Lin et al. (2010)

[j] Yamamoto et al. (2007)

was spiked with the highest concentrations of each pharmaceutical, initially prepared in 3-L glass beakers, mixed by homogenization using a Teflon spatula, and subsequently mixed for 30 min in a bottle roller. Pharmaceutical-spiked sediments were incubated for 7 days (at 4 °C in the dark) to ensure equilibration of the test substance between water and sediment in the preparation sealed bottle (Francis et al. 1984; Löffler et al. 2005). After the equilibration period, spiked sediment was diluted with the reference sediment to obtain the concentrations proposed for the bioassay.

The following pharmaceutical compounds were tested until 100× greater than concentrations found in the environment: CBZ, IBP, and PRO (500, 50, 5, 0.5, and 0.05 ng g^{-1}), FX and EE2 (100, 10, 1, 0.1, and 0.01 ng g^{-1}), and CAF (1,500, 150, 15, 1.5, and 0.15 ng g^{-1}). Environmental concentrations were previously reported in the following studies: Ternes et al. 2002; Hernando et al. 2006; Schultz et al. 2010; Pintado-Herrera et al. 2013; Zhou and Broodbank 2014.

Determination of Pharmaceutical Concentrations in Spiked Sediments

The highest concentrations of the six selected pharmaceuticals were measured in spiked sediments in the first day of the bioassay according to a modification of the method proposed by Jelic et al. (2009). Briefly, 2 g of sediment were place inside an 11-mL stainless steel cells and extracted by pressurized liquid extraction using an ASE 200 Accelerated Solvent Extractor unit (Dionex, Sunnyvale, CA) and methanol and water (1:2 v/v) as solvent. After extraction, samples were evaporated and reconstituted in methanol and water (25:75 v/v), and internal standards (CBZ [d10], atenolol [d7], and naproxen [d3]) were added at 50 ng mL^{-1}. Further details can be found in Jelic et al. (2009). The main modification of the method was that a different ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry system was used, which consisted of a Bruker EvoQ Elite liquid chromatographer coupled to a UPLC Bruker Advance tandem mass spectrometer (Bruker Corporation, Freemont, CA). Separation of target compounds was achieved using a Bruker Intensity Trio C18 column (100 × 2 mm × 1.9 µm internal diameters) and methanol (A) and water (B) as solvents. The gradient was as follows (flow = 0.4 mL min^{-1}): 5 % B for 0.4 min, increased linearly to 30 % in 0.1 min, increased linearly to 95 % in 4.5 min, and then held for 3 min. Multiple monitoring reaction (MRM) transitions had to be optimized for target compounds (Table 3). CBZ, FX, PRO, and CAF were monitored in positive ionization mode, whereas IBP and EE2 were determined in negative ionization mode. Source parameters were spray voltage 4,500 V, cone temperature 250 °C, cone gas flow 20 mL min^{-1}, probe temperature 450 °C, probe gas flow 50 mL min^{-1}, and nebulizer gas flow 60 mL min^{-1}. Measured concentrations of target compounds in spiked sediments are listed in Table 3 (Maranho et al. 2014). Therefore, nominal concentrations are presented throughout this article.

Table 3 Nominal versus measured concentrations for target pharmaceuticals in spiked sediments (ng g^{-1})

Target compound	MRM transitions	Nominal concentration	Measured concentration
CBZ	237.1 > 193.8 (22 V)	500	505 ± 10
	237.1 > 192.4 (31 V)		
IBP	205.1 > 162.1 (5 V)	500	405 ± 50
	205.1 > 160.0 (5 V)		
FX	310.1 > 44.6 (10 V)	100	90 ± 8
	310.1 > 148.4 (7 V)		
EE2	295.3 > 145.2 (36 V)	100	121 ± 25
	295.3 > 158.3 (36 V)		
PRO	260.1 > 116.2 (17 V)	500	515 ± 47
	260.1 > 72.9 (21 V)		
CAF	195.1 > 137.3 (17 V)	1500	1407 ± 76
	195.1 > 138.9 (18 V)		

Pharmaceuticals vary among their rates of degradation with reported half-lives (Guler and Ford 2010). In the present study, no degradation products were assumed because the half-lives of CBZ (Lam et al. 2004; Löffler et al. 2005), IBP (Beausse 2004), FX (Lam et al. 2004), EE2 (Beausse 2004; De Mes et al. 2005), PRO (Andreozzi et al. 2003), and CAF (Bradley et al. 2007) were all >10 days.

Bioindicator Species

Ampelisca brevicornis is a crustacean amphipod found in the Iberian Peninsula and has been reported as a suitable bioindicator species of the Spanish Atlantic coast (Casado-Martínez et al. 2006, 2007; Morales-Caselles et al. 2008; Ramos-Gómez et al. 2009). This crustacean has burrowing *domiculous* behaviour (species that build their own tubes in sediment) and are deposit and filter feeders. These characteristics allow them to be constantly in contact with all phases of sediment and overlying water. This bioassay is considered inexpensive, simple, and easy reproducible.

Bioassay Approach

Individuals of *A. brevicornis* were obtained from a reference area at the Bay of Cádiz (SW Spain) (36°29′16″N; 6°15′52″W). Environmental conditions were checked on the day that amphipods were sampled in the field (pH 7.90 ± 0.3, temperature 15 °C, salinity 35, dissolved oxygen 8 mg L^{-1}). Individuals were acclimatized for 1 week

before the bioassay started. The bioassay was performed with 3-L glass beakers containing sediment and water (1:4). Thirty amphipods per beaker were exposed for 10 days (ASTM 1993; Ramos-Gómez et al. 2009). The bioassay was performed in triplicate in an acclimatization room (18 °C ± 2 °C) in the dark. The pH and salinity were monitored every 2 days (pH 7.60 ± 0.33, salinity 36.94 ± 1.04). Oxygen saturation was always >80 %. After the exposure period, sediment from each beaker was sieved and amphipod mortality checked. Surviving amphipods were counted and separated into pools of 15 organisms, according to the replicates, for biomarker analysis.

Determination of Sublethal Responses

Each pool of amphipods were homogenized with ultra-turrax tool in a buffer solution of 140 mM NaCl, 25 mM HEPES–NaOH, 0.1 mM ethylene diamine tetraacetic acid, and 0.1 mM dithiothreitol (pH 7.5) (Gagné et al. 2007). Homogenate samples (HF) were used for the determination of DNA damage (strand breaks) and LPO responses. An aliquot of HF was centrifuged at 15,000×*g* for 20 min at 4 °C (S_{15}). Enzymatic activities of EROD, DBF, GST, GPX, and GR were determined in S_{15} extract. Another aliquot of HF was centrifuged at 3000×*g* for 20 min at 4 °C (S_3) to determine the AChE enzymatic activity. Samples of HF, S_3, and S_{15} were stored at −80 °C until biochemical analyses. Protein concentration was analysed according to Bradford methodology (Bradford 1976). All biomarkers responses were normalized by the total protein content of the correspondent extract (HF, S_3, or S_{15}).

EROD Activity

Concerning mixed-function oxidase (MFO–CYP1A2) activity, EROD activity was determined using the adapted assay described by Gagné and Blaise (1993). EROD enzymatic activity was determined by fluorescence using 485 nm (excitation) and 580 nm (emission) filters. Determination of EROD activity was performed using a standard calibration curve of 7-hydroxyresorufin concentration. MFO–CYP3A4 activity concerning DBF activity was determined as previously described by Gagné et al. (2007). Fluorescence was measured at 485 nm (excitation) and 516 nm (emission) filters. Both reactions were started by the addition of 50 µM nicotinamide adenine dinucleotide phosphate (NADPH) and incubation for 0, 15, 30, 45, and 60 min at 30 °C. Results were expressed as pmol min^{-1} mg^{-1} total protein.

GST Activity

The procedure applied for the determination of the GST activity was described by McFarland et al. (1999). Two substrates were used: 42 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH. GST activity was measured by absorbance at 340 nm every 30 s for 3 min. The results were expressed as OD (optical density) GST min^{-1} mg^{-1} total protein.

GPX and GR Activities

GPX and GR activities were determined applying the method described by McFarland et al. (1999). GPX activities were measured using 1 mM cumene hydroperoxide as substrate. GR activities were estimated by NADPH oxidation, and the substrate was oxidized glutathione (GSSG). GPX and GR activities were measured by absorbance at 340 nm every 2 min for 10 min. Results were expressed as pmol min^{-1} mg^{-1} total protein.

AChE Activity

AChE activity was determined using the method described by Ellman et al. (1961) adapted by Guilhermino et al. (1996). AChE enzymes were degraded acetylthiocholine in thiocholine and acetate. Absorbance was measured at 412 nm every 5 min for 20 min. Results were expressed as nmol DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid)) min^{-1} mg^{-1} total protein.

Lipid peroxidation

Lipid peroxidation (LPO) was measured according to Wills' (1987) protocol. Thiobarbituric acid reactants (TBARS) were determined by fluorescence at 530 nm (excitation) and 630 nm (emission). Results were expressed as µg TBARS mg^{-1} total protein.

DNA Damage (Strand Breaks)

DNA damage (strand breaks) was assessed by alkaline precipitation assay (Olive 1988) based on K-SDS precipitation of DNA–protein cross-link followed by detection of DNA strands (Gagné et al. 1995). Salmon sperm DNA standards were used for calibration. Fluorescence readings were taken at 360 nm (excitation) and 460 nm (emission). Results were expressed as µg DNA mg^{-1} total protein.

Statistical Analyses

Statistical analyses were performed with the SPSS/PC 21.0 + statistical package (IBM, USA). Normality and homogeneity of variances were verified with Welch and Levene's test, respectively. For normal and homogeneous data, significant differences between individuals exposed to

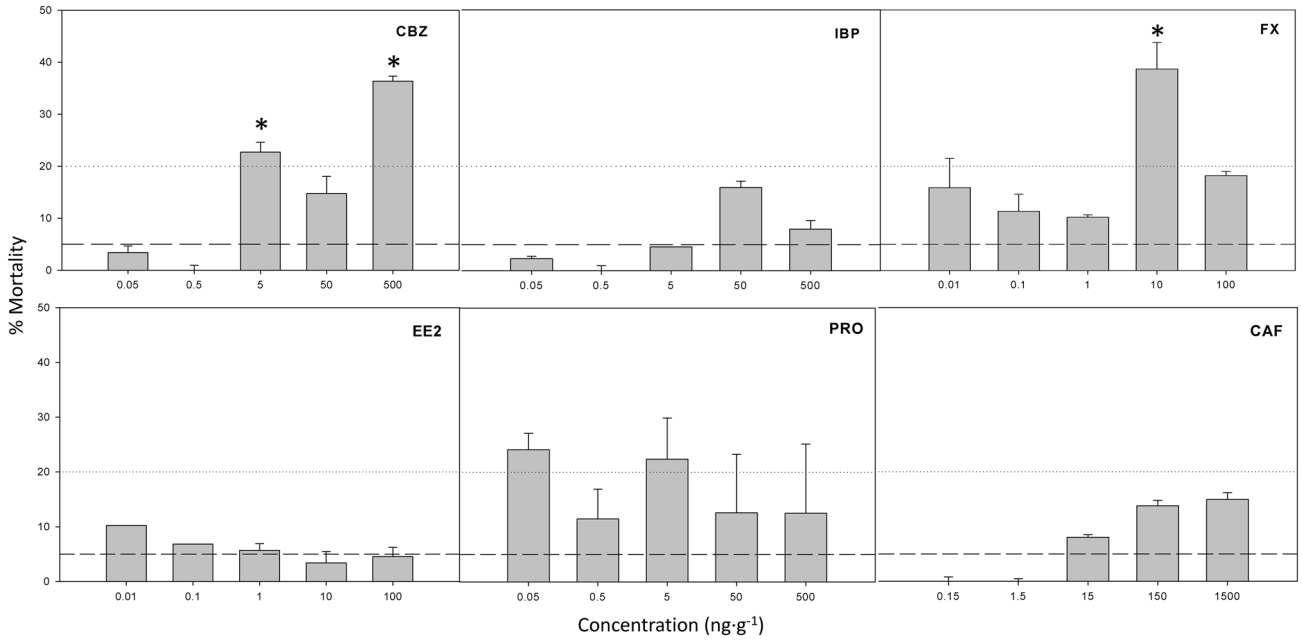


Fig. 1 Mortality rate of amphipods *A. brevicornis* exposed to six pharmaceutical compounds spiked in whole sediment samples. *Significant difference set at $p < 0.05$. There was no mortality of amphipods in the control samples. *Long-dashed line* (—) = mortality of the reference sediment spiked with DMSO 0.001 % (v/v). *Dotted line* (···) = mortality 20 % greater than the control sediment (USEPA 1998b; Environment Canada 2000; Casado-Martínez et al. 2006)

control (reference) sediment and individuals exposed to pharmaceutical-spiked sediments were determined using one-way analysis of variance followed by multiple comparison Dunnett's test. When the data did not assume normality or homogeneity, Dunnett's T3 test was applied. Significance level was set at $p < 0.05$. Correlation between biomarker responses, concentration, and mortality was undertaken according to Spearman's correlation analysis. Significance level for Spearman analysis was set at $p < 0.01$ and $p < 0.05$.

Results

Mortality of Amphipods *A. brevicornis* Exposed to Sediment Samples Spiked with Pharmaceutical Products

No significant differences ($p < 0.05$) were observed between control (reference) sediment and solvent control (DMSO-spiked sediment 0.001 % v/v) (Fig. 1). The concentrations of 500 ng·g^{-1} (mortality average of three replicates = 34.48 ± 0.9 %), 5 ng g^{-1} (mortality average of three replicates = 20.69 ± 1.9 %) CBZ, and 10 ng g^{-1} (mortality average of three replicates = 37.93 ± 3.1 %) FX caused significant amphipod mortality compared with the control ($p < 0.05$). Mortality was positively correlated with CBZ ($r = 0.807$, $p < 0.01$), IBP ($r = 0.846$, $p < 0.01$), FX ($r = 0.813$, $p < 0.01$), PRO ($r = -0.702$, $p < 0.05$), and CAF ($r = 0.929$, $p < 0.01$) concentrations. The concentration of CBZ responsible for 20 % mortality (LC_{20}) was 186.52 ng g^{-1}. For FX, the LC_{20} value was 62.28 ng g^{-1}.

Some agencies and investigators have classified sediment samples as toxic based on a value of 20 % mortality greater than that produced by the control sediment (USEPA 1998a, b; Environment Canada 2000; Casado-Martínez et al. 2006). According to this classification, PRO concentrations of 5 ng g^{-1} (mortality average of three replicates = 22.4 ± 7.5 %) and 0.05 ng g^{-1} (mortality average of three replicates = 24.1 ± 3 %) were considered toxic concentrations for this species. The data variability did not permit the calculation of an LC_{20} value for propranolol.

Sublethal Responses of Amphipods *A. brevicornis* Exposed to Sediment Samples Spiked with Pharmaceutical Products

No significant differences were observed between control and solvent control (DMSO-spiked sediment 0.001 % v/v) ($p < 0.05$). Results of the biochemical responses obtained from control organisms and amphipods exposed for 10 days to CBZ-, IBP-, FX-, EE2-, PRO-, and CAF-spiked sediment samples are represented in Figs. 2, 3, 4, 5, 6 and 7. Spearman coefficients (Table 4) showed that 38 of the total 270 correlations between biomarker responses, concentration,

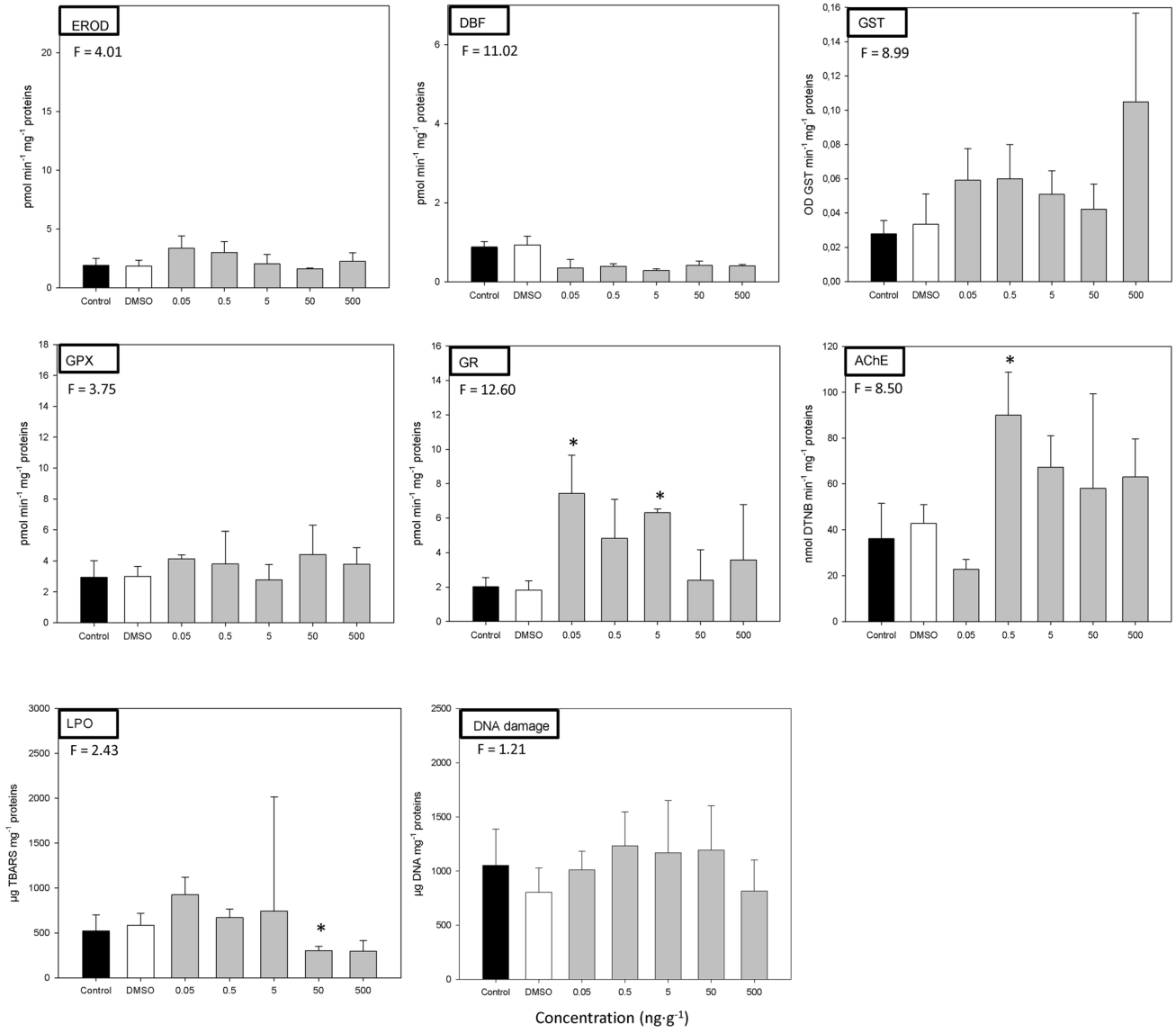


Fig. 2 CBZ exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipod *A. brevicornis* after 10 days of laboratory exposure compared with the control, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % (v/v) and CBZ-spiked sediment samples at 500, 50, 5, 0.5, and 0.05 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). degrees of freedom (df) = 6 in all groups

and mortality were highly significant ($p < 0.01$). According to Bisquera (Bisquerra 1987), 14.07 % of these correlations were moderate ($0.4 < r < 0.6$), and 21.85 % were high ($0.6 < r < 0.8$) or very high ($0.8 < r < 1$).

Figure 2 shows the biochemical data determined in *A. brevicornis* after CBZ-spiked sediment exposure. Phases I and II of the metabolism did not respond to CBZ. Nevertheless, DBF ($r = -0.403$, $p < 0.05$) and GST ($r = 0.622$, $p < 0.01$) activities were correlated with CBZ concentration. As consequence, CBZ activated the antioxidant system in amphipods. Significantly greater GR activities (5 ng g^{-1}, 0.05 ng g^{-1}) were observed compared with the control ($p < 0.05$). CBZ concentration ($r = 0.436$, $p < 0.05$) and GST activity ($r = 0.552$, $p < 0.05$) were positively correlated with the antioxidant system (GR activity). CBZ caused a significant decrease in LPO in amphipods exposed to 50 ng g^{-1} ($p < 0.05$). Increased DNA damage was not observed in amphipods. The environmental concentration of 0.5 ng g^{-1} was neurotoxic, *i.e.*, it was positively correlated with the concentration ($r = 0.536$, $p < 0.01$), which showed the possible neurotoxic effect of this compound for amphipods ($p < 0.05$).

Environmental concentrations of IBP (0.5, 0.05 ng g^{-1}) were metabolized by phase I of the metabolism, thus

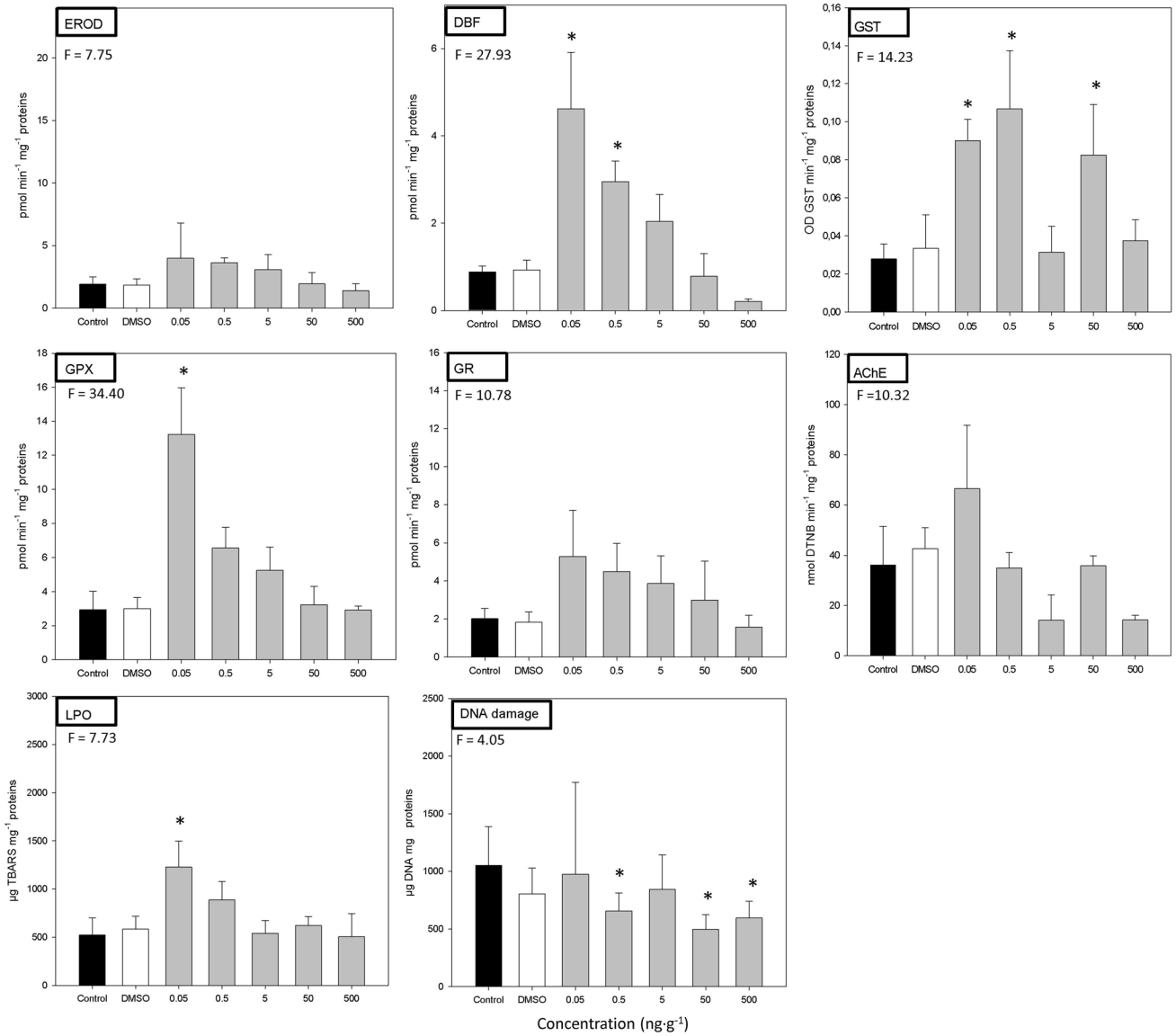


Fig. 3 IBP exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipod *A. brevicornis* after 10 days of laboratory exposure compared with the control, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % [v/v] and IBP-spiked sediment samples at 500, 50, 5, 0.5, and 0.05 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). $df = 6$ in all groups

showing the significant increase of the DBF activity compared with the control ($p < 0.05$). DBF activity was negatively correlated with concentration ($r = -0.556$, $p < 0.05$) and mortality ($r = -0.480$, $p < 0.05$) and positively correlated with phase II of the metabolism ($r = 0.618$, $p < 0.01$), the antioxidant system (GPX $r = 0.744$, $p < 0.01$; GR $r = 0.759$, $p < 0.01$), and AChE activity ($r = 0.703$, $p < 0.01$). IBP concentrations (50, 0.5, and 0.05 ng g^{-1}) were conjugated by phase II of the metabolism ($p < 0.05$). GPX enzymatic activity significantly increased compared with the control in amphipods exposed to the sediment spiked with an IBP environmental concentration (0.05 ng g^{-1}) ($p < 0.05$). Antioxidant enzymes were positively correlated between them ($r = 0.678$, $p < 0.01$). However, a significant increase of LPO was determined in organisms after exposure to sediment spiked with IBP (only for the environmental concentration of 0.05 ng g^{-1}) ($p < 0.05$). LPO was positively correlated with GR ($r = 0.449$, $p < 0.05$) and AChE ($r = 0.544$, $p < 0.05$) activities. DNA strand breaks significantly decreased in amphipods after exposure to IBP (500, 50, and 0.5 ng g^{-1}) ($p < 0.05$). DNA damage was positively correlated with concentration ($r = 0.638$, $p < 0.01$) and AChE activity ($r = 0.660$, $p < 0.01$).

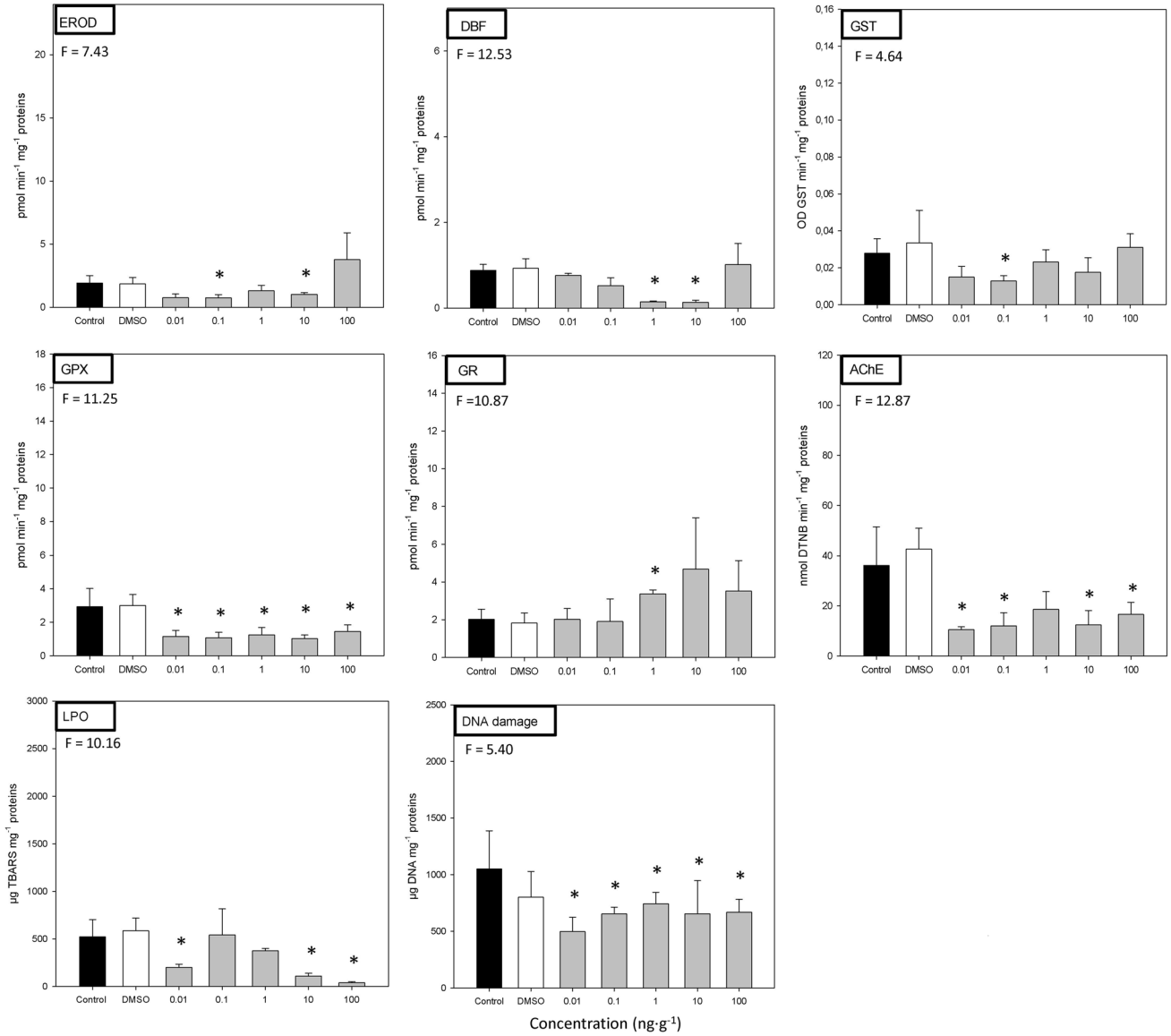


Fig. 4 FX exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipod *A. brevicornis* after 10 days of laboratory exposure compared with the control, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % [v/v] and FX-spiked sediment samples at 100, 10, 1, 0.1, and 0.01 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). $df = 6$ in all groups

FX (Fig. 4) significantly decreased phase I metabolism activities concerning EROD (10 and 0.1 ng g^{-1}) and DBF (10 and 1 ng g^{-1}) ($p < 0.05$). EROD activity was positively correlated with GST ($r = 0.772$, $p < 0.01$). Phase II metabolism significantly decreased in amphipods exposed to sediment spiked with 0.1 ng g^{-1} of FX ($p < 0.05$). Phase II metabolism was significantly correlated with AChE enzymatic activity ($r = -0.680$, $p < 0.01$). A significant decrease of antioxidant enzymatic activity (GPX) was determined in amphipods exposed to FX (all concentrations) compared with the control ($p < 0.05$). GPX activity was negatively correlated with mortality ($r = -0.512$, $p < 0.01$). GR showed significantly increased activity for amphipods exposed to FX (1 ng g^{-1}) ($p < 0.05$). GR activity was negatively correlated with LPO ($r = -0.763$, $p < 0.01$), and positively correlated with concentration ($r = 0.817$, $p < 0.01$) and mortality ($r = 0.624$, $p < 0.01$). Therefore, a significant decrease of LPO was determined in amphipods exposed to the same concentrations (100, 10, and 0.01 ng g^{-1}) ($p < 0.05$). LPO was negatively correlated with concentration ($r = -0.851$, $p < 0.01$) and mortality ($r = -0.832$, $p < 0.01$). FX (all concentrations) caused a significant decrease of DNA strand breaks ($p < 0.05$). FX was

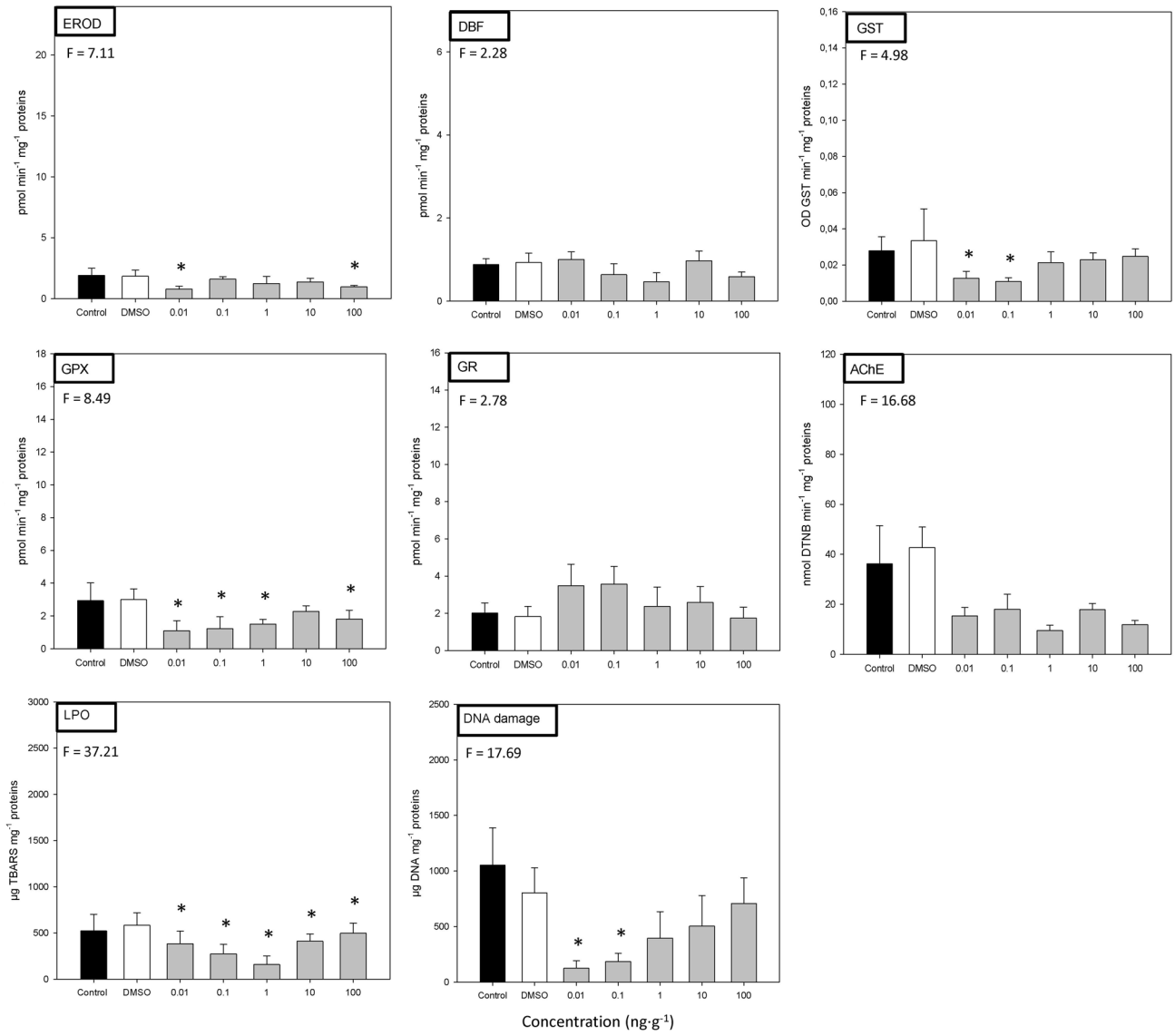


Fig. 5 EE2 exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipods *A. brevicornis* after 10 days of laboratory exposure compared with the control, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % [v/v] and EE2-spiked sediment samples at 100, 10, 1, 0.1, and 0.01 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). $df = 6$ in all groups

significantly neurotoxic to amphipods (100, 10, 0.1, and 0.01 ng g^{-1}) ($p < 0.05$).

Biomarker responses determined in amphipods exposed to EE2-spiked sediment are shown in Fig. 5. Detoxification metabolism (EROD activity) was significantly decreased compared with the control in amphipods exposed to EE2 (100 and 0.01 ng g^{-1}) ($p < 0.05$). Likewise, DBF did not respond to EE2. Phase II (GST activity) was significantly decreased compared with the control in amphipods exposed to EE2 concentrations (0.1 and 0.01 ng g^{-1}). Mortality was negatively correlated with phase II metabolism ($r = -0.702$, $p < 0.01$). A significant decrease of GPX activity was determined after the organism's exposure to EE2 (100, 1, 0.1, and 0.01 ng g^{-1}) ($p < 0.05$). GPX activity was negatively correlated with mortality ($r = -0.792$, $p < 0.01$). Individuals exposed to all concentrations of EE2 showed significantly decreased LPO compared with the control ($p < 0.05$). LPO was positively correlated with DNA damage ($r = 0.637$, $p < 0.01$) and negatively correlated with mortality (LPO $r = -0.623$, $p < 0.01$; DNA $r = -0.838$, $p < 0.01$). Nevertheless, exposure to EE2 resulted in a significant decrease of DNA strand breaks in amphipods exposed to environmental concentrations (0.1 and 0.01 ng g^{-1}) ($p < 0.05$). EE2 was not neurotoxic to amphipods.

Biochemical responses observed in amphipods exposed to PRO concentrations spiked in sediment samples are

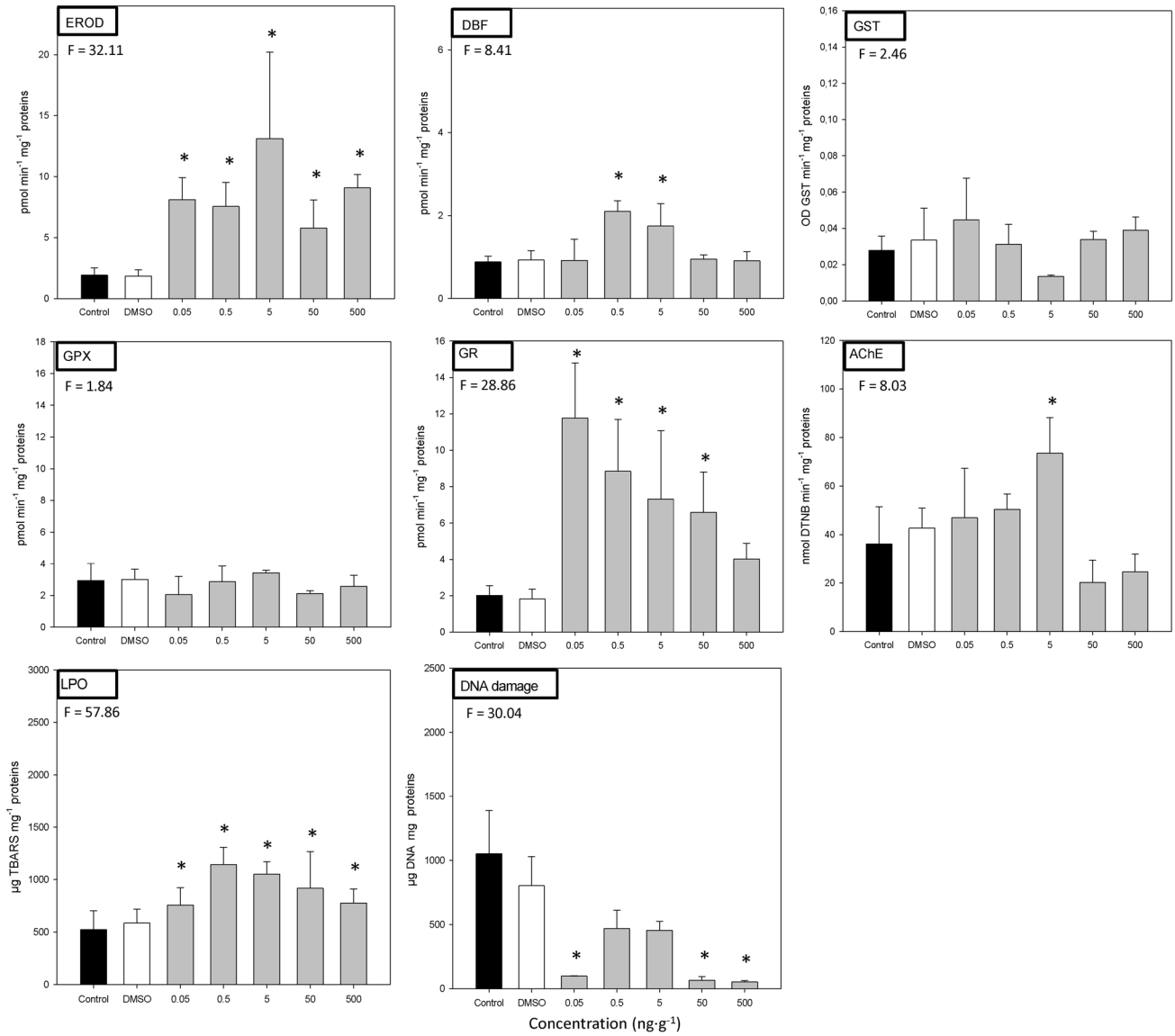


Fig. 6 PRO exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipod *A. brevicornis* after 10 days of laboratory exposure compared with controls, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % (v/v) and PRO-spiked sediment samples at 500, 50, 5, 0.5, and 0.05 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). $df = 6$ in all groups

shown in Fig. 6. PRO was detoxified by phase I metabolism by EROD and DBF enzymatic activities. These responses were positively correlated between them ($r = 0.633$, $p < 0.01$). EROD activity was significantly induced compared with the control for amphipods exposed to all concentrations ($p < 0.05$). Only the PRO environmental concentrations (5 and 0.5 ng g^{-1}) were detoxified by DBF enzyme ($p < 0.05$). Phase II metabloism did not respond to PRO. GR was the main antioxidant response in amphipods exposed to PRO concentrations (50, 5, 0.5, and 0.05 ng g^{-1}) ($p < 0.05$). EROD and GR activities were positively correlated between them ($r = 0.633$, $p < 0.01$) and with mortality (EROD $r = 0.711$, $p < 0.01$; GR $r = 0.811$, $p < 0.01$). Oxidative effects showed significantly greater LPO in organisms exposed to PRO-spiked sediment (all concentrations) ($p < 0.05$). Amphipods exposed to concentrations of 500, 50, and 0.05 ng g^{-1} showed significant decrease of DNA strand breaks compared with the control ($p < 0.05$).

Biochemical responses determined in amphipods after exposure to CAF-spiked sediments are shown in Fig. 7. CAF was not detoxified by the phase I metabolism because EROD activity was significantly lower in organisms exposed to CAF (0.15 ng g^{-1}) compared with the control

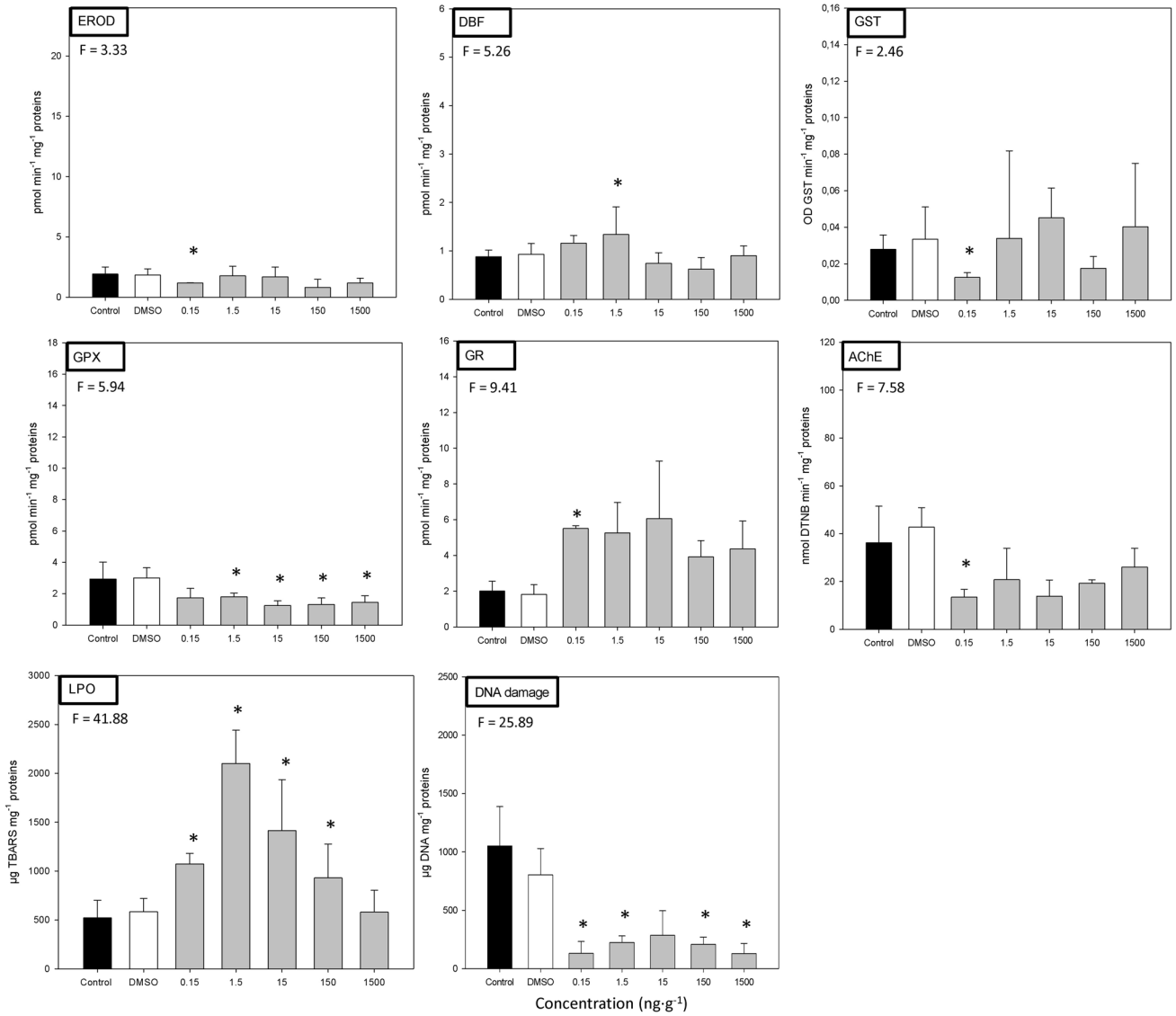


Fig. 7 CAF exposure. Mean and SD of EROD, DBF, GST, GR, GPX, and AChE enzymatic activities, LPO, and DNA damage (strand breaks) values analysed in amphipod *A. brevicornis* after 10 days of laboratory exposure compared with controls, *i.e.*, reference sediment, and reference sediment spiked with DMSO 0.001 % (v/v) and CAF-spiked sediment samples at 500, 50, 5, 0.5, and 0.05 ng g^{-1}. *Significant difference compared with the control (reference sediment) ($p < 0.05$). $df = 6$ in all groups

organisms ($p < 0.05$). Except for the concentration of 1.5 ng g^{-1}, which showed significantly greater DBF activity ($p < 0.05$); all other concentrations were not significantly different compared with the control. Phase II metabolism did not respond to CAF concentrations. Amphipods exposed to the concentration of 0.15 ng g^{-1} showed significantly lower GST activity compared with the control ($p < 0.05$). Concerning the antioxidant system, a significant decrease of GPX activity was determined in amphipods exposed to CAF (1500, 150, 15, and 1.5 ng g^{-1}) compared with the control ($p < 0.05$). GPX activity was negatively correlated with mortality ($r = -0.624$, $p < 0.01$) and LPO ($r = -0.712$, $p < 0.01$). Nevertheless, a significant increase of GR activity was determined for amphipods exposed to 0.15 ng g^{-1} ($p < 0.05$). A significant oxidative effect (LPO) was observed in amphipods exposed to CAF (150, 15, 1.5, and 0.15 ng g^{-1}) ($p < 0.05$). LPO was negatively correlated with the antioxidant system (GPX activity) ($r = -0.712$, $p < 0.01$). In contrast, significantly lower DNA strand breaks compared with the control were observed in amphipods exposed to CAF (1500, 150, 1.5, and 0.15 ng g^{-1}) ($p < 0.05$). DNA strand breaks was positively correlated with AChE activity ($r = 0.700$, $p < 0.01$). The CAF environmental concentration (0.15 ng g^{-1}) was neurotoxic to amphipods ($p < 0.05$).

Table 4 Spearman coefficients correlating biomarker responses, the mortality of *A. brevicornis* and the concentration of pharmaceutical products spiked in sediment samples

		Concentration	EROD	DBF	GST	GPX	GR	AChE	LPO	DNA damage	Mortality
Carbamazepine	Concentration	1	–.187	*–.403**	**.614****	.334	*.436**	*.536***	*–.520***	–.005	**.807****
	EROD		1	.223	.178	.175	.371	.035	*.596***	.128	–.227
	DBF			1	–.235	.171	*–.468**	.164	.344	.198	*–.529***
	GST				1	.248	*.574***	.314	–.064	–.221	*.485**
	GPX					1	*.487**	*.417**	–.087	.122	.106
	GR						1	.193	.083	.094	.355
	AChE							1	–.176	.311	.134
	LPO								1	.119	–.315
	DNA damage									1	–.086
	Mortality										1
Ibuprofen	Concentration	1	–.204	*–.556**	.193	–.101	–.009	**.702****	.062	**.638****	**.846****
	EROD		1	**.875****	*.416*	*.584***	**.626****	*.591***	.321	.382	–.217
	DBF			1	**.618****	**.744****	**.759****	**.703****	*.433*	**.349**	*–.480**
	GST				1	*.435**	*.449**	*.434*	.329	–.069	.106
	GPX					1	**.678****	.351	.378	.104	–.128
	GR						1	.353	*.449**	.063	–.051
	AChE							1	*.544**	**.660****	*–.400*
	LPO								1	*.408*	.020
	DNA damage									1	*–.450**
	Mortality										1
Fluoxetine	Concentration	1	–.048	–.298	–.009	*–.464**	**.817****	–.175	**–.851****	–.101	**.813****
	EROD		1	*.444*	**.772****	*.540**	–.060	*.577**	.000	.307	–.272
	DBF			1	.274	.320	*–.418*	.156	–.024	.037	–.176
	GST				1	*.518***	.073	**.680****	.066	*.490**	–.174
	GPX					1	–.202	*.451**	*.440*	.333	*–.512***
	GR						1	.104	**–.763****	–.232	**.624****
	AChE							1	.346	*.487**	*–.450**
	LPO								1	*.507**	**–.832****
	DNA damage									1	–.352
	Mortality										1

Table 4 continued

		Concentration	EROD	DBF	GST	GPX	GR	AChE	LPO	DNA damage	Mortality
17α-ethynylestradiol	Concentration	1	*.505***	–.229	–.292	–.170	–.080	**.600****	–.392	.206	.155
	EROD		1	.024	*.477**	.270	–.017	.356	*.451**	.274	*–.597***
	DBF			1	.199	.036	–.041	*.461**	.273	–.048	–.069
	GST				1	*.418*	*–.435*	*.488**	*.479*	*.523**	**–.702****
	GPX					1	–.329	.282	*.413*	*.579***	**–.792****
	GR						1	–.105	–.284	*–.529***	*.426**
	AChE							1	*.569**	.082	–.285
	LPO								1	**.637****	**–.623****
	DNA damage									1	**–.838****
	Mortality										1
Propranolol	Concentration	1	**.634****	–.057	.107	–.194	.346	*.557***	*.427**	**.832****	*.447**
	EROD		1	**.633****	.042	.077	**.639****	.218	*.574***	–.232	**.771****
	DBF			1	–.235	*.592***	.372	*.517**	*.505**	.068	.343
	GST				1	*–.453*	.218	–.111	–.202	–.300	.234
	GPX					1	–.036	.365	.088	.094	–.051
	GR						1	.120	*.479**	–.222	**.811****
	AChE							1	*.418*	*.538**	.150
	LPO								1	.117	.323
	DNA damage									1	–.390
	Mortality										1
Caffeine	Concentration	1	*.556***	–.287	.008	**–.696****	*.493**	–.194	.369	*–.459**	**.929****
	EROD		1	.302	.371	.357	–.161	*.596**	–.310	.358	*–.541***
	DBF			1	–.091	.185	*.489**	–.025	.228	–.078	*–.434**
	GST				1	.091	–.203	.375	.017	.238	.075
	GPX					1	*–.481**	*.407*	**–.712****	*.441*	**–.624****
	GR						1	*–.411*	*.486**	*–.495**	.358
	AChE							1	*–.408*	**.700****	.068
	LPO								1	–.262	.246
	DNA damage									1	–.307
	Mortality										1

Bold text indicates high ($0.6 < r < 0.8$) or very high ($0.8 < r < 1$) correlations; italic text indicates moderate ($0.4 < r < 0.6$) correlations; and plain text indicates low ($0.2 < r < 0.4$) or very low ($0 < r < 0.2$) correlations (Bisquerra 1987)

* $p < 0.05$

** $p < 0.01$

Discussion

Lethal Response

In this study, procedures were described for laboratory testing with infaunal amphipods exposed to six frequently used pharmaceutical compounds spiked in whole marine sediment samples to evaluate their lethal and sublethal toxicity. The present data were compared with the mode of action (MOA) described for humans and other mammals and with previous studies on freshwater and marine organisms (when data were available). There is a lack of information about environmental risk assessment of pharmaceuticals bound to sediments in marine environments.

Short-term toxicity bioassays determining the mortality rate of amphipods are recognized worldwide as being a useful tool to evaluate marine and estuarine ecosystem impacts by different sources of contamination (ASTM 1991; USEPA 2001; Casado-Martínez et al. 2007; Ramos-Gómez et al. 2009). The amphipod test is included in protective environmental regulation frameworks (CEDEX 1994; USACE 1998; USEPA 1998a, b; GIPME 2000; SedNet European Sediment Network 2003). The short-term bioassay was a suitable tool to analyse the mortality rate of amphipods exposed to CBZ, FX, and PRO. In addition, sublethal responses were observed in amphipods after exposure to all pharmaceutical products tested.

The Metabolism and Adverse Effects of Pharmaceutical Products

The battery of biomarkers was an effective tool to assess the impact of pharmaceutical products in amphipods. Concentrations of a single pharmaceutical compound activated specific isoenzymes but deactivated others. Therefore, activation or deactivation may result in a considerable alteration of isoenzymes and effects. Nonmonotonic dose responses are characteristic of hormesis response, in which the cell, tissue, or organism being tested responds to low concentrations of a given drug but responds negatively to high concentrations (Fong and Ford 2014). An increasing number of studies are finding biological effects at lower concentrations but not at higher concentrations, which can also lead to adaptation. However, the physiological mechanism by which hormesis occurs is still unknown.

Once the xenobiotic is inside the organism, phase I metabolism is responsible for transforming the compound into a more soluble and easily excreted one. Modulation of CYP-mediated metabolism is the most important drug-interaction mechanism (Quinn et al. 2004). Several cytochromes are involved in the metabolisation of pharmaceutical products. CYP3A4 is the most abundant P450 isoform in human liver: It has very broad substrate specificity and is responsible for the metabolism of >60 % of all clinically relevant drugs (Lin et al. 2002) including all drugs studied in the present work.

CBZ is a strong inducer of CYP1A2 (EROD activity) and 3A4 (DBF activity) in humans (Quinn et al. 2004). However, no significant increase of EROD or DBF activities was observed in the present study. CBZ might not be metabolized by CYP1A2 or CYP3A4 enzyme in amphipods; nevertheless, more research should be performed in this direction. Phase II (GST enzymatic activity) metabolism was not activated in amphipods exposed to CBZ. In contrast, increased GST enzymatic activities in aquatic invertebrates exposed to CBZ was reported by previous studies (Martín-Díaz et al. 2009; Vernouillet et al. 2010). GST enzymatic activity was induced in the amphipod species *Gammarus fasciatus* (Gélinas et al. 2013) and *Monoporeia affini* (Gorokhova et al. 2010) exposed to environmental pollutants. Antioxidant enzymes, such as GPX and GR, prevent adverse effects of oxidative stress in cells (Li et al. 2011). In this study, GR activity in *A. brevicornis* suggested that CBZ caused an increase of antioxidant enzymes as a defence mechanism. The oxidative stress of CBZ is indeed a side effect that has been shown in human patients, and it has also observed in marine mussels (Martín-Díaz et al. 2009). In contrast, there was no increase of LPO or DNA damage due to the exposure to CBZ. As in humans (Andreazza et al. 2007), CBZ did not induce DNA damage in amphipods. CBZ showed neurotoxic potential to amphipods. This data are in agreement with Mizuno et al. (2000), *i.e.*, that observed the increase of intracellular AChE levels in striatum and hippocampus of rats due to acute and chronic administrations of CBZ (25 and 50 mg g^{-1}/d).

IBP is considered inhibitor of CYP1A2 and 3A4 in humans. CYP enzyme activities decreased in amphipods exposed to increasing concentrations of IBP. IBP showed a similar-shaped trend concerning phase I metabolism and antioxidant systems with a concentration-dependent decrease of LPO. IBP was conjugated by phase II metabolism. The similar-shaped trend may be due the activation of several genes involved in the metabolism of xenobiotics (*e.g.*, GST, CYP450) as previously shown in marine bivalves *Ruditapes philippinarum* exposed to IBP (Milan et al. 2013). Oviedo-Gómez et al. (2010) exposed amphipods *Hyalella azteca* to sediment spiked with a nonsteroidal anti-inflammatory drug (NSAID) (diclofenac) and observed oxidative stress due to high increases of GPX activity and LPO. For *A. brevicornis*, low concentrations of IBP were metabolized by phases I and II metabolism and also increased antioxidant enzyme activity (GPX), which resulted in LPO. Although the environmental concentration level was low, IBP and its metabolites, which are probably

more harmful, may present a potential hazard for aquatic ecosystem health (Illés et al. 2013). IBP had a considerable effect on the activities of antioxidant and detoxifying enzymes in *A. brevicornis* as shown in oxidative status imbalances in the freshwater mussel *Dreissena polymorpha* (Parolini et al. 2011). For amphipods, there was a concentration-dependent decrease of metabolism rate, antioxidant activities, and LPO. These data corroborated those of the previous study, in which IBP solution first increased the toxicity of irradiation because of first degradation products and then decreased the toxicity with an increase of absorbed dose (Illés et al. 2013). Due to the large number of by products, it is difficult to claim which of them can influence substantial responses. DNA strand breaks decreased in amphipods exposed to IBP concentrations. However, Parolini et al. (2011) showed IBP genotoxicity in freshwater mussels *D. polymorpha* primarily through marked genetic injuries by passing DNA fragmentation. Previous studies on the exposure of the same bivalve to another NSAID drug, paracetamol (Parolini et al. 2011), and the fish *Danio rerio* exposed to IBP (Rocco et al. 2010), showed a similar pathway. These investigators observed homogeneous IBP-induced DNA fragmentation as shown by the low percentage of DNA in the comet tail, suggesting alternating DNA damage and repair and a time-dependent increase in apoptotic cell frequency (Rocco et al. 2010; Parolini et al. 2011). In contrast, IBP could have the same effects on the DNA material of individuals of *D. polymorpha* exposed to water and amphipods exposed to spiked sediment samples. According to the literature and currently available evidence, enzymatic systems may repair minor DNA damage caused by IBP exposure. However, when remarkable genetic injuries occur, the apoptotic pathway is triggered, and the production of micronuclei is enhanced (Parolini et al. 2011). Further studies concerning the role of NSAIDs in DNA damage and the production of micronuclei in marine invertebrates should be elucidated. IBP was not neurotoxic to amphipods. However, neurotoxic effects of IBP were previously shown in marine bivalve molluscs (Milan et al. 2013).

FX is an inhibitor of CYP1A2 and 3A4. A decrease of CYP1A2 and 3A4 enzymatic activities was expected in amphipods exposed to FX concentrations, and such a decrease was observed. FX-specific effects were explored based on the biological read-across hypothesis that a pharmaceutical may elicit similar effects in invertebrates by acting through conserved MOA in humans (Brooks et al. 2003; Huggett et al. 2003; Gunnarsson et al. 2008; Franzellitti et al. 2013). This feature was also determined in zebrafish because EROD activity was strongly inhibited after exposure to FX (Smith et al. 2012). Phase II enzymatic activity was insufficient to conjugate FX. Antioxidant activity was inhibited in amphipods exposed to FX concerning GPX enzymatic activity. However, GR enzymatic activity increased in amphipods exposed to the environmental concentration tested (1 ng g^{-1}). This finding corroborates with no clear antioxidant responses detected in digestive gland of marine mussels exposed to FX, whereas significant dose-dependent increases of catalase and GST activities were observed in gills (Franzellitti et al. 2013). The decrease of LPO in amphipods exposed to FX might be indicative of other oxidative stress in cells because of the accumulation of insoluble granules of lipofuscins in the lysosomes, which is strongly correlated to lysosomal damage (Viarengo et al. 2007). Studies concerning histopathological observations could bring more information about the decrease of LPO and the presence of granules in the lysosomes. LPO assessed as malondialdehyde content was evaluated in digestive gland of marine mussels after a 7-day treatment with FX, and this also decreased with increasing concentrations (Franzellitti et al. 2013). FX did not induce DNA damage (Andreazza et al. 2007). Similar effects in amphipods, *i.e.*, acting through conserved MOA in humans, were found because DNA damage in amphipods exposed to FX was lower than that in control organisms. FX was an important source of AChE-inhibiting activity and neurotoxicity for amphipods. Considerable evidence indicated that SSRIs inhibit AChE activity in clams (Munari et al. 2014), marine mussels (Franzellitti et al. 2013), fish (Park et al. 2012), and humans (Müller et al. 2002). The presented data are also in agreement with the neurotoxicity reported in vertebrates and bivalves because a significant decrease of AChE activity was detected in amphipods after FX exposure. The combination of biological end points and bioaccumulation can provide a step forward in this field (Franzellitti et al. 2013).

