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NUEVAS APROXIMACIONES TOXICOLÓGICAS PARA LA DETECCIÓN DE DISRUPTORES ENDOCRINOS Y LIPÍDICOS EN ECOSISTEMAS MEDITERRÁNEOS Y SISTEMAS CELULARES

María Blanco Rubio

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Directora de Tesis:

Dra. Cinta Porte Visa

“En la vida no hay cosas que temer,
sólo hay cosas que comprender”

Marie Curie

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Abreviaturas

17P4 – 17-hidroxiprogesterona
17P5 – 17-hidroxipregnenolona
A – Amposta
AB – Alamar Blue
AD - Androstenediona
AhR – Receptor de hidrocarburos de arilo
APs – Alquilfenoles
ARNT – Translocador nuclear AhR
BE – Benifallet
BFCOD–7-benziloxi-4-trifluorometil-coumarin*O*-debenziloxilasa
BNF – β-Naftoflavona
BPA – Bisfenol A
CE – Ésteres de colesterol
CF – Factor de condición
CFDA-AM–5-carboxifluoresceina diacetato acetoximetil éster
COS-7 – Línea celular derivada de riñón de *Cercopithecus aethiops*
DDT – Diclorodifeniltricloroetano
DE – Disruptores endocrinos
DG – Diacilgliceroles
DMSO – Dimetil sulfóxido
DRO – Drospirenona
d.w. – Peso fresco
E2 – Estradiol
EE2 – Etinilestradiol
ER – Receptor de estrógenos
EROD – Etoxiresorufina-O-deetilasa
ESI – Ionización por electrospray
FA – Ácidos grasos
FBS – Suero fetal bovino
FIA – Injección en flujo
FL – Flix
GC-MS/MS–Cromatografía de gases acoplada a espectrometría de masas
HCB – Hexaclorobenzeno
HCH – Hexaclorociclohexano
HHCB – Galaxolide
LC-MS/MS–Cromatografía líquida acoplada a espectrometría de masas

NP – Nonilfenol
OCs – Compuestos organoclorados
OP – Octilfenol
P4 – Progesterona
P5 – Pregnenolona
PAH –Hidrocarburos aromáticos policíclicos
PC – Fosfatidilcolina
PCA – Análisis de componentes principales
PCB – Bifenilos policlorados
PC-P – PC-plasmalógeno
PE – Fosfatidiletanolamina
PE-P – PE-plasmalógeno
PG – Fosfatidilglicerol
PI – Fosfatidilinositol
PLHC-1 – Línea celular derivada de un carcinoma hepatocelular de *Poeciliopsis lucida*
PLS-DA – Análisis discriminante mínimos cuadrados parciales
PPAR–Receptor proliferador de peroxisomas
PUFA – Ácidos grasos poliinsaturados
PXR – Receptor X de pregnano
RA – Ácido retinoico
$\mathbf{R_{BNF}}$ – Concentración de sedimento requerida para inducir la actividad EROD equivalente a 1 μM de β-naftoflavona
$\mathbf{REC_{20}}$ – Concentración de compuesto que produce una respuesta igual a un 20% de la respuesta máxima inducida por clotrimazol en el ensayo PXR
ROS – Especies reactivas de oxígeno
ROSI – Rosiglitazona
RR – Ribaroja
RXR–Receptor X de retinoides
RYA – Ensayo recombinante de levadura
SM – Esfingomielina
T – Testosterona
TBT –Tributilo de estaño
TG – Triacilglicerol
TOF – Analizador de tiempo de vuelo
UDPGT – Uridina difosfato glucuronil transferasa
VTG – Vitelogenina
w.w. – Peso seco
zfPxr – Receptor de pregnano de pez cebra

Resumen de la Tesis

Los compuestos químicos derivados de actividades humanas llegan a los sistemas acuáticos, donde quedan disponibles para los organismos que los habitan. Tradicionalmente, la evaluación del riesgo ambiental se ha basado en el análisis químico de contaminantes prioritarios en matrices ambientales. Sin embargo, la necesidad de evaluar el efecto de estos contaminantes en los organismos, ha llevado a desarrollar biomarcadores que informen de cambios fisiológicos y bioquímicos en los organismos como consecuencia de la exposición a xenobióticos. Entre los biomarcadores utilizados en esta Tesis, se incluyen biomarcadores de exposición a xenobióticos y biomarcadores de disrupción endocrina. Recientemente, los avances analíticos y bioinformáticos han hecho posible la aplicación de tecnologías ómicas para descubrir nuevos biomarcadores a nivel molecular. Además, debido a la necesidad de reducir el número de animales en los estudios experimentales y de mejorar las prácticas llevadas a cabo en los laboratorios, los experimentos de exposición y los estudios de campo se están sustituyendo por ensayos *in vitro*, donde el uso de líneas celulares se está aplicando con resultados muy favorables.

La combinación de biomarcadores tradicionales (enzimas metabólicas, cambios en expresión génica, análisis de esteroides, metabolitos en bilis), junto con el análisis del lipidoma, ha sido una herramienta eficiente para determinar el impacto de la contaminación en ríos mediterráneos y en juveniles de lubina expuestos en laboratorio al progestágeno sintético drospirenona. Por otro lado, los ensayos *in vitro* con líneas celulares, han permitido determinar la presencia de contaminantes bioactivos en una zona costera mediterránea en Croacia y alteraciones en el perfil lipídico de las células PLHC-1 cultivadas en diferentes medios y expuestas a agonistas del receptor RXR. El uso de estos modelos *in vitro* permitirá en el futuro reducir considerablemente el uso de organismos en Toxicología Ambiental.

Abstract

Chemical compounds derived from human activities continuously reach aquatic systems, where they are available to the organisms that inhabit them. Traditionally, environmental risk assessment has been based on the chemical analysis of priority pollutants in environmental matrices. However, the need to evaluate the effect of these contaminants in organisms has led to the development of biomarkers that report physiological and biochemical changes in organisms as a consequence of exposure to xenobiotics. Biomarkers used in this Thesis include biomarkers of exposure to xenobiotics and biomarkers of endocrine disruption. Recently, analytical and bioinformatic advances have made possible the application of omic technologies to discover new biomarkers at the molecular level. In addition, due to the need to reduce the number of animals in the experimental studies and to improve the practices carried out in laboratories, exposure experiments and field studies are being replaced by *in vitro* tests, where the use of lines cell phones is being applied with very favorable results.

The combination of traditional biomarkers (metabolic enzymes, changes in gene expression, analysis of steroids, metabolites in bile), together with the analysis of lipidoma, has been an efficient tool to determine the impact of pollution in Mediterranean rivers and juveniles of sea bass exposed in laboratory to the synthetic progestin drospirenone. On the other hand, *in vitro* tests with cell lines have allowed determining the presence of bioactive contaminants in a Mediterranean coastal area in Croatia and alterations in the lipid profile of PLHC-1 cells cultured in different media and exposed to RXR agonists. The use of these *in vitro* models will allow in the future to considerably reducing the use of organisms in Environmental Toxicology.

1. INTRODUCCIÓN GENERAL

1.1. Contaminantes y evaluación ambiental

La producción y uso de compuestos químicos ha incrementado exponencialmente en las últimas décadas. Actualmente, existen 143.000 sustancias químicas registradas por la Agencia Europea de Sustancias Químicas (ECHA, 2017) y 85.000 han sido incluidas en la Ley de Control de Sustancias Tóxicas de la EPA (USEPA, 2016). Estos compuestos químicos pueden llegar a los ecosistemas acuáticos a través de las aguas residuales, debido a la ineficiente eliminación en los tratamientos de depuración, pero también a través de efluentes industriales, agrícolas, vertidos directos, deposición atmosférica y escorrentías, entre otros. Una vez en los sistemas acuáticos, los contaminantes se distribuyen en diferentes compartimentos bióticos y abióticos en función de la matriz donde se liberan y de las propiedades físico-químicas de los propios contaminantes, como presión de vapor, coeficiente de reparto aoctanol-agua (Kow) y solubilidad en agua.

Los compuestos químicos, sus productos de transformación, y/o sus mezclas pueden producir efectos negativos en la calidad del agua de ríos, lagos y mares, y en los organismos que las habitan, incluyendo los peces (Loos et al., 2009; Kuzmanovic et al., 2015). Los compuestos altamente lipofílicos tienden a almacenarse en los tejidos grasos de los organismos. En músculo, hígado, gónadas o bilis de peces se han llegado a encontrar concentraciones en el rango de ng/g y µg/g. La bioamplificación y bioacumulación de estos contaminantes en los niveles superiores de la cadena trófica conlleva la dispersión de compuestos lejos de su punto de origen, como por ejemplo, en peces de aguas profundas y en zonas árticas, y pueden significar concentraciones elevadas en los organismos tras años de exposición. Algunos compuestos como el DDT, utilizado masivamente

como insecticida y plaguicida, y sus metabolitos, se siguen encontrando hoy en día en sedimentos y organismos acuáticos en el rango de ng/g, pese a que su uso se prohibió en los años 70 (Agencia para las Sustancias Tóxicas y Riesgo de Enfermedades, 2002).

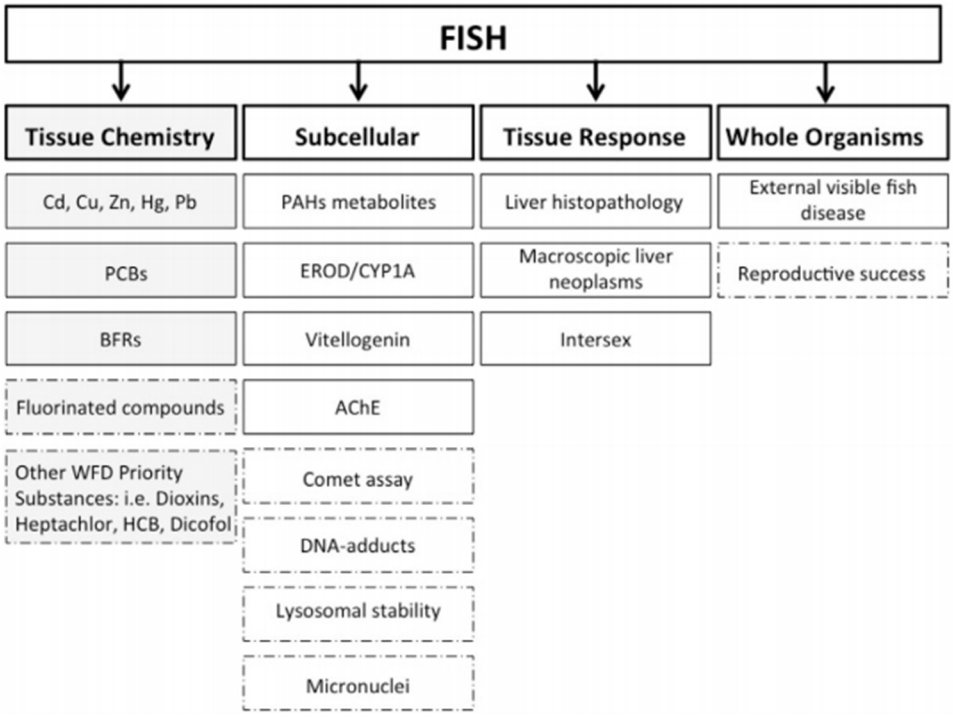
En la evaluación del riesgo ambiental, el análisis químico de contaminantes en matrices ambientales proporciona información sobre la concentración y bioacumulación de dichos compuestos en el medio ambiente y en organismos. Sin embargo, no permite la identificación individual de todos los compuestos químicos presentes y no proporciona información sobre su biodisponibilidad y su impacto biológico en organismos acuáticos. Los compuestos más abundantes o analizados con mayor frecuencia no son necesariamente los que más efectos producen y los posibles efectos sinérgicos o antagónicos en los organismos no se describen (Brack et al., 2005).

La evaluación del impacto de contaminantes en los organismos se impulsó en los años 80 con el uso de biomarcadores, definidos como respuestas moleculares, celulares, histológicas, fisiológicas o de comportamiento, medibles y adoptados como “señales de alerta temprana” del desequilibrio de los organismos (Wu et al, 2005; Galloway, 2006). A partir de entonces, en Europa se empezaron a implantar estrategias de evaluación de los efectos de los contaminantes en los ecosistemas acuáticos que incluían mediciones de respuestas biológicas (Hylland et al., 2017).

Actualmente, la Directiva Marco 2008/56/CE sobre la estrategia marina establece que los Estados miembros deben adoptar medidas necesarias para lograr y mantener un buen estado medioambiental del medio acuático antes de 2020. El Plan de Acción del Mediterráneo (PAM), aprobado en el Convenio de Barcelona y el Convenio OSPAR de Naciones

Unidas, sobre la protección del medio ambiente marino del Atlántico Norte, constituyen los marcos de política regional para el desarrollo sostenible del medio marino en la zona mediterránea. El Convenio OSPAR pone de relieve la necesidad de integración de biomarcadores químicos, de organismos completos, de tejidos y de muestras celulares, y detalla metodologías, protocolos y criterios, para la evaluación ambiental de los efectos biológicos en el medio acuático (Vethaak et al., 2017). Estos enfoques están siendo muy aceptados en foros internacionales y se han convertido en herramientas sólidas en la evaluación del riesgo ambiental. La Figura 1 recoge las principales estrategias desarrolladas en el Convenio OSPAR para la evaluación del riesgo ambiental en peces. Aunque este Convenio está enfocado en zonas marinas del Atlántico Norte, los parámetros y protocolos que recoge pueden aplicarse a otras zonas acuáticas. En esta Tesis se han aplicado en zonas mediterráneas.

Figura 1. Parámetros y protocolos incluidos en el Convenio OSPAR sobre la protección del medio ambiente marino del Atlántico Norte (Vethaak et al., 2017).



Líneas sólidas, métodos centrales; Líneas discontinuas, métodos adicionales.
BFR, retardantes de llama bromados; AchE, acetilcolinesterasa.

1.2. Metabolismo de xenobióticos

La evaluación del impacto de xenobióticos en los organismos acuáticos es un reto difícil debido a la complejidad de los sistemas biológicos y a los factores ambientales que influyen (Hylland et al., 2009; Vestheim et al., 2012). En la monitorización de la contaminación acuática los peces son ampliamente utilizados como especies centinela por el conocimiento de su taxonomía, su papel funcional en el ecosistema y la similitud de algunos de sus sistemas enzimáticos con el de los mamíferos, que permite advertir de efectos sobre la salud humana (Friberg et al., 2011; Birk et al., 2012). Especies autóctonas (*Anguilla anguilla*, *Mugil cephalus*, *Liza ramada o Barbus barbus*) e introducidas (*Cyprinus carpio*) han sido extensamente estudiadas en zonas mediterráneas, siendo el músculo y el hígado los tejidos más analizados (28 y 37 %, respectivamente) (Colin et al., 2016). Los peces están expuestos a compuestos químicos presentes en el agua o en la materia particulada (plancton y/o sedimentos) a través de la respiración, osmorregulación, ingesta, contacto dérmico y/o transferencia materna (Mills and Chichester, 2005). Los xenobióticos con características lipofílicas tienen la capacidad de cruzar membranas celulares fácilmente, y conllevan un riesgo toxicológico más elevado. Una vez dentro del organismo, estos compuestos experimentan una serie de reacciones de biotransformación catalizadas por diferentes sistemas enzimáticos (ej. citocromo P450 y enzimas de conjugación).

Citocromo P450: enzimas CYP1A y CYP3A

Los **citocromos P450** son complejos enzimáticos descubiertos en los años 80, distribuidos universalmente desde bacterias a mamíferos. Participan en una gran variedad de procesos metabólicos (Tabla 1), incluyendo la

biotransformación de un amplio número de compuestos exógenos. Este citocromo supone la primera línea de defensa contra xenobióticos potencialmente tóxicos incluyendo fármacos, disolventes orgánicos, pesticidas, tintes, hidrocarburos, alcoholes, antioxidantes y sustancias carcinogénicas, entre otros (Lewis et al., 2006; Uno et al., 2012).

Tabla 1. Nomenclatura y funciones de los isoenzimas del citocromo P450 (Lewis, 2001).

Familia	**Funciones o características**
CYP1	Metabolismo de xenobióticos
CYP2	Metabolismo de xenobióticos
CYP3	Metabolismo de xenobióticos
CYP4	Metabolismo de xenobióticos
CYP5	Síntesis de tromboxanos (eicosanoides)
CYP6	Isoforma de insectos
CYP7	Metabolismo de esteroides
CYP8	Biosíntesis de prostaglandinas
CYP9	Isoforma en insectos
CYP10	Isoforma en moluscos
CYP11	Biosíntesis de esteroides
CYP17	Biosíntesis de esteroides
CYP19	Biosíntesis de esteroides
CYP21	Biosíntesis de esteroides
CYP24	Metabolismo de vitamina D
CYP26	Metabolismo de retinoides
CYP27	Biosíntesis de ácidos biliares

El metabolismo de desintoxicación se lleva a cabo principalmente en el retículo endoplasmático de los hepatocitos, pero también en células de intestino, riñón, pulmones, cerebro o piel, entre otros (Stegeman and Hahn, 1994; Husøy et al., 1994; Ortiz-Delgado et al., 2002). Mediante reacciones de oxidación, reducción, hidrólisis y/o hidratación, los xenobióticos se

transforman en moléculas más polares, facilitando así su eliminación del organismo.

CYP1A es una isoforma del citocromo P450 que desempeña un importante papel en el metabolismo y biotransformación de una amplia variedad de sustratos, incluyendo xenobióticos y compuestos endógenos. La inducción de CYP1A en peces, ha recibido especial atención como indicador de exposición a compuestos orgánicos, incluyendo PAHs, PCB, furanos y dioxinas. Por otro lado, el **isoenzima CYP3A** es responsable del metabolismo de una amplia variedad de sustancias lipofílicas y participa en la hidroxilación de esteroides y ácidos biliares, y en oxidaciones de xenobióticos. En humanos, CYP3A representa el grupo más importante de enzimas involucrados en el metabolismo de fármacos, siendo capaz de metabolizar más del 60% de drogas y fármacos existentes (Pelkonen, 2002; Burk and Wojnowski, 2004). Trabajos recientes han puesto de manifiesto la importancia de CYP3A en el metabolismo de fármacos como ibuprofeno, carbamazepina, propanolol o fenofibrato en peces (Crespo and Solé, 2016).

Receptores nucleares: AhR y PXR

La inducción de los isoenzimas CYP1A y CYP3A está regulada principalmente por los receptores nucleares **AhR (receptor de hidrocarburos de arilos)** y **PXR (receptor X de pregnano)** respectivamente (Whyte et al., 2000; Wassmur et al., 2010; Creusot et al., 2015). El AhR, identificado en los años 80, fue uno de los primeros factores de transcripción en ser descubierto, estudiado y clonado (Nebert et al., 1981; Nebert and Eisen, 1984). Por su parte, PXR se describió por primera vez en 1998 en ratones y posteriormente en humanos (hPXR).

Después fue clonado en diversas especies de mamíferos y aves, y recientemente en especies de peces incluyendo el pez cebra (*Danio rerio*) (Ihunnah et al., 2011; Bainy et al., 2013).

Ambos receptores, AhR y PXR, actúan como sensores de xenobióticos, detectando la presencia de sustancias exógenas en las células y activando la expresión de los sistemas de biotransformación (Kliewer et al., 1998). Estructuralmente, AhR y PXR presentan un dominio de unión a ligando y un dominio de unión a ADN. De forma general, la unión ligando-receptor se transloca del citosol al núcleo formando un heterodímero transcripcionalmente activo con ARNT (receptor nuclear translocador de hidrocarburos de arilo) en el caso del AhR (AhR-ARNT), y con el receptor RXR de ácido retinoico en el caso del PXR (PXR-RXR). Estos heterodímeros activos se unen a la región de elemento de respuesta del ADN, induciendo la expresión de determinados genes y su transcripción (Fig. 2). Como consecuencia de la activación de AhR se induce la expresión de los isoenzimas CYP1A1, CYP1A2 y CYP1B1 (enzimas de Fase I), así como glutatión-S-transferasas (GST) o UDP-glucuroniltransferasas (UDPGT) (enzimas de Fase II) (Reen et al., 2002). La activación de PXR promueve la inducción de CYP3A4, enzimas de Fase II (sulfotransferasas (SULT), GST, UDPGT) y enzimas de Fase III (proteínas transportadoras de membrana (ABC)) (Ihunnah et al., 2011).

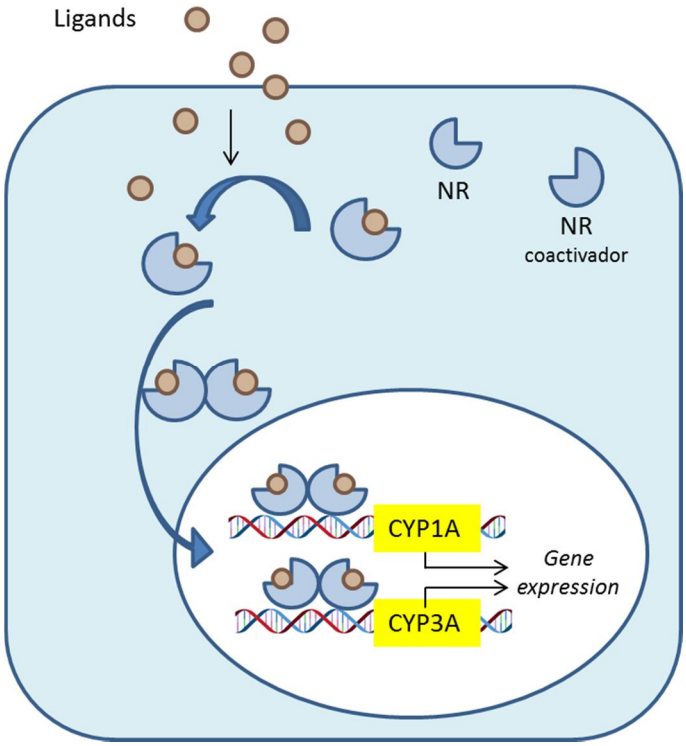


Figura 2. Mecanismo general de señalización de receptores nucleares (NR).

La bioactividad de xenobióticos y compuestos endógenos está mediada por la afinidad en la unión entre ligando y receptor. AhR se une a ligandos generalmente planares, con anillos aromáticos e hidrofóbicos, e incluyen dibenzo-*p*-dioxinas tetracloradas (TCDDs), PCBs, PAHs, furanos y algunos plaguicidas organoclorados (Stegeman and Lech, 1991; Fent, 2001; Uno et al., 2012; Colin et al., 2016). PXR está considerado un receptor promiscuo, que tiene como ligandos hormonas naturales y xenobióticos ambientales como pesticidas, fármacos y esteroides sintéticos, entre otros (Lemaire et al., 2006).

El papel de AhR y PXR en la biotransformación de xenobióticos, puede contribuir al aumento de la toxicidad de ciertos compuestos, dependiendo de los productos de biotransformación. Por ejmplo, AhR unido a algunos ligandos como PAHs, puede producir la activación de genes asociados con la formación de tumores, inmunotoxicidad, fallo

hepático, carcinogénesis , toxicidad reproductiva y neurotoxidad (Puga et al., 2000; Machala et al., 2001).

Biomarcadores de exposición

7-etoxiresorufina O-desetilasa (EROD) es el biomarcador más sensible en la determinanción de la inducción/inhibición del enzima CYP1A en peces y se considera un indicador eficaz de exposición a hidrocarburos aromáticos halogenados/policíclicos planares (PHH/PAH) y otros compuestos estructuralmente relacionados (Whyte et al., 2000; Gagnon and Rawson, 2017). Una gran cantidad de experimentos de laboratorio y estudios de campo han examinado la inducción de EROD en más de 150 especies de peces. La actividad EROD puede verse influida por la temperatura y el estado de maduración del pez, factores importantes en la interpretación de los estudios de campo que utilizan este biomarcador (Whyte et al., 2000).

Por otro lado, el biomarcador **BFCOD (7-benziloxi-4-trifluorometilcoumarin *O*-debenzilasa)** ha sido utilizado con éxito para monitorizar la actividad del isoenzima CYP3A (Creusot et al., 2015). Aunque existen pocos trabajos que traten sobre este biomarcador, en los últimos años se ha utilizado junto con EROD, como biomarcador de exposición en estudios de campo. En este sentido, Habila et al. (2017) detectaron una alta inducción de BFCOD (junto con una inducción de EROD de más de 10 veces) en hígado de barbos (*Barbus callensis*), como consecuencia del vertido de aguas residuales domésticas e industriales en la presa más importante de Argelia. Quesada-García et al. (2013) también reportaron un incremento de BFCOD en *Oncorhynchus mykiss* recolectados en una piscifactoría española, llegando a valores de hasta 15

veces más altos en septiembre respecto al resto del año. BFCOD también ha sido utilizado en ensayos *in vitro* con embriones (*Danio rerio*), larvas y células de peces (Creusot et al., 2015; Habila et al., 2017; Oziolor et al., 2017). En células de hígado de trucha (RTL-W1) concentraciones de 0.1 nM de TCDD y policlorodibenzodioxinas (PCDD) y de 0.3 µM de PAHs produjeron una inducción de hasta 34 veces la actividad BFCOD después de 24 h de exposición, mientras que conocidos agonistas de PXR como clotrimazol y ketoconazol indujeron 6 y 7 veces, respectivamente, dicha actividad a una concentración de 3 µM. Este estudio sugiere un papel potencial de AhR en la regulación de BFCOD, por lo que podría llegar a ser considerado un biomarcador no sólo de CYP3A, sino también de CYP1A (Creusot et al., 2015). De hecho, existen indicios de que CYP3A podría estar regulado, además de por PXR, por AhR/ARNT, aunque el mecanismo subyacente sigue sin conocerse (Tseng et al., 2005; Chang et al., 2013).

La activación del receptor PXR puede detectarse mediante ensayos *in vitro*, utilizando células transfectadas con plásmidos que contienen los vectores de interés, incluyendo el PXR de pez cebra (zfPxr) (Bainy et al., 2013). Contaminantes ambientales como APs, fenoles clorados, ftalatos, BPA, OCs, y compuestos organoestánicos, entre otros, han demostrado su capacidad para activar el receptor zfPxr (Milnes et al., 2008). Además de la presencia de un ligando, la activación de este receptor también depende de la especie en que PXR se haya clonado (Ekins et al., 2008; Lille-Langøy et al., 2015). Por ejemplo, compuestos como carbamazepina o tocoferoles activan el hPXR hasta 4 y 5 veces, respectivamente, mientras que no activan el zfPxr (Ekins et al., 2008).

Metabolismo de Fase II

Los metabolitos resultantes de la Fase I pasan a ser sustratos del metabolismo de Fase II (Fig. 3). El metabolito resultante de la Fase I se acopla mediante enlaces covalentes a un grupo endógeno más polar (ácido glucurónico, glutatión o sulfato), aumentando el tamaño de la molécula y su hidrosolubilidad y facilitando así su excreción a través de la bilis, orina o branquias de los peces (Clarke et al., 1991).

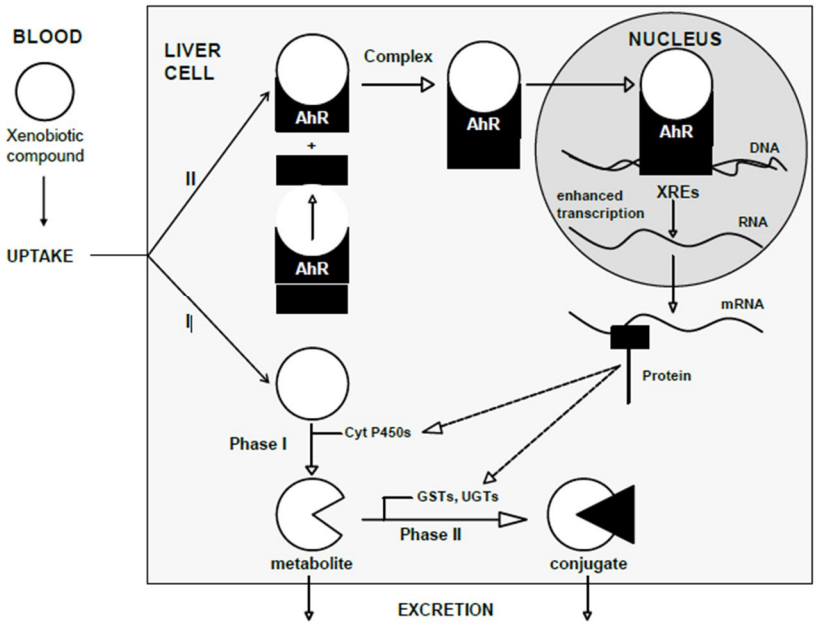


Figura 3. Esquema del metabolismo de Fase I y Fase II en una célula hepática (Van der Oost et al., 2003).

Entre los enzimas que catalizan las reacciones de Fase II, destacan las SULT y GST, localizadas principalmente en el citosol de las células, y UDP-glucuronil transferasas, que se encuentran en el retículo endoplasmático. **UDP-glucuronil transferasas (UDPGT)** catalizan la transferencia de UDPGA (sal trisódica del ácido 5'-difosfoglucurónico de uridina) a una extensa variedad de sustratos para formar principalmente *O*-glucurónidos (Mulder, 1990). La eliminación de estos conjugados depende

de su tamaño molecular y está influenciada por factores como sexo, estación, pH y temperatura. Así, la síntesis de glucurónidos es la principal ruta de inactivación y posterior excreción de xenobióticos en peces.

UGT se considera biomarcador de exposición y es utilizado en la determinación de la actividad UDPGT en peces (Clarke et al., 1991; Van der Oost et al., 2003). Numerosos estudios en laboratorio han demostrado la capacidad de algunos PAHs, PCBs y PCDDs para inducir la actividad UGT en peces (Celander et al, 1993; Otto and Moon, 1995; Fenet et al., 1998). En estudios de campo también se ha observado una mayor actividad UGT en hígado de peces muestreados en zonas contaminadas, comparado con peces de zonas más limpias (Porte et al., 2002; Gaworecki et al., 2004). Esta actividad está mediada por la activación de AhR, aunque su respuesta a contaminantes no es tan sensible como la respuesta de actividades de Fase I también reguladas por este receptor, (ej. EROD) (Clarke et al., 1992).

Dado que algunas vías enzimáticas de Fase I y Fase II son comunes para el metabolismo de xenobióticos así como de sustratos endógenos, la exposición a compuestos contaminantes que activen la actividad de los enzimas implicados en el metabolismo de xenobióticos puede incrementar la eliminación de biomoléculas, incluyendo hormonas, con negativas consecuencias en el desarrollo y la reproducción de los organismos (Stegeman et al., 1991). De igual manera, una inhibición de estas vías enzimáticas puede producir acumulación de sustratos endógenos, como la bilirrubina (Clarke et al., 1992).

1.3. Disrupción endocrina

Disruptores endocrinos

En la década de los 90 se puso de manifiesto la relación entre el incremento de la producción y uso de compuestos químicos, y el aumento de varios trastornos endocrinos en humanos y animales (Kabir et al., 2015). En 2002, el Programa Internacional de Seguridad de las Sustancias Químicas de Naciones Unidas, definió los **disruptores endocrinos (DE)** como "sustancias exógenas o combinaciones de ellas que alteran las funciones del sistema endocrino y, por lo tanto, tienen efectos perjudiciales para la salud de un organismo, su progenie, o poblaciones" (OMS, 2002). Los DE interfieren con la producción, liberación, transporte, metabolismo, unión, acción o eliminación de hormonas naturales responsables del mantenimiento de la homeóstasis y la regulación de los procesos de desarrollo y, en consecuencia, alteran la concentración y el equilibrio hormonal (Kavlock et al., 1996). Los DE, aunque pueden tener un origen natural, incluyen una amplia gama de compuestos antropogénicos. 564 sustancias químicas han sido propuestas en la Comisión Europea como sospechosas de DE, aunque finalmente sólo 66 se han incluido en la lista de compuestos prioritarios por tener claras evidencias de actividad disruptiva endocrina. Otras 52 sustancias están bajo revisión por ser potenciales DE. Algunos de los DE regulados por la Comisión Europea se detallan en la Tabla 2.

Tabla 2. Ejemplos de DE regulados por la Comisión Europea con riesgo en organismos acuáticos por exposición.

Grupo	Sustancia	AVP[1] / persistente	Preocupación
Pesticidas/Herbicidas	Clordano	Muy persistente	Alta
	DDT y derivados	AVP, muy persistente	Alta
	Lindano	AVP	Alta
	Atrazina	AVP	Alta
	Alacloro	AVP	Alta
Plástificantes	Bisfenol A	AVP	Alta
Biocidas/aditivos	Tributilo de estaño y derivados	AVP	Alta
Detergentes	4-*tert*-octilfenol	AVP	Media
	Nonilfenol	AVP	Media

[1]AVP = Alto volumen de producción.

Los efectos de los DE en los organismos están influenciados por una serie de factores, como la edad de los organismos expuestos, siendo más vulnerables en etapas de desarrollo tempranas; el tiempo de latencia desde la exposición, ya que las consecuencias de la exposición en las etapas tempranas de desarrollo pueden manifestarse en la edad adulta; los efectos aditivos o sinérgicos de los contaminantes (Crews et al., 2003); la dinámica dosis-respuesta, ya que bajas dosis de un contaminante pueden tener más efecto que dosis altas (Sheehan et al., 1999; Vom Saal et al., 2007); la capacidad de producir efectos transgeneracionales y epigenéticos (Anway and Skinner, 2006) y la diversidad y complejidad de los mecanismos de acción (Rasier et al., 2007). Una vez el interior de los organismos, los DE imitan o antagonizan efectos de hormonas endógenas (estrógenos y andrógenos); alteran el patrón de síntesis y el metabolismo de hormonas; modifican los niveles de receptores hormonales (Soto et al., 1995); y/o interferirien con las proteínas transportadoras de hormonas (Pait and Nelson, 2003).

Receptor de estrógenos

Uno de los mecanismos de acción de los DE implica su unión a un receptor celular, que pueden ser receptores nucleares, de membrana, para neurotransmisores o huérfanos (como el AhR). El **receptor de estrógenos (ER)** es una proteína perteneciente a la superfamilia de receptores nucleares que actúan como factores de transcripción, cuyo mecanismo de acción se basa en el explicado en el apartado anterior (Fig. 2) (Carson-Jurica et al., 1990; Tsai and Oimalley, 1994). En el caso del ER, tras la unión con el ligando, el receptor forma un homodímero activo que es capaz de reconocer y asociarse con las secuencias del ADN llamadas "estrogen responsive elements" (ERE), produciendo la expresión de genes estrógeno-dependientes (Tsai and Oimalley, 1994). La activación de ER por ligandos como fármacos, surfactantes y plaguicidas se ha convertido en un marcador importante de disrupción endocrina.

Una de las técnicas utilizadas para determinar la activación del ER se basa en el uso de la **levadura recombinante *Saccharomyces cerevisiae***, modificada genéticamente en 1996 con la inserción de la secuencia de ADN que codifica para el receptor estrogénico humano (hER-α) en el cromosoma de la levadura (Routledge and Sumpter, 1996). Cuando un ligando se une y activa el receptor hormonal (hER), se estimula la expresión del gen reportero, que conlleva la producción del enzima β-galactosidasa, cuya producción es medida mediante el sustrato Mu-Gal, que es transformado por β-galactosidasa en un compuesto fluorescente (Noguerol et al., 2006b). Esta técnica ha permitido evaluar la detección de DE como NP, OP, PCBs o diuron en distintas matrices ambientales (Noguerol et al., 2006ab).

Disrupción endocrina en peces

Los peces están considerados como el taxón más sensible a los efectos adversos producidos por estrógenos sintéticos en el medio acuático (Young et al., 2004; Sumpter and Johnson, 2005). La exposición a DE puede producir inducción de vitelogenina, alteración en la determinación del sexo, disminución en la tasa de crecimiento, retrasos en la reproducción y alteración en el comportamiento de los peces, entre otros (Ismail et al., 2017).

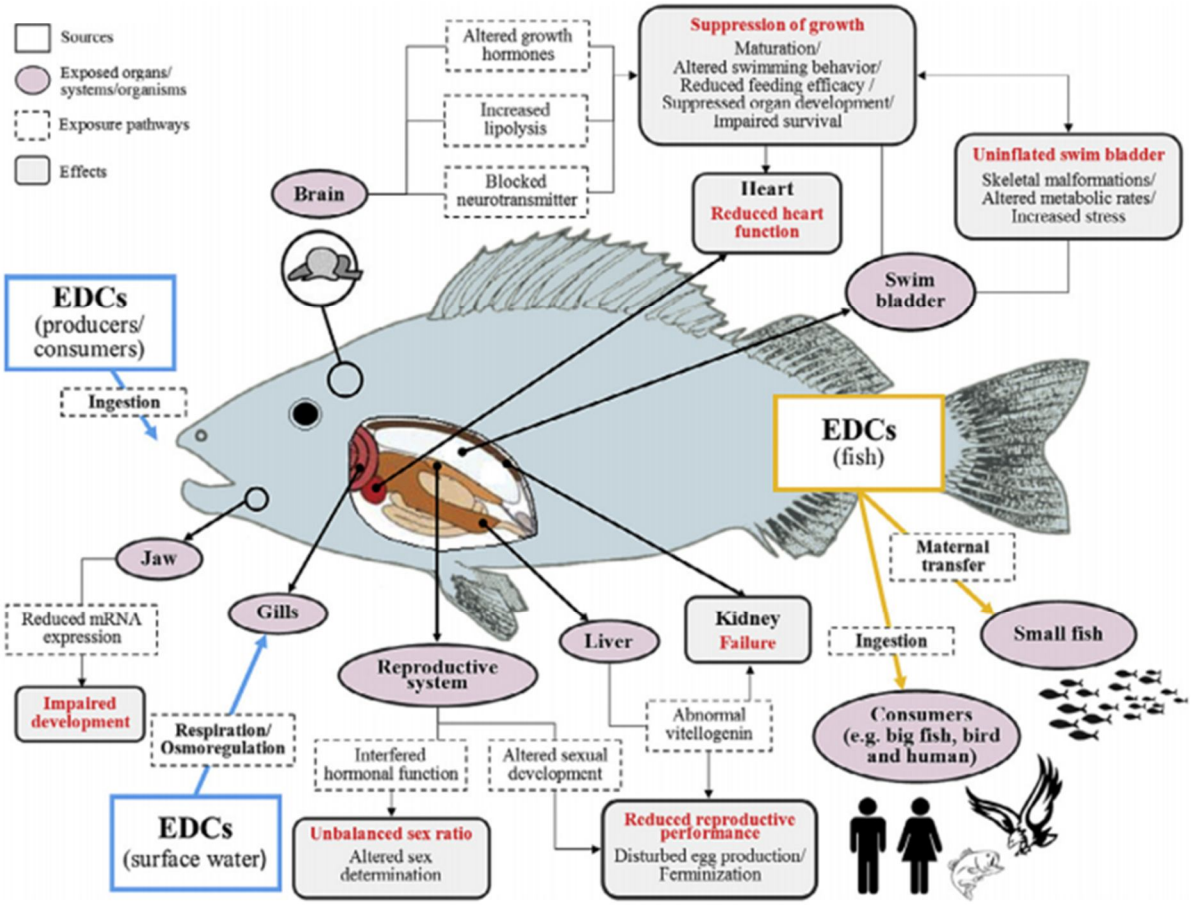


Figura 4. Vías de exposición y efectos potenciales de DE en peces (Ismail et al., 2017).

Vitelogenina en machos

Los primeros estudios de disrupción endocrina en peces se realizaron en Reino Unido en los años 90, donde se observó un aumento en los niveles de **vitelogenina (VTG)** en machos de trucha arcoíris (*Oncorhynchus mykiss*), carpa (*C. carpio*) y *Rutilus rutilus* tras la exposición a efluentes de origen urbano contaminados con etinilestradiol (EE2) y alquilfenoles (APs) (Purdom et al., 1994; Harries et al., 1996, 1997; Jobling et al., 1998). La VTG es una lipofosfoproteína sintetizada en el hígado como respuesta a los niveles plasmáticos de estradiol (E2) circulante. Cuando un compuesto estrogénico activa el ER, se induce la producción de VTG en hígado, que se transporta hasta los ovarios a través de la sangre, y se incorpora en los ovocitos como sustancia de reserva. En condiciones normales, pese a que también poseen el ER en el hígado, los machos no sintetizan esta proteína (Hyllner et al., 1991). Sin embargo, debido a la exposición a compuestos estrogénicos, la VTG se ha detectado en peces macho, incluso niveles similares que en hembras. Por ello, la presencia de VTG en peces macho se ha consolidado como un biomarcador ampliamente utilizado de exposición a compuestos estrogénicos.

Síntesis de esteroides

Andrógenos, estrógenos y progestágenos están implicados en procesos de gametogénesis, ovogénesis y espermatogénesis, y juegan un papel crucial en la diferenciación gonadal y la maduración sexual en peces (Devlin and Nagahama, 2002). Las hormonas esteroideas regulan diversos procesos que se dan en el organismo y fluctúan en respuesta a cambios físicos o patológicos. La interferencia de los xenobióticos con la síntesis de esteroides puede modificar la concentración y biodisponibilidad de

esteroides activos y, en consecuencia, afectar a la diferenciación sexual, crecimiento y maduración de gametos de peces (Fernandes and Porte, 2013). Por ello, el estudio de la alteración de las vías de síntesis de esteroides puede ser una herramienta útil para detectar la exposición a DE en peces.

La biosíntesis de esteroides comienza con el metabolismo de colesterol por isoenzimas hidroxiesteroide deshidrogenasas (HSD) del citocromo P450 (Fig. 5). De forma resumida, el colesterol se transporta desde el citosol a la membrana mitocondrial donde CYP11A cataliza su conversión en pregnenolona (P5), que a su vez, es metabolizada a progesterona (P4) por 3β-HSD, ésta se metaboliza a androstenediona (AD) por CYP17, que a su vez es metabolizada a testosterona (T) por el enzima 17β-HSD, y T es aromatizada por CYP19 para obtener estrona o E2. Así, el isoenzima CYP19 es clave para la conversión de andrógenos en estrógenos (Skolness et al., 2013).

El **isoenzima CYP17**, codificado por el gen *cyp17* localizado en el retículo endoplasmático, tiene un papel importante en la síntesis de esteroides en gónadas de peces teleósteos, donde se han identificado dos formas de CYP17: CYP17A1 y CYP17A2 (Ding et al., 2012). El CYP17A1 posee actividades hidroxilasa y liasa, mientras que CYP17A2 sólo tiene actividad hidroxilasa. La actividad 17α-hidroxilasa cataliza la hidroxilación de P5 a 17α-P5y de P4 a 17α- P4, mientras que la actividad liasa, cataliza la conversión de 17α-P5 en DHEA y de 17α-P4 en AD. El isoenzima CYP17 desempeña un papel clave en la orientación de la esteroidogénesis hacia la biosíntesis de glucocorticoides como cortisol, si CYP17 sólo manifiesta actividad 17α-hidroxilasa, o de esteroides sexuales, si manifiesta ambas actividades, 17α-hidroxilasa y 17,20-liasa (Miller, 2002).

El **isoenzima CYP11β** se localiza en la membrana mitocondrial y está implicado en la conversión de P4 a cortisol y en la formación de andrógenos. AD es un precursor de T, y ambas pueden ser transformadas en los testículos en metabolitos 11-hidroxilados por CYP11β (11β-hidroxiandrostenediona y 11β-hidroxitestosterona). Estos metabolitos hidroxilados son precursores de 11-ketoandrostenediona y 11-ketotestosterona, ambos considerados como andrógenos activos en teleósteos.

El **isoenzima CYP19 (P450 aromatasa)** cataliza la conversión de andrógenos (T y AD) en estrógenos (estrona y E2) y desempeña un papel clave en el control de los niveles de estrógenos durante los procesos de diferenciación sexual, así como en el control de la reproducción y el desarrollo gonadal. En peces, las principales isoformas son CYP19A y CYP19B, esencialmente presentes en ovarios y cerebro, respectivamente.

Se ha descrito que CYP17 y CYP11β, son sensibles a la modulación por xenoestrógenos, detectándose por lo general una inhibición tras la exposición a EE2 (Filby et al., 2007; Garcia-Reyero et al., 2009). CYP19 representa una diana potencial para un amplio rango de DE, capaces de alterar la expresión y/o la actividad de CYP19 en experimentos *in vivo* e *in vitro*. Por ejemplo, la actividad P450 aromatasa en cerebro de perca y en ovario de trucha arcoíris se ha visto inhibida tras la exposición a TBT y algunos fungicidas, produciendo un incrementeo en los niveles de andrógenos relacionados con masculinización en peces (Cheshenko et al., 2008). En carpas capturadas en un canal de riego y después de una planta de tratamiento de aguas residuales, una inhibición de hasta 2 veces de la actividad CYP19 se ha observado (Lavado et al., 2004). CYP19 principalmente se regula vía ER, aunque algunos estudios apuntan a una

posible modulación a través del receptor AhR o incluso del receptor proliferador de peroxisomas (PPAR) (Cheshenko et al., 2008).