EE2, a major constituent of many oral contraceptives, inhibited EROD enzymatic activity in amphipods exposed to the highest and lowest concentrations tested. EROD activity was strongly inhibited in carp after the administration of EE2 (Solé et al. 2000). A different pattern was observed for DBF activity, in which the concentration–response exhibited a plateau and was not significantly different for any concentration tested. This fact can be due to the fact that EE2 is an effective mechanism-based inhibitor of CYP 3A4, which is dependent on NADPH and thus irreversible (Lin et al. 2002). Phase II metabolism was not activated by EE2 concentrations; nevertheless, environmental concentrations significantly inhibited GST activity. According to previous studies, EE2 (approximately 10 ng L^{-1} in water) significantly decreased GST activity in calanoid copepods (Clubbs and Brooks 2007). No clear EE2-related response was observed in cytosolic GST measured in carp *Cyprinus carpio* after EE2 injection (Solé et al. 2000). Little is known about the effects of

estrogenic compounds on phase II metabolism and antioxidant enzymes. Solé et al. (2000) indicated an unclear antioxidant response in carp injected with 500 ng L^{-1} of EE2. Therefore, amphipods exposed to EE2-spiked sediment presented a significant decrease of GPX enzymatic activity for all concentrations tested ($p < 0.05$). However, GR and GST activities were not activated after exposure to EE2. Antioxidant enzymes did not show any significant relation with LPO and DNA damage. These data are in agreement with the research performed with Japanese sea bass *Lateolabrax japonicus* after 30 days of exposure to EE2 (Thilagam et al. 2010). LPO significantly decreased in amphipods exposed to sediment samples spiked with EE2. Metabolism of EE2 might lead to the production of semiquinones and quinones, which in turn produces free radicals and oxidative stress through redox cycling (Cavalieri et al. 2000). Although antioxidant enzymes play a key role in preventing cellular damage, cellular ROS accumulation can lead to oxidative stress and result in various types of tissue and genetic damage (Thilagam et al. 2010). Due to this, DNA damage was significantly decreased for the lowest concentrations tested and thus they were dose-responsive concentrations. In contrast, previous studies observed that EE2 causes oxidative DNA damage in fish (Rempel-Hester et al. 2009; Thilagam et al. 2010) and mammals (Wellejus et al. 2004). EE2 was not neurotoxic to amphipods, which corroborated the results of a previous study on freshwater copepods (Souza et al. 2013). Bioaccumulation study on the amphipod *H. azteca* indicated a negligible contribution from sediments to the uptake of EE2 (Dussault et al. 2009). Their results suggested that consumption of invertebrate food items could provide an additional source of exposure to estrogenic substances in vertebrate predators (Dussault et al. 2009).

According to the European Commission directive 93/67/EEC and regulation no. 1488/94, PRO is classified as being "very toxic to aquatic organisms" (Cleuvers 2005). Human and other vertebrates' β-adrenergic receptors are similar, although little is known regarding the presence of this receptor in invertebrates (Solé et al. 2010). Studies performed in crustaceans point to a lack of β-adrenergic receptor (Stanley et al. 2006). However, freshwater cladocerans and amphipods exposed to PRO showed negative growth and reproductive negative effects (Huggett et al. 2002). Therefore, PRO could be eliciting its effect in amphipods through one or multiple receptor classes, for example, the 5HT-1 receptor described for marine mussels (Franzellitti et al. 2014). Concerning CYP1A2, an increase of EROD enzymatic activity was determined in amphipods after exposure to PRO. CYP3A4 activity was significantly increased compared with the control for two concentrations of PRO (0.5 and 5 ng g^{-1}) ($p < 0.05$). Marine mussels exposed for 10 days to a PRO concentration (147 ng L^{-1}) showed enhanced phase I metabolism as measured by carboxylesterases, which also plays a significant role in drug metabolism in humans and vertebrates (Solé et al. 2010). Responses of phase II (GST) enzymatic activity were less pronounced compared with those of phase I. Phase II metabolism and antioxidant enzyme GPX were not activated in amphipods exposed to PRO concentrations. A previous study showed inhibition of GST activity in mantle/gonads and GST induction in digestive gland of marine mussels *M. galloprovincialis* exposed to PRO (Franzellitti et al. 2014). Oxidative reactions of phases I and II metabolism produce free radicals, and normal GSH-to-GSSG ratios can be maintained due to increased GR activities (Van der Oost et al. 2003) because GR activities in amphipods exposed to PRO were significantly greater compared with those of the control organisms ($p < 0.05$) except for at the highest concentration (500 ng g^{-1}). The interaction of ROS with cell membrane produced more LPO, which is a manifestation of oxidative stress (Thilagam et al. 2010). Amphipods exposed to all concentrations of PRO spiked in sediment samples showed a significant increase of LPO ($p < 0.05$). Nevertheless, PRO exposure did not cause oxidative stress (CAT and LPO) in digestive gland of *M. provinciallis* (Solé et al. 2010). Interestingly, clinical and epidemiological studies have suggested that β-blockers have additional human therapeutic benefits (Hara et al. 2013). Hara et al. (2013) showed that administration of the β-blocker PRO prevents behavioural stress-induced accumulation of DNA damage in mice. The present study also showed that PRO significantly decreased DNA strand breaks in amphipods exposed to concentrations of this pharmaceutical product spiked in sediment samples ($p < 0.05$). PRO was not neurotoxic to amphipods. This is in agreement with previous study about the exposure of marine mussels *M. galloprovincialis* to PRO concentrations, in which AChE activity determined in gill tissues was not different compared with the control (Solé et al. 2010). In our study with *A. brevicornis*, sublethal responses associated with PRO exposure suggested its metabolism [increased phase I activity (EROD), oxidative stress (GR), and effects (LPO)] at all concentrations assayed.

CAF is the most widely used drug in the world, and it is a particularly good marker for wastewater contamination (Potera 2012). CYP1A2 is responsible for >95 % of the primary metabolism of CAF (Kalow and Tang 1993). Nevertheless, a decrease of CYP1A2 EROD enzymatic activity was observed in amphipods exposed to the lowest CAF concentration tested. For fish, EROD activity determined in gills filaments was inhibited after CAF exposure (Beijer et al. 2010). CYP3A4 is also part of CAF metabolism (Kalow and Tang 1993). A previous study reported the induction of EROD and DBF activities in gills of crab *Carcinus maenas* exposed to CAF (Aguirre-Martínez et al.

2013b). Therefore, EROD and DBF activities determined in amphipods did not respond to CAF. The removal rate of toxic metabolites by conjugation may be relatively decreased as to the rate of their formation by oxidative reactions (Van der Oost et al. 2003), which may lead to an increase of GST activity. Literature exists regarding the increase of GST enzymatic activities in aquatic invertebrates due to different types of contaminants including pharmaceutical products such as CAF (Martín-Díaz et al. 2009). In contrast, GST activity was not induced in amphipods; the antioxidant system represented by GPX activity was inhibited; and the lowest concentration tested induced GR activity. This metabolism resulted in membrane impairment (LPO) in amphipods. Previous studies with marine crustaceans exposed to CAF-spiked water showed dose-dependent responses for GST, GPX, LPO, and DNA damage (Aguirre-Martínez et al. 2013b). Studies on interactions between CAF, sediment, and benthic biota should elucidate the differences in the CAF dose–response relationship in crustaceans. CAF might inhibit DNA repair and potentiates mutagenic and teratogenic effects (Selby and Sancar 1990; Nehlig and Debry 1994). Studies on other types of DNA damage should elucidate its decrease in amphipods exposed to sediment spiked with CAF concentrations. The lowest concentration of CAF was neurotoxic to amphipods. AChE inhibition can be due the *N*-methyl of the pyrrolidine ring, which is an important binding receptor to AChE, and it makes part of the structural feature of CAF (Karadsheh et al. 1991).

Considerations About Each Biomarker Response

Drugs are generally recognized to induce phase I and II biotransformation enzymes, which results in oxidative stress during the biotransformation process (Laville et al. 2004). Indeed, direct or indirect deactivation of CYP450 can affect the capacity for xenobiotic metabolism or endogenous functions. Further studies could bring more information about the role of EROD and DBF activities in amphipods metabolism of organic contaminants. There have been no previous studies on the determination of DBF activity in amphipods. These responses determined indirectly the presence of CYP450 enzymes in amphipods as previously reported for crustaceans by Aguirre-Martínez et al. (2013a).

A number of studies have shown potential for antioxidant enzymes responses in invertebrate species (Martín-Díaz et al. 2009; Ramos-Gómez et al. 2011). Little attention is given to studies undertaken on crustaceans (Aguirre-Martínez et al. 2013b) and about GR activity in amphipods. The results of the current study suggest further investigations concerning the importance of GR activity response in amphipods as well as more studies about other antioxidant responses in crustaceans that could be activated by pharmaceutical products.

Between the free-radical formation and consequences, the present study determined the possible effects on transmission of nervous impulses (AChE activity), LPO of membranes, and genetic damage (formation of DNA strand breaks). The AChE enzyme is involved in the proper coordination of neuromuscular transmission; plays a role in the deactivation of acetylcholine at nerve endings, thus preventing continuous nerves firings; and has a vital function for normal functioning of the sensory neuromuscular system (Van der Oost et al. 2003). In the present study, a significant increase or decrease of AChE activity in animals exposed to pharmaceutical-spiked sediments suggested neurotoxic impact, whereas significant correlations between AChE and oxidative biomarkers (GPX) indicated connections with redox state regulation, which was previously reported by Montserrat et al. (2007). All of the pharmaceutical products showed a potential for neurotoxicity and are important sources of AChE-inhibiting and -inducing compounds.

Of particular interest are the reduction products of molecular oxygen, which may react with critical cellular macromolecules, thus possibly leading to enzyme inactivation, LPO, DNA damage, and ultimately cell death (Winston and Di Giulio 1991). Except for CBZ, all pharmaceutical products tested in the present study showed a decrease of DNA strand breaks. Exposure to toxic chemicals may cause secondary concomitant types of DNA alterations. The repair of DNA strand breaks could be considered because the genetic damage measured was in the mitochondrial DNA. However, an increase in the level of DNA repair can be considered as damage (Shugart et al. 1992) leading to mutations and diseases. Studies concerning DNA strand break repair are scarce even for humans. Further studies concerning the role of pharmaceuticals in DNA damage repair in aquatic organisms should be elucidated.

Conclusion

In summary, amphipods have a number of characteristics that make them useful indicators of exposure, such as sensitivity to environmental disturbance such as pharmaceutical product pollution. The short-term assay to determine amphipod mortality rate is a frequently used test and is a suitable tool for monitoring pharmaceuticals bound to sediments. Lethal and sublethal effects of pharmaceutical-spiked sediments were determined in adult individuals of *A. brevicornis* exposed under laboratory conditions. Biomarkers were helpful for gaining insights regarding the mechanisms and observed effects of chemicals on whole-

organism performance. Our results showed that pharmaceutical products released in marine systems may affect physiological mechanisms that decrease organism fitness, which is of great concern in genotoxic and neurotoxic risks. Pharmaceutical products at concentrations currently found in the environment are sufficient to cause a wide variety of effects (based on laboratory studies). The assessment of long-term exposure to environmentally relevant levels of pharmaceuticals is recommended to characterize the ecological significance of pharmaceutical contamination in the environment. The battery of biomarkers tested herein showed bioavailability of the following pharmaceutical products to the benthic biota as follows: phases I and II detoxification enzymatic activities were involved in the metabolism of IBP and PRO; oxidative stress was caused by PRO and CAF; alteration of DNA strand breaks in amphipods exposed to IBP, FX, EE2, PRO, and CAF; and neurotoxicity of FX. In this study, pharmacodynamic effects may potentially induce or inhibit degradation metabolism or effects in amphipods compared with the MOA known for human and other mammals. Additional information is necessary before biomarker responses can be understood correctly. Thus, studies on bioaccumulation, molecular indicators, and the toxicity of the metabolites should bring complementary information to the present study.

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Bioavailability, oxidative stress, neurotoxicity and genotoxicity of pharmaceuticals bound to marine sediments. The use of the polychaete *Hediste diversicolor* as bioindicator species

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ABSTRACT

A set of "early warning responses", measured as biomarkers of exposure and effect, was applied in the marine bioindicator *Hediste diversicolor*, in a way to assess the environmental quality of sediment affected by pharmaceutical contamination. Sublethal responses were determined in the sea-worms after 14-days of exposure to sediment spiked with some of the most representative pharmaceutical products found in the environment: carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethynylestradiol (EE2) and propranolol (PRO), including the environmental concentrations. Phases I (ethoxyresorufin O-deethylase – EROD and dibenzylfluorescein dealkylase – DBF) and II (glutathione S-transferase – GST) of the metabolism, antioxidant system (glutathione peroxidase – GPX and glutathione reductase – GR), neurotoxicity (acetylcholinesterase – AChE) and oxidative effects (lipid peroxidation – LPO and DNA damage *strand breaks*) were selected to evaluate the sublethal responses in the sea-worms. FX, EE2 and PRO were detoxified by the phase I of the metabolism (EROD activity). On the other hand, phase II (GST-activity) did not respond in sea-worms exposed to pharmaceutical products, except for the environmental concentrations of CBZ (activation) and PRO (deactivation). Neurotoxicity was induced in sea-worms exposed to EE2 (only the environmental concentrations), FX, IBP and CBZ. Oxidative effect determined as LPO increased in sea-worms exposed to environmental concentrations of IBP, EE2 and PRO. Genetic damage increased in sea-worms exposed to IBP and diminished for FX, EE2 and PRO. Our results indicated the toxicity of pharmaceutical products and recommended the battery of biomarkers and the bioindicator specie *H. diversicolor* for the environmental quality assessment of sediment affected by pharmaceutical contamination.

1. Introduction

Pharmaceutical products are found in the sediment at concentrations much higher than the water column or wastewater treatment plants (WWTPs) effluents (Ternes et al., 2002; Löffler and Ternes, 2003; Hernando et al., 2006; Pintado-Herrera et al., 2013). This means that sediment can work as a sink and long-term source of these compounds in the environment (Gilroy et al., 2012). However, there is a lack of knowledge about the bioavailability and possible adverse effects on the aquatic biota. Guidelines for sediment quality assessment and monitoring did not include pharmaceuticals as contaminants of concern (CEDEX, 1994; USACE, 1998; USEPA, 1998a, 1998b; GIPME (Global Investigation of Pollution in the Marine Environment), 2000; SEDNET, 2003). Therefore, previous studies confirmed the possible adverse effects in non-target organisms (Martín-Díaz et al., 2009; Franzellitti et al., 2012, 2013; Aguirre-Martínez et al., 2013a, 2013b). The biological effects can be established based on laboratory tests that determine toxic responses (Moralles-Caselles et al., 2007). Analytical protocols established by USEPA (1994) and USACE (1991, 1998) for risk assessment of dredged material recommended short-term acute toxicological bioassays of sediments on aquatic invertebrates, including polychaetes.

However, acute toxicity is often not prominent at low concentrations of contaminants as pharmaceuticals, which due to their

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persistence in the environment may contribute to sublethal toxicity. The lacks of knowledge and suitable tools to evaluate sublethal responses in marine benthic species impede a proper ecotoxicological assessment for the sediment quality monitoring of emergent pollutants.

Hediste diversicolor has been the subject of wide research and recommended for sediment sublethal toxicity studies (Durou et al., 2007; Lewis and Galloway, 2008; Solé et al., 2009; Kalman et al., 2010; Gomes et al., 2013). Sublethal measurements are useful to evaluate the bioavailability of pharmaceuticals in the aquatic environment and the possible oxidative exposure and effects. It is not from our knowledge, previous studies concerning responses of sea-worms exposed to pharmaceutical products.

Pharmaceutical compounds have been recently identified as potentially toxic for aquatic organisms (Quinn et al., 2008a, 2008b; Martín-Díaz et al., 2009; Eades and Waring, 2010; Oviedo-Gómez et al., 2010; Parolini et al., 2011; Aguirre-Martínez et al., 2013a, 2013b). The present paper focused on compounds with different chemical properties and modes of action (MOA), representative of a range of pharmaceutical classes: carbamazepine (anti-epileptic and neuroactive drug, CBZ), ibuprofen (non-steroidal anti-inflammatory drug – NSAIDs, IBP), fluoxetine (selective serotonin reuptake inhibitor – SSRIs, anti-depressive drug, FX), 17α-ethynylestradiol (oral female contraceptive, steroid drug, EE2) and propranolol (β-blocker drug, PRO). These compounds are among the most frequently detected in the environment (Daughton and Ternes, 1999; Heberer, 2002; Beausse, 2004; Díaz-Cruz and Barceló, 2004; Hernando et al., 2006). They become neutral or ionized (positive or negative) depending on the pH of the environment. Sorption of pharmaceuticals onto sediment is highly influenced by the type and degree of ionization. The limited information available on their sorption/desorption onto/from sediments point to a special concern for their environmental fate (Martínez-Hernández et al., 2014).

Once the pharmaceutical compound is inside the organism, the activities of the biotransformation enzymes of the phases I (ethoxyresorufin O-deethylase – EROD and dibenzylfluorescein dealkylase – DBF) and II (glutathione S-transferase – GST) of the metabolism are activated to transform the parent compound in an easier extractable one. Phase I activity (DBF) was induced in gill and hepatopancreas tissues of crabs exposed to CBZ (Aguirre-Martínez et al., 2013b) and in mussels injected with CBZ (Martín-Díaz et al., 2009). Phase II activity (GST) was activated by IBP in gill, hepatopancreas and muscle of crabs (Aguirre-Martínez et al., 2013a). Also PRO and FX were responsible for the increase of phase II activity in mussels *Mytilus galloprovincialis* (Franzellitti et al., 2011, 2013).

Phases I and II are formed undergo a variety of reactions which produces oxygen free radicals and metabolites. In this way, the antioxidant system (glutathione peroxidase – GPX and glutathione reductase – GR) plays an especial role in protecting membranes from damage. Aguirre-Martínez et al. (2013b) observed an increase of GPX activity following the CBZ concentration in crabs *Carcinus maenas*. Li et al. (2011) also observed higher antioxidant activities of SOD, CAT, GR and GPX after 96 h of exposure of fish *Oncorhynchus mykiss* to CBZ.

Biochemical biomarkers are useful tools for predicting significant or permanent long-term damage, as neurotoxicity (determined here by acetylcholinesterase – AChE activity), lipid peroxidation (LPO) and DNA damage (*strand breaks*). The increase of LPO and DNA damage in crabs *C. maenas* exposed to increasing concentrations of IBP and CBZ were described by Aguirre-Martínez et al. (2013a). LPO was induced in mussels *Elliptio complanata* injected with CBZ (Martín-Díaz et al., 2009). Considerable evidence indicated that SSRIs drugs inhibit AChE activity in humans (Müller et al., 2002), fishes (Park et al., 2012), marine mussels (Franzellitti et al., 2013) and clams (Munari et al., 2014).

The aims of the following research were (1) to assess potential toxicity of different pharmaceutical products spiked on marine sediment samples integrating biochemical responses; (2) to evaluate the feasibility of the biomarker approach and the use of the bioindicator *H. diversicolor* for coastal sediment quality assessment related to pharmaceutical pollution.

2. Materials and methods

2.1. Pharmaceutical compounds

Briefly, an overview of physical chemical characteristics of CBZ, IBP, FX, EE2 and PRO was shown in Table 1. All reagents used in the present study were purchased from Sigma-Aldrich® (Madrid, Spain).

Such compounds are largely prescribed for human use. They were ranked in the top 300 prescriptions list in the United States (http://www.rxlist.com/script/main/art.asp?articlekey=79510). Previous studies detected these pharmaceuticals in marine sediment compartment (Ternes et al., 2002; Hernando et al, 2006; Schultz et al., 2010; Pintado-Herrera et al., 2013; Zhou and Broodbank, 2014). Selected pharmaceuticals have different modes of action (MOA) in target organisms. In the environment, they differ in water solubility rates (Sw), lipophilic characteristics, $\log K_{ow}$ (octanol–water distribution coefficient), and $\log K_{oc}$ (adsorption coefficient). USEPA (2003) classifies $\log K_{oc}$ values in "very strong" (>4.5), "strong" (3.5–4.5), "moderate" (2.5–3.4), "low" (1.5–2.4), and "negligible" (<1.5) sorption capacities, suggesting that CBZ has "moderate", IBP has "low", PRO has "strong", and EE2 and FX have "very strong" sorption capacities.

Pharmaceuticals vary among their rates of degradation with reported half-lives (Guler and Ford, 2010). The half-lives determined inside the target organism are different of the half-lives of such compounds in the sediment, under controlled temperature and in the dark. For example, CBZ, IBP and PRO have the half-life inside the human body of around 36, 3 and 5 h. Nevertheless, a previous study showed that the half-lives of these compounds, after photolysis and biodegradation experiments, were much higher than that inside the organism (Yamamoto et al., 2009). It was assumed that no degradation products were transformed, since the half-lives of CBZ (Löffler et al, 2005; Lam et al., 2004), IBP (Beausse, 2004), FX (Lam et al., 2004), EE2 (Mes et al., 2005; Beausse, 2004) and PRO (Andreozzi et al., 2003) were higher than the time of exposure used in the present study.

2.2. Sediment and spiking the test substances

Spiking of the pharmaceutical products was performed using the natural marine sediment sampled in an intertidal creek area that is part of the Natural Park "Bahía de Cádiz" (SW, Spain). This area was considered reference by previous studies (Pérez et al., 2004; Solé et al., 2009; Basallote et al., 2012; De Orte et al., 2013).

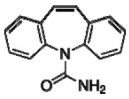
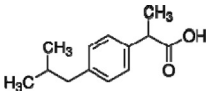
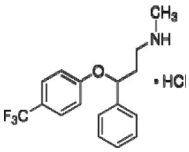
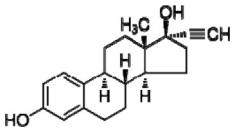
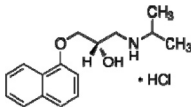
The topmost 10 cm layer of the sediment was sampled in the field and sieved through a 2-mm mesh to remove large debris and other living organisms. Sediment samples were dried at 70 °C in a way to eliminate water, part of the organic matter and volatile compounds. Dry-sediment is recommended for sediment spiking procedure (OECD, 2000). The same volume of seawater lost was added back to the sample. Overlying seawater was obtained from "Experimental Marine Aquaculture Plant – Cultivos Marinos" at the University of Cádiz. This water has been used for the culture and maintenance of test organisms (fish, molluscs and plankton) since 2002.

Physical chemical characteristics of the reference sediment from Río San Pedro were summarized in Table 2. For sediment grain size, an aliquot of dry sediment was analyzed by following the methodology recommended by Poppe et al. (2013 U. S. Geological Survey Open-File Report 00-358). Total organic carbon (TOC) and organic matter (OM) content were determined applying the methods reported by USEPA (2002).

Pharmaceutical-spiked sediments were initially prepared in 3-L glass beakers, mixed by homogenization using a Teflon spatula and subsequently mixed for 30 min in a bottle roller. This methodology was an adaptation of ASTM (2000), USEPA (2001) and OECD (2004) protocols. The pharmaceutical-spiked sediments were incubated for 7 days (4 °C in the dark) to ensure equilibration of the test substance between water and sediment in the preparation sealed bottle (Francis et al., 1984; Löffler et al, 2005). After the equilibration period, it was done the sediment dilution to obtain the concentrations proposed for the bioassay.

Five nominal concentrations of each pharmaceutical product were tested, including the environmental concentrations (underlined) based on previous studies (Ternes et al., 2002; Hernando et al, 2006; Schultz et al., 2010; Pintado-Herrera et al., 2013; Zhou and Broodbank, 2014): CBZ, IBP and PRO (500 ng g^{-1}, 50 ng g^{-1}, 5 ng g^{-1}, 0.5 ng g^{-1}, 0.05 ng g^{-1}), FX and EE2 (100 ng g^{-1}, 10 ng g^{-1}, 1 ng g^{-1}, 0.1 ng g^{-1}, 0.01 ng g^{-1}). Pharmaceutical stock solutions were prepared using the solvent DMSO 0.001% (v/v) as recommended by Quinn et al. (2008a, 2008b), Eades and Waring (2010) and Aguirre-Martínez et al. (2013a, 2013b). Two

Table 1
Overview of physical chemical characteristics of pharmaceuticals products (carbamazepine – CBZ, ibuprofen – IBP, fluoxetine – FX, 17α-ethynylestradiol – EE2, propranolol – PRO). Sw = water solubility, p*Ka* = acidity constant, log K_{ow} = octanol–water distribution coefficient, log K_{oc} = adsorption coefficient.

CAS no.	**CBZ** 298-46-4	**IBP** 15687-27-1	**FX** 56296-78-7	**EE2** 57-63-6	**PRO** 318-98-9
Therapeutic class	Anticonvulsant	Anti-inflammatory	Antidepressant	Oral contraceptive	Cardiac disorder
Developed formula					
Molecular weight (g mol^{-1})	236.27	206.28	345.79	296.4	295.8
Sw (mg L^{-1})	Insoluble[a]	100,000[a]	4000[a]	4.8–11.3[f]	50,000[a]
p*Ka*	14[b]	4.52[b]	9.39[e]	10.4	9.5[h]
log K_{ow}	1.51[b]	2.48[b]	1.22[d]	3.6–4.2[f]	1.2–3.48[i]
log K_{oc}	2–3.42[b]	2.14–2.21[b]	5.18–4.31[d]	3.19–3.86[m]	9.4[l]
Pharmacokinetic excretion rate	1–2% unchanged[c]	1–8% unchanged[c]	NA	Up 80% not-metabolized[g]	NA
Total removal via STP	7%[c]	60%[j]	NA	NA	96%[j]

NA: no data available.

[a] www.sigmaaldrich.com.
[b] Scheytt et al. (2005).
[c] Ternes et al. (1998).
[d] Kwon and Armbrust (2008).
[e] Vasskog et al. (2006).
[f] Nagpal and Meays (2009).
[g] Mes et al. (2005).
[h] Yu and Chu (2009).
[i] Lin et al. (2010).
[j] Yamamoto et al. (2007).
[l] Yamamoto et al. (2009).
[m] Sun et al. (2012).

Table 2
Physical chemical properties of sediment used for the pharmaceutical spiking procedure.

Properties	**Percentage (%)**
Gravel	0.84
Sand	57.39
Fines	41.77
OM	8.07 ± 0.34
TOC	1.20 ± 0.09

controls were run in parallel with the experiment: reference sediment (control) and reference sediment spiked with DMSO 0.001% (v/v) (solvent control).

2.3. Determination of pharmaceutical concentrations in spiked sediments

The highest concentration of the selected pharmaceuticals was measured in spiked sediments following a modification of the method proposed by Jelic et al. (2009). Briefly, 2 g of sediment were place inside 11 mL stainless steel cells and extracted by pressurized liquid extraction using a ASE 200 unit (Dionex) and methanol/water 1/2 (v/v) as solvent. After extraction, samples were evaporated and reconstituted in methanol/water 25/75 (v/v) and internal standards (carbamazepine d10, atenolol d7, naproxen d3) were added at 50 ng mL^{-1}. Further details can be found in Jelic et al. (2009). The main modification of the method was that a different ultra-performance liquid chromatography (UPLC)–tandem mass spectrometry (MS/MS) system was used, consisting of a Bruker EvoQ Elite coupled to a UPLC Bruker Advance. Separation of target compounds was achieved using a Bruker Intensity Trio C18 column (100 × 2 mm^2, 1.9 μm internal diameters) and methanol (A) and water (B) as solvents. The gradient was as follows (flow = 0.4 mL min^{-1}): 5% B for 0.4 min, increased linearly to 30% in 0.1 min, increased linearly to 95% in 4.5 min, and then kept for 3 min. Multiple monitoring reaction (MRM) transitions had to be optimized for target compounds. CBZ, FX and PRO were monitored in positive ionization mode, whereas IBP and EE2 were determined in negative ionization mode. Source parameters were spray voltage 4500 V, cone temperature 250 °C, cone gas flow 20 mL min^{-1}, probe temperature 450 °C, probe gas flow 50 mL min^{-1} and nebulizer gas flow 60 mL min^{-1}. Measured concentrations of target compounds in spiked sediments and MRM transitions are shown in Table 3.

Table 3
Nominal and real concentrations (ng g^{-1}) of the pharmaceutical products spiked in the sediment (Day-0). MRM transitions are also shown.

Target compound	MRM transitions	Nominal concentration	Measured concentration
CBZ	237.1 > 193.8 (22 V) 237.1 > 192.4 (31 V)	500	505 ± 10
IBP	205.1 > 162.1 (5 V) 205.1 > 160.0 (5 V)	500	405 ± 50
FX	310.1 > 44.6 (10 V) 310.1 > 148.4 (7 V)	100	90 ± 8
EE2	295.3 > 145.2 (36 V) 295.3 > 158.3 (36 V)	100	121 ± 25
PRO	260.1 > 116.2 (17 V) 260.1 > 72.9 (21 V)	500	515 ± 47

Concentrations of the five selected pharmaceuticals in non-spiked sediments were below 1 ng g^{-1}. Therefore, nominal concentrations are presented throughout this manuscript.

2.4. Species selection

Burrowing benthic invertebrates play an important role in modifying their habitat reducing sediment stability. The marine intertidal polychaetes *H. (=Nereis) diversicolor* (O.F. Müller, 1976) is widely distributed in intertidal soft-sediments in European and Atlantic North American coasts. It is an opportunistic omnivore and can be a predator, surface deposit or suspension feeders, depending on the impacts on fauna and environment around them. In areas impacted by sewage inputs, polychaetes are predominantly surface deposit feeders on organic matter and benthic algae, whereas at cleaner sites where benthic food is less abundant, it will rely more on suspension feeding (Aberson, 2010). Polychaetes have been extensively used for measuring the toxicity of water and sediment matrices associated with marine/estuarine environments, frequently restricted to acute lethality of trace metals and organic contaminants (PAHs and PCBs) (Environment Canada, 2001).

2.5. Exposure conditions

Individuals of *H. diversicolor* were gently hand-picked at low tide from intertidal estuarine mudflats between Chiclana de la Frontera and San Fernando (SW, Spain). This area is located at the Natural Park "Bay of Cádiz". After collection, individuals were transported to the laboratory placed in cold containers, with wet algae from the site of origin. Specimens were maintained in aerated aquariums filled with the natural filtered seawater and reference sediment for less than one week before testing.

Test vessels were 4-L glass aquaria containing the proportion 1:4 (sediment: water). A total of 10 sea-worms were randomly selected and added to each test vessel. The 14-days worm sediment test was conducted according to the methods provided in "Standard guide for conducting sediment toxicity test with polychaetes annelids" (American Society for Testing and Materials (ASTM), 2009; Thain and Bifield, 2001). Three replicates per treatment were performed. Vessels were maintained at controlled temperature (18 ± 2 °C), in the dark, under laboratory conditions. Physicalchemical parameters (oxygen saturation$=7.3 \pm 0.3$ mg L^{-1}, pH$=8.13 \pm 0.1$, salinity$=35.6 \pm 0.8$) were monitored each 2 days until day-14. In the end of the bioassay, the organisms were recovered from the replicates and counted. Endpoint measured at the completion of the assay was mortality. All living organisms were transferred to clean test vessels, and maintained overnight in clean seawater for removal of sediment from the animals' digestive tract. After the depuration period, animals were carefully rinsed with clean seawater and stored at −80 °C till further analysis.

2.6. Samples procedure for biochemical determination

Organisms from the same replicate were pooled before homogenization. Each poll was homogenized in 1:3 volumes of buffer (pH 7.5) containing 100 mM NaCl, 25 mM Hepes-NaOH, 1 mM EDTA and 1 mM dithiotreitol (DTT) (Gagné et al., 2007). Homogenized, S3 (homogenized samples centrifuged at 3000*g* for 20 min) and S15 (homogenized samples centrifuged at 15,000*g* for 20 min) fractions were used for biochemical determinations. All the process was carried out on ice.

2.6.1. Protein concentration

Total protein content was determined following the method described by Bradford (1976), using bovine serum albumin as standard. All the biomarkers responses were normalized by the total protein content according to their respective extract (homogenized, S3 or S15).

2.6.2. Biomarkers of exposure

Phase I was determined through EROD and DBF enzymatic activities. Ethoxyresorufin O-deethylase (EROD) activity was evaluated by methodology adapted by Gagné and Blaise (1993). The transformation of 7-hydroxyresorufin in resorufin at fluorescence 485 nm (excitation) and 580 nm (emission) during the oxidation of NADPH to $NADP^+$ was the indicative of the EROD enzymatic activity.

Dibenzylfluorescein dealkylase (DBF) activity was evaluated by methodology adapted by Gagné et al. (2007). The transformation of dibenzylfluorescein in fluorescein was measured at 485 nm (excitation) and 516 nm (emission) during the oxidation of NADPH to $NADP^+$. The results of both enzymatic activities were expressed as pmol min^{-1} m g^{-1} proteins.

Phase II was determined following the procedure used for glutathione S-transferase (GST) activity determination, described by McFarland et al. (1999). The enzymatic activity was measured by the detoxification reaction of chloro-dinitrobenzene (CNDB) through glutathione S-transferase. Absorbance (340 nm) was determined each 5 min for 30 min. The activity was expressed as OD GST min^{-1} m g^{-1} proteins.

Antioxidant defense enzymatic activities were determined following the methodology proposed by McFarland et al. (1999). Glutathione peroxidase (GPX) enzymatic activity was determined by the reduction of hydroperoxides (ROOH) forming oxidized glutathione (GSSG). The absorbance (340 nm) was measured every 30 s for 30 min. Glutathione reductase (GR) enzymatic activity was the measurement of the reduced glutathione (GSH) regeneration. The absorbance (340 nm) was measured every 2 min for 10 min. GPX activity was expressed as μmol min^{-1} m g^{-1} proteins, and GR activity was expressed as pmol min^{-1} m g^{-1} proteins.

Acetylcholinesterase (AChE) activity was determined using the method described by Ellman et al. (1961), adapted by Guilhermino et al. (1996). AChE enzymes degrade acetylcholine in ticoline and acetate. The absorbance was measured at 412 nm every 5 min for 20 min. Results were expressed as nmol DTNB min^{-1} m g^{-1} proteins.

2.6.3. Biomarkers of effect

Lipid peroxidation (LPO) was determined by absorbance (540 nm) using thiobarbituric acid (TBA). The method was described by Wills (1987). LPO was expressed as μg TBARS m g^{-1} proteins.

DNA damage was assessed by alkaline precipitation assay based on the K_2SDS precipitation of DNA–protein crosslink. DNA *strand breaks* were quantified by fluorescence at 360 nm (excitation) and 460 nm (emission) following the method described by Gagné et al. (1995). DNA damage was expressed as μg DNA m g^{-1} proteins.

2.7. Statistical approach

At least six polychaetes ($n \geq 6$) from each replicate were used for the biomarker determination. Biomarkers responses were analyzed using the SPSS/PC 21.0+statistical package. Data sets were evaluated to determine normality of distribution and homogeneity of the data. Mortality and sublethal responses of *H. diversicolor* were subsequently evaluated using one-way Analysis of Variance (ANOVA) followed by Dunnett's test. Statistical difference was set up at $p < 0.05$. Significant correlations were examined by Spearman's rank correlation analysis. The significance level was set up at $p < 0.01$ and $p < 0.05$.

Biological index was calculated for the environmental concentrations (0.5 ng g^{-1} for CBZ, IBP and PRO; 0.1 ng g^{-1} for FX and EE2). The biochemical responses were subdivided in 4 groups: exposure (phases I and II), oxidative stress (antioxidant enzymes), effects (LPO and DNA damage) and neurotoxicity (AChE enzymatic activity). To calculate the index, the response of each biomarker replicate was divided by the control average. It was applied the one-way Analysis of Variance (ANOVA) followed by Dunnett's test.

3. Results

3.1. Mortality of polychaetes

Following the experimental period of 14 days, significant mortality of *H. diversicolor* was observed in replicates of sediment samples spiked with CBZ (500 ng g^{-1}, 50 ng g^{-1}), FX (1 ng g^{-1}) and EE2 (100 ng g^{-1}, 10 ng g^{-1}) (Fig. 1) ($p < 0.05$). Concentrations of CBZ ($r = -0.949$) and EE2 ($r = -0.900$) were negatively correlated with mortality ($p < 0.05$).

3.2. Biochemical biomarkers

Specimens of *H. diversicolor* were analyzed to determine biochemical biomarkers responses after 14 days of exposure to sediments spiked with human pharmaceutical products. The results indicated no significant difference of the investigated biomarkers in sea-worms exposed to two controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) ($p < 0.05$).

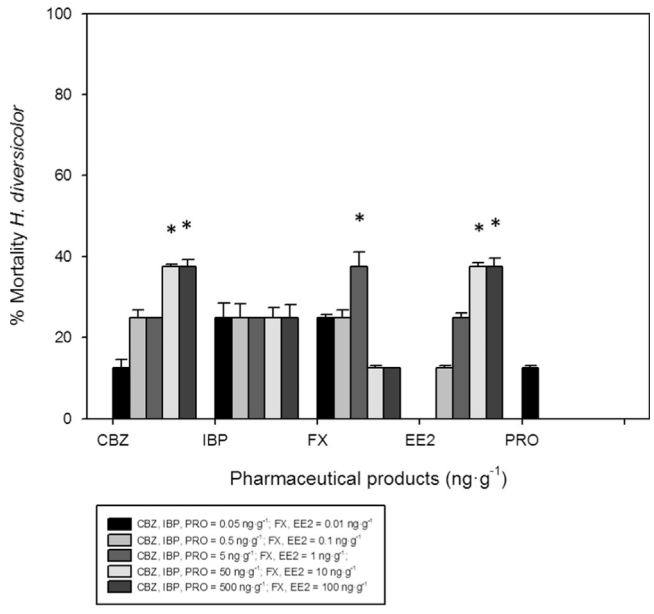


Fig. 1. Mortality rate of polychaetes *H. diversicolor* exposed to five pharmaceutical compounds spiked in whole sediment samples. *=significance difference set up at $p < 0.05$. There was no mortality of polychaetes in the control samples.

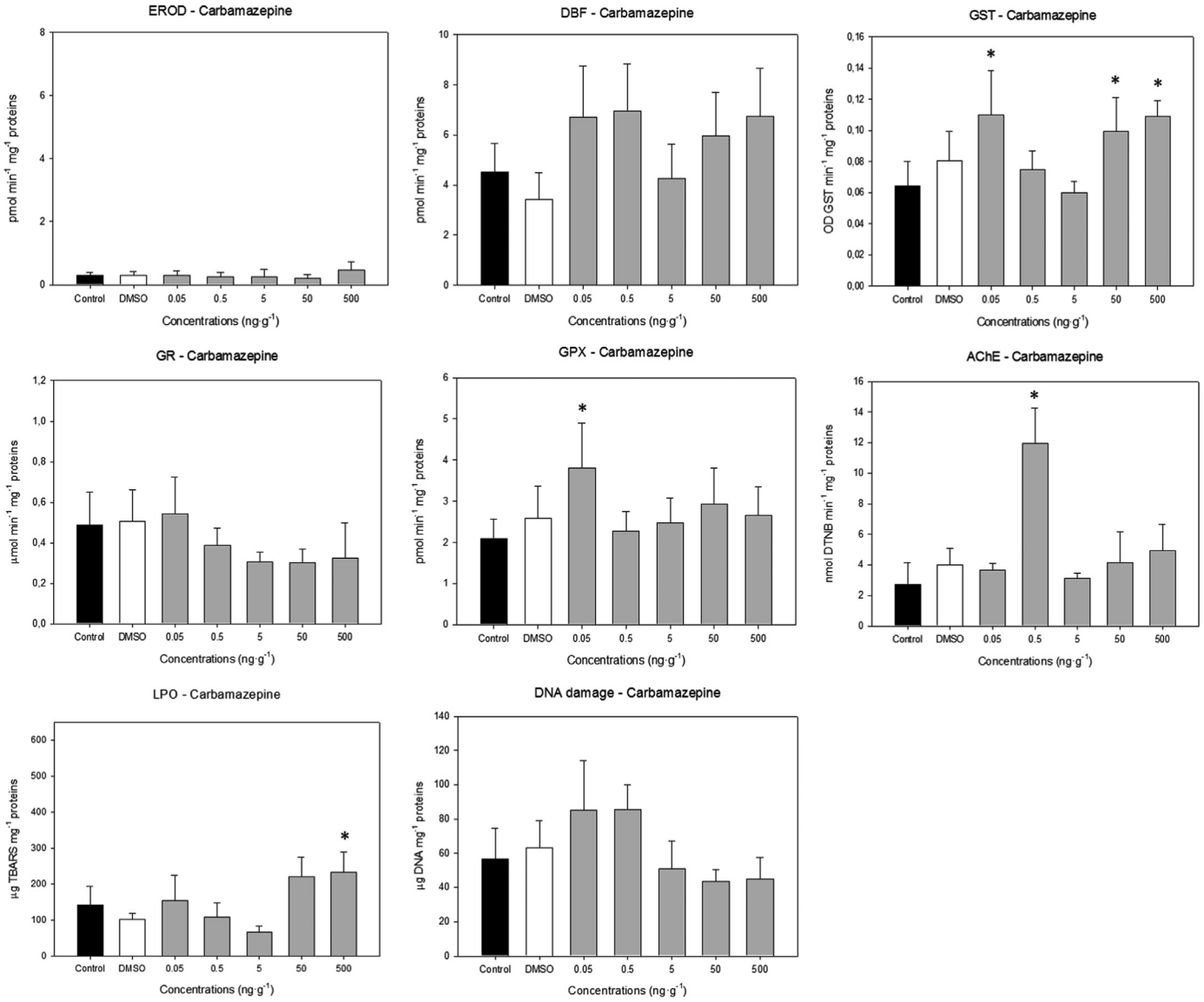


Fig. 2. Mean and standard deviation values of ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), acetylcholinesterase (AChE) enzymatic activities and lipid peroxidation (LPO) and DNA damage (*strand breaks*) values, analyzed in *Hediste diversicolor* after 14-days laboratory exposure to controls and spiked sediment samples [Carbamazepine (CBZ): 500 ng g^{-1}, 50 ng g^{-1}, 5 ng g^{-1}, 0.5 ng g^{-1} and 0.05 ng g^{-1}]. * represent significant differences compared between the control (reference sediment) ($p < 0.05$) and the pharmaceutical concentration, for each biochemical parameter.

The metabolism of CBZ (Fig. 2) activated the phase I concerning EROD and mainly DBF activities. However, no significant induction compared with the control was observed. Phase II was induced by CBZ. Significant induction was observed after exposure to the lowest (0.05 ng g^{-1}) and the highest (50 ng g^{-1} and 500 ng g^{-1}) exposure concentrations ($p < 0.05$). The most activated antioxidant enzyme for this pharmaceutical was GPX activity, which showed significantly increased activity for polychaetes exposed to 0.05 ng g^{-1} ($p < 0.05$). LPO increased with the concentration and DNA damage decreased following the concentration. Significant LPO compared with the control was determined for the highest (500 ng g^{-1}) CBZ exposure concentration ($p < 0.05$). CBZ did not cause DNA damage, which decreased following the concentration, although no significant differences were observed when compared with the control. CBZ did not produce neurotoxicity, except for the increase of the enzymatic activity in polychaetes exposed to the concentration of 0.5 ng g^{-1} ($p < 0.05$). AChE was positively correlated with DBF activity ($r = 0.900$, $p < 0.05$).

Regarding IBP (Fig. 3), phases I and II were not induced. Significant decrease of DBF activity was determined in polychaetes exposed to IBP concentrations of 500 ng g^{-1} and 0.5 ng g^{-1} ($p < 0.05$). EROD activity was broadly similar to the control. Phase II (GST) enzymatic activity decreased with the concentration in polychaetes exposed to IBP. For the antioxidant system, IBP concentration was negatively correlated with GR activity ($r = 0.900$, $p < 0.05$). However, GPX activity did not respond to the concentrations tested of IBP. GPX and DBF enzymatic activities were negatively correlated ($r = -0.900$, $p < 0.05$). Oxidative stress that may not be reduced properly by the antioxidant metabolism might lead to LPO and DNA damage. Individuals exposed to 5 ng g^{-1} of IBP showed significantly higher LPO than the control ($p < 0.05$). Sea-worms exposed to the concentration of 500 ng g^{-1} and 5 ng g^{-1} showed significantly higher DNA damage compared with the control ($p < 0.05$). AChE activity and DNA damage were positively correlated ($r = 0.900$, $p < 0.05$). AChE activity measured in polychaetes exposed to IBP was significant different compared

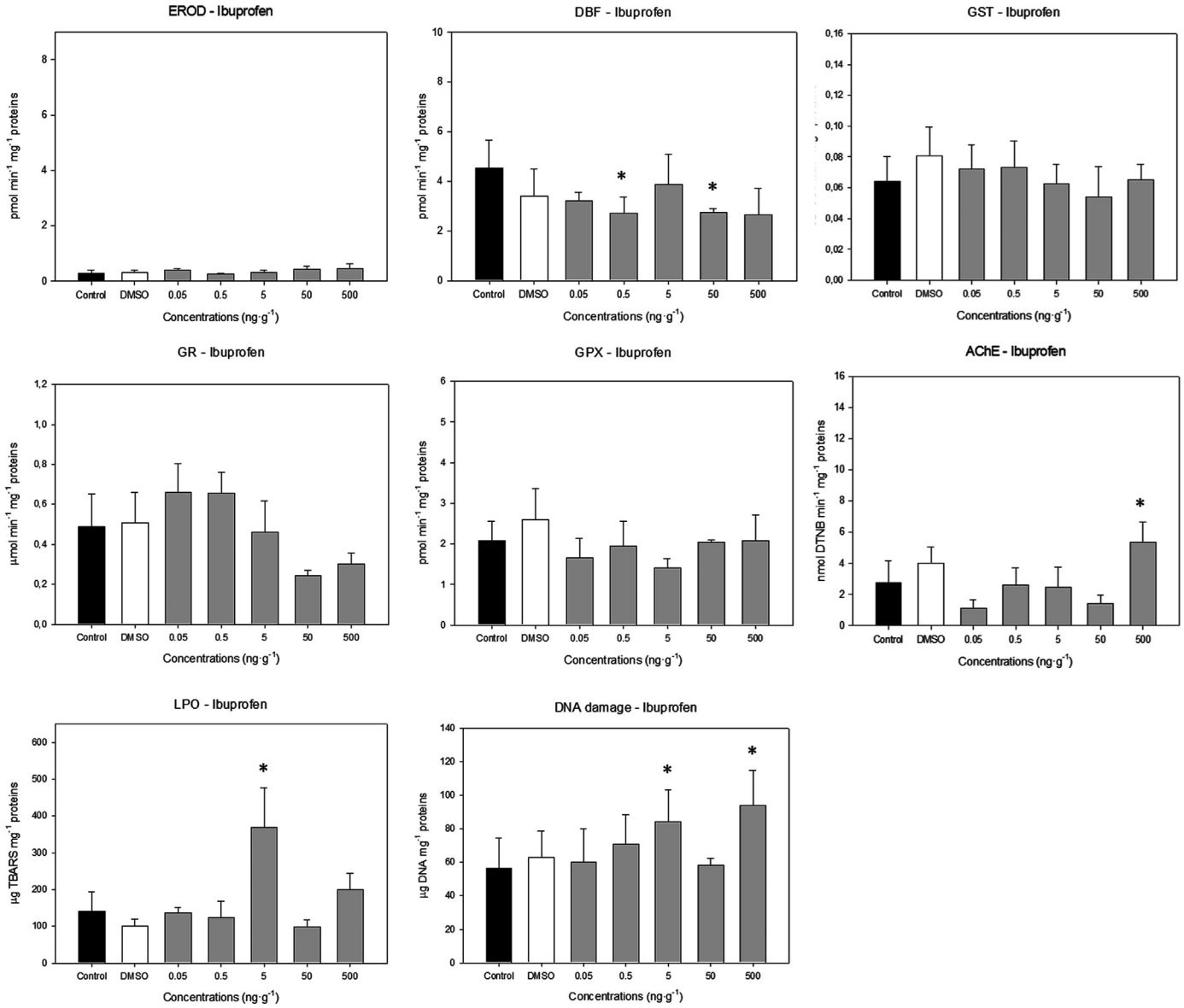


Fig. 3. Mean and standard deviation values of ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), acetylcholinesterase (AChE) enzymatic activities and lipid peroxidation (LPO) and DNA damage (*strand breaks*) values, analyzed in *Hediste diversicolor* after 14-days laboratory exposure to controls and spiked sediment samples [Ibuprofen (IBP): 500 ng g^{-1}, 50 ng g^{-1}, 5 ng g^{-1}, 0.5 ng g^{-1} and 0.05 ng g^{-1}]. * represent significant differences compared between the control (reference sediment) ($p < 0.05$) and the pharmaceutical concentration, for each biochemical parameter.

with the control only for polychaetes exposed to 500 ng g^{-1} ($p < 0.05$).

Biochemical responses determined after the exposure to FX are shown in Fig. 4. FX was detoxified by the phase I (EROD) enzymatic activity. Significant induction compared with the control was observed for all concentrations tested ($p < 0.05$). DBF activities showed similar values with the control. Phase II was not activated. Between the antioxidant enzymes, GR activity responded better with the increase of the FX concentration than GPX activity. Significant GR activity activation was observed after exposure to 1 ng g^{-1} of FX ($p < 0.05$). GST and GR enzymatic activities were positively correlated ($r = 0.900$, $p < 0.05$). LPO and DNA damage increased with the concentration, nevertheless, this increase was not significant. LPO and DBF activity were positively correlated ($r = 1.00$, $p < 0.01$). Sea-worms exposed to the highest concentration of FX (100 ng g^{-1}) showed significantly higher LPO than the control ($p < 0.05$). Oxidative effects measured through DNA damage *strand breaks* significantly decreased in polychaetes exposed to FX all concentrations tested ($p < 0.05$). FX concentration was positively correlated to AChE and GPX activities ($r = 0.900$, $p < 0.05$). Significant induction of AChE activity compared with the control ($p < 0.05$) was observed in polychaetes exposed to 100 ng g^{-1} and 10 ng g^{-1} of FX. AChE and GPX activities were positively correlated ($r = 1.00$, $p < 0.01$).

Fig. 5 showed the biomarker responses determined in polychaetes after the exposure to sediment spiked with EE2. EE2 was detoxified by the phase I of the metabolism (EROD activity), which showed significant activation compared with the control for the sea-worms exposed to 10 ng g^{-1}, 1 ng g^{-1}, 0.1 ng g^{-1} and 0.01 ng g^{-1} ($p < 0.05$). Individuals exposed to EE2 did not present significant increase of DBF activity. Phase II was not activated. GST activity and mortality were negatively correlated ($r = -0.900$, $p < 0.05$). Between the antioxidant enzymes, GR activity responded better with the increase of the EE2 concentration than GPX activity. GR activity was significantly activated when compared with the control for 0.1 ng g^{-1} EE2 concentration ($p < 0.05$).

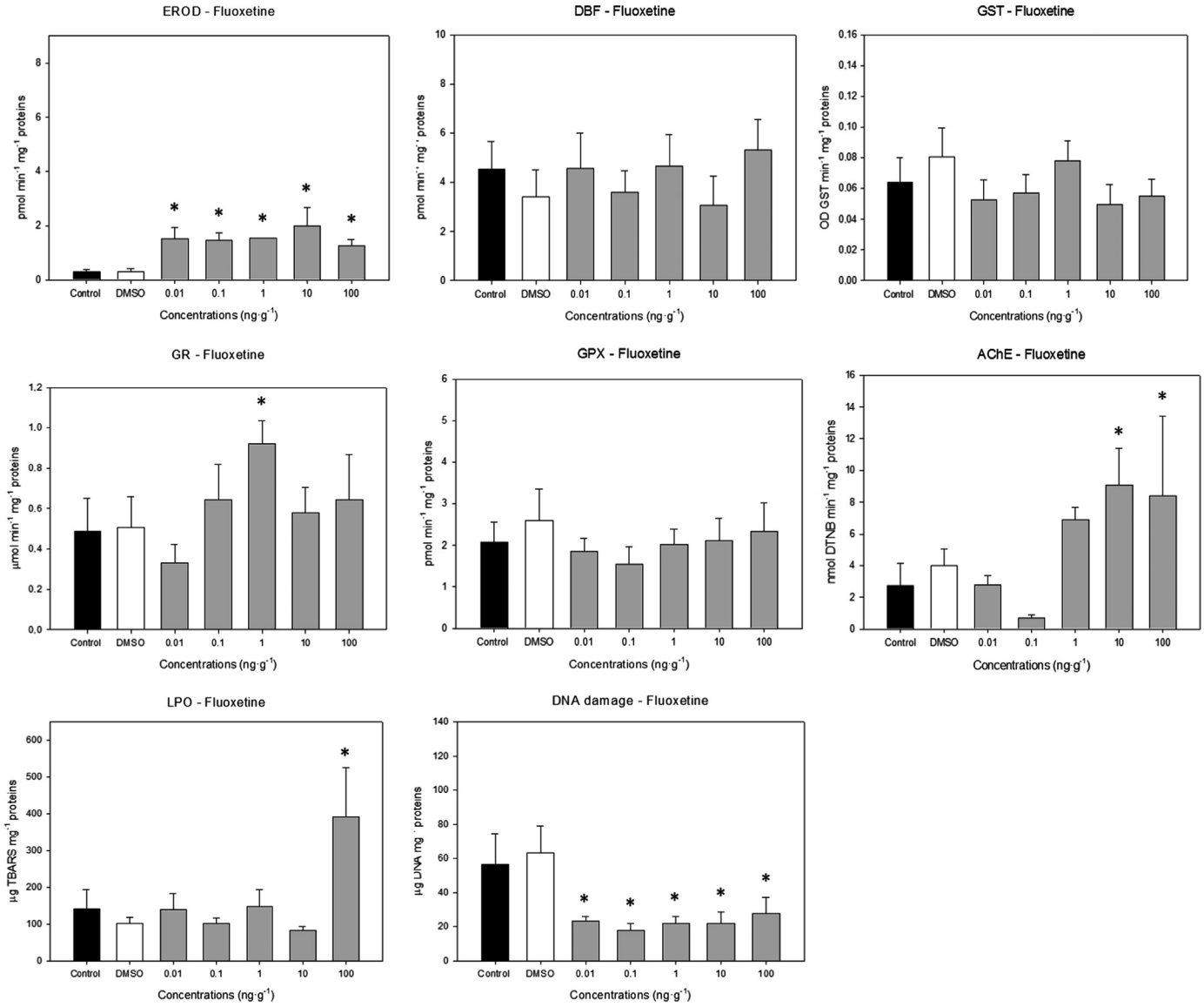


Fig. 4. Mean and standard deviation values of ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), acetylcholinesterase (AChE) enzymatic activities and lipid peroxidation (LPO) and DNA damage (*strand breaks*) values, analyzed in *Hediste diversicolor* after 14-days laboratory exposure to controls and spiked sediment samples [Fluoxetine (FX): 100 ng g^{-1}, 10 ng g^{-1}, 1 ng g^{-1}, 0.1 ng g^{-1} and 0.01 ng g^{-1}]. * represent significant differences compared between the control (reference sediment) ($p < 0.05$) and the pharmaceutical concentration, for each biochemical parameter.

GR and EROD activities were positively correlated ($r = 1.00$, $p < 0.01$). LPO and DNA damage decreased with the concentration. Sea-worms exposed to 0.01 ng g^{-1} concentration showed significant LPO compared with the control individuals ($p < 0.05$). Mitochondrial genetic damage measured as DNA damage *(strand breaks)* was significantly lower in sea-worms exposed to EE2 concentrations of 10 ng g^{-1}, 1 ng g^{-1}, 0.1 ng g^{-1} and 0.01 ng g^{-1} when compared with the control ($p < 0.05$). AChE activity decreased with increasing concentrations. Sea-worms exposed to the lowest concentrations of 1 ng g^{-1}, 0.1 ng g^{-1} and 0.01 ng g^{-1} of EE2 showed significant increase of AChE activity compared with the control ($p < 0.05$). AChE and DBF activities were positively correlated ($r = 0.900$, $p < 0.05$).

Means and standard deviations of the biomarkers determined in sea-worms after the exposure to PRO are shown in Fig. 6. PRO was detoxified by the phase I of the metabolism (EROD activity). EROD activity was significantly activated compared with the control for polychaetes exposed to all concentrations ($p < 0.05$). On the other hand, DBF enzymatic activity was significantly lower compared with the control for all PRO concentrations ($p < 0.05$). GST activities were significantly lower than the control for sea-worms exposed to 500 ng g^{-1}, 5 ng g^{-1} and 0.5 ng g^{-1} ($p < 0.05$). Antioxidant enzymes did not respond. Oxidative effects measured as LPO showed significantly increased values for the lowest PRO concentrations tested (5 ng g^{-1} g^{-1}, 0.5 ng g^{-1} and 0.05 ng g^{-1}) ($p < 0.05$). Sea-worms exposed to the concentration of 50 ng g^{-1} showed significantly lower DNA damage compared with the control ($p < 0.05$). PRO was not neurotoxic for polychaetes. AChE and GPX activities were positively correlated between them ($r = 1.00$, $p < 0.01$), and negatively correlated with mortality ($r = -0.900$, $p < 0.05$).

3.3. Environmental concentration

Taking into consideration the biological index (Fig. 7), the biochemical analyses were subdivided in groups related to biomarkers of the phases I and II of the metabolism called here as exposure (DBF, EROD and GST enzymatic activities), antioxidant

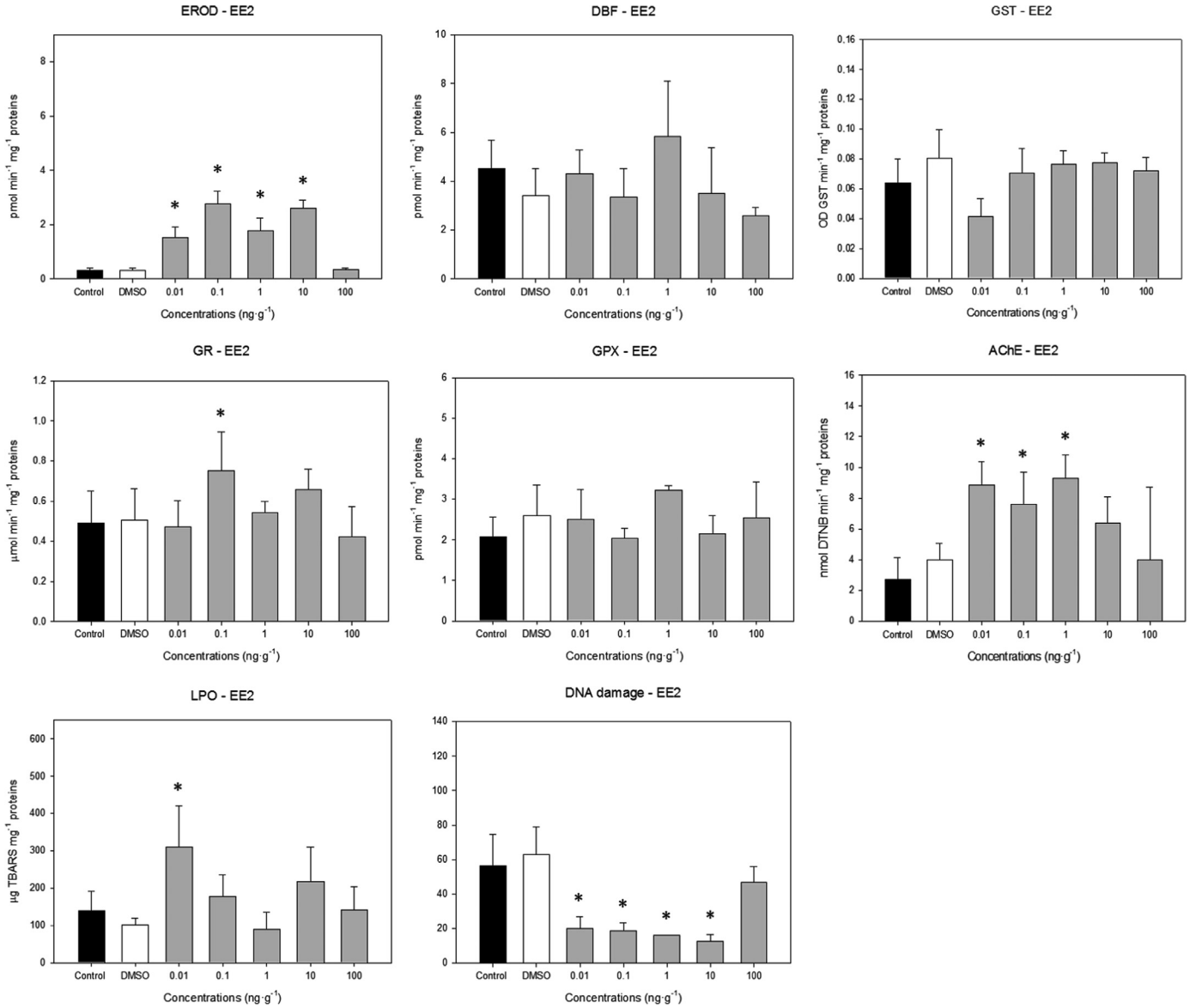


Fig. 5. Mean and standard deviation values of ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), acetylcholinesterase (AChE) enzymatic activities and lipid peroxidation (LPO) and DNA damage (*strand breaks*) values, analyzed in *Hediste diversicolor* after 14-days laboratory exposure to controls and spiked sediment samples [17-α-ethynylestradiol (EE2): 100 ng g^{-1}, 10 ng g^{-1}, 1 ng g^{-1}, 0.1 ng g^{-1} and 0.01 ng g^{-1}]. * represent significant differences compared between the control (reference sediment) ($p < 0.05$) and the pharmaceutical concentration, for each biochemical parameter.

responses (GR and GPX enzymatic activities), neurotoxicity (AChE enzymatic activity) and effects (DNA damage and LPO). The significance was set at $p < 0.05$. The environmental concentration chosen for the index was 0.5 ng g^{-1} of CBZ, IBP and PRO and 0.1 ng g^{-1} of FX and EE2. These concentrations were based in previous studies about the pharmaceutical products occurrence in sediments previous described.

The environmental concentration of 0.5 ng g^{-1} was significantly neurotoxic compared with the control for polychaetes exposed to CBZ spiked sediments ($p < 0.05$). The environmental concentration of 0.5 ng g^{-1} IBP did not show significant responses concerning the four subdivided biomarker groups. For FX, 0.1 ng g^{-1} significantly activated the degradation metabolism ($p < 0.05$), but not the phase II of the metabolism. Oxidative stress was not reduced properly by the antioxidant metabolism which possibly led to effects (LPO and DNA damage) ($p < 0.05$). EE2 was detoxified by the phase I of the metabolism, but the phase II was not activated. This group was significantly different compared with the control ($p < 0.05$). The degradation metabolism was significantly activated in polychaetes exposed to PRO ($p < 0.05$). This metabolism produces free radicals and stress. Antioxidant system did not properly reduce the oxidative stress, which might lead to effects (LPO and DNA damage) ($p < 0.05$).

4. Discussion

In the present study, the reference sediment chosen for the spiking procedure showed good environmental quality, since there was not significant mortality of polychaetes exposed to the two controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)).