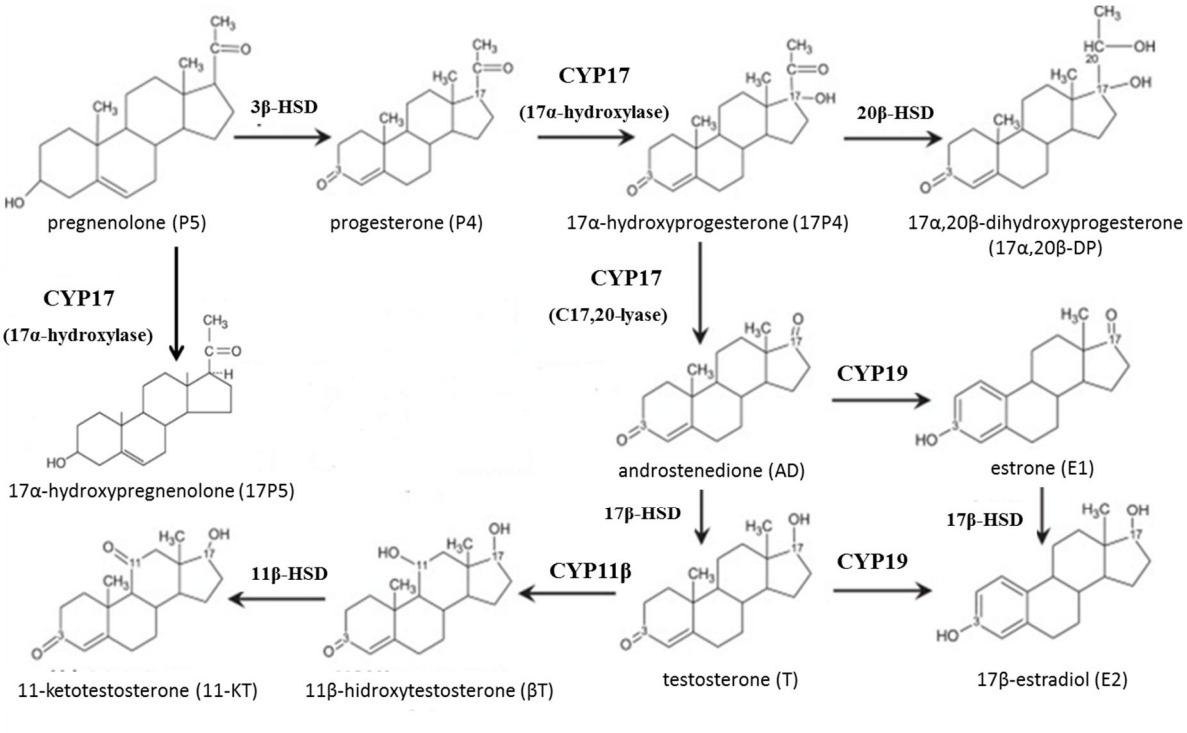


Figura 5. Vías metabólicas y hormonas esteroideas involucradas en la biosíntesis de esteroides en peces.

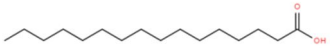
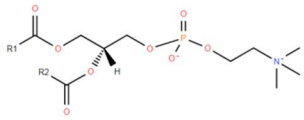
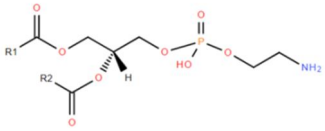
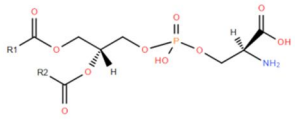
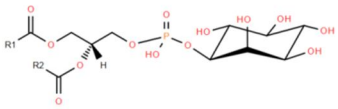
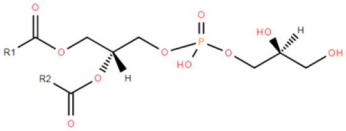
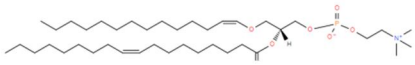
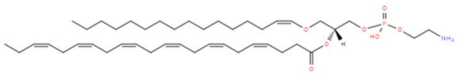
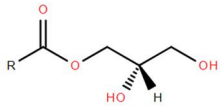
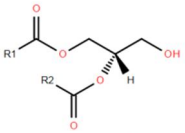
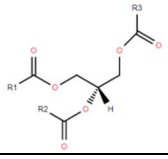
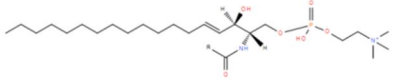
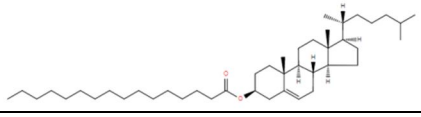
1.4. Disrupción lipídica

Lípidos

Los lípidos son un grupo de biomoléculas muy diverso y complejo. En general, están estructuralmente formados por una cabeza polar y cadenas alifáticas apolares, que varían en longitud (números de átomos de carbono), grado de insaturación, ubicación de dobles enlaces y ramificaciones (Han et al., 2012). Los lípidos son los principales constituyentes orgánicos de los peces, junto con las proteínas (Tocher, 2003). La International Lipid Classification and Nomenclature Committee bajo el patrocinio del Consorcio de LIPID Metabolites and Pathways Strategy (LIPID MAPS®), clasifica los lípidos en 8 grupos: ácidos grasos (FA), glicerofosfolípidos (GP), glicerolípidos (GL), esfingolípidos (SP), esteroles, prenoles, sacarolípidos y poliquétidos (Fahy et al., 2005; 2009). En la Tabla 3 se muestran los principales grupos de lípidos analizados en esta Tesis.

Los lípidos están involucrados en la formación de membranas celulares y en la producción y almacenamiento de energía, pero también tienen un papel importante en diferentes funciones biológicas, como síntesis de hormonas, procesos de señalización celular, apoptosis, crecimiento y supervivencia celular (Van Meer, 2005; Orešič et al., 2008). Se ha observado que la interrupción del metabolismo de lípidos y de las vías de señalización asociadas, alteran la función celular, dando como resultado una serie de trastornos y enfermedades importantes como obesidad, enfermedades cardiovasculares, diabetes, alteraciones neurodegenerativas y cáncer (Shi and Burn, 2004; Shui et al., 2007; Cífková et al., 2015).

Tabla 3. Categorías de lípidos y principales subclases estudiadas en esta Tesis, según la clasificación establecida por el sistema LIPID-MAPS.

Categoría	Subclase	Estructura
Ácidos grasos (FA)		
Glicerofosfolípidos (GP)	Fosfatidilcolina (PC)	
	Fosfatidiletanolamina (PE)	
	Fosfatidilserina (PS)	
	Fosfatidilinositol (PI)	
	Fosfatidilglicerol (PG)	
	PC-Plasmalógeno (PC-P)	
	PE-Plasmalógeno (PE-P)	
Glicerolípidos (GL)	Monoacilglicerol (MG)	
	Diacilglicerol (DG)	
	Triacilglicerol (TG)	
Esfingolípidos (SP)	Esfingomielina (SM)	
Esteroles (ST)	Colesterol éster (CE)	

Lipidómica

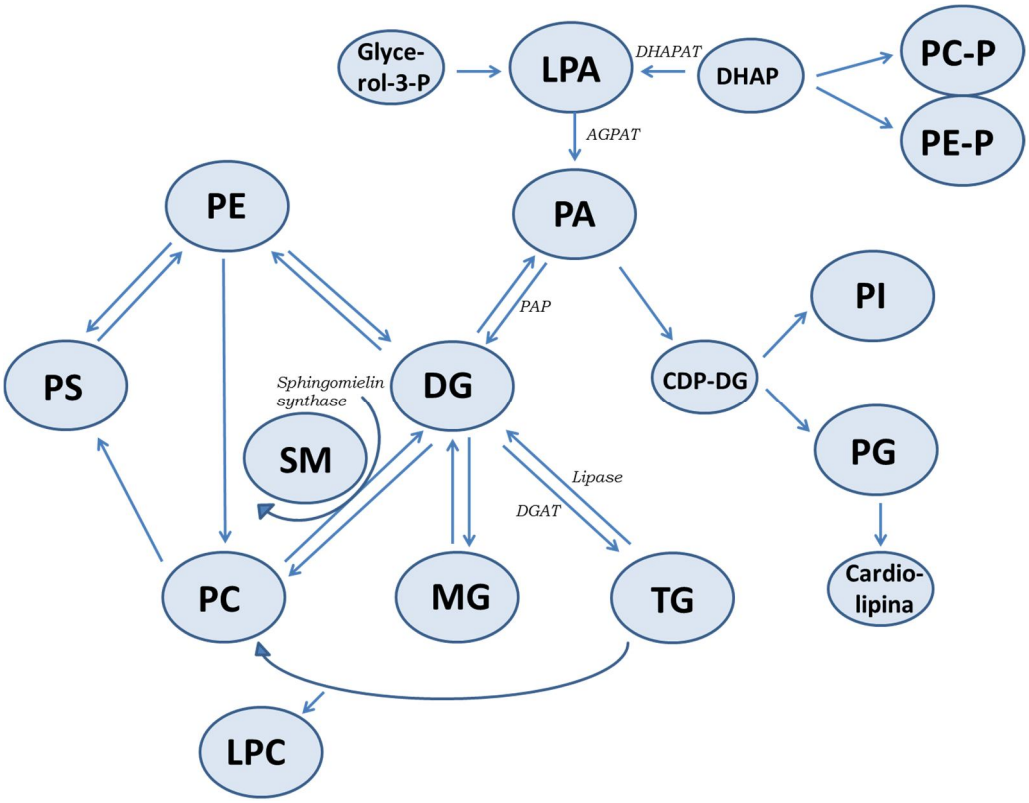
En los años 50 se obtuvieron los primeros indicios de que compuestos xenobióticos podían producir un aumento de la grasa corporal en ratas (Lesser et al., 1952). Estos estudios se desarrollaron principalmente con metodologías gravimétricas y colorimétricas. Sin embargo, desde el año 2003, avances en el campo de la espectrometría de masas (MS), como el reemplazo de cuadrupolos convencionales por analizadores como Orbitrap o tiempo de vuelo (ToF), junto con mejoras en bioinformática, han facilitado el rápido progreso y el éxito de los estudios lipidómicos. Actualmente se consiguen detectar y medir moléculas con una alta sensibilidad, resolución y precisión en células aisladas, levaduras, moluscos y peces, entre otras matrices (Sommer et al., 2006; Scano et al., 2008; Ejsing et al., 2009; Blanksby and Mitchell, 2010; Gorrochategui et al., 2014; Liu et al., 2017). Sin embargo, el número de moléculas actualmente detectadas refleja sólo una fracción de la diversidad total de lípidos presentes en los organismos, ya que las predicciones apuntan a que existen de miles a cientos de miles de especies en el lipidoma celular (Han and Jiang, 2009; Shevchenko et al., 2010; Yore et al., 2014).

Biosíntesis de lípidos

El equilibrio entre absorción, transporte, almacenamiento, biosíntesis, metabolismo y catabolismo de lípidos definen el lipidoma de un organismo, célula o tejido. La síntesis de FA se lleva a cabo en el citosol. Mientras que prácticamente todas las células son capaces de sintetizar fosfolípidos, la biosíntesis de TG tiene lugar principalmente en hígado y tejido adiposo. Los FA se sintetizan a partir de una molécula de Acetil-CoA., que se transporta desde la mitocondria al citosol en forma de citrato, mediante una

proteína transportadora, iniciando así la síntesis *de novo*. Una vez formados, los FA pueden entrar a formar parte de las distintas subclases de lípidos en función de las necesidades de la célula. Así, por ejemplo, se pueden acumular en forma de TG, para el almacenamiento de energía metabólica, o bien pueden incorporarse a los GP para la formación de membranas celulares. Los lípidos mayoritarios en células eucariotas son PC y PE, y se distribuyen asimétricamente en la membrana plasmática: la mayor parte de PC están localizadas en el folíolo externo, mientras que PE se enriquecen en el folíolo interno (Devaux, 1991). A partir de la información recopilada en numerosos artículos, las vías de formación de las diferentes subclases se recogen en la Figura 6.

Figura 6. Propuesta de esquema del metabolismo de lípidos en peces.



Receptores de lípidos

Existen evidencias de que el metabolismo lipídico está regulado por el factor proliferador de peroxisomas (PPAR) y el receptor de retinodes (RXR). Hay tres isoformas diferentes de PPAR (α/β/γ) que se distinguen por su distribución en tejidos, la especificidad del ligando y sus funciones fisiológicas. PPARα y PPARβ se expresan principalmente en hígado y músculo, respectivamente, y facilitan la combustión de energía, mientras que PPARγ se expresa preferiblemente en el tejido adiposo y contribuye al almacenamiento de energía mediante la inducción de la adipogénesis (Grygiel-Górniak, 2014). Por otro lado, el receptor X de retinoides (RXR), del cual también existen 3 isoformas (α/β/γ), participa en el desarrollo, diferenciación, metabolismo y muerte celular. RXRα abunda en epidermis, intestino, riñón e hígado; RXRβ está presente en prácticamente todos los tejidos, y RXRγ predomina en cerebro y músculo (Dawson and Xia, 2012).

Los receptores PPAR pueden unirse al ADN interactuando con secuencias específicas, conocidas como elementos de respuesta al receptor del proliferador peroxisomal (PPRE), regulando así la expresión de genes implicados en el metabolismo de lípidos (Sandoval et al., 2009). Los PPAR también forman heterodímeros con RXR, que pueden ser activados por ligandos endógenos (ácidos grasos y eicosanoides) y exógenos (ftalatos, herbicidas, fármacos, fibratos, PAHs y PCBs) (Berger and Moller, 2002; Cajaraville et al., 2003; Kota et al., 2005; Christodoulides and Vidal-Puig, 2010). Por su parte, el RXR puede ensamblar tanto homodímeros como heterodímeros con el receptor de ácido retinoide (RAR) y el PPAR, entre otros. El principal agonista de RXR es el 9-*cis*-ácido retinoico (Dawson and Xia, 2012).

Disrupción lipídica

Algunos contaminantes ambientales, incluyendo DE, pueden alterar el balance de lípidos en vertebrados e invertebrados (Grün and Blumberg, 2006; Chamorro-Garcia et al., 2013). BPA, piriproxifen y la hormona natural 20-hidroxiecdisona favorecen la acumulación de TGs en *Daphnia magna*, mientras que metopreno, fenarimol y NP la disminuyen (Jordão et al, 2016). BPA, el herbicida atrazina, TBT y TPT también interfieren en el metabolismo de lípidos en anfibios (Grün et al., 2006; Higley et al. 2013; Capitão et al., 2017). En ratones, el hexabromociclododecano, PCB 153, TBT y TPT, algunos ftalatos y BPA estimulan la adipogenesis, alteran el balance de glucosa en hígado, inducen la acumulación de lípidos en tejido adiposo, promueven la obesidad y/o agraban enfermedades como la de 'hígado graso' (Hurst and Waxman, 2003; Masuno et al., 2005; Wahlang et al., 2013; Pereira-Fernandes et al., 2013; Yanagisawa et al., 2014).

El TBT es el compuesto del que más información se dispone como disruptor lipídico en peces teleósteos. Se han descrito incremento en el contenido lipídico, en niveles de TGs, aumento del tamaño de adipocitos y alteraciones en el peso corporal tras exposiciones puntuales o crónicas a este compuesto en *Oncorhynchus tshawytscha*, *Danio rerio, Carassius auratus, Sebastiscus marmoratus* (Capitão et al., 2017). La exposición a BPA y sus derivados también produce incremento en la acumulación de lípidos y aumento de peso en *Danio rerio* y *Sparus aurata* y una disminución en el contenido de TGs en suero en hembras de *Gobiocypris rarus* (Maradonna et al., 2015; Riu et al., 2011, 2014; Guan et al., 2016). OP fomenta la acumulación de lípidos en hígado de *Sparus aurata* y la exposición a organofosfatos incrementa el contenido de TG en hígado de *Carassius gibelio* (Xu et al., 2012). Otros compuestos incluyendo ftalatos,

fibratos, herbicidas, DDTs o PCBs también alteran el balance de lípidos en peces (Yadetie et al., 2014; Capitão et al., 2017). El mecanismo de acción que activa las alteraciones en el metabolismo lipídico se basa en la unión de xenobióticos con los receptores PPARγ y RXR, principalmente (Ouadah-Boussouf and Babin, 2016; Capitão et al., 2017).

La investigación de alteraciones lipídicas y de los mecanismos de acción de los contaminantes, puede contribuir a la detección de biomarcadores de exposición para revelar el efecto de disruptores lipídicos.

1.5. Uso de líneas celulares en toxicología ambiental

La investigación con animales en los últimos años ha sido fuente de controversia. En España, 808.827 animales fueron utilizados en el año 2014 en experimentación científica (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (Mapama), 2017). El Tratado de Funcionamiento de la Unión Europea (TFUE) reconoce que "los animales son seres sensibles" y establece que "se han de tener plenamente en cuenta las exigencias de bienestar animal en investigación y desarrollo tecnológico". En este contexto, la estrategia de las 3R (Reemplazo, Reducción y Refinamiento) se plantea como el principio que debe regir la investigación con animales. Con estas premisas, el uso de órganos, explantes de tejidos, células, componentes celulares, proteínas y/o biomoléculas en ensayos *in vitro*, se han desarrollado como alternativas al uso de ánimales. Aunque plantean ciertos inconvenientes, estos métodos permiten controlar las condiciones ambientales y son más rápidos, más reproducibles, más baratos, producen menos residuos tóxicos y a menudo son más sensibles que los ensayos *in vivo.* La implantación de estas nuevas estrategias ha permitido reducir hasta un 40% el número de animales utilizados en investigación (Mapama, 2017).

El primer nivel de acción del compuesto tóxico en el organismo empieza con la interacción entre célula y xenobiótico. Los ensayos *in vitro* basados en la utilización de líneas celulares son una herramienta útil para estudios toxicológicos porque permiten estudiar los mecanismos de acción de xenobióticos aislados y de mezclas, y su potencial tóxico, incluyendo efectos aditivos, sinérgicos o antagónicos. La Agencia de Validación de Métodos Alternativos (ECVAM por sus siglas en inglés) acepta los cultivos celulares como métodos alternativos válidos en investigación.

La primera línea celular de peces, se estableció a partir de las gónadas de trucha arco iris juvenil (RTG-2). Desde entonces, casi 300 líneas celulares derivadas de peces se han establecido (Wolf and Quimby, 1962; Lakra et al., 2011). La mayoría de las utilizadas en toxicología acuática provienen de especies de agua dulce, principalmente de salmónidos o ciprínidos (RTG-2); procedentes de tronco caudal de *Lepomis macrochirus* (BF-2), de embriones de salmón (CHSE-124), de tejido epitelial de fathead minnow (EPC) y, más recientemente, de hígado de pez cebra (ZFL), pero también se utilizan cultivos procedentes de peces marinos como el derivado de aleta caudal de *Sparius aurata* (SAF-1) (Lakra et al., 2011, Morcillo et al., 2016).

La utilización de líneas celulares se ha aplicado con éxito en toxicología, en ensayos de viabilidad, morfología, metabolismo, permeabilidad de membrana celular, proliferación o cinética de crecimiento (Fent, 2001). Actualmente, entre otras aplicaciones, su uso está generando interés para determinar alteraciones en la composición intracelular de lípidos después de la exposición a xenobióticos ambientales. Por ejemplo, Dimastrogiovanni et al. (2015), reportaron que BPA y el ftalato DEHP aumentaban significativamente la acumulación intracelular de TGs, mientras TBT, 4-NP, BPA y DEHP alteraban la composición de los lípidos de membrana (PC y PC-P) en la línea celular de hígado de trucha arco iris (RTL-W1).

Línea celular PLHC-1

La línea celular PLHC-1, derivada de un carcinoma hepatocelular de hembra adulta de la especie de ciprínido *Poeciliopsis lucida* (Fig. 7), tiene la capacidad de expresar el receptor AhR. Se ha detectado inducción de

CYP1A tras la exposición de estas células a extractos de sedimentos acuáticos, escorrentías urbanas, muestras de suelos contaminados y contaminantes aislados (Babich et al., 1991; Hahn et al., 1996; Fent and Batscher, 2000; Thibaut and Porte, 2008; Traven et al., 2008; Louiz et al., 2008; Zaja et al., 2013; Pérez-Albaladejo et al., 2016). PLHC-1 también se ha utilizado para determinar repuestas del isoenzima CYP3A, enzimas antioxidantes (SOD, CAT, GPx, glutatión reductasa (GR) y glutatión -S-transferasa (GST)), peroxidación lipídica (LPO), inducción de metalotioneinas, cito- y geno-toxicidad y alteración de propiedades de membranas (Choi and Oris, 2000; Puerto et al., 2009; Schnell et al., 2009; Creusot et al., 2015; Habila et al., 2017). Recientemente, esta línea celular se ha utilizado para evaluar la toxicidad de nanopartículas (Bermejo-Nogales et al., 2016).

En 2008 se detectó por primera vez la presencia de los tres receptores PPAR (α/β/γ) en PLHC-1 (Caminada and Fent, 2008). Aunque esta información no está confirmada, con estos indicios la línea celular PLHC-1 se plantea como un buen modelo para la detección de disruptores lipídicos.

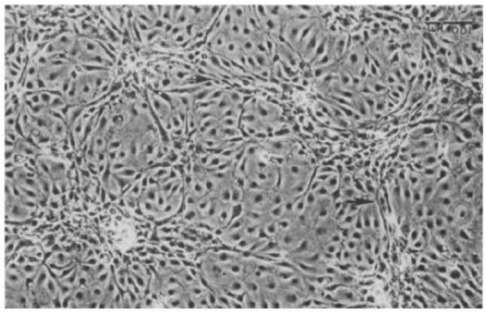


Figura 7. Imagen de células PLHC-1, derivadas de un carcinoma hepatocelular de *Poeciliopsis lucida* (100x).

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2. OBJETIVOS

El **objetivo general** de esta Tesis es investigar el impacto de xenobióticos en el medio acuático, con especial atención al estudio de disruptores endocrinos y lipídicos, mediante el uso combinado de biomarcadores y nuevas aproximaciones toxicológicas basadas en la utilización de líneas celulares.

Concretamente esta Tesis persigue los siguientes objetivos específicos:

1. Evaluar el estado ambiental de ríos mediterráneos (Ebro y Ripoll) mediante el uso combinado de análisis químico de contaminantes prioritarios y biomarcadores moleculares y subcelulares en especies autóctonas de peces.

2. Evaluar el potencial del progestágeno sintético drospirenona de actuar como disruptor endocrino en juveniles de lubina, durante un periodo de desarrollo particularmente sensible a esteroides exógenos.

3. Caracterizar la calidad ambiental de sedimentos marinos de una zona costera mediterránea mediante el uso combinado de la línea celular PLHC-1, una línea celular transfectada con zfPxr y ensayos con levaduras recombinantes.

4. Caracterizar el lipidoma de la línea celular PLHC-1, su respuesta a compuestos modelo y la influencia de diversos medios de cultivo, mediante espectrometría de masas de alta resolución.

En conjunto, en este trabajo se ilustra a través del uso de diversos bioensayos, experimentos de exposición y estudios de campo, la complementariedad de estas aproximaciones toxicológicas para investigar el impacto de contaminantes en el medio ambiente acuático.

La Tesis se divide en 3 capítulos, presentados como 1 o varios artículos científicos, dirigidos a abordar los objetivos específicos descritos anteriormente.

Capítulo 1.

Artículo 1. "The combined use of chemical and biochemical markers to assess the effect of dredging in the lower course of the Ebro River". Sometido en *Ecotoxicology and Environmental Safety,* Nov-2017.

Artículo 2. "Multistress assessment including new lipidomic approaches in the Ripoll River: a field study", *In Preparation*.

Capítulo 2.

Artículo 3. "Drospirenone intake alters plasmatic steroid levels and *cyp17a1* expression in gonads of juvenile sea bass", publicado en *Environmental Pollution* 213, 541-548 (2016). Factor de Impacto: **5.099**.

Capítulo 3.

Artículo 4. "Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays" publicado en *Science of The Total Environment* (2017), *In Press.* Factor de Impacto: **4.900**.

Artículo 5. "Lipidomic analysis of PLHC-1 cells: the effects of culture medium, retinoic acid, and rosiglitazone", *In Preparation*.

3. RESULTADOS

CAPÍTULO 1

Combinación de biomarcadores para la evaluación ambiental de ríos mediterráneos

Artículo 1

'The combined use of chemical and biochemical markers to assess the effect of dredging in the lower course of the Ebro River'

Maria Blanco[1], Denise Fernandes[1], Juliane Rizzi[2], David Huertas[1], Nuno Caiola[3], Pilar Fernández[1], Cinta Porte[1]

[1]Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

[2]PhD Program in Water Resources and Environmental Engineering, Federal University of Paraná, 81531-980 Curitiba, Paraná, Brasil.

[3]IRTA Aquatic Ecosystems, Ctra. de Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain.

Ecotoxicology and Environmental Safety, submitted

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ABSTRACT

The lower course of the Ebro River is polluted with high concentrations of mercury and organochlorine compounds dumped by a chloro-alkali plant during the last century. A remediation plan, including building of a protective wall, removal and disposal of polluted sediments started in 2012. With the aim of assessing the effects of dredging of contaminated sediments and potential alterations of water quality, areas located upstream (RR) and downstream (BE, A) the chemical plant (FL) were monitored prior (October 2012) and during dredging (June 2013) using roach (Rutilus rutilus) as sentinel organisms. Concentrations of organochlorine compounds (OCs) in fish muscle and biliary levels of polycyclic aromatic hydrocarbons (PAHs), galaxolide (HHCB) and alkyphenols (APEs) were determined together with selected biomarkers (7-ethoxyresorufin-O-deethylase (EROD), 7-benzyloxy-4-trifluoromethyl-coumarin *O*-debenzyloxylase (BFCOD) and UDP-glucuronyltransferase (UGT)) in the liver. The obtained results proved the effectiveness of the wall retaining suspended particles and avoiding further contamination of downstream sites as fish sampled at downstream sites showed up to 9-fold higher concentrations of OCs in muscle during wall construction than during dredging. EROD and UGT activities were induced in fish from downstream

sites; however, no clear response to the observed pollution gradient was detected.

Key words: *Rutilus rutilus, Ebro River, organochlorine compounds, biomarkers, bile.*

1. Introduction

The Ebro River (NE Spain) with a drainage area of about 85,000 km² and a population of three million people living in the watershed, receives significant amounts of industrial and urban discharges together with agricultural inputs (Fernández et al., 1999; Claver et al., 2006; Silva et al., 2011). Flix, located in the low course of the river, is one of the most heavily polluted areas due to discharges from a chloro-alkali plant. This area has received over a hundred years industrial wastes containing a mixture of metals (e.g. Cd, Hg, As, Cr, Cu, Zn, Pb, Se) and organochlorine compounds (OCs) (hexachlorobenzene (HCB), pentachlorobenzene (PeCB), DDTs, polychlorobiphenyls (PCBs), polychloronaphtalenes (PCNs) and polychlorostirens (PCEs)), among other pollutants (Suárez-Serrano et al., 2010). This resulted in the accumulation of 500,000 tons of wastes in the adjacent riverbed and the consequent contamination of river sediments with concentrations of Hg, PeCB, PCBs, DDTs and PCNs in the range from 1 to 640 µg/g (Grimalt et al., 2003; Palanques et al., 2014). Moreover, high concentrations of metals, PCBs, PeCBs, HCB, HCHs, DDE and DDTs have also been reported in sediments located downstream Flix, confirming the transport of pollutants to the lower part of the river (Bosch et al., 2009). In addition, significant amounts of metals and OCs have been reported in biota from Flix and downstream areas (Lavado et al., 2006; Faria et al., 2010; Soto et al., 2011; Alcaraz et al., 2011; Huertas et al., 2016).

Biomarkers have been successfully applied in Mediterranean rivers to monitor the environmental status and the potential effects of pollutants in aquatic organisms (Colin et al., 2016). Thus, a significant increase in EROD activity together with a depletion of acetylcholinesterase (AChE) activity and high levels of metallothioneins have been reported in carps from Flix and associated to exposure to PCBs, DDTs, nonylphenol and mercury, among other pollutants (Lavado et al., 2006). Elevated CYP1A gene expression was detected in barbels and carps from Flix and related to exposure to dioxin-like PCBs and other OCs (Quirós et al., 2007, Olivares et al., 2010, Eljarrat et al., 2008). Similarly, Faria et al. (2010) reported high EROD activity, lipid peroxidation and DNA damage in mussels and crayfish from the area.

Due to this severe pollution, an ambitious remediation plan started in 2010 with the construction of a retaining wall to isolate the contaminated sludge. The building of the retaining wall finished in 2012 and dredging of the contaminated sediments started in March 2013. These sediments were removed, subsequently processed in a nearby treatment plant and disposed in a landfill (Quesada et al., 2014).

As dredging activities could represent a considerable release of toxic compounds accumulated in sediments to the river water, posing a risk to aquatic organisms, this study was designed to investigate the potential impact of dredging in aquatic fauna using roach (*Rutilus rutilus*) as sentinel species, as this species has been successfully used in a number of biomonitoring studies all over Europe to assess the impact of water quality in aquatic fauna (Bjerregaard et al., 2006; Gerbron et al., 2014). Sampling campaigns were performed in October 2012 (wall construction) and in June 2013 (during dredging) in four representative stations: Ribarroja, a potential reference site located upstream of Flix (Navarro et al., 2009);

Flix, the main focus of pollution, and two downstream stations, Benifallet and Amposta. Combined chemical analysis of selected contaminants in muscle and bile, together with several biochemical markers including 7-ethoxyresorufin *O*-deethylase (EROD) activity, CYP3A catalytic probe (BFCOD) and phase II enzyme UDP-glucuronyltransferase (UGT)) were selected for the study, since they are induced by a variety of xenobiotics, and catalyze the oxidative metabolism and conjugation of both xenobiotics and endogenous compounds.

2. Material and methods

2.1. Chemicals

Uridine 5'-diphosphoglucuronic acid (UDPGA), *p*-nitrophenol (pNP), NADPH, 7-ethoxyresorufin (7-ER), bovine serum albumin (BSA; fatty acid free, ≥ 99% purity), methyl tert-butyl ether (MTBE) and hydroxylamine hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin (7-BFC) was purchased from Cypex (Dundee, Scotland, UK). Cellulose extraction cartridges were obtained from Whatman Ltd. (UK). External standard mixtures of PeCB, HCB, HCHs (α-HCH, β-HCH, γ-HCH and δ-HCH), DDTs (2,4'-DDE, 4,4´-DDE, 2,4'-DDD, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT) and PCBs (congeners 28, 52, 101, 118, 138, 153 and 180) and the internal standards PCB 200 and PCB 142 were purchased from Dr. Ehrenstofer (Wesel, Germany). 1,2,4,5-tetrabromobenzene (TBB) was from Aldrich-Chemie (Steinheim, Germany) and all solvents and other reagents were obtained from Merck (Darmstadt, Germany).

2.2. Sample collection and preparation

Male and female roach (*Rutilus rutilus*) were collected by DC electrofishing from four stations along the Ebro River: Ribarroja (RR) a relatively clean site located 6 km upstream from the dredging site; Flix (FL), the historically polluted site; Benifallet (BE) and Amposta (A), located 42 and 82 km downstream from Flix, respectively (Fig. 8). Samplings were carried out in October 2012 and June 2013. Immediately after collection, fish were killed by severing the spinal cord. Total length and weight were measured (Table 4). Liver, muscle and bile were dissected and immediately frozen in liquid nitrogen, and stored at -80ºC. A subsample from the central part of gonad tissue was fixed in 10% formaldehyde buffered with 100 mM sodium phosphate at pH 7.4 for histological examination.

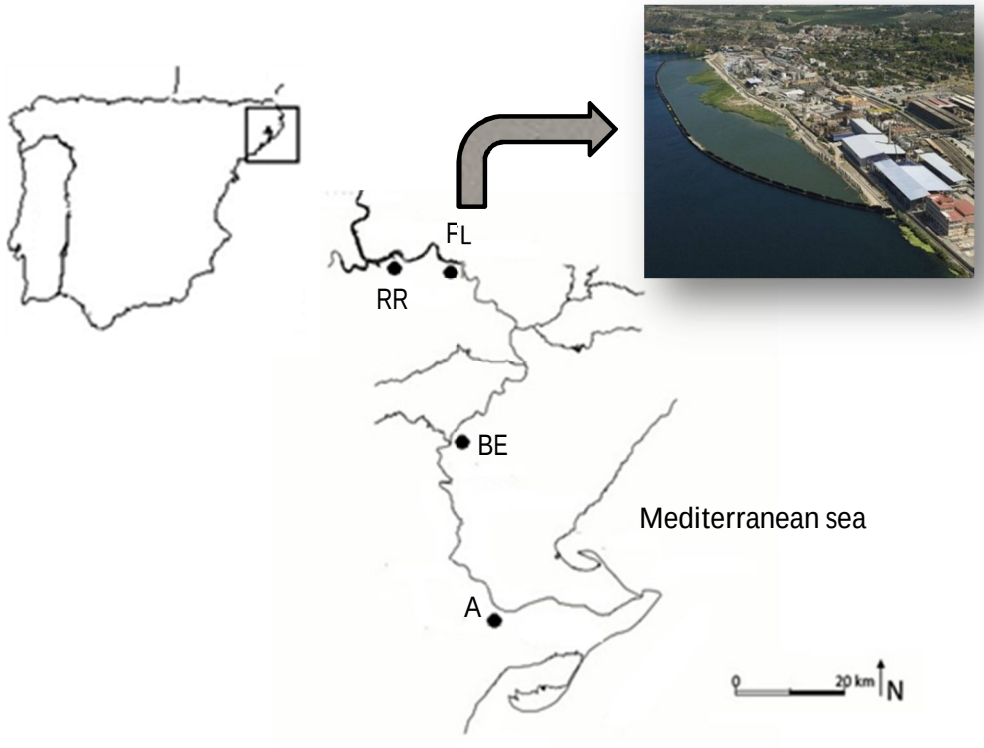

Figure 8. Map of the sampling sites in Ebro River. RR: Ribarroja; FL: Flix; BE: Benifallet; A: Amposta.

2.3. Histological analysis of gonads

Gonad samples fixed in 10% formalin for 24 h were dehydrated with ethanol, cleared in Histo-Clear (National Diagnostic, Atlanta, USA) and embedded in paraplast (Sigma–Aldrich, Steinheim, Germany). Tissue sections (7 μM) were stained with haematoxylin-eosin Y and examined by light microscopy. Gonads were sorted into four stages of sexual maturation (0- undeveloped, I- early maturation, II- mid maturation, III- final maturation) following a modification of Geraudie et al. (2010).

2.4. Chemical analysis

Analysis of bile samples

Hydroxylated metabolites of PAHs, alkylphenols and galaxolide (HHCB) were determined in bile samples following the method described in Escartín and Porte (1999) with some modifications. Briefly, 20 to 100 mg of bile were incubated for 1 h at 40ºC in 0.4 M acetic acid/sodium acetate buffer pH 5.0 containing 2000 units of β-glucoronidase and 50 units of sulphatase. Hydrolysed metabolites were extracted with ethyl acetate; the extracts were recombined and concentrated under a nitrogen stream. Dry residues were derivatized by the addition of 100 μL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1 h at 70ºC, dried and reconstituted in ethyl acetate (1:10, w:v.). Analyses were carried out by gas chromatography-mass spectrometry (GC-MS) operating in electron impact (EI) and selected ion monitoring (SIM) modes. The equipment was an Agilent 6890 series GC system with an Agilent 5973 Network mass selective detector. The column, a TRB-5MS 30 m x 0.20 mm i.d., film thickness 0.25 μm (Teknokroma Analítica SA, Spain), was programmed

from 90ºC to 140ºC at 10°C/min and from 140ºC to 300ºC at 4ºC/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 250°C and the ion source and the analyser were maintained at 230°C and 150°C, respectively. Target compounds were identified by comparison of the retention times and spectra of reference compounds. The ions of silyl derivatives used for the monitoring and quantification were: *m/z* 216, 201 for 1-naphthol, *m/z* 290 for 1-pyrenol, *m/z* 207,193 for 4-nonylphenol (NP), *m/z* 207 for 4-*tert*-octylphenol (OP) and *m/z* 243, 258 for HHCB. Quantification was performed by external standard method. Concentrations for all compounds are expressed as ng/mL of bile.

Analysis of organochlorine compounds in muscle

Three pooled samples (between 2 and 3 individuals per pool) of fish muscle were analyzed for each sampling site. The extraction of organic pollutants was performed as described in Koenig et al. (2013). Briefly, lyophilized muscle tissue (2-4 g) was ground and homogenized with anhydrous Na_2SO_4 and soxhlet-extracted with 100 mL of dichloromethane: hexane (1:4) for 18 h. TBB (1,2,4,5-tetrabromobenzene) and PCB 200 were added as recovery standards. Extracts were further purified with concentrated sulfuric acid. The cleaned extracts were concentrated by evaporation, transferred to vials, evaporated to near dryness under a gentle stream of nitrogen and re-dissolved in 100 µL of PCB 142 in isooctane, as internal standard, prior to the determination of OC levels.

To determine levels of PCBs (7 congeners: IUPAC # 18, 52, 101, 118, 138, 153, 180), DDTs (2,4'-DDE, 4,4´-DDE, 2,4'-DDD, 4,4'-DDD, 2,4'-DDT, 4,4'-DDT), PeCB, HCB and HCHs (α-, β-, γ-, δ-HCH), samples were analyzed using a gas chromatograph (Model HP-6890) equipped with an electron-capture detector (µ-ECD). A 60 m x 0.25 mm DB-5 column

(J&W Scientific, Folsom, CA, USA) coated with 5% diphenylpolydimethylsiloxane (film thickness 0.25 µm) was used for separation. The oven temperature was programmed to increase from 90°C (holding time 2 min) to 130°C at a rate of 15°C/min and finally to 290°C at 4°C/min, holding the final temperature for 20 min. The injector and detector temperatures were 280°C and 320°C, respectively. Injection was performed in splitless mode and helium was used as carrier gas (30 psi). OC levels were determined by internal standard method. Procedural blanks were performed for every set of six samples. Blank values were used to establish method detection (MDL) and quantification limits (MQL), which were defined as the mean of the blanks plus three times (MDL) or five times (MQL) the standard deviation. They were in the order of 0.02 and 0.59 ng/g dry weight (d.w.) (MDL) and 0.02 to 0.91 ng/g d.w. (MQL), depending on the compound. Extraction and analytical performances were evaluated by surrogate standard recoveries, which were 58±10% and 80±23% for TBB and PCB 200, respectively. Values reported in this study were corrected by surrogate recoveries.

2.5. Biochemical determinations

Preparation of microsomal fractions

After weighing, livers were flushed with ice-cold 1.15% KCl and homogenized in 1:4 w/v of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 x *g* for 15 min, and the obtained supernatant centrifuged at 12,000 x *g* for 20 min. The resulting supernatant was further centrifuged at 100,000 x *g* for 60 min to obtain the microsomal

pellet, which was resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Biomarkers

7-Ethoxyresorufin *O*-deethylase (EROD) activity was assayed by incubating 0.1 mg of liver microsomal protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 at 30ºC for 10 min. The reaction was stopped by adding 400 μL of ice-cold acetonitrile. After centrifugation, an aliquot of the supernatant was transferred into a 96-multiwell plate. Fluorescence of 7-hydroxyresorufin was read at the excitation/emission wavelength pairs of 537/583, using a Varioskan microplate reader (Thermo Electron Corporation). Quantification was performed using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute.

Benzyloxy-4-trifluoromethyl-coumarin-O-debenzyloxylase (BFCOD) activity was analysed according to the procedure described by Thibaut et al. (2006). The assay consisted in incubating 25 μg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 at 30ºC for 10 min. The reaction was stopped by addition of acetonitrile (20:80, v/v) and the fluorescence was read in a 200 μL aliquot transferred into a 96-multiwell plate at the excitation/emission wavelength pairs of 409/530 nm, using a Varioskan microplate reader. The activity was

calculated as the amount of 7-hydroxy-4-(trifluoromethyl)-coumarin (pmol) generated per milligram of protein per minute.

Hepatic UDP-glucuronyltransferase (UGT) was assayed by a modification of the method described in Clarke et al. (1992). Briefly, 0.25 mg of liver microsomal proteins pre-treated with Triton X-100 were incubated with 3.15 mM of UDPGA in 30 mM Tris/$MgCl_2$ buffer pH 7.4. The reaction was initiated by the addition of 80 µM p-nitrophenol (pNP), run for 30 min at 30ºC and stopped by the addition of 0.2 M ice-cold trichloracetic acid, centrifuged (1,500 x *g*; 15 min), alkalinized with 0.1 mL of 10 N KOH and the remaining pNP measured spectrophotometrically at 405 nm. The activity was calculated as the amount of pNP (nmol) consumed per milligram of protein per minute of reaction time.

2.6. Statistical analysis

Biochemical activities were determined individually in 6-12 organisms per site and run per duplicate. Chemical analyses were conducted in pooled muscle tissue of three individuals (n = 2 to 3 pools per site) and bile samples were analyzed individually (n = 5 to 9). Values are presented as mean ± SEM. When data followed a normal distribution One-way ANOVA followed by multiple independent group comparison (Tukey's test) were used for the statistical analysis. When normality was not met, non-parametric analyses (Kruskal Wallis followed by Mann-Whitney's test) were used. All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and STATA SE/12.1 and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Morphometric data of samples

Length, weight and condition factor (CF) of the sampled fish are reported in Table 4. Fish collected in RR in October 2012 were significantly bigger (13.8 ± 0.3 cm; 29.5 ± 0.7 g) than those from A (11.5 ± 0.6 cm; 22.5 ± 4.0 g); while in the second sampling (June 2013), fish from A were significantly bigger (16.3 ± 0.3 cm and 67.5 ± 3.5 g) than the rest. Nonetheless, no significant differences were observed among sampling sites in terms of CF of the sampled individuals, indicating a similar nutritional state of the fish.

Table 4. Total length, body weight and condition factor (CF) of *R. rutilus* collected in the Ebro River in October 2012 and June 2013. Values are mean ± SEM (n = 6-12).

	Sampling	Station			
		RR	**FL**	**BE**	**A**
Total length (cm)	Oct-12	13.8 ± 0.3^{a}	13.1 ± 0.3^{ab}	12.2 ± 0.2^{ab}	11.5 ± 0.6^{b}
	Jun-13	12.3 ± 0.3^{a}	11.6 ± 0.1^{a}	13.6 ± 0.1^{b}	16.3 ± 0.3^{c}
Body weight (g)	Oct-12	29.5 ± 0.7^{a}	22.2 ± 1.7^{b}	22.7 ± 1.0^{b}	22.5 ± 4.0^{b}
	Jun-13	30.0 ± 2.6^{ab}	21.3 ± 1.3^{a}	37.1 ± 1.3^{b}	67.5 ± 3.5^{c}
CF*	Oct-12	1.2 ± 0.1^{a}	0.97 ± 0.1^{a}	1.2 ± 0.04^{a}	1.1 ± 0.1^{a}
	Jun-13	1.5 ± 0.05^{a}	1.3 ± 0.1^{a}	1.5 ± 0.03^{a}	1.5 ± 0.04^{a}

*CF calculated as (weight / length^3) x 100.
Distinct letters indicate significant differences between sites ($p < 0.05$).

3.2. Analysis of bile samples

The concentrations of organic contaminants measured in bile are shown in Figure 9. NP was the most abundant pollutant detected (450-2250 ng/mL bile), followed by the synthetic fragrance HHCB (57-280 ng/mL bile). No

significant differences among sampling sites were observed for the levels of NP, OP, 1-naphtol and 1-pyrenol in fish bile in any of the two samplings. In contrast, high levels of HHCB were measured in October 2012 in fish from FL (195 ± 67 ng/mL bile) in comparison to BE (57 ± 7 ng/mL bile), while in June 2013, the highest concentration of HHCB was detected in the bile of fish from RR (281 ± 44 ng/mL bile) and the lowest at A (157 ± 15 ng/mL bile).

When comparing the levels of chemicals in bile between the two sampling periods, a significant increase of 3 to 5-fold for HHCB was observed in June 2013 in fish from RR and BE, respectively. No significant temporal differences were observed for the other chemicals.