Eight biochemical responses related to the metabolism of xenobiotics and possible effects were determined in polychaetes exposed for 14-days to pharmaceutical-spiked sediments. Lethal and sublethal responses were evaluated in a way to relate the

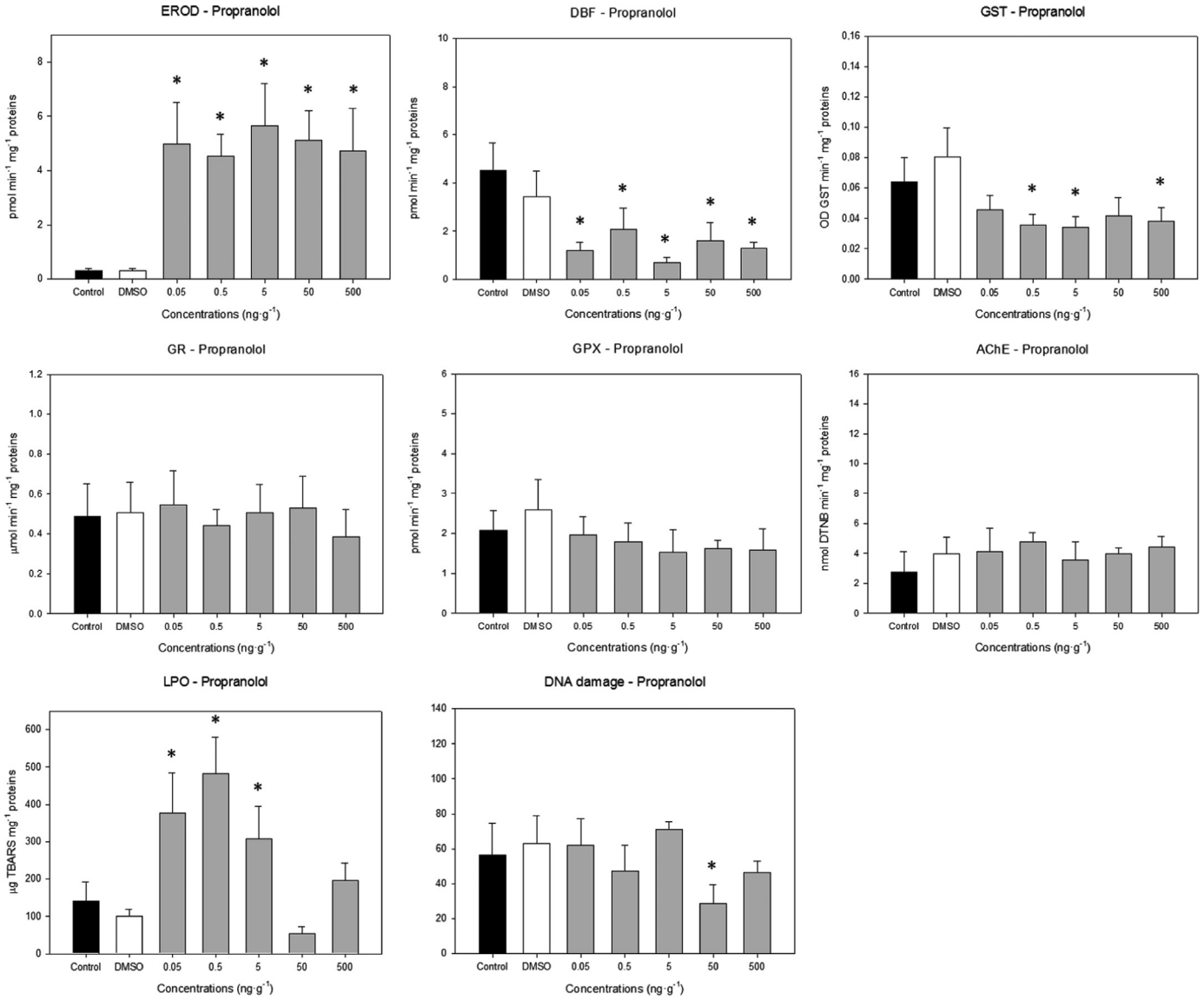


Fig. 6. Mean and standard deviation values of ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), acetylcholinesterase (AChE) enzymatic activities and lipid peroxidation (LPO) and DNA damage (*strand breaks*) values, analyzed in *Hediste diversicolor* after 14-days laboratory exposure to controls and spiked sediment samples [Propranolol (PRO): 500 ng g^{-1}, 50 ng g^{-1}, 5 ng g^{-1}, 0.5 ng g^{-1} and 0.05 ng g^{-1}]. * represent significant differences compared between the control (reference sediment) ($p < 0.05$) and the pharmaceutical concentration, for each biochemical parameter.

bioavailability and possible adverse effects of pharmaceutical products on benthic biota due to the contamination of pharmaceutical products. The present data was compared with the mode of action (MOA) described for humans and mammals. The comparison was also made with others invertebrates, when the data was available. Therefore, there is a lack of information about lethal and sublethal responses on marine invertebrates affected by pharmaceutical products. It is not from our knowledge studies about the exposure of marine polychaetes to pharmaceutical-spiked sediment samples.

Previous studies reported the use of *H. diversicolor* as biomonitor of environmental quality via measurement of biomarkers (AChE, catalase, GST and LPO) and accumulated concentrations of contaminants validated in the field (Durou et al., 2007). The environmental quality of different areas of the Southwest Iberian Coast was previously evaluated focusing on trace metal contamination (Cd, Cu, Ni, Pb and Zn) in *H. diversicolor* whole tissues though a multi-biomarker approach (catalase, superoxide dismutase, GPX, metallothionein and LPO) (Gomes et al., 2013). *H. diversicolor* was also used to measure different biomarkers (catalase, GPX, GST, EROD, DT-diaphorase and AChE) after the exposure to a pollution gradient caused by untreated domestic discharges at the Bay of Cádiz (Pérez et al., 2004). As observed by previous studies, domestic discharges bring loads of pharmaceutical products to the environment (Farré et al., 2001; Andreozzi et al., 2002; Ternes et al., 2002; Metcalfe et al, 2003; Hernando et al, 2006). Solé et al. (2009) confirmed their adequacy as sentinel species and to evaluate the impact of domestic discharges. Polychaetes *H. diversicolor* was considered a suitable bioindicator for the sublethal assessment of possible impacts of pharmaceutical pollution.

4.1. Metabolism of pharmaceutical products in polychaetes *H. diversicolor*

CBZ works in the nervous system, and it is prescribed for epilepsy and bipolar disorder. This pharmaceutical product is a strong inducer of the CYP 1A2 and 3A4 in vertebrates. Polychaetes

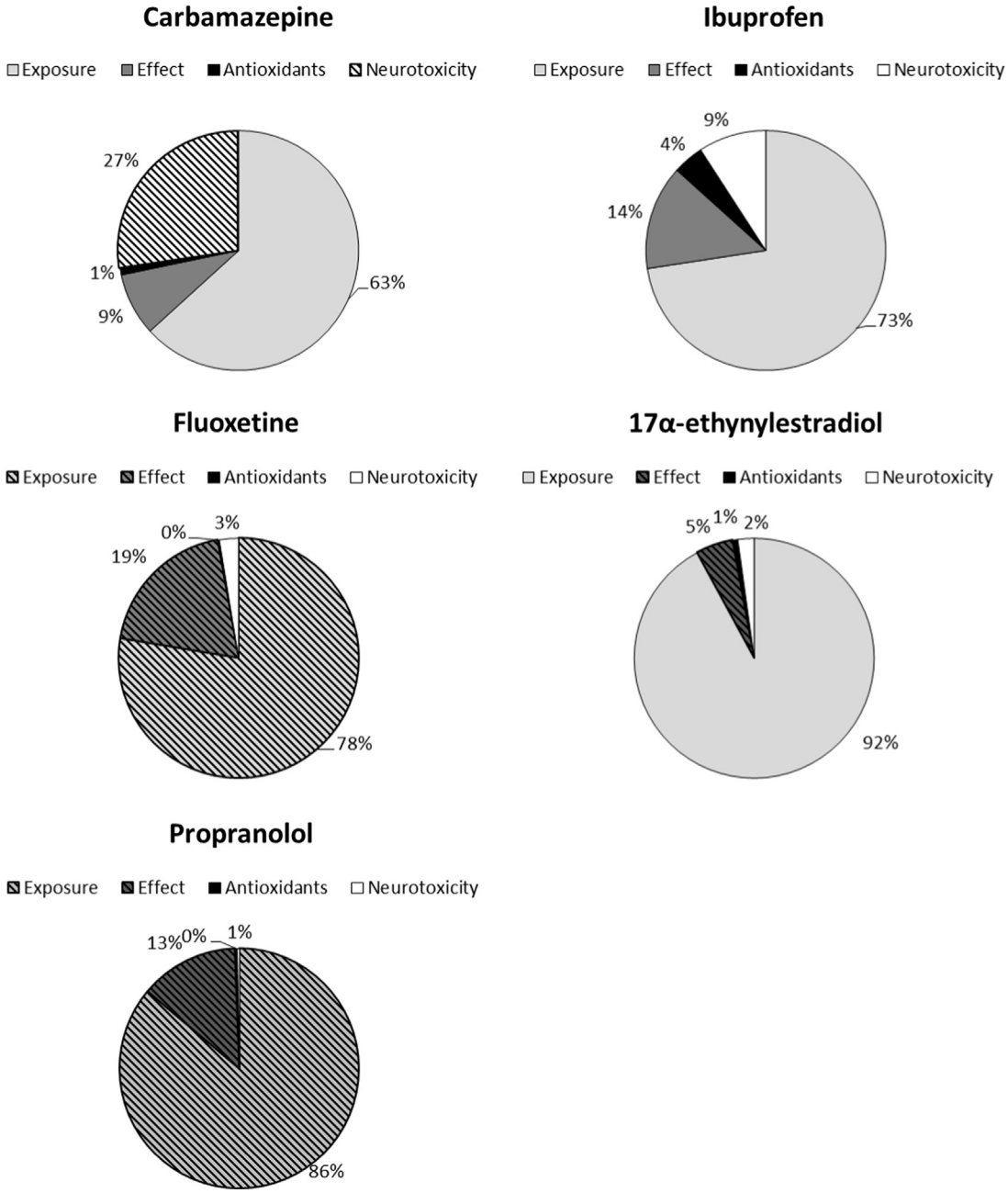


Fig. 7. Pie charts of biochemical responses index concerning the environmental concentrations of 0.5 ng g^{-1} CBZ, IBP and PRO and 0.1 ng g^{-1} FX and EE2 analyzed in *Hediste diversicolor* after 14-days of laboratory exposure. It was considered the percentage of each group of responses subdivided in exposure (DBF, EROD and GST enzymatic activities), antioxidants (GR and GPX enzymatic activities), effect (DNA damage and LPO) and neurotoxicity (AChE enzymatic activity). Diagonal stripes represent significant differences between control and the pharmaceutical concentration, for each group of biochemical parameters.

exposed to CBZ-spiked sediment showed that EROD (CYP 1A2) activity was not significantly different compared with the control, although the highest enzymatic activity was related to the highest CBZ concentration (500 ng g^{-1}). EROD activity was induced in gill, hepatopancreas, muscle and gonad of crab *C. maenas* exposed to CBZ (Aguirre-Martínez et al., 2013b). DBF (CYP 3A4) activities were high but not significantly different compared with the control for polychaetes exposed to CBZ. Aguirre-Martínez et al. (2013b) observed the induction of DBF activity in crabs *C. maenas* after the exposure to environmental concentrations of CBZ. The highest concentrations should be tested to observe the possible induction of phase I activities in marine invertebrates exposed to CBZ. Phase II of the metabolism (GST) was responsive for CBZ concentrations. The increase of GST enzymatic activities in aquatic invertebrates exposed to CBZ was reported by previous studies (Martín-Díaz et al., 2009; Vernouillet et al., 2010; Aguirre-Martínez et al., 2013b). After 14-days of exposure to CBZ, antioxidant enzymes did not respond to the increase of concentrations, except for GPX activity highly induced in polychaetes exposed to the lowest concentration tested (0.05 ng g^{-1}) ($p > 0.05$). Aguirre-Martínez et al. (2013b) observed an increase of GPX activity with the CBZ concentration in *C. maenas*. After 96 h of exposure to CBZ, activities of SOD, CAT, GR and GPX in *Oncorhynchus mykiss* were higher in intestine, muscle and liver (Li et al., 2011). GR activity was slightly inhibited in polychaetes exposed to CBZ concentrations up to 0.5 ng g^{-1} which could lead to accumulation of oxidative substances, suggesting inadequate compensation for the presence of CBZ. LPO increased with the concentration of CBZ, being significantly higher than the control for the maximum concentration tested ($p > 0.05$). The increase of oxidative stress

indices was reported in *C. maenas* after the exposure to CBZ (Aguirre-Martínez et al., 2013b) and *O. mykiss* (Li et al., 2011). CBZ did not induce DNA damage in humans (Andreazza et al., 2007). In polychaetes, DNA damage showed a slight decrease, without significance compared with the control. A decrease of DNA damage related to the increasing CBZ concentrations was also observed by Aguirre-Martínez et al. (2013b). The slight increase of AChE enzymatic activity following the concentration showed the neurotoxic potential of CBZ. However, only the environmental concentration of 0.5 ng g^{-1} was significantly neurotoxic ($p > 0.05$). CBZ environmental concentrations were conjugated by the phase II of metabolism, with the activation of antioxidant response (GPX activity), and it was also neurotoxic for polychaetes.

IBP is a non-steroidal anti-inflammatory drug (NSAIDs) widely used in the treatment of pain, dysmenorrhea, inflammation and fever (Parolini et al., 2011). IBP is known to inhibit CYP450 enzymes, which was demonstrated in the present study. Also the phase II and antioxidant enzymes did not respond to this pharmaceutical product. GR activity was inhibited with increasing concentration of pharmaceutical, which could lead to oxidative stress and accumulative oxidative reactants in the cells. Overall, antioxidant enzyme SOD and AChE activities were generally lower in IBP-exposed clams *Ruditapes philippinarum* than in controls (Milan et al., 2013). Polychaetes exposed to IBP showed significantly increased LPO compared with the control for the concentration of 5 ng g^{-1} ($p > 0.05$). DNA damage significantly increased with the concentration in polychaetes exposed to sediment spiked with 5 ng g^{-1} and 50 ng g^{-1} ($p > 0.05$). AChE activity increased with the concentration, being the highest concentration significantly higher than the control ($p > 0.05$). AChE induction plays an important role in apoptosis (Zhang et al., 2002). The battery of biomarkers did not permit to clarify the exposure route of the IBP metabolism and excretion. However, IBP exposure resulted in DNA damage and increased AChE levels. IBP, and it is probably more harmful metabolites, may present a potential hazard for the aquatic ecosystem health (Illés et al., 2013). Aguirre-Martínez et al. (2013a) observed the increase of phases I and II, antioxidant system, LPO and DNA damage in crabs *C. maenas* exposed to increasing concentrations of IBP. IBP had considerable effects on antioxidant and detoxifying enzymes activities in zebra mussel *Dreissena polymorpha* due to oxidative status imbalances (Parolini et al., 2011). Although environmental concentrations of IBP did not activate any enzymatic activity, the increase of DNA damage and LPO was observed.

FX is a SSRI drug prescribed for bipolar disorders and depression. This pharmaceutical product was degraded by the phase I (EROD activity) of the metabolism in polychaetes exposed to spiked-sediments; even FX is considered an inhibitor of CYP450 in humans. Phase II was not responsive in polychaetes exposed to this pharmaceutical product. For mussels *M. galloprovincialis* exposed to FX, GST activity was induced (Franzellitti et al., 2013). However, antioxidant enzymes slightly increased with the concentration which may culminate in LPO. No clear antioxidant responses were previous detected in digestive gland of marine mussels exposed to FX, but lipid peroxidation (malondialdehyde content) were lower than the control organisms (Franzellitti et al., 2013). DNA *strand breaks* significantly decreased with the control for all FX concentrations tested ($p > 0.05$). Concentration of FX was positively correlated to AChE activity ($p > 0.05$). Considerable evidence indicated that SSRIs inhibit AChE activity in humans (Müller et al., 2002), fish (Park et al., 2012), marine mussels (Franzellitti et al., 2013) and clams (Munari et al., 2014) which was not the case for polychaetes. FX environmental concentrations were detoxified by the phase I of the metabolism, which increased the GR activity and decreased the DNA *strand breaks*.

EE2 is widely used as contraceptive and considered a neuroendocrine disruptor for invertebrates. In polychaetes, EE2 was mainly degraded by EROD enzymatic activity. Phase II and antioxidant enzymes (except for GR at concentration of 0.1 ng g^{-1}) were not responsive for this pharmaceutical. No clear EE2-related response was observed in cytosolic GST and antioxidant enzymes measured in carps *Cyprinus carpio* after EE2 injection (Solé et al., 2000). LPO significantly increased related with the low concentration tested and after this decrease with the concentration. DNA damage was significantly lower than the control for the concentrations down to 100 ng g^{-1} ($p > 0.05$). AChE enzymatic activity increased for polychaetes exposed to the environmental concentrations (0.01 ng g^{-1}, 0.1 ng g^{-1} and 1 ng g^{-1}) ($p > 0.05$), and decreased for the highest concentrations tested. EE2 environmental concentrations were detoxified by the phase I, activated the antioxidant responses concerning the GR activity, and caused oxidative effect determined as the LPO high values. Nevertheless, DNA *strand breaks* decreased compared with the control. Also neurotoxicity was observed.

PRO is an antagonist β-blocker (adrenergic receptor) used to treat high blood pressure and related heart illnesses. EROD activity was the main responsible for the degradation of PRO in polychaetes, even DBF activity was significantly inhibited for all concentrations tested ($p > 0.05$). Marine mussels exposed for 10-days to PRO concentration showed enhanced phase I, measured by carboxylesterases (CbE), which also play a significant role in drug metabolism in humans and vertebrates (Solé et al., 2010). Phase II was inhibited by PRO concentrations. *M. galloprovincialis* exposed to PRO showed the inhibition of GST activity in mantle/gonads and the induction in digestive gland (Franzellitti et al., 2014). Antioxidant enzymes did not respond for PRO concentrations. LPO significantly increased for the environmental concentrations of 0.05 ng g^{-1}, 0.5 ng g^{-1} and 5 ng g^{-1} ($p > 0.05$). PRO did not cause DNA damage, and the concentration of 50 ng $\cdot$ g^{-1} showed a significant decrease ($p > 0.05$). PRO was not neurotoxic for polychaetes. However, the PRO environmental concentrations were mainly detoxified by the phases I (EROD activity) and II (GST activity) of the metabolism. High levels of LPO were observed in polychaetes exposed to PRO environmental concentrations.

The quantification of biochemical parameters provided important insights on toxicant-induced changes in key physiological mechanisms and the potential toxicity of different pharmaceutical products. The feasibility of the biomarker approach and the use of the *H. diversicolor* are highly recommendable for coastal sediment quality assessment related to pharmaceutical contamination.

All the pharmaceutical products tested in the present study significantly altered biomarker responses in the polychaetes *H. diversicolor*. It has been observed that inhibition of phase I of the metabolism due to increasing concentrations of pharmaceuticals may lead to bioaccumulation. More responses on this direction should be performed for FX and EE2. On the other hand, an activation of the degradation metabolism produced oxidative stress, which may not be reduced properly by the antioxidant metabolism. This might lead to LPO and DNA damage. This behavior has been observed after the exposure to IBP and PRO. The activation of the phase II after the exposure to CBZ might produce free radicals and cause oxidative stress, only confirmed for the highest concentration tested. Furthermore, our study highlighted the importance of integrating responses to understand the mechanistic bases of stress responses and interpret their consequences.

5. Conclusions

Although not able to induce mortality, pharmaceutical compounds may still be able to induce sublethal changes that can

affect the physiology of the exposed organisms. The battery of biomarkers showed the possible bioavailability of pharmaceuticals to the benthic biota. However, more research should be performed with other CYP 450 isoforms and phase II enzymes, mainly concerning the exposure to CBZ and IBP. Additional studies about other enzymes involved in the antioxidant system should be performed to better understand the role of this system to prevent the oxidative stress produced by pharmaceutical products contamination. Nevertheless, oxidative effect LPO was observed in polychaetes exposed to IBP, EE2 and PRO. However, only IBP at the environmental concentration produced DNA damage. Further studies should be done in a way to clarify the influence of pharmaceutical products in the decrease of DNA *strand breaks*. This study has the relevance to underline biomarkers as early warning tools, which can be applied for bioremediation purposes and for sediment quality assessment, with possible application in sediments involved in dredging activities, since WWTPs can be associated to dredging areas.

As a whole, this preliminary investigation highlighted the need of focusing future research efforts on possible physiological impairments caused by long-term exposure of marine benthic species to pharmaceutical products. As a suggestion, enzyme assays should be used in association with other biomarkers, as the lysosome membrane stability and lipofuscin accumulation analysis. The effects of metabolites should bring relevant information about the toxicity of pharmaceutical compounds. It is also important to examine contaminant mobilization and microbial degradation of these compounds in the sediment compartment, as the biotransformation in benthic organisms. Considering our results, further studies are needed to understand the bioaccumulation and trophic transfer of pharmaceutical compounds.

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In preparation

Toxicological evaluation of sediment samples spiked with human pharmaceutical products. Changes in the energy status, neuroendocrine effects, gametogenesis and inflammation process in marine polychaetes *Hediste diversicolor*.

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ABSTRACT

There is a lack of studies about the ecotoxicology of pharmaceutical products on marine environment. In a way to predict possible adverse effects of pharmaceutical products on benthic biota, polychaetes *Hediste diversicolor* were exposed for 14-days to pharmaceutical-spiked sediments under laboratory conditions. Carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol and propranolol, including environmental concentrations, were spiked in marine sediment samples. After the exposure, changes in cellular energy status (total lipids content – TLP; and mitochondrial electron transport activity - MET), alkali-labile phosphates level (ALP), metabolism of monoamines (monoamine oxidase activity - MAO) and inflammation properties (cyclooxygenase activity - COX) were examined in polychaetes. Carbamazepine increased TLP content and MET activity, and decreased MAO activity in polychaetes. Ibuprofen did not interfere on the TLP and ALP levels, but on the MET and MAO activities (environmental concentrations). Fluoxetine did not cause changes in the energy status of this organism. Therefore, environmental concentration diminished the ALP levels and MAO activity. 17α-ethynylestradiol did not change the energy status and ALP level, however, MAO activity was significantly lower in polychaetes exposed to environmental concentration. Propranolol increased TLP level in polychaetes, but not MET activity and ALP level. MAO activity was significantly lower for polychaetes exposed to environmental concentration of propranolol. Except fluoxetine, all pharmaceuticals tested showed anti-inflammatory properties confirmed by the decrease of COX activity. The data revealed pharmaceutical products could influence *H. diversicolor* physiology. As benthic top predator, adverse effects on sea-worms can culminate in ecosystem perturbations.

Keywords: *Hediste diversicolor*, pharmaceutical compounds, immunotoxicity, neuroendocrine assessment, marine sediment.

1. INTRODUCTION

Marine and estuarine environments are the major receptor of wastewater discharges from coastal areas, where are located the most urban populated areas worldwide. Municipal effluents contain pharmaceutical products including neuroactive drugs (e.g. carbamazepine and fluoxetine), non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen), steroids (e.g. 17α-ethynylestradiol) and β-blockers drugs (e.g. propranolol) (Metcalfe et al., 2003, Andreozzi et al., 2002, Farré et al., 2001). Pharmaceuticals are extremely bioactive compounds designed to alter physiology at low doses in target organisms. Interestingly, these drugs are commonly found at concentrations that often reach $ng \cdot g^{-1}$ in sediments (Zhou and Broodbank, 2014, Pintado-Herrera et al., 2013, Schultz et al., 2010, Hernando et al., 2006, Ternes et al., 2002). In addition, pharmaceuticals are constantly released into the environment, and sediments can act as a sink and/or source for a variety of these compounds. Nowadays, they are considered particularly emergent and potent contaminants, and some of these compounds can persist in the environment for months to years (Daughton, 2001). In fact, acute toxicity tests have generally failed to detect the subtle action elicited by these compounds at environmental concentrations (Schnell et al., 2009, Daughton and Ternes, 1999). It is agreed the need for sublethal responses to predict ecological consequences due to pharmaceutical products exposure (Schmidt et al., 2011, Santos et al., 2010, Crane et al., 2006, Fent et al., 2006). Even in low concentrations, pharmaceuticals may have significant consequences to the aquatic organisms.

Nonetheless, there is a lack of data on the marine and estuarine biota exposed to environmental concentrations of pharmaceutical products and wastewater discharges.

Daughton and Ternes (1999) suggested that bioassays or biomarkers should focus on specific mechanisms of pharmaceutical action on non-target biota. The class of biomarkers proposed are those related to the mode of action (MOA) of the drug in question, with possible effects on an organism's survival, immune function and reproduction (Gagné et al., 2007b).

Currently studies about freshwater environments have applied this class of biomarkers. The occurrence of inflammation syndrome was observed in freshwater mussels *Elliptio complanata* exposed to urban wastewater discharges (Gagné et al., 2008, Gagné et al., 2007 a, Gagné et al., 2005) measured by cyclooxygenase activity (COX). This enzymatic activity could be blocked by NSAIDs such as ibuprofen. Another study confirmed the neurotoxicity potential of wastewater discharges on *E. complanata* through the application of a battery of biomarkers indicative of neuroendocrinal perturbations including monoamine oxidase activity (MAO) (Gagné et al., 2007 a, b) and related the neurotoxicity and estrogenic effects (vitellogenin-like proteins - Vtg) (Gagné et al., 2007 b). MAO activity is responsible for the breakdown of monoamines as serotonin, noradrenaline and dopamine, fully related to neuroactive (e.g. fluoxetine and carbamazepine) and β-blockers drugs (e.g. propranolol). Steroid hormones as progesterone, estrogen and androgens induced MAO enzymatic activity and vitellogenesis in spotted ray (Prisco et al., 2008). Serotonin and dopamine is highly related to the enhancement of vitellogenin synthesis in freshwater prawns (Kuo et al., 2009).

For the enzymatic activities as MAO and MET occurring in the mitochondrial membrane, and for Vtg-like proteins synthesis and COX activity could properly work, the organism needs energy reserves which are mainly stored as lipids content. Mitochondrial electron transport chain extracts energy via redox reactions through oxidation of sugars

and cellular respiration, which result in the concomitant release of reactive oxygen species and oxidative stress. The increase of energy reserves expenditure were observed in freshwater organisms exposed to contaminated habitats by effluents and determined by mitochondrial electron transport activity (MET) and lipid reserves (TLP) reduction (Gagné et al., 2007 a, Smolders et al., 2004). The set of biomarkers described above is relevant for invertebrates and can be related to the MOA of different drugs. Effects on organism's immune function, energy status and neuroendocrine system can affect the survival, reproduction and population maintenance (Gagné et al., 2007 b). Nonetheless, little is known about marine benthic biota and the possible effects due to pharmaceutical products.

The benthic worm *Hediste* (= *Nereis*) *diversicolor* appears as a key species in estuarine ecosystems considered predators as they feed also on bivalves. Moreover, bioturbation caused by the sea-worm greatly affects the biogeochemical cycle of nutrients and contaminants (Banta and Andersen, 2003, Blaise et al., 2013). This bioindicator is considered an essential component of ecotoxicological toolbox for sediment quality assessment, due to their abundance, ecological relevance and constantly contact with contaminants in the sediment and water column (Lewis and Watson, 2012). More recently, new and emerging anthropogenic contaminants such as microplastics, pharmaceuticals and nanoparticles have become the focus of ecotoxicological research (Lewis and Watson, 2012). Polychaetes have already shown themselves to be highly useful bioindicators for research into the modes of toxicity for such emergent compounds (Galloway et al., 2010). *H. diversicolor* has been recommended for sediment sublethal toxicity studies concerning biomarker analysis (Gomes et al., 2013, Kalman et al., 2010, Lewis and Galloway, 2008, Solé et al., 2009, Durou et al., 2007). Nevertheless,

biochemical and reproduction responses of therapeutic drugs on this bioindicator are largely unstudied.

An attempt was made to relate the MOA of pharmaceuticals with possible effects on survival, immune and neuroendocrine functions and reproduction of the benthic sea-worm *H. diversicolor*. Organisms were exposed for 14-days to marine sediment spiked with different concentrations of pharmaceutical products used worldwide representing a wide variety of therapeutic classes with different MOAs: carbamazepine (antiepileptic, CBZ), ibuprofen (anti-inflammatory, IBP), fluoxetine (antidepressant, FX), 17α-ethynylestradiol (oral contraceptive, EE2) and propranolol (anti-hypertensive, PRO). Changes in the energy status were examined by following mitochondrial electron transport (MET) activity (energy expenditure) and total lipids (TLP) (energy reserve) methods. Inflammation potential of each pharmaceutical product was determined applying cyclooxygenase activity (COX) method. Monoamine oxidase activity (MAO) and alkali-labile phosphates in high molecular weight proteins (ALP) were analysed to evaluate the neuroendocrine and gametogenic effects of these pharmaceutical products, respectively. The application of the battery of biomarkers had the aim to evaluate the suitability of such sublethal responses and the use of this bioindicator related to pharmaceutical pollution in marine ecosystems.

2. MATERIALS AND METHODS

2.1. Polychaetes collection and maintenance

Individuals of *H. diversicolor* were collected by hand at low tide at the intertidal estuarine mudflats located between the urban areas of Chiclana de la Frontera and San Fernando (SW, Spain), within the Natural Park "Bahía de Cádiz", far from the urban areas. After collection, individuals were transported to the laboratory in cold containers, with wet algae from the site of origin. Sea-worms were maintained in aerated aquariums

filled with natural filtered seawater and reference sediment from Río San Pedro (the same sediment used for the spiking procedure) for one week before testing.

2.2. Sediment spiking procedure

Spiking of pharmaceutical products used in this study was performed in the natural marine sediment (total organic carbon = 1.20% ± 0.09; organic matter = 8.07% ± 0.34; texture = 0.84% gravel, 57.39% sand, 41.77% fines) sampled from Río San Pedro (SW, Spain) (36°31′53″ N; 6°12′48″ W), an intertidal creek area that is part of the Natural Park "Bahía de Cádiz". This place was considered reference by previous studies (De Orte et al., 2013, Basallote et al., 2012, Solé et al., 2009, Pérez et al., 2004).

The topmost 10 cm layer of the sediment was sampled and sieved through a 2-mm mesh to remove large debris and other living organisms. Sediment samples were dried at 70^{o}C (OECD, 2000). The same volume of water lost in the dry sediment procedure was replaced in the sample by clean seawater. Overlying seawater was obtained from "Central Service of Mariculture Research", University of Cádiz, where the same seawater has been used for the culture and maintenance of test organisms (fish, molluscs and plankton) since 2002.

Five concentrations of each pharmaceutical product were tested, including the environmental concentrations (underlined) based on previous studies (Zhou and Broodbank, 2014, Pintado-Herrera et al., 2013, Schultz et al., 2010, Hernando et al, 2006, Ternes et al., 2002): CBZ, IBP and PRO (500 ng·g^{-1}, 50 ng·g^{-1}, <u>5 ng·g^{-1}, 0.5 ng·g^{-1}, 0.05 ng·g^{-1}</u>), FX and EE2 (100 ng·g^{-1}, 10 ng·g^{-1}, <u>1 ng·g^{-1}, 0.1 ng·g^{-1}, 0.01 ng·g^{-1}</u>). Pharmaceutical stock solutions were prepared using the solvent DMSO 0.001% (v/v) as recommended by Aguirre-Martínez et al. (2013 a, b), Eades and Waring (2010) and

Quinn et al. (2008 a, b). Two controls were run in parallel with the experiments: reference sediment and reference sediment spiked with DMSO 0.001% (v/v).

The methodology used for the spiking procedure was an adaptation of OECD (2004), USEPA (2001) and ASTM (2000) protocols. Pharmaceutical-spiked sediments were initially prepared in 3-L glass beakers, mixed by homogenization using Teflon spatula, and subsequently mixed for 30 min on a bottle roller. The pharmaceutical-spiked sediments were incubated for 7 days (4^{o}C in the darkness) to ensure equilibration of the test substance between water and sediment in the preparation sealed bottle (Löffler et al., 2005, Francis et al., 1984). After the equilibration period, the toxicity bioassay started (day-0).

2.3. Determination of pharmaceutical concentrations in spiked sediments.

Sediment from day-0 was analyzed to quantify the highest pharmaceutical concentration spiked, since this spiked-sediment was diluted with reference sediment to reach the concentrations proposed for the bioassay. The highest concentration of the selected pharmaceuticals was measured in spiked sediments following a modification of the method proposed by Jelic et al. (2009). Detailed methodology and the measured concentrations of target compounds in spiked sediments are shown in Maranho et al. (2014). Concentrations of the five selected pharmaceuticals in non-spiked sediments were below 1 ng·g^{-1}. Therefore, nominal concentrations are presented throughout this manuscript.

Pharmaceuticals vary among their rates of degradation with reported half-lives (Guler and Ford, 2010). In the present study, it was assumed that no degradation products were transformed, since the half-lives of CBZ (Loofler et al., 2005, Lam et al., 2004), IBP

(Beausse, 2004), FX (Lam et al., 2004), EE2 (Mes et al., 2005, Beausse, 2004) and PRO (Andreozzi et al., 2003) were higher than the time of exposure used in the present study.

2.4. Exposure of polychaetes and tissue preparation

H. diversicolor individuals were placed in 4L-test vessels containing the proportion of 1: 4 of sediment: water. Static renewal of the overlying water provided approximately 1: 4 volumes of water/ week to each aquarium. Briefly, 10 polychaetes were randomly placed to each test vessels. The 14-days worm sediment test was conducted according to the methods provided in "Standard guide for conducting sediment toxicity test with polychaetes annelids" (ASTM, 2009) and described by Thain and Bifield (2001). Three replicates per treatment were performed. Vessels were maintained at 18 ± 2^{o}C in the dark under laboratory conditions. Physical-chemical parameters (oxygen saturation = 7.3 ± 0.3 mg·L^{-1}, pH = 8.13 ± 0.1 and salinity = 35.6 ± 0.8 ‰) were monitored each 2 days until the day 14. At the end of the exposure period, living organisms were depurated in clean seawater overnight in 4 L aquaria at 18 ± 2^{o}C. After the depuration period, animals were stored at -80^{o}C till further analysis.

Organisms from the same replicate were pooled before tissue homogenization. The homogenization buffer (pH 7.5) consisted of 100 mM NaCl, 25 mM Hepes-NaOH, 1 mM EDTA and 1 mM dithiothreitol (DTT) (Gagné et al., 2007 a). The homogenates were stored at -80°C until further analysis. Total protein content was determined using the principle of protein-dye binding with serum bovine albumin for calibration (Bradford, 1976).

2.5. Cellular energy status

Energy reserves were determined by levels of total lipids (TLP) in homogenate samples of polychaetes according to the phosphovanillin method (Frings et al., 1972). Homogenate samples were incubated in the presence of H_2SO_4 and phosphovanilin for 10 min at 80^{o}C. Calibration was achieved with olive oil as the standard. The absorbance (540 nm) was measured. The data was expressed as μg total lipids/ mg proteins.

Mitochondrial energy consumption was determined applying mitochondrial electron transport (MET) activity according to the reduction of p-iodonitrotetrazolium dye method (Smolders et al., 2004, King and Packard, 1975). Briefly, homogenate was centrifuged at 3,000g at 4°C for 20 min and the supernatant mixed with one volume of 0.1 M Tris–HCl (pH 8.5) containing 0.1 mM $MgSO_4$, 0.1% Triton X-100 and 5% polyvinylpyrrolidone for 1 min before adding 1 mM NADH and 0.2 mM NAPDH. The reaction started by adding 1 mM of p-iodonitrotetrazolium. The absorbance readings were taken at 520 nm at 5 min of intervals. The data were expressed as A 520 nm/min/mg proteins.

2.6. Phosphate reserves

Alkali-labile phosphate (ALP) levels were determined in homogenized extracts applying the method described by Gagné et al. (2003). Proteins were precipitated by acetone (35% v/v) and centrifuged at 10,000g for 5 min at 4 ^{o}C. The pellet was washed in 50 % acetone and re-centrifuged. The pellet was resuspended in 100 μL of 1M NaOH and incubated for 30 min at 60 ^{o}C. Levels of inorganic phosphates liberated from NaOH treatment were determined by phosphomolybdate assay (Stanton, 1968). Absorbance was measured at 444 nm (some cases with interferences in the colour, the reading was taken at 815 nm). Data were expressed as μg Phosphate/ mg proteins.

2.7.Metabolism of monoamines

Monoamine oxidase (MAO) activity was determined using the serotonin analogue tryptamine as the substrate (Gagné et al., 2007 b). Homogenate samples were incubated in the presence of 10 µM dichlorofluorescein, 100 µM tryptamine in 10 mM Hepes-NaOH (pH 7.4), containing 140 mM NaCl, 1 mM aminotriazole and 0.1 µg/mL of horseradish peroxidase. The reaction was allowed to proceed at 30^{o}C for 0, 15, 30 and 60 min. Fluorescence for fluorescein was measured at 485 nm (excitation) and 535 nm (emission). Standard solutions of fluorescein were used for calibration. MAO enzymatic activity was expressed as nmol fluorescein/ min/ mg proteins.

2.8. Inflammation biomarker

Inflammation was tracked by measuring the activity of arachidonic acid cyclooxygenase (COX) activity determined in homogenate centrifuged at 15,000g at 4°C for 20 min. COX activity was determined by following the oxidation of 2, 7-dichlorofluorescein in the presence of arachidonate (Fujimoto et al., 2005). The incubation buffer (pH 8) consisted of 50 mM Tris- HCl, 0.05% Tween-20, 50 µM arachidonate, 2 µM dichlorofluorescein and 0.1 µg/mL horseradish peroxidase. The formation of fluorescein was measured at 485 nm (excitation) and 530 nm (emission). Standard solution of fluorescein prepared in the incubation buffer was used for calibration. The data were expressed as RFU/ min/ mg proteins.

2.9. Statistical analysis

A number of six polychaetes ($n \geq 6$) from each replicate were used for the biomarker responses determination. Biomarkers responses were analyzed using the SPSS/PC 21.0 + statistical package. Data were examined using a one-way Analysis of

Variance (ANOVA) followed by Dunnett's t test ($p < 0.05$), after confirming for homogeneity of variance and normality in the data distribution with Bartlett and Shapiro Wilks´s tests respectively. Significant correlations between mortality, pharmaceutical concentrations in sediments and sublethal responses in *H. diversicolor* were examined by Spearman´s rank correlation analysis. The significance level for the Spearman´s rank correlation was set at $p < 0.05$. The integrated biomarker response (IBR) was calculated following the methodology described by Beliaeff and Burgeot (2002) for the all concentrations of the pharmaceutical products tested. However, environmental concentrations were grouped in one group (0.05 $ng \cdot g^{-1}$, 0.5 $ng \cdot g^{-1}$, 5 $ng \cdot g^{-1}$ of CBZ, IBP and PRO; 0.01 $ng \cdot g^{-1}$, 0.1 $ng \cdot g^{-1}$, 1 $ng \cdot g^{-1}$ of FX and EE2), and the two highest concentrations were calculated separately. Visualization was possible between pharmaceutical products and concentrations since biomarker responses in polychaetes were displayed as IBR star plots.

3. RESULTS AND DISCUSSION

3.1. Mortality of polychaetes exposed to pharmaceutical products

Following the experimental period of 14 days, significant mortality ($p < 0.05$) of *H. diversicolor* was observed in replicates of sediment samples spiked with CBZ (500 $ng \cdot g^{-1}$, 50 $ng \cdot g^{-1}$), FX (1 $ng \cdot g^{-1}$) and EE2 (100 $ng \cdot g^{-1}$, 10 $ng \cdot g^{-1}$). Concentrations of CBZ ($p < 0.01$), IBP ($p < 0.01$), FX ($p < 0.05$) and EE2 ($p < 0.01$) were positively correlated with mortality.

3.2. Biochemical responses in polychaetes exposed to pharmaceutical products

During times when energy intake exceeds the maintenance, growth and reproductive requirements of an organism, energy is stored as glycogen or/ and lipids.

Lipids provide an essential and readily available source of energy for aquatic organisms. Their partitioning into lipids, adsorption to environmental matrices, and bioavailability at trace levels are important transport characteristics that determine the ultimate fate of pharmaceutical products in the environment (Boxall and Ericson, 2012).

The storage of total lipids (TLP) measured in this study (Figure 1) significantly increased in polychaetes exposed to CBZ (500 $ng \cdot g^{-1}$, 0.5 $ng \cdot g^{-1}$, 0.05 $ng \cdot g^{-1}$) and PRO (500 $ng \cdot g^{-1}$, 0.05 $ng \cdot g^{-1}$) ($p < 0.05$). TLP levels were positively correlated with increasing concentrations of CBZ ($p < 0.01$). CBZ was described as responsible for the increase of lipids levels in studies performed in humans (Eirís et al., 2000, Isojärvi et al., 1993). Other studies reporting relationships between CBZ and high levels of cholesterol and triglycerides have been described (Brown et al., 1992, Demircioglu et al., 2000, Voudris et al., 2006). TLP was also positively correlated with concentration for polychaetes exposed to PRO ($p < 0.01$). This finding is in agreement with the studies showing PRO chronic treatments produced the increase of triglycerides and decrease of HDL cholesterol (Ayatollahi and Kholasehzadeh, 2013).

Physical and chemical stressors can cause changes in the concentration of stored energy reserves of lipids in organisms. During times of increased stress, energy demand is mobilized (Gagné et al., 2007 a, Smolders et al., 2004). TLP was negatively correlated ($p < 0.01$) with the IBP concentration. Dabhi et al. (2006) showed IBP reduced total cholesterol, triglycerides, LDL cholesterol and atherogenic index in hypercholesterolemic mammals.

Polychaetes exposed to FX and EE2 did not show alterations of the TLP levels after 14-days of exposure. Data about EE2 exposure of polychaetes did not agree with studies on vertebrates which the exposure to estrogenic compounds affected lipid metabolism. Palace et al. (2001) observed TLP content was elevated in fish gonads

treated with the highest dose tested (125 ng·L^{-1}) of EE2 compared with controls. Energy reserves (TLP) and consumption (MET activity) can be considered a general indicator of the energetic status of the organisms exposed to pharmaceutical compounds.

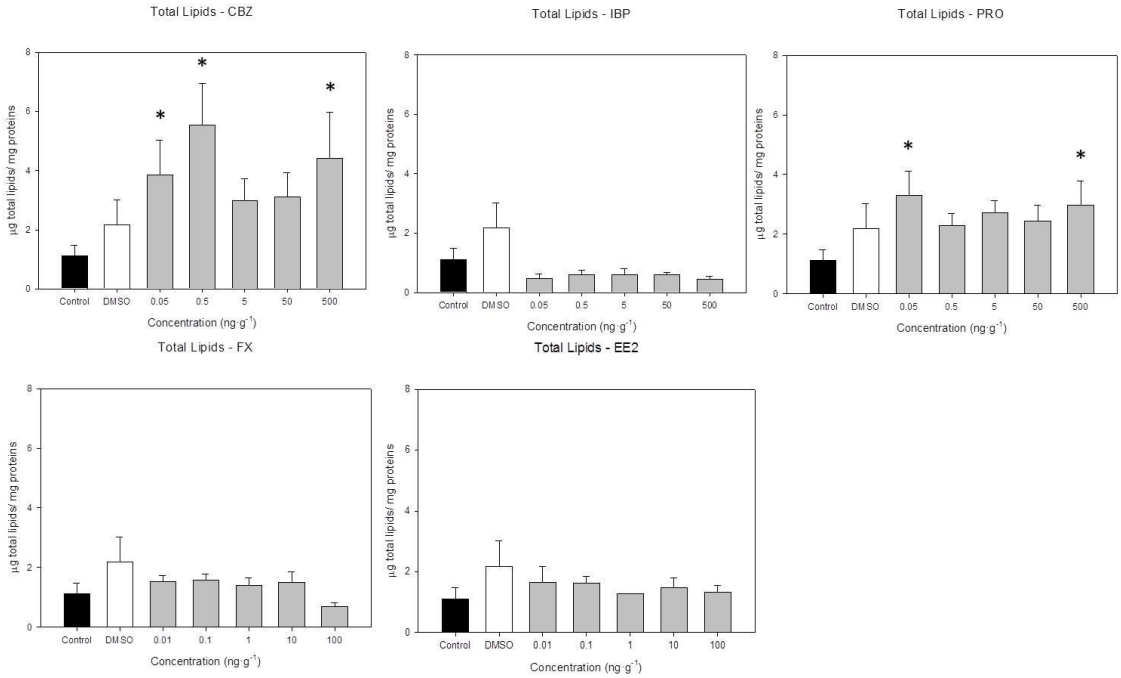

Figure 1. Mean (± standard deviation) of total lipids analysed in *Hediste diversicolor* after 14-days of laboratory exposure to controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) and spiked sediment samples with carbamazepine (CBZ), ibuprofen (IBP) and propranolol (PRO) [500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1} and 0.05 ng·g^{-1}], fluoxetine (FX) and 17α-ethynylestradiol (EE2) [100 ng·g^{-1}, 10 ng·g^{-1}, 1 ng·g^{-1}, 0.1 ng·g^{-1} and 0.01 ng·g^{-1}]. Environmental concentrations are underlined.
* represent significant differences between controls ($p < 0.05$) and the pharmaceutical concentrations.

Mitochondrial electron transport (MET) activity is shown in the Figure 2. Polychaetes exposed to CBZ showed high levels of TLP and MET activity: CBZ concentrations of 500 ng·g^{-1} and 0.5 ng·g^{-1} were able to significant increase MET activity when compared with the control ($p < 0.05$). Polychaetes exposed to IBP were not reserving energy. On the other hand, MET activity measured in polychaetes exposed to IBP concentration of 5 ng·g^{-1} was significantly lower compared with the control ($p < 0.05$). The increase of MET chain can induce cell death through generating reactive oxygen species (ROS) (Chen et al., 2007). FX and EE2 did not show interference with the

MET activity or TLP. Polychaetes exposed to PRO (500 ng·g^{-1} and 0.05 ng·g^{-1}) were reserving energy, but not spending them through MET activity.

Chronic toxicant effects may drain the energy during exposure to stressors mobilizing energy stores (Gagné et al., 2007 a, Smolders et al., 2004). The ability of PPCPs to increase the rate of mitochondrial electron transport (MET activity) was previously observed in mussels (Gagné et al., 2006). Reductions in survival, growth and reproduction occur via an energetic route. Freshwater mussels exposed to concentrations of CBZ, IBP and FX showed an increase of the MET activity (Gagné et al., 2006).

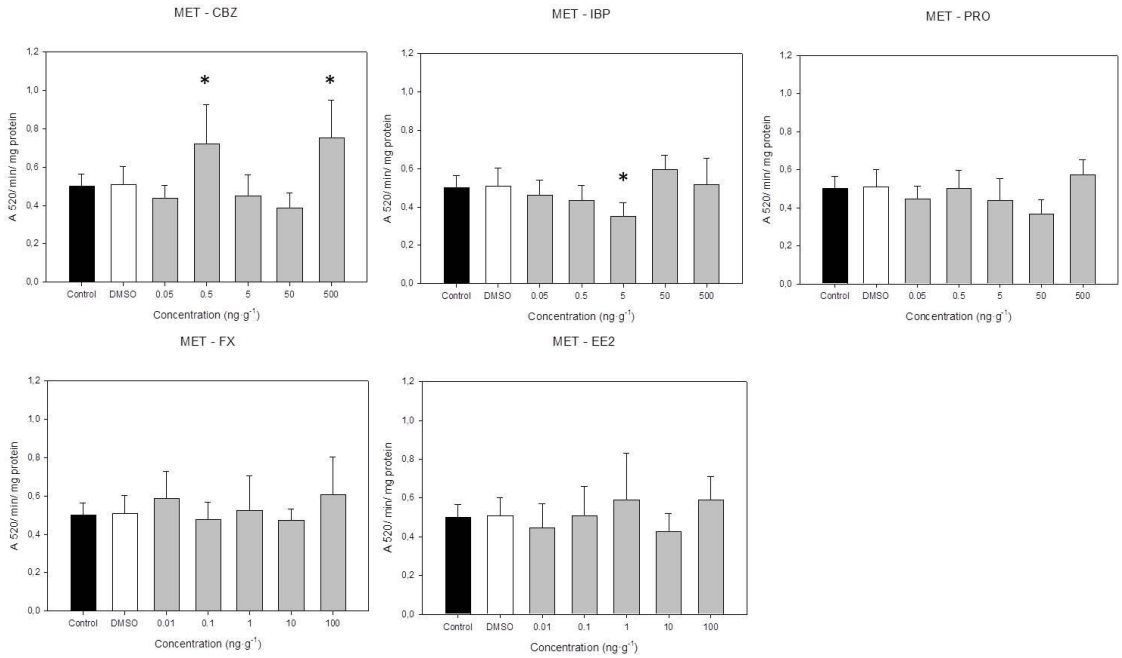

Figure 2. Mean (± standard deviation) of mitochondrial electron transport (MET) activity analysed in *Hediste diversicolor* after 14-days of laboratory exposure to controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) and spiked sediment samples with carbamazepine (CBZ), ibuprofen (IBP) and propranolol (PRO) [500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1} and 0.05 ng·g^{-1}], fluoxetine (FX) and 17α-ethynylestradiol (EE2) [100 ng·g^{-1}, 10 ng·g^{-1}, 1 ng·g^{-1}, 0.1 ng·g^{-1} and 0.01 ng·g^{-1}]. Environmental concentrations are underlined.
* represent significant differences between controls ($p < 0.05$) and the pharmaceutical concentrations.

Data concerning alkali-labile phosphate (ALP) levels is shown in the Figure 3. For *H. diversicolor* exposed to the concentrations of pharmaceutical products, ALP levels

were significantly decreased after the exposure to FX (0.01 $ng \cdot g^{-1}$) compared with the control ($p < 0.05$). Previous studies observed that FX stimulated reproduction in the amphipods *Hyalella azteca* (Brooks et al., 2003) and induced mussel spawning (Fong et al., 1998). No effects of FX concentrations, higher than used in the present study, were observed on egestion rates, body weight and size-specific egestion rates of the marine-estuarine polychaete *Capitella* (Mendéz et al., 2013). On the other hand, FX favoured the occurrence of males with abnormal genital spines which suggested important reproductive implications (Mendéz et al., 2013).

ALP is cleaved to form yolk proteins, a large molecular weight lipophosphoprotein (Melancon et al., 1992). The impacts of pharmaceuticals may be significant for polychaetes such as Nereids that have sensitive steroidal-influenced reproductive systems (Lewis and Watson, 2012). Previous studies described endocrine regulation of reproduction of *H. diversicolor* related to neuroendocrinology aspects (Durou and Mouneyrac, 2007). Decrease of ALP levels can be due the inhibition of its production. Therefore, ALP can accumulate in the oocytes of young *H. diversicolor* females without undergoing any significant structural modification (Bonnier and Baert, 1992). This suggests that the activation of the ALP processing in *Nereid* oocytes depends upon the developmental stage. All the polychaetes chosen in the present study were near the maturation phase. Although, there was no increase of ALP levels between the different concentrations of pharmaceutical products tested.

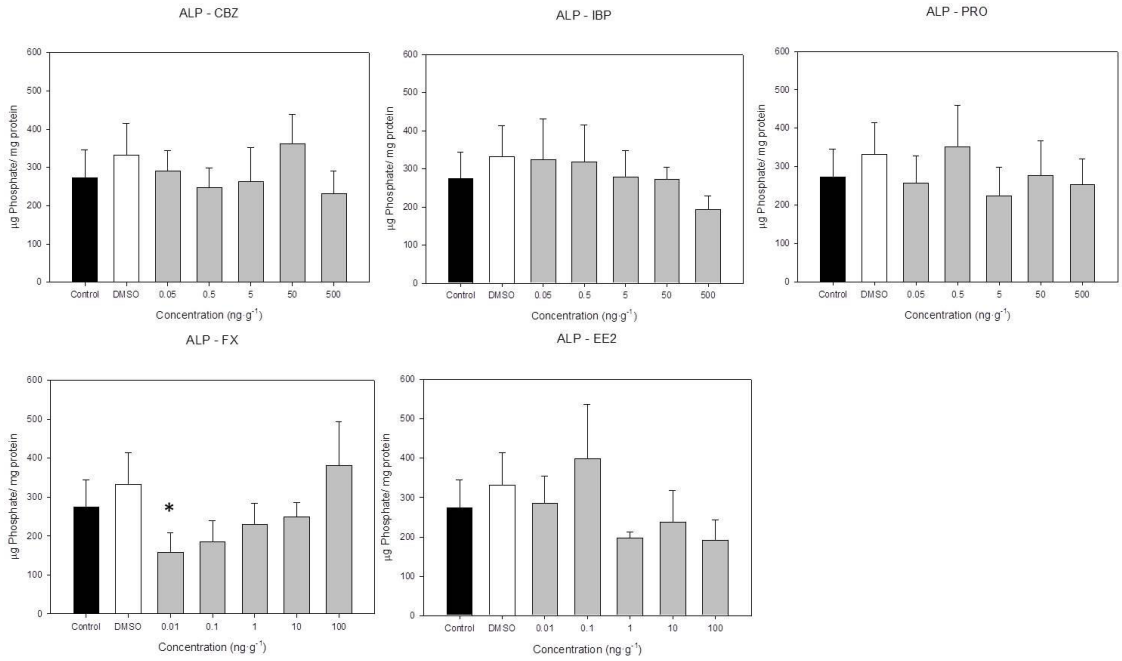

Figure 3. Mean (± standard deviation) of alkali-labile phosphate (ALP) levels analysed in *Hediste diversicolor* after 14-days of laboratory exposure to controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) and spiked sediment samples with carbamazepine (CBZ), ibuprofen (IBP) and propranolol (PRO) [500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1} and 0.05 ng·g^{-1}], fluoxetine (FX) and 17α-ethynylestradiol (EE2) [100 ng·g^{-1}, 10 ng·g^{-1}, 1 ng·g^{-1}, 0.1 ng·g^{-1} and 0.01 ng·g^{-1}]. Environmental concentrations are underlined.
* represent significant differences between controls ($p < 0.05$) and the pharmaceutical concentrations.

The data concerning MAO activities measured in polychaetes is shown in the Figure 4. High level of MAO activity was determined in polychaetes exposed to IBP (0.5 ng·g^{-1}) ($p < 0.05$). Organisms exposed to CBZ (500 ng·g^{-1}, 50 ng·g^{-1}), FX (1 ng·g^{-1}, 0.01 ng·g^{-1}), EE2 (1 ng·g^{-1}) and PRO (5 ng·g^{-1}, 0.05 ng·g^{-1}) showed significant decrease of MAO activity compared with the control ($p < 0.05$). Several mechanisms have been suggested to explain CBZ efficacy in epilepsy, including the regulation of brain monoamine levels (Bazinet et al., 2006). FX is a selective serotonin reuptake inhibitor (SSRI) and was identified as a weak inhibitor of MAO (Brooks et al., 2003). Inhibitors of MAO have therapeutic value in the treatment of depressive illness, for example Parkinson disease and depression (Bazinet et al., 2006). There is a paucity of investigations about monoamine metabolism in polychaetes, but the present study could inform that some

pharmaceutical compounds can interfere in the MAO activity responses. MAO activity is involved in the catabolism of biogenic amines such as serotonin, dopamine and adrenaline and could serve as indicator of neuroendocrine disruption. The presence of 5-HT, dopamine and noradrenaline has been reported in polychaetes nervous tissue (Sloley, 2004). Monoamines (e.g., serotonin and dopamine) are important mediators of gamete maturation and spawning (Gagné et al., 2007 b).

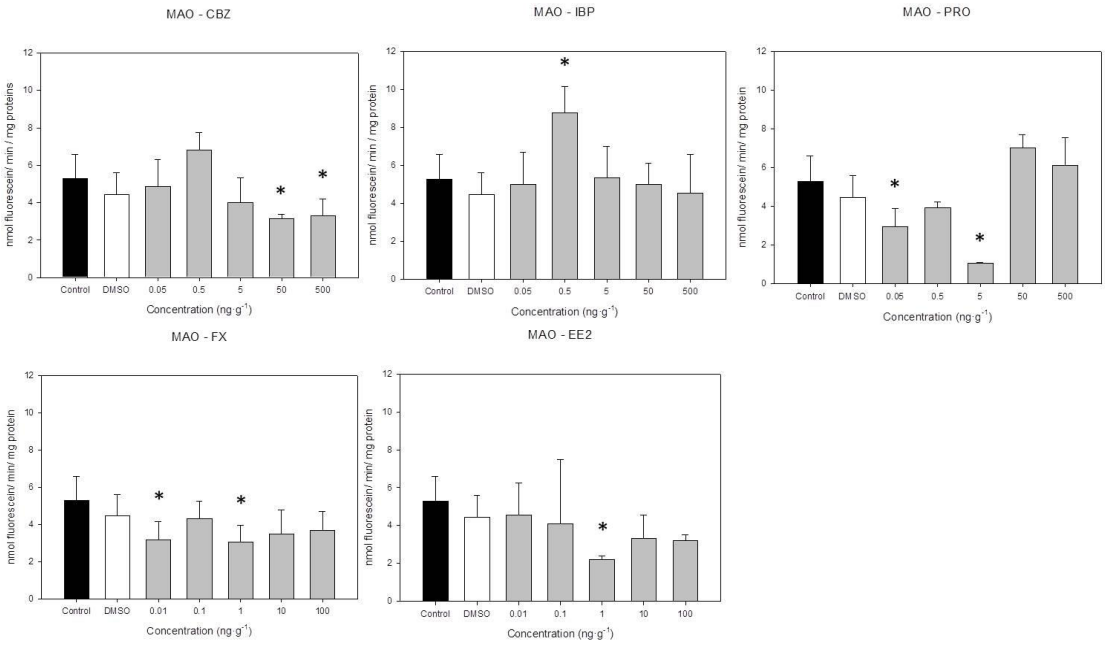

Figure 4. Mean (± standard deviation) of monoamine oxidase (MAO) activity analysed in *Hediste diversicolor* after 14-days of laboratory exposure to controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) and spiked sediment samples with carbamazepine (CBZ), ibuprofen (IBP) and propranolol (PRO) [500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1} and 0.05 ng·g^{-1}], fluoxetine (FX) and 17α-ethynylestradiol (EE2) [100 ng·g^{-1}, 10 ng·g^{-1}, 1 ng·g^{-1}, 0.1 ng·g^{-1} and 0.01 ng·g^{-1}]. Environmental concentrations are underlined.
* represent significant differences between controls ($p < 0.05$) and the pharmaceutical concentrations.

The data concerning COX activities is shown in the Figure 5. IBP exerts its effects through inhibition of COX activity, as in the present study when all the concentrations of IBP were significantly lower than the control ($p < 0.05$). COX was highly positive correlated with TLP for IBP ($p < 0.01$). The immune system provides resistance to

infectious agents, destruction of neoplastic cells and rejection of nonself-components (Siwickiia et al., 1998). Spawning in invertebrates is activated by serotonin and prostaglandin released by the induction of COX (Matsutani and Nomura, 1987). Inhibition of this enzymatic activity can provide relief from the symptoms of inflammation and pain.

FX has recently been found to possess anti-inflammatory properties proved in rats (Branco-de-Almeida et al., 2012), which was not observed in polychaetes. An experiment with long time of exposure is recommended. COX was positively correlated with TLP for FX ($p < 0.05$).

CBZ down regulates the kinetics of arachidonic acid in brain phospholipids (Bazinet et al., 2006). Polychaetes showed to down regulate COX activity significantly lower than the control ($p < 0.05$). Cyclooxygenase (COX) is an enzyme that provides the formation of important biological mediators, including prostaglandins, prostacyclin and thromboxane (Dubois et al., 1998). For polychaetes, all the individuals exposed to PRO concentrations presented significantly lower COX activity than the control ($p < 0.05$). Nevertheless, COX activity was negatively correlated with TLP for CBZ and PRO ($p < 0.01$). All polychaetes exposed to EE2 concentrations showed significantly lower COX activity than the control ($p < 0.05$). Previous study reported COX-2 expression significantly inhibited in the glandular epithelium of women who used ethynylestradiol (Maia et al., 2010).

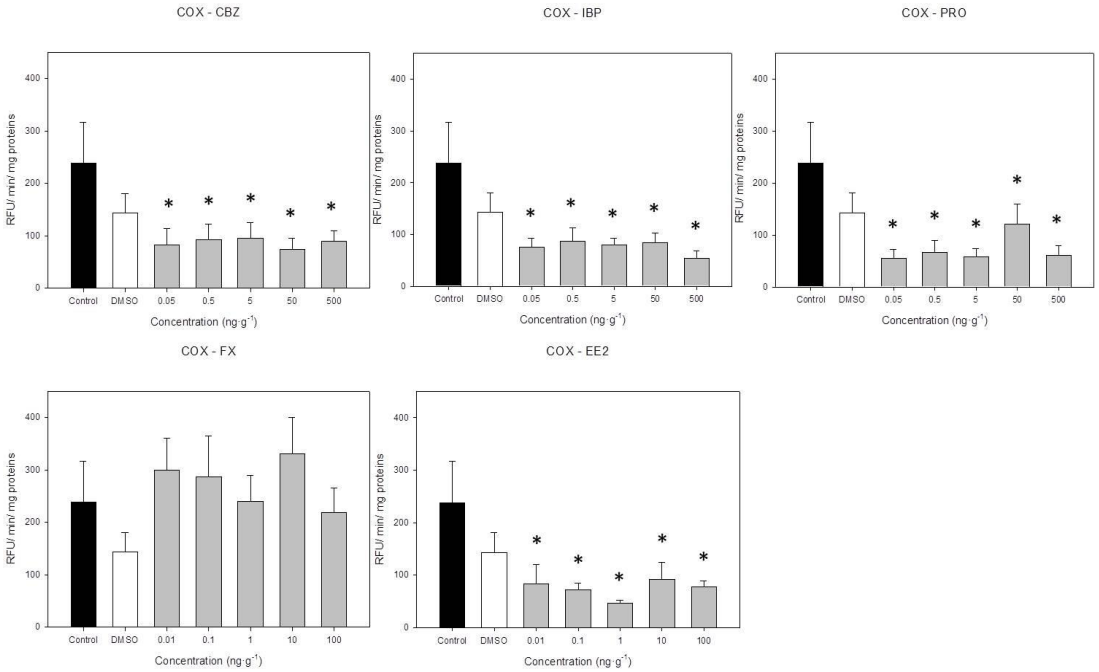

Figure 5. Mean (± standard deviation) of cyclooxygenase (COX) activity analysed in *Hediste diversicolor* after 14-days of laboratory exposure to controls (reference sediment and reference sediment spiked with DMSO 0.001% (v/v)) and spiked sediment samples with carbamazepine (CBZ), ibuprofen (IBP) and propranolol (PRO) [500 ng·g^{-1}, 50 ng·g^{-1}, 5 ng·g^{-1}, 0.5 ng·g^{-1} and 0.05 ng·g^{-1}], fluoxetine (FX) and 17α-ethynylestradiol (EE2) [100 ng·g^{-1}, 10 ng·g^{-1}, 1 ng·g^{-1}, 0.1 ng·g^{-1} and 0.01 ng·g^{-1}]. Environmental concentrations are underlined.
* represent significant differences between controls ($p < 0.05$) and the pharmaceutical concentrations.