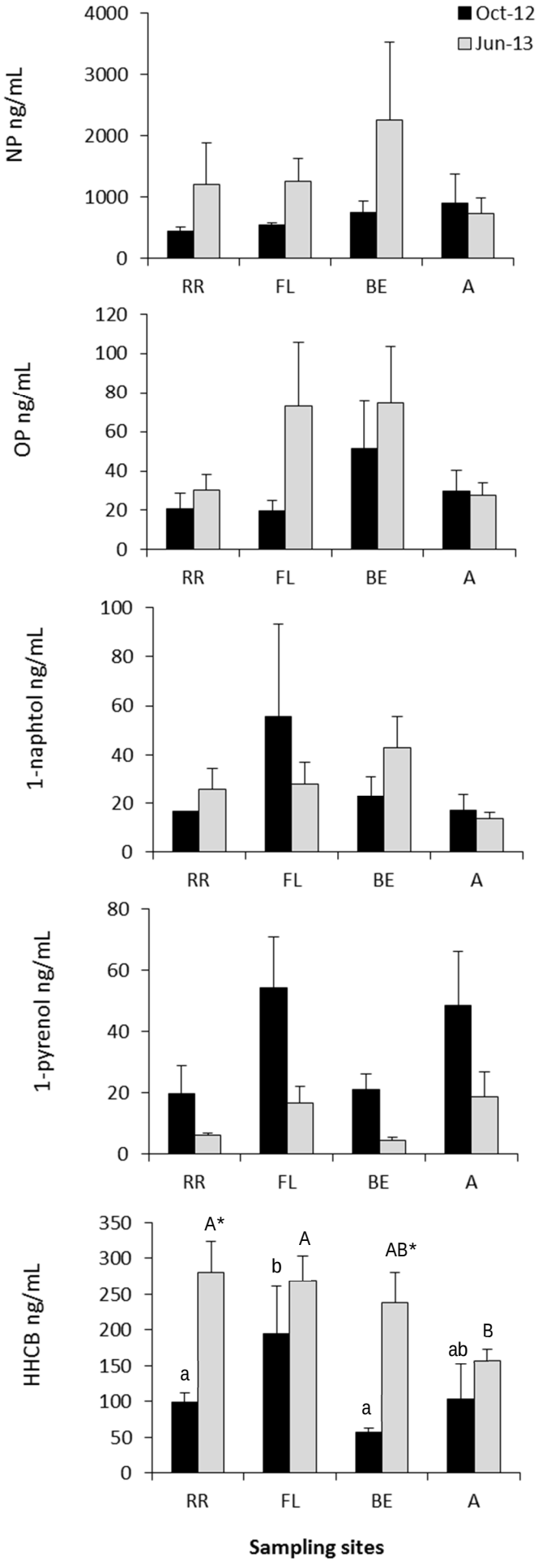
Oct-12
Jun-13
NP ng/mL
4000
3000
2000
1000
0
RR
FL
BE
A
OP ng/mL
120
100
80
60
40
20
0
1-naphtol ng/mL
1-pyrenol ng/mL
HHCB ng/mL
350
300
250
200
150
100
50
A*
A
b
AB*
a
ab
B
Sampling sites

Figure 9. Biliary levels (mean ± SEM, n = 5-9) of NP, OP, 1-naphthol, 1-pyrenol and HHCB in *R. rutilus* collected in October 2012 and June 2013 in the Ebro River. Distinct letters indicate significant differences between sites. *Significant differences between October 2012 and June 2013 samplings ($p < 0.05$).

3.3. Organochlorine compounds in muscle

OC levels in the muscle of roach are shown in Figure 10 and summarised in Table 5. Detailed information about specific congeners is reported at Table 7. Regarding the spatial distribution, significant differences among sampling sites were observed for DDTs, HCHs, HCB and PCBs concentrations in October 2012; the highest concentrations were detected in fish from BE (1317 ± 105; 9 ± 2; 68 ± 1 and 1111 ± 22 ng/g d.w. of muscle, respectively). In June 2013, fish collected at downstream sites (BE, A) showed higher residues of PCBs and DDTs; whereas no significant differences among sampling sites were observed for the other compounds (HCHs, HCBs, PeCBs).

Regarding temporal trends, 4 to 9-fold higher concentrations of all OCs, with the exception of PeCB, were found in BE during barrier construction (2012) in comparison to the dredging period (2013) (Table 5). Similarly, DDTs, HCHs, HCB and PCBs were found at higher concentrations prior than during dredging in fish collected in FL. No differences in OC concentrations in fish from RR, the reference site, were found between sampling periods.

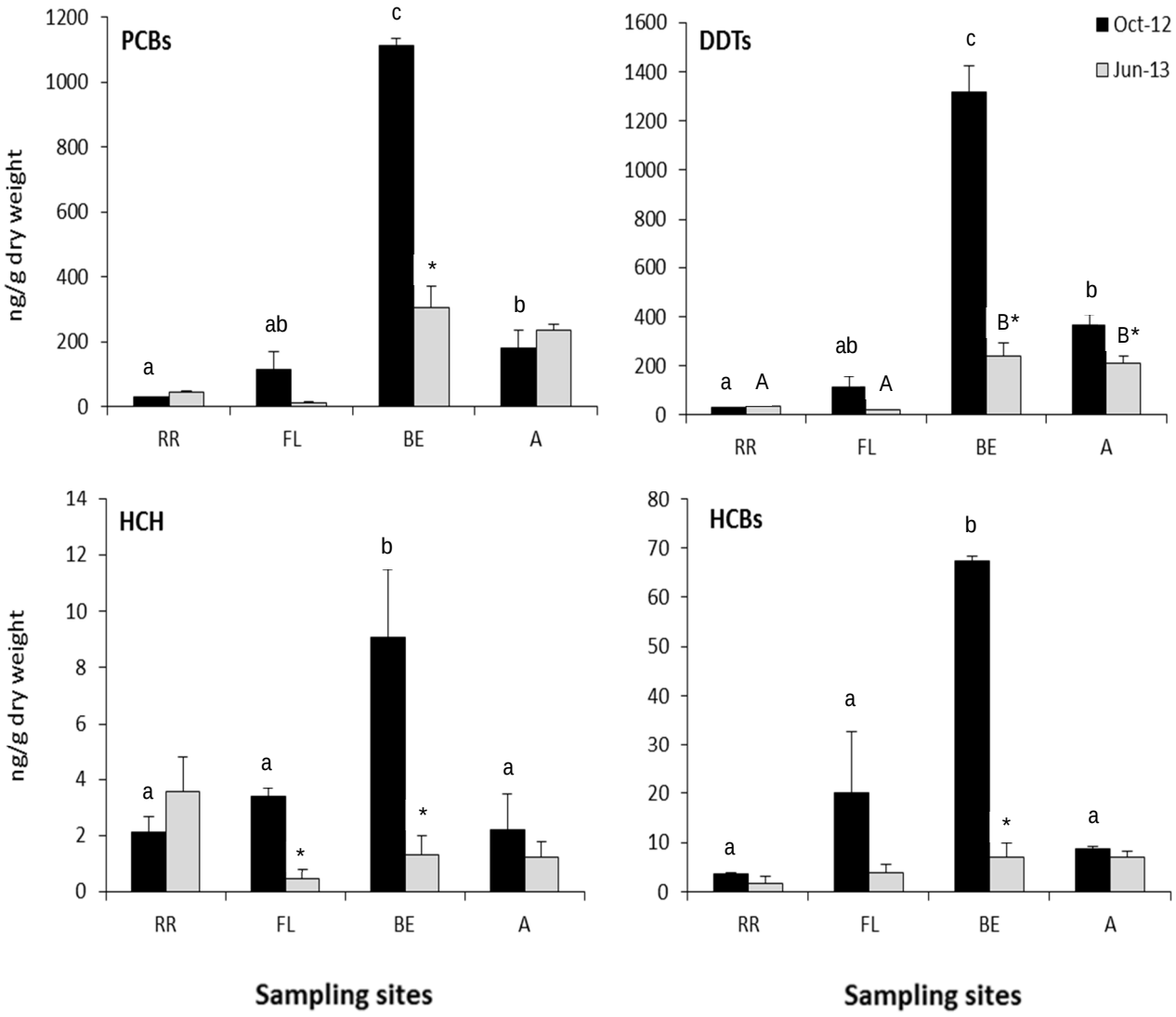

Figure 10. Levels of DDTs, HCHs, HCBs, PCBs and PeCBs (ng/g d.w.) (mean ± SEM, n = 2-3) analysed in muscle of *R. rutilus* collected in October 2012 and June 2013 in the stations RR, FL, BE and A. Each sample corresponds to a pool of 3 individual fish. Distinct letters indicate significant differences between sites.*Indicates significant differences between October 2012 and June 2013 samplings ($p < 0.05$).

DDTs were the most abundant pollutants in fish muscle, followed by PCBs, HCB, HCHs and PeCB. Among DDT metabolites, 4,4'-DDE was the dominant compound, comprising on average 50-70% of the total DDT content, while 4,4'-DDT contributed only to 4-7%. Samples from FL had higher 4,4'-DDT contribution than those from the other stations. Among the seven PCB congeners determined, PCB 153 (35%), PCB 138 (22%)

and PCB 180 (15%), were the most prevalent. The dominant HCH isomer detected in muscle of all specimens was γ-HCH (52%), with the exception of fish collected in RR in October 2012, which showed higher proportion of β-HCH (80%).

Table 5. Total concentrations of OCs (DDTs, HCHs, PCBs, HCB, PeCB) reported in muscle of *R. rutilus* (ng/g d.w.) collected in Flix and downstream areas.

	Flix			**50-65 km downstream**			**90-100 km downstream**		
	2006[a]	**2012**	**2013**	**2006[a]**	**2012**	**2013**	**2006[a]**	**2012**	**2013**
∑DDT	477	111 ± 46	19 ± 3	227*	1317 ± 105	242 ± 53	226*	364 ± 45	212 ± 27
∑HCH	5.0*	3 ± 0.3	0.5 ± 0.4	3.5*	9 ± 2	1.3 ± 0.7	2*	2 ± 1	1.2 ± 0.6
∑PCB	408*	114 ± 57	11 ± 4	196*	1111 ± 22	305 ± 67	141*	183 ± 53	237 ± 18
HCB	1292*	20 ± 12	4 ± 2	37*	68 ± 1	7.2 ± 3	39*	9 ± 1	7 ± 1
PeCB	116*	7 ± 3	1 ± 0.2	2.5*	2.4 ± 0.02	1.3 ± 0.8	2.5*	0.3 ± 0.03	0.6 ± 0.4

[a]Data from Huertas et al. (2015) originally reported as ng/g wet weight and multiplied by a factor of 5 to estimate concentrations in dry weight (considering water content of 80% in muscle tissue).

3.4. Biochemical responses

In October 2012, EROD activity was significantly increased in fish from A (16 ± 6 pmol/min/mg protein), approximately 2-fold when compared to RR and FL, while no differences among sampling sites were detected for BFCOD and UGT activities (Fig. 11). In June 2013, the same tendency was observed, although only UGT activity was significantly elevated in fish from A (450 ± 32 pmolmin/mg protein) when compared to RR and FL (341 and 360 pmol/min/mg protein, respectively). On the other hand, no significant differences were observed for the selected biomarkers between 2012 and 2013, with the exception of EROD activity that was 1.7-fold higher in October 2012 than in June 2013 in fish from A.

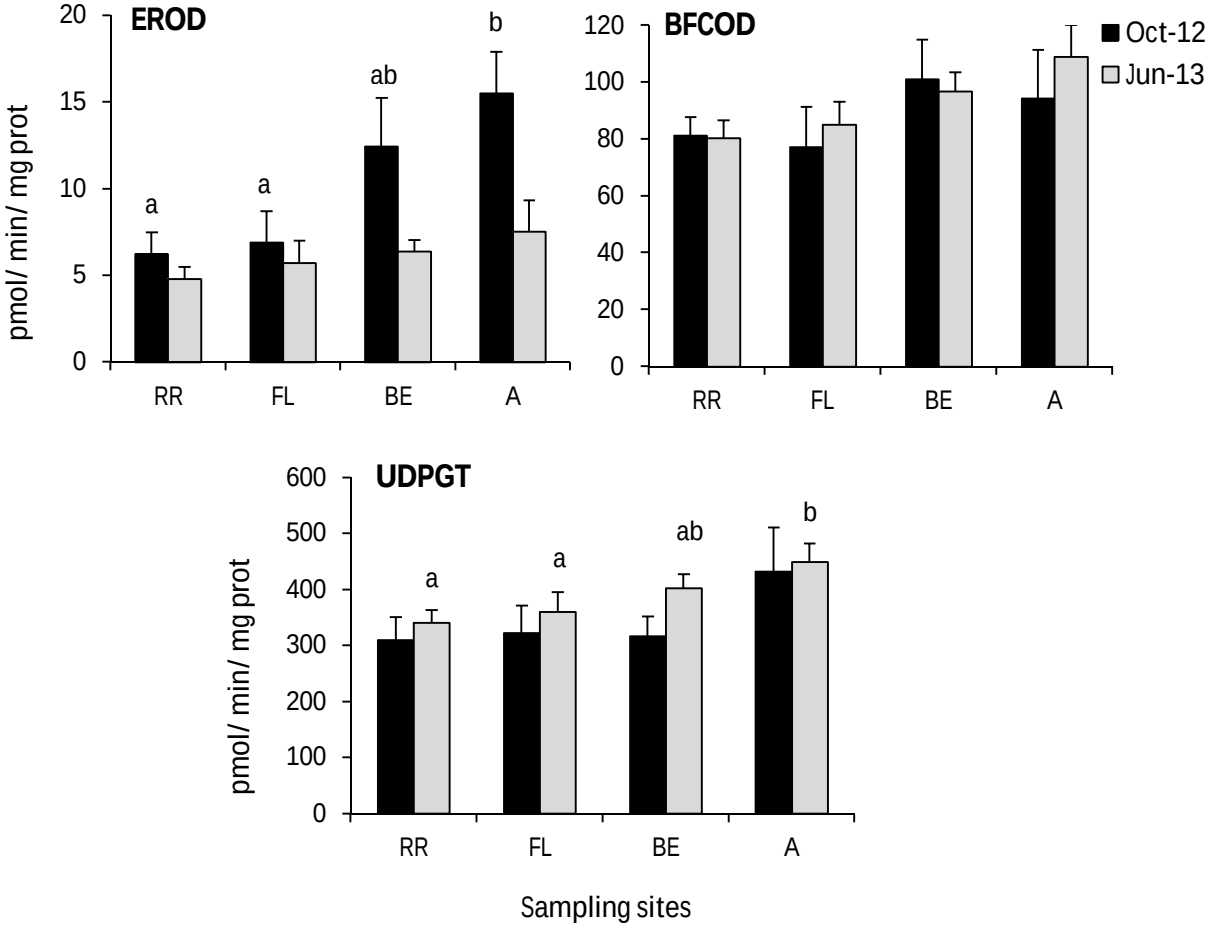

Figure 11. EROD, BFCOD and UGT activities (mean ± SEM, n = 6-12) determined in the liver of *R. rutilus* collected in October 2012 and June 2013 along the Ebro River (RR, FL, BE and A). Distinct letters indicate significant differences between sites ($p < 0.05$).

3.5. Histological analysis of the gonads

No significant abnormalities within gonad tissue of males or females were observed (Fig. 12). However, during barrier construction, female roach from the upper course of the river (RR and FL) had gonads at undeveloped stage (SMS-0), while females sampled downstream had gonads at advanced stages of sexual maturation. Thus, primary oocytes with perinuclear and cortical alveoli (SMS-I) and some secondary oocytes with yolk granules (SMS-II) were observed in females from BE, while 25% of

females collected in station A had already oocytes completely filled with yolk granules (SMS-III). In contrast, no differences on maturation stage were observed for males, which had undeveloped gonads (SMS-0).

In June 2013, females from FL had undeveloped gonads (SMS-0), whereas those collected upstream (RR) and downstream (BE and A) had 50 to 100 % of the gonads classified as SMS-I. Regarding males, those collected upstream (RR, FL) had 50-70% of the gonads classified as SMS-0, while those collected downstream (BE, A) were mostly classified as SMS-I (early gametogenesis).

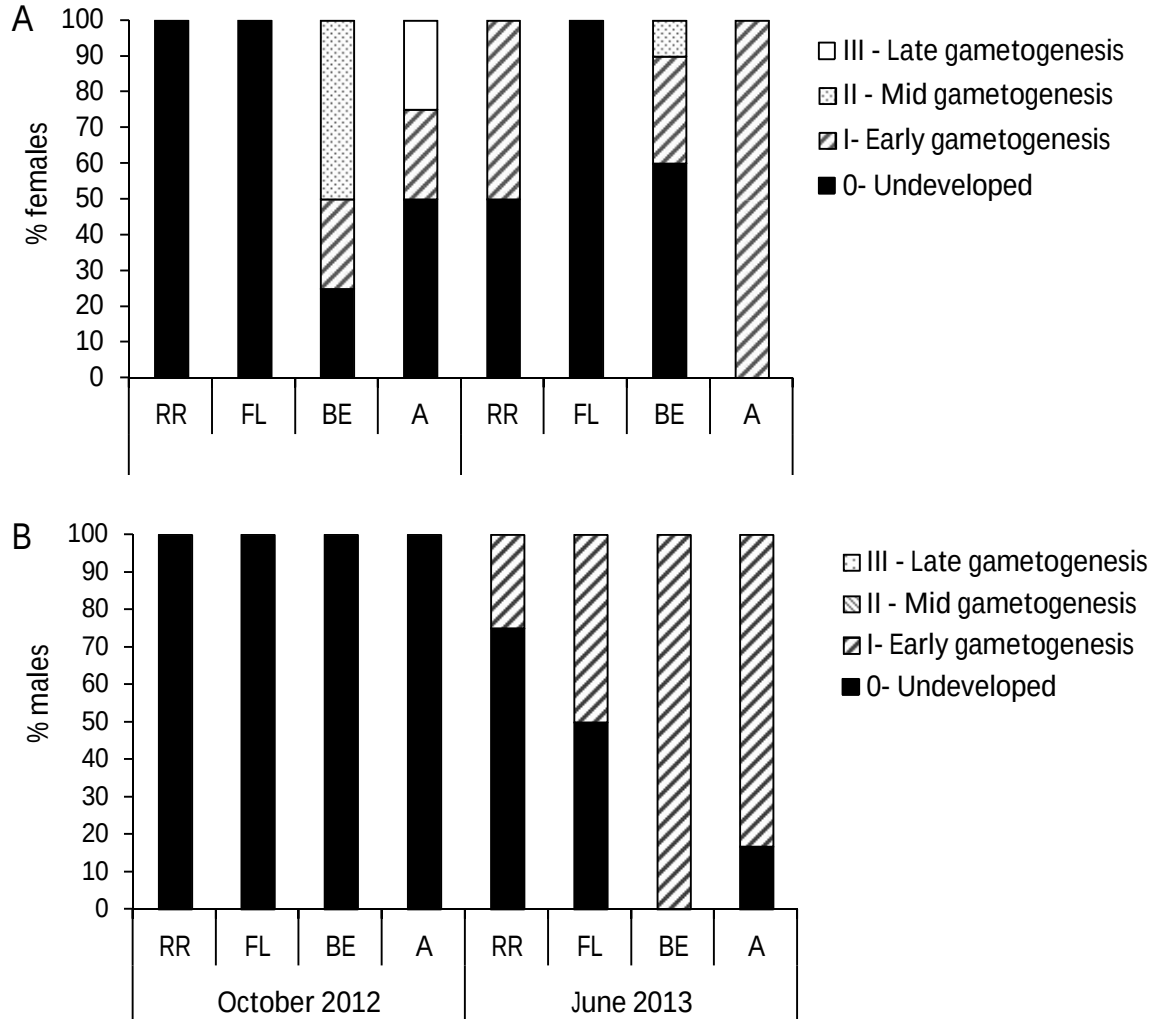

Figure 12. Percentage of roach fish (A, females; B, males) classified by gonad maturation stage collected in both October 2012 and June 2013 sampling along the Ebro River.

4. Discussion

Although sediments act as a sink for hydrophobic and persistent organic contaminants in aquatic systems, several processes such as physical disturbances induced by water currents, dredging or other activities can trigger the resuspension of contaminants back into the water column making them available to aquatic organisms (Latimer et al., 1999). Both, the construction of the wall and dredging activities could have enhanced the mobility of contaminants from FL towards the lower course of the Ebro River. The first sampling of roach took place in October 2012, two years from the beginning of the construction of the wall. When comparing OC levels in the muscle of roach collected downstream FL in October 2012 with those obtained in a previous study in 2006 (Table 5), a significant increase in the concentration of PCBs and DDTs was observed (up to 5.6-fold) in October 2012, and associated to the release of OCs during wall construction. The direct influence of FL contamination down to BE is further supported by the strong correlation observed between all analyzed OCs in fish from both stations, indicating a common pollution source (Table 6). This strong correlation was not observed further downstream (A) and this was attributed to the presence of additional pollution sources together with the decoupling of the compounds during transport.

However, during the second sampling (June 2013), the construction of the barrier in FL had finished and sediments resuspended as a consequence of dredging were expected to be retained within the barrier. The effectiveness of the barrier was evidenced by the 4- to 9-fold lower concentrations of OCs found in muscle of fish from FL and BE in comparison to 2012. Although fish size/length was pretty homogenous, some differences were observed among sampling sites. Size differences

could be a source of variability regarding OC concentrations in muscle, and may have acted as a confounding factor. Some studies have reported a positive relationship between length and OC levels in freshwater fish, while others reported no clear relationship or even the opposite trend due to the so called dilution effect (Devaux and Monod, 1987; Manchester-Neesvig et al., 2001; Covaci et al., 2006). In our study, no clear relationship between fish length and OC accumulation was observed.

Table 6. Pearson correlation coefficients between log-transformed concentrations of OCs in the sampling sites (October 2012).

			HCB	DDTs	HCHs	PCBs		HCB	DDTs	HCHs	PCBs
RIBARROJA	HCB	*r*		-0.558	-0.648	-0.582	BENIFALLET		0.991**	0.900**	0.964**
		p		0.25	0.164	0.225			0.000	0.006	0.000
		N		6	6	6			7	7	7
	DDTs	*r*	-0.558		0.846*	0.728		0.991**		0.890**	0.968**
		p	0.25		0.034	0.101		0.000		0.007	0.000
		N	6		6	6		7		7	7
	HCHs	*r*	-0.648	0.846*		0.662		0.900**	0.890**		0.942**
		p	0.164	0.034		0.152		0.006	0.007		0.001
		N	6	6		6		7	7		7
	PCBs	*r*	-0.582	0.728	0.662			0.964**	0.968**	0.942**	
		p	0.225	0.101	0.152			0.000	0.000	0.001	
		N	6	6	6			7	7	7	
FLIX	HCB	*r*		0.923**	0.797	0.948**	AMPOSTA		0.271	0.689	-0.086
		p		0.009	0.057	0.004			0.604	0.13	0.871
		N		6	6	6			6	6	6
	DDTs	*r*	0.923**		0.802	0.985**		0.271		0.425	0.017
		p	0.009		0.055	0.000		0.604		0.401	0.974
		N	6		6	6		6		6	6
	HCHs	*r*	0.797	0.802		0.884*		0.689	0.425		0.269
		p	0.057	0.055		0.019		0.13	0.401		0.606
		N	6	6		6		6	6		6
	PCBs	*r*	0.948**	0.985**	0.884*			-0.086	0.017	0.269	
		p	0.004	0.000	0.019			0.871	0.974	0.606	
		N	6	6	6			6	6	6	

r, Pearson correlation coefficient; *p*, significance level; N, number of samples.
*Statistically significant at 95% confidence level.
**Statistically significant at 99% confidence level.
PeCB is not included as it showed any relationship.

PCBs 138, 153 and 180 were the most prevalent congeners in roach muscle. These congeners have a high degree of chlorination (hexa and hepta-PCB) and consequently greater tendency to adsorb in sediments and to bioaccumulate in organisms than the less chlorinated ones. Regarding DDTs, 4,4'-DDE was the dominant isomer in all samples; its predominance is indicative of old DDT residues that progressively degrade into 4,4'-DDE (Shaw et al., 2005). Another characteristic feature is the significant amount of DDDs found in FL and its area of influence, which has been also reported by other authors (Huertas, 2015). These relatively higher concentrations of DDDs are due to the anaerobic conditions of the sediments in FL, which facilitate the transformation of DDT into DDD instead of DDE. The highest percentage of DDDs was observed in samples from FL and downstream sites in 2012, with a strong decrease in 2013 in BE and A (Fig.13). Values in the reference site (RR) were lower and remain uniform among samplings. These results further evidence a strong pollution load from FL to downstream areas in 2012 (during wall construction) that was significantly reduced in 2013 (dredging).

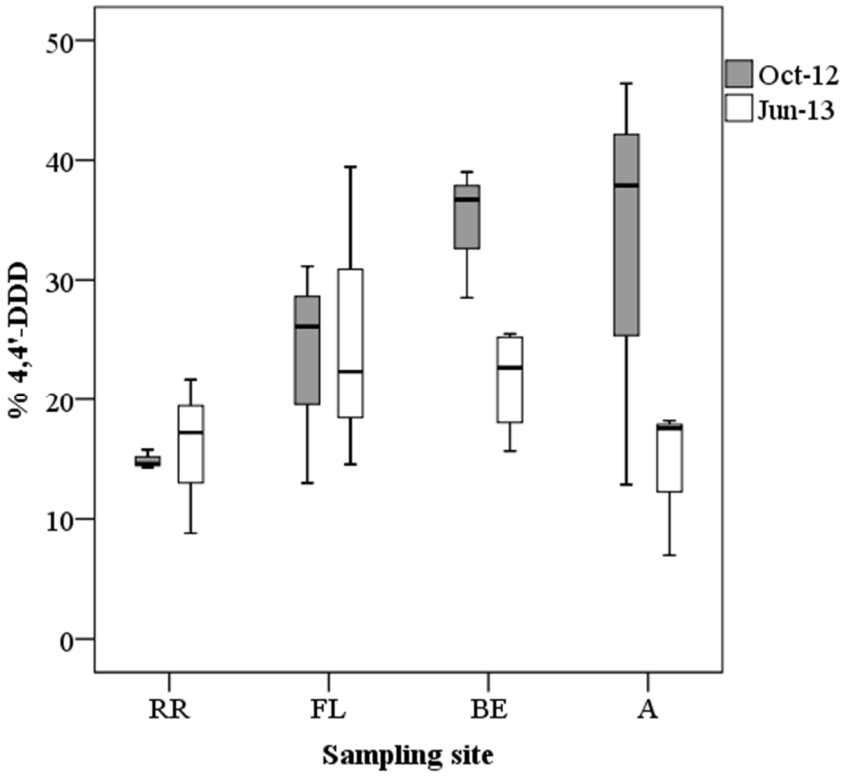

Figure 13. Box-plot diagram for 4,4'-DDD (%) in *R. rutilus* collected in both October 2012 and June 2013 sampling along the Ebro River. Horizontal line shows median values and box is interquartil range.

While significant spatial and temporal differences were detected for OCs in roach muscle, bile analysis did not evidence substantial differences among sites or samplings, mainly due to a high inter-individual variability. High concentrations of octyl- and nonylphenol were previously detected in the bile of carps from FL (NP: 16.5 and OP: 0.3 µg/g of bile), indicating a continuous and significant input of these compounds in the area (Lavado et al., 2006). APs act as endocrine disruptors on aquatic organisms by binding to the estrogen receptor. Consequently, presence of immature cells into the lumen, macrofage aggregates in testes, and depressed levels of testosterone and estradiol in plasma were observed in male carps, while delayed maturation was reported in females (Lavado et al., 2006). Moreover, high concentrations of OCs, namely PCBs (95 ng/g wet weight (w.w.)), DDTs

(29 ng/g w.w.) and HCB (1.65 ng/g w.w.) were detected in the muscle of these carps, suggesting that not only APs, but also OCs might be responsible for the significant endocrine alterations detected (Lavado et al., 2004). In the present study, no significant alteration in roach gonads was observed, nor strong evidences of maturation delay in roach from FL or downstream sites. Certainly, biliary levels of NP and OP were 3 to 5-fold lower in roach than in carp, and OCs residues in muscle, including PCBs, HCBs and DDTs were up to 43-, 2-, and 8-fold lower in roach, indicating reduced exposure in comparison to carps.

Among biomarkers, EROD activity has been successfully used as biomarker of exposure to a wide variety of organic pollutants, including polycyclic aromatic hydrocarbons, dioxin-like PCBs and many others (Whyte et al., 2000). EROD activity was significantly elevated in roach from downstream sites (BE, A) during wall construction, while during dredging, EROD activity in the liver of fish from downstream sites had decreased, and no significant differences among sites was observed. Similarly, the determination of BFCOD activity did not show significant differences among sampling sites. BFCOD is a measure of CYP3A activity, which is induced by steroids, bile acids and different xenobiotics, e.g. pharmaceuticals, pesticides, among others. Reports on the induction of BFCOD activity in fish are scarce and few studies have applied this biomarker in field studies (Quesada-García et al., 2013; Habila et al., 2017). Regarding UGT activity, a significant increase was observed in roach from station A during dredging. Generally, the transcriptional induction of UGTs via AhR has been described in the liver of fish (Christen and Fent, 2014). This leads to induction of many UGT isoforms together with CYP1A enzymes that will conjugate a broad range of both

endogenous (bilirubin, bile acids, estrogens, androgens, thyroid hormones) and exogenous (phenols, non-steroidal anti-inflammatory drugs) substrates.

Overall, the construction works of the barrier in Flix produced the resuspension and subsequent mobilization of contaminants (OCs) downstream as evidenced by the high accumulation of organochlorinated compounds found in the muscle of fish in BE in October 2012, during wall construction. The OC profiles were strongly related to those detected in FL, indicating a common pollution source for OCs. This observation was supported by increased EROD activity in fish from downstream areas. However, during dredging (June 2013), the wall efficiently retained the resuspended pollutants as shown by the decreased concentration of OCs in muscle of fish in FL and downstream sites in comparison to previous studies. In addition, the lack of induction of EROD activity in fish from downstream sites during dredging indicates no significant release of CYP1A inducers and further proves the effectiveness of the barrier.

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Supporting Information

Table 7. Concentration (mean ± SEM) of congeners of OCs analysed in muscle of *R. rutilus* collected in October 2012 and June 2013 along the Ripoll River.

	2012				2013			
	RR	**FL**	**BE**	**A**	**RR**	**FL**	**BE**	**A**
HCB	3.5 ± 0.2	20.2 ± 7.0	47.3 ± 11.3	8.6 ± 0.4	1.7 ± 0.8	3.8 ± 0.96	7.1 ± 1.6	7.1 ± 0.6
α-HCH	n.d	n.d	1.1 ± 0.1	0.5 ± 0.05	n.d	n.d	n.d	n.d
β-HCH	1.8 ± 0.2	0.9 ± 0.08	1.6 ± 0.4	0.6 ± 0.07	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.08	0.3 ± 0.05
γ-HCH	0.2 ± 0.08	1.4 ± 0.03	3.8 ± 1.5	1.1 ± 0.4	2.4 ± 0.7	0.1 ± 0.06	0.8 ± 0.3	0.7 ± 0.2
δ-HCH	0.2 ± 0.01	0.6 ± 0.06	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.005	0.2 ± 0.06	0.3 ± 0.01	0.3 ± 0.07
2,4'-DDE	1.05 ± 0.3	2.9 ± 0.4	5.9 ± 1.8	8.5 ± 2.3	3.3 ± 0.8	0.7 ± 0.3	14.3 ± 7.5	4.5 ± 2.2
4,4´-DDE	20.2 ± 0.9	63.2 ± 12.3	497 ± 102.4	197.3 ± 26.4	19.6 ± 1.8	8.3 ± 0.3	136.5 ± 21.1	151.1 ± 12.1
2,4'-DDD	4 ± 0.3	14.3 ± 4.05	137.3 ± 33.9	34.1 ± 5.7	4.3 ± 0.4	4.8 ± 0.9	37.5 ± 8.1	18.2 ± 2.8
4,4'-DDD	3.5 ± 0.1	25.2 ± 8.06	282.9 ± 62.6	93.5 ± 14.7	4 ± 0.4	3.4 ± 0.7	42.7 ± 8.4	29.1 ± 5.6
2,4'-DDT	n.d	n.d	n.d	13.3 ± 1.6	n.d	n.d	3.4 ± 0.8	n.d
4,4'-DDT	n.d	5.8 ± 0.9	42.8 ± 5.3	21.9 ± 2.3	2.5 ± 0.3	1.2 ± 0.06	10.1 ± 2.5	9.1 ± 0.5
PeCB	2.6 ± 0.7	6.7 ± 1.6	3.8 ± 0.8	7.04 ± 3.8	2.1 ± 0.9	0.7 ± 0.09	1.3 ± 0.5	0.6 ± 0.2
PCB 101	5.7 ± 0.5	11.5 ± 2.6	68.5 ± 14.2	19.9 ± 0.4	3.6 ± 0.5	2.4 ± 0.8	25.7 ± 3.04	16 ± 2.8
PCB 28	1.5 ± 0.2	5.4 ± 0.4	58.7 ± 13.4	10.3 ± 1.3	2.2 ± 0.2	0.8 ± 0.1	12.9 ± 2.2	8.6 ± 0.6
PCB 52	3.9 ± 0.1	5.4 ± 0.7	52.1 ± 11.6	12.6 ± 0.5	3.3 ± 0.3	0.6 ± 0.1	15.5 ± 2.5	14.9 ± 1.05
PCB 118	2.5 ± 0.1	8.7 ± 1.5	44.5 ± 9	11.7 ± 1.9	6.2 ± 1	0.8 ± 0.4	32.7 ± 10.5	21.6 ± 3.9
PCB 153	7.2 ± 0.3	37.2 ± 12	293.4 ± 59.8	68.1 ± 16.1	14.7 ± 1	1.4 ± 0.03	97.6 ± 11	88.2 ± 4.2
PCB 138	5.4 ± 0.3	24.4 ± 6.9	179.7 ± 36.9	38.4 ± 9.3	10.3 ± 0.9	2.2 ± 0.6	72.5 ± 10.3	53 ± 2.4
PCB 180	2.9 ± 0.9	21.1 ± 8.6	117.4 ± 24.4	28.3 ± 5.8	5.5 ± 0.3	2.4 ± 0.7	48.7 ± 5.8	34.9 ± 3.01
∑DDTs	28.8 ± 1.6	111.3 ± 25.9	965.8 ± 202.5	364.2 ± 25.4	33.6 ± 2.3	18.9 ± 1.6	242 ± 29.7	212 ± 15.3
∑HCHs	2.1 ± 0.3	3.4 ± 0.2	6.9 ± 1.8	2.2 ± 0.7	3.6 ± 0.7	0.5 ± 0.2	1.3 ± 0.4	1.2 ± 0.3
∑PCBs	29.2 ± 0.8	113.8 ± 32.4	814.3 ± 167.8	182.7 ± 30	45.8 ± 2.5	10.7 ± 2.5	305.3 ± 37.7	237.2 ± 10.1

n.d: below detection limit.

CAPÍTULO 1

Combinación de biomarcadores para la evaluación ambiental de ríos mediterráneos

Artículo 2

'Multistress assessment including new lipidomic approaches in the Ripoll River: a field study'

Maria Blanco[1], Juliane Rizzi[2], Denise Fernandes[1], Nicole Colin[3], Alberto Maceda-Veiga[3], Cinta Porte[1]

[1]Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

[2]PhD Program in Water Resources and Environmental Engineering, Federal University of Paraná, 81531-980 Curitiba, Paraná, Brasil.

[3]Department of Animal Biology & Biodiversity Research Institute (IRBio), Faculty of Biology, University of Barcelona, ES-08028 Barcelona, Spain.

In preparation

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'Multistress assessment including new lipidomic approaches in the Ripoll River: a field study'

Maria Blanco[1], Juliane Rizzi[2], Denise Fernandes[1], Nicole Colin[3], Alberto Maceda-Veiga[3], Cinta Porte[1]

[1]Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

[2]PhD Program in Water Resources and Environmental Engineering, Federal University of Paraná, 81531-980 Curitiba, Paraná, Brasil.

[3]Department of Animal Biology & Biodiversity Research Institute (IRBio), Faculty of Biology, University of Barcelona, ES-08028 Barcelona, Spain.

ABSTRACT

The Ripoll River is a small Mediterranean river strongly affected by pollution and water scarcity. Over the summer period, urban and industrial discharges arrive into the river with little dilution. In order to assess the water quality, two fish species, *Barbus meridionalis* and *Squalius laietanus*, were collected from six sites along the Ripoll River. PAHs metabolites, alkylphenols (nonylphenol and octylphenol) and the musk galaxolide were determined in bile in conjunction with different biochemical responses including, 7-ethoxyresorufin-O-deethylase (EROD) and 7-benzyloxy-4-trifluoromethyl-coumarin O-debenzyloxylase (BFCOD). Additionally, the activity CYP19 aromatase was determined in the ovaries of *B. meridionalis* as a potential marker of endocrine disruption. Ultrahigh resolution mass spectrometry was applied to determine changes in lipids including phosphatidylcholines (PC), PC-plasmalogens (PC-P), cholesterol esters (CE), triacylglycerols (TG), diacylgliceros (DG) and sphingomielins (SM) in muscle tissue. The analysis of bile indicated that fish from the lower course of the river were strongly exposed to pollutants. Accordingly, a significant induction of EROD (9 to 10-fold) and BFCOD (3 to 5-fold) activities were detected in both fish species together with an increased P450-aromatase activity in females of *B. meridionalis* from the

most pollutes sites. Principal component analysis (PCA) and Volcano plot were applied to separate the muscle lipid profiles of fish from polluted and reference areas. A decrease of the most polyunsaturated PCs together with an accumulation of CEs in B. *meridionalis* and TGs in S. *laietanus* from polluted sites was observed. Overall, the study highlights the impact of urban and industrial effluents in common Mediterranean species and points out different strategies of adaptation after exposure to environment stressors.

Key words: *Fish, Mediterranean rivers, pollution, biomarkers, endocrine disruption, bile, lipids, LC-MS*.

1. Introduction

The Ripoll River is a small Mediterranean river located in the NE of the Iberian Peninsula with a long history of anthropogenic disturbance, including water abstraction and overexploitation, sewage discharge, modification of riparian coverage, pollution and/or erosion caused by an increasing human pressure over decades (Maceda-Veiga et al., 2013). Water flow has continuously decreased since 1999, the lowest flow recorded in 2015 (135 L/s). Consequently, during drought periods, when water flow is significantly reduced, water quality can be extremely poor as the river receives urban, industrial and agricultural effluents with a very low dilution factor, which increases the environmental pressure on aquatic fauna. Therefore, in a context of global warming, this and other Mediterranean rivers are particularly vulnerable, as most climatic models predict scenarios characterized by less precipitation and higher temperatures, and a further reduction of river flow (Pascual et al., 2015).

A combination of chemical and biological tools is frequently used to assess water quality in rivers. While chemical monitoring identifies a fraction of environmental pollutants, often those included in ‘priority lists’,

the use of biomarkers in sentinel species enables assessing the overall effects of pollutants and their interaction with natural and other anthropogenic stressors (Birk et al., 2012). Chemicals discharged into the river, directly or via sewage treatment plants (STPs), may be taken up by fish and either accumulated in different tissues or metabolized and subsequently excreted through the bile as hydroxylated and/or conjugated metabolites (Koenig et al. 2013). Thus, the bile represents the major excretion route for many xenobiotics, and the analysis of biliary metabolites has been successfully used as a marker of recent exposure to pollutants in fish (Beyer et al., 2011). Among the metabolic system, CYP1A plays a key role in the biotransformation of many pollutants, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, and many others. CYP1A induction has been successfully used as a biomarker of pollution exposure, often through the determination of 7-ethoxyresorufinO-deethylase (EROD) activity. An induction of EROD activity over 10-fold was detected in common carps exposed to sewage treatment plant effluents in Mediterranean rivers. The increased EROD activity was positively correlated with exposure to organic pollutants, e.g. PAHs and organochlorinated compounds (Fernandes et al., 2002; Lavado et al., 2006).

Endocrine disruption has been reported in fish living in or nearby highly contaminated effluents, such as STPs. Thus, increased levels of plasmatic vitellogenin were detected in male carps living in a small Mediterranean river strongly influenced by discharges of STP (Solé et al., 2002). This occurred together with the presence of intersex individuals and testicular atrophy and was attributed to exposure to high concentration of nonylphenol (up to 600 µg/L) (Solé et al., 2000). Lavado et al. (2004) reported the presence of plasmatic vitellogenin and low levels of

testosterone together with low gonadosomatic index and several histological alterations in gonads of male carps collected in the Ebro River, downstream a STP. Important alterations (viz. delayed maturation in females, indications of arrested spermatogenesis in males) were detected in carps near a heavily industrialized area (chlor-alkali plant).

The Ripoll River has a long history of sewage pollution, which eradicated fish and invertebrate species in mid and downstream reaches for decades. After modernization of the current STPs, water quality improved substantially. However, chemical analyses still reveal black spots where the concentrations of metals (Cu, Hg, Pb, Zn), nitrites and chlorides exceeded the reference values established in Spanish legislation (Maceda-Veiga et al., 2012). As a consequence of pollution, reduced haematocrit and haemoglobin levels were reported in autochthonous fish (Maceda-Veiga et al., 2013). Nonetheless, there is no information on the presence of organic pollutants in the area and the potential consequences for aquatic species.

It is recognized that lipid composition influences tissue distribution of hydrophobic chemicals (Nichols et al., 1998), but also, exposure to some pollutants may alter lipid metabolism and consequently lipid composition. Indeed, Grün and Blumberg (2006) pointed out the existence of a number of chemicals, termed obesogens, which inappropriately regulate lipid metabolism and promote adipogenesis. Lipids are involved in many biological functions, as signal transduction, hormonal regulation, compartmentalization, energy production and storage, and cell-signalling, among others. Therefore, lipid alterations may have negative consequences for fish in terms of growth, reproduction and movement.

The autochthones species, barbel -*Barbus meridionalis*- and chub -*Squalius laietanus*- were selected for the study; both cyprinids typically

inhabit upstream (*B. meridionalis*) and mid and downstream reaches (*S. laietanus*) of Mediterranean rivers and have been previously used to assess water quality (Mas-Martí et al., 2010). In Mediterranean rivers many ecotoxicological studies have focused on relatively tolerant exotic species, such as the common carp (*Cyprinus carpio*), the red swamp crayfish (*Procambarus clarkii*) and the zebra mussel (*Dreissena polymorpha*) (Fernandes et al., 2002; Lavado et al., 2004; Faria et al., 2010).

The aim of this study was to assess the water quality of the Ripoll River by integrating the chemical analysis of selected contaminants (polycyclic aromatic hydrocarbons (PAHs), alkylphenols (APs) and synthetic musks (galaxolide)) in fish bile with the determination of CYP isoenzymes involved in the metabolism of both, exogenous and endogenous substrates, namely, EROD activity, a classical biomarker widely used to assess exposure to organic contaminants, and 7-benzyloxy-4-trifluoromethyl-coumarin *O*-debenzyloxylase (BFCOD), a catalytic probe for CYP3A. Additionaly, CYP19 aromatase activity was determined in the ovaries of *B. meridionalis* as a marker of endocrine alteration (Lavado et al., 2004; Hinfray et al., 2010). Finally, different lipid species (phosphatidylcholines (PC), PC-plasmalogens (P-PL), cholesterol esters (CE), triacylglycerols (TG), diacylgliceros (DG) and sphingomielins (SM)) were analyzed in the muscle of fish in order to detect potential alterations as a consequence of pollution exposure.

2. Material and methods

2.1. Chemicals

4-Nonylphenol (NP) technical mixture (99% purity) and 4-*tert*-octylphenol (OP) (97% purity) were purchased from Aldrich Chemicals, UK.

Galaxolide (HHCB), 7-ethoxyresorufin (7-ER), bovine serum albumin (BSA; fatty acid free, ≥ 99% purity), and NADPH were obtained from Sigma-Aldrich (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin (7-BFC) was purchased from Cypex (Dundee, Scotland, UK). [1β-^{3}H(N)]Androst-4-ene-3,17-dione was purchased from Perkin-Elmer Life Sciences (Boston, USA). All solvents were from Merch (Darmstadt, Germany). Lipid standards 16:0 D31-18:1 PC; 1,2,3-17:0 TG, 1,3-17:0 D5 DG and17:0 CE were from Avanti Polar Lipids (Alabaster, AL, USA). Standard of PC-P and SM were not available.

2.2. Sample collection

The Ripoll River is located in the NE of the Iberian Peninsula (Catalonia, Spain) (Fig. 14). Sampling sites were selected along the Ripoll River to include upstream areas (R1, R2, R3), and sampling sites located after point sources of urban and industrial effluents (P1, P2, P3). P1 receive both urban and industrial sewage effluents and P3 is located 1 km after point source of an urban effluent. Both, STPs treat approximately 30,000 m^3/day and the maximum flow of the river is of 4,800 m^3/day in summer. Thus, river flow is mainly determined by the effluent of the STPs. No further sampling downstream P3 was possible because *B. meridionalis* was almost absent (Maceda-Veiga et al., 2012). Fish movements in the study area are restricted due to the existence of physical barriers (e.g. weirs, small dams) at each sampling site.

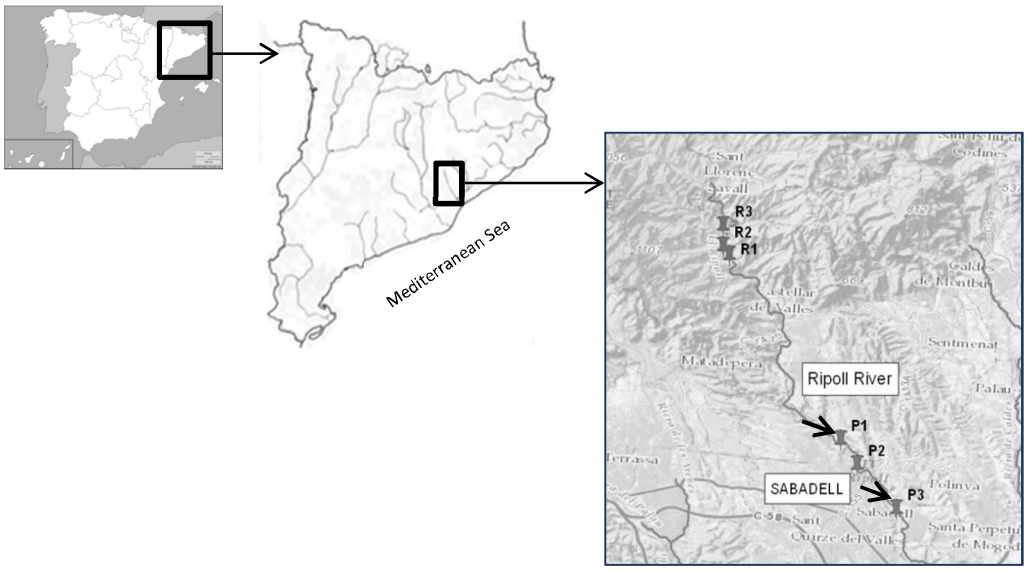


Figure 14. Map of the sampling sites in the Ripoll River (Catalunya, NE Spain). Arrows indicate effluent inputs from sewage treatment plants.