Pharmaceutical compounds are biologically active compounds that are designed to interact with a receptor in the target organisms. Polychaetes showed responses related to the MOAs of pharmaceutical products which corroborates with the fact that such compounds may affect organisms in the environment.

3.3. Correlations between biomarker responses and the environmental concentrations of each pharmaceutical product tested.

Polychaetes exposed to environmental concentrations of CBZ increased TLP levels and spent the energy through MET activity. There was no influence on the metabolism of monoamines (neuroendocrine effect determined as MAO activity).

However, CBZ also presented anti-inflammation properties even at low concentrations, as evidenced by the inhibition of COX activity. Mortality was positively correlated with the CBZ concentrations ($p < 0.01$), energy reserve (TLP) ($p < 0.01$) and negatively correlated with monoamine activity (MAO) and inflammation properties (COX) ($p < 0.01$).

For IBP, environmental concentration did not interfere on the energy demand (TLP), but on the energy spend (MET activity). ALP levels did not change. IBP is an anti-inflammatory widely used worldwide, and the environmental concentrations tested showed the inhibition of COX activity. Mortality was positively correlated with concentrations ($p < 0.01$) and negatively correlated with energy demand (TLP) and inflammation properties (COX) ($p < 0.01$).

FX may bind to sediments and affect benthic organisms (Brooks et al., 2003). Nevertheless, benthic macroinvertebrates responses to marine sediment FX exposures have not been reported. Environmental concentrations did not show energy demand (TLP) or spend (MET activity) by the polychaetes exposed to FX spiked-sediment samples. Serotonin may stimulate ecdysteroids, ecdysone, and juvenile hormone in invertebrates, which are responsible for controlling oogenesis and vitellogenesis. The lower concentrations seemed to significantly decreased metabolism of monoamines (MAO activity) and consequently ALP levels, which could be related to the inhibition of serotonin re-uptake (Brooks et al., 2003). Environmental concentrations of FX did not interfere in the COX activity. Mortality was positively correlated with FX concentrations ($p < 0.05$) and energy reserves (TLP) ($p < 0.01$), and negatively correlated with ALP levels ($p < 0.01$) and metabolism of monoamines (MAO activity) ($p < 0.05$).

Polychaetes exposed to environmental concentrations of EE2 did not show changes in the energy status (TLP or MET activity) or ALP levels. Neuroendocrine effects MAO and COX activities were significantly lower in polychaetes exposed to EE2

environmental concentrations. Mortality was positively correlated with concentrations ($p < 0.01$) and energy reserve (TLP) ($p < 0.05$), and negatively correlated with neuroendocrine effects (MAO and COX activities) ($p < 0.01$).

Environmental concentrations of PRO showed the increase of energy reserve (TLP) but not the increases of energy spend (MET activity). PRO did not interfere in ALP levels of polychaetes exposed to environmental concentrations. Neuroendocrine effect (MAO activity) was significantly lower for polychaetes exposed to environmental concentrations of PRO. PRO showed anti-inflammatory properties, since the environmental concentrations tested inhibited COX activity. Mortality was positively correlated with energy store TLP ($p < 0.01$) and negatively correlated with neuroendocrine effects (MAO ($p < 0.01$) and COX activities ($p < 0.05$)).

3.4. Integrated biomarker response - IBR index

The integrated biomarker responses (IBR) index was calculated for the all concentrations of the pharmaceutical products tested. Environmental concentrations were grouped in one group (0.05 $ng \cdot g^{-1}$, 0.5 $ng \cdot g^{-1}$, 5 $ng \cdot g^{-1}$ of CBZ, IBP and PRO; 0.01 $ng \cdot g^{-1}$, 0.1 $ng \cdot g^{-1}$, 1 $ng \cdot g^{-1}$ of FX and EE2), and the two highest concentrations were calculated separately.

IBR index was shown in the Figure 6. The area circulating in black integrates the IBR for environmental concentrations and the two highest concentrations tested of each pharmaceutical product represented as star plots. IBR values showed large range of variation between the pharmaceutical responses and concentrations, varying from -4 to 1 for environmental concentrations, from -6 to 2 for the second highest concentrations, and from -50 to 10 for the highest concentrations tested. CBZ, IBP, FX and EE2 affected the energy status (MET activity and TLP) for the highest two concentrations (50 $ng \cdot g^{-1}$ and

500 ng·g^{-1} for CBZ and IBP; 10 ng·g^{-1} and 100 ng·g^{-1} for FX and EE2). However, the two highest concentrations of PRO (50 ng·g^{-1} and 500 ng·g^{-1}) were mainly related to energy status (MET activity) and neuroendocrine effect (MAO activity). Inflammation properties (COX activity) was inhibited for the environmental concentrations of all pharmaceutical products tested, which means that they have anti-inflammatory properties in polychaetes exposed to the spiked-sediment samples.

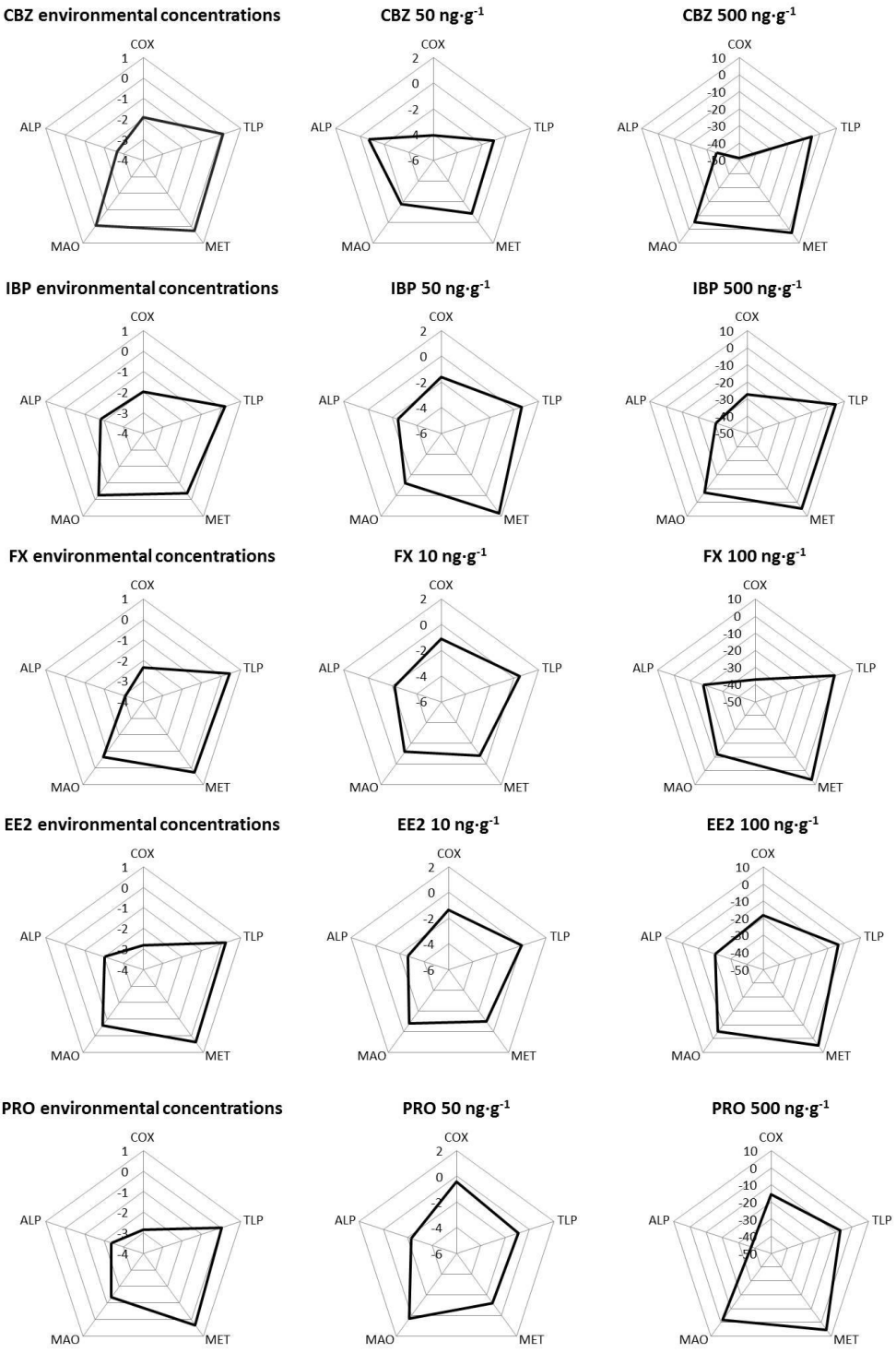

Figure 6. Integrated biomarker response (IBR) and pollutant star plots: environmental concentrations of each pharmaceutical product [(CBZ, IBP, PRO: 5 ng·g^{-1}, 0.5 ng·g^{-1}, 0.05 ng·g^{-1}) and (FX, EE2: 1 ng·g^{-1}, 0.1 ng·g^{-1}, 0.01 ng·g^{-1})], the second highest concentration [(CBZ, IBP, PRO: 50 ng·g^{-1}) and (FX, EE2: 10 ng·g^{-1})] and the highest concentration tested [(CBZ, IBP, PRO: 500 ng·g^{-1}) and (FX, EE2: 100 ng·g^{-1})]. Biomarker responses considered for the IBR index were: COX (cyclooxygenase activity), TLP (total lipids), MET (mitochondrial electron transport activity), MAO (monoamine oxidase activity) and ALP (alkali-labile phosphate levels).

4. CONCLUSION

This study emphasizes the importance of selecting a battery of biomarkers that are potential monitors for stress on marine organisms. The selection of biomarker responses related to the MOAs of pharmaceutical products is important, with possible profound effects on an organism's survival, immune function and reproduction. Polychaetes are part of the dominant benthic fauna in the marine environment and play an important role in biomonitoring programs for the marine sediment quality. This organism is a suitable bioindicator for the assessment of the chronic toxicity of pharmaceutical products in the marine environment. The battery of biomarker responses used in the present study is a recommended tool for the characterization of the effects of each pharmaceutical product in the organism. These findings can be applied to better predict long-term impacts of pharmaceuticals in the marine and estuarine environments.

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CAPÍTULO 4

Evaluación de la calidad ambiental de sedimentos marinos afectados por vertidos de agua residuales en la Bahía de Cádiz: ensayos de toxicidad agudos y crónicos en laboratorio. Efecto de la estacionalidad.

Las zonas costeras de todo el mundo se encuentran entre los ecosistemas más amenazados por las actividades antrópicas. Estuarios y aguas costeras reciben contaminantes a partir de múltiples fuentes y vertidos, que producen un escenario complejo, en que la heterogeneidad del medio ambiente natural combina la presencia de decenas o cientos de contaminantes. En este contexto, la evaluación de los efectos de la contaminación se convierte en una tarea difícil, ya que la mayoría de los estudios son dedicados a establecer las concentraciones máximas permitidas. Por lo tanto, los efectos de las combinaciones complejas de múltiples contaminantes son casi desconocidos.

Hay varias herramientas disponibles para evaluar la contaminación acuática y sus efectos en la biota. Los análisis químicos se utilizan para identificar la presencia de posibles contaminantes y sus concentraciones, mientras que se recomiendan testes de toxicidad conjuntos con los análisis químicos, para evaluar los efectos potencialmente perjudiciales (Carr et al., 2008), ya que proporcionan respuestas resultantes de mezclas complejas de contaminantes, que normalmente no es posible cuando los análisis químicos se usan solos.

La evaluación de la calidad de la descarga proveniente de estaciones depuradoras de aguas residuales (EDAR) en los sistemas acuáticos se debe basar en la caracterización integrada entre diferentes líneas de evidencia: caracterización química, toxicidad aguda y crónica en laboratorio y en campo (*in situ*). La integración de estos resultados mediante el análisis multivariado permite visualizar la contaminación en el medio ambiente y los riesgos para la biota y salud humana, en el cual se permite diseñar una herramienta para la toma de decisiones en la gestión de estos compuestos. Los estudios que se centraron en la contaminación por fármacos son especialmente importantes en vista de su inclusión en un marco normativo que pueden contribuir positivamente a llenar el vacío con respecto al riesgo ambiental de tales fuentes de contaminación.

Actualmente está bien establecido el hecho de que los fármacos utilizados durante los tratamientos médicos puedan ser excretados parcialmente sin metabolizar, pasando por los sistemas de drenaje urbanos, y que todavía sobreviven a los tratamientos de aguas residuales. Por lo tanto, las estaciones de tratamiento de aguas residuales son la principal fuente para la introducción de productos farmacéuticos en el medio acuático (Beausse, 2004, Buchberger, 2007).

La Bahía de Cádiz (Andalucía, España) constituye un ambiente estuario marino con alto valor ecológico e importancia socio-económica. La Bahía está rodeada de marismas, y gran parte de estos ambientes están protegidos por ley (Parque Natural de la Bahía de Cádiz desde 1989). Por otro lado, vertidos de distintas EDAR descargan sus efluentes diariamente en la Bahía, que conllevan un conjunto de diferentes contaminantes en la región. La Bahía de Cádiz constituye un mosaico con muchas partes interesadas compartiendo el mismo medio ambiente: la pesca, el turismo, industrias, puertos, puertos deportivos, vertederos domésticos e industriales y barriadas. Adicional a este panorama, hay el incremento de la población en verano, con el aumento de aproximadamente 30%

(INE, 2011). Las grandes cantidades de residuos sólidos, materia orgánica, metales, hidrocarburos, fármacos y otros contaminantes que se introducen en la Bahía pueden acumularse en los sedimentos. Por lo tanto, la evaluación de la calidad de sedimentos es importante para la gestión de tales ecosistemas acuáticos.

En este capítulo está incluida la evaluación de la calidad de sedimento en laboratorio, con análisis químicas, toxicidad aguda y crónica. Un importante factor llevado en consideración fue la estacionalidad, ya que la composición y concentración de los contaminantes en sedimento variaran con las estaciones del año, inverno y verano. Fueran evaluados la calidad del sedimento de seis distintos puntos (P1 – P6) de la Bahía de Cádiz, muestreados con una draga Van Veen o con la ayuda de buzos, cuando necesario (Figura 12). Los muestreos se llevaran a cabo al final del invierno y verano de 2011, a fin de comparar las distintas respuestas en ambas estaciones del año.

Figura 12. Muestreo de sedimento en barco y por tierra.

P1, P2 y P5 son puntos directamente afectados por vertidos de estaciones depuradoras (EDAR), siendo que P3 y P4 son afectados por estaciones de bombeo de aguas residuales (EBAR). El sexto punto (P6) está localizado en Rota, lejos de tales fuentes de contaminación y con alta renovación de agua, y fue considerado como punto control. La localización de los distintos puntos está en el mapa (Figura 13) de los siguientes artículos e ilustrados en la Figura 14.

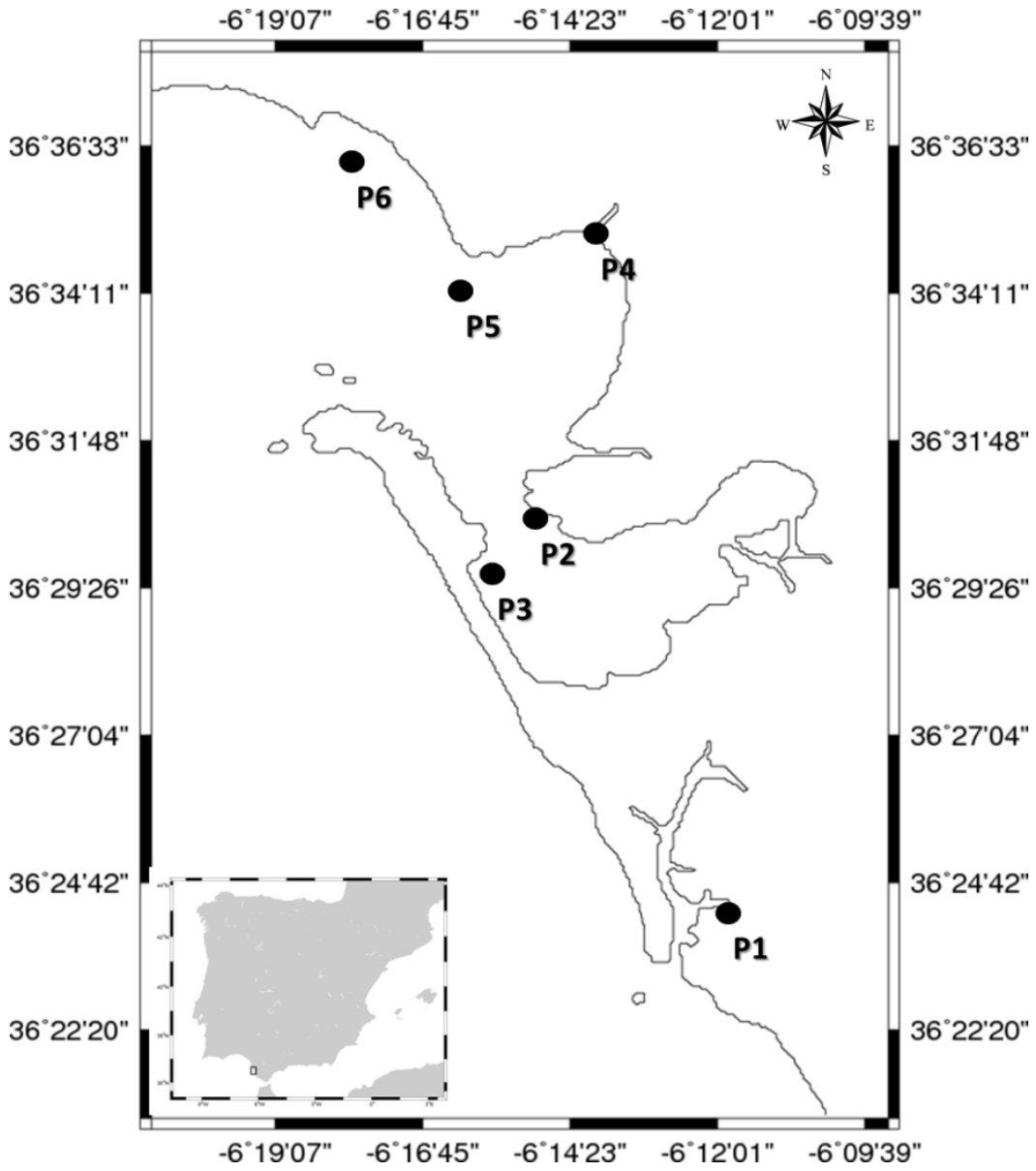

Figura 13. Localización de los distintos puntos de muestreo en la Bahía de Cádiz.

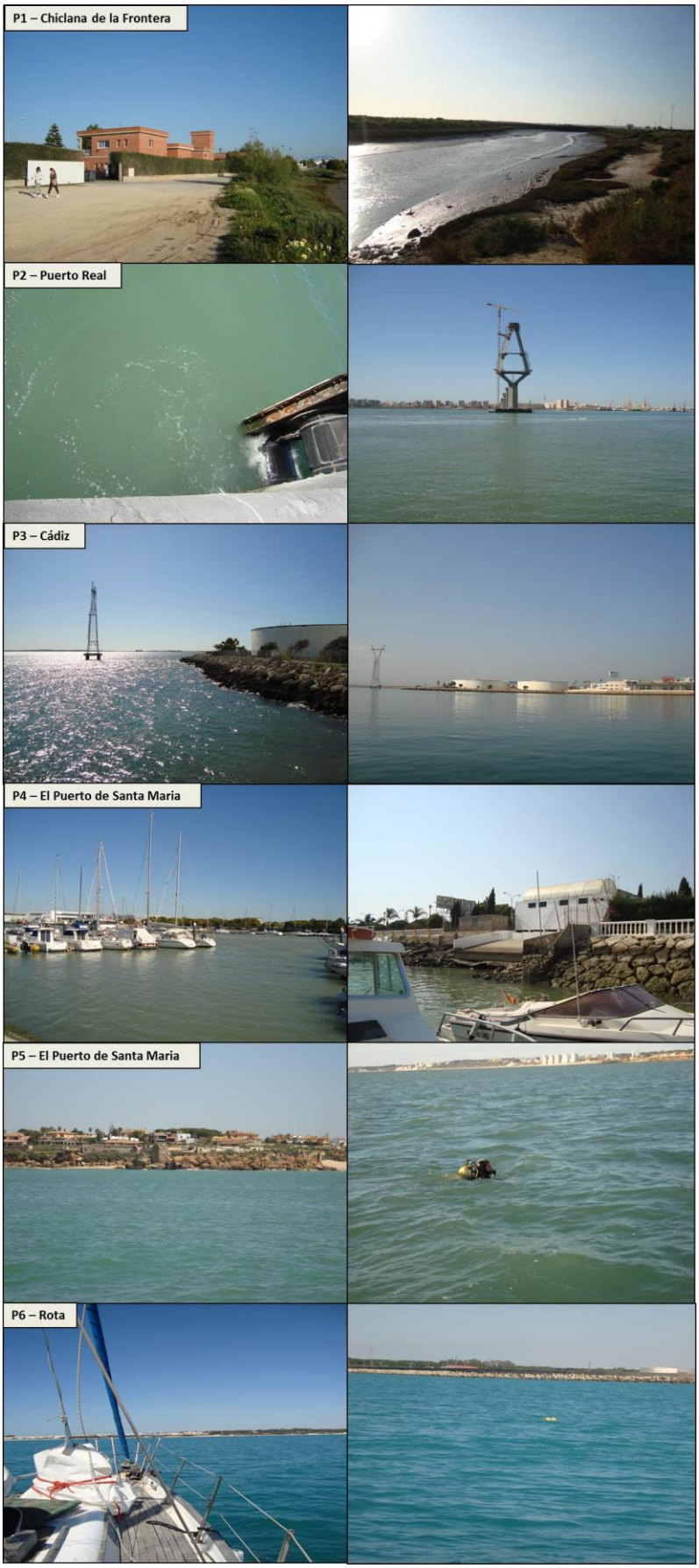

Figura 14. Los distintos puntos de muestreo en la Bahía de Cádiz.

Fueran determinados las concentraciones de metales (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Hg, Zn), hidrocarburos aromáticos policíclicos (PAH), fármacos (anti-inflamatorios: acetaminofeno, diclofenaco, fenoprofeno; anti-hipertensivos: atenolol, propranolol; reguladores de lípido: ácido clofíbrico, gemfibrozil; drogas psiquiátricas: carbamazepina, fluoxetina, amitriptilina, cafeína; antibióticos: cloranfenicol, cefdinir, tiamulina, eritromicina, claritromicina, azitromicina, roxitromicina, lincomicina, flumequina, clindamicina, esparfloxacina, novobiocina, metronidazol ornidazol, sulfadiazina, sulfametoxipiridazina, sulfatiazol, trimetoprim, monensina; antiácidos: famotidina, ranitidina; y otros: glibenclamida, hidroclorotiazida) y surfactantes (en inglés: secondary alkane sulfonates - SAS) en las muestras de sedimento. Carbono orgánico total (en inglés: total organic carbon – TOC), granulometría y contenido de materia orgánica fueran determinados en cada muestra (Figura 15).

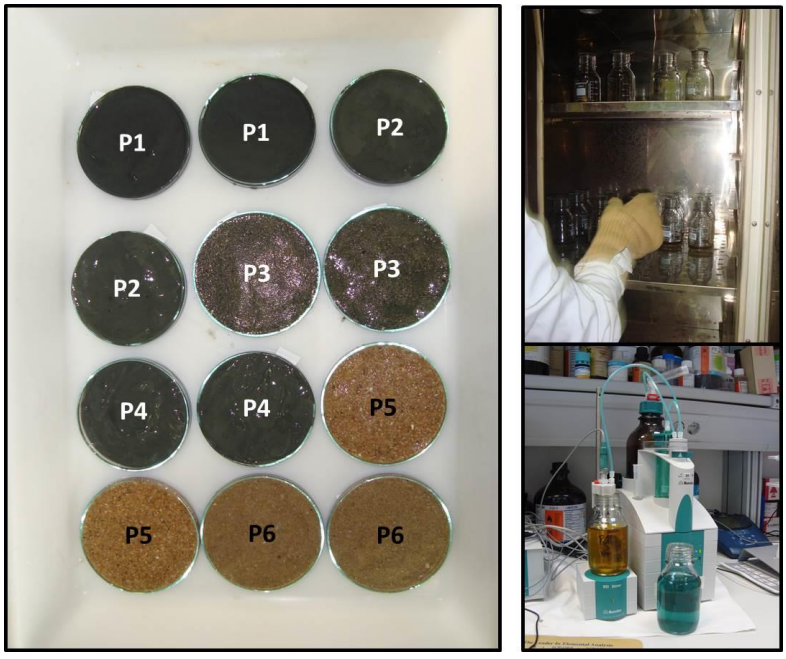

Figura 15. Muestras de sedimento de los distintos puntos de muestreo (P1 – P6). TOC fue determinado en las distintas muestras.

El articulo V trata de los efectos agudos relacionados a la contaminación de sedimento directamente afectado por vertidos de aguas residuales. Los distintos bioensayos referentes a diferentes fases del sedimento están ilustrados en la Figura 16. Los bioensayos con sedimento bruto fueran realizados para determinar la tasa de mortalidad de anfípodos *Ampelisca brevicornis* y la inhibición de la bioluminiscencia de la bacteria *Vibrio fischeri*. Los lixiviados provenientes del sedimento bruto fueran analizados a través de la inhibición de la bioluminiscencia de la bacteria *Vibrio fischeri*, los efectos en la fecundación y en desarrollo embriolarval de erizos de mar *Paracentrotus lividus*, y también de la tasa de crecimiento de las microalgas *Isochrysis galbana* y *Tetraselmis chuii*.

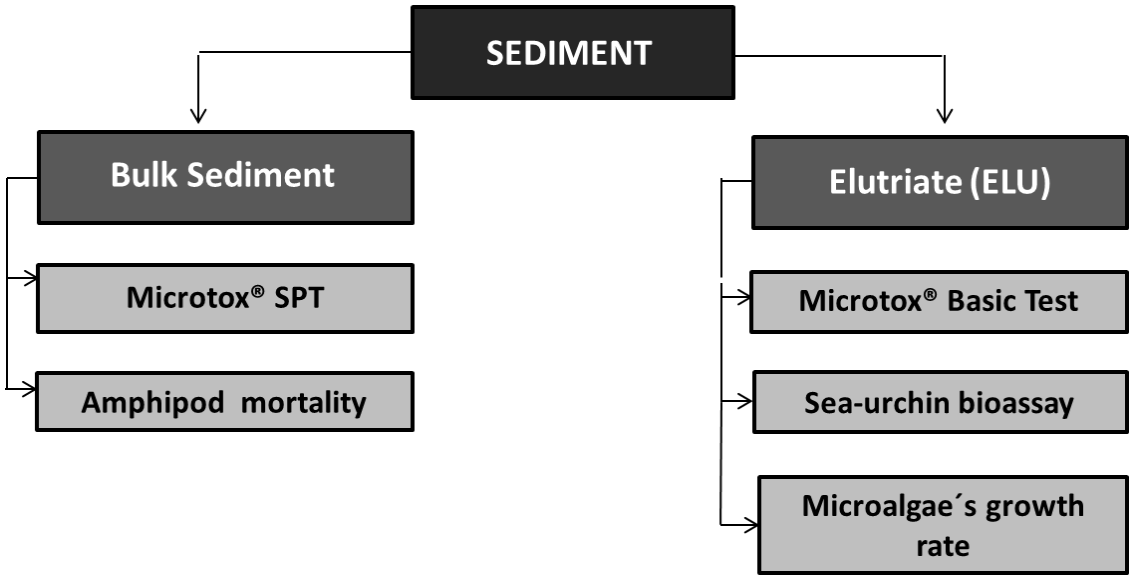

Figura 16. Bioensayos para evaluación de sedimento bruto (en inglés: bulk sediment) y lixiviados (en inglés: elutriate). SPT = Solid Phase Test.

A través del análisis multivariado fue posible determinar los principales contaminantes responsables por los efectos en la biota, incluyendo fármacos y surfactantes, lo que es extremamente novedoso ya que no hay estudios que relacionen tales contaminantes con los efectos agudos en la biota acuática. Fue posible relacionar los

contaminantes que pueden volver a la columna de agua por ressuspensión del sedimento, causada por factores naturales (e.j. tempestades) o antropogénicos (e.j. dragaje), con efectos agudos en la biota acuática. Estos datos están incluidos en el artículo V.

Es de gran relevancia la inclusión de estudios con biomarcadores en el diagnóstico y la vigilancia del medio ambiente con el fin de la pesca, maricultura y la protección de la biota, así como para regular las concentraciones seguras de xenobióticos que son introducidos en el medio ambiente. Varios organismos acuáticos han sido empleados en estudios sobre la vigilancia de la calidad ambiental de las zonas costeras, principalmente el uso de los moluscos bivalvos, como las almejas.

En esta parte de la Tesis, fueran utilizados almejas de la especie *Ruditapes philippinarum* para la exposición en laboratorio a las muestras de sedimento bruto colectadas en campo. Los organismos fueran comprados de una acuicultura localizada en Chiclana de la Frontera, aclimatados en laboratorio y expuestos a los sedimentos en duplicado (Figura 17), por un período de 14-días sobre condiciones controladas.

Figure 17. Aclimatación y exposición de almejas *Ruditapes philippinarum* a sedimentos muestreados en campo, directamente afectados por vertidos de aguas residuales.

Pasados los 14 días, las almejas pasaran por un proceso de depuración, y en seguida fue determinado la estabilidad de la membrana lisosómica (en inglés: lysosomal membrane stability – LMS) de la células de la hemolinfa (artículo VI). Las almejas fueran congeladas para posterior homogenización de los distintos tejidos y análisis de los biomarcadores en glándula digestiva y gónadas (Figura 18). Este parte de la Tesis dio origen a los artículos VII y VIII.

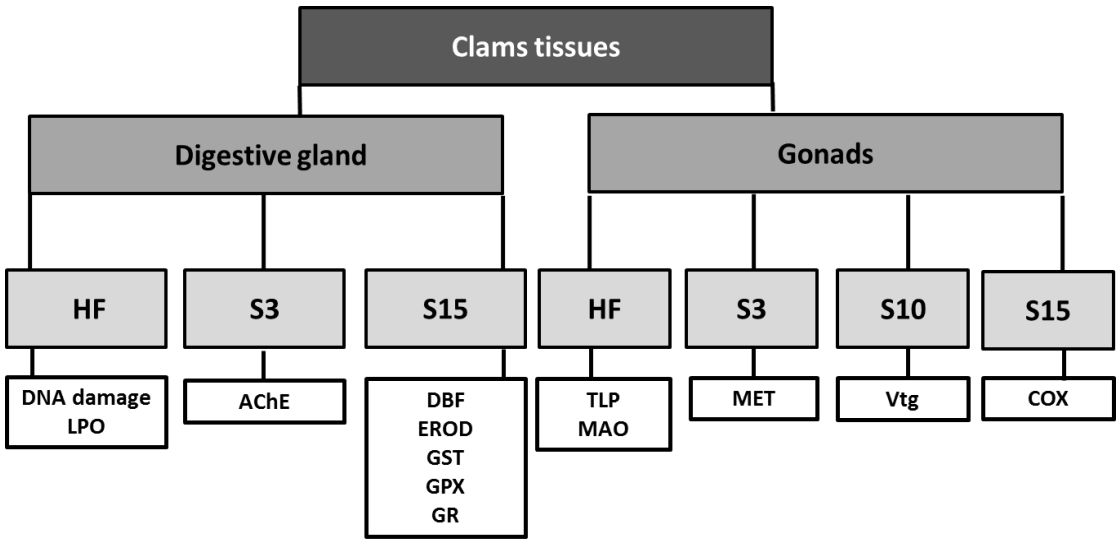

Figura 18. HF = fracción homogeneizada. S3 = HF centrifugado a 3.000g por 20 min a 4 ^{o}C. S10 = HF centrifugado a 10.000g por 5 min a 4 ^{o}C. S15 = HF centrifugado a 15.000g por 20 min a 4 ^{o}C. Los biomarcadores determinados en cada extracto están descritos en las cajas blancas.

Las ventajas de la aplicación de una batería adecuada de biomarcadores posee la capacidad de predecir la toxicidad, la elucidación de la amplia gama de contaminantes, así como su biodisponibilidad para la evaluación del estado ecológico y la salud de la biota afectada (Martín-Díaz et al., 2008). Por lo tanto, el uso de tales métodos son particularmente recomendados como parte de técnicas complementarias o herramientas aplicadas en el estudio de "peso de evidencias" (en inglés: weight of evidence approach - WOE) en las evaluaciones ambientales (Martín-Díaz et al., 2008).

En cuanto a los daños causados por xenobióticos en orgánulos, la integridad de la membrana lisosomal se ha utilizado como un biomarcador de estrés químico (Figura 19). El ensayo de tiempo de retención del colorante rojo neutro (en inglés: Neutral Red Retention Time – NRRT) en los hemocitos de los bivalvos (Lowe, 1995) se ha utilizado con el fin de detectar cambios en la permeabilidad de la membrana lisosomal, que pueden ocurrir debido a la exposición a contaminantes, lo que causa el aumento de los procesos de autofagia seguidos por degeneración celular. Una considerable evidencia sugiere que esta es una respuesta generalizada al estrés químico, y debido a su alta sensibilidad a la mayoría de los grupos de contaminantes y la extrapolación probable de daños a los niveles más elevados de organización biológica, el uso de este biomarcador se ha recomendado en la vigilancia ambiental. NRRT ya fue incluido en programas ambientales como el Mediterranean Action Plan (UNEP/MAP, 2007).

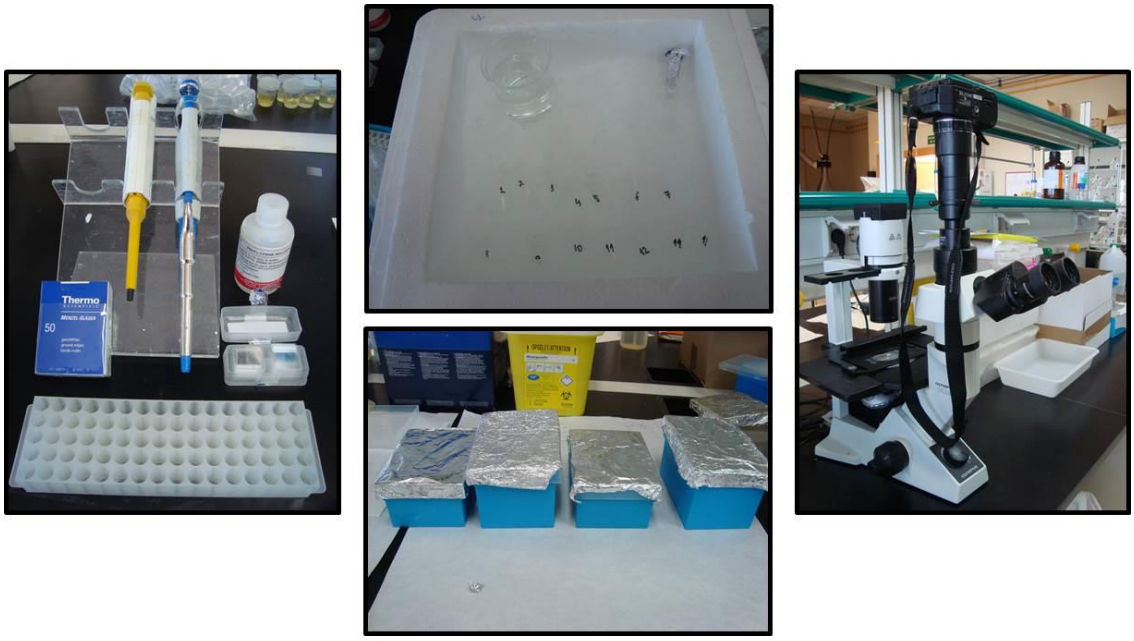

Figura 19. Ensayo del tiempo de retención del rojo neutro (NRRT) en almejas *Ruditapes philippinarum*.

Biomarcadores de estrés y efecto (EROD, DBF, GST, GPX, GR, AChE, LPO, DNA damage), y NRRT fueran correlacionados con concentraciones ambientales de PAHs, metales, fármacos y surfactantes en sedimento muestreados en dos estaciones del año: inverno y verano. Los biomarcadores medidos fueran aquellos relacionados con la fase I (EROD y DBF) and II (GST) del metabolismo, repuestas antioxidantes (GPX y GR), neurotóxicas (AChE), de efecto en membrana (LPO) y daño genético (DNA *strand breaks*). Todos las analices bioquímicas fueran determinadas en tejidos de la glándula digestiva, y el NRRT en hemolinfa. Estos datos son parte del artículo VII.

Con el objetivo de determinar interferencias de la contaminación por aguas residuales en la reproducción, estado energético y sistemas endocrino y inmunológico de bivalvos marinos, la segunda clase de biomarcadores fue determinada (COX, MAO, MET, TLP, dopamine, Vtg-like proteins). Estos datos son parte del artículo VIII.

Por lo tanto, los artículos V, VI, VII y VIII muestran los efectos adversos que la contaminación por aguas residuales puede causar en la biota acuática y en especial a los bivalvos marinos, cuando expuestos en condiciones controladas de laboratorio. Este estudio es de especial relevancia ya que las almejas utilizadas son apreciadas en la culinaria mediterránea. Es cierto que efectos adversos pueden estar ocurriendo en otros organismos marinos de la Bahía, que también son de consumo humano. Estudios adicionales deben ser priorizados para obtener información sobre posible bioacumulación, biomagnificación, y sus posibles consecuencias a la biota acuática y a la salud humana de los consumidores de pescado de la Bahía de Cádiz.

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Ecotoxicology

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Are WWTPs effluents responsible for acute toxicity? Seasonal variations of sediment quality at the Bay of Cádiz.

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ABSTRACT

The effects of wastewater treatment plants (WWTPs) on the sediment quality in the Bay of Cádiz (SW, Spain) were evaluated by analysing a battery of acute bioassays and the chemical contamination. Five sites directly affected by WWTPs effluents and one control site were chosen. Results evidenced clear deterioration of the ecological sediment quality parameters and possible effects on the aquatic communities towards WWTPs areas. Acute toxicity and chemical contamination varied significantly across the studied sites and differed between winter and summer seasons. The Bay of Cádiz is contaminated by PAHs, metals, detergents (SAS) and pharmaceutical products. Principal Component Analyses indicated metals, SAS and pharmaceutical products as the major environmental stresses. Sea-urchin embryo-larval and microalgae growth rate were the most sensitive bioassays to evaluate the ressuspension of contaminants (elutriate) from the bulk sediment. Amphipods mortality and Microtox® Solid Phase Test (SPT) bioassays were recommended to evaluate the bulk sediment quality. Therefore, the use of multiple-bioassays sensitive to sediment pollution may provide complementary information to diagnose environmental factors that can impair aquatic communities. The use of a battery of bioassays is recommendable to assess and monitor the marine sediment directly affected by the mixture of contaminants released from WWTPs.

Keywords: wastewater, marine sediment, acute bioassays, contamination, seasonality.

1. INTRODUCTION

Municipal effluents are considered important sources of contamination discharged in the Bay of Cádiz (SW, Spain). Industrial, domestic and agriculture residues can be harmful for the local marine and estuarine environments. According to the European Directive (Urban Wastewater Treatment Directive - 92/271/EEC), Spain should have collecting systems and secondary treatment, or equivalent process, in towns with more than 10,000 inhabitants. Population around Bay of Cádiz comprises 460,000 inhabitants, which increases around 30% in the summer period (INE 2011). The wastewater treatment system capacity can be insufficient to treat all the wastewater produced, including the increase of population in summer or the increase of rain in winter.. Wastewater composition and consequently possible adverse effects on biota change according to the seasons. Bay of Cádiz is classified as "sensitive area" in the European Directive. Nevertheless, the European Directive did not include monitoring of contaminants as metals, organic or emergent compounds related to wastewater discharges. There is a lack of information about adverse impacts of wastewater treatment plants (WWTPs) on environmental quality of coastal areas.

Nutrients, metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), detergents (secondary alkane sulfonates - SAS), pesticides and pharmaceutical and personal care products (PPCPs) are traditionally found in wastewater discharges. Removal efficiency of contaminants from WWTPs influents varies greatly with seasonality, quantities of individual active pharmaceutical ingredients being used for the population, properties of individual contaminant, antagonism/ synergism behaviours of mixtures, and type of wastewater treatment being applied. Contaminants, including emergent compounds, can cause risk to human health and to environment associated with their presence and frequency of occurrence (Ferrari et al. 2003; Fang et al. 2012).

Contaminants can accumulate at bottom sediments which play an important environmental role as a sink and/or source of such compounds. Sediments can be remobilized, and contaminants may be bioavailable to the water column affecting associated biota (Cesar et al. 2007; Torres et al. 2009).

Several tools are available to assess aquatic contamination and its effects on the biota. To correctly estimate the ecotoxicological potential of the sediment is necessary to evaluate the main routes of exposure to contaminants (Cesar et al. 2007). Evaluation of bulk sediment samples represents the exposure through the direct contact with solid phase and pore water. Elutriate is a way to assess the potential of contaminants transferred from sediment to adjacent water column by re-suspension process, whether by natural or anthropogenic causes (e.g. dredging, storms), which may result in deleterious effects on aquatic organisms.

Chemical analyses are used to quantify potential contaminants in the environment, whereas sets of toxicity tests are recommended to evaluate potential harmful effects (Carr et al. 2008). Ecotoxicological assessments provide ecological responses and integrate the effects of contaminants, including those not considered or detected by chemical analyses. Furthermore, a battery of bioassays covers different routes of exposure, and could be useful to discriminate the effects or no–effects at several trophic levels (Riba et al. 2004). Guidelines of sediment quality assessment recommend toxicity tests (CEDEX 1994; USEPA 1998 a, b; USACE 1998; GIPME 2000; SEDNET 2003). Standardized single species toxicity tests are conducted to screen the potential hazards of aquatic contaminants and to develop environmental quality criteria (Brooks et al. 2003). Nevertheless, little attention is given to seasonal variation of bioavailability and effects of contaminants bound to sediments, and even less regarding the emergent compounds released from the WWTPs to the aquatic systems.

The aim of the present study was to evaluate the environmental quality of areas directly affected by WWTPs located at the Bay of Cádiz (SW, Spain). In order to evaluate possible acute toxicity effects on the biota associated to sediment affected by WWTPs, bulk sediment toxicity tests were applied to estimate toxicity in two organisms and two endpoints: *Ampelisca brevicornis* amphipod mortality and bioluminescence inhibition of the bacteria *Vibrio fischeri* (Microtox® Solid Phase Test - SPT). Sediment elutriates were conducted to predict toxicity in four organisms and three endpoints: bioluminescence inhibition of *V. fischeri* (Microtox® Basic Test - BT), embryogenesis success of sea-urchin *Paracentrotus lividus* and growth rate of microalgae *Isochrysis galbana* and *Tetraselmis chuii.*

A comparison between environmental qualities of sediments near five WWTPs located at the Spanish coast was performed, highlighting the differences between winter and summer seasons. Contamination by PAHs, metals, SAS and pharmaceutical compounds and the suitability of standardized acute tests were taken into consideration for sediment quality assessment. An integrated method was applied in order to derive site-specific quality values for the ecosystems studied (Sediment Quality Guidelines - SQG).

2. MATERIAL AND METHODS

2.1. Sampling approach

The Bay of Cádiz is important from an ecological point of view, which justified its qualification as a Natural Park since 1996 (Solé et al. 2009). However, ship, offshore, car and aerospace manufacturing, agriculture and tourism are the main economic activities in this zone. Summer in South Spain is a touristic season with increase of the population around 30%, and consequent increase of water consumption. Previous studies demonstrated the Bay of Cádiz has been directly affected by wastewater discharges

(Ponce et al. 2000; Carrasco et al. 2003; Lara-Martín et al. 2008), as the maps provided by Junta de Andalucía (Spanish Government).

In this study, sediment was sampled at six sites at the Bay of Cádiz (Figure 1) including five areas of salt-marsh directly affected by wastewater discharges (P1 – P5), and one control site (P6). Control site was located far from WWTP discharges and with high water circulation. Cities involved in this study are located around the Bay of Cádiz (SW, Spain): Chiclana de la Frontera - P1, Puerto Real - P2, Cádiz - P3, El Puerto de Santa Maria - P4 and P5, and Rota - P6.

Seawater was also sampled *in situ*. Once in the laboratory, pH, salinity, dissolved oxygen and ammonium NH_4^+ (adaptation of the method described by Hansen and Koroleff 2007) were determined.

Several sediment grabs were taken from each site in a way to cover the area nearby WWTPs discharges and control site. Approximately 40 kg of sediment was sampled in each site in the end of winter (March) and summer (September) 2011. Sampling was made from an inflatable launch on an ebbing tide by means of Van Veen grab (when it was possible) taking the topmost 10 cm layer. In deep places, sediment sampling was performed with scuba divers help. Once in the laboratory, sediment samples were kept at 4^oC in the dark for maximum three days, up to their use for the sediment toxicity tests and chemical analyses.

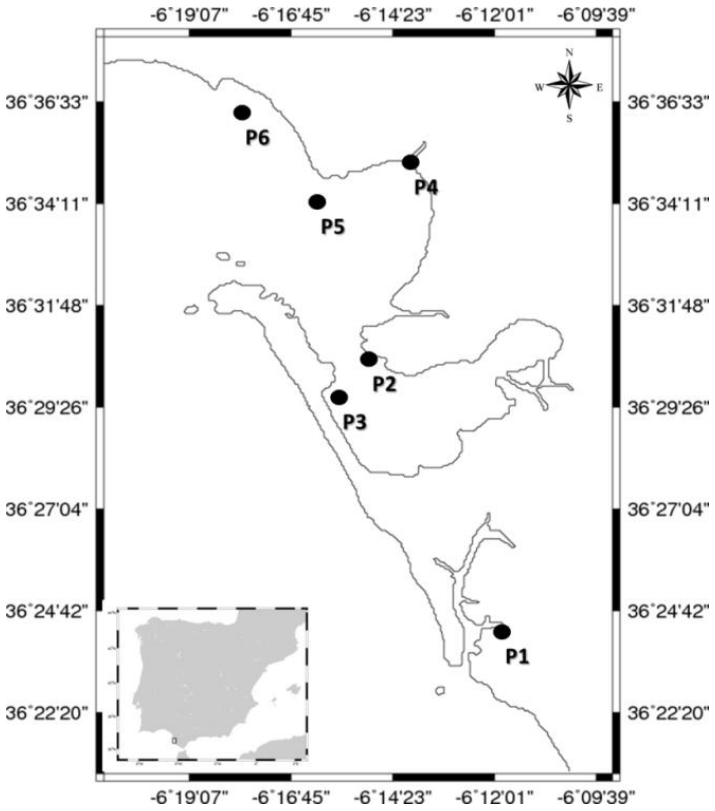

Fig. 1. Map of the coastal area of the Bay of Cádiz showing the locations of the studied sites (P1 – P6).

2.2. Chemical analyses

For sediment grain size analysis, aliquots of dry sediment were analysed following the method described by USGS (2000). Total organic carbon (TOC) and organic matter (OM) content were determined following the methods reported by USEPA (2002). Aqua regia extraction method (ISO11466 1995) was applied for metal analyses. Metal concentrations (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). LECO AMA 254 analyser was used to determine Hg concentrations. Results were checked using MESS-1 NRC reference material. PAHs content was analysed according to USEPA SW-846 method 8270D (2007).

Selected pharmaceuticals and secondary alkane sulfonates (SAS), an anionic surfactant used in dishwashing and other cleaning products, were measured following

methods proposed by Jelic et al. (2009) and Baena-Nogueras et al. (2013). Briefly, 2 grams of sediment were place inside 11 mL stainless steel cells and extracted by pressurized liquid extraction using a ASE 200 unit (Dionex) and methanol/water 1/2 (v/v) (for pharmaceuticals) or methanol/dichloromethane 1/1 (v/v) (for SAS) as solvent. After extraction, samples were evaporated and reconstituted in methanol/water 25/75 (v/v) and internal standards (carbamazepine d10, atenolol d7, naproxen d3, C_{12}SAS) were added at 50 ng mL^{-1}. Determination of target compounds was achieved by ultra-performance liquid chromatography (UPLC) – tandem mass spectrometry (MS/MS) on a Bruker EvoQ Elite equipped with electrospray interface (ESI) coupled to UPLC Bruker Advance. Separation of target compounds was carried out using Bruker Intensity Trio C18 column (100x2 mm, 1.9 µm internal diameters), and methanol (A) and water (B) as solvents. The gradient was as follows (flow = 0.4 mL min^{-1}): 5% B for 0.4 min, increased linearly to 30% in 0.1 min, increased linearly to 95% in 4.5 min, and then kept for 3 min. Source parameters were: spray voltage 4500 V for ESI+ and 3000 V for ESI-, cone temperature 250 ºC, cone gas flow 20 mL min^{-1}, probe temperature 450 ºC, probe gas flow 50 mL min^{-1} and nebulizer gas flow 60 mL min^{-1}. Recoveries were between 60 and 100% for most target compounds, and detection limits under 0.1 ng g^{-1}.

2.3. Toxicity tests

Bulk sediment samples were sieved through 2-mm mesh in order to remove any associated macrofauna. Elutriates were obtained with rotator mixing sediment: water (1:4) at 60 rpm for 30 min in airtight glass flasks. After overnight settling at 4°C in the dark, liquid phase was siphoned into a separate recipient, and gently aerated before the bioassays. Temperature, salinity, pH, dissolved oxygen and ammonia NH_4^+ (Hansen and Koroleff 2007, measured for elutriates) were controlled during toxicity tests. Seawater used for elutriates, dilution and overlying water was obtained from “Experimental Marine

Aquaculture Plant – Cultivos Marinos" at University of Cádiz. The seawater has been used for culture and maintenance of test organisms since 2002. A summary of test conditions is presented in the Tables 1.

Table 1. Toxicity testing conditions used to evaluate sediment quality under the direct influence of WWTPs effluents.

Parameters	Species			
	Amphipod *Ampelisca brevicornis*	**Bacteria** *Vibrio fischeri*	**Sea-urchin** *Paracentrotus lividus*	**Microalgae´s** *Isochrysis galbana*, *Tetraselmis chuii*
Exposure	BS	BS / ELU	ELU	ELU
Salinity (‰)	30 - 40	30 - 40	30 - 40	30 - 40
Water temperature (°C)	18 ± 2	15	20 ± 2	24 ± 0.1
Photoperiod	Natural photoperiod	Dark	1 h light: 23h dark	Continuous white light
Exposure design	200 g sediment, 800 mL of seawater	7 g sediment, 35 mL Microtox® Solid Phase Test or Basic Test diluents	20 mL ELU	12 mL ELU
Number of replicates	3	2	5	3
Number of organisms per replicates	20	10 µL of the bacteria suspension	20 - 30 embryos per mL	Maximum of 10^4 cells·mL^{-1}
Aeration	Gentle aeration of the overlying water	No aeration	Gentle aeration before starting the test	Gentle aeration before starting the test
Water source	Filtered seawater	-	Filtered seawater	Filtered seawater
Test duration	10 days	30 minutes	48 hours	96 hours
Endpoint	Mortality	Bioluminescence inhibition (IC_{50})	Embryogenesis success	Growth rate
Acceptability criteria	≥ 80% survival in the control	Initial light output (Io) higher than 100	≥ 80% of normal *pluteus* in the control	Continuous algae growth (96h) of the control

BS = bulk sediment, ELU = elutriate

2.3.1. Amphipods

Adults of crustacean amphipods *A. brevicornis* (body length ~ 8 – 10 mm) were obtained from a reference area at the Bay of Cádiz (SW, Spain) (36°29′16″N; 6°15′52″W). Previous studies reported this species as suitable bioindicator of the Spanish Atlantic coast (Casado-Martínez et al. 2007, 2006a; Morales-Caselles et al. 2007; Ramos-Gómez et al. 2009). Sediment from the same area of organisms' collection was used as toxicity control (Casado-Martínez et al. 2007). Sediment toxicity test was performed in 3-L glass beakers containing the proportion 1: 4 (sediment: water). Twenty amphipods were placed per beaker and exposed for 10 days (ASTM 1993; Riba et al. 2004; Ramos-Gómez et al. 2009). Amphipods bioassay was conducted in triplicate. After the exposure period, sediment samples were sieved and amphipods mortality was checked.

2.3.2. Bacteria

Bioluminescence inhibition assay with the bacteria *V. fischeri* were conducted with bulk sediment (Solid Phase Test - SPT) and elutriate (Basic Test - BT) samples. Commercial Microtox® apparatus (model 500) was used following standard protocols (AZUR, 1998). Bulk sediment and elutriates were diluted to series of nine concentrations in the incubator wells. Bacteria suspension was prepared and I0 was recorded. Bacteria reagent was dispensed in all incubator wells which contained the sample dilutions. Light readings were recorded at 5, 15 and 30 min. In this study, only IC_{50} concerning 30 min of exposure was reported. Regression statistics of concentration (logC) on the gamma parameter were used to estimate correlation, which gives nominal toxic effect (IC_{50}).

2.3.3. Sea-urchins

Adult sea-urchins were collected in low tide from rocky intertidal platform at Getares (SW, Spain) (Carballeira et al. 2011, 2012). *In vitro* fertilization was conducted following the

methodology described by Fernandez and Beiras (2001). Gametes were obtained by direct extraction. Once fecundation was successfully completed, embryos were introduced in 25 mL vials with sediment elutriates to a density of 20–30 embryos per mL. Five replicates per dilution were conducted. Two toxicity controls were tested: 1. seawater used for the preparation of elutriates; 2. seawater from the place where sea-urchins were sampled. Different dilutions of the environmental samples (100%, 50%, 25% and 12.5%) were applied to minimize possible interferences. After 48h at 20^{o}C (± 2) in the dark, replicates were fixed with formaldehyde. Results were expressed as percentage of abnormal *pluteus*.

2.3.4. Microalgae

Inoculums of *I. galbana* and *T. chuii* were from "Experimental Marine Aquaculture Plant – Cultivos Marinos" at the University of Cádiz. Microalgae were cultivated under aseptic conditions in a nutritive medium composed of synthetic seawater (USEPA 2002) and a supply of nutrients and vitamins according to the f/2 nutritive medium (Guillard and Ryther 1962). The experimental protocol followed was according to the method 1003.0 (absorbance) for measuring the toxicity of effluents and receiving waters with the microalgae *S. capricornotum* (USEPA 2002). Bioassay was performed in sterilized vials of borosilicate glass under continuous illumination (cold white light of 11000 lux) and temperature (24 ^{o}C ± 0.1). Four dilutions of each elutriate sample were applied in order to find the adequate range of toxicity for each microalgae species. All dilutions were performed in triplicate. Two controls were disposed: synthetic seawater used in the dilutions and natural seawater used to prepare the elutriate samples. Thirty minutes after the inoculation of the microalgae to the replicates (*t0*) and each day at the same hour until 96h of exposure time, biomass concentrations of the treatments were measured in terms of optical density (USEPA 2002) at wavelength of 690 nm in a colorimeter adapted for direct measurements of the test vials (Nannocolor® PT-3 Macherey-Nagel). For the microalgae assay, different dilutions of

environmental samples (100%, 50%, 25% and 12.5%) were applied to minimize possible interferences. Growth rate was normalized by the control. Areas under the growth curves were calculated and growth or inhibition percentage was obtained from these data. Percentage of response was calculated through the equation [(Ns - Nc)/ Nc] x100, where Ns was the cell number in the sample and Nc was the cell number in the control (Mucha et al. 2003). Negative response values mean inhibition of algal growth while positive values mean stimulation of algal growth.

2.4. Statistical analyses

Toxicity tests data met assumptions for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). Statistical differences compared with the control were analysed by one-way ANOVA followed by Dunnett´s test ($p < 0.05$). Sediment physical chemical and toxicity results were normalized to conduct the principal component analysis (PCA) calculated with SPSS/PC+ statistical package. For the PCA analysis, % of fines was calculated and different PAHs were grouped in one group. Pharmaceutical products were grouped according to human prescription: anti-inflammatories (acetaminophen, diclofenac, fenoprofen), antihypertensive (atenolol, propranolol), lipid regulators (clofibric acid, gemfibrozil), psychiatric drugs (carbamazepine, fluoxetine, amitriptyline, caffeine), antibiotics (chloramphenicol, cefdinir, tiamulin, erythromycin, clarithromycin, azithromycin, roxithromycin, lincomycin, flumequine, clindamycin, sparfloxacin, novobiocin, metronidazole, ornidazole, sulfadiazine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, monensin), antacids (famotidine, ranitidine) and others (glibenclamide, hydrochlorothiazide). Two PCAs were conducted with toxicity and chemical results, one for winter, the other one for summer. Only the variables whose coefficient was ≥ 0.5 (Comrey's 1973) were considered components of the factors.

3. RESULTS

3.1. Sediment characterization

Physical-chemical characterization data are included in the Table 2. Grain size was classified according to Wentworth scale, with predominance of sand (0.063 mm - 2mm). The highest levels of TOC and OM were observed at P2 during winter and summer seasons. The highest concentration of ammonium (NH_4^+) was determined at P1 in both seasons.

Table 2. Physical-chemical results of the different sediment phases and seawater of the sampling sites.

		Winter - Summer					
Parameter	**Sediment Phase**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
% Gravel (> 2mm)	BS	1.76	0	1.64	0.97	0.01	0.35
% Sand (2mm – 0.063mm)	BS	94.55	94.59	97.75	95.01	98.77	99.03
% Silt – Clay (< 0.063mm)	BS	3.69	5.41	0.61	4.02.	1.22	0.61
% TOC	BS	1.46 - 1.59	2.67 - 2.48	1.63 - 2.63	1.42 - 1.06	0.51 - 0.37	0.60 - 0.42
% OM	BS	11.53 - 13.78	19.18 - 16.95	1.07 - 4.44	16.31 - 15.95	1.34 - 1.41	0.82 - 2.15
pH	Seawater	7.65 - 7.41	7.89 - 7.96	8.17 - 7.95	7.82 - 7.84	8.00	7.71 - 7.68
Salinity (‰)	Seawater	29.67 - 14.73	36.43 - 30.25	35.35 - 33.93	33.61 - 25.53	33.21 - 34.13	35.78 - 33.97
O_2 ($mg \cdot L^{-1}$)	Seawater	9.95 - 6.83	6.89 - 7.53	8.95 - 7.89	9.07 - 7.39	7.78	13.91 - 7.94
NH_4	Seawater	2.87 - 2.24	0	0 - 0.02	0.15 - 0.20	0 - 0.01	0 - 0.08
	ELU	0.33 - 1.31	0.11 - 0.74	0.06 - 0.56	0.23 - 0.56	0	0

TOC = total organic carbon, OM = organic matter, BS = bulk sediment, ELU = elutriate.

Results of contaminant concentrations in sediments are shown in the Table 3. Contaminants that exceeded any SQGs were highlighted. The highest metal concentration was Al, followed by Fe, Mn, Zn, Cr, Cu, Pb, Ni, As and Cd. Se concentration was below the ICP-OES detection limit (< 2.00 $mg \cdot kg^{-1}$ dry weight). Between sites, control site (P6) showed the lowest concentrations of metals.

To evaluate the sediment contamination and potential ecotoxicological effects associated with observed concentrations of metals, published SQGs were applied: CEDEX (1994), CCME (1995), USEPA (2004) and Riba et al. (2004). CEDEX (1994) and Riba et al. (2004) brings information about SQGs for the Spanish coast.

The highest PAHs levels were detected in sediment sampled at P2 in both seasons. Benzo [a] anthracene and benzo [a] pyrene surpassed the CCME (1999) in sediment sampled at P2. Sediment sampled at P3 also surpassed the levels described in CCME guideline for benzo [a] anthracene in summer.

Sediment sampled at P1, P2, P3, P4 and P5 were contaminated by metals and PAHs. P6 was characterized by no-metal or PAHs contamination. Concentrations of such compounds did not significantly differ between seasons. Otherwise, the total concentration of SAS in sediment at the Bay of Cádiz was higher in summer than winter. Concerning pharmaceutical products, the highest total concentration was observed in sediment sampled at winter. Seasonal fluctuation was observed in concentration of psychiatric drugs (mainly caffeine) and antibiotics (mainly flumequine) with the highest levels in winter. The group of analgesic and anti-inflammatories drugs showed the third highest concentration in both seasons without seasonal fluctuation.

Table 3. Sediment chemical characterization (n = 2): metals (mg·kg^{-1} dry weight), polycyclic aromatic hydrocarbons (PAHs) (ng·g^{-1}), pharmaceutical compounds (ng·g^{-1}) and surfactants (SAS) (ng·g^{-1}). Sediment was sampled at six studied sites located at the Bay of Cádiz (SW, Spain) in winter and summer seasons: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa Maria and P6 – Rota (control).

		Winter – Summer					
	Parameters	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Metals	Hg	0.13 – 0.05	0.17 – 0.18 [a. b]	0.32 – 0.17 [a. b]	0.24 [a. b] – 0.09	0.04 – 0.08	0.01 – 0.03
	Al	53494.66 – 57723.92	52632.41 – 48587.24	19069.62 – 8001.16	46548.53 – 49139.60	15310.81 – 15161.31	6874.08 – 13005.66
	Fe	27367.32 – 28946.51	28236.89 – 27071.63	8392.90 – 6330.39	24927.43 – 27062.05	10444.91 – 12671.31	3234.01 – 8368.41
	Mn	362.80 – 351.06	389.21 – 410.89	158.75 – 200.14	336.44 – 330.16	408.23 – 499.10	125.64 – 422.92
	Cr	71.42 – 78.08 [a. b]	73.48 – 70.34 [a. b]	32.10 – 19.98	71.81 – 78.66 [a. b]	26.93 – 30.36	5.19 – 14.09
	Cu	37.55 – 40.52 [a. b]	39.25 – 42.17 [a. b]	26.51 – 44.31 [a. b]	35.98 – 31.02 [a. b]	7.04 – 6.09	0 – 3.75
	Ni	33.77 – 34.16	33.94 – 31.15	10.69 – 6.17	31.57 – 34.46	7.46 – 5.02	2.83 – 6.76
	Zn	99.75 – 105.73	106.90 – 111.58	60.66 – 76.60	95.33 – 127.10 [a. b]	28.94 – 26.70	7.88 – 36.49
	Pb	26.66 – 21.66	31.88 [a. b] – 30.20	25.70 – 34.38 [a. b]	23.37 – 18.40	10.30 – 9.69	5.45 – 14.00
	Cd	0.34 – 0.39	0.28 – 0.26	1.32 – 1.11 [a. b. c. d]	0.57 – 0.43	0.64 – 0.73 [a, b, c, d]	0.59 – 0.69
	As	7.88 – 6.68	8.46 – 7.34	4.50 – 2.98	5.20 – 4.32	5.87 – 5.81	5.82 – 6.56
	Se	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
	Anthracene	<2.00	25.36 – 32.74	7.76 – 30.94	<2.00 – 6.62	< 2.00	<2.00
PAHs	Benzo [a] anthracene	6.63 – 7.45	244.00 - 281.40[e]	66.5 – 102.00[e]	19.21 – 18.26	5.54 - < 2.00	<2.00
	Benzo [a] pyrene	<2.00	359.30 - 399.30[e]	59.86 - 76.76	10.94 - 9.34	< 2.00	<2.00
	Benzo [b] fluoranthene	4.41 – <2.00	81.52 - 120.20	65.74 - 95.68	13.79 - 14.53	4.53 - < 2.00	<2.00
	Benzo [c] fluorene	<2.00	22.94 – 23.16	7.70 – 8.85	<2.00	< 2.00	<2.00
	Benzo [g, h, i] perylene	4.60 – 4.31	393.40 – 516.00	51.62 – 67.08	9.71 – 12.29	< 2.00	<2.00
	Benzo [j] fluoranthene	<2.00	41.58 – 51.88	30.98 – 46.04	6.94 – 6.74	< 2.00	<2.00
	Benzo [k] fluoranthene	<2.00	27.98 – 33.32	30.94 – 44.58	6.62 – 6.03	< 2.00	<2.00
	Dibenzo [a, h] anthracene	<2.00	132.20 – 164.92	15.27 -21.78	<2.00	< 2.00	<2.00
	Fluoranthene	5.70 - <2.00	35.86 – 24.84	98.22 – 169.00	26.94 – 15.30	8.38 – 5.19	<2.00
	Indene [1, 2, 3 – c,d] pyrene	<2.00	85.72 – 129.80	40.88 – 56.40	7.64 -9.16	< 2.00	<2.00
	5-metylchrysene	<2.00	205.00 – 229.50	4.56 – 6.73	<2.00	< 2.00	<2.00
Anti-inflammatories	Acetaminophen	27.10 – 28.50	8.50 - < 0.10	< 0.10	15.90 - < 0.10	< 0.10 – 1.40	2.20 – 7.50
	Diclofenac	< 0.10 – 1.50	< 0.10 – 0.10	< 0.10	0.50 – 0.20	0.60 – 0.10	< 0.10
	Fenoprofen	< 0.10 – 0.30	0.90 - < 0.10	2.90 – 0.90	< 0.10 – 0.60	1.30 – 0.70	3.70 – 0.80
Anti-hypertensive	Atenolol	0.20 – 0.30	< 0.10 – 0.10	0.10 - < 0.10	0.20 – 0.30	0.10 - < 0.10	< 0.10 – 0.10
	Propranolol	0.30 - 0.20	0.10 – 0.20	0.10 – 0.60	0.90 – 0.30	0.10 - 0.10	0.10 – 0.20

Lipid regulators	Clofibric acid	0.10 - 0.10	0.10 - 0.10	< 0.10	< 0.10	< 0.10	< 0.10
	Gemfibrozil	0.90 - 0.30	< 0.10	< 0.10	0.10 – 0.10	< 0.10	< 0.10
Psychiatric drugs	Carbamazepine	0.20 - 0.20	< 0.10	< 0.10	0.90 - < 0.10	88.80 – 0.50	0.10 - < 0.10
	Fluoxetine	0.10 - 0.60	< 0.10 – 0.10	< 0.10	0.70 – 0.50	< 0.10	< 0.10
	Amitriptyline	0.20 - 0.20	0.10 – 0.10	< 0.10 – 0.20	0.40 – 0.30	< 0.10	< 0.10
	Caffeine	3.50 - 12.20	1.90 – 3.00	3.50 – 4.50	7.00 – 8.10	8.70 – 4.50	8.80 – 3.30
Antibiotics	Chloramphenicol	0.10 – 1.00	4.30 - < 0.10	0.40 – 0.10	0.10 – 0.20	0.20 - < 0.10	0.60 - < 0.10
	Cefdinir	0.10 - 0.10	0.10 – 0.10	0.10 – 0.10	0.20 – 0.10	0.10 – 0.10	0.10 - < 0.10
	Tiamulin	< 0.10	0.10 - < 0.10	0.10 - < 0.10	0.10 - < 0.10	0.10 - < 0.10	0.10 - < 0.10
	Erythromycin	0.40- 0.30	1.60 – 0.20	1.00 – 0.20	0.20 – 0.10	0.50 - 0.10	1.00 – 0.20
	Clarithromycin	0.30 - 0.3	1.0 – 0.1	0.70 – 0.10	0.70 – 0.60	0.30 - 0.10	0.30 – 0.10
	Azithromycin	0.20 - 0.20	0.40 – 0.10	0.30 - < 0.10	0.30 – 0.30	< 0.10	0.10 – 0.10
	Roxithromycin	0.20 - 0.20	0.60 – 0.10	0.60 – 0.10	0.90 – 0.20	0.20 – 0.50	0.40 – 0.50
	Lincomycin	0.10 – < 0.10	0.10 - < 0.10	0.10 - < 0.10	0.10 – 0.10	0.10 - 0.10	0.10 - < 0.10
	Clindamycin	0.10 - 0.10	0.10 – 0.10	0.20 – 0.10	0.10 – 0.10	0.10 - 0.10	0.60 – 0.10
	Flumequine	0.80 - 0.30	7.10 – 0.40	6.70 – 0.20	0.40 – 0.30	21.40 – 0.60	2.90 – 0.40
	Sparfloxacin	< 0.10	1.00 - < 0.10	0.10 - < 0.10	< 0.10	< 0.10	< 0.10
	Novobiocin	0.20 - 0.10	0.50 – 0.20	0.40 - < 0.10	< 0.10	0.10 - < 0.10	0.50 - < 0.10
	Metronidazole	< 0.10 – 0.20	0.40 – 0.10	0.10 – 0.30	< 0.10	< 0.10	0.40 – 0.10
	Ornidazole	< 0.10	2.30 – 0.10	2.00 - < 0.10	0.10 - < 0.10	0.50 - < 0.10	1.20 – 0.10
	Sulfadiazine	0.60 – 0.60	0.60 – 0.30	0.20 – 0.40	0.40 – 0.30	1.90 – 0.40	0.20 - < 0.10
	Sulfamethoxypyridazine	0.10 – 0.10	0.10 – 0.10	0.10 - < 0.10	0.10 - < 0.10	< 0.10	0.10 – 0.30
	Sulfathiazole	0.30 – 0.10	0.10 – 0.20	0.20 – 0.20	0.80 – 0.20	0.20 - 0.10	0.20 - < 0.10
	Trimethoprim	< 0.10	0.20 - < 0.10	0.10 – 0.10	0.10 - < 0.10	0.10 - 0.10	< 0.10 – 0.10
	Monensin	< 0.10	< 0.10	< 0.10	< 0.10	3.00 - < 0.10	< 0.10
Antacids	Famotidine	< 0.10	0.10 - < 0.10	< 0.10	0.10 - < 0.10	< 0.10	< 0.10
	Ranitidine	0.40 – 0.70	0.60 – 0.30	0.10 – 0.30	1.00 – 0.20	0.30 – 0.30	0.20 – 0.10
Others	Glibenclamide	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
	Hydrochlorothiazide	1.10 – 2.10	1.20 – 1.50	0.30 – 1.00	1.10 – 0.80	0.20 - < 0.10	< 0.10 – 0.10
Surfactants	SAS	1978.65 - 1521.00	61.10 - 77.40	73.90 - 44.70	1147.90 - 2344.80	400.70 – 159.30	623.20 - 118.70

a. USEPA. Marine Screening Benchmarks 2004.
b. CCME 1995.
c. CEDEX 1994.
d. Riba et al. 2004.
e. CCME 1999.

3.2. Acute toxicity

Results of the bioassays are shown in the Table 4. Bioassays data validated P6 as a suitable control site for ecotoxicological analyses since no pollution was determined. Significantly higher percentage of amphipod mortality in comparison with control was observed at P2 and P4 during both seasons, and P1 only in the summer season ($p < 0.05$). Some agencies classified as toxic sample when amphipod mortality is 20% higher than observed in the control sediment (USEPA 1998 a, b; Environment Canada 2000; Casado-Martínez et al. 2006 a). According to this classification, sediment sampled at P1, P2, P3, P4 and P5 in winter was considered toxic for *A. brevicornis.*

Concerning Microtox® assays, IC_{50} was determined after 30 min of exposure. Microtox® BT was applied for the evaluation of elutriates, but hormesis was detected. Concerning bulk sediment samples, sediments were classified according to the level of toxicity: P4 > P2 > P3 > P1 for winter, and P1 > P4 > P2 > P3 for summer. "Highest effect" was observed in sediment sampled at P5 and P6, which the recommendation was to concentrate the samples. Since they were environmental samples, it was not possible to concentrate them. P5 and P6 samples were assumed as no-toxic for *V. fischeri*. Toxicity tests applying Microtox® SPT is recommended by different international frameworks to evaluate environmental quality of sediment. Canadian standards (Environment Canada, 2002) considers the IC_{50} limit of 1000 $mg \cdot kg^{-1}$. According to the Spanish standards (CEDEX 1994; Casado-Martínez et al. 2006 b; Morales-Caselles et al. 2007), IC_{50} is associated with the concentration of 750 $mg \cdot kg^{-1}$. Based on these standards, sediment sampled at P1 (summer), P2 (winter) and P4 (winter and summer) were toxic for the bacteria *V. fischeri*.

Dilutions of the environmental samples (100%, 50%, 25% and 12.5%) were applied to minimize possible interferences in the sea-urchins and microalgae assays.. For sea-urchin embryo-larval development assay, sediments sampled at P1, P4 and P5 were toxic in the

winter season. Sediment sampled at P1, P3 and P5 were toxic in the summer season ($p < 0.05$). These samples surpassed the Spanish standard limit of toxicity (20% of abnormal embryo-larval development) based on previous studies (DelValls et al. 2003; Casado-Martínez et al. 2006 c).

Results concerned the microalgae bioassays also differed according to the species and seasons. *I. galbana* grew much more in summer than winter, rather than *T. chuii* grew in winter and inhibited the growth in summer season. P1, P2 and P4 were significantly different compared with control site in winter and summer seasons for both microalgae species ($p < 0.05$).

Table 4. Summarized results of the acute toxicity tests (average ± standard deviation) for the sediment quality assessment of five sites (P1-P5) and one control (P6) in winter and summer. P1-P5 were directly affected by wastewater discharges. Microtox® SPT IC_{50} = $mg \cdot kg^{-1}$

		Toxicity tests					
		Bulk sediment			Elutriate		
		Amphipods (% mortality)	Microtox® SPT IC_{50} (30 min)	Sea-urchins (% abnormal development)	Microalgae *I. galbana* (% growth)	Microalgae *T. chuii* (% growth)	Microtox® BT IC_{50} (30 min)
Winter	P1	21.0 ± 3.6	10256.0	41.6 ± 9.1 *	22.5 ± 4.3 *	42.3 ± 4.9 *	Hormesis
	P2	38.6 ± 1.5*	328.0	10.7 ± 6.0	22.7 ± 3.8 *	52.7 ± 6.9 *	Hormesis
	P3	21.0 ± 0	54072.0	8.4 ± 3.2	9.8 ± 7.7	22.0 ± 5.4	Hormesis
	P4	43.9 ± 3.0*	312.0	75.3 ± 2.6 *	13.5 ± 0.1 *	47.7 ± 4.9 *	Hormesis
	P5	24.6 ± 0.5	0	29.7 ± 7.1 *	0.6 ± 2.0	18.7 ± 3.2	Hormesis
	P6	0	0	0	0	0	Hormesis
Summer	P1	38.2± 1.1*	539.2	100.0 ± 0*	562.7 ± 31.8 *	-30.4 ± 4.9 *	Hormesis
	P2	40.0 ± 1.0*	1334.8	0	322.5 ± 7.3 *	-40.8 ± 6.9 *	Hormesis
	P3	10.9 ± 0.5	18489.8	100.0 ± 12.5*	163.0 ± 10.7 *	-10.1 ± 5.4	Hormesis
	P4	43.6 ± 1.5 *	547.8	1.7 ± 0	424.2 ± 4.4 *	-35.8 ± 4.9 *	Hormesis
	P5	5.4 ± 2.0	0	100.0 ± 5.4*	90.1 ± 5.8	-6.6 ± 3.2	Hormesis
	P6	0	0	0	0	0	Hormesis

* = significantly difference compared with the control site (P6) ($p < 0.05$).

3.3. Integrated approach

The first PCA analysis was performed with chemical and acute toxicity data from winter. Results were presented in the Table 5 and Figure 2, where two factors explained 100% of the total variance. Positive correlations of the factor 1 (PC1 > 0) explained 61.97% of variance, represented contamination by compounds as PAHs, metals (Al, Fe, Mn, Cr, Cu, Ni, Zn, Pb, Cd, As), pharmaceutical products (antibiotics, others) and SAS. Negative correlations of the factor 1 (PC1 < 0) represented toxicity of elutriate (abnormal embryo-larval development of sea-urchin *P. lividus* and growth rate of the microalgae *I. galbana*) and contamination for metals (Hg, Cd) and pharmaceutical compounds (anti-hypertensive, psychiatric drugs, antacids). Factor 2 accounted 38.03% of variances. Positive correlations (PC2 > 0) related contamination by PAHs, metals (Hg, Cr) and pharmaceuticals (antibiotics, antacids, others) associated with TOC and OM content, and toxicity of bulk sediment besides amphipods mortality and bioluminescence inhibition of *V. fischeri* (Microtox® SPT). Negative correlations (PC2 < 0) were found for pharmaceuticals (anti-inflammatories, lipid regulators), SAS, NH_4 and growth rate of *T. chuii*.

Table 5. Principal component analysis (PCA) based on chemical contamination and acute toxicity of sediments directly affected by wastewater discharges at the Bay of Cádiz (SW, Spain) in winter.

Winter		
Variables	**#1** **61.97%**	**#2** **38.03%**
PAHs	.715	.699
Hg	-.775	.632
Al	.909	
Fe	.998	
Mn	.981	
Cr	.619	.785
Cu	.971	
Ni	.962	
Zn	.940	
Pb	.944	
Cd	-.994	
As	.989	
Amphipods		.909
Microtox® SPT		.968
Sea-urchin	-.984	
Isochrysis galbana	-.957	
Tetraselmis chuii		-.957
Anti-inflammatories		-.981
Anti-hypertensives	-.996	
Lipid regulators		-.979
Psychiatric drugs	-.999	
Antibiotics	.705	.709
Others	.745	.667
Antacid	-.726	.687
SAS	.853	-.522
% Fines	.930	
TOC	.763	.646
OM		.985
NH_4		-.987

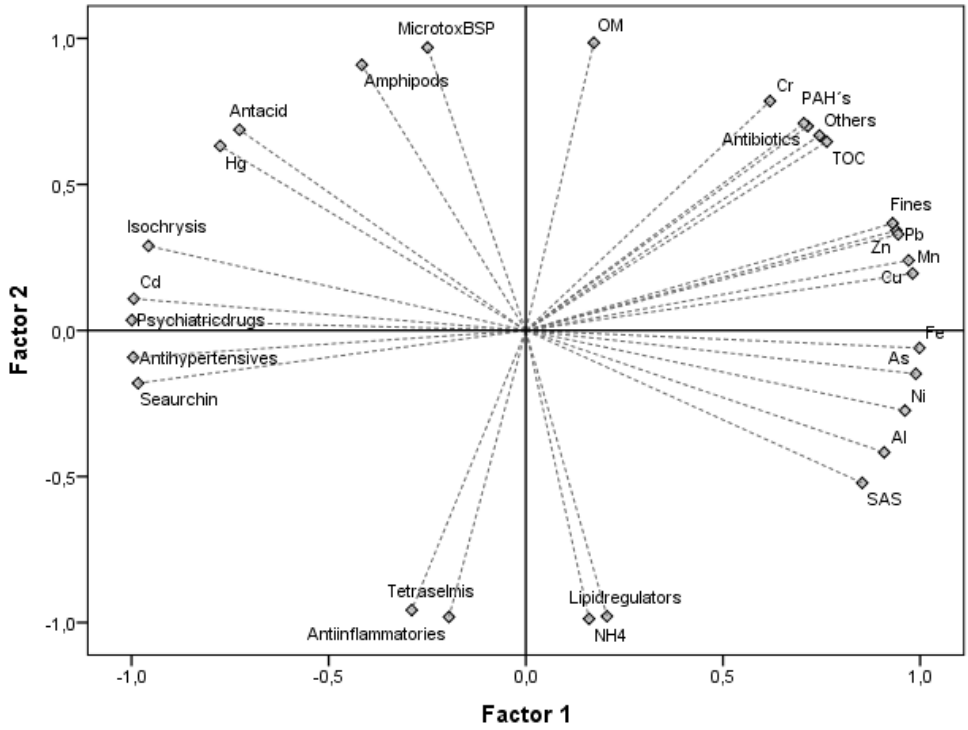

Fig. 2. Ordination results of the PCA based on chemical contamination and acute toxicity data in winter.

The second PCA analysis was performed with chemical and acute toxicity data from summer. Results were presented in the Table 6 and Figure 3. Two factors explained 100% of the total variance. Factor 1 represented 59.64% of variance. Positive correlations (PC1 > 0) were related chemical contamination by metals (Al, Fe, Mn, Cr, Ni, Zn, As) associated with OM, and toxicity of bulk sediment was showed by amphipods mortality and bioluminescence inhibition of the bacteria *V. fischeri*. Factor 1 negative correlations (PC1 < 0) linked chemical contamination by PAHs and metals (Hg, Cu, Pb, Cd) with % of fines and TOC in the sediment, and toxicity of elutriates, demonstrated by abnormal embryo-larval development of sea-urchin *P. lividus*, growth of *I. galbana* and *T. chuii* growth inhibition. Positive correlations of the factor 2 (PC2 > 0) explained 40.36% of the variance, and related chemical contamination by As, pharmaceuticals (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids, others), SAS and NH_4 with toxicity of elutriate (abnormal

embryo-larval development of sea-urchin *P. lividus*). Negative correlations (PC2 < 0) related contamination by metals (Hg) and pharmaceutical compound (anti-hypertensive) with toxicity of elutriate (growth rate of microalgae *I. galbana*).

Table 6. PCA on chemical contamination and acute toxicity of sediments directly affected by wastewater discharges at the Bay of Cádiz (SW, Spain) in summer.

Summer		
Variables	**#1** 59.64%	**#2** 40.36%
PAHs	-.895	
Hg	-.772	-.636
Al	.869	
Fe	.909	
Mn	.885	
Cr	.941	
Cu	-.917	
Ni	.941	
Zn	.997	
Pb	-.987	
Cd	-.917	
As	.509	.861
Amphipods	.980	
Microtox® SPT	.932	
Sea-urchin	-.770	.638
Isochrysis galbana	-.762	-.647
Tetraselmis chuii	-.988	
Anti-inflammatories		.986
Anti-hypertensives		-.986
Lipid regulators		.917
Psychiatric drugs	.632	.775
Antibiotics		.877
Others		1.00
Antacid		1.00
SAS		.998
% Fines	-1.00	
TOC	-.985	
OM	.999	
NH_4		.971

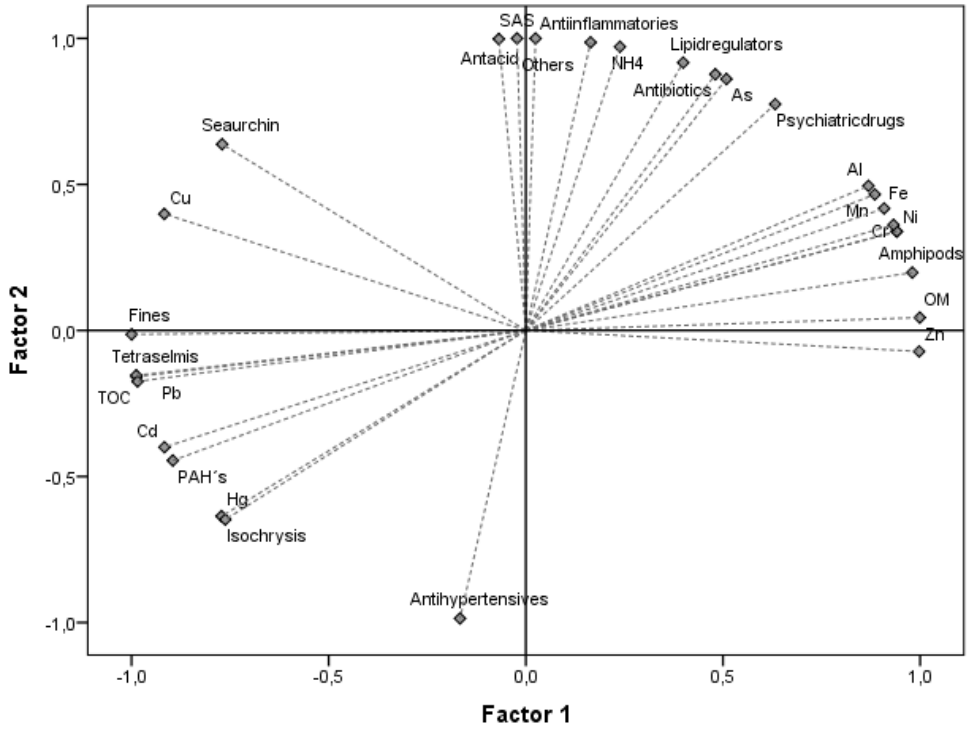

Fig. 3. Ordination results of the PCA based on chemical contamination and acute toxicity data in summer.

3.4. Deriving site-specific sediment quality guidelines

Derivation of site-specific SQGs followed the methodology reported by DelValls and Chapman (1998). Factor scores are included in the Figure 4. In winter, factor 1 was predominant at P2 (positive) and P4 (negative) to a lesser extent at P1. Factor 2 was prevalent at P1 (negative), P2 (positive) and very low at P4. In summer, factor 1 was dominant at P3 (negative), followed by P4 (positive), and very low at P1 (positive). Factor 2 was prevalent at P1 (positive), P4 (negative) to a lesser extent at P3 (negative).

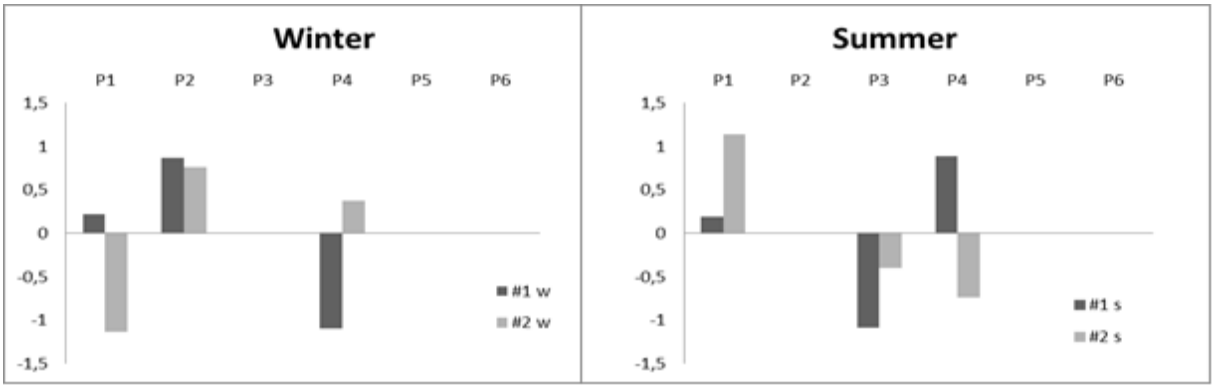

Fig. 4. Factor scores (relevance) of the two factors obtained after performing the PCA in the six sites in winter and summer seasons.

4. DISCUSSION

4.1. Time-trend of sediment quality: chemical contamination and acute toxicity

The presence of high levels of metals as Hg, Cr, Cu, Zn, Pb, Cd and As in some studied sites at the Bay of Cádiz suggested possible sources of industrial contamination in the vicinity. Hg was high at P2, P3 and P4 (winter). The primary Hg-human-related sources include: coal combustion, chlorine alkali processing, waste incineration, and metal processing. Cr was high at P1, P2 and P4 in both seasons. Industrial use of Cr includes metal alloys such as stainless steel, protective coatings on metal, magnetic tapes and pigments. Cu was high at P1, P2, P3 and P4 in both seasons. Zn was markedly high, particularly at P5 in summer, which would imply a direct link with discharges of anthropic nature. Pb values were high at P2 (winter) and P3 (summer), exceeding the limit set by SQGs. Carrasco et al. (2003) observed high concentration of Pb in sediments sampled near P2. Cd concentration was high at P3 in both seasons. As was high at P2 (both seasons), and P1 in winter.

PAHs compounds benzo [a] pyrene and benzo [a] anthracene surpassed the SQGs values. Such compounds are related to burning of fossil fuels and with the presence of wastewater discharges. Anti-inflammatory acetaminophen was the predominant pharmaceutical product found at P1 (both seasons), P2 (winter) and P5 (winter). Acetaminophen is a major ingredient in numerous cold and flu remedies. High concentrations

of SAS were observed at P1 and P4. SAS may act as detergents, emulsifiers and dispersants, and can be considered as chemical marker that indicates the presence of wastewater discharges. The highest NH_4 concentration was observed in seawater sampled at P1 in both seasons, which is consider a marker of wastewater discharges occurrence. High chemical concentration determined in sediment and seawater from the sampling points are due to the fact that the Bay of Cádiz is the main receiving point of wastewater generated in cities around the Bay, and located at upstream regions of the province.

Regarding toxicity of bulk sediment, P2 and P4 were toxic for amphipods in both seasons, and P1 was toxic only in summer season. Results obtained in previous studies reported the absence of amphipods *A. brevicornis* mortality exposed to sediment sampled at P3 (Cesar et al. 2007; Ramos-Gómez et al. 2011). However, sediment samples were classified according to the corresponding toxicity for the bacteria *V. fischeri*: P4 > P2 >P1 >P3 in winter and P1 > P4 > P2 > P3 in summer.

Sediment sampled at P1 was toxic for all bioassays in both seasons. P2 and P4 were toxic for microalgae assays and P5 was toxic for sea-urchin embryo-larval development assay in both seasons. P3 was toxic for the sea-urchin embryo-larval development and for microalgae *I. galbana* assay in summer. It was excluded the confounding factor ammonia that can contribute to the toxicity (Chapman et al. 2002). NH_4 concentration was verified in the beginning of short-term bioassays.

4.2. Integrated approach

Physical chemical and toxicological results (n=24 and n=5, respectively) were integrated by means of factor analyses in order to reveal the relationships among them, and in short, the data structure in winter and summer (Table 7). PAHs were not associated to biological effect in any case, as previous reported at the Bay of Cádiz by Ramos-Gómez et al. (2011).

For metals and pharmaceutical products grouped with their highest loadings (> 0.9) (Riba et al. 2004) in factor 1 and 2, SQGs were developed by following procedures reported by DelValls and Chapman (1998). Factor scores were shown in the Figure 5 for studied sites. For winter, chemical data considered in factor 1 were: metals (Al, Fe, Mn, Cu, Ni, Zn, Pb, As), pharmaceutical products (antibiotics, others) and SAS. Factor 2 corresponded only for lipid regulators. For summer, factor 1 was related to metals (Fe, Cr, Ni, Zn). Highest loadings in factor 2 represented metal (As), pharmaceutical products (anti-inflammatories, anti-hypertensives, lipid regulators) and SAS.

It should be emphasized that this approach is based on comparing those chemicals grouped under the same factor as the toxicity parameters. The assumption is that these chemicals presumably are correlated in cause-and-effect manner (Riba et al. 2004). In this sense, when factor score is ≤ 0, it suggests a correlation between chemicals and biological adverse effects. The maximum concentrations of toxic chemicals at any of those sites represent the maximum chemical concentrations that are not associated with adverse effects. These are considered concentrations which biological effects are low or minimal and indicated as "not polluted". In contrast, to establish the minimal concentrations which biological effects are always high, those minimal concentrations at sites where factor scores were ≥ 0 were selected and described as "highly polluted". Also, an intermediate range of chemical concentrations representing an area of uncertainty was shown and described as "moderately polluted".

Table 7. Summary of benchmark SQGs proposed to evaluate sediment quality of the Bay of Cádiz (SW, Spain) for trace metals ($mg \cdot kg^{-1}$), pharmaceutical compounds ($ng \cdot g^{-1}$) and SAS ($ng \cdot g^{-1}$) associated with toxic effects.

Winter				Summer			
Chemical	**Highly polluted**	**Not polluted**	**Moderately polluted**	**Chemical**	**Highly polluted**	**Not polluted**	**Moderately polluted**
Al	52632.41	46548.53	52632 - 46548	**Fe**	27062.05	6630.39	27062 – 6630
Fe	27367.32	24927.43	27367 – 24927	**Cr**	78.66	19.98	78.66 – 19.98
Mn	362.80	336.44	362.8 – 336.44	**Ni**	34.46	6.17	34.46 – 6.17
Cu	37.55	35.98	37.55 – 35.98	**Zn**	127.10	76.60	127.10 – 76.60
Ni	33.77	31.57	33.77 – 31.57	**As**	6.68	4.32	6.68 – 4.32
Zn	99.75	95.33	99.75 – 95.33	**Anti-inflammatories**	30.3	0.8	30.3 – 0.8
Pb	26.66	23.37	26.66 – 23.37	**Lipid regulators**	0.4	0.1	0.4 – 0.1
As	7.88	5.20	7.88 – 5.20	**SAS**	1521	2344.8	1521 – 2344.8
Cd	0.57	0.34	0.57 – 0.34				
Anti-hypertensives	1.1	0.5	1.1 – 0.5				
Psychiatric drugs	9	4	9 – 4				
Lipid regulators	1	0.1	1 – 0.1				
SAS	1978.65	1147.9	1978 – 1147.9				

5. CONCLUSION

Sediments directly affected by WWTP discharges at the Bay of Cádiz are contaminated by metals, PAHs, pharmaceutical products and SAS. Such contaminants were associated to biological effects. Adverse effects were observed in bulk sediment and elutriate, which means some contaminants were associated with the solid phase, and others can be bioavailable to the water column due to resuspension process, because of anthropogenic or natural causes. This fact demonstrated that policies for contamination control were ineffective to deal with population expansion and economic activities, since contaminants continue to be released into the environment and caused toxicity. There was no environmental legislation about pharmaceutical products or SAS impacts; therefore, the present study demonstrated that these compounds can be associated with adverse effects on the aquatic biota. The integrated analysis of two lines of evidence allowed noticing differences between toxicity and contamination according to seasonality.

6. ACKNOWLEDGMENTS

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Environmental Science and Pollution Research

Under review

Seasonal variations of cell damage and oxidative stress in clams *Ruditapes philippinarum* exposed under laboratory conditions to sediment affected by wastewater discharges.

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ABSTRACT

Marine clams *Ruditapes philippinarum* were exposed under laboratory conditions to sediments sampled from five sites directly affected by wastewater effluents at the Bay of Cádiz (SW, Spain), in winter and summer seasons. Chemical contamination was determined in sediment samples. Lysosomal membrane stability (LMS) and different biomarkers of exposure of early biological stress were determined in clams. Phases I and II of metabolism, and antioxidant system differed according to the seasons. LMS was significant lower in summer than winter. In winter, antioxidant system and DNA damage were related to the concentrations of trace metals and pharmaceutical products. Phases I and II, and oxidative effect (LPO) were related to the concentration of trace metals, PAHs, SAS and antibiotics. In summer, phase I and antioxidant system were related to PAHs and SAS contamination. GR, LPO and LMS were related to contamination by pharmaceutical products. Phase II was related to trace metals and pharmaceuticals. Present work revealed the seasonality of sediment contamination and biological responses related to wastewater effluents. The use of this bioindicator and the set of biomarkers were validated as potential tools for sediment quality assessment.

Keywords: seasonality, *Ruditapes philippinarum*, biomarkers, cell damage, oxidative stress, sediment, wastewater discharges.

1. INTRODUCTION

The Bay of Cádiz (SW, Spain) is constantly threatened by pollution due to the proximity to urban areas and their associated activity. The main industries located in this zone are related with ship, offshore, car and aerospace manufacturing. Agriculture and tourism activities are also important socio-economic activities at the Bay of Cádiz. Summer in South Spain is a touristic season with increase of the population around 30% and the consequent increase of water consumption. However, the occurrence of big storms in winter promotes the resuspension of sediments, which can bring back the pollutants accumulated in the sediment to the water column, and become the contaminants bioavailable to the aquatic biota. The Bay of Cádiz is also important from an ecological point of view, which justified its qualification as a Natural Park since 1996 (Montserrat et al. 2009). Despite this qualification, the Bay receives constantly loads of wastewater treatment plants (WWTPs). The removal efficiency of pollutants from the influents of WWTPs varies greatly with the seasonality, the quantities of individual active ingredients that are being used for the population, the function of the individual contaminants properties and the type of wastewater treatment being used. Emergent compounds (e.g. pharmaceutical and personal care products – PPCPs) are of special concern, since little is known about their possible adverse effects on the aquatic ecosystem.

Lethal and sublethal effects can be the consequence of exposure to a variety of contaminants. Wastewater discharges are a plethora of trace metals, nutrients, pesticides and organic compounds (PAHs, PCBs and PPCPs). The application of a battery of biomarkers seemed more appropriate than only chemical analysis or acute toxicity tests, to evaluate the sublethal effects of pollutants in aquatic environments (Cajaraville et al. 2000). The success of a multibiomarker approach in invertebrates to evaluate polluted sites had been applied in previous studies (Rank et al. 2007; Morales-Caselles et al. 2008b; Solé et al. 2009; Cotou et

al. 2013). This approach has been recommended for environmental international agencies such as UNEP, OSPAR, OECD, ICES and IOC (Cajaraville et al. 2000; Solé et al. 2009).

Between the benthic organisms, bivalves have been used to assess chronic water/sediment pollution. Bivalves are considered suitable bioindicators to evaluate the impacts of municipal effluents (Box et al. 2007; Solé et al. 2009; Franzellitti et al. 2010; Gagné et al. 2011). The bivalve clam *Ruditapes philippinarum* has been extensively used in biomonitoring studies (Moralles-Caselles et al. 2008a; Moschino et al. 2011) and sublethal measurements as histopathology and accumulation (Riba et al. 2005; Martín-Díaz et al. 2005; 2008), biochemical responses (Martín-Díaz et al. 2007) and lysosomal membrane stability assays (LMS) (Nigro et al. 2006; Coughlan et al. 2009; Buratti et al. 2010; 2012).

The metabolism of xenobiotics in bivalves can be investigated by the application of biochemical analysis concerning biomarkers of exposure and effect (Van Der Oost et al. 2003). Biomarkers of exposure are mainly enzymatic activities involved with the phase I (metabolism of detoxification), phase II (metabolism of conjugation) and antioxidant enzymes. A set of enzymatic biomarkers of exposure, namely cytochrome (CYP) P450 as a family of phase I enzymes (ethoxyresorufin *O*-deethylase - EROD and dibenzylfluorescein dealkylase – DBF), glutathione S-transferase (GST) as one of the phase II enzymes, antioxidant enzymes (glutathione peroxidase - GPX and glutathione reductase - GR) and measurement of neurotoxic (acetylcholinesterase - AChE) were analysed in the present study. The metabolism of xenobiotics produces free radicals responsible for oxidative stress and effects. Biomarkers of effect as lipid peroxidation of cellular membranes (LPO) and mitochondrial DNA damage (*strand breaks*) were chosen to evaluate the oxidative stress of clams exposed to sediment affected by wastewater discharges. Lysosomal membrane stability (LMS) was determined as target of oxidative and chemical toxicity due to wastewater discharges.

The aim of this work was to investigate the exposure and effects on benthic biota exposed to sediment directly affected by WWTPs effluents. It was investigated the seasonal fluctuations of the battery of biomarkers in clams (*Ruditapes philippinarum*) exposed to sediment sampled near five wastewater discharges during winter and summer 2011. The use of *R. philippinarum* as a bioindicator and the suitability of the battery of biomarkers applied were questioned in the evaluation of sublethal exposure and effects. The results obtained (physical chemical characterization and toxicity) were integrated, which allowed the environmental quality assessment of the Bay of Cádiz taking into account the seasonal variations.

2. MATERIALS AND METHODS

2.1. Study areas and sample collections

Sediment samples were collected near wastewater discharges located at the Bay of Cádiz (SW, Spain) during winter and summer 2011 (Figure 1). Six sampling sites were chosen representing different cities, being five of them affected by wastewater discharges: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María. P6 (Rota) was chosen as a control site, since it was located far from known wastewater sources. Previous studies demonstrated that these areas were directly affected by wastewater discharges (Ponce et al. 2000; Carrasco et al. 2003; Lara-Martín et al. 2008) as the maps provided by Junta de Andalucía (Spanish Government). A previous characterization of each sampling area was described below:

- Chiclana de la Frontera (P1): sediment was sampled at Iro River. Previous study speculated the discharge of anticholinergic agents, such as pesticides (Solé et al. 2009). This river receives water from agricultural sources as well as the urban wastewater discharge. Although the wastewater is treated in a treatment plant before

the discharge, a high level of contamination by nutrients and pathogens were measured in the aquatic ecosystem (Garrido-Pérez et al. 2003).

- Puerto Real (P2): characterized by the WWTP discharge from agricultural, urban and industrial sources, moderate metal contamination (Carrasco et al. 2003) and significant shipping activity.
- Cádiz (P3): this area support a season wastewater pumping and storage station, that send the wastewater to be treated in another WWTP located at San Fernando. However, there are occasional discharges of wastewater from this station in the Bay of Cádiz.
- El Puerto de Santa María (P4 and P5): P4 is characterized by season wastewater pumping, and also receives the effluents coming from the upper part of the Guadalete River. Surfactants (SAS) were previously detected in sediment in this area which correlated their usage and the presence of wastewater discharges (Lara-Martín et al. 2006). In this point, it is located some marinas. P4 might be considered as the main receiving point for wastewater generated in the upstream regions of the province of Cádiz. P5 is a sewage outfall located in the north of the Bay of Cádiz, at Puerto Sherry, which receives WWTP effluents from the city. There are only occasional untreated discharges since the WWTP which treats the wastewater and discharges into the ocean has existed for several years (Lara-Martín et al. 2006).
- Rota (P6): this area is located far from known wastewater discharges, near the Chorillo sandy beach.

Sediments were sampled from an inflatable launch on an ebbing tide by means of a Van Veen grab (when it was possible) or scuba divers help, taking the topmost 10 cm layer of the sediments. In the laboratory, sediment samples were sieved to remove large debris and

other animals, and kept at 4°C in the dark for maximum 2 days up to their use for the sediment toxicity tests and chemical analyses.

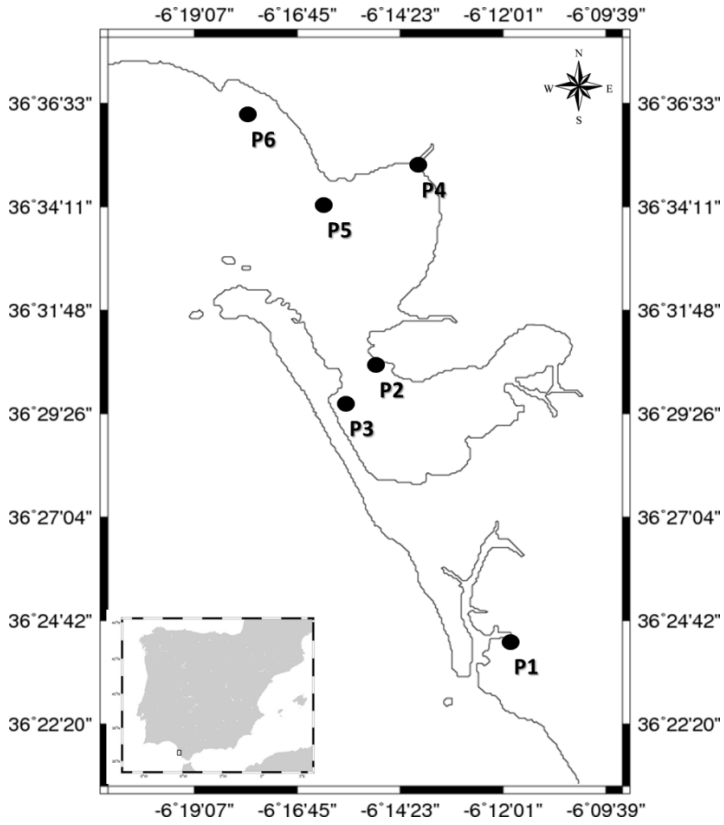

Figure 1. Geographic locations of sediment sampling sites across the Bay of Cádiz (SW, Spain). Five sampling sites representing urban areas were selected to the sediment collection near wastewater discharges: Chiclana de la Frontera (P1), Puerto Real (P2), Cádiz (P3), El Puerto de Santa Maria (P4 and P5), and ending with the control site at Rota (P6).

2.2. Physical chemical characteristics of the sediments

For sediment grain size analysis, an aliquot of dry sediment was analysed by following the methodology recommended by USGS (2013). Total organic carbon (TOC) and organic matter (OM) content were determined by using the methods reported by USEPA (2002). For trace metal analyses, it was applied the method of aqua regia extraction (ISO11466 1995). Trace metal concentrations (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). LECO AMA 254 analyser was used to determine Hg concentrations. The results were checked using MESS-1

NRC reference material. Grain size, TOC, OM and trace metals concentrations are shown in the Table 1.

Concerning organic compounds, PAHs content in sediment samples was analysed according to USEPA SW-846 method 8270/8082 (Table 2). Selected pharmaceuticals and secondary alkane sulfonates (SAS) were measured following the methods proposed by Jelic et al. (2009) and Baena-Nogueras et al. (2013) (Table 3).

2.3. *Ruditapes philippinarum* exposure under controlled conditions

Specimens of clam's *R. philippinarum* were bought from an aquaculture farm at Chiclana de la Frontera (SW, Spain). The animals were acclimatized for one week at clean seawater and under controlled conditions. Before the bioassay started, lysosomal membrane stability (LMS) was determined in some specimens from the acclimated aquarium, to certify the health status of the organisms that were exposed to the sediment sampled in the field.

Sediment samples were placed in 20-L aquarium filled with filtered seawater. Duplicates were used. Twenty-eight individuals were placed to each aquarium (day-0). Conditions of the bioassay were verified each two days (pH: 8.1 ± 0.3, dissolved oxygen: 7.3 $mg \cdot L^{-1}$ ± 0.2, salinity: 35.3 ± 1.4). 1: 3 of the seawater was renewed each three days. The exposure period lasted 14 days at 18 ± 2 ^{o}C, under constant aeration. At the end of the exposure period, clams were counted and placed overnight to acclimatization aquariums under controlled conditions.

2.4. Lysosomal membrane stability

Haemolymph was collected from six specimens of *R. philippinarum* from each replicate and LMS test was immediately processed. LMS was determined by neutral red retention time (NRRT) method (Lowe and Pipe 1994).

2.5. Sample procedure and biochemical determination

Digestive gland mass were dissected out and homogenized for each pool of clams. This procedure was made on ice using Teflon pestle tissue grinder apparatus in homogenization buffer (pH 7.5) containing 100 mM NaCl, 25 mM Hepes-NaOH, 0.1 mM EDTA and 0.1 mM dithiotreitol (DTT) (Gagné et al., 2007). A portion of homogenate was centrifuged at 3,000g at 4^{o}C for 20 min (S3 fraction), and other aliquot centrifuged at 15,000g at 4^{o}C for 20 min (S15 fraction), and the supernatant was used for the biomarker determinations. The homogenate, S3 and S15 fractions were stored at -80^{o}C until further analysis.

Total protein content (mg) was determined to each extract according to Bradford method (1976) using serum bovine for calibration.

EROD activity was evaluated by the transformation of 7-ethoxyresorufin in resorufin at fluorescence 485 nm (excitation) and 580 nm (emission) during the oxidation of NADPH to $NADP^{+}$ (Gagné and Blaise 1993).

DBF activity was evaluated by the transformation of dibenzylfluorescein in fluorescein was measured at 485 nm (excitation) and 516 nm (emission) during the oxidation of NADPH to $NADP^{+}$ (Gagné et al. 2007).

GST activity was measured by the detoxification reaction of chloro-dinitrobenzene (CNDB) through glutathione S-transferase in absorbance at 340 nm each 5 min for 30 min (McFarland et al. 1999).

GPX enzymatic activity was determined by the reduction of hydroperoxides (ROOH) by glutathione peroxidase forming oxidized glutathione (GSSG). The absorbance at 340 nm was measured every 30 sec for 30 min (McFarland et al. 1999).

GR enzymatic activity was the measurement of the reduced glutathione (GSH) regeneration by glutathione reductase. The absorbance (340 nm) was measured every 2 min for 10 min (McFarland et al. 1999).

AChE activities were determined by the degradation of acetylcholine in ticoline and acetate. The absorbance was measured at 412 nm every 5 min for 20 min (Guilhermino et al. 1996).

LPO was determined by absorbance at 540 nm using thiobarbituric acid (TBA) (Wills 1987).

DNA damage (*strand breaks*) was assessed by alkaline precipitation assay based on the K_2SDS precipitation of DNA-protein crosslink quantified by fluorescence at 360 nm (excitation) and 460 nm (emission) (Gagné et al. 1995).

2.6. Data analysis

Twelve clams (n = 12) from each replicate were used for the biomarker responses determination. Data sets were evaluated to determine normality of distribution and homogeneity. Mortality and sublethal responses of *R. philippinarum* were subsequently evaluated using one-way Analysis of Variance (ANOVA) followed by Dunnett's test. Statistical difference was set up at $p < 0.05$. The present study used star plots to display results for the panel of biomarkers used for each site and season following the methodology described by Beliaeff and Burgeot (2002) adapted by Sanchez et al. (2013). Integrated biomarker response (IBR) was then computed as the star plot area.

Significant correlations were examined by Spearman´s rank correlation analysis. The significance level was set up at $p < 0.01$ and $p < 0.05$.

Principal Component Analyses (PCA) on biochemical and LMS responses and on the whole dataset of chemical measurements was performed. It was considered all replicates

within sites to classify the studied sample collections according to their biochemical response patterns. PAHs and pharmaceutical products detected in the sediment samples were grouped. Pharmaceutical products were grouped according to the human prescription: anti-inflammatories (acetaminophen, diclofenac, fenoprofen), anti-hypertensive (atenolol, propranolol), lipid regulators (clofibric acid, gemfibrozil), psychiatric drugs (carbamazepine, fluoxetine, amitriptyline, caffeine), antibiotics (chloramphenicol, cefdinir, tiamulin, erythromycin, clarithromycin, azithromycin, roxithromycin, lincomycin, flumequine, clindamycin, sparfloxacin, novobiocin, metronidazole, ornidazole, sulfadiazine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, monensin), antacids (famotidine, ranitidine) and others (glibenclamide, hydrochlorothiazide). Two separate PCAs were conducted on the biological and chemical results, one for winter, the other one for summer. Statistical analysis was performed using the IBM SPSS/ PC 21.0 + statistical package.

3. RESULTS

3.1. Sediment characterization

Summarized results for sediment physical chemical characterization were shown in the Tables 1, 2 and 3. Parameters as TOC, OM content and grain size percentages were found to vary from the study sites to the control site. Grain size distribution analysis indicated that, despite a certain variability in the Bay bottoms at the sites chosen for sampling, three overall groupings predominated, with essentially clayed silt sediments on the one hand (P1, P2 and P4), medium sand (P3) and the predominance of sand (P5 and P6) with a fairly significant gravel and sand content. TOC and OM content varied between sites, but not between seasons. P2 presented the highest levels of TOC and OM during winter and summer seasons.

From the twelve trace metals analysed in the sediment samples, only Se was not detected (Table 1). The highest trace metal concentration was observed for Al, followed by

Fe, Mn, Zn, Cr, Cu, Pb, Ni, As and Cd. Of all the stations, the control site (P6) showed the lowest levels of trace metals. To evaluate the sediment contamination and potential ecotoxicological effects associated with the observed concentrations of trace metals, different published Sediment Quality Guidelines (SQGs) were consulted: USEPA (2004), CCME (1995), CEDEX (1994) and Riba et al. (2004). CEDEX (1994) and Riba et al. (2004) is about SQGs for the Spanish coast. In the Table 1, the contaminants that exceeded any SQG were highlighted. The letter superscripted indicated the metal concentrations which SQGs were surpassed.

Table 1. Sediment physical chemical characterization (n = 2) which includes the percentages of fines (% dry weight), the total organic carbon (TOC) (% dry weight), organic matter (OM) (% dry weight) and the concentration of contaminants [trace metals ($mg \cdot kg^{-1}$)]. Sediment was sampled at six studied sites located at the Bay of Cádiz (SW, Spain) in winter (w) and summer (s) seasons: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María, P6 – Rota (control).

		Fines	TOC	OM	Hg	Al	Fe	Mn	Cr	Cu	Ni	Zn	Pb	Cd	As	Se
Winter	P1	49.08	1.46	11.53	0.13	53494.66	27367.32	362.80	71.42[a.b]	37.55[a.b]	33.77	99.75	26.66	0.34	7.88[a.b]	-
	P2	64.90	2.67	19.18	0.17[a.b]	52632.41	28236.89	389.21	73.48[a.b]	39.25[a.b]	33.94	106.90[a.b]	31.88[a.b]	0.28	8.46[a.b]	-
	P3	65.72	1.63	1.07	0.32[a.b]	19069.62	8392.90	158.75	32.10	26.51[a.b]	10.69	60.66	25.70	1.32[a.b.c.d]	4.50	-
	P4	40.46	1.42	16.31	0.24[a.b]	46548.53	24927.43	336.44	71.81[a.b]	35.98[a.b]	31.57	95.33	23.37	0.57	5.20	-
	P5	97.43	0.51	1.34	0.04	15310.81	10444.91	408.23	26.93	7.04	7.46	28.94	10.30	0.64	5.87	-
	P6	68.45	0.60	0.82	0.01	6874.08	3234.01	125.64	5.19	ND	2.83	7.88	5.45	0.59	5.82	-
Summer	P1	49.08	1.59	13.78	0.05	57723.92	28946.51	351.06	78.08[a.b]	40.52[a.b]	34.16	105.73	21.66	0.39	6.68	-
	P2	64.90	2.48	16.95	0.18[a.b]	48587.24	27071.63	410.89	70.34[a.b]	42.17[a.b]	31.15	111.58	30.20	0.26	7.34	-
	P3	65.72	2.63	4.44	0.17[a.b]	8001.16	6330.39	200.14	19.98	44.31[a.b]	6.17	76.60	34.38[a.b]	1.11[a.b.c.d]	2.98	-
	P4	40.46	1.06	15.95	0.09	49139.60	27062.05	330.16	78.66[a.b]	31.02[a.b]	34.46[a.b]	127.10[a.b]	18.40	0.43	4.32	-
	P5	97.43	0.37	1.41	0.08	15161.24	12671.31	499.10	30.36	6.09	5.02	26.70	9.69	0.73[a.b.c.d]	5.81	-
	P6	68.45	0.42	2.15	0.03	13005.66	8368.41	422.92	14.09	3.75	6.76	36.49	14.00	0.69	6.56	-

(-) – not detected

a. USEPA, Marine Screening Benchmarks, 2004.

b. CCME, 1995.

c. CEDEX, 1994.

d. Riba et al., 2004.

Table 2. Concentrations of target polycyclic aromatic hydrocarbons (PAHs) compounds ($ng \cdot g^{-1}$) determined in sediment sampled at six studied sites located at the Bay of Cádiz (SW, Spain) in winter (w) and summer (s) seasons: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María, P6 – Rota (control).

	LOQ	P1w	P1s	P2w	P2s	P3w	P3s	P4w	P4s	P5w	P5s	P6w	P6s
Anthracene	2	-	-	25.36	32.74	7.76	8.11	-	-	-	-	-	-
Benzo [a] anthracene	2	6.63	7.45	244[a]	281.4[a]	66.5	102[a]	19.21	18.26	5.54	-	-	-
Benzo [a] pyrene	2	-	-	359.3[a]	399.3[a]	59.86	76.76	10.94	9.34	-	-	-	-
Benzo [b] fluoranthene	2	4.41	-	81.52	120.2	65.74	95.68	13.79	14.53	4.53	-	-	-
Benzo [c] fluorene	2	-	-	22.94	23.16	7.7	8.85	-	-	-	-	-	-
Benzo [g, h, i] perylene	2	4.6	4.31	393.4	516	51.62	67.08	9.71	12.29	-	-	-	-
Benzo [j] fluoranthene	2	-	-	41.58	51.88	30.98	46.04	6.94	6.74	-	-	-	-
Benzo [k] fluoranthene	2	-	-	27.98	33.32	30.94	44.58	6.62	6.03	-	-	-	-
Dibenzo [a, h] anthracene	2	-	-	132.2	164.92	15.27	21.78	-	-	-	-	-	-
Fluoranthene	2	5.7	-	35.86	24.84	98.22	169	26.94	15.3	8.38	5.19	-	-
Indene [1, 2, 3 – c,d] pyrene	2	-	-	85.72	129.8	40.88	56.4	7.64	9.16	-	-	-	-
5-metylchrysene	2	-	-	205	229.5	4.56	6.73	-	-	-	-	-	-

(-) – not detected

a. CCME, 1999.

Table 3. Concentrations of target pharmaceutical compounds ($ng \cdot g^{-1}$) in sediment sampled at six studied sites located at the Bay of Cádiz (SW, Spain) in winter (w) and summer (s) seasons: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María, P6 – Rota (control).

THERAPEUTIC CLASS	DRUG	P1 w	P1 s	P2 w	P2 s	P3 w	P3 s	P4 w	P4 s	P5 w	P5 s	P6 w	P6 s
Anti-inflammatories	Acetaminophen	27.1±7.8	28.5±15.2	8.5±0.2	-	-	-	15.9±0.1	-	-	1.4±0.7	2.2±1.2	7.5±0.7
	Diclofenac	-	1.5±0.3	-	0.1±0.1	-	-	0.5±0.1	0.2±0.1	0.6±0.2	0.1±0.1	-	-
	Fenoprofen	-	0.3±0.2	0.9±0.6	-	2.9±0.8	0.9±0.3	-	0.6±0.3	1.3±0.8	0.7±0.3	3.7±0.2	0.8±0.4
Anti-hypertensive	Atenolol	0.2±0.1	0.3±0.0	-	0.1±0.0	0.1±0.0	-	0.2±0.0	0.3±0.0	0.1±0.0	-	-	0.1±0.0
	Propranolol	0.3±0.1	0.2±0.0	0.1±0.1	0.2±0.0	0.1±0.0	0.6±0.1	0.9±0.1	0.3±0.0	0.1±0.1	0.1±0.0	0.1±0.1	0.2±0.0
Lipid regulators	Clofibric acid	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	-	-	-	-	-	-	-	-
	Gemfibrozil	0.9±0.1	0.3±0.2	-	-	-	-	0.1±0.1	0.1±0.0	-	-	-	-
Psychiatric drugs	Carbamazepine	0.2±0.1	0.2±0.1	-	-	-	-	0.9±0.5	-	88.8±17.5	0.5±0.3	0.1±0.0	-
	Fluoxetine	0.1±0.0	0.6±0.1	-	0.1±0.0	-	-	0.7±0.0	0.5±0.0	-	-	-	-
	Amitriptyline	0.2±0.0	0.2±0.2	0.1±0.0	0.1±0.0	-	0.2±0.2	0.4±0.1	0.3±0.0	-	-	-	-
	Caffeine	3.5±0.2	12.2±0.2	1.9±0.6	3.0±0.4	3.5±1.1	4.5±0.8	7.0±0.2	8.1±0.2	8.7±0.8	4.5±1.7	8.8±1.0	3.3±0.2
Antibiotics	Chloramphenicol	0.1±0.1	1.0±0.6	4.3±2.6	-	0.4±0.3	0.1±0.1	0.1±0.1	0.2±0.0	0.2±0.0	-	0.6±0.1	-
	Cefdinir	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	-
	Tiamulin	-	-	0.1±0.1	-	0.1±0.0	-	0.1±0.0	-	0.1±0.0	-	0.1±0.0	-
	Erythromycin	0.4±0.0	0.3±0.0	1.6±0.8	0.2±0.0	1.0±0.1	0.2±0.0	0.2±0.1	0.1±0.0	0.5±0.1	0.1±0.0	1.0±0.4	0.2±0.0
	Clarithromycin	0.3±0.1	0.3±0.0	1.0±0.5	0.1±0.0	0.7±0.2	0.1±0.1	0.7±0.3	0.6±0.2	0.3±0.1	0.1±0.0	0.3±0.0	0.1±0.0
	Azithromycin	0.2±0.0	0.2±0.0	0.4±0.3	0.1±0.0	0.3±0.4	-	0.3±0.0	0.3±0.2	-	-	0.1±0.0	0.1±0.0
	Roxithromycin	0.2±0.1	0.2±0.0	0.6±0.4	0.1±0.0	0.6±0.2	0.1±0.1	0.9±0.4	0.2±0.0	0.2±0.1	0.5±0.4	0.4±0.3	0.5±0.2
	Lincomycin	0.1±0.1	-	0.1±0.1	-	0.1±0.0	-	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.0	-
	Clindamycin	0.1±0.0	0.1±0.0	0.1±0.2	0.1±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.6±0.3	0.1±0.0
	Flumequine	0.8±0.0	0.3±0.0	7.1±0.9	0.4±0.0	6.7±0.1	0.2±0.0	0.4±0.1	0.3±0.0	21.4±1.1	0.6±0.0	2.9±0.2	0.4±0.0
	Sparfloxacin	-	-	1.0±0.4	-	0.1±0.0	-	-	-	-	-	-	-
	Novobiocin	0.2±0.1	0.1±0.0	0.5±0.5	0.2±0.0	0.4±0.2	-	-	-	0.1±0.1	-	0.5±0.0	-

	Metronidazole	-	0.2±0.2	0.4±0.2	0.1±0.0	0.1±0.1	0.3±0.0	-	-	-	0.4±0.3	0.4±0.3	0.1±0.1
	Ornidazole	-	-	2.3±0.5	0.1±0.0	2.0±0.5	-	0.1±0.0	-	0.5±0.4	-	1.2±0.7	-
	Sulfadiazine	0.6±0.2	0.6±0.1	0.6±0.1	0.3±0.0	0.2±0.0	0.4±0.0	0.4±0.1	0.3±0.0	1.9±0.4	0.2±0.0	0.2±0.0	0.3±0.0
	Sulfamethoxypyridazine	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.0	-	0.1±0.0	-	-	-	0.1±0.0	-
	Sulfathiazole	0.3±0.0	0.1±0.1	0.1±0.1	0.2±0.0	0.2±0.0	0.2±0.0	0.8±0.1	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.1
	Trimethoprim	-	-	0.2±0.3	-	0.1±0.0	0.1±0.1	0.1±0.0	-	0.1±0.1	0.1±0.0	-	0.1±0.1
	Monensin	-	-	-	-	-	-	-	-	3.0±1.8	-	-	-
Antacids	Famotidine	-	-	0.1±0.1	-	-	-	0.1±0.0	-	-	-	-	-
	Ranitidine	0.4±0.1	0.7±0.1	0.6±0.1	0.3±0.1	0.1 ± 0.0	0.3±0.0	1.0±0.0	0.2±0.1	0.3±0.0	0.3±0.0	0.2±0.0	0.1±0.0
Others	Glibenclamide	-	-	-	-	-	-	-	-	-	-	-	-
	Hydrochlorothiazide	1.1±0.3	2.1±0.2	1.2±0.1	1.5±0.9	0.3±0.1	1.0±0.3	1.1±0.1	0.8±0.2	0.2±0.0	-	-	0.1±0.1
TOTAL		37.6	50.8	34.1	7.7	20.4	9.4	33.4	13.9	128.9	10.0	23.9	14.1
Anionic surfactant	SAS	1978.65	1521.0	61.1	77.4	73.9	44.7	1147.9	2344.8	400.7	159.3	623.2	118.7

(-) – not detected

The highest PAHs levels were detected in sediment samples from P2 in both seasons. In the Table 2, the contaminants that exceeded the SQG were highlighted. Benzo [a] anthracene and benzo [a] pyrene surpassed the CCME (1999) in the sediment sampled at P2. P3 also surpassed the levels described in this guideline for benzo [a] anthracene in summer.

The chemical results indicated that P1, P2, P3, P4 and P5 were contaminated by a variety of organic and inorganic contaminants (trace metals and PAHs). The control P6 was characterized by no trace metal or PAHs contamination. Trace metal and PAHs concentrations in the sediment samples did not significantly differed between the seasons.

Otherwise, the concentration of SAS and flumequine (antibiotic) were higher in summer than winter. The highest concentration of SAS in sediment samples observed in winter was P1, and in summer was P4. The highest concentration of pharmaceutical products was detected in sediment samples from P1 in winter and P5 in summer season.

3.2. Biochemical responses of *Ruditapes philippinarum*

There was no mortality of clams exposed to sediment sampled in the summer season. However, increasing of clams' mortality was observed during the winter season: P5 – 7.14%, P2 – 21.4%, P3 – 26.7%, P1 – 32.1% and P4 – 50%. The mortality observed in clams exposed to sediment sampled at P6 was 15%.

LMS is an indicator of general stress and health status. During winter, P1 and P5 showed significant reduction of the LMS compared with the control ($p < 0.05$). However, in summer season the organisms exposed to sediment sampled at P1, P2, P3 and P5 showed significant reduction of the LMS compared with the control organisms ($p < 0.05$).