Fish (*B. meridionalis* and *S. laietanus*) were sampled in July 2012 with a portable electrofishing unit which generated up to 200V and 3A pulsed D.C. Immediately after collection, fish were anesthetized and killed by severing the spinal cord. Total length and weight were recorded and liver, gonad and bile were dissected, immediately frozen in liquid nitrogen and stored at -80ºC upon arrival at the laboratory. A subsample from the central part of the gonad was fixed in 10% formaldehyde buffered with 100 mM sodium phosphate at pH 7.4 for histological examination. An international standardised fish sampling method (CEN standards EN 14962 and EN 14011) driven by the European Water Framework Directive was followed.

2.3. Bile analysis

Bile samples were pooled (up to 4 individuals per site) and hydrolysed as described in Fernandes et al. (2013). Briefly, bile (100 mg) was incubated for 1 h at 40ºC in the presence of 1 mL 0.4 M acetic acid/sodium acetate

buffer pH 5.0, containing 2000 units of β-glucoronidase and 50 units of sulfatase. Hydrolysed metabolites were extracted with 1 mL of ethyl acetate (x 3), the extracts recombined and reduced to ~100 μL under a nitrogen stream. The extracts were derivatized by the addition of 100 μL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1h at 70ºC, and further evaporated under a nitrogen stream. Analyses were carried out by gas chromatography-mass spectrometry electron impact mode (GC-MS-EI). The equipment was a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. The column, a 30 m X 0.25 mm i.d. HP-5MS crosslinked 5% PH ME siloxane (Hewlett-Packard, USA) was programmed from 90 to 140 at 10ºC/min and from 140 to 300 at 4ºC/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 250ºC and the ion source and the analyser were maintained at 200ºC and 250ºC, respectively. Just prior to sample analysis, calibration curves were performed with reference compounds and operating in selected ion monitoring mode (SIM). Metabolites were identified and quantified by comparison of retention times and spectra of reference compounds: *m/z* 243, 258 for HHCB; *m/z* 216 and 201 for 2-naphthol;; *m/z* 290 for 1-pyrenol; *m/z* 207 and 193 for 4-nonylphenol (NP) and *m/z* 207 for 4-*tert*-octylphenol (OP). Concentrations are expressed as ng/g of bile.

2.4. Biochemical determinations

After weighting, livers and gonads were flushed with ice-cold 1.15% KCl and homogenized in 1:4 w/v of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 x *g* for 15 min, and the obtained supernatant centrifuged at 12,000 x *g*

for 20 min. The resulting supernatant was further centrifuged at 100,000 x *g* for 60 min to obtain the microsomal pellet, which was resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Microsomal proteins were measured in microsomal subcellular fraction using bovine serum albumin as standard (Bradford, 1976).

7-Ethoxyresorufin O-deethylase (EROD) activity was determined as described in Burke and Mayer (1974). Briefly, 0.1 mg of liver microsomal protein was incubated with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 (final volume of 250 μL) at 30ºC for 10 min and the reaction was stopped by adding 400 μL of ice-cold acetonitrile. After centrifugation, an aliquot of the supernatant was transferred into a 96-multiwell plate. Fluorescence of 7-hydroxyresorufin was read at the excitation/emission wavelength pairs of 537/583 using a Varioskan microplate reader (Thermo Electron Corporation).

BFC-*O*-debenzyloxylase (BFCOD) activity was analysed according to Thibaut et al. (2006). The assay consisted in incubating 25 μg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume of 250 μL) at 30ºC for 10 min. The reaction was stopped by addition of 75 μL of acetonitrile (20:80, v/v), and the fluorescence was read in a 200 μL aliquot transferred into a 96-multiwell plate at the excitation/emission wavelength pairs of 409 and 530 nm, using a Varioskan microplate reader (Thermo Electron Corporation).

Aromatase activity was determined by the tritiated-water release method as described in Lavado et al. (2004). For the assay, 0.1 mg of

ovarian microsomal proteins were incubated for 1 h at 25ºC in the presence of 100 mM Tris–HCl, pH 7.6, 0.2 mM NADPH and [1β-3H]androstenedione (40 pmol, 1 μCi). The reaction was stopped using 3 mL of ice cold methylene chloride and the excess of substrate was further eliminated from the aqueous phase by extraction with methylene chloride (x3). The remaining tritiated steroids were further eliminated by the addiction of a suspension of 2.5% (w/v) of activated charcoal and 0.25% dextran in mili-Q water. The solution was centrifuged 60 min at 3,600 rpm, and the amount of titrated water formed was counted in two aliquots of the aqueous phase (Tri-Carb 2100TR, Packard).

2.5. Gonad histology

Both female and male gonad subsamples were fixed in buffered formalin for 24 h, dehydrated through a graded ethanol series, cleared in histoclear and embedded in liquid paraplast. Tissue sections (7μm) were stained with Hematoxylin-eosin, mounted and examined by light microscopy. Sexual maturation stages were identified according to Bucholtz et al. (2008), with slight modifications. Namely, those ovaries showing a mixture of both perinucleolar and cortical alveoli oocytes were classified as stage I-early maturation; ovaries with secondary growth oocytes containing yolk granules were classified as stage II-Mid maturation, whereas those containing secondary growth oocytes in the nuclear migration stage, filled with yolk were classified as stage III-late maturation. Those in which most oocytes had hydrolysed yolk granules and the nucleus is no longer visible were classified as stage IV-spawning prepared. Finally, those ovaries containing numerous post-ovulatory follicles, primary growth oocytes and a thick ovary wall were classified as stage V-spent/regeneration. For males, testes with thick germinal epithelium containing spermatogonia, primary

and secondary spermatocytes and spermatids were classified as stage I- early maturation; those containing spermatozoa in the centre of most of the tubules with spermatogonia, primary and secondary spermatocytes and spermatids also visible were classified as stage II- mid maturation. Testes that mostly had a thin germinal epithelium with only scattered spermatogenic activity were classified as stage III; When spermatozoa were uniformly distributed in the tubules and detached from the tubule walls, tested were classified as stage IV- spawning. Finally, when residual sperm was present in some tubules and testicular and tubule walls were thick, testes were classified as stage V- spent/regeneration.

2.6. Lipid analysis

In order to obtain homogeneous samples, 20 mg of the dorsal muscle tissue of 4 fish was pooled and freeze-dried. Three pools were prepared per sampling site. Lipids were extracted by a modification of the method of Christie (1985). A solution of methanol:chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT) was added to the 5 mg of freeze-dried muscle, vortexed and after 1 h at room temperature, extracted in an ultrasonic bath for 5 min (x2). The extracts were evaporated to dryness, reconstituted in 1.6 mL of methanol and an aliquot further diluted, previous addition of ~ 200 pmol of the internal standard mix, covering each lipid family.

All analyses were performed with an ultrahigh performance liquid chromatography (UHPLC) system using an octyl carbon chain (C8)-bonded silica column. Chromatographic parameters, such as column temperature, injection volume, flow rate, mobile phases, and gradient elution programs were chosen according the conditions described in Gorrochategui et al. (2014) with some modifications. The liquid

chromatography-mass spectrometer consisted of a Waters Acquity UHPLC system connected to a Waters LCT Premier XE Time of Flight Mass Spectrometer (Waters, Millford, MA), operated in positive electrospray ionization mode (ESI+). Full scan spectra from 200 to 1000 Da were obtained. Mass accuracy and reproducibility were maintained by using an independent reference spray via LockSpray. The mass resolving power of the TOF-MS (determined from the $[M+H]^+$ ion of leucine at m/z 556.2771) was 10,000 FWHM (full with at half maximum). A 100mm x 2.1mm id, 1.7µm C8 Acquity UPLC BEH (Waters, Ireland) analytical column was used at 30ºC. The capillary voltage was 3.0kV, with a desolvatation temperature of 350ºC and a desolvatation gas flow of 600 l/h. The mobile phases were 1 mM ammonium formate in methanol (phase A) and 2 mM ammonium formate in H_2O (phase B), both phases with 0.2% of formic acid. The programmed gradient was: 0 min, 80% A; 3 min, 90% A; 6 min, 90% A; 15 min, 99% A; 19 min, 99% A; 21 min, 80% A held for 2 min. The flow rate was 0.3 mL/min and the injection volume was 8µL. Quantification was carried out using the extracted ion chromatogram obtained. The linear dynamic range was determined by injection of standard mixtures. As quality control the RSD % of the internal standard was measured, values between 9 and 26% were found. Positive identification of lipids was based on the accurate mass measurement with an error < 10 ppm and the retention time, compared to that of the standards (± 2) analyzed under the same chromatographic conditions (Garanto et al., 2013). An inventory of lipids, containing PC, PC-P, LPC, TG, DG and CE, based on reported identified species was first generated. Their theoretical exact masses were determined using a spectrum simulation tool of MassLynx software and LIPID MAPS Lipidomics Gateway, and the obtained list was further used as a referential database. Individual chromatographic peaks of distinct lipid species were isolated from full-scan

MS spectra when selecting their theoretical exact masses, extracted from the database. Then, a list of possible candidates fitting the specific exact mass was generated using formula determination tools (elemental composition search) of Micromass MassLynx software.

2.7. Statistical analysis

Values are presented as mean ± SEM. Comparisons between groups were made using a one-way ANOVA followed by multiple independents group comparison (Duncan and Tukey's test). All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and p values lower than 0.05 were considered statistically significant. In all instances, transformations of the data were performed when the assumption of normality of residuals was not met.

Lipid profile was analysed by using MATLAB R2016a (MathWorks, Natick, Massachusetts) and Metaboanalyst 3.0 Software (McGill University). Data were autoscaled by mean-centering and dividing by standard deviation of each variable. Principal component analysis (PCA) was performed to compare the lipid profile of the fish from references and polluted areas. Loadings were used to interpret relationships between lipid metabolites and fish from R and P sites. Moreover, a univariate study was carried out using Volcano analysis. Volcano shows a graphic representation that allows to sort the lipids along two dimensions, the biological, represented by the fold change (FC) and the statistic represented by the negative logarithm of the p value; and allowed to define significantly changed lipids as those with thresholds of $FC > 2$ and $p < 0.05$. Prior to Volcano analysis data were normalized by median and scaled by mean-centering and dividing by standard deviation of each variable.

3. Results

3. 1. Biological parameters

Size, weight and condition factor of the sampled individuals of *B. meridionalis* and *S. laietanus* are given in Table 8. All the individuals were homogeneous (size and weight), except *B. meridionalis* from station R2 that were bigger (33 ± 4 g) and *S. laietanus* from R3, which were smaller (19 ± 5 g) than the rest. No significant differences were observed for the condition factor among sampling stations.

Table 8. Biological parameters (mean ± SEM) of *B. meridionalis* and *S. laietanus* collected in reference sites (R1-R3) and impacted sites (P1-P3) along the Ripoll River.

Fish specie	Site	N	Length (cm)	Weight (g)	CF[1]
B. meridionalis	R3	10	11.3 ± 9.1	21.0 ± 5.1	1.3 ± 0.03
	R2	10	13.5 ± 6.0	32.7 ± 3.9*	1.3 ± 0.02
	R1	10	11.4 ± 4.7	19.6 ± 2.8	1.3 ± 0.12
	P1	10	10.4 ± 5.3	15.7 ± 3.3	1.3 ± 0.05
	P2	10	11.0 ± 4.2	18.6 ± 2.6	1.3 ± 0.02
	P3	10	10.1 ± 2.9	15.0 ± 1.3	1.4 ± 0.03
S. laietanus	R3	6	11.4 ± 7.7	18.5 ± 4.6*	1.1 ± 0.10
	R2	10	13.8 ± 2.4	31.5 ± 1.5	1.2 ± 0.04
	R1	10	13.9 ± 7.4	33.6 ± 6.6	1.1 ± 0.03
	P1	10	12.5 ± 4.6	26.3 ± 2.8	1.3 ± 0.02
	P2	15	12.2 ± 9.0	31.7 ± 5.4	1.3 ± 0.07
	P3	10	12.6 ± 3.2	26.2 ± 2.1	1.3 ± 0.03

*Significant differences ($p < 0.05$).
[1]Condition factor (CF) calculated as (weight/length^3) x 100.

3.2. Chemical analysis

The analysis of bile samples by GC-MS allowed the identification and quantification of OP, NP, HHCB and two hydroxylated PAHs (2-naphtol and 1-pyrenol) in both species (Fig. 15). NP concentrations are reported as the sum of 11 isomers, and their identification was shown unequivocally by the complete match of profiles between samples and the NP standard mixture. Among the detected compounds, NP and 1-pyrenol were above the detection limit in all the analysed samples. However, OP, HHCB and 2-naphtol were below detection limit in some fish individual from R sites. Levels of all the detected compounds were significantly higher in the bile of fish collected in P than those from R sites. Thus, the highest levels of NP were detected in P2 (1557 ± 176 and 2157 ± 292 ng/g, respectively) in both *B. meridionalis* and *S. laietanus*, whereas high concentrations of 2-naphtol (156 ± 29 and 226 ± 22 ng/g), 1-pyrenol (34 ± 5 and 56 ± 10 ng/g) and HHCB (1277 ± 470 and 1907 ± 990 ng/g) were found in P3. Generally, higher concentrations of all analysed compounds were detected in the bile of *B. meridionalis* in comparison to *S. laietanus*.

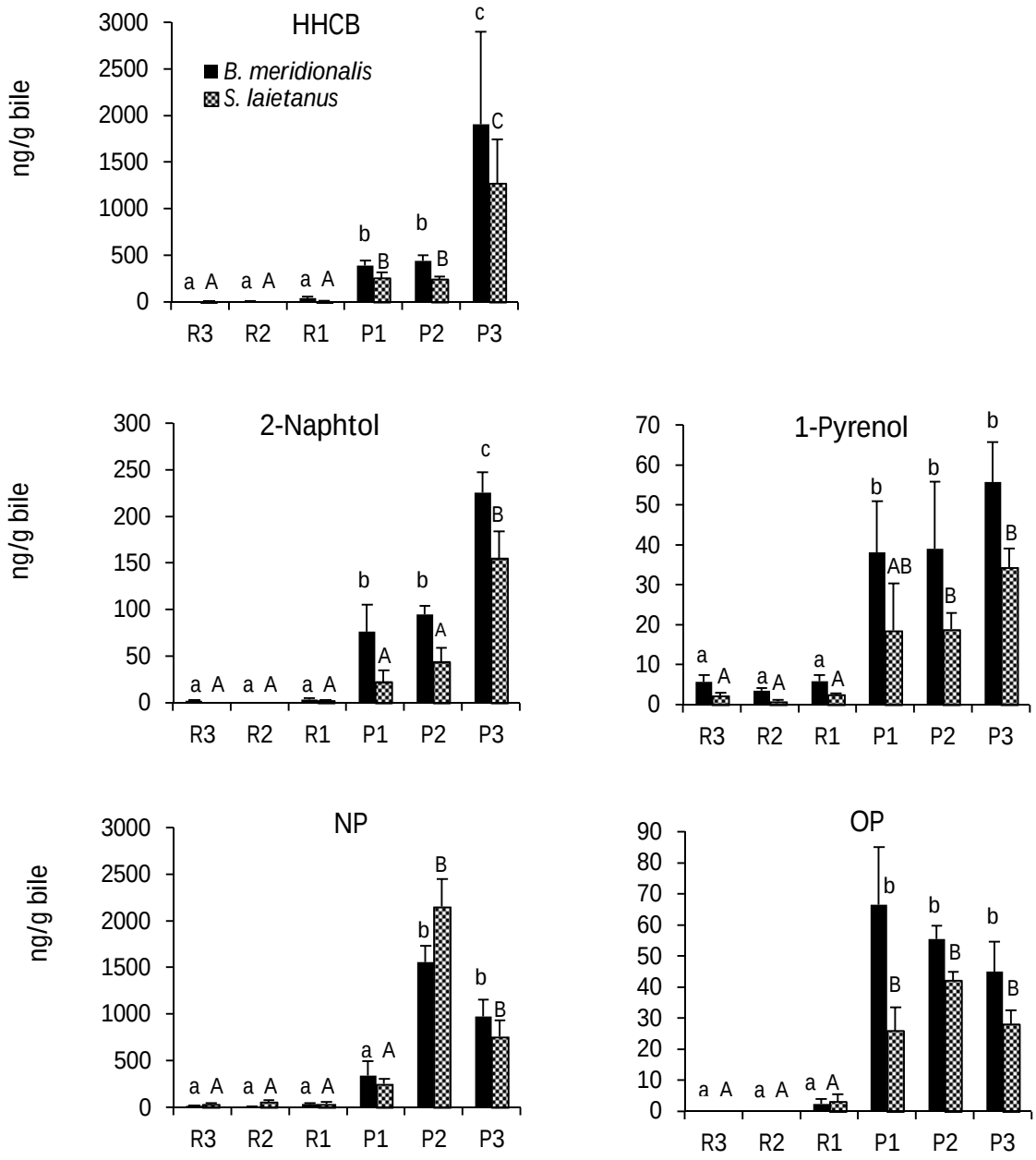


Figure 15. Biliary levels of HHCB, hydroxylated PAHs (2-napthol and 1-pyrenol) and alkylphenols (NP and OP), detected in *B. meridionalis* and *S. laietanus* collected along the Ripoll River. Values are expressed as mean ± SEM (n = 3-8, each sample is a pool of 2-4 individuals). Distinct letters indicate statistically significant differences among sampling sites ($p < 0.05$). Lower case letters, *B. meridionalis*; capital letters, *S. laietanus*.

3.3. Biochemical markers

The determination of EROD and BFCOD activity allowed the detection of clear differences between R and P stations (Fig. 16). EROD activity was significantly elevated in both, *B. meridionalis* (120 ± 18 to 184 ± 32 pmol/min/mg protein) and *S. laietanus* (48 ± 12 to 104 ± 26 pmol/min/mg protein) from P sites in comparison to individuals sampled upstream (20 ± 3 - 35 ± 6 and 10 ± 2 - 11 ± 1 pmol/min/mg protein). Similarly, increased BFCOD activity was detected in P sites (357 ± 34 to 431 ± 40 and 155 ± 18 to 250 ± 24 pmol/min/mg protein in *B. meridionalis* and *S. laietanus*, respectively) in comparison to R stations (144 ± 22 to 188 ± 30 *B. meridionalis* and 50 ± 7 to 57 ± 8 pmol/min/mg protein *S. laietanus*). EROD and BFCOD activities were higher in *B. meridionalis* than in *S. laietanus*.

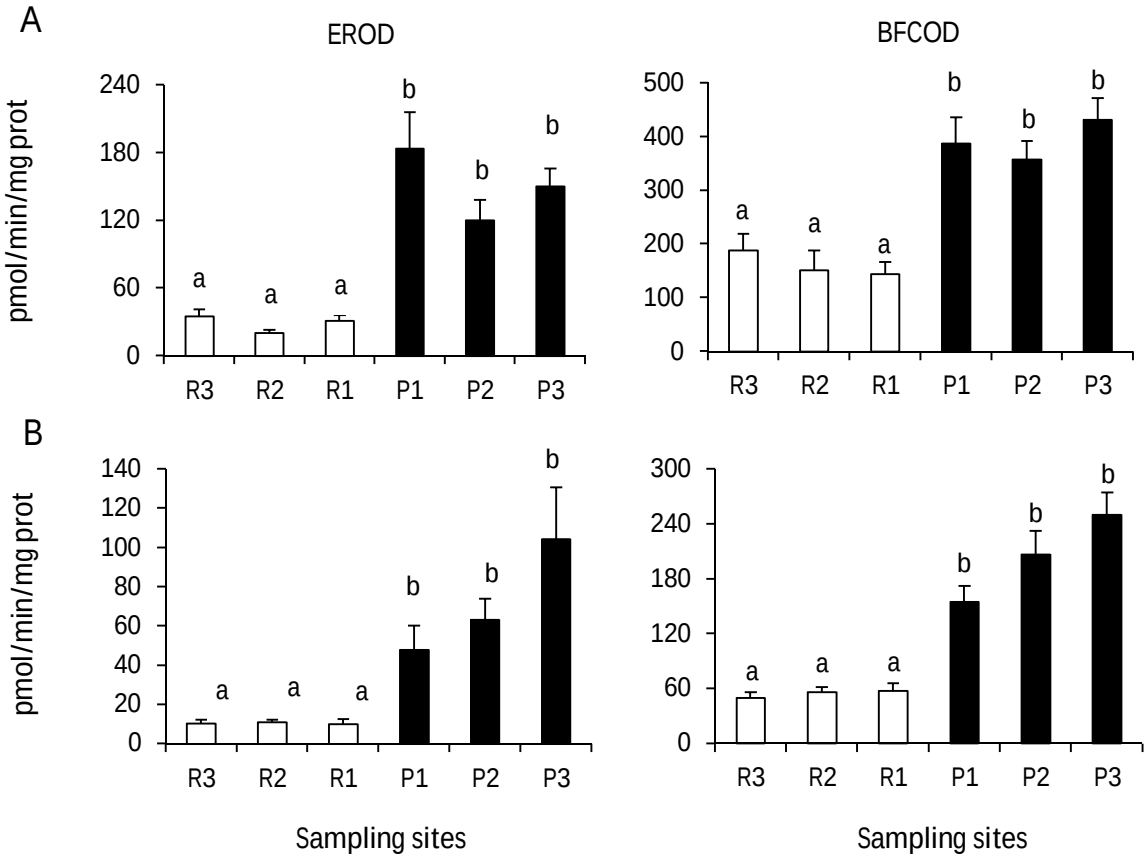


Figure 16. EROD and BFCOD activities determined in liver of *B. meridionalis* (A) and *S. laietanus* (B) collected along the Ripoll River. Values are expressed as mean ± SEM (n = 6-7). Different letters denote significant differences between sampling sites ($p < 0.05$).

3.4. Histological analysis and ovarian aromatase activity

Histological analysis showed no abnormalities within the gonadal tissue (Fig. 17). Most of the females of *B. meridionalis* (70-80%) had ovaries at mid and late stages of gametogenesis (stages II and III), while females of *S. laietanus* (66-80%) had less developed ovaries containing perinuclear and cortical alveoli oocytes that were classified as stage I. No significant differences between R and P sites were observed for females. Regarding males, 70% of *B. meridionalis* collected in P sites had testes at mid and late spermatogenesis (stages II and III), while those collected in R sites (81%) had mature testes classified as stages IV and V that correspond to testes in spawning and regeneration. In contrast, no significant differences between P and R sites were observed for males of *S. laietanus*, about 55% of males had testes at stages IV and V, and the rest were at different stages of gametogenesis.