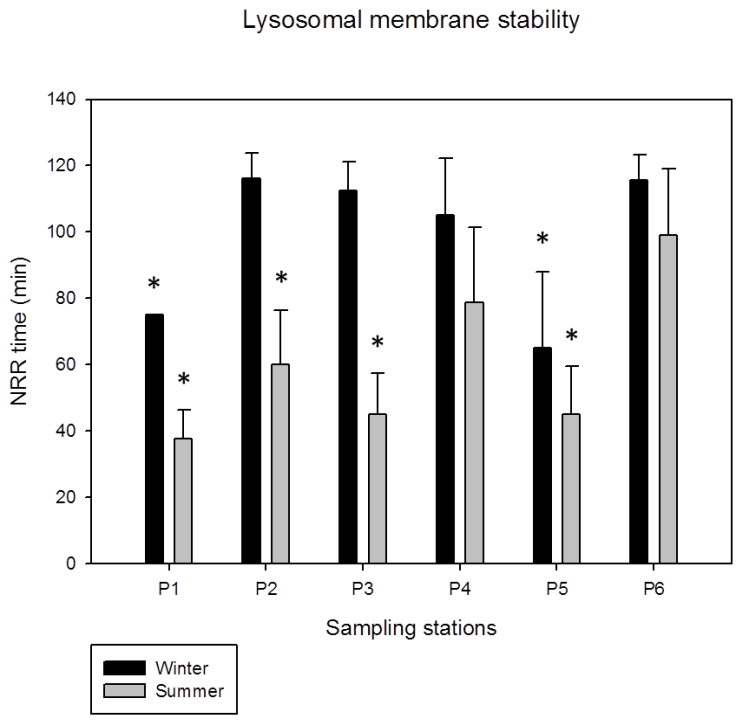

Figure 2. Seasonality of lysosomal membrane stability (LMS) assessed through neutral red retention (NRR) assay in haemocytes of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites (P1 – P5) directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

Some enzymatic activities corresponding to the phases I (EROD and DBF activities), II (GST activity) and antioxidant system (GR activity) showed significant differences as a function of seasonal period of exposure ($p < 0.05$). These differences were represented in the graphs as $p < 0.05$ under the control responses.

In Figure 3 – 10, the results for biomarkers in this specie per site and seasonal period were presented. Phase I of the metabolism had the maximal variation concerning EROD (Figure 3) and DBF (Figure 4) enzymatic activities in summer. Seasonal differences were particularly evident for EROD activities in clams. P1 and P2 showed significant increase of EROD enzymatic activity during winter ($p < 0.05$). Phase II of the metabolism (GST enzymatic activity) was not significant for any site in summer or winter (Figure 5).

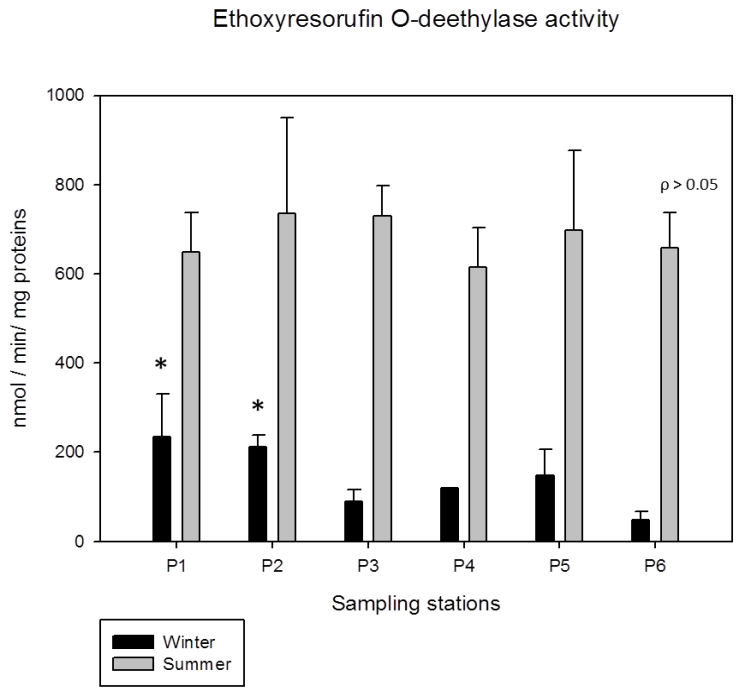

Figure 3. Seasonality of EROD enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sitess directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means that there was significance difference between winter and summer in the control responses.

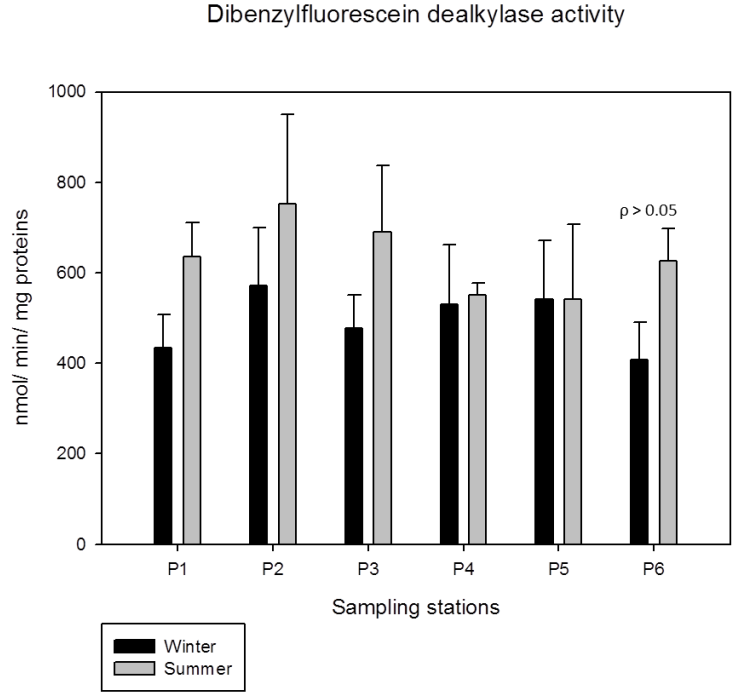

Figure 4. Seasonality of DBF enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days.

Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means that there was significance difference between winter and summer in the control responses.

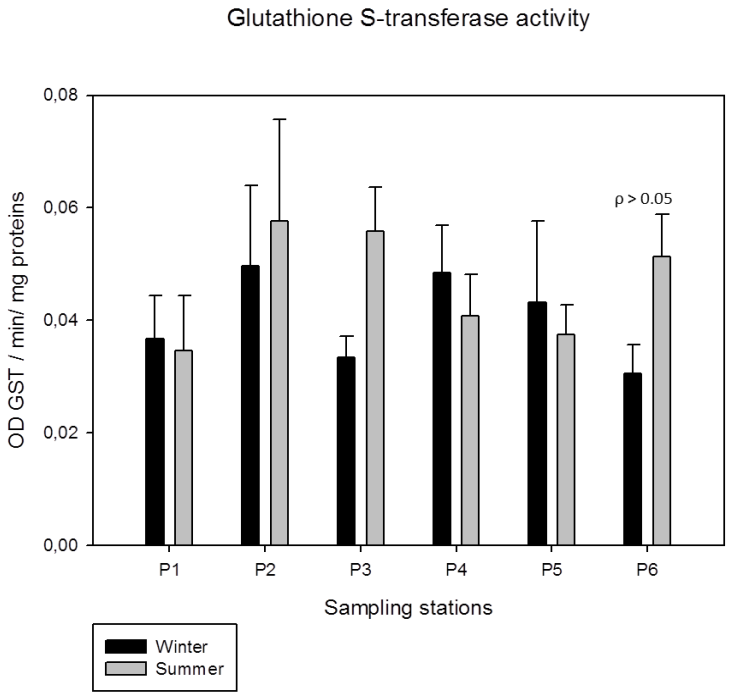

Figure 5. Seasonality of GST enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means that there was significance difference between winter and summer in the control responses.

Among oxidative responses, GPX (Figure 6) exhibited higher values in winter and GR (Figure 7) during summer. P2 and P4 showed significantly increased GPX values compared with the control in winter ($p < 0.05$). In summer, clams exposed to P5 presented significant higher GPX activities compared with the control ($p < 0.05$). There was no significant difference compared with the control organisms for the GR responses.

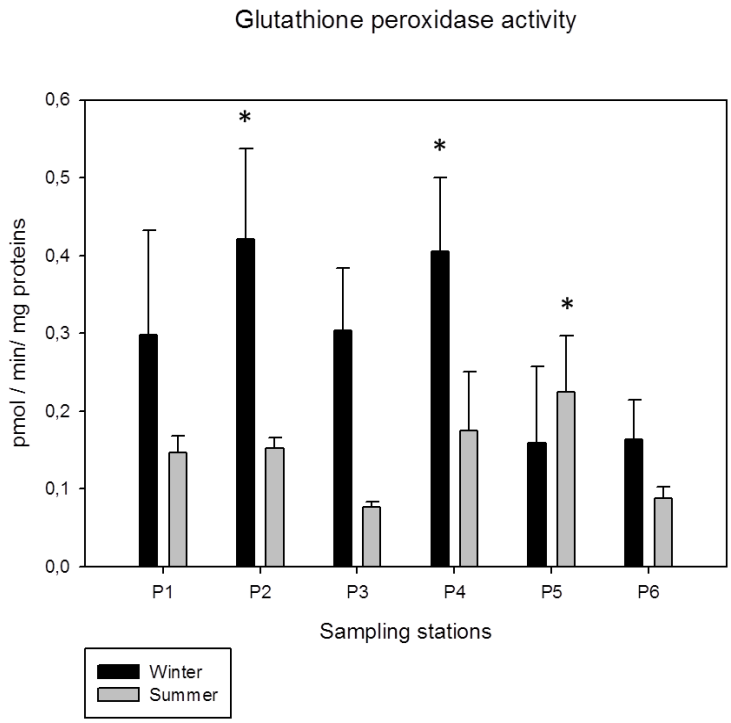

Figure 6. Seasonality of GPX enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

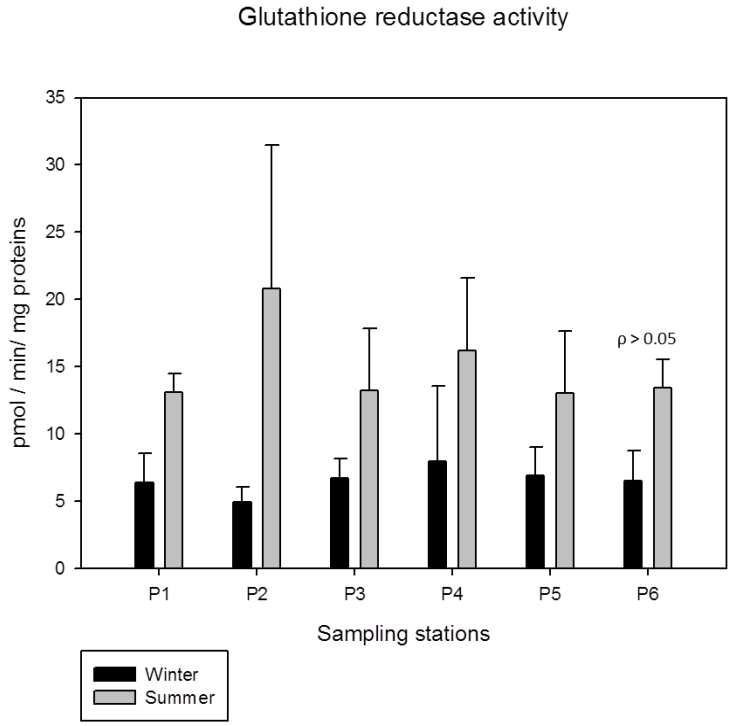

Figure 7. Seasonality of GR enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for

P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means that there was significance difference between winter and summer in the control responses.

AChE activity (Figure 8) was higher in winter than summer, and clams exposed to P1 showed significant AChE activity compared with the control ($p < 0.05$). Clams exposed to P3 during winter presented neurotoxicity due to the significant inhibition of AChE enzymatic activity when compared with the control ($p < 0.05$).

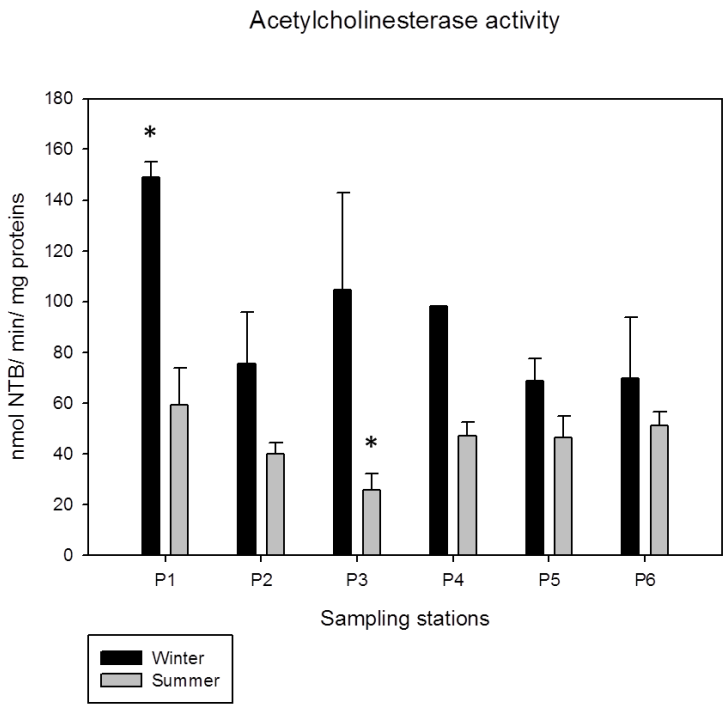

Figure 8. Seasonality of AChE enzymatic activities determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

Effects of LPO (Figure 9) and DNA damage (Figure 10) showed no seasonal variations in clams exposed to sediment samples. Clams exposed to P2 during winter showed significant increase of LPO compared with the control ($p < 0.05$). DNA damage significantly

increased during summer period for clams exposed to sediment sampled at P2 and P5 compared with the control ($p < 0.05$).

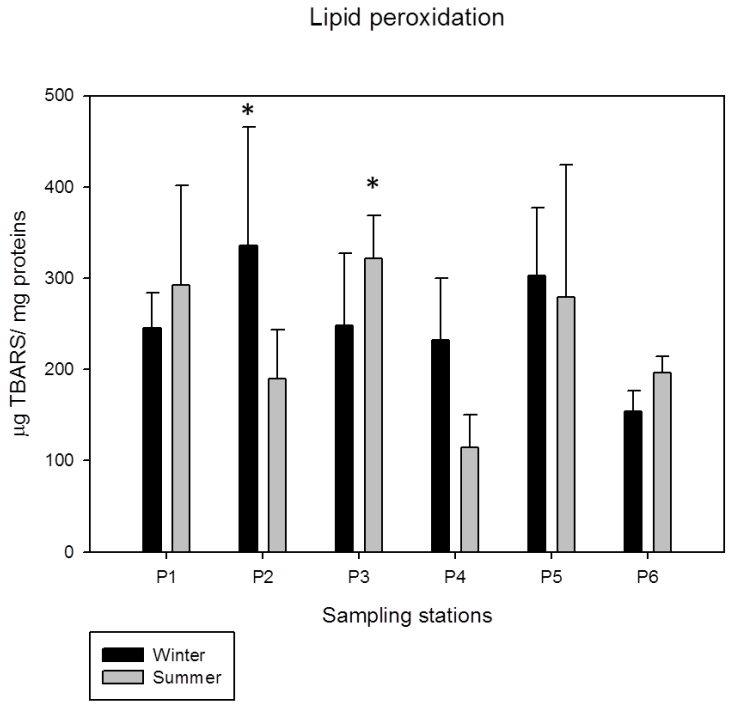

Figure 9. Seasonality of lipid peroxidation (LPO) determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

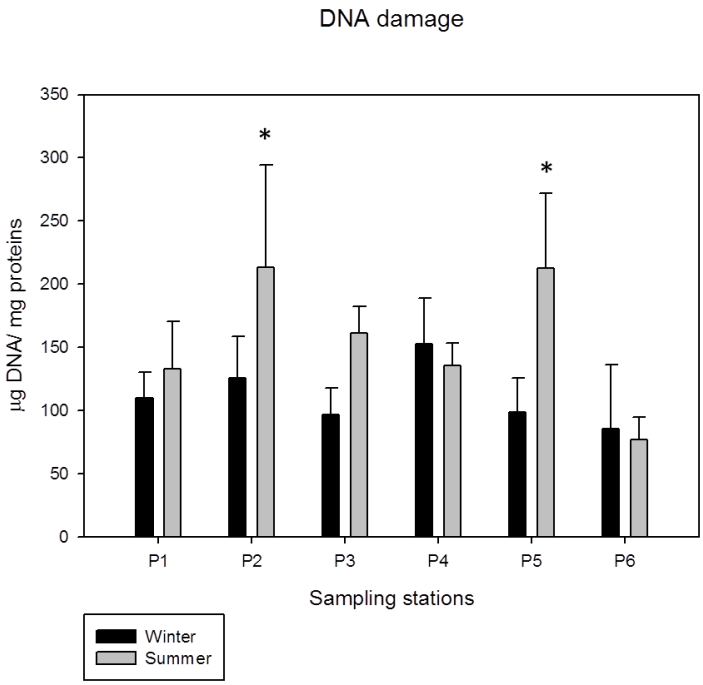

Figure 10. Seasonality of genetic damage (DNA *strand breaks*) determined in digestive gland of clams *R. philippinarum*. Individuals were exposed to marine sediments under laboratory conditions for 14-days. Sediment samples were collected in five sites directly affected by wastewater discharges (except for P6 that was the control site) in winter and summer. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

3.3. Correlation between the biological responses according to the seasons

Correlation coefficients amongst the biological endpoints measured in digestive gland of clam's *R. philippinarum* exposed to sediment sampled near WWTPs at the Bay of Cádiz (SW, Spain) were shown in the Table 4.

Table 4. Correlation coefficients amongst the biological endpoints measured in digestive gland of clams *Ruditapes philippinarum* exposed to sediment sampled near WWTP´s at the Bay of Cádiz (SW. Spain). Lysosomal membrane stability measured in haemocytes was included in the analysis (see text for further details).

Winter

	LPO	GST	GR	GPX	EROD	DBF	AChE	LMS
DNA	.608**	.586**	-0.125	.486*	.504*	.517*	0.096	-0.235
LPO		.494*	-0.032	0.206	.526*	.500*	-0.359	-0.332
GST			0.063	.559**	0.363	.894**	0.054	-0.037
GR				-0.242	-0.307	0.155	-0.087	-0.320
GPX					0.371	.663**	0.430	0.194
EROD						0.353	0.015	-0.441
DBF							0.060	-0.153
AChE								-0.105

Summer

DNA	0.423	0.048	0.194	0.355	0.407	0.234	-0.310	-0.291
LPO		0.113	-0.217	0.128	0.149	0.118	-0.214	-.459*
GST			0.002	-.675**	0.374	.483*	-.605**	0.399
GR				0.088	0.454	.758**	0.032	0.098
GPX					-0.108	-0.228	0.072	-0.168
EROD						0.454	-0.402	-0.181
DBF							-0.193	0.052
AChE								-0.045

DNA, DNA damage; LPO, lipid peroxidation; GST, glutathione S-transferase; GR, glutathione reductase; GPX, glutathione peroxidase; EROD, ethoxyresorufin O-deetylase; DBF, dibenzylfluorescein dealkylase; AChE, acetylcholinesterase; LMS, lysosomal membrane stability.
* $p < 0.05$ according to the Spearman`s correlation test; ** $p < 0.01$ according to the Spearman's correlation test

During winter, biomarkers of effect (LPO and DNA damage) were positively correlated between them ($p < 0.01$) and with the phase I (EROD and DBF activities) ($p < 0.05$) and II (GST activity) of the metabolism ($p < 0.01$ for DNA damage and $p < 0.05$ for LPO). DNA damage ($p < 0.05$) and phase II ($p < 0.01$) were positively correlated with antioxidant system (GPX activity). Phase II (GST activity) and antioxidant enzyme GPX were positively correlated with the phase I (DBF activity) ($p < 0.01$).

Clams exposed to sediment sampled in summer showed different correlations between the responses than that observed in winter. Oxidative effect (LPO) was negatively correlated with LMS ($p < 0.05$). Phase II (GST activity) was negatively correlated with antioxidant enzymatic activity (GPX activity) and neurotoxicity (AChE activity) ($p < 0.01$), and positively correlated with phase I (DBF activity) ($p < 0.05$). Antioxidant response (GR activity) was also positively correlated with phase I (DBF activity) ($p < 0.05$).

3.4. Relationships between physical chemical and biological factors

Multivariate analyses were determined using the integrative biomarker, discriminant, principal component analyses and the IBR index (Figure 11). Two PCAs were performed on up to 31 biological and physical chemical parameters defined three interpretable components that explained 86.44% of data variance in winter, and four interpretable components that explained 92.05% of data variance in summer. For this work, only variables whose coefficient was ≥ 0.5, close to Comrey's (1973) cut off value of 0.55, were considered components of the factors.

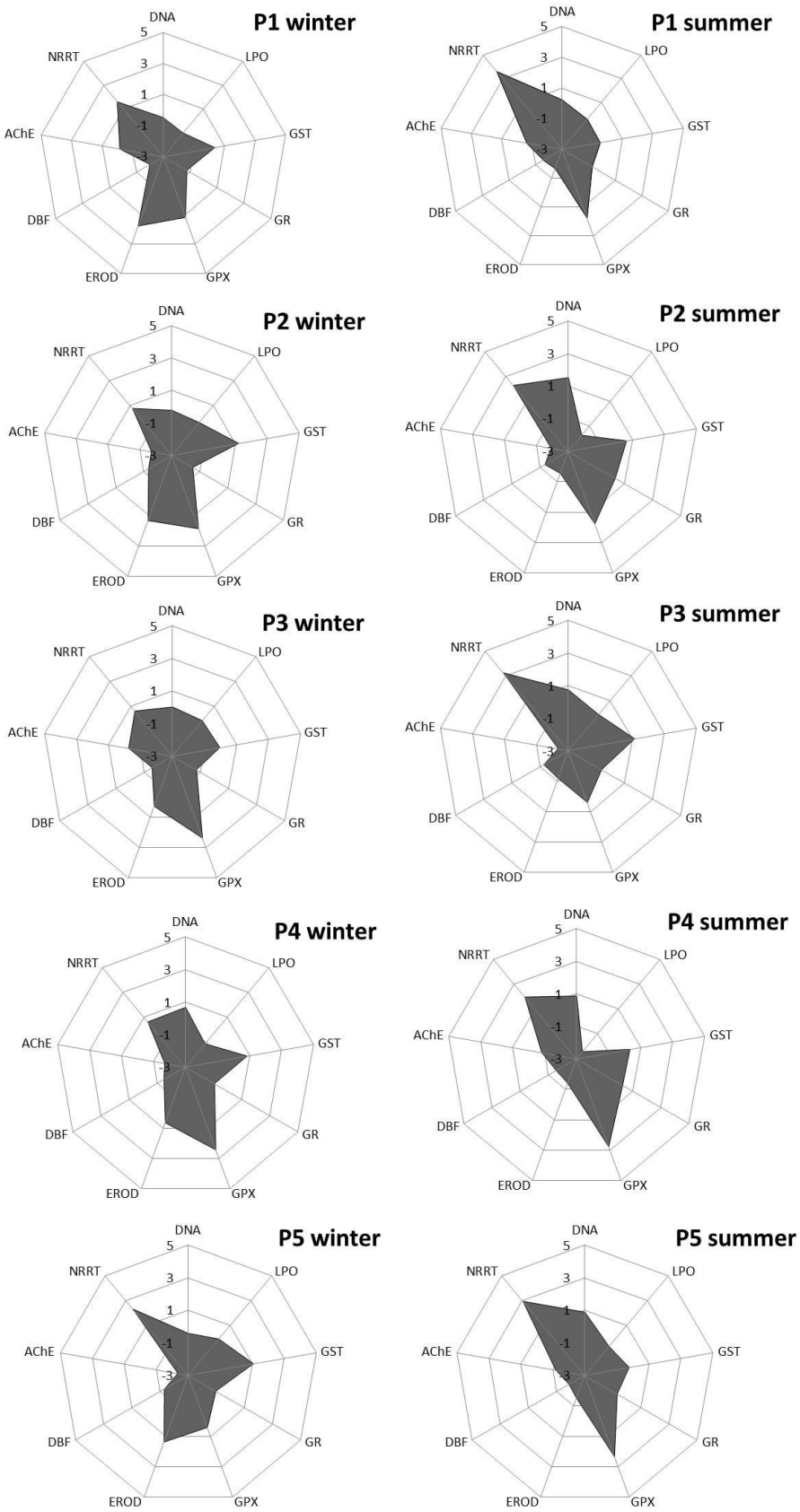

Figure 11. Integration of biomarker responses (IBR index) across the sites at the Bay of Cádiz (P1 – P5) and seasons (winter and summer).

The IBR index showed the physiological biomarkers NRRT, phase I (especially EROD) and antioxidant (especially GPX) enzymatic activities were more variable than the other biomarkers of effects or phase II of the metabolism.

Concerning winter analysis (Table 5), factor #1 was the most important since it explained 42.4% of the results total variance. The positive loadings presented in this factor exhibited values for antioxidant system (GPX activity) and oxidative effect (DNA damage). These biological responses were related to the concentrations of trace metals (Al, Fe, Cr, Cu, Ni, Zn and Pb) and some groups of pharmaceutical products (anti-inflammatories, anti-hypertensive, antacids and others). TOC and OM content had strong influence in the concentration of these contaminants in the sediments. Factor #2 explained 24.21% of the total variance. Positive loadings indicated the relationship of biological responses of phase I of the metabolism (EROD activity) and oxidative effect (LPO), with the concentration of trace metals (Al, Fe, Mn and As) and SAS. Factor #2 negative loadings indicated the relationship between the antioxidant system (GR activity) and Cd. Factor #3 accounted for 19.82% of the variance. It expressed the relationship (positive loadings) between biochemical responses of the phases I (EROD activity) and II (GST activity) of the metabolism, oxidative effect (LPO) and the presence of PAHs and antibiotics in the sediment. Negative loadings explained the relationship between neurotoxicity (AChE activity) and the presence of anti-inflammatories and lipid regulators in the sediment samples.

Table 5. Sorted rotated factor loadings of the 31 variables determined in winter on the three principal factors resulting from the PCA analysis.

Percentage Explained Variance	#1	#2	#3
	42.40%	24.21%	19.82%
DNA damage	.872		
LPO		.670	.664
GST			.822
GR		-.814	
GPX	.944		
EROD		.934	
DBF			.952
AChE			-.970
LMS	-.671		
PAHs			.532
Al	.832	.509	
Fe	.796	.560	
Mn		.690	
Cr	.892		
Cu	.905		
Ni	.870		
Zn	.908		
Pb	.728		
Cd		-.783	
As		.968	
Anti-inflammatories	.589		-.668
Anti-hypertensive	.628		
Lipid regulators			-.846
Psychiatric drugs	-.786		
Antibiotics	-.694		.670
Others	.884		
Antacid	.809		

SAS	.843
Fines	-.898
TOC	.639
OM	.878

Only loadings greater than 0.5 are shown. Factors are in explained variance decreasing order.

Concerning summer analysis (Table 6), factor #1 was the most important since it explained 32.78% of the results total variance. Positive loadings explained the relationship between antioxidant response (GR activity) and the concentrations of trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (lipid regulators and antacids). The concentration of these contaminants in the sediment was related to the TOC and OM content. Negative loadings of the factor #1 related the oxidative effect (LPO), the presence of Cd and the % of fines. Factor #2 accounted for 20.52% of the original variance and it grouped, with positive loadings, LPO, LMS and the concentrations of pharmaceuticals products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and others). Besides, negative loadings of factor #2 showed the inhibition of the phase II (GST activity) of the metabolism. Factor #3 explained 20.12% of the total variance. Positive loadings of factor #3 showed that trace metal (Pb), PAHs and SAS were present in the sediment and related to TOC, which seemed to be detoxified by the phase I of the metabolism (EROD and DBF activities), activated the antioxidant defences (GR activity), but culminated in DNA damage (strand breaks). Negative loading showed the inhibition of AChE activity. Factor #4 explained 18.63% of the total variance. Positive loadings of factor #4 showed that trace metals (Cu and Pb) and pharmaceutical products (anti-hypertensive drugs) were responsible for the increase of phase II (GST) enzymatic activity. Negative loadings grouped antioxidant

responses (GPX activity), neurotoxicity (AChE activity) and the concentration of trace metal (Mn) in the % of fines in the sediment.

Table 6. Sorted rotated factor loadings of the 31 variables determined in summer on the four principal factors resulting from the PCA analysis.

Percentage Explained Variance	#1	#2	#3	#4
	32.78%	20.52%	20.12%	18.63%
DNA damage			.810	
LPO	-.653	.683		
GST		-.632		.542
GR	.723		.537	
GPX				-.765
EROD			.923	
DBF			.700	
AChE			-.516	-.548
LMS		.902		
PAHs			.883	
Al	.947			
Fe	.946			
Mn				-.976
Cr	.928			
Cu				.723
Ni	.966			
Zn	.832			
Pb			.578	.809
Cd	-.921			
As				
Anti-inflammatories		.760		
Anti-hypertensive				.888
Lipid regulators	.595	.746		
Psychiatric drugs		.752		

Antibiotics		.828		
Others		.945		
Antacid	.589	.563		
SAS			.821	
Fines	-.707			-.608
TOC	.566		.686	
OM	.953			

Only loadings greater than 0.5 are shown. Factors are in explained variance decreasing order.

The weight of each factor at each site, concerning the winter season, was shown in the Figure 12. Factor #1 exhibited a positively predominant score for P4 and a negative predominant score for P5. While factor #2 was positively dominant in the case of P2, P3 and P4 showed the negative predominance. Factor #3 grouped variables with positive coefficients predominant in P2, and negative variables predominant in P1. The weight of each factor at each site concerning the summer season was shown in the Figure 13. Factor #1 variables with positive loadings were important in P1, P2 and P4, and negative loadings were important in P3 and P5. Factor #2 was positively dominant in P1. Factor #3 showed positive scores and a high dominance in P2. Factor #3 score was predominant and negative in P4. Factor #4 positive loadings were predominant in P3, and negative loadings were predominant in P5.

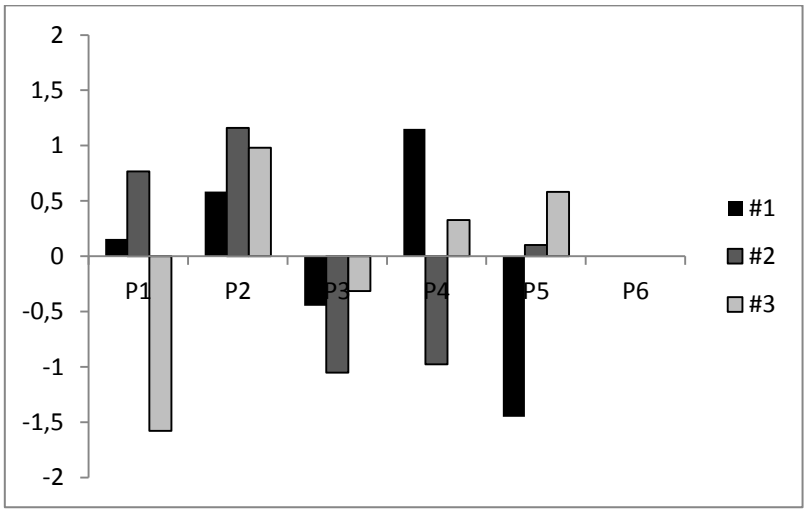

Figure 12. Factor scores of the five sediment sampling sites evaluated at the Bay of Cádiz (P1, P2, P3, P4 and P5) and that of the control site (P6) during winter season.

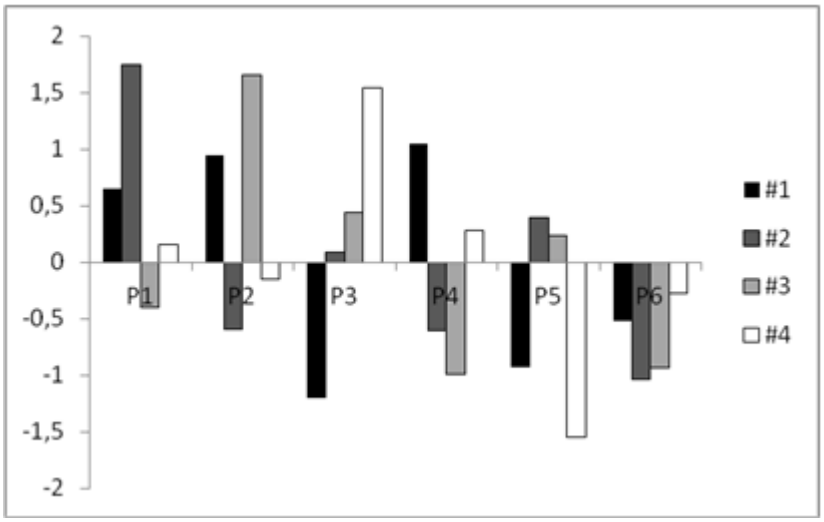

Figure 13. Factor scores of the five sediment sampling sites evaluated at the Bay of Cádiz (P1, P2, P3, P4 and P5) and that of the control site (P6) during summer season.

4. DISCUSSION

4.1. Seasonal variations

Sediment quality in coastal areas of Spain directly affected by wastewater discharges was assessed by determining biochemical biomarkers and health status in specimens of *Ruditapes philippinarum* exposed to field sediments under laboratory conditions. Through PCA analysis, biological responses were related to specific physical

chemical characteristics of the sediment so as to identify the possible cause for such responses. Chemical analysis showed that the composition varied widely with seasonality. These results corroborates with characteristic of Mediterranean regions (Damásio et al. 2011), where intensive water resource use is frequently linked to the lack of water flow due to climatic constrains, and rivers can receive effluents from cities, industries and agriculture with null or scarce dilution in summer. The same happened at the Bay of Cádiz, characterized by dry summer and rainy winter.

The battery of biomarkers chosen in the present study has been recommended for pollution biomonitoring studies (Viarengo et al. 2007; Van Der Oost et al. 2003; Cajaraville et al. 2000). They revealed the close relationship between the toxicity of sediment affected directly by wastewater discharges and the impairment of cellular redox status and possible oxidative effects. Variations of the antioxidant defence may have several consequences including the alterations of LMS, occurrence of membranes lipid peroxidation, genetic alteration and neurotoxicity. Nevertheless, interactions between biochemical responses and pollutants are complex, and potentially influenced by seasonal natural factors.

Previous studies showed the fluctuations between biomarker responses in bivalves according to the seasonality (Bocchetti et al. 2008) and temporal variation (Solé et al. 2009). This study clearly evidences the occurrence of seasonal trends of biochemical responses in this species. The phases I and II of the metabolism and antioxidant system (GR activity) increased the activities in *Ruditapes philippinarum* exposed during summer. Nevertheless, in the present study, it was not observed significant responses concerning the phase II of metabolism. However, previous studies showed that scallops have exceptionally high activities of phase II enzymes during summer due to their sediment-dwelling feeding strategy (Regoli et al. 1998). Between the antioxidant responses, GR

increased the activity in summer. Summer variations of antioxidant efficiency have been indicated in several Mediterranean organisms as a typical short-term response to the seasonal increase of pro-oxidant challenge due to higher seawater temperature and intensity of light irradiance (Regoli et al. 2004). AChE activity was higher in winter than summer. On the other hand, any of the biomarkers of effect (LPO and DNA damage) or subcellular effect level (LMS) measured showed significant differences according to the seasonality.

4.2. Integration of sediment characteristics and biological responses

An extensive investigation of eight biochemical biomarkers, lysosomal membrane stability (LMS), concentrations of trace metals, PAHs, pharmaceutical products and SAS have been conducted in order to monitor the responses of *Ruditapes philippinarum* to WWTPs-induced stress. A further analysis combining cellular, biochemical and physical chemical characteristics of sediment identified different associations and contributing factors. In addition, the interrelations among biomarkers and contaminants concentrations were discussed in order to evaluate the significance of the studied parameters in a relatively enclosed bay such as the Bay of Cádiz. Moreover, the use of a large set of biochemical and cellular responses may allow the identification of potential hazardous contaminants in the environment (Van der Oost et al. 2003), and also to produce relevant information for Water Authorities to taken actions to prevent further deterioration of ecological status (Damásio et al. 2010) due to the constantly wastewater discharges.

In winter, antioxidant system (GPX activity) was significantly induced in clams exposed to sediment sampled at P2 and P4 ($p < 0.05$). However, DNA damage was not significant different compared with the control organisms. These responses were related to the presence of trace metals (Al, Fe, Cr, Cu, Ni, Zn and Pb) and pharmaceutical

products (anti-inflammatories, anti-hypertensive, antacid and others). Previous studies showed that trace metals (Regoli et al. 1998; Tsangaris et al. 2007) and pharmaceutical products as anti-inflammatories drugs (Oviedo-Gómez et al. 2010; Aguirre-Martínez et al. 2013a) can cause oxidative stress and possible effects as DNA damage (*strand breaks*) in invertebrates.

Lysosomal parameters of clams exposed to sediment sampled at the reference site remained stable during the both seasons. The LMS value for control clams was about 100 min, in agreement with a good general health status (Lowe et al. 1995). During winter, clams exposed to sediment sampled at sites P1 and P5 showed significantly lower LMS than the control organisms ($p < 0.05$). However, P5 showed the highest weight for the negative score. The LMS values were higher than 60 min for these organisms. In the PCA analysis, LMS was related to the presence of psychiatric drugs and antibiotics, probably trapped in the sediment % of fines. Franzellitti et al. (2010) observed the reduction of LMS time mussels *Mytilus galloprovincialis* after 14 days of transplantation to contaminated sites. Ibuprofen (anti-inflammatory), novobiocin (antibiotic), carbamazepine and caffeine (psychiatric drugs) diminished the LMS in clams' *R. philippinarum* exposed to these compounds (Aguirre-Martínez et al. 2013b).

Phase I (EROD activity) was significantly induced during winter in clams exposed to sediment sampled at sites P1 and P2 ($p < 0.05$). Also, LPO in clams exposed to sediment sampled at site P2 was significantly higher than the control organisms during winter ($p > 0.05$). During this season, factor #2 related the biochemical responses of the phase I (EROD activity) and oxidative effect (LPO) with the presence of trace metals (Al, Fe, Mn and As) and SAS in sediment. EROD activity was induced also in the digestive gland of *Elliptio complanata* and in whole soft tissues of *Dreissena polymorpha* exposed downstream of municipal sewage treatment plant (Gagné et al. 2002). Gagné et al. (2007)

observed a significant correlation between LPO and CYP40 1A1 activities (EROD activity) in freshwater mussels exposed to domestic wastewater aeration lagoons.

Cd was related to the % of fines in the sediment, which can cause oxidative stress and the activation of the antioxidant defences (GR activity). This response was mainly related with clams exposed to P3 and P4, even GR activity was not significant different compared to the control. There are numerous studies about the influence of Cd in GR responses in many phylum (Crupkin and Menone 2013; Llugany et al. 2013).

The oxidative effect LPO, the phases I (DBF activity) and II (GST activity) of the metabolism were related to the presence of PAHs and antibiotics in the sediment samples. The overt CYP3A4 response, the activation of phase II and the consequent oxidative effect LPO may be indicative of low process efficiency of the WWTPs located near P1 and P2 during the winter season. CYP450 3A4 plays a major role in the biotransformation of pharmaceutical products. DBF enzymatic activity was previously related to the detoxification metabolism of antibiotics in *R. philippinarum* (Aguirre-Martínez et al. 2013a). Antibiotics as sulfamethoxazole (Nie et al. 2013) and novobiocin (Aguirre-Martínez et al. 2013a) can induce GST activity. Different pharmaceutical products, including antibiotics, were responsible for LPO in freshwater mussel *Elliptio complanata* (Gagné et al. 2006) and in *R. philippinarum* (Aguirre-Martínez et al. 2013a).

AChE activity was strongly induced in clams exposed to sediment sampled at site P1 during winter. AChE induction plays an important role in apoptosis (Zhang et al. 2002). AChE activity was related to pharmaceutical products (anti-inflammatories and lipid regulators), that demonstrated to be potential neurotoxic for clams, even though anti-inflammatories were considered potential AChE inhibitors (Richardson et al. 2013).

During summer, the antioxidant enzyme GR was related to the presence of trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (lipid regulators and

antacids). These xenobiotics were trapped in the sediment in the TOC and OM sediment compartments. It was considered that GR enzyme is also involved in antioxidant defence in the same way as GPX enzyme (Martín-Díaz et al. 2008b). Antioxidant system (GPX activity) was significantly induced during summer in clams exposed to sediment sampled at P5 ($p < 0.05$). Cd related with the % of fines in the sediment was responsible for the oxidative effect of LPO in clams exposed to sediment sampled at P3 in summer season. LPO is a very important consequence of oxidative stress (Van Der Oost et al. 2003).

Oxidative effect LPO and the decrease of LMS were linked with pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and others) mainly in clams exposed to sediment sampled at P1. Novobiocin (antibiotic), carbamazepine and caffeine (psychiatric drugs) were previously related to the increase of LPO in freshwater mussel *E. complanata* (Martín-Díaz et al. 2009) and marine crabs *Carcinus meaenas* (Aguirre-Martínez et al. 2013a) exposed under laboratory conditions. Ibuprofen (anti-inflammatory), novobiocin (antibiotic), carbamazepine and caffeine (psychiatric drugs) caused the decreased of LMS in clams *R. philippinarum* exposed to these pharmaceutical products (Aguirre-Martínez et al. 2013b).

Phase I (EROD and DBF activities) and antioxidant response (GR activity) were involved in the biotransformation metabolism of PAHs, Pb and SAS. These compounds were related to the TOC in sediment. These findings showed the enhancement of the detoxification and antioxidant defence systems which resulted in oxidative effect (DNA damage). This study corroborates with Capello et al. (2013) which showed a direct correlation between the high concentrations of PAHs in digestive glands of mussels *Mytilus galloprovincialis* and significantly altered activity of phases I and II biotransformation enzymes (Capello et al. 2013). DNA damage significantly increased in clams exposed to sediment sampled at sites P2 and P5 during summer ($p < 0.05$). DNA

damage significantly increased also in the digestive gland of mussels *Elliptio complanata* and in whole soft tissues of mussels *Dreissena polymorpha* exposed downstream of municipal sewage treatment plant (Gagné et al. 2002).

Trace metals (Cu and Pb) and pharmaceutical products (anti-hypertensive and antacids) were conjugated by the phase II of the metabolism (GST activity) in clams exposed to sediment sampled at P3. Trace metal (Mn) related to the % of fines in sediment sampled at P5 was responsible for the oxidative stress, the significant induction of GPX activity and the AChE enzymatic activity.

4.3. Sediment toxicity classification

The biological responses tested allowed the assessment of sediment contamination and pollution by WWTPs, not taken into consideration by CEDEX guidelines or proposed by Spanish legislation, as SAS, PAHs and pharmaceutical products. The integrated use of chemical data, a battery of biomarkers and subcellular response measured in *R. philippinarum* exposed under laboratory conditions to sediments sampled in different areas of the Bay of Cádiz, allowed the toxicological characterization of sediments affected by wastewater discharges and related to the seasonality. Moreover, the relationship between WWTPs activities and effluents characteristics are showed:

- Chiclana de la Frontera (P1): in winter, sediments were mainly characterized by pharmaceutical products (anti-inflammatories and lipid regulators) contamination that can be considered neurotoxic for clams *R. phillipinarum*. In summer, sediment showed contamination by additional pharmaceutical compounds (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and other) which culminated in oxidative effect (LPO) and the decrease of the health status (decrease of LMS);

- Puerto Real (P2): trace metals (Al, Fe, Mn and As) and SAS contamination were detoxified by EROD activity and resulted in oxidative effect (LPO) in *R. philippinarum* exposed in winter. The biotransformation metabolism (EROD and DBF activities) of PAHs, Pb and SAS associated to the TOC of the sediment produced free radicals, which increased the antioxidant defence activity (GR activity). Therefore, DNA damage was observed in clams exposed in summer season;

- Cádiz – P3: antioxidant defence (GR activity) was induced by Cd contamination, associated to the % of fines of the sediment in clams exposed in winter. However, trace metals (Cu and Pb) and pharmaceutical products (anti-hypertensive drugs) contamination were conjugated by the phase II of metabolism in clams exposed in summer;

- El Puerto de Santa María – P4: the antioxidant defence (GPX activity) was due to the contamination by trace metals (Al, Fe, Cr, Cu, Ni, Zn and Pb), pharmaceutical products (anti-inflammatories, anti-hypertensive, antacid and others) coming from the wide variety of human activities that have developed in the area and by a oxidative effect (DNA damage) to *R. philippinarum* exposed in winter. In summer, P4 sediments were distinguished by trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical (lipid regulators and antacids) contamination bound to TOC and OM content of the sediments. These compounds initiated biochemical defence responses (GR activity) in clams exposed to sediment in summer season;

- El Puerto de Santa María – P5: sediments were distinguished by pharmaceutical contamination (psychiatric drugs and antibiotics) which was related to the decrease of LMS in clams exposed in winter. Mn contamination related to the % of fines, influenced the antioxidant defence activity (GPX activity). Mn was neurotoxic (AChE inhibitor) for clams exposed in summer.

To conclude, this work has revealed the suitability of the selected battery of biomarkers measured in *R. philippinarum* as they showed sensitivity responding significantly to contamination compared with the control organisms. Also, it was underlined the usefulness of integrating results through a PCA to verify the occurrence of contamination and pollution in the marine environment due to wastewater discharges.

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In preparation

Toxicity potential of marine sediment affected by wastewater discharges: evaluation of neuroendocrine effects, energy budget and inflammation processes in clams *Ruditapes philippinarum*.

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ABSTRACT

The present study investigated the possible adverse outcomes in the marine clams *Ruditapes philippinarum* exposed to sediment affected by wastewater discharges at the Bay of Cádiz (SW, Spain). Six different locations representing five cities were chosen for the sediment sampling during the winter and summer seasons: P1 – Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa María, P6 – Rota (control site). An evaluation of biochemical biomarkers for sub-lethal toxicity was explored in clams after 14-days of exposure under controlled conditions, that included changes in cellular energy status (total lipids content – TLP and mitochondrial electron transport activity - MET), gametogenic activity (dopamine and Vtg-like proteins levels), metabolism of monoamines (monoamine oxidase activity - MAO) and inflammation and spawning properties (cyclooxygenase activity - COX). Wastewater discharges reduced the health status of clams and induced energy budget alterations, as suggested by MET decrease (P4 and P5) and accumulation of TLP (P1, P2 and P3) in gonads. Vtg-like proteins (P1, P2 and P3), dopamine (P2) and COX activity (P1, P2, P3, P4 and P5) decreased in clams after the exposure to summer sediments. MAO increased in clams exposed to winter (P1 and P2) and summer (P3 and P4) sediments. Wastewater discharges composition changed between different seasons, mainly leading to oxidative stress, inflammation (COX activity and Vtg-like proteins) and spawning delay in summer. This study highlights the importance of considering reproduction and health status of marine biota when assessing adverse effects of wastewater discharges. Thus, the continuous release of wastewaters adequately threated or not in aquatic ecosystems poses a health status risk to the local benthic biota.

Keywords: seasonality, *Ruditapes philippinarum*, wastewater discharges, immunotoxicity, neuroendocrine assessment, gametogenic effects, marine sediment.

1. INTRODUCTION

Rivers, wastewater discharges and sewage outfalls drive large amounts of effluents to estuaries and marine environments, which contain a plethora of chemicals including trace metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, steroids, surfactants, nutrients and pharmaceutical and personal care products (PPCPs). Marine and estuarine environments are the major receptor of wastewater discharges from coastal areas and where is located the most urban populated areas in the world. Although a variety of PPCPs have been currently detected in the marine environment (water column and sediment), their ecological significance remains unknown. Only few studies have addressed the wastewater exposure and effects on non-target species, and even less on marine benthic biota.

There are now regulatory arguments in the Water Framework Directive (WFD) to recommend the use of biota for assessing contamination trends in water bodies, particularly for hydrophobic organic substances (Gust et al., 2014). Sensitivity of the molluscs to various pollutants has been explored to evaluate the impacts of municipal effluents (Box et al., 2007, Solé et al., 2009, Franzellitti et al., 2010, Gagné et al., 2011).

Data about biochemical responses related to the mode of action (MOA) of pharmaceutical compounds in non-target biota are scarce. This response group involves the assessment of neuroendocrine effects (monoamine oxidase activity – MAO, dopamine levels), immunotoxicity (cyclooxygenase activity - COX), reproduction (vitellogenin-like protein levels - Vtg) and energy status (total lipids content – TLP, mitochondrial electron transport – MET). Linked biomarkers, known to be affected by wastewater treatment plant (WWTP) effluents were assessed previously in freshwater molluscs, where the

exposure to primary-treated municipal effluents produced an inflammation syndrome in mussels *Elliptio complanata* (Gagné et al., 2008 a, Gagné et al., 2007 a, Gagné et al., 2005) measured by cyclooxygenase activity (COX), and in snails *Lymnaea stagnalis* (Gust et al., 2013). Another study confirmed the neuroendrocrine toxicity potential and estrogenic activity of primary-treated municipal effluents on *E. complanata* through the determination of monoamine oxidase activity (MAO) (Gagné et al., 2007 a, b) and Vtg-like proteins (Gagné et al., 2007 b). Evidence of feminization was confirmed in *E. complanata* exposed to municipal effluents through the measurements of COX activity, serotonin and dopamine levels, and Vtg-like proteins (Gagné et al., 2011). However, activity of the pro-inflammatory enzyme COX and levels of Vtg-like proteins were not significantly changed in *E. complanata* exposed to secondary-treated municipal effluent (Farcy et al., 2011). Other studies showed freshwater organisms exposed to contaminated habitats by effluents increased expenditure of energy reserves as determined by mitochondrial electron transport activity (MET) and lipid reserves (TLP) reduction (Gagné et al., 2007 a, Smolders et al., 2004). However, such biological responses can be influenced by seasonality, and natural variability needs to be distinguished by the effects caused by xenobiotics.

Marine bivalves, such as the clams *Ruditapes philippinarum* have shown to be sensitive bioindicators exposed to chronic water/sediment pollution. This bioindicator species has been extensively used in biomonitoring studies (Morales-Caselles et al., 2008, Moschino et al., 2011) and sublethal measurements (Buratti et al., 2010, 2012, Coughlan et al., 2009, Martín-Díaz et al., 2008 b, 2007, 2005). Research of immune and neuroendocrine responses in marine bivalves to stressors and their application in pollution biomonitoring studies is not as extensive on biochemical biomarkers and changes in the reproductive function concerning Vtg-like proteins (Gagné at al., 2008 b, Matozzo and

Marin, 2007). Such aspects have particular relevance to monitor population dynamics (Gust et al., 2014). Reproduction aspects were impaired in contaminated environments (Solé et al., 2003, Martín-Díaz et al., 2008 a, Matozzo et al., 2008).

The present study explored the possibilities of extrapolating effects at cellular level in clams exposed to marine sediment affected by wastewater discharges sampled during winter and summer at the same locations by means of changes in energy budgets, inflammatory properties, neuroendocrine and reproduction effects. The research described herein aims to evaluate differences in sublethal stress responses of bivalve sentinel organisms and the suitability of this bioindicator to evaluate sediment affected by wastewater discharges. Five different locations (P1 - P5) representing four cities (SW, Spain) were selected under directly influence of wastewater discharges and compared with a reference site (P6). 14-days bioassay with marine clams (*R. philippinarum*) was performed under laboratory conditions. Effects of the exposure were measured as changes in total lipids (energy reserves), mitochondrial electron transport (energy consumption), cyclooxygenase activity (inflammation properties), monoamine oxidase activity and dopamine levels (neuroendocrine effects), and Vtg-like proteins (reproduction). Observed effects were examined in gonads taking into account the seasonality and contamination.

2. MATERIALS AND METHODS

2.1. General approach

The Bay of Cádiz (SW, Spain) comprises a population of 460,000 habitants, but during the summer season the population increases by 30% compared with winter owing to tourisms. There is an increase of some PPCPs use (e.g. sunscreen in summer) in each season, and the decrease of others (e.g. cold medications in summer). Summer in South Spain is a dry season, and consequently, water consumption increases. However, winter

comes with strong storms, which deal with sediment resuspension in the studied areas, and as a consequence, contaminants accumulated in the sediment compartment tend to be bioavailable to the aquatic biota. Main industries located in this zone are related with ship, offshore, car and aerospace manufacturing. Agriculture and tourism are important socio-economic activities at the Bay of Cádiz.

Six points were chosen located at the Bay of Cádiz (SW, Spain) (Figure 1) for the assessment of sediment toxicity. Five of them were under the influence of wastewater discharges from urban areas: P1 – Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4 and P5 – El Puerto de Santa Maria. P6 was located at Rota, and chosen as the control site because it was far from any wastewater discharge.

- Chiclana de la Frontera (P1): the sample point was located at Iro River. Previous study speculated the discharge of anticholinergic agents, such as pesticides (Solé et al., 2009). This river receives water from agricultural sources as well as urban wastewater discharge. Although the waste is treated in a treatment plant before the discharge, a high level of contamination by nutrients and pathogens were measured in the aquatic ecosystem (Garrido-Pérez et al., 2002).
- Puerto Real (P2): characterized by the wastewater treatment plant discharge, moderate metal contamination (Carrasco et al., 2003) and significant shipping activity.
- Cádiz (P3): this area support a seasonal wastewater pumping and storage station, that send the wastewater to be treated in another WWTP located at San Fernando. However, there are occasional discharges of wastewater from this station to the Bay of Cádiz.
- El Puerto de Santa Maria (P4 and P5): P4 is characterized by season wastewater pumping, and also receives the effluents coming from the upper part of the

Guadalete River. SAS were previously detected in sediment in this area which correlated their usage and the presence of wastewater discharges (Lara-Martín et al., 2006). In this point are located some marinas and small harbours. P4 might be considered the main receiving point for wastewater generated in the upstream regions of the province of Cádiz. P5 is a sewage outfall located in the north of the Bay of Cádiz, at Puerto Sherry, which receives WWTP effluents from the city. There are only occasionally untreated discharges since WWTP has existed for several years (Lara-Martín et al., 2006).

- Rota (P6): this area is located far from known wastewater discharges, near the Chorillo sandy beach.

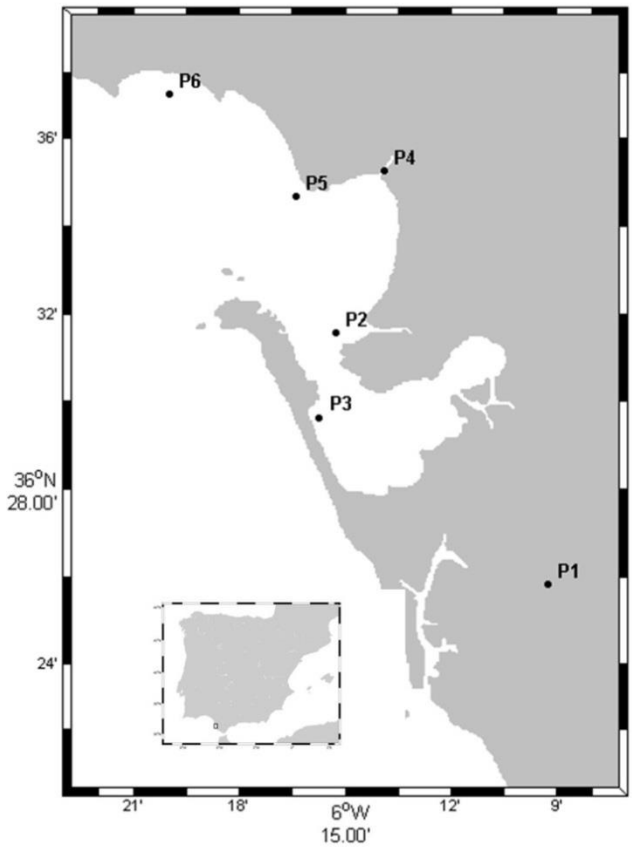

Figure 1. Geographic locations of sampling regions across the Bay of Cádiz (SW, Spain). Five sampling sites representing urban areas were selected to the sediment collection near wastewater discharges: Chiclana de la Frontera (P1), Puerto Real (P2), Cádiz (P3), El Puerto de Santa Maria (P4 and P5), and ending with the control site in Rota (P6).

2.2. Sediment sampling

Sediment was sampled during winter and summer 2011, from an inflatable launch on an ebbing tide by means of Van Veen grab (when it was possible) or scuba divers help, taking the topmost 10 cm layer of the sediments. Samples were brought to the laboratory, sieved to remove large debris and other animals, and kept at 4 ^{o}C in the dark for maximum two days until the clams' exposure. Sediment samples were placed in 20-L aquarium filled with filtered seawater.

Physical chemical characteristics (total organic carbon - TOC, organic matter - OM and grain size) of the sediment sampled at P1 – P6 in winter and summer seasons were described in the previous study (Maranho et al., 2014xxx). The methodology to measure PAHs, trace metals (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se, Zn and Hg), surfactants (SAS) and pharmaceuticals concentrations (anti-inflammatories: acetaminophen, diclofenac, fenoprofen; anti-hypertensive: atenolol, propranolol; lipid regulators: clofibric acid, gemfibrozil; psychiatric drugs: carbamazepine, fluoxetine, amitriptyline, caffeine; antibiotics: chloramphenicol, cefdinir, tiamulin, erythromycin, clarithromycin, azithromycin, roxithromycin, lincomycin, flumequine, clindamycin, sparfloxacin, novobiocin, metronidazole, ornidazole, sulfadiazine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, monensin; antacids: famotidine, ranitidine; and others: glibenclamide, hydrochlorothiazide), and the respective real values found in the sediment samples were detailed in this previous study. However, these data was included for the statistical analysis of the present study.

2.3. Clams exposure and tissues preparation

Specimens of *R. philippinarum* were bought from an aquaculture farm located at Chiclana de la Frontera (SW, Spain). These specimens were acclimatised in the

laboratory for 7 days before exposure. After the acclimation period, 28 individuals were placed to each aquarium (day-0). Duplicates were used. Conditions of the bioassay were verified each two days (pH: 8.1 ± 0.3, dissolved oxygen: 7.3 $mg \cdot L^{-1}$ ± 0.2, salinity: 35.3 ± 1.4). 1: 3 of the seawater was renewed each three days. Exposure period lasted 14 days at 18 ± 2 ^{o}C, photoperiod 16: 8, under constant aeration. At the end of the exposure period, clams were counted and placed overnight to aquariums filled with filtered seawater at temperature of 18 ± 2 ^{o}C. Gonad mass were dissected out and homogenized for each pool of clams on ice using Teflon pestle tissue grinder apparatus in homogenization buffer (pH 7.5) containing 140 mM NaCl, 25 mM Hepes-NaOH, 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT) (Gagné et al., 2007 a). A portion of homogenate was centrifuged at 3,000g at 4 ^{o}C for 20 min and the supernatant fraction carefully collected (S_3 fraction). Other aliquot of homogenized fraction was centrifuged at 15,000g at 4 ^{o}C for 20 min, and the supernatant used for the biomarker determinations (S_{15} fraction). Homogenate, S_3 and S_{15} fractions were stored at -80 ^{o}C until further analysis.

Total protein content (mg) was determined to each extract according to Bradford method (1976) using serum bovine for calibration.

2.4. Neuroendocrine parameters – dopamine levels and monoamine oxidase activity

Dopamine levels were determined in the S_{15} fraction using a competitive enzyme-linked immunosorbent assay (ELISA) (Kim et al., 2008) with modifications (Gagné et al., 2011). First, 96-well luminescence plates (Microlite 2, Thermo Fisher Scientific, ON, Canada) were coated with 0.5 μg of BSA-conjugated dopamine (US Biological, Boston, MA, USA) in 50 mM Tris–HCl (pH 8.5) at 4 °C overnight. The plates were washed three times with PBS (5 mM KH_2PO_4, 1 mM $NaHCO_3$, 150 mM NaCl, pH 7.4) and incubated in blocking buffer (1% dry milk in PBS) for 90 min at room temperature with constant

shaking. Dopamine standard were diluted in buffer (0.5% dry milk in PBS) in ranging concentration from 1 to 1000 μM and 0.5 to 1000 μM, respectively. Plates were washed with PBS and standards, and pre-diluted samples were added to the wells followed by an addition of primary antibody 1:5000 (Rabbit polyclonal to dopamine ab888, Abcam, MA, USA). Plates were washed three times with PBS and incubated with HRP conjugated goat anti-rabbit IgG (1:10,000, Stressgen, MI, USA) for an hour after which unbound HRP-conjugate antibodies were removed. Wells were washed three times with PBS and HRP substrate solution (BM Chemiluminescence ELISA Substrate, Roche Diagnostics, QC, Canada) was added in the microplate. Chemiluminescence intensity was measured using a Chameleon plate reader (Hidex, Finland). Data were expressed as μmol dopamine/mg protein.

MAO activity was determined using serotonin analogue tryptamine as the substrate (Gagné et al, 2007 b). Homogenate extracts were incubated in a solution of 10 μM dichlorofluorescein, 1 mM tryptamine in 10 mM Hepes-NaOH (pH 7.4), containing 140 mM NaCl, 10 mM aminotriazole and 0.1 $\mu g \cdot ml^{-1}$ of horseradish peroxidase for 0, 15, 30 and 60 min at 30 ^{o}C. Fluorescence was measured at 485 nm and 535 nm. Activity was expressed as nmol RFU/ min/ mg proteins.

2.5. Estrogenic activity assessment

Vtg-like proteins were determined in homogenized extracts applying alkali-labile phosphate (ALP) method described by Gagné et al. (2003). Proteins were precipitated by acetone (35% v/v) and centrifuged at 10,000g for 5 min at 4 ^{o}C. The pellet was washed in 50 % acetone and re-centrifuged. The pellet was resuspended in 100 μL of 1M NaOH and incubated for 30 min at 60 ^{o}C. Levels of inorganic phosphates liberated from NaOH treatment were determined by phosphomolybdate assay (Stanton, 1968). Absorbance was

measured at 444 nm (some cases with interferences in the colour, the reading was taken at 815 nm). Data were expressed as μg ALP/ mg proteins.

2.6. Inflammation biomarker

Cyclooxygenase (COX) activity was tracked by measuring the oxidation of 2, 7 – diclorofluorescein in the presence of arachidonate in S_{15} fractions (Fujimoto et al., 2005). The S15 fraction was incubated in 50 mM Tris-HCl, 0.05% Tween-20, 50 μM arachidonate, 2 μM dichlorofluorescein and 0.1 $\mu g \cdot ml^{-1}$ horseradish peroxidase. Fluorescence was measured at 485 nm and 530 nm. COX activity was expressed as RFU/ min/ mg proteins.

2.7. Energy budget

Total lipids (TLP) were determined in homogenate samples according to the phosphovanillin method (Frings et al., 1972). Samples were incubated for 10 min at 80 ^{o}C in the presence of H_2SO_4 and phosphovanilin reagent. The appearance of a pink color was measured at 540 nm. Standard solutions of olive oil were used for calibration. The data were expressed as μg total lipids/ mg proteins.

Mitochondrial electron transport (MET) activity was determined according to the reduction of p-iodonitrotetrazolium dye method (Smolders et al., 2004, King and Packard, 1975). The S_3 fractions were mixed with buffer composed by 0.1 M Tris–HCl containing 0.1 mM $MgSO_4$, 0.1% Triton X-100 and 5% polyvinylpyrrolidone (pH 8.5) for 1 min, followed by the addition of 1 mM NADH, 0.2 mM NAPDH and 1 mM p-iodonitrotetrazolium. Absorbance readings were taken at 520 nm each 5 min for 30 min. Data were expressed as A 520 nm/ min/ mg proteins.

2.8. Data analysis

Biomarker data were produced from 12 clams from each duplicate (n = 24). Normality and homogeneity were tested using the Shapiro Wilk and Bartlett´s tests respectively. Then, data were subjected to two-way Analysis of Variance (ANOVA) followed by Dunnett's t test to compare with the controls. Significance was set at $p < 0.05$. Spearman´s rank correlation was performed to detect significant trends between biomarker responses determined in each sediment sample point and between seasons. Significance was set at $p < 0.05$. Two separate PCAs were conducted on the biological and chemical results (Maranho et al., 2014XXX), one for winter, and the other one for summer. Only the variables whose coefficient was ≥ 0.5 (Comrey's, 1973) were considered to be components of the factors. All responses were analyzed using the SPSS/PC 21.0 + statistical package.

3. RESULTS

3.1. Biomarker responses

Neuroendocrine parameters, energy budget and the inflammation state of clams exposed to marine sediments contaminated by effluent discharges from wastewater treatment plants located at the Bay of Cádiz (SW, Spain) were investigated. Although there was no mortality of clams exposed to sediment sampled in the summer season, increasing of clam's mortality was observed during the winter season: P1 – 32.1%, P2 – 21.4%, P3 – 26.7%, P4 – 50%, P5 – 7.14%, P6 – 15% in the control site.

Biomarker responses were examined in clams exposed to sediment samples affected by wastewater discharges (Figures 2 – 7). There was no significant difference of dopamine levels in clams exposed to marine sediment in winter season (Figure 2). Clams

exposed to sediments from P2 showed significantly lower dopamine levels (1-fold) compared with the control during the summer season ($p < 0.05$).

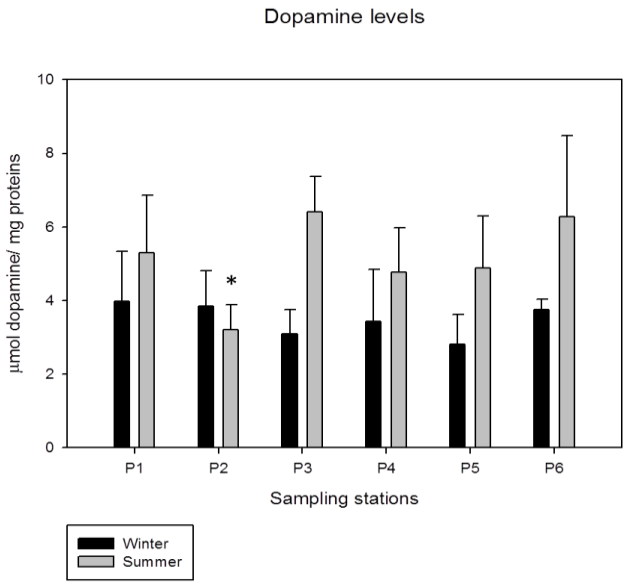

Figure 2. Dopamine levels in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-days under laboratory conditions and analysed for dopamine levels. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

During the winter season, clams exposed to sediments affected by wastewater discharges P1 and P2 showed significant MAO activity reaching 1-fold higher compared with the control ($p < 0.05$) (Figure 3). However, clams exposed to sediments from P3 and P4 significant increased MAO activity ($p < 0.05$) in summer, which was found with 0.9 and 1.6-fold higher than the control, respectively.

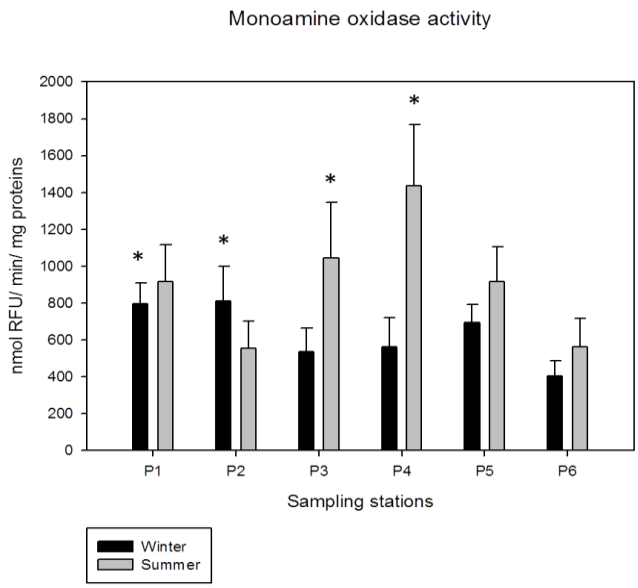

Figure 3. Monoamine oxidase (MAO) activity in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-days under laboratory conditions and analysed for monoamine oxidase activity. RFU: relative fluorescein units. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

Vtg-like proteins were higher in summer than winter (Figure 4). P1 (1.2-fold), P3 (0.6-fold) and P4 (1.2-fold) were significantly lower compared with the control ($p < 0.05$) during winter.

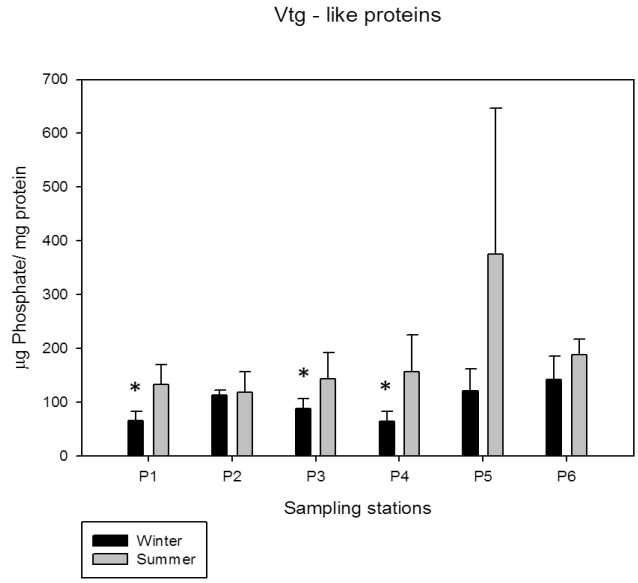

Figure 4. Vitellogenin-like proteins (Vtg-like proteins) in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-

days under laboratory conditions and analysed for gonad activity. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

COX activity (Figure 5) increased in summer compared with winter including clams exposed to sediment sampled at the control site. Nevertheless, COX activity was significantly decreased compared with the control ($p < 0.05$) during the summer season for all stations (P1: 1.3-fold, P2: 2.5-fold, P3: 0.9-fold, P4: 1.9-fold, P5: 0.9-fold).

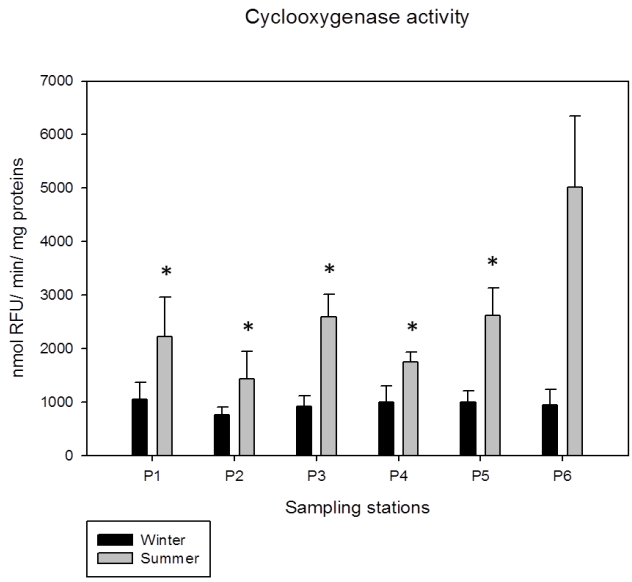

Figure 5. Cyclooxygenase (COX) activity in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-days under laboratory conditions and analysed for cyclooxygenase activity. RFU: relative fluorescein units. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

TLP (Figure 6) are essential for gametogenesis as with many other vital functions. Clams exposed to sediments sampled in winter showed higher TLP content, being P1 (0.9-fold), P2 (1.5-fold) and P3 (0.9-fold) significantly increased compared with the control ($p < 0.05$). Clams showed lower TLP in summer compared with winter, however, P1 (1.4-fold) showed significantly higher TLP content compared with the control clams ($p < 0.05$).

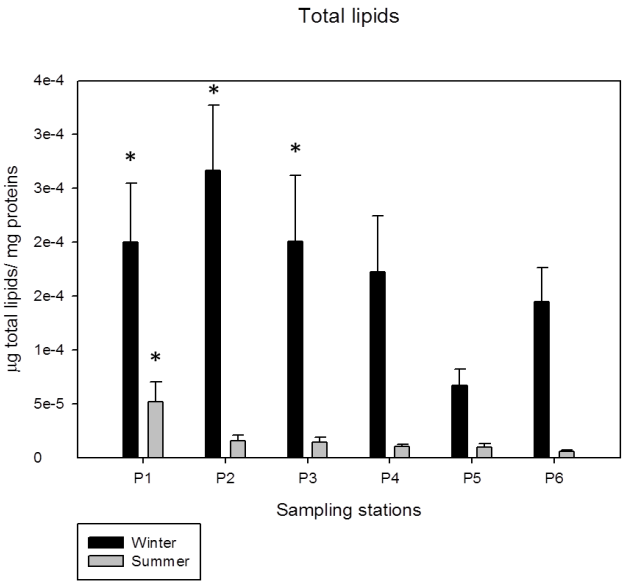

Figure 6. Total lipids (TLP) content in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-days under laboratory conditions and analysed for total lipids content. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

Respiration rates of clams exposed to marine sediments affected by wastewater discharges was determined by mitochondrial electron transport (MET) activity (Figure 7). During winter, there was no significant difference for any station compared with the control. However, P4 and P5 (1.1-fold) showed significant decrease in MET activity in summer ($p < 0.05$).

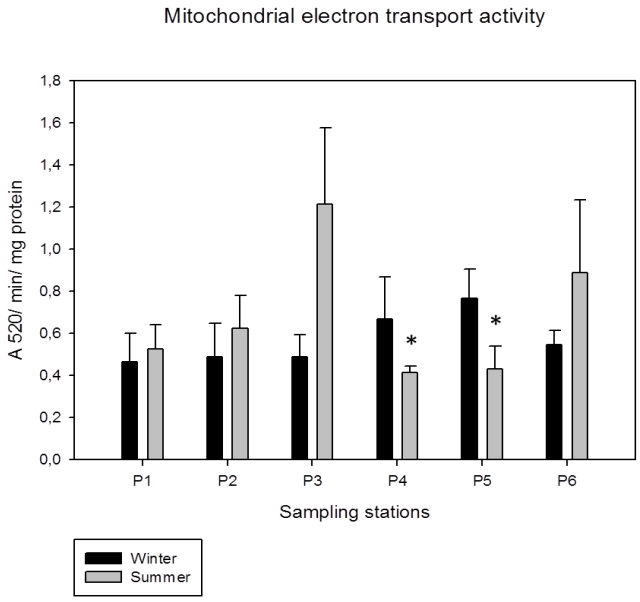

Figure 7. Mitochondrial electron transport (MET) activity in clams exposed to marine sediment affected by wastewater discharges. Clams *R. philippinarum* were exposed to the sediments for 14-days under laboratory conditions and analysed for mitochondrial electron transport activity. A 520: absorbance at 520 nm. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. The data represent the mean with standard deviation from n = 24 animals.

3.2. Correlation between biomarker responses

Concerning P1 in winter, there was no correlation between biomarker responses. In summer, TLP was positively correlated with dopamine, Vtg-like proteins and MAO activity ($r = 1$, $p < 0.01$). TLP and COX activity were negatively correlated ($r = -1$, $p < 0.01$). Dopamine was also positively correlated with MET activity ($r = 1$, $p < 0.01$).

Biomarker responses determined in clams exposed to P2 in winter showed positive correlation of MET activity, TLP ($r = 0.9$, $p < 0.05$) and COX activity ($r = 1$, $p < 0.01$). MET activity was also negatively correlated with MAO activity ($r = -1$, $p < 0.01$). Dopamine and Vtg-like proteins levels were negatively correlated ($r = -1$, $p < 0.01$). Clams exposed in summer showed positive correlation between COX activity and TLP ($r = 0.9$, $p < 0.05$). Vtg-like proteins were positively correlated with MAO activity ($r = 1$, $p < 0.01$), and negatively correlated with dopamine ($r = -1$, $p < 0.01$).

Positive correlation was observed between TLP and dopamine in clams exposed to sediment sampled at P3 in winter ($r = 1$, $p < 0.01$). No correlation was observed in summer season.

Clams exposed to sediment sampled at P4 in winter showed positive correlation between dopamine, MET and COX activities ($r = 1$, $p < 0.01$). In summer, COX activity was negatively correlated with Vtg-like proteins and MET activity ($r = -1$, $p < 0.01$). MAO activity was positively correlated with dopamine and MET activity ($r = 1$, $p < 0.01$), and negatively correlated with Vtg-like proteins ($r = -1$, $p < 0.01$).

In winter, clams exposed to P5 showed positive correlation between Vtg-like proteins and COX activity ($r = 0.9$, $p < 0.05$), and negative correlation with MET activity ($r = -0.9$, $p < 0.05$). In summer, MAO activity and Vtg-like proteins were negatively correlated ($r = -1$, $p < 0.01$).

P6 was considered the control site, and the clams exposed to this sediment samples showed positive correlation between COX and MAO activities ($r = 1$, $p < 0.01$). However, MAO and MET activities, and TLP and Vtg-like proteins were negatively correlated ($r = -1$, $p < 0.01$). In summer, TLP was positively correlated with dopamine, MAO and MET activities ($r = 1$, $p < 0.01$). MET activity was also positively correlated with Vtg-like proteins ($r = 1$, $p < 0.01$).

3.3. Integrated approach

The first PCA analysis was performed with chemical and biomarker responses data from winter. Results were presented in the Table 1, where three factors axes explained 90.34% of the total variance. Positive correlations to factor 1explained 32.55% of variance, and related TLP and dopamine levels with contamination by PAHs, trace metals (Al, Cu, Ni, Zn, Pb) and pharmaceutical products (others) associated with TOC

and OM in the sediment. Negative correlations linked MET and COX activities with psychiatric drugs associated with % of fines in the sediment. Factor 2 accounted for 31.51% of variances, and positive correlations explained the relationship between MAO activity and dopamine levels related with contamination by trace metals (Al, Fe, Mn, Cr, Zn, Ni, As), pharmaceuticals (anti-inflammatories, lipid regulators, others) and SAS associated with OM content in the sediment. Factor 3 explained 26.28% of the variance. Positive loadings linked COX activity with contamination by trace metals (Cr, Cu) and pharmaceutical products (anti-inflammatories, anti-hypertensive, lipid regulators). Negative loadings grouped Vtg-like proteins with PAHs and pharmaceutical products (antibiotics) associated with % of fines in the sediment.

Table 1. Principal component analysis (PCA) based on chemical contamination and biomarker responses of clams exposed under controlled conditions to sediments affected by wastewater discharges at the Bay of Cádiz (SW, Spain) in winter.

Winter			
Variables	**#1** 32.55%	**#2** 31.51%	**#3** 26.28%
TLP	.974		
MET	-.844		
MAO		.882	
Dopamine	.613	.669	
Vtg-like proteins			-.974
COX	-.686		.623
PAH	.760		-.542
Al	.503	.725	
Fe		.805	
Mn		.888	
Cr		.680	.511
Cu	.785		.510
Ni	.503	.694	
Zn	.709	.516	
Pb	.957		
Cd		-.992	
As		.895	
Anti-inflammatories		.558	.760
Anti-hypertensive drugs			.886
Lipid regulators		.500	.509
Psychiatric	-.876		
Antibiotics			-.938
Antacids			
Others	.513	.695	
SAS		.748	
% Fines	-.505		-.849
TOC	.951		
OM	.506	.686	

The second PCA was concerning summer data (Table 2). Factor 1 explained 31.65% of the results total variance. Positive loadings represented the contamination by PAHs, trace metals (Al, Fe, Cr, Ni, Zn), SAS and pharmaceutical products (others) associated with TOC and OM in the sediment. Negative loadings of the factor 1 showed

the relationship between dopamine, MET and COX activities due the contamination by Cd. Factor 2 accounted for 27.30% of the original variance and it grouped, with positive loadings, TLP and the concentrations of trace metals (Al, Fe, Cr, Ni) and pharmaceuticals products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids,others). Factor 3 explained 24.02% of the total variance. Positive loadings of factor 3 showed that trace metal (Cu, Zn, Pb) and pharmaceutical products (anti-hypertensive drugs and others) were present in the sediment related to TOC, which affected MET activity. Negative loading showed Vtg-like proteins related with trace metals (Mn, As) and % of fines in the sediment.

Table 2. Principal component analysis (PCA) based on chemical contamination and biomarker responses of clams exposed under controlled conditions to sediments affected by wastewater discharges at the Bay of Cádiz (SW, Spain) in summer.

Summer			
Variables	**#1** 31.65%	**#2** 27.30%	**#3** 24.02%
TLP		.822	
MET	-.537		.547
MAO			
Dopamine	-.959		
Vtg-like proteins			-.764
COX	-.701		
PAH	.700	-.559	
Al	.762	.606	
Fe	.811	.551	
Mn			-.960
Cr	.769	.556	
Cu			.860
Ni	.752	.532	
Zn	.638		.629
Pb			.880
Cd	-.848		
As			-.551
Anti-inflammatories		.869	
Anti-hypertensive drugs			.883
Lipid regulators		.878	
Psychiatric		.968	
Antibiotics		.964	
Antacids		.756	
Others	.520	.510	.578
SAS	.852		
% Fines			-.617
TOC	.756		.609
OM	.905		

The weight of factors at each sample site concerning the winter season was shown in the Figure 8. Factor 1 exhibited a positively predominant score for P2 and a negatively predominant score for P5. While factor 2 was positively dominant in the case of P1 and P2, P3 showed the negative predominance. Factor 3 grouped variables with positive coefficients predominant in P1 and P4, and negative variables predominant in P2 and P5. The weight of factors at each sample site concerning the summer season was shown in the Figure 9. Factor 1 variables with positive loadings were important in P2, and negative loadings were important in P3. Factor 2 was positively dominant in P1, and negatively dominant in P2 and P3. Factor 3 showed positive scores and high dominance in P3. Factor 3 score was negatively predominant in P5.

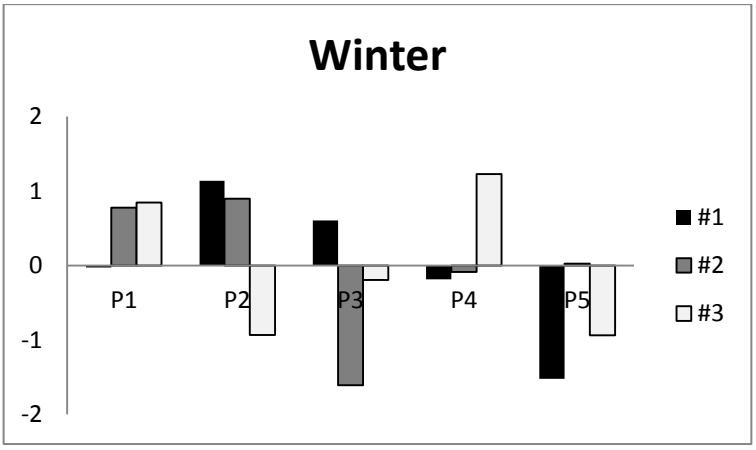

Figure 8. Factor scores of the five sediment sampling sites evaluated at the Bay of Cádiz (P1, P2, P3, P4 and P5) and that of the control site (P6) during winter season.

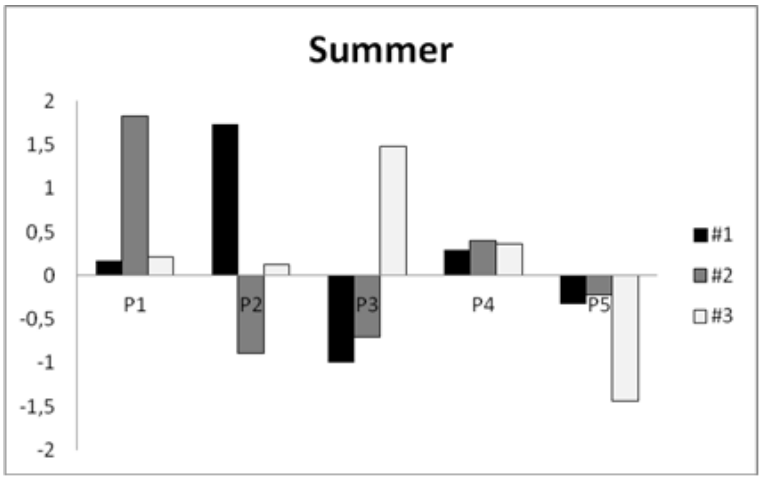

Figure 9. Factor scores of the five sediment sampling sites evaluated at the Bay of Cádiz (P1, P2, P3, P4 and P5) and that of the control site (P6) during summer season.

4. DISCUSSION

Increase of the population during summer season is directly related with the mass exodus of tourists visiting these locations. For Cádiz - San Fernando (sharing water treatment system) is usual that summer population increase more than 30% compared with winter. According to data from the Ministry of Environment of the Junta de Andalucía (Spain), these municipalities spend up water during summer an average of 59% over winter months which reflect directly to the volume of wastewater discharges. Wastewater treatment consists of a set of physical, biological and chemical operations that seek to eliminate organic matter and contaminants before discharge, so that the levels of contamination remaining in the treated effluents meet the existing legal limits. However, even after the treatments, wastewater presented toxicity to organisms exposed to the effluents (Gagné et al., 2007 a, b, Gagné et al., 2008 a, b).

Marine sediment sampled near different WWTPs discharges at the Bay of Cádiz affected the clams in a different way depending the locations and seasons. Seasonal changes in the biochemical components have been studied in *R. philippinarum* (Fernandez-Reiriz et al., 2007) in North Spain. Fernandez-Reiriz et al. (2007) observed

that clams *R. philippinarum* used their own energy reserves (carbohydrates and glycogen) for sexual development in high temperature situations (18 °C), while on low temperature conditions (14 °C) was produced an accumulation of reserves. The same pattern was observed for *R. philippinarum* in the present study, since clams exposed during the winter increased the lipids content (TLP) in gonads when compared with clams exposed during the summer season. Therefore, positive energy balances permitted clams gonadal development and reserves accumulation of both low and high temperature conditions (Fernandez-Reiriz et al., 2007). Gonadal development was slow and energy reserves accumulated in clams exposed to low temperature situations. When clams were conditioned at high temperatures, gonadal development was fast and energy reserves were consumed, which was shown by the correlation between Vtg-like proteins, TLP and MET activity in clams exposed to control sediment (P6) in summer.

In general, the pattern followed by each biomarker response observed in clams exposed to the control site (P6) was closely related to the state of sexual maturity and the use of previously stored reserves. Clams exposed to sediment sampled at control site located at Rota (P6) showed different correlations according to the seasons. In winter, COX and MAO activities were positively correlated ($p < 0.01$). MAO activity has the function to break down neurotransmitters such as noradrenaline, serotonin and dopamine. Increase of MAO enzymatic activity can provide free radicals due to the oxidation of neurotransmitters, culminating on harm the neurons that produce dopamine. Evidence for MAO activity in bivalve molluscs is compelling based on previous studies (Sloley et al., 2004). Increase of COX activity deals with inflammatory properties, but also with the spawning phase which normally occurs during summer for this specie. Decrease of COX activity could be due the increase of NSAIDs use for the population during this season. However, TLP were negatively correlated with MAO activity and Vtg-like proteins ($p <$

0.01) in winter, which means that the energy is transferred into vitellogenesis. MAO activity was negatively correlated with MET activity ($p < 0.01$). MET chain is responsible to transfer electrons via redox reactions across the membrane, involved in the oxidative phosphorylation and ATP synthesis.

Clams exposed in summer season showed positive correlation between TLP, MAO and MET activities and dopamine levels ($p < 0.01$). Under stress, TLP content is expected to be low which happened in summer. Vtg-like protein was also positively correlated with dopamine and MET activity ($p < 0.01$). Therefore, two phases were distinguished in the gonadal cycle of *R. philippinarum*: resting phase in winter, and gametogenesis and spawning phase during summer.

Bivalve molluscs have been shown seasonal changes in biomass of soft tissue and its relation to the reproductive cycle reflects a complex interaction between external environmental factors and endogenous response (regulated by neuroendocrine system). Within the same species, periods of reserves mobilization may change between locations, once the stages of reproductive cycle and energy reserves may be influenced by exogenous factors. Lipids form part of the reserves and are also important component of bivalve oocytes. Invertebrate reproduction is energy intensive (Brooks et al., 2003). Their maximum levels occur in the pre-spawning period before summer. The accumulation of reserves, allocation of stored energy to somatic growth or to the germinal pathway, and importance of each biochemical response to the reproductive process play a special role in the strategies to environmental stresses. Temperature and food availability seems the main factors that can influence the clams' reproduction (Fernandez-Reiriz et al., 2007, Ojea et al., 2004). In the present study, clams were exposed to the same controlled temperature and there was no scarce of food resources, since the sediment affected by wastewater discharges were fulfilled of nutrients. However, wastewaters discharges

produced different effects at all levels examined within the same season on different sites due to contamination.

The Bay of Cádiz (SW, Spain) is an area with great ecological and economic relevance. Differences on biochemical responses between sites in the same season should be due the different wastewater treatments. Volume of wastewater discharged per day by each WWTP or pumping stations was different, based on the webpage of the agencies responsible for the services. However, volume along the seasons in the year of 2011 did not vary so much, because the rain in winter might be compensated by the increase of population and water discharge during the summer. Composition of the wastewater discharges changed, being the main stress for the organisms, even the volume kept around the same along the year.

To extrapolate the same behaviour for *R. philippinarum*, and considering P6 as a good control site, clams exposed to sediments sampled at P1 (Chiclana de la Frontera) did not show correlation between biomarker responses during winter. In summer, COX activity was negatively correlated with TLP ($p < 0.01$), which was positively correlated with Vtg-like proteins ($p < 0.01$). However, the energy required to support anti-inflammatory properties by endogenous or exogenous inputs of NSAIDS contaminants represents expenditure for bivalves, as corroborated by the negative correlation between COX activity and TLP. In summer, there was no significant increase of Vtg-like proteins levels compared with the control. The same happened at the Seine estuary, since Vtg-like protein levels in mussels from polluted sites were not significantly induced compared with the least-polluted site (Gagné et al., 2008 b). Vtg-like proteins are the precursor of the energy reserves for the embryos, composed by lipid phosphorylated proteins. The exposure of clams to sediment affected by wastewater discharges produced marked increase in energy reserves (lipids in the gonads) which was positively related to Vtg-like

proteins in summer. In winter, Vtg-like proteins were significantly lower than the control ($p < 0.05$) and MAO activity was significant higher than the control ($p < 0.05$). Previous study showed mussels exposed to urban contaminants produced marked increase in energy reserves and lipids in the gonads (Gagné et al., 2001). Wastewater discharges contained high amounts of estrogen compounds (Hernando et al, 2006, Metcalfe et al, 2003, Ternes et al., 2002, Andreozzi et al., 2002, Farré et al., 2001) capable of interacting with mollusc estradiol-binding sites.

The most contaminated site was P2 which presented a modified granulometric distribution and under influence of uncontrolled discharges (Carrasco et al., 2003). Lara-Martín et al. (2008) measured various organic compounds including linear alkylbenzene sulfonates (LAS), nonylphenol polyethoxylates (NPEOs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorinated pesticides in sediments of the Bay of Cádiz. In general, the highest concentrations of these organic compounds were found in surface sediments near the untreated urban effluents (Lara-Martín et al., 2008). Variations on trace metal concentrations (Pb, Zn, Cd, Cu) were observed with the distance from the urban and industrial effluents at the Bay of Cádiz (Ponce et al., 2000). Diversity and abundance of xenobiotics (e.g. xenoestrogens) in aquatic environments suggests significant potential for adverse effects on reproduction (Doyle et al., 2013). MAO activity and TLP were significantly higher than the control in winter ($p < 0.05$). In the summer season, sediments from P2 showed significantly lower dopamine levels and COX activity compared with the control ($p < 0.05$).Reduced levels of dopamine in clams exposed to P2 during summer might represent loss of susceptibility and an adaptive response to oxidative stress, as previously reported for mussels exposed to municipal effluents (Gagné et al., 2007 a).

Positive correlation was observed between TLP and dopamine levels in clams exposed to sediment sampled at P3 (Cádiz) in winter ($p < 0.05$). In this season, clams seemed to reserve lipids and to produce Vtg-like proteins. However, there was no significant correlation between biomarker responses in clams exposed in summer. Energy reserves seemed to be more associated towards MAO and COX enzymatic activities.

Previous study with some sites in common with the present study classified the Bay of Cádiz as moderated contaminated (Carrasco et al., 2003). A station located near P4 showed high faecal coliforms values, which in conjunction with physical–chemical analyses, confirmed the existence of domestic and industrial uncontrolled discharges in this area (Carrasco et al., 2003). Concerning two points located at the city of El Puerto de Santa Maria (P4 and P5), clams exposed in winter to sediment collected at P4 showed positive correlation between COX activity and dopamine levels ($p < 0.01$), which dopamine was positively correlated with MET activity and Vtg-like proteins ($p < 0.01$). Energy required to support vitellogenesis and by endogenous or exogenous inputs of estrogenic contaminants represents another major expenditure for bivalves, thus contributing to the increase in energy demands (Smolders et al., 2004), as corroborated by the correlation between Vtg-like proteins and MET activity. Previous study on the scallop *Argopecten purpuratus* during vitellogenesis showed the increase of dopamine and serotonin levels, and COX enzymatic activity for final gamete maturation, fertilization and spawning steps (Martinez and Rivera, 1994). However, COX activity decreased in clams exposed during summer, MAO increased, but dopamine levels did not increase which means no spawning.

In winter, COX activity was positively correlated with Vtg-like proteins ($p < 0.05$), which was negatively correlated with MET activity ($p < 0.05$) in clams exposed to sediment sampled at P5. A secondary role of Vtg-like proteins might be involved in the

immune response. Gagné et al (2011) observed a significant correlation between Vtg-like proteins and the phagocytic efficiency index, involved in the immunocompetence of mussels exposed to municipal effluents. Vtg-like proteins have been associated with infection-resistant response which plays an integrative function in regulating immunity via its pleiotropic effects on both recognizing pathogen-associated molecular patterns and promoting macrophage phagocytosis in fish (Li et al., 2008).

MAO activity was negatively correlated with Vtg-like proteins ($p < 0.01$) in clams exposed to sediment from P5 in summer. MAO activity significantly increased in clams exposed to P1 and P2 in winter, and P3 and P4 in summer ($p < 0.05$). Previous study showed that MAO activity was induced in caged mussels exposed to urban effluent plume (Gagné and Blaise, 2003). The main role of MAO activity is the oxidative catabolism of important amine neurotransmitters, including serotonin, dopamine and adrenaline. Monoamines (e.g., serotonin and dopamine) are important mediators of gamete maturation and spawning (Gagné et al., 2007 b). Selective serotonin reuptake inhibitor (SSRIs), as anti-depressive drugs, works as MAO inhibitors. The increase of MAO activity can be due progesterone products, therefore, reduce dopamine levels, increase free radicals and oxidative stress (Edmondson et al., 2009), and it is associated to oocyte maturation (Brooks et al., 2003).

Indeed, the correlation between dopamine levels and Vtg-like proteins in winter suggests gametogenesis where a neuroendocrine interaction between signalling and dopamine metabolism in clams exposed to sediment affected by WWTP effluents (P2 and P4). At the end of gametogenesis, dopamine levels drops and serotonin increases to finalize gamete maturation, to initiate egg release as with COX activity (Gagné et al., 2011). However, COX activity significantly decreased in summer for clams exposed to all stations compared with the control ($p < 0.05$). Estrogenic signalling may keep the clams

in gametogenesis stage rather than spawning (Matozzo et al., 2008). This could be due the fact that municipal effluent contaminants such as estrogenic compounds and non-steroidal anti-inflammatory drugs (NSAIDs) present in wastewater discharges could inhibit COX activity in summer, which could result in negative impacts on spawning. Previous studies showed the presence of NSAIDs in wastewater discharges (Hernando et al., 2006, Scheytt et al., 2005, Loffler and Ternes, 2003). The class of compounds detected at highest concentrations in sediments sampled in Ebro River were analgesics/anti-inflammatory drugs followed by β-agonists and some antibiotics, which ibuprofen was the most abundant pharmaceutical product detected (Da Silva et al., 2011).

5. CONCLUSION

This study underscores the contamination of the Bay of Cádiz by anthropic activities and the impact of wastewater discharges, responsible for effects related to reproduction and health status of the biota exposed. Such effects are associated to oxidative metabolism, stress endpoints and the reproductive stage, and could culminate in long-term changes affecting the population. Wastewater discharges composition changed between different seasons, as the biochemical responses, mainly leading to oxidative stress and immunotoxicity (COX activity). Observed effects are consistent with the occurrence of pharmaceutical products. The exposure to municipal effluents seems to keep clams in a gametogenesis stage and delayed spawning based on the presented results. Further investigation should encompass the spawning activity in clams exposed to municipal effluent and to determine whether this process is disrupted as well. Thus, the continuous release of wastewater discharges adequately threated or not in aquatic ecosystems poses a health status risk to the local biota.

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CAPÍTULO 5

Evaluación de la calidad ambiental de sedimentos marinos afectados por vertidos de aguas residuales en la Bahía de Cádiz: ensayos de toxicidad crónicos *in situ*. Efecto de la estacionalidad.

Los datos ecotoxicológicos obtenidos en los estudios de laboratorio son a menudo difíciles de traducir a predicciones precisas de los posibles efectos adversos en campo. Dado que pueden ocurrir tanto sobreestimación o subestimación de los efectos, los resultados de laboratorio son mejor validados por la investigación de campo (Van der Oost et al., 2003). El trasplante de organismos para evaluar la calidad ambiental del sedimento evita tales inconvenientes y permite la integración de la evaluación de campo con la de laboratorio. Estudios previos incluyen el análisis de biomarcadores en distintos organismos enjaulados como ostras (Maranho et al., 2012), almejas (Martín-Díaz et al., 2005, 2007), mejillones (Gagné et al., 2007, 2011), cangrejos (Martín-Díaz et al., 2007) y poliquetas (Ramos-Gómez et al., 2008).

A pesar de muchos estudios sobre el efecto tóxico de vertidos domésticos e industriales, los productos químicos agrícolas entre otros compuestos xenobióticos relacionados con los efectos nocivos sobre los organismos acuáticos, poco se sabe sobre el efecto subletal combinado de esos productos químicos en el campo (O'neill et al., 2004). Estudios *in situ* utilizando el trasplante de organismos desde las zonas no contaminadas a zonas sospechosas de contaminación han sido ampliamente aplicados (Odzak et al., 2000, Bainy et al., 2000, Pereira et al., 2007, 2008). Este procedimiento

puede proporcionar información sobre la acción y la bioacumulación de contaminantes en condiciones naturales, la promoción de los niveles de control típicos en experimentos de laboratorio, pero con el realismo de los estudios de campo (Romeo et al. 2003, Kalpaxis et al. 2004).

En este contexto, los bivalvos han adquirido importancia mundial como bioindicadores, y se han empleado en los programas de monitoreo de aguas costeras, como lagunas (Lafontaine et al., 2000), estuarios (Nasci et al., 2002), y agua dulce (Gagné et al., 2007). Los bivalvos poseen distribución geográfica amplia, disponibilidad directa través de la acuicultura, y uso efectivo en los experimentos de introducción de jaulas a lo largo de las líneas costeras (Cajaraville et al., 2000).

Este capítulo trata de la exposición de almejas *Ruditapes philippinarum* a los seis puntos de muestreo de sedimento (P1 – P6) anteriormente citados en el capítulo 4. Jaulas hechas con PVC fueran ancladas con la ayuda de un buzo, y permanecerían en estos distintos puntos por 14 días (Figura 19). Cada jaula contenía 30 almejas, y fueran expuestas en duplicado. La exposición *in situ* ocurrió en inverno y verano de 2011, al mismo tiempo que se transcurría el bioensayo con las almejas en laboratorio.

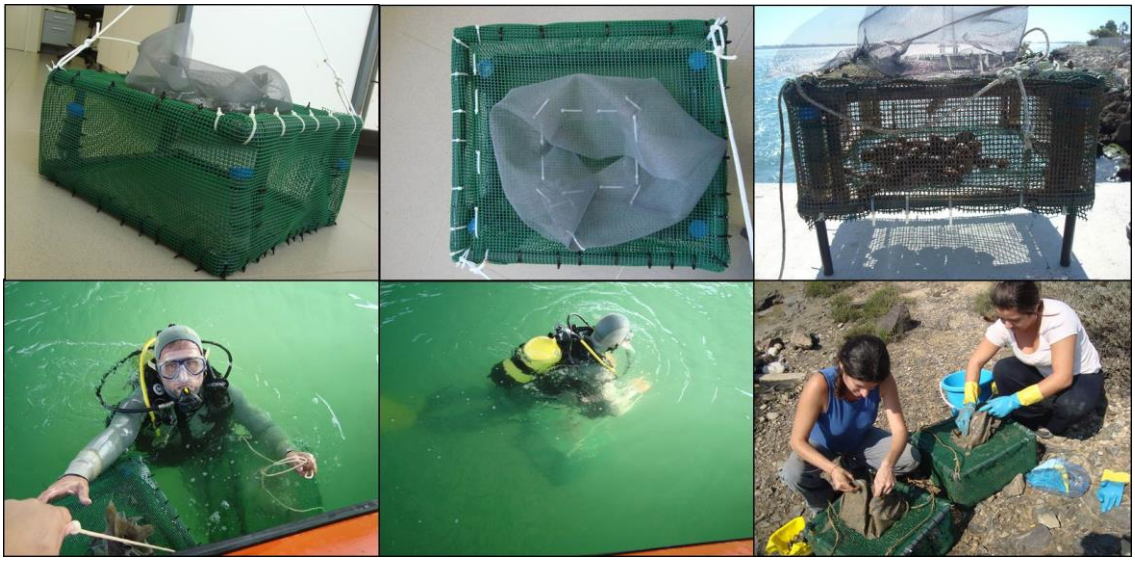

Figura 19. Jaulas utilizadas para la exposición de *R. philippinarum* y su instalación con la ayuda de un buzo.

Una vez en laboratorio, NRRT y los distintos biomarcadores (los mismos determinados en las almejas expuestas en laboratorio) fueran determinados en hemolinfa, glándula digestiva y gónadas. A través del analice multivariada fue posible relacionar las distintas respuestas biológicas con niveles de contaminación del sedimento por metales, PAHs, fármacos y surfactantes.

El principal objetivo de este capítulo fue la validación de los resultados obtenidos en laboratorio en campo, para certificar que los efectos adversos causados en las almejas expuestas en laboratorio serian lo mismo que en campo. Pero se puedo verificar que los efectos en campo son muy distintos de los efectos en laboratorio, ya que en campo los animales estaban en constante contacto con el sedimento contaminado, pero también con el constante aporte de aguas residuales y otros interferentes ambientales. El artículo IX trae con más detalles la metodología y los resultados de la evaluación en campo.

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In preparation

Seasonal variation of multi-biomarker responses in caged clams *Ruditapes philippinarum*.

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ABSTRACT

Sediment quality was determined by using *in situ* caged clams *Ruditapes philippinarum* taking into account the seasonality. Clams were caged in sediment directly affected by wastewater discharges at four sites (P1, P2, P3, P4) in the Bay of Cádiz (SW, Spain), and one control site (P6). Exposure to contaminated sediments was confirmed by measurement of trace metals, PAHs, pharmaceutical products and surfactants (SAS) in bottom sediments. Biological effects were determined by following biomarkers of early effects (enzymatic activities of 7-ethoxyresorufin O-deethylase - EROD, dibenzylfluorescein dealkylase - DBF, glutathione S-transferase - GST, glutathione peroxidase - GPX, glutathione reductase - GR and acetylcholinesterase - AChE), damage (lysosomal membrane stability - LMS, DNA damage and lipid peroxidation - LPO), energy status (total lipids - TLP and mitochondrial electron transport - MET), and involved in the mode of action of pharmaceutical products (monoamine oxidase activity - MAO, vitellogenin-like proteins - Vtg-like proteins and cyclooxygenase activity - COX). Results revealed that levels of sediment-bound PAHs, pharmaceutical products and SAS were higher in summer than winter season. In winter, urban effluents were detoxified by phase I biotransformation (CYP3A-like activity), phase II (GST), and the activation of antioxidant defence enzymes (GR). Urban effluents lead to the detoxification metabolism (CYP1a-like), oxidative effects (LPO and DNA damage), neurotoxicity (AChE) and neuroendocrine disruption (COX and Vtg-like proteins) involved in inflammation (P1 and P2) and changes in reproduction as spawning delay (P3 and P4) in clams exposed in summer. Adverse effects on biota exposed to sediment directly affected by wastewater discharges depend on the chemical contamination level and also on the reproductive cycle according to seasonality.

Keywords: *Ruditapes philippinarum,* caged system, wastewater discharges, biochemical responses, lysosomal membrane stability, biomonitoring, seasonality, marine sediment.

1. INTRODUCTION

Wastewater treatment plants (WWTPs) must treat a wide variety of natural and industrial products since they receive wastewater from both domestic and industrial sources. Domestic wastewater discharges are frequently observed at the Spanish coast. According to the European Directive 92/271/EEC, Spain should have collecting systems and secondary treatments (or equivalent process) in towns with more than 10,000 inhabitants. The population around the Bay of Cádiz (SW, Spain) comprises approximately 460,000 inhabitants, which increases by 30% during the summer months (INE, 2011). The volume of wastewater effluents discharged daily in the Bay of Cádiz does not change along the year, however, the composition vary widely. Summer is a dry season in this area, and the main driver of contamination comes from the touristic population. Winter is a rainy season, which brings about water overflows, the ressuspension of sediments, and the increase of drainage from the other cities. Hence, municipal effluents from the cities located around the Bay of Cádiz are considered important sources of contamination for the local marine and estuarine environments by industrial, domestic and agriculture residues.

Biomonitoring is an important tool for understanding the linkages between chemicals exposures and the potential health outcomes in contaminated environments. The sediment compartment is a well-known sink and long-term source of xenobiotics. Previous studies reported the concentrations of different xenobiotics in this Bay, including pharmaceutical and personal care products (Pintado-Herrera et al., 2013), trace metals (Ramos-Gómez et al., 2011, 2008, Moralles-Caselles et al., 2008, Martín-Díaz et al., 2008a, Riba et al., 2004), surfactants (Lara-Martín et al., 2006, González-Mazo et al., 1999), nutrients (Ribas-Ribas et al., 2013), PAHs (Ramos-Gomez et al., 2011, 2008, Martín-Díaz et al., 2008a, Riba et al., 2004) and PCBs (Martín-Díaz et al., 2008a, Riba et al., 2004). However, contaminants can be bioavailable in this Bay, as reported by previous study about caged organisms such as

polychaetes *Arenicola marina* (Ramos-Gómez et al., 2008). Through a number of studies published on wastewater ecotoxicology, the chemical characterization of the released discharges and its ecotoxicological effects *in situ* are generally less understood (Farcy et al., 2011).

In situ exposure approaches, such as clams caging, allow the determination of both exposure and effects in the natural environment (Farcy et al., 2011). Within the Water Framework Directive, there are regulatory arguments in favour of using biota for assessing contamination trends (Gust et al., 2014), highlighting the relevance of caged invertebrates (Besse et al., 2012).

The real consequences of sediment contamination on biota can be determined by the measurement of biomarkers (Ramos-Gómez et al., 2008). Biomarkers are suitable tools recommended for environmental international agencies such as UNEP, OSPAR, OECD, ICES and IOC guidelines applied around the world (Cajaraville et al., 2000, Solé et al., 2009). In addition to the relevance of the studied biological responses, as potential biomarkers of environmental stress, the influence of external variables such as temperature and seasonal changes needs to be understood for biomonitoring application (Guerlet et al., 2007).

Several endpoints can be assessed in field studies. Gagné and Blaise (2003) proposed two types of biomarkers to measure PPCPs effects in field studies. The first group are biomarkers encompassing the effects of drugs with no assumption of their mechanism of action and specificity. This group can be subdivided in two subgroups: biomarkers of early effects and biomarkers of damage. Biomarkers of early effects involve the metabolism of xenobiotics (phases I and II), antioxidant enzymes and neurotoxicity (AChE activity). Phase I is composed by detoxification enzymatic activities: ethoxyresorufin *O*-deethylase (EROD) and dibenzylfluorescein dealkylase (DBF). Phase II is based on the conjugation of xenobiotics by glutathione S-transferase (GST) enzymatic activity. Glutathione peroxidase (GPX) and

glutathione reductase (GR) are part of the antioxidant system. Acetylcholinesterase activity (AChE) represents the neuronal effect. Biomarkers of damage are related to the oxidative effects, consequence of free radicals production, as lysosomal membrane stability (LMS), lipid peroxidation (LPO) and to some extent DNA damage (*strand breaks*). LMS is considered a suitable tool to analyse the health status of populations in different environmental compartments, including the exposure to sediment samples (Buratti et al., 2012). Other endpoints are related to the energy status of the organism, measured as total lipids content (TLP) and mitochondrial electron transport (MET).The second group of biochemical responses involves the mode of action (MOA) of different drugs, with possible profound effects on an organism´s survival, immune function and reproduction: monoamine oxidase activity (MAO) (for MAO inhibitor drugs as fluoxetine), cyclooxygenase activity (COX) (for COX inhibitors drugs as ibuprofen), vitellogenin-like proteins levels (Vtg-like proteins) (for estrogenic compounds).

In the present study, a suite of biomarkers of exposure, effects and damage were evaluated in the clams *Ruditapes philippinarum*, proposed as sentinel species to monitor the environmental quality of the Southern Iberian Peninsula (Cajaraville et al., 2000, Blasco and Puppo, 1999, Bebianno et al., 1993, 1994). This bioindicator has been extensively used in biomonitoring studies (Morales-Caselles et al., 2008, Moschino et al., 2011) and sublethal measurements (Buratti et al., 2012, 2010, Coughlan et al., 2009, Martín-Díaz et al., 2008a, 2007, 2005). As mussels, clams are at high risk of exposure to such contamination because these benthic bivalves are sedentary, they live at the sediment/ water interface and they are filter feeders, including suspended materials. Manila clams (*Ruditapes philippinarum*) are species of subtropical to boreal low-latitude western Pacific and introduced in the Atlantic, being well distributed in temperate Europe (Goulletquer, 2005). This specie has considerable

commercial value. Aquaculture production is mainly located in Ireland, Portugal, Spain and Italy (Goulletquer, 2005).

The main aim of the present study was to assess the suitability of biochemical and cellular tools applied in clam's *R. philippinarum* to evaluate the adverse effects of WWTPs on the benthic biota, taking into consideration two distinct seasons: winter and summer. Sediment quality of the Bay of Cádiz was evaluated, including the traditional approach which involved chemical analysis and the ecotoxicological effects of sediment contamination. This purpose has been carried out by means of an *in situ* approach of 14-days toxicity test.

2. MATERIAL AND METHODS

2.1. Test species

Adults of *R. philippinarum* were obtained from an aquaculture farm. Specimens were acclimatized for one week under laboratory conditions, before the exposure for 14-days to five stations located at the Bay of Cádiz (SW, Spain) in winter and summer seasons. In winter, the cage systems located in P3 and P4 (Figure 1) disappeared and it was not possible to recover the animals from the cages. Spawning period of *R. philippinarum* occurs in summer (Kanazawa and Sato, 2007) which can interfere in some of the biomarkers responses, and also be influenced by contamination (Blaise et al., 2002).

2.2. Exposure sites

The Bay of Cádiz is characterized by salt-marsh areas and watershed covering about 36.6 km^2, and average depth of 4 m (Forja et al., 1994), which drains a dense urban area in the Southern of Spain. The main industries located in this zone are related with ship, offshore, car and aerospace manufacturing. Agriculture and tourism activities are also important socio-economic activities. The WWTPs treats the wastewaters from 460,000 inhabitants, which

increase approximately 30% in the summer period (INE, 2011). Exposure of caged clams was performed on five stations located at the Bay of Cádiz. Four stations (P1 – P4) were chosen where the WWTPs effluent discharges occur, comprising the cities of Chiclana de la Frontera - P1, Puerto Real - P2, Cádiz - P3 and El Puerto de Santa María - P4. P6 located in Rota was considered the control site (Figure 1).

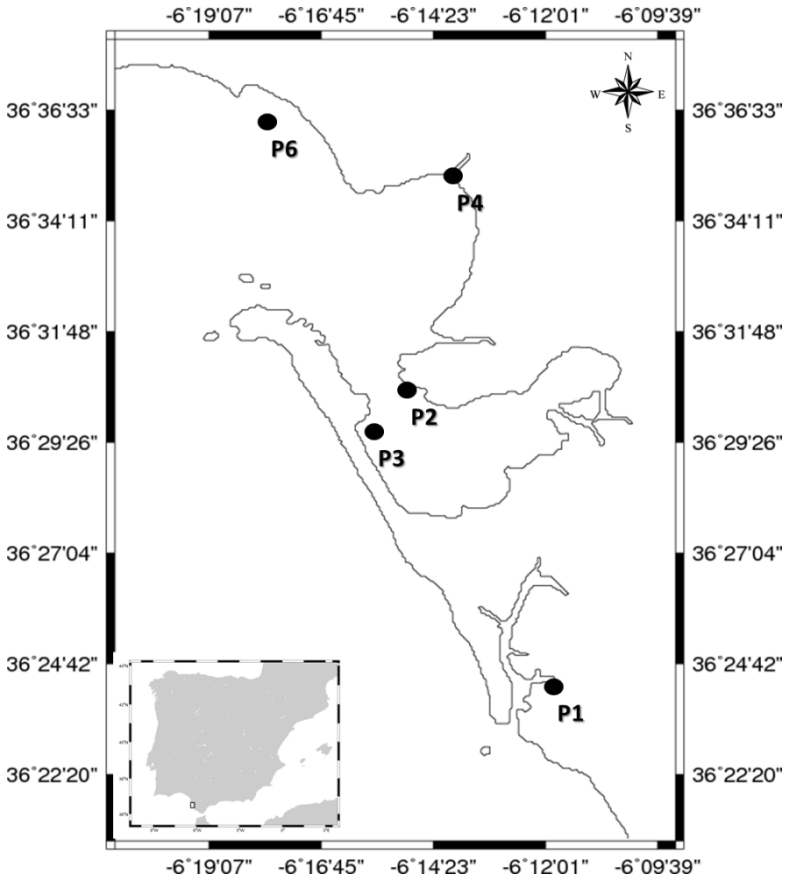

Figure 1. Geographic locations of sampling regions across the Bay of Cádiz (SW, Spain). Four exposure sites were selected, directly affected by wastewater discharges: Chiclana de la Frontera (P1), Puerto Real (P2), Cádiz (P3), El Puerto de Santa Maria (P4), and the control site in Rota (P6). Scale: 1:250.000.

2.3. Experimental clams' exposure

The cage skeleton was previously described by Ramos-Gómez et al. (2008), which was built using PVC tubes. The box cages consisted of four edges (25 cm) to compose the square base, four edges (25 cm) to compose the square top and four more edges (15 cm) to

join the vertexes of the base and the top. The structure was covered with a plastic netting (5 mm for the outer layer). Cages were provided with four PVC legs (± 10 cm of tubes) to be anchored to the bottom sediment, and also with an anchor line. The cage design allows the water to flow through the cage system. The cage system was placed in the stations with the scuba dive help. The cages remained anchored to the bottom sediment and enough sediment was soaked in for the clams to burry. Duplicate were used containing 28 animals each cage. After 14 days of exposure, cages were retrieved and the organisms were immediately placed in a portable fridge. Once in the laboratory, clams were kept in filtered seawater aquariums under controlled laboratory conditions for the depuration during overnight. After the depuration, organisms were frozen in a -80^{o}C freezer.

2.4. Sediment characterization

Surface sediment samples (10 cm upper layer) were collected at the five exposure sites with a 0.025 m^2 Van Veen grab, or with the scuba dive help when it was necessary. Samples were brought to the laboratory and subsampled for physical–chemical characterization. For sediment grain size, an aliquot of dry sediment was analysed by following the methodology recommended by USGS (2013). Total organic carbon (TOC) and organic matter (OM) content were determined applying the methods reported by USEPA (2002).

Trace metals content (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) in the different sediment samples was determined following methods of inductively coupled plasma optical emission spectrometry (ICP-OES). For trace metal analyses, aqua regia extraction was used (ISO11466, 1995). LECO AMA 254 analyser was used to determine Hg concentrations. Results were checked using MESS-1 NRC reference material. PAHs content was analysed according to USEPA SW-846 method 8270D (2007).

Selected pharmaceuticals and secondary alkane sulfonates (SAS), an anionic surfactant used in dishwashing and other cleaning products, were measured following methods proposed by Jelic et al. (2009) and Baena-Nogueras et al. (2013). Briefly, 2 grams of sediment were place inside 11 mL stainless steel cells and extracted by pressurized liquid extraction using a ASE 200 unit (Dionex) and methanol/water 1/2 (v/v) (for pharmaceuticals) or methanol/dichloromethane 1/1 (v/v) (for SAS) as solvent. After extraction, samples were evaporated and reconstituted in methanol/water 25/75 (v/v) and internal standards (carbamazepine d10, atenolol d7, naproxen d3, C_{12}SAS) were added at 50 ng mL^{-1}. Determination of target compounds was achieved by ultra-performance liquid chromatography (UPLC) – tandem mass spectrometry (MS/MS) on a Bruker EvoQ Elite equipped with electrospray interface (ESI) coupled to a UPLC Bruker Advance. Separation of target compounds was carried out using a Bruker Intensity Trio C18 column (100x2 mm, 1.9 µm internal diameters) and methanol (A) and water (B) as solvents. The gradient was as follows (flow = 0.4 mL min^{-1}): 5% B for 0.4 min, increased linearly to 30% in 0.1 min, increased linearly to 95% in 4.5 min, and then kept for 3 min. Source parameters were: spray voltage 4500 V for ESI+ and 3000 V for ESI-, cone temperature 250 ºC, cone gas flow 20 mL min^{-1}, probe temperature 450 ºC, probe gas flow 50 mL min^{-1} and nebulizer gas flow 60 mL min^{-1}. Recoveries were between 60 and 100% for most target compounds, and detection limits under 0.1 ng g^{-1}.

2.5. Lysosomal membrane stability

Neutral Red Retention Time (NRRT) assay was carried out following the method described by Lowe et al. (1995). This method is considered non-destructive, employed haemolymph withdrawn from the posterior adductor muscle of living bivalves. The clam's haemolymph was mixed to physiological saline solution (pH 7.3 containing 4.77 $g \cdot L^{-1}$

HEPES, 25.48 $g \cdot L^{-1}$ NaCl, 13.06 $g \cdot L^{-1}$ $MgSO_4$, 0.75 $g \cdot L^{-1}$ KCl, 1.47 $g \cdot L^{-1}$ $CaCl_2$). Haemolymph was spread on slides and transferred to a lightproof and humid chamber, where it remained for 15 min to allow cells attachment. Excess liquid was removed, Neutral Red (NR) dye was added to the cell monolayer and a cover slip was placed. After 15 min incubation period, slides were examined every 15 min by optical microscopy (400x) for NR dye loss from the lysosomes to the cytosol. The endpoint was the time when at least 50% of the examined cells exhibited these characteristics.

2.6. Tissues preparation

Gonads and digestive glands from each individual were excised, homogenized (homogenized fraction - HF) and centrifuged. The following fractions were obtained: S_3 (3,000g for 20 min at 4°C) and S_{15} (15,000g for 20 min at 4°C). All the biomarker responses were normalized by the total proteins content of each extract corresponding to digestive gland or gonads tissues. Biomarkers determined in digestive gland were named as "set of biomarkers I", while the biomarkers determined in gonads were named as "set of biomarkers II". Total protein content (mg) was determined to each extract according to Bradford method (1976), using serum bovine for calibration. All the biochemical responses were normalized by the total protein content according to the tissue and extract.

2.7. Biochemical responses determined in digestive gland tissue: set of biomarkers I

2.7.1. Detoxification metabolism

Mixed function oxidase activity was measured using the adapted methodology for ethoxyresorufin O-deethylase (EROD) and dibenzylfluorescein dealkylase (DBF) activities (Gagné and Blaise, 1993, Gagné et al., 2007a). In dark microplates, duplicates of S_{15} were added to 160 µM of 7-ethoxyresorufin and 10 µM reduced NADPH in 100 mM KH_2PO_4

buffer (pH 7.4). Fluorescent determination of 7-hydroxyresorufin was carried out using a standard calibration curve of resorufin (5 mM). The reaction was initiated by the addition of 10 µl of 20 mM NADPH using a multichannel pipette and stopped by the addition of 100 µl of 0.1 M NaOH. The 7- hydroxyresorufin was determined at 0, 15, 30, 45 and 60 min at 30^{o}C by a fluorimeter using 485 nm (excitation) and 580 nm (emission) filters. Results were expressed as nmol /min /mg proteins.

Dibenzylfluorescein dealkylase activity (DBF) was determined in dark microplates, which duplicates of S_{15} were added to the substrate dibenzylfluorescein 50 µM, and incubated with a solution of 100 µM NADPH in a test solution (125 mM NaCl contained 10 mM HEPES–NaOH, pH 7.4). Fluorescence was measured at 485 nm (excitation) and 516 nm (emission). Results were expressed as nmol /min /mg proteins.

2.7.2. Conjugation metabolism

Glutathione S-transferase activity (GST) was determined by the application of the methodology adapted from McFarland et al. (1999) protocol. In transparent microplate, S_{15} was added to 200 µL of 1mM GSH and 1mM 1-chloro-2.4-dinitrobenzene in a buffer of 10mM Hepes-NaOH (pH 6.5) containing 125 mM NaCl. The rate of reaction was measured by absorbance spectrophotometer at 340 nm at every 5 min for 30 min. Results were expressed as OD /min /mg proteins.

2.7.3. Antioxidant system

Glutathione peroxidase activity (GPX) was determined following the methodology proposed by McFarland et al. (1999). S_{15} samples were mixed with 200 µl of the daily assay mixture (3mM GSH and 1mM NADPH with 67 units of glutathione reductase in 200 mM KH_2PO_4 buffer at pH 7, kept at 30 ^{o}C) and placed in the transparent microplates. In addition, a

fresh substrate of 1 mM cumene hydroperoxide was prepared, added to the microplate and incubated for 2 min at 30°C. Readings were taken at each 41 sec for 3 min. GPX activity measures the decrease in absorbance at 340 nm resulting from the consumption of NADPH by the GR reaction. Results were expressed as pmol /min /mg proteins.

Glutathione reductase activity (GR) was determined following the methodology proposed by McFarland et al. (1999). GR assay buffer contains 200 mM monosodium phosphate and 200 mM disodium phosphate (pH 7.6). In addition, two daily assay mixtures were prepared containing 10 mM oxidized glutathione (solution A) and NADPH (solution B), both dissolved in GR buffer. These solutions were mixed with GR buffer and heated at 30°C. S_{15} sample was run in duplicate in transparent microplates to which the final daily assay mixture was added. Decrease in NADPH absorbance was measured at 340 nm during the oxidation of NADPH to NADP. GR activities were measured in a spectrophotometer every 2 min for 10 min. Results were expressed as pmol /min /mg proteins.

2.7.4. Neurotoxicity

Acetylcholinesterase enzymatic activity (AChE) was determined using the method described by Ellman et al. (1961) and adapted by Guilhermino et al. (1996). Three mixture solutions were prepared: (1) 10 mM K_2HPO_4 and 10 mM KH_2PO_4, (2) 0.75 mM acetylthiocholine iodide, (3) sodium bicarbonate and 10 mM 5-5´dithiobis (2-nitrobenzoic acid) (DTNB). Daily assay mixture was the addition of these three solutions. 166.6 µl of this mixture was added to each replicate of S_3 in the transparent microplates. AChE enzymatic activity was measured in a spectrophotometer at 412 nm every 5 min for 20 min. Results were expressed as nmol DTNB/min /mg proteins.

2.7.5. Oxidative effects

Lipid peroxidation (LPO) was assessed by the method described by Wills (1987). 10% trichloroacetic acid (TCA), 1 mM $FeSO_4$ and 0.67% thiobarbituric acid solution (TBAR) were added to the sample, and incubated in a water bath at 70^{o}C for 10 min. Lastly, this mixture was added to each replicate of HF sample in the dark microplates. Standard solutions were made in different concentrations (i.e. 0, 0.6, 1.5, 3, 4, 6, 10 mM of 0.001% of tetramethoxypropane - TMP). Measurements were done in a spectrophotometer, with the absorbance at 540 nm in order to set the standard curve of TMP. The data were expressed as µM TBARS /mg proteins.

DNA damage was assessed by the alkaline precipitation assay (Olive, 1988) based on the K-SDS precipitation of DNA–protein crosslinks and using fluorescence to quantify the DNA *strand breaks* (Gagné et al., 1995). HF sample was mixed with 200 µl of 2% SDS containing 10 mM EDTA, 10 mM Tris-base and 40 mM NaOH. After mixing by inversion, the solution was stocked at room temperature for 1 min, 200 µL of 0.12 M KCl was added and the solution heated to 60^{o}C for 10 min. The solution was incubated at 4^{o}C for 30 min to precipitate the genomic DNA bound to SDS associated with nucleoproteins. This mixture was centrifuged at 8,000g for 5 min at 4^{o}C. Supernatant fraction was added to 150 µl of Hoechst dye at a concentration of 100 nM (diluted with 100 mM Tris–HCl, containing 400 mM NaCl and 4 mM sodium cholate, pH 8.5), in the dark microplate. Salmon sperm DNA standards were used for the calibration curve, with different concentrations (i.e., 0, 0.91, 2.27, 4.55, 9.09, 27.30 and 45.55 µg·ml^{-1}). Fluorescence readings were taken at 360 nm (excitation) and 450 nm (emission). The data were expressed as µg DNA strands/mg proteins.