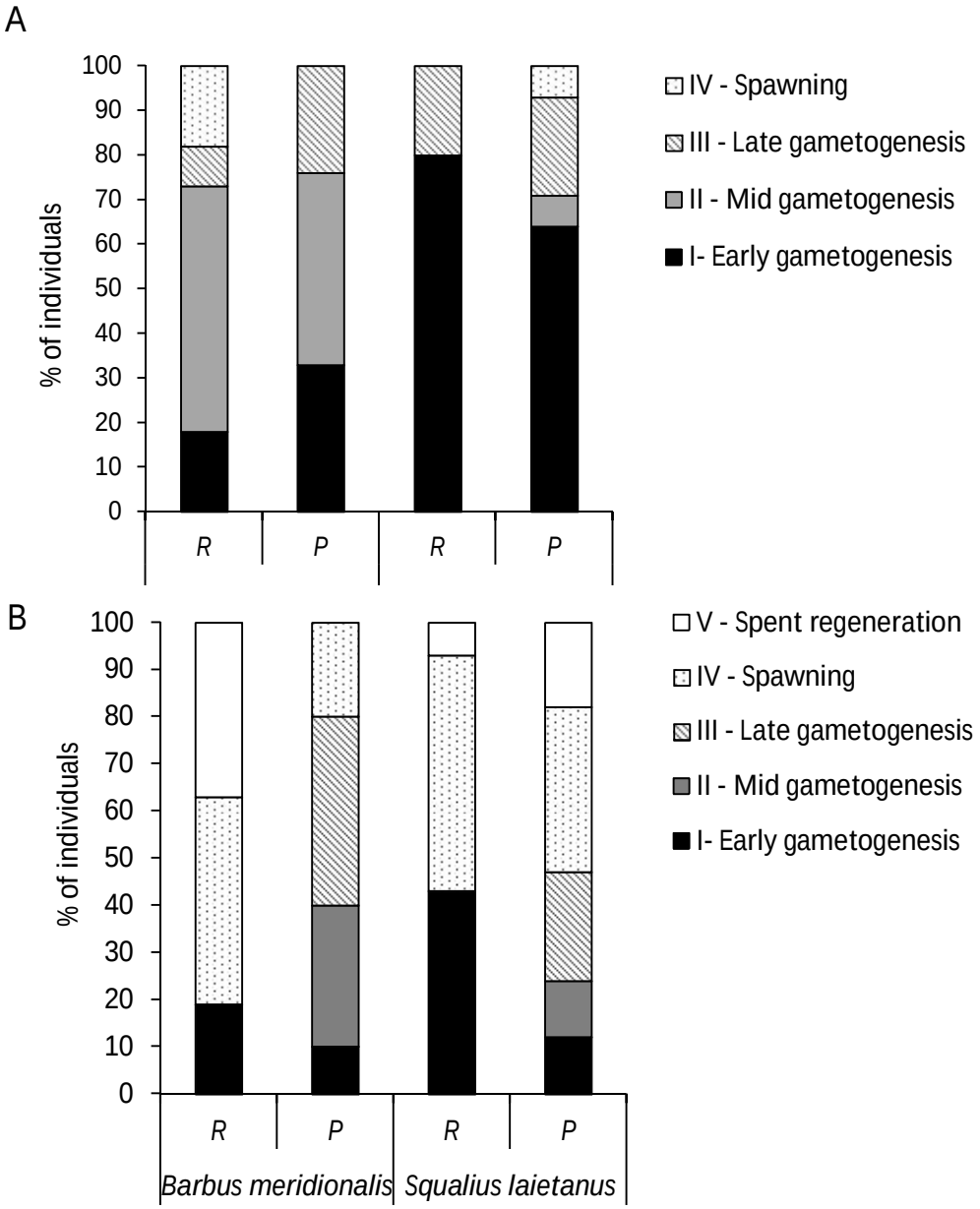


Figure 17. Percentage of females (A) and males (B) classified by gonad maturation stage collected along the Ripoll River in P and R sites. *B. meridionalis*: n = 32 females, 25 males; *S. laietanus*: n = 19 females, 26 males.

P450-aromatase activity was only determined in females of *B. meridionalis*, as most of the females of *S. laietanus* were at early stages of gametogenesis and P450-aromatase activity was very low. Aromatase activity was tightly related to the sexual maturation stage of the ovaries; the activity increased over the maturation process and reached a maximum in

ovaries classified as stage III (3.1 ± 0.2 to 13 ± 3 pmol/h/mg protein), to further decrease during spawning (1.4 ± 0.08 to 4.02 pmol/h/mg protein) (Fig. 18). Aromatase was significantly higher in female of *B. meridionalis* collected in P sites regardless of the maturation stage of the ovaries. This increase was particularly evident in ovaries classified as stage III (late gametogenesis), for which aromatase activity was up to 4-fold higher in females sampled in P sites in comparison to R sites.

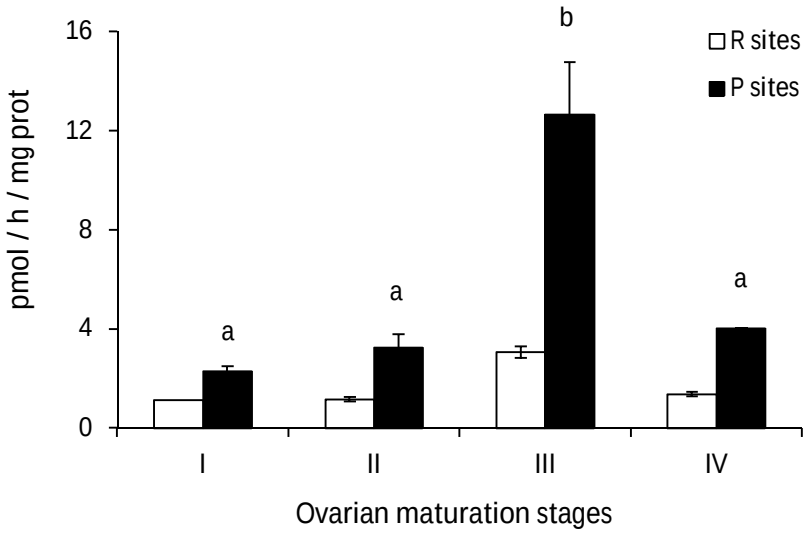


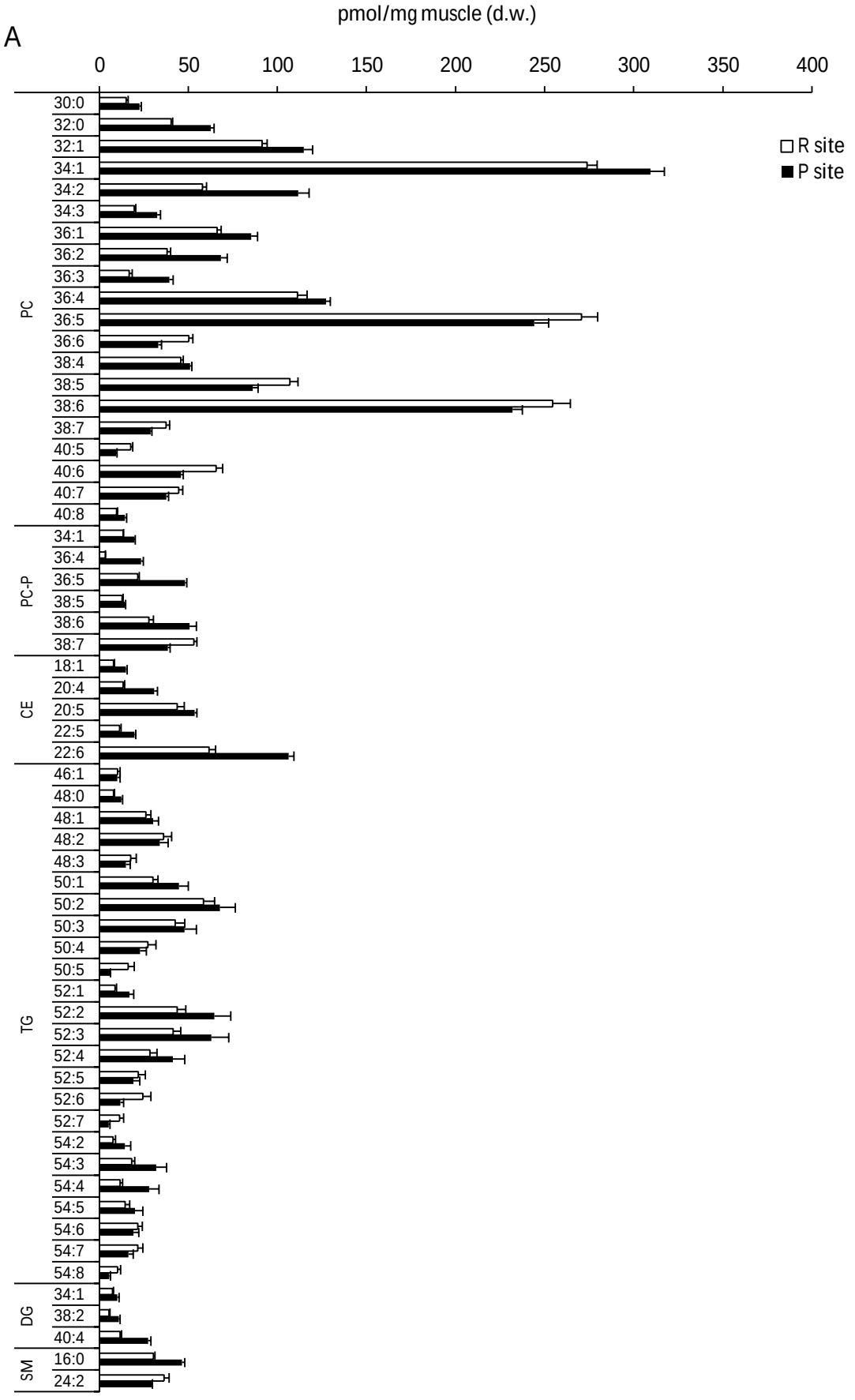
Figure 18. P450-aromatase activity (mean ± SEM (n = 1-9)) determined in ovarian microsomal fraction of *B. meridionalis* collected along the Ripoll River related to their gonadal maturation stage (I- Early maturation, II- Mid Maturation, III- Final maturation, IV-Spawning).

3.5. Lipid analysis

PC, PC-P and SM were the first groups of lipids to elute and appeared at the early 10 min of the UHPLC-ToF chromatogram. These lipids were totally resolved from DG, TG and CE which eluted in the subsequent 15-20 min. Individual lipid species unresolved in the total ion chromatogram were successfully isolated when their exact masses were selected. TG, DG and CE were detected as $[M+NH_4]^+$ adducts, whereas the rest of lipids were

identified in the protonated form, $[M+H]^+$. The analysis by UPLC-ToF in the muscle tissue from both fish species allowed the identification and quantification about 130 lipids, including 36 PC, 23 PC-P, 12 CE, 40 TG, 11 DG and 8 SM. Molecules were identified under the criteria of molecular formula, accurate mass, error (< 5 ppm) and retention time (Table 9).

The use of internal standard in lipid samples allowed the semi quantification of all lipid molecules. As no standard was available for SM and PC-P, PC standard was used to calculate its concentration. Semi quantitative analysis of the detected lipids evidenced that the muscle tissue of fish from reference sites was characterized by a high abundance of PC 34:1, 36:5, 38:6; TGs 50:2, 52:2, 52:3; CE 20:5, 22:6, SM 16:0 in both fish species; together with PC-P 38:7; DG 40:4; SM 24:2 in *B. meridionalis* and PC-P 36:5; DG 34:1 in *S. laietanus* (Fig. 19A,B).


A
pmol/mg muscle (d.w.)
0
50
100
150
200
250
300
350
400
R site
P site
PC
30:0
32:0
32:1
34:1
34:2
34:3
36:1
36:2
36:3
36:4
36:5
36:6
38:4
38:5
38:6
38:7
40:5
40:6
40:7
40:8
PC-P
34:1
36:4
36:5
38:5
38:6
38:7
CE
18:1
20:4
20:5
22:5
22:6
TG
46:1
48:0
48:1
48:2
48:3
50:1
50:2
50:3
50:4
50:5
52:1
52:2
52:3
52:4
52:5
52:6
52:7
54:2
54:3
54:4
54:5
54:6
54:7
54:8
DG
34:1
38:2
40:4
SM
16:0
24:2

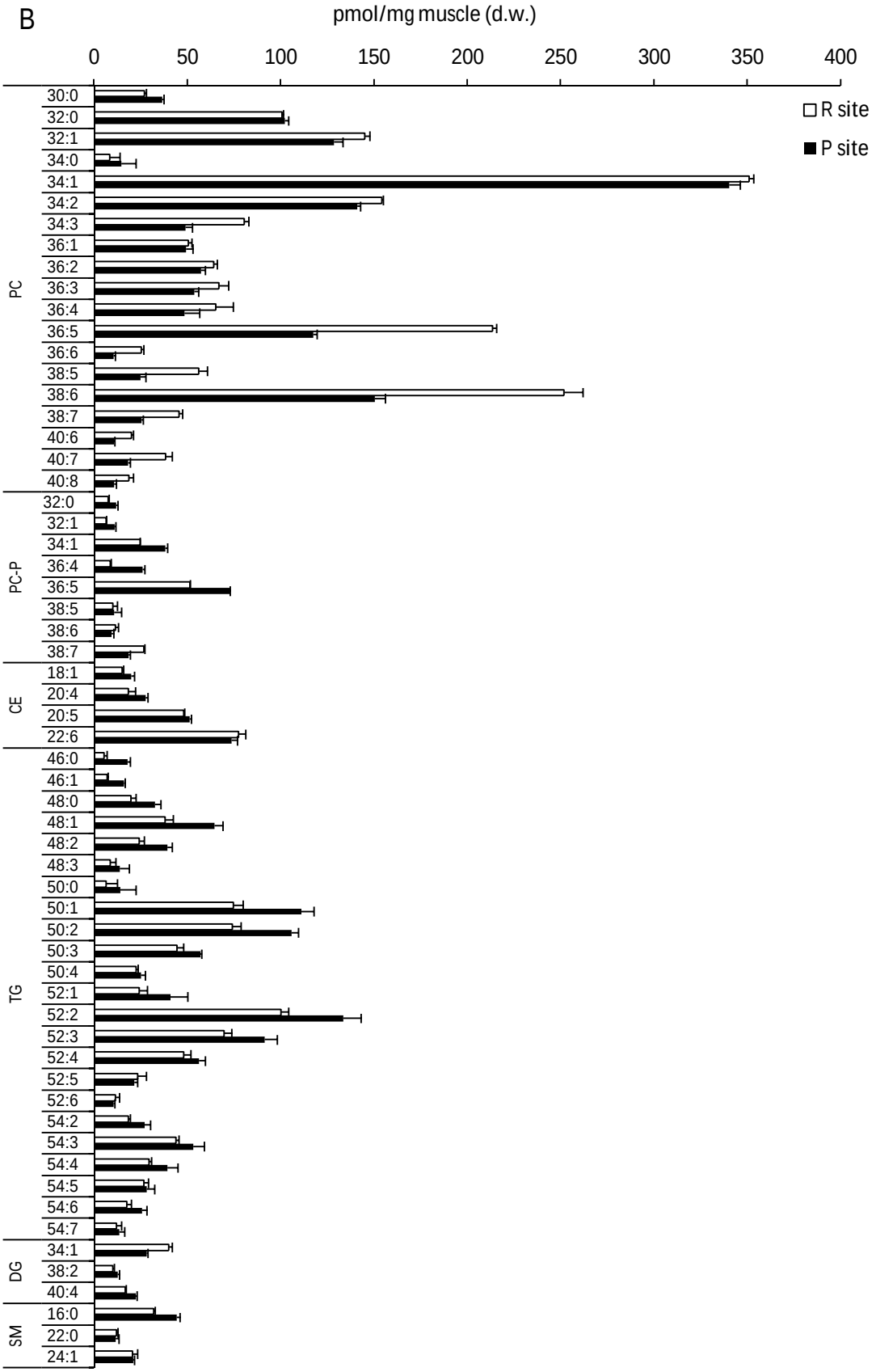


Figure 19. Lipid profile (mean ± SEM) in muscle of *B. meridionalis* (A) and *S. laietanus* (B) from R and P sites. Lipids under 10 pmol/mg of muscle are not presented.

Principal component analysis (PCA) was used to evaluate differences in lipid composition among fish collected from R and P sites. PCA results explained 60.3% of total variance and allowed the separation of the lipidome of *B. meridionalis* collected from P and R sites, with two principal components (Fig. 20A). PC1, which assumed 38% of the explained variance, gave high positive loadings for PC with low number of unsaturations (32:0, 34:0, 34:2, 36:1, 36:2), PC-P 34:1, 34:2, 38:4, unsaturated SM, CE 22:2, 22:4 and TG less polyunsatured (48:0, 50:1, 52:1; 54:4; 58:4) in the muscle tissue of *B. meridionalis* from P sites (Fig. 20B). In contrast, PCs with a higher number of double bounds (viz. 34:5, 36:6, 40:6, 40:5) were positively related with *B. meridionalis* from R sites.

A

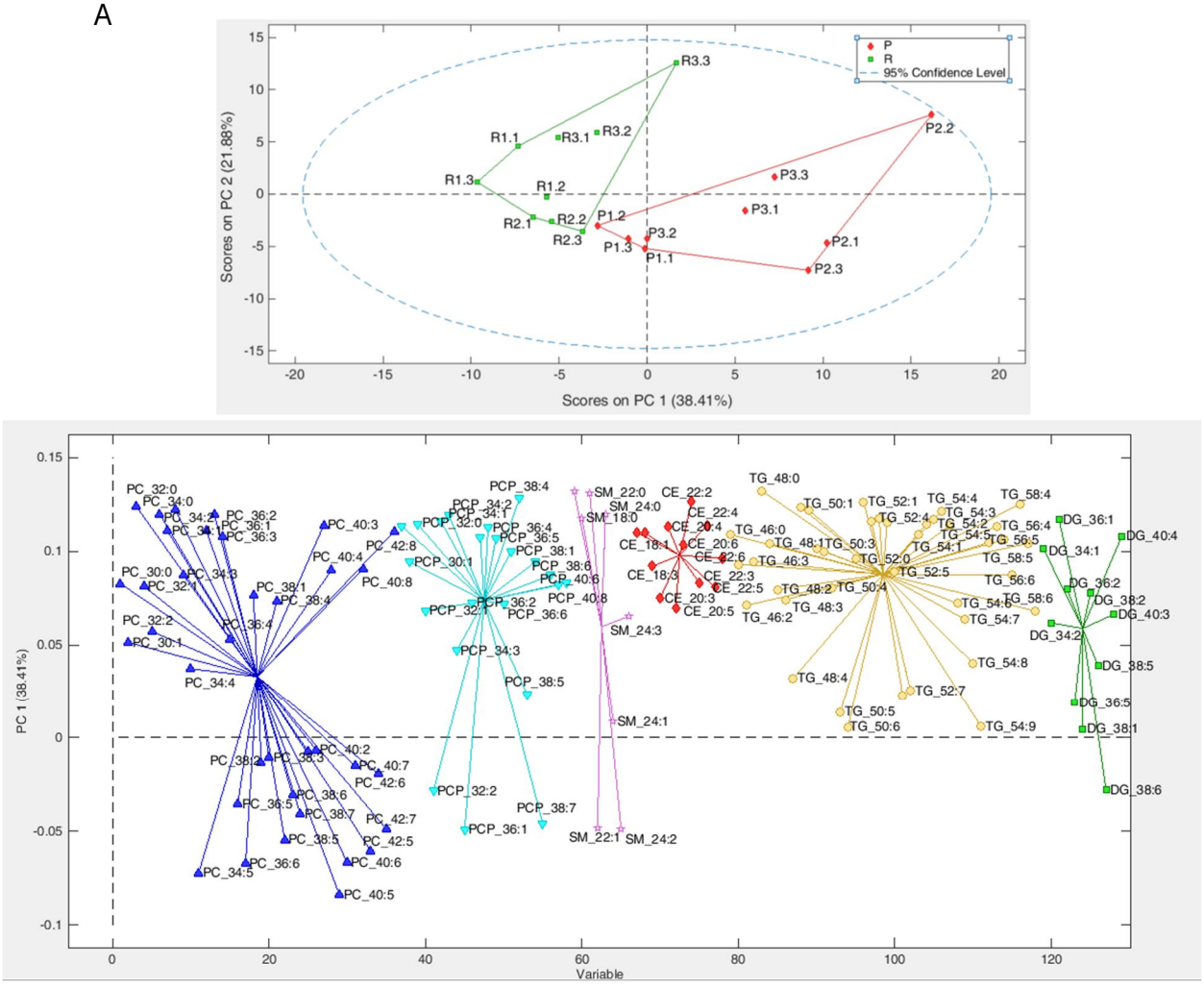
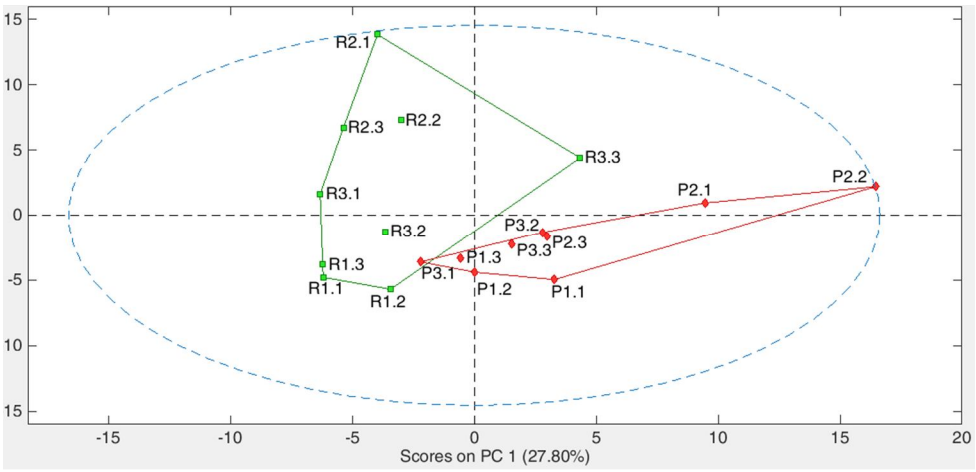


Figure 20. PCA analysis of muscle of *B. meridionalis* from R and P sites (A) and loadings of variables (B) resulting from autoscaled pmol.

PCA analysis executed for *S. laietanus* assumed 49% of the total variance (Fig. 21A). PC1 (27.8%) allowed differentiating fish collected from P and R sites. TG molecules were the lipids