2.8. Biochemical responses determined in gonad tissue: set of biomarkers II

2.8.1. Endocrine status of the gametogenesis

Monoamine oxidase activity (MAO) was determined using the serotonin analogue tryptamine as the substrate (Gagné et al, 2007b). The HF was incubated in a solution of 10 µM dichlorofluorescein, 1 mM tryptamine in 10 mM Hepes-NaOH (pH 7.4), containing 140 mM NaCl, 10 mM aminotriazole and 0.1 $\mu g \cdot ml^{-1}$ of horseradish peroxidase for 0, 15, 30 and 60 min at 30^{o}C. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission). The data were expressed as nmol RFU/ min/ mg proteins.

Vtg-like proteins were determined in HF applying alkali-labile phosphate method described by Gagné et al. (2003). Proteins were precipitated by acetone (35% v/v) and then, centrifuged at 10,000 for 5 min at 4^{o}C. The pellet was dissolved in 200 µl of 1 mM NaOH at 60^{o}C for 30 min. The levels of inorganic phosphates were determined using the phosphomolybdenum method (Stanton, 1968). Rainbow trout vitellogenin was used for the standard calibration curve. Absorbance was measured at 444 nm (some cases with interferences in the colour, the reading was taken at 815 nm). Vtg-like proteins data were expressed as µg Phosphate/ mg proteins

Cyclooxygenase activity (COX) was tracked by measuring the oxidation of 2, 7 – dichlorofluorescein substrate in the presence of arachidonate in S_{15} fractions (Fujimoto et al., 2005) incubated in a assay mixture containing 50 µM arachidonate, 2 µM dichlorofluorescein and 0.1 $\mu g \cdot ml^{-1}$ horseradish peroxidase, in 50 mM Tris-HCl buffer (pH 8) containing 0.05% Tween-20. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). The data were expressed as RFU/ min/ mg proteins.

2.8.2. Energy status

Total lipids (TLP) were determined in HF according to the phosphovanillin method (Frings et al., 1972). Samples were incubated for 10 min at 80^{o}C in the presence of H_2SO_4 and phosphovanilin. Absorbance was measured at 540 nm. Standard solutions of olive oil were used for calibration. The data were expressed as µg total lipids/ mg proteins.

Mitochondrial electron transport (MET) activity was determined according to the reduction of p-iodonitrotetrazolium dye method (Smolders et al., 2004, King and Packard, 1975). The S_3 fractions were mixed with a buffer composed by 0.1 M Tris–HCl containing 0.1 mM $MgSO_4$, 0.1% Triton X-100 and 5% polyvinylpyrrolidone (pH 8.5) for 1 min, and later 1 mM NADH, 0.2 mM NAPDH and 1 mM p-iodonitrotetrazolium were added. Absorbance readings were taken at 520 nm each 5 min for 30 min. The data were expressed as A520 nm/ min/ mg proteins.

2.9. Statistical analyses

The biomarker responses were analyzed using the SPSS/PC 21.0 + statistical package. Significant differences between individuals exposed to control site and individuals exposed to sites affected by wastewater discharges, and between the same responses in different seasons, were determined using a two-way ANOVA followed by a multiple comparison of Dunett's t tests. Significance level was set at $p < 0.05$. Principal component analysis (PCA) was performed to determine major responses to sediment contaminant levels and thus linked sediment contamination and biochemical and LMS responses. For PCA analysis, different groups of PAHs were grouped in one, and pharmaceutical products were divided in different groups according to the human prescription. Only variables whose coefficient was ≥ 0.5 (Comrey's, 1973) were considered components of the factors. Significant correlations were examined by Spearman´s rank correlation analysis. Significance level was set up at $p < 0.05$.

3. RESULTS

3.1. Physical chemical characterization of the sediment samples

Total organic carbon (TOC), organic matter (OM), grain size, trace metals, PAHs and pharmaceutical products results are shown in the Table 1. Grain size distribution analysis indicated that, despite some variability in the Bay bottoms at the sites chosen for sampling, three overall groupings predominated, with essentially clayed silt sediments in P1, P2 and P4, medium sand in P3 and the predominance of sand with a fairly significant gravel and sand content in P6. Variability of OM between winter and summer seasons was observed in sediment sampled at P3 (3.14-fold higher in summer than winter) and P6 (1.64-fold higher in summer than winter). Also P3, TOC content was 0.61-fold higher in summer than winter.

Chemical characterization of the sediment samples indicated a number of contaminants containing trace metals, PAHs and pharmaceuticals. The highest trace metal concentration was found for Al, followed by Fe, Mn, Zn, Cr, Cu, Pb, Ni, As and Cd. Concentration of Se was below the ICP-OES detection limit. Of all the sites, the control site (P6) showed the lowest concentrations of trace metals, PAHs and total concentration of pharmaceutical products. To evaluate the sediment contamination and potential ecotoxicological effects associated with observed concentrations of contaminants, different published Sediment Quality Guidelines (SQGs) concerning trace metals have been used for comparison: USEPA (2004), CCME (1995), CEDEX (1994) and Riba et al. (2004). CEDEX (1994) and Riba et al. (2004) brings information about SQGs for the Spanish coast. Results of PAHs concentrations were compared with the CCME (1999). In the Table 1, contaminants that exceed any SQG were highlighted. The letter that follows the concentration indicates which SQG was surpassed.

Table 1. Sediment physical chemical characterization (n = 2) which includes the percentages of fines (% dry weight), total organic carbon (TOC) (% dry weight), organic matter (OM) (% dry weight) and concentrations of contaminants: trace metals ($mg \cdot kg^{-1}$), polycyclic aromatic hydrocarbons (PAHs) ($ng \cdot g^{-1}$), target pharmaceutical compounds ($ng \cdot g^{-1}$) and surfactants (SAS) ($ng \cdot g^{-1}$) in sediment sampled at five studied sites located at the Bay of Cádiz (SW, Spain) in winter and summer seasons: P1 - Chiclana de la Frontera, P2 – Puerto Real, P3 – Cádiz, P4– El Puerto de Santa María and P6 – Rota (control).

	Winter - Summer				
Parameters	**P1**	**P2**	**P3**	**P4**	**P6**
% Fines	49.1	64.9	65.7	40.5	68.4
% TOC	1.5 - 1.6	2.7 - 2.5	1.6 - 2.6	1.4 - 1.1	0.6 - 0.4
% OM	11.5 - 13.8	19.2 - 16.9	1.1 - 4.4	16.3 - 15.9	0.8 - 2.1
Hg	0.13 – 0.05	0.17 – 0.18 [a. b]	0.32 – 0.17 [a. b]	0.24 [a. b] – 0.09	0.01 – 0.03
Al	53494.66 – 57723.92	52632.41 – 48587.24	19069.62 – 8001.16	46548.53 – 49139.60	6874.08 – 13005.66
Fe	27367.32 – 28946.51	28236.89 – 27071.63	8392.90 – 6330.39	24927.43 – 27062.05	3234.01 – 8368.41
Mn	362.80 – 351.06	389.21 – 410.89	158.75 – 200.14	336.44 – 330.16	125.64 – 422.92
Cr	71.42 – 78.08 [a. b]	73.48 – 70.34 [a. b]	32.10 – 19.98	71.81 – 78.66 [a. b]	5.19 – 14.09
Cu	37.55 – 40.52 [a. b]	39.25 – 42.17 [a. b]	26.51 – 44.31 [a. b]	35.98 – 31.02 [a. b]	0 – 3.75
Ni	33.77 – 34.16	33.94 – 31.15	10.69 – 6.17	31.57 – 34.46	2.83 – 6.76
Zn	99.75 – 105.73	106.90 – 111.58	60.66 – 76.60	95.33 – 127.10 [a. b]	7.88 – 36.49
Pb	26.66 – 21.66	31.88 [a. b] – 30.20	25.70 – 34.38 [a. b]	23.37 – 18.40	5.45 - 14
Cd	0.34 – 0.39	0.28 – 0.26	1.32 – 1.11 [a. b. c. d]	0.57 – 0.43	0.59 – 0.69
As	7.88 – 6.68	8.46 – 7.34	4.50 – 2.98	5.20 – 4.32	5.82 – 6.56
Se	ND	ND	ND	ND	ND
Anthracene	<2.00	25.36 – 32.74	7.76 – 30.94	ND – 6.62	ND
Benzo [a] anthracene	6.63 – 7.45	244 - 281.4 [e]	66.5 – 102 [e]	19.21 – 18.26	ND
Benzo [a] pyrene	<2.00	359.3 - 399.3 [e]	59.86 - 76.76	10.94 - 9.34	ND
Benzo [b] fluoranthene	4.41 – <2.00	81.52 - 120.2	65.74 - 95.68	13.79 - 14.53	ND
Benzo [c] fluorene	<2.00	22.94 – 23.16	7.7 – 8.85	ND	ND
Benzo [g. h. i] perylene	4.6 – 4.31	393.4 – 516	51.62 – 67.08	9.71 – 12.29	ND
Benzo [j] fluoranthene	<2.00	41.58 – 51.88	30.98 – 46.04	6.94 – 6.74	ND
Benzo [k] fluoranthene	<2.00	27.98 – 33.32	30.94 – 44.58	6.62 – 6.03	ND
Dibenzo [a. h] anthracene	<2.00	132.2 – 164.92	15.27 -21.78	ND	ND
Fluoranthene	5.7 - <2.00	35.86 – 24.84	98.22 - 169	26.94 – 15.3	ND
Indene [1. 2. 3 – c.d] pyrene	<2.00	85.72 – 129.8	40.88 – 56.4	7.64 -9.16	ND
5-metylchrysene	<2.00	205 – 229.5	4.56 – 6.73	ND	ND
Acetaminophen	27.1 – 28.5	8.5 - ND	ND	15.9 - ND	2.2 – 7.5
Diclofenac	ND – 1.5	ND – 0.1	ND	0.5 – 0.2	ND

Fenoprofen	ND – 0.3	0.9 - ND	2.9 – 0.9	ND – 0.6	3.7 – 0.8
Atenolol	0.2 – 0.3	ND – 0.1	0.1 - ND	0.2 – 0.3	ND – 0.1
Propranolol	0.3 - 0.2	0.1 – 0.2	0.1 – 0.6	0.9 – 0.3	0.1 – 0.2
Clofibric acid	0.1 - 0.1	0.1 - 0.1	ND	ND	ND
Gemfibrozil	0.9 - 0.3	ND	ND	0.1 – 0.1	ND
Carbamazepine	0.2 - 0.2	ND	ND	0.9 - ND	0.1 - ND
Fluoxetine	0.1 - 0.6	ND – 0.1	ND	0.7 – 0.5	ND
Amitriptyline	0.2 - 0.2	0.1 – 0.1	ND – 0.2	0.4 – 0.3	ND
Caffeine	3.5 - 12.2	1.9 – 3.0	3.5 – 4.5	7.0 – 8.1	8.8 – 3.3
Chloramphenicol	0.1 - 1	4.3 - ND	0.4 – 0.1	0.1 – 0.2	0.6 - ND
Cefdinir	0.1 - 0.1	0.1 – 0.1	0.1 – 0.1	0.2 – 0.1	0.1 - ND
Tiamulin	ND	0.1 - ND	0.1 - ND	0.1 - ND	0.1 - ND
Erythromycin	0.4- 0.3	1.6 – 0.2	1.0 – 0.2	0.2 – 0.1	1.0 – 0.2
Clarithromycin	0.3 - 0.3	1.0 – 0.1	0.7 – 0.1	0.7 – 0.6	0.3 – 0.1
Azithromycin	0.2 - 0.2	0.4 – 0.1	0.3 - ND	0.3 – 0.3	0.1 – 0.1
Roxithromycin	0.2 - 0.2	0.6 – 0.1	0.6 – 0.1	0.9 – 0.2	0.4 – 0.5
Lincomycin	0.1 - ND	0.1 - ND	0.1 - ND	0.1 – 0.1	0.1 - ND
Clindamycin	0.1 - 0.1	0.1 – 0.1	0.2 – 0.1	0.1 – 0.1	0.6 – 0.1
Flumequine	0.8 - 0.3	7.1 – 0.4	6.7 – 0.2	0.4 – 0.3	2.9 – 0.4
Sparfloxacin	ND	1.0 - ND	0.1 - ND	ND	ND
Novobiocin	0.2 - 0.1	0.5 – 0.2	0.4 - ND	ND	0.5 - ND
Metronidazole	ND – 0.2	0.4 – 0.1	0.1 – 0.3	ND	0.4 – 0.1
Ornidazole	ND	2.3 – 0.1	2.0 - ND	0.1 - ND	1.2 – 0.1
Sulfadiazine	0.6 – 0.6	0.6 – 0.3	0.2 – 0.4	0.4 – 0.3	0.2 - ND
Sulfamethoxypyridazine	0.1 – 0.1	0.1 – 0.1	0.1 - ND	0.1 - ND	0.1 – 0.3
Sulfathiazole	0.3 – 0.1	0.1 – 0.2	0.2 – 0.2	0.8 – 0.2	0.2 - ND
Trimethoprim	ND	0.2 - ND	0.1 – 0.1	0.1 - ND	ND – 0.1
Monensin	ND	ND	ND	ND	ND
Famotidine	ND	0.1 - ND	ND	0.1 - ND	ND
Ranitidine	0.4 – 0.7	0.6 – 0.3	0.1 – 0.3	1.0 – 0.2	0.2 – 0.1
Glibenclamide	ND	ND	ND	ND	ND
Hydrochlorothiazide	1.1 – 2.1	1.2 – 1.5	0.3 – 1.0	1.1 – 0.8	ND – 0.1
SAS	1978.65 - 1521.0	61.1 - 77.4	73.9 - 44.7	1147.9 - 2344.8	623.2 - 118.7

ND = not detected

a. USEPA, Marine Screening Benchmarks, 2004.
b. CCME, 1995.
d. CEDEX, 1994
e. Riba et al., 2004.
c. CCME, 1999

Sediment sampled at P2 was characterized by the highest concentrations of trace metals and PAHs that surpassed the guidelines, such as Hg, Cr, Cu, Pb, As, benzo [a] anthracene and benzo [a] pyrene, followed by sediment sampled at P3, that was predominantly characterized by contamination due to Hg, Cu, Pb, Cd and benzo [a] anthracene (summer). Contamination by trace metals such as Hg, Cr, Cu and Zn was found in sediment sampled at P4. High concentrations of Cr, Cu and As were observed in sediment sampled at P1. Low concentrations of trace metals and PAHs were observed in sediment sampled in the control site (P6).

Except for P1 and P6, all the other sites presented higher concentration of total pharmaceuticals in winter than summer season. The highest values for SAS were detected in sediment sampled at P1 and P4. Otherwise, concentration of PAHs, SAS and flumequine (antibiotic) were higher in summer than winter.

3.2. Biomarker responses

No significant mortality compared with the control site was observed. Lysosomal membrane stability is a general measure of stress. The mean value of neutral red retention time (NRRT) observed in lysosomes from control clams (n = 12) in summer and winter did not differ, while P1, P2 and P3 were under significant stress during the summer season ($p < 0.05$). Adopting the LMS criteria previous reported for other species (Viarengo et al, 2007, Martínez-Gómez et al., 2008, Aguirre-Martínez et al., 2010), and the previous work employed in *R. philippinarum (*Aguirre-Martínez et al., 2013, Buratti et al., 2010), the threshold values used in this study were as follows: clams were considered healthy if NRRT was ≥ 80 min; stressed but compensated if NRRT was between 80 min and 45 min; and in a stressed status if NRRT was < 45 min. P1, P2 and P3 were

considered stressed clams (Figure 2). This condition may be reversible if the clams were removed and places in clean sediments.

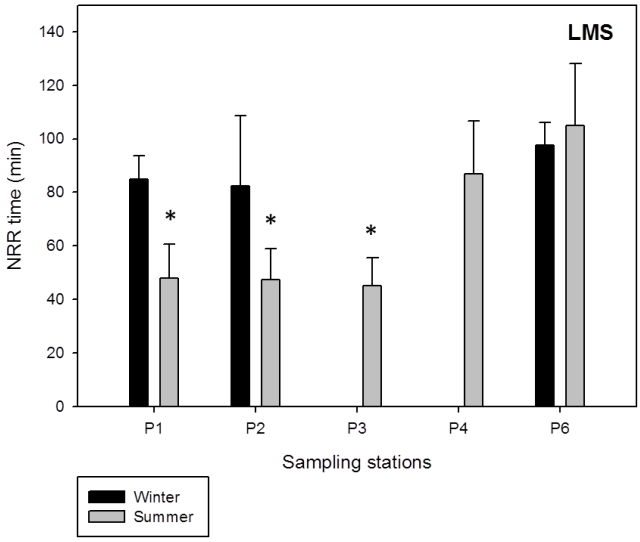

Figure 2. Lysosomal membrane stability (LMS) assessed through neutral red retention (NRRT) assay in haemocytes from clams *R. philippinarum*. Individuals were exposed *in situ* for 14-days to sediment directly affected by wastewater discharges in winter (P1, P2) and summer (P1 – P4). P6 was the control site. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams.

Concerning all the biomarker responses determined in digestive glands named as "set of biomarkers I" (Figure 3), only GPX differed significantly between summer and winter for the control clams ($p < 0.05$). Phase I of the metabolism significantly increased in clams exposed to P1 (during winter and summer), P2 and P3 (during summer season) ($p < 0.05$). However, DBF activity significantly increased in clams exposed to P1 and P2 in winter ($p < 0.05$). Phase II of the metabolism was activated in clams exposed to P1 in winter, but seemed not activated in clams exposed in summer. Between the antioxidant system, GPX activity was not different compared with the control for any exposure site, but GR activity was activated in winter in clams exposed to P1 and P2 ($p < 0.05$). Neurotoxicity (AChE) was determined in clams exposed to P1 and P2 in winter ($p < 0.05$), and P3 during summer season ($p < 0.05$). Clams exposed to all sites in summer and

winter showed higher oxidative effect (LPO) compared with the control organisms ($p < 0.05$). Also DNA damage was observed in clams exposed to all stations during summer season ($p < 0.05$).

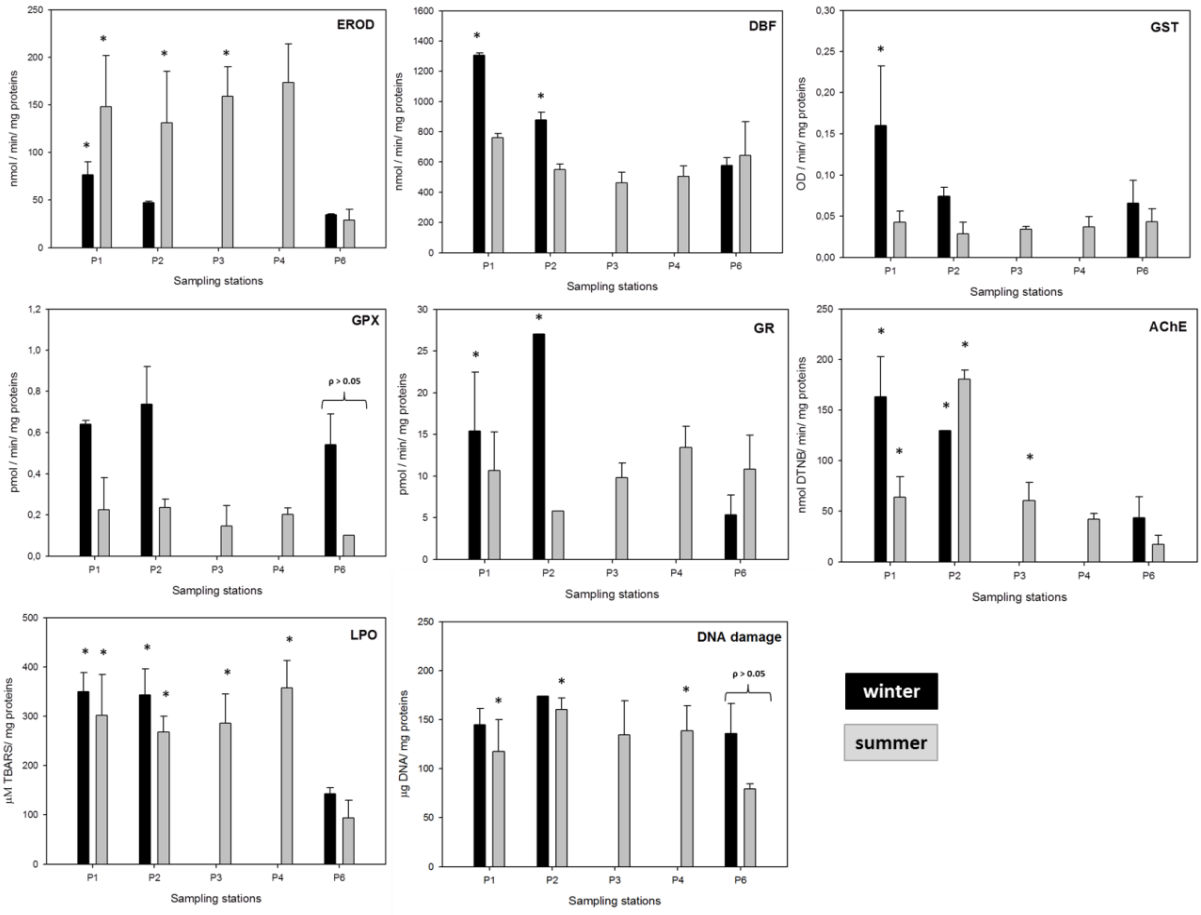

Figure 3. Set of biomarkers I. Phases I (EROD and DBF enzymatic activities) and II (GST enzymatic activities) of the metabolism, antioxidant defences (GR and GPX enzymatic activities), neurotoxicity (AChE enzymatic activity) and oxidative effects (LPO and DNA damage) determined in digestive gland of clams *R. philippinarum*. Individuals were exposed *in situ* for 14-days to sediment directly affected by wastewater discharges in winter (P1, P2) and summer (P1 – P4). P6 was the control site. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means significance difference between winter and summer in the control responses.

Unlike the "set of biomarkers I", all the biochemical responses named as "set of biomarkers II" (Figure 4) showed significantly higher activities or content during winter than summer season ($p < 0.05$). However, TLP and MAO activity were not significant

different compared with the control. Clams exposed to P3 during summer showed significantly higher MET activity compared with the control ($p < 0.05$). Vtg-like proteins were higher in clams exposed during winter than summer, nevertheless, all clams exposed during summer showed significantly higher Vtg-like proteins levels than the control organisms ($p < 0.05$). Clams exposed to P1 and P2 during winter showed significantly higher COX activities when compared with the control organisms ($p < 0.05$).

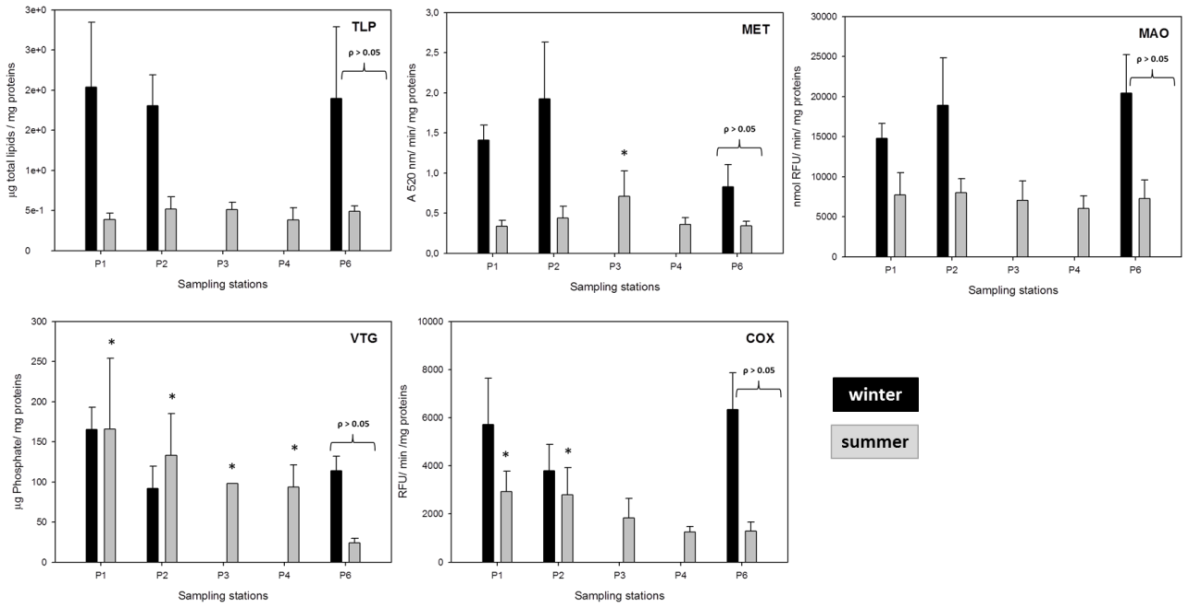

Figure 4. Set of biomarkers II. Changes in energy status (TLP content and MET activity), reproduction (Vtg-like proteins), neuroendocrine effects (MAO activity) and immunotoxicity (COX activity) were determined in gonads of clam's *R. philippinarum*. Individuals were exposed *in situ* for 14-days to sediment directly affected by wastewater discharges in winter (P1, P2) and summer (P1 – P4). P6 was the control site. Asterisks (*) indicate significance at $p < 0.05$ level in respect to controls clams. $p < 0.05$ under the controls means significance difference between winter and summer in the control responses.

Spearman´s test showed higher correlations between biomarker responses in winter ($r = 1$, $p < 0.01$) than summer ($r = 0.9$, $p < 0.05$). In winter, phase I (EROD and DBF) and II (GST) enzymatic activities, neurotoxicity (AChE) and oxidative effect (LPO) were positively correlated between them, and negatively correlated with monoamine oxidase activity (MAO) ($p < 0.01$). Antioxidant enzymes (GR and GPX);

energy expenditure (MET), lysosomal (LMS) and DNA damages were positively correlated between them, but negatively correlated with COX activity ($p < 0.01$). In summer, phase I EROD activity was positively correlated with oxidative effect LPO ($p < 0.05$), and phase I DBF activity was negatively correlated with energy expenditure (MET) ($p < 0.05$). Phase II GST activity was negatively correlated with DNA damage ($p < 0.05$). Antioxidant enzyme GR was negatively correlated with COX activity ($p < 0.05$), and GPX was positively correlated with AChE activity ($p < 0.05$). Vtg-like proteins was positively correlated with AChE and COX activities ($p < 0.05$).

To better understand the results and be able to interpret them alongside, a relationship among chemical concentrations in sediment and biological responses was established. Principal component analysis (PCA) was conducted using the dataset for 37 variables defined by different sites (P1, P2 and P6 in winter; P1, P2, P3, P4 and P6 in summer). Different PAHs were grouped in one group. Pharmaceutical products were grouped according to the human prescription: anti-inflammatories (acetaminophen, diclofenac, fenoprofen), anti-hypertensive (atenolol, propranolol), lipid regulators (clofibric acid, gemfibrozil), psychiatric drugs (carbamazepine, fluoxetine, amitriptyline, caffeine), antibiotics (chloramphenicol, cefdinir, tiamulin, erythromycin, clarithromycin, azithromycin, roxithromycin, lincomycin, flumequine, clindamycin, sparfloxacin, novobiocin, metronidazole, ornidazole, sulfadiazine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, monensin), antacids (famotidine, ranitidine) and others (glibenclamide, hydrochlorothiazide). Two separate PCAs were conducted on the biological and chemical results, one for winter, the other one for summer.

After PCA was undertaken, original variables could be described by two new factors for winter and three new factors for summer (Table 2). The criteria selected to interpret variables associated with a particular factor was loading of 0.5 (Comrey's, 1973).

Each component was described according to the dominant group of variables. Concerning the winter results, 100% of the total variance was divided in two factors. Factor 1 explained 63.50% of the variance following by factor 2, which explained 36.50% of the variance:

Factor 1: combined the increase of oxidative effects (DNA damage and LPO), antioxidant defences (GPX and GR activities), neurotoxicity (AChE enzymatic activity), immunotoxicity (the inhibition of COX), energy expenditure (MET activity) and lysosomal membrane stability (LMS) with the exposure to PAHs, all trace metals determined in sediment, surfactant (SAS) and pharmaceutical products (psychiatric drugs, antibiotics, antacid and other drugs). Contaminants and biological effects were related to TOC and OM content in the sediment.

Factor 2: associated variables which described the induction of phases I (EROD and DBF) and II (GST) enzymatic activities, neurotoxicity (AChE enzymatic activity), the increase of energy reserve (TLP), Vtg-like proteins and inhibition of breakdown of monoamines (serotonine and dopamine) (MAO). Such responses were related with exposure to PAHs, pharmaceutical products (anti-inflammatories, anti-hypertensive, lipid regulators and antibiotics) and surfactants (SAS) determined in the sediment samples. These compounds were related to the % of fines in the sediment.

Concerning summer results, variables explained 88.18% of the total variance. Factor 1 explained 37.42%, following by factor 2 that explained 30.23%, and factor 3 that explained 20.53% of the variance:

Factor 1: linked the activation of phase I (DBF activity) and antioxidant defences (GPX enzymatic activity) resulting in oxidative effects (LPO) associated with activation of COX activity, the decrease of energy status (TLP and MET activity) and the increase of Vtg-like proteins levels. These biological responses were related to the trace metal

concentrations (Al, Fe, Cr, Ni, Zn and Cd), pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacid and others) and % of fines in the sediment.

Factor 2: combined the inhibition of phase II (GST activity), the activation of GPX and the inhibition of GR activities, and neurotoxicity (AChE activity) resulting in genetic damage (DNA *strand breaks*). This factor also related activation of COX activity, the increase of energy reserve (TLP), the neuroendocrine effects (activation of MAO activity) and the lysosomal membrane stability (LMS). Such biological responses were linked to the presence of PAHs, trace metals (Cu, Pb and Hg) and surfactants (SAS) associated with TOC and OM content in the sediment samples.

Factor 3: combined oxidative effects (LPO and DNA damage) with phase I of the metabolism (activation of EROD activity and deactivation of DBF activity), the neuroendocrine effects (deactivation of MAO activity) and also with energy expenditure (MET activity). Such biological responses were related to the presence of trace metals (Mn, Cu, Zn, Pb and As) and anti-hypertensive drugs, associated with OM content in the sediment samples.

Table 2. Sorted rotated factor loadings (pattern) of 37 variables for three principal factors resulting from the multivariate analysis of results obtained from biochemical and LMS responses of *Ruditapes philippinarum* and physical chemical characteristics of the sediment during summer and winter 2011.

	Winter		Summer		
	Factor 1	**Factor 2**	**Factor 1**	**Factor 2**	**Factor 3**
% Variance	**63.50**	**36.50**	**37.63**	**28.68**	**21.55**
DNA damage	.934			.747	.534
LPO	.885		.577		.788
GST		.988		-.891	
GR	.994			-.923	
GPX	.998		.832		
EROD		.930			.847
DBF		.881	.564		-.647
AChE	.745	.667		.955	
COX	-.935		.606	.632	
TLP		.942	-.761	.600	
MAO		-.945		.592	-.626
MET	.999		-.567		.603
Vtg-like proteins		.974	.791		
LMS	.963			.703	
PAHs	.835	-.550		.979	
Al	.892		.954		
Fe	.912		.924		
Mn	.935				-.869
Cr	.910		.915		
Cu	.916			.614	.616
Ni	.900		.916		
Zn	.926		.735		
Pb	.965			.781	
Cd	-.967		-.764		
As	.971				-.939
Hg	.979			.860	
Anti-inflammatories		.975	.641		
Anti-hypertensive		.998			.920
Lipid regulators		.987	.922		
Psychiatric drugs	-.986		.830		
Antibiotics	.621	-.784	.878		
Others		-.998	.761		
Antacid	.983		.787		
SAS	.776	.630		.867	
% Fines		-.971	-.729		
TOC	.986			.924	
OM	1.00		.710		

Graphical representations of estimated factor values corresponding to each season were presented in order to confirm the descriptions of these new factors for each sampling site (Figure 5).

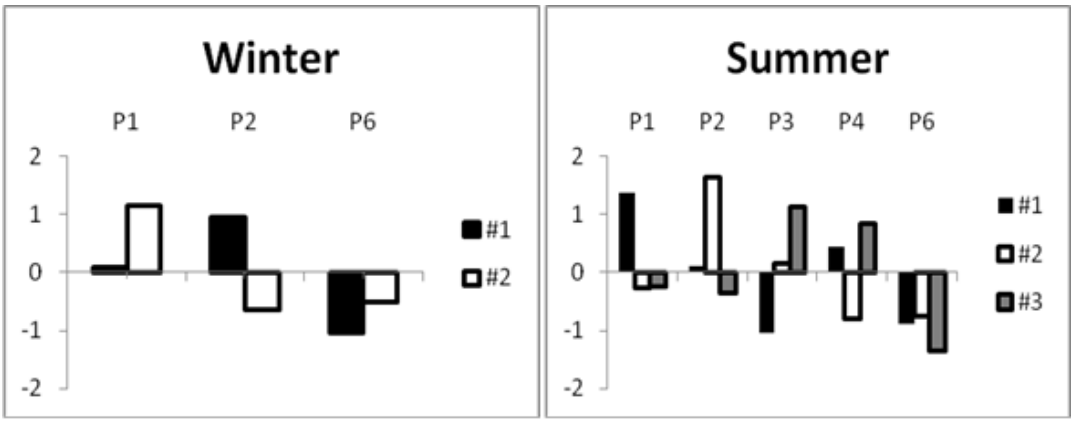

Figure 5. Representation of factor scores estimation for each season and the respective exposure points. P1: Chiclana de la Frontera, P2: Puerto Real, P3: Cádiz, P4: El Puerto de Santa María, P6: Rota (control). Physical chemical characteristics of the sediments from the different exposure sites were used to link with the biological adverse effects observed in clams *Ruditapes philippinarum*.

With respect to winter, factor 1 values indicated the increase of oxidative stress (GR and GPX activities) and effect (DNA damage and LPO), neurotoxicity (AChE activity) and the energy expenditure (MET activity). There was the decrease of LMS, which means the decrease of the health status. These responses were determined in exposed individuals to P2, due to the presence of trace metals (except Cd), pharmaceutical products (antibiotics, antacids and others) and surfactants (SAS). Indeed, this site was characterized by high concentrations of trace metals, in particular Hg, Cr, Cu and Pb. Meanwhile, factor 2 positive values were related to the activation of phases I (EROD and DBF activities) and II of the metabolism (GST activity), neurotoxicity (AChE activity), energy status (TLP) and Vtg-like proteins, due to the presence in sediments of a distinct groups of pharmaceutical products (anti-inflammatories, anti-hypertensive and lipid regulators) and surfactants (SAS). Highest values for this factor were found at P1, which showed the highest values of SAS concentrations in sediment during winter.

During summer, positive values for factor 1 related the induction of phase I (DBF activity), antioxidant system (GPX activity) and COX activity, oxidative effects of LPO and Vtg-like proteins levels with the presence of trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics and others) concentrations in P1, characterized by high concentrations of trace metals (Cr and Cu) and pharmaceutical products. Negative values of the factor 1 represented the decrease of energy status (TLP and MET activity) in clams exposed to P3 due to the presence of Cd associated with the % of fines in the sediment.

Factor 2 was related to the increase of DNA damage, antioxidant defences (GPX activity), neurotoxicity (AChE activity), COX activity, changes in the energy status (TLP), neuroendocrine effects (MAO activity) and decrease of lysosomal membrane stability (LMS) in clams exposed to P2. These biological changes were associated with the presence of PAHs, trace metals (Cu, Pb and Hg) and surfactants (SAS) linked to TOC and OM content in sediments. On the other hand, negative values of factor 2 demonstrated the decrease of GST and GR activities in clams exposed to P4.

Finally, factor 3 was associated with the increase of phase I of the metabolism (EROD enzymatic activity), energy expenditure (MET activity) and oxidative effects (DNA damage and LPO) due to trace metals (Cu, Zn and Pb) and anti-hypertensive drugs concentrations linked to OM content of the sediment sampled in P3.

4.DISCUSSION

The exposure of caged bivalves in impacted areas is commonly used for the environmental quality evaluation in coastal environments (Pereira et al., 2014, Maranho et al., 2012, Nigro et al., 2006, Nasci et al., 2002). A monitoring program using multiple biological responses and chemical measurements is able for the identification of impacted

environments. Biomarkers are important tools for understanding the linkages between external contaminant exposures, internal doses, and the associated potential health outcomes. Sediment quality assessments using sublethal responses of benthic organisms, such as changes in the metabolism of xenobiotics, neurotoxicity, oxidative stress and effects, and effects on growth and reproduction, have been used to evaluate contaminated areas (Gust et al., 2014, Gagné et al., 2007 a, b, Guerlet et al., 2007). In previous studies, exposure to wastewater effluents has been found to alter the oxidative state of cells thereby increase oxidative stress and effects (Gagné et al., 2006a, Dizer et al., 2002). In addition, alterations in energy status, reproduction (neuroendocrine effects) and immunotoxicity have been previously reported in bivalves exposed to wastewater effluents around the world (Gagné et al., 2007b, Hemming et al., 2001).

Neutral Red Retention Time (NRRT) assay is a non-destructive biomarker when practiced in the haemolymph, and as such, it allows repeated measurements and the application of other biomarkers in the same individual (Martínez-Gómez et al., 2008). Lysosomal membrane stability (LMS) is thought to be a non-specific and general measure of stress. NRRT has been applied as screening tool in order to detect adverse changes in health status associated with contaminated environments (Buratti et al., 2012, Domouhtsidou et al., 2004). Previous studies reported the success of the NRRT assay in clams *Ruditapes philippinarum* (Aguirre-Martínez et al., 2013, Coughlan et al., 2009). However, little is known about the application and validation of NRRT in sediment bioindicator species under field conditions. In the present study, LMS decreased in clams exposed *in situ* to sediment directly affected by wastewater discharges, mainly concerning the summer season, when the population of the Bay of Cádiz increases around 30% due to tourism, and the wastewater composition varied compared with the winter season. LMS was positively correlated with PAHs, trace metals (except Cd), antacids, TOC and OM (p

< 0.01), and negative correlated with Cd and psychiatric drugs in winter ($p < 0.01$). Buratti et al. (2012) observed a significant correlation between NRRT assay responses in clams *R. decussatus* and trace metals concentration (Cr, Ni, Zn, Cd and Cu ($p < 0.01$) and As and Pb ($p < 0.05$)). This previous study reported the exposure of clams in the Bay of Cádiz, near the P2 of the present study. On the other hand, this previous study demonstrated a significant correlation between the decrease in NRRT and PAHs total concentration ($p < 0.01$). In addition, significant reduction of NRRT during the summer season in clams exposed to P1, P2 and P3 ($p < 0.05$) was related to the contamination by PAHs, trace metals (Cu, Pb and Hg) and SAS associated with TOC and OM content in the sediment. Trace metals (Cu and Pb) ($p < 0.01$) and TOC ($p < 0.05$) were positively correlated with LMS.

LMS was also considered an effective indicator of health status in bivalves (e.g. mussels *Mytilus galloprovincialis*) along the Iberian coast and related to body burden of organic contaminants, particularly PAHs and PCBs (Martínez-Gómez et al., 2008). Nigro et al. (2006) observed the decrease of LMS and the bioaccumulation of different trace metals, including Cu and Pb, in mussels *Mytilus galloprovincialis* transplanted to a contaminated estuary. LMS measurements are currently included in the general guidelines for monitoring programs (JAMP by OSPAR) and also proposed as a marine pollution index to evaluate stress responses in molluscs by the Mediterranean Pollution Programme (MEDPOL) (Martínez-Gómez et al., 2008). In this respect, clams *Ruditapes philippinarum* is a recommended species for the application of LMS assay in monitoring programs concerning the Iberian Atlantic coast. The present study corroborates with Martínez-Gómez et al. (2008), which demonstrated NRRT assay for LMS as an effective and rapid screening tool for determining the health status in bivalves, and therefore can be easily included in the Spanish national monitoring programme.

Summer season is the spawning period for this species. Bivalve reproductive cycle (vitellogenesis and spawning) can modulate the expression of several biochemical biomarkers (Blaise et al., 2002). On the other hand, the temporal *in situ* survey conducted at five sites in the Bay of Cádiz yielded a variety of differences in biomarkers levels generally higher in winter than summer controls ($p < 0.05$): GPX, DNA damage, TLP, MET; MAO, Vtg-like proteins and COX. However, only DBF, GR and LPO were significantly higher in clams exposed to P1 and P2 than the control site in winter ($p < 0.05$). Low biomarker levels were expected in winter because of lower water temperatures should correspond to a time when clams have slowed down metabolically (Blaise et al., 2002). Nevertheless, during the gametogenesis period in summer, correlations between biomarker responses were weaker than winter. The influence of contaminant composition and input, and the temperature variation may have modulated biomarker response in clams during different seasons.

Phase I detoxification metabolism was significantly increased compared with the control organisms in clams exposed to P1 (EROD and DBF activities) and P2 (DBF activity) ($p < 0.05$) in winter. The activation of EROD and DBF activities in winter was related to the presence of pharmaceutical products (anti-inflammatories, anti-hypertensive and lipid regulators) and SAS in sediment. Typically, the bioavailability of the drug molecule is modulated by cytochrome P450 (Risks et al., 2009): CYP 1A2 (EROD activity) is responsible for around 15%, and mainly CYP 3A4 (DBF activity) which is responsible for around 50% of the pharmaceutical metabolism in humans (Okita and Masters, 2004). EROD is also related to PAHs metabolism (Van Der Oost et al., 2003). SAS are organic compounds and constitute the most important group of detergent components, which could be metabolized by the phase I of detoxification metabolism. EROD activity in clams exposed in summer season was related to trace metals (Cu, Zn

and Pb), anti-hypertensive drugs and OM content in the sediment. The relationship of EROD activity with other organic compounds (PAHs and PCBs) has been widely reported (Van der Oost et al., 2003). CYP 1A2 may also play a role in the anti-hypertensive drug metabolism (e.g. anti-hypertensive propranolol) (Laville et al., 2004). Clams exposed in summer to P1, P2 and P3 showed significant increase of the EROD activity ($p < 0.05$). EROD was positively correlated with anti-inflammatories drugs and negative correlated with Mn and As in summer ($p < 0.05$). However, anti-inflammatories drugs were reported to inhibit somewhat EROD activity when exposed separately (Laville et al., 2004). However, the mixture of contaminants can change this panorama, and the induction of EROD activity can happen, as based on previous studies about the exposure to wastewater effluents (Gagné et al., 2007a).

Phase II was not induced in summer, but in winter clams exposed to P1 showed significantly higher activity than compared with the control organisms ($p < 0.05$). GST activity was related to the presence of pharmaceutical products (anti-inflammatories, anti-hypertensive and lipid regulators) and SAS in sediment. Phase II was negatively correlated with PAHs, Hg and TOC in summer ($p < 0.05$). Gagné et al. (2008) observed the significant increase of GST activity in mussels exposed to wastewater effluents in four different estuaries in France and Canada. Phases I and II enzymatic activities were positively correlated with oxidative effect (LPO), neurotoxicity (AChE activity), trace metal (Al), pharmaceutical products (anti-inflammatories drugs and lipid regulators) and SAS ($p < 0.01$) in winter. Phases I and II enzymatic activities were negatively correlated with neuroendocrine effect (MAO activity) and % fines of the sediment ($p < 0.01$) in winter.

Antioxidant system, concerning GPX and GR activities, was not activated in clams exposed during summer. GR enzyme is also involved in antioxidant defence in the same

way as GPX enzyme (Martín-Díaz et al., 2008b). Oxidative stress increases in high temperature, which was expected in summer. However, GR activity significantly increased in clams exposed to P1 and P2 in winter ($p < 0.05$). The increase of GR activity was due the exposure to PAHs, trace metals (except Cd), pharmaceutical products (antibiotics, antacids and others) and SAS, present in the sediment (TOC and OM content). In winter, antioxidant system was positively correlated with DNA damage, neuroendocrine effect (MET activity) and LMS ($p < 0.01$). If activated defences are insufficient to protect tissues from damage, lipid peroxidation (LPO), DNA damage, or immunosuppression alterations (COX activity) may prevail (Blaise et al., 2002). In winter, the antioxidant system of the clams was negatively correlated with inflammation (increase of COX activity) and pharmaceutical products (psychiatric drugs) ($p < 0.01$). However, the inactivation of the antioxidant system was positively correlated with the concentration of PAHs, trace metals (except Al), pharmaceutical products (antacids), TOC and OM content ($p < 0.01$).

The increase of AChE enzymatic activity may indicate alterations in cholinergic neurotransmission and consequently associated with impairments at the neuromuscular and ganglia nervous systems (Zhang and Greenberg, 2012). In addition, the increase of AChE activity plays an important role in apoptosis, being first synthetized in the cytosol and then accumulated in the nucleus (Zhang and Greenberg, 2012). Clams exposed to P1 and P2 showed significant increase of AChE enzymatic activity when compared with the control organisms ($p < 0.05$) in winter. The inhibition of AChE activity was previously reported as related with PAHs contamination (Bocchetti et al., 2008). Nevertheless, the increase of AChE activity in winter was related to PAHs, trace metals (except Cd), pharmaceutical products (anti-inflammatories, anti-hypertensive and lipid regulators), SAS, TOC and OM content in the sediment.

Sediment directly affected by wastewater discharges caused oxidative effects in clams exposed to both seasons. Clams exposed to all exposure sites showed significantly higher LPO compared with the control organisms ($p < 0.05$). In winter, LPO was positively correlated to antioxidant system (GPX and GR activities) that was not activated, with neurotoxicity (AChE activity) and energy expenditure (MET activity). AChE inhibition plays an important role in the preservation of the integrity of the red cell in humans, therefore the increase of AChE activity can be related to reactive oxygen species (ROS) production and consequently to LPO (Santi et al., 2011). The oxidative effect of LPO was related to metabolic activity due to the exposure to PAHs, trace metals (except Cd), pharmaceutical products (antibiotics, antacids and others) and SAS associated with TOC and OM content in the sediment. The enhancement of ROS formation in winter resulted in oxidative stress, since the balance between prooxidant forces and antioxidant defences seemed to be overcame (Winston, 1991). ROS lead to oxidative effects as DNA damage (*strand breaks*), lipid peroxidation (LPO), lysosomal alterations (LMS) and also the energy expenditure (MET activity). In winter, DNA damage was positively correlated with antioxidant defences, energy expenditure (MET activity) and LMS due to the exposure to PAHs, trace metals (except Al and Cd) and pharmaceutical products (antacids) associated with TOC and OM content in the sediment ($p < 0.01$). In summer, LPO was positively correlated with phase I (EROD activity) and trace metal (Cd) ($p < 0.05$), and negatively correlated with % of fines in the sediment ($p < 0.05$). The activation of the detoxification metabolism (phase I) and the failure of the antioxidant system in summer resulted in energy expenditure (MET activity) and oxidative effects as LPO and DNA damage. ROS was associated to xenobiotic transformation further supported by an analysis of covariance of phase I, LPO and DNA

damage using MET activity as covariate, which showed that LPO remained significantly elevated at impacted sites in summer.

Previous studies observed DNA damage in organisms exposed near WWTPs (Blaise et al., 2002, Gagné et al., 2006b, Gagné et al., 2007b). DNA damage was higher in winter, but clams exposed to P1, P2 and P4 showed significantly increased compared with the control organisms in summer ($p < 0.05$). DNA damage was negatively correlated with phase II ($p < 0.05$). The inefficiency of phase II during summer season and the contamination by PAHs, trace metals (Cu, Pb and Hg) and SAS associated with TOC and OM content in sediment can be the responsible for the increase of DNA damage in clams exposed to P1, mainly P2 (factor 2) and P4.

However, there was no significant difference for the energy reserve (TLP) compared with the control for any season. In winter, clams had more TLP content in gonads than in summer season which is logical following the gametogenesis. However, TLP was positively correlated with Vtg-like proteins in winter ($p < 0.01$) when the vitellogenesis occurs.

Exposure to pharmaceutical products and urban effluents were shown to increase MET activity in freshwater mussels (Gagné et al., 2006b). Interestingly, the energy expenditure of the clam's gonads was significantly lower than the control organisms only for P3 in summer season ($p < 0.05$). The decrease of MET activity in P3 was mainly related to trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and others). In winter, MET activity was positively correlated with DNA damage, antioxidant system (GR and GPX activities), LMS, PAHs, trace metals (except Al and Cd) and pharmaceutical products (antacids), associated with TOC and OM content in sediment ($p < 0.01$). Energy expenditure (MET activity) was negatively correlated with

immunotoxicity (COX activity), trace metals (Cd) and pharmaceutical products (psychiatric drugs) ($p < 0.01$).

MAO activity has a vital function because it plays a role in the inactivation of neurotransmitters (e.g. noradrenaline, serotonin, dopamine). Domestic wastewater aeration lagoons exposure of freshwater mussels *Elliptio complanata* resulted in neuroendocrine effects by increasing MAO activity (Gagné et al., 2007b). On the other hand, the exposure to sediment directly affected by wastewater effluents did not seem to cause changes in MAO activity in marine clams for any season.

Vtg-like proteins levels increase vitellogenesis followed by the development of oocytes in female clams, characterized by the pre-spawning period (Blaise et al., 2002). Vtg-like proteins are an egg yolk protein precursor, usually silent in males, but that can be induced by estrogen exposure (Gagné et al., 2011). Clams exposed to P1, P2, P3 and P4 in summer season showed significantly higher Vtg-like proteins levels compared with the control organisms ($p < 0.05$). However, Vtg-like proteins levels were significantly higher in winter than summer when compared the controls ($p < 0.05$), which means that contamination may delay spawning event in this species. Important perturbations in either the oocyte maturation process or Vtg-like proteins expression occurred in summer. Vtg-like proteins in summer were related to sediment contamination by municipal effluents as evidenced by trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and others). The mixtures of carbamazepine (psychiatric drugs), fenofibric acid (lipid regulators), propranolol (anti-hypertensive), sulfamethoxazole (antibiotic) and trimethoprim (antibiotic) in the Douro River estuary caused increases in Vtg levels in male zebrafish (Madureira et al., 2012). Urban effluents lead to neuroendocrine disruption may be involved in sex differentiation (feminization) and other changes in reproduction in clam's

populations in summer. Feminization of mussels exposed downstream of municipal effluents was previously reported by Gagné et al. (2011). Vtg-like proteins was positively correlated with energy reserve (TLP) and negatively correlated with pharmaceutical products (antibiotics) ($p < 0.01$) in winter. Vtg-like proteins was positively correlated with COX activity and negatively correlated with antacids and other drugs ($p < 0.01$) in summer.

COX activity is an enzymatic activity involved in inflammation, and also responsible for formation of important biological mediators as prostanglandins, which is considered as a spawning agent (Gagné et al., 2007b). The induction of this activity is related to spawning and inflammation when associated to LPO (Gagné et al., 2007b). The inhibition of this activity can cause relief of pain and inflammation in mammals or vertebrates. COX activity increased in winter than summer for both controls, but during summer, clams exposed to P1 and P2 showed significantly higher activity than the control organisms ($p < 0.05$). COX activity in clams exposed to P1 was mainly related to trace metals (Al, Fe, Cr, Ni and Zn) and pharmaceutical products (anti-inflammatories, lipid regulators, psychiatric drugs, antibiotics, antacids and others). COX activity in P2 was mainly related to PAHs, trace metals (Cu, Pb and Hg) and SAS associated with TOC and OM content in the sediment. Contamination at P1 and P2 may be the responsible for the inflammation, since Vtg-like proteins, LPO and COX activity were significantly higher in clams exposed to P1 and P2 than the control ($p < 0.05$).

Moreover, COX activity was also readily increased in freshwater mussels exposed to aeration lagoon for wastewater treatment (Gagné et al., 2007b). COX activity was positively correlated with Vtg-like proteins and antacids in summer ($p < 0.05$). Previous study showed the correlation between COX enzymatic activity, LPO and Vtg-like proteins for freshwater mussels exposed to aeration lagoons for wastewater treatment

(Gagné et al., 2007b). The later stage of gametogenesis in P3 and P4 can be supported by the significantly higher levels of Vtg-like proteins compared with controls ($p < 0.05$), and no increase of COX activity. To support this information about delay of spawning or inflammation, phagocytosis biomarker is recommended to clarify this response.

5.CONCLUSIONS

In summary, the battery of biochemical responses and the NRRT assay were suitable tools to evaluate the environmental quality of sediments directly affected by wastewater effluents *in situ*. The chemical composition of the sediment directly affected by wastewater effluents suffered seasonal changes, which reflected in the health status of the benthic organisms. Nevertheless, even with the seasonal fluctuations, this methodology is recommended for the environmental evaluation of contaminated areas. In winter, urban effluents were detoxified by the phase I (DBF activity of the metabolism), conjugated by the phase II (GST activity), activated the antioxidant defences (GR activity) and the exposure resulted in neurotoxicity (AChE activity) and lipid peroxidation (LPO). WWTP effluents lead to the detoxification metabolism (EROD activity), oxidative effects (LPO and DNA damage), neurotoxicity (AChE activity) and neuroendocrine disruption (COX activity and Vtg-like proteins) involved in inflammation and spawning delay in clams' populations in summer. Adverse effects varied according to the contamination level, and are also depend on the reproductive cycle of the clams.

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CAPÍTULO 6

Conclusiones

1. Spiked marine sediment with pharmaceutical products including environmental concentrations: carbamazepine (CBZ), ibuprofen (IBP), fluoxetine (FX), 17α-ethynylestradiol (EE2), propranolol (PRO) and caffeine (CAF), showed acute toxicity in organisms belonging to different trophic levels. Acute toxicity tests, recommended by different organizations (CEDEX, USEPA, USACE, GIPME, SEDNET) for sediment quality assessment, and applied for selected pharmaceuticals pollution refuted the fact that Microtox® (Basic Test), amphipods mortality (*Ampelisca brevicornis*) and microalgae growth rate (*Isochrysis galbana* and *Tetraselmis chuii*) acute bioassays were sufficient for pharmaceutical products monitoring. Sea-urchins embryotoxicity assay is recommended for the acute toxicity assessment of pharmaceutical products associated to the marine sediment matrix.

2. Chronic toxicity of marine sediments spiked with selected pharmaceuticals was observed. Biomarkers related with metabolism (phases I – EROD and DBF, and II - GST enzymatic activities), neurotoxicity (AChE activity), oxidative stress (lipid peroxidation - LPO and antioxidant enzymes activities – GPX and GR) and genetic damage (DNA *strand breaks*) were determined in both organisms amphipods (*Ampelisca brevicornis*) and polychaetes (*Hediste diversicolor*). Comparing the metabolism of selected pharmaceuticals, amphipods´ detoxification metabolism seemed to be stronger affected than polychaetes´, what might show higher

availability of these compounds to amphipods. On the other hand, the second battery of biomarkers was examined in polychaetes in order to determine changes in cellular energy status (total lipids - TLP and mitochondrial electron transport - MET), and associated to the mode of action of pharmaceutical products (alkali-labile phosphates level - ALP, metabolism of monoamines - MAO and cyclooxygenase activity - COX). Except for FX, all pharmaceuticals tested showed anti-inflammatory properties confirmed by the decrease of COX activity. COX and MAO activities were the most sensitive responses between the second battery of biomarkers. This study suggested pharmaceutical products at environmental concentrations might cause a wide variety of adverse effects (based on laboratory studies).

3. In order to determine contamination and associated acute effects caused by WWTP effluents, sediment quality at the Bay of Cádiz (SW, Spain) was evaluated through a battery of acute bioassays in the laboratory and chemical contamination data. Results evidenced a clear deterioration of ecological sediment quality parameters and adverse effects on aquatic communities towards WWTPs areas. Acute toxicity and chemical contamination varied significantly across the studied sites and differed between winter and summer seasons. The Bay of Cádiz is contaminated by PAHs, metals, detergents (SAS) and pharmaceutical products. However, Principal Component Analyses indicated metals, SAS and pharmaceutical products as the major environmental stresses. Sea-urchin embryo-larval and microalgae growth rate bioassays were the most sensitive to evaluate the ressuspension of contaminants (elutriate) from bulk sediment. Amphipods mortality and Microtox® Solid Phase Test (SPT) were recommended to evaluate bulk sediment quality. Therefore,

multiple-bioassays and chronic exposure are recommendable to assess and monitor environmental quality of marine sediment directly affected by the mixture of contaminants released from WWTPs.

4. Biomarkers determined in clams *Ruditapes philippinarum* exposed for 14-days to sediment directly affected by WWTP effluents under laboratory conditions were underlined as early warning tools. Early warning tools can be applied for bioremediation purposes and sediment quality assessment, with possible application in sediments involved in dredging activities, since WWTPs can be associated to dredging areas. Contamination by WWTP discharges at the Bay of Cádiz is responsible for adverse effects related to reproduction and health status of the exposed clams. Adverse effects were associated to oxidative metabolism and stress endpoints, and might culminate in long-term changes affecting the population. WWTP discharges composition changed between different seasons, mainly leading to oxidative stress and inflammation process (COX activity). Observed effects are consistent with the occurrence of pharmaceutical products as NSAIDs, estrogens and selective serotonin-reuptake inhibitor (SSRI).

5. Through the *in situ* exposure of caged benthic organisms, seasonality of biological responses in *R. philippinarum* was observed. Even with seasonal fluctuations, the proposed battery of biomarkers is recommended for environmental evaluation of contaminated areas. In winter, WWTPs effluents were detoxified by phase I (DBF activity), conjugated by phase II (GST activity), antioxidant defences were activated (GR activity) but the exposure resulted in neurotoxicity and lipid peroxidation (LPO). WWTPs effluents lead to the detoxification metabolism (EROD activity),

oxidative effects (LPO and DNA damage), neurotoxicity (AChE activity) and neuroendocrine disruption (COX activity and Vtg-like proteins) involved in inflammation and spawning delay in clams' populations in summer. Chemical concentration of PAHs and metals did not vary according to the season. On the other hand, total concentration of pharmaceutical products was higher in winter, and detergents were higher in summer. Adverse effects on the biota also showed seasonality.

The responses observed in clams exposed *in situ* differed from clams exposed under controlled conditions. Clams exposed *in situ* seemed to be more affected due to constant discharge of WWTP effluents in addition with environmental conditions. Nevertheless, adverse effects on biota exposed to sediment directly affected by WWTP discharges depend also on chemical contamination and reproductive cycle according to seasonality. Mixture of pharmaceutical products and other contaminants in the environment can cause adverse effects that cannot be determined under controlled conditions.

6. The integrative battery of chemical and biological responses selected for the evaluation of marine sediments affected by pharmaceutical products contamination in a matrix of diverse contaminants at the Bay of Cadiz (SW, Spain) validated for environmental monitoring of these compounds in the laboratory. Thus, the continuous release of WWTPs discharges adequately threated or not to aquatic ecosystems poses health status risk to local biota.

Environmental risk assessment of marine sediments affected by contamination of pharmaceutical products: studies in laboratory and *in situ*.

Recently, pharmaceutical products have been identified as potential emerging contaminants in the environment whose main source of pollution is wastewater discharges. Exposure to these compounds can affect the health of the exposed organisms, increasing oxidative stress, genotoxicity and producing impaired reproduction. To date, European Water Framework Directive (WFD) includes such emerging substances as contaminants of interest. This directive requires that a risk assessment should be part of the approval process of new substances. However, this legislation recommends a battery of ecotoxicological tests for evaluating the acute and chronic toxicity in freshwater and terrestrial environments, but without regard to the marine environment. This thesis deals with the design and implementation of an integrated methodology to evaluate the environmental quality of marine benthic ecosystems affected by pharmaceuticals, and identification of new risks in the laboratory and *in situ*. This methodology includes three Lines of Evidence (LOEs), studied in marine sediment spiked with different drugs and in areas affected by wastewater discharges at the Bay of Cádiz (SW, Spain): pollution; determination of laboratory toxicity (acute toxicity, assessment of bioavailability and sublethal effects) and *in situ* (bioavailability and sublethal effects on organisms exposed in the field), using species belonging to different trophic levels. Integrating LOEs allowed determination of the bioavailability of pharmaceuticals and associated adverse effects. This integrated approach could contribute to improving the regulatory frameworks relating to the marine environment, in order to minimize and prevent future environmental risks caused by emerging contaminants. As a result, the Bay of Cádiz (SW, Spain) were contaminated by PAHs, metals, detergents (SAS) and pharmaceuticals. The mixture of pollutants, including pharmaceutical products, bound to marine sediments affected by wastewater discharges, can cause adverse effects on the biota measured in the laboratory and *in situ* (according to seasonality), leading to oxidative stress, neurotoxicity, genotoxicity and reproductive effects.

Evaluación del riesgo ambiental de sedimentos marinos afectados por la contaminación de productos farmacéuticos: estudios en laboratorio e *in situ*.

Recientemente, los productos farmacéuticos se han identificado como contaminantes emergentes en el medio ambiente, cuya principal fuente de contaminación son las aguas residuales. La exposición a estos compuestos puede afectar a la salud de los organismos expuestos, aumentando el estrés oxidativo, genotoxicidad, neurotoxicidad y produciendo alteraciones en la reproducción. Hasta la fecha, la Directiva Europea del Marco del Agua (WFD) incluye estas sustancias emergentes como contaminantes de interés. Esta directiva prescribe que una evaluación de riesgos debe ser parte del procedimiento de aprobación de nuevas sustancias. Sin embargo, esta legislación recomienda una batería de ensayos ecotoxicológicos apropiados para evaluar la toxicidad aguda y crónica en ambientes de agua dulce y terrestre, pero sin tener en cuenta el ambiente marino. La presente tesis aborda el diseño y aplicación de una metodología integrada que permita la evaluación de la calidad ambiental de los ecosistemas marinos bentónicos afectados por productos farmacéuticos, y la identificación de nuevos riesgos en laboratorio e *in situ*. Esta metodología incluye tres líneas de evidencia (LOEs), estudiadas en sedimento dopado con distintos fármacos y en áreas afectadas por vertidos de aguas residuales de la Bahía de Cádiz (SO, España): contaminación; determinación de toxicidad en laboratorio (toxicidad aguda, la evaluación de la biodisponibilidad y efectos subletales) e *in situ* (biodisponibilidad y efectos subletales en los organismos expuestos en campo), utilizando especies pertenecientes a diferentes niveles tróficos. La integración de LOEs permitió la determinación de la biodisponibilidad de los productos farmacéuticos y los efectos adversos asociados. Esta metodología integrada podría contribuir a la mejora del marco regulador referente al ambiente marino, con el fin de minimizar y prevenir riesgos ambientales futuros causados por estos contaminantes emergentes. Como resultado, la Bahía de Cádiz presentó contaminación por HAP, metales, detergentes (SAS) y productos farmacéuticos. La mezcla de contaminantes, incluidos los productos farmacéuticos, presentes en los sedimentos marinos afectados por los vertidos de aguas residuales, podrían provocar efectos adversos en la biota, medidos en condiciones de laboratorio e *in situ* (de acuerdo a la estacionalidad), tales como estrés oxidativo, neurotoxicidad, genotoxicidad y efectos en la reproducción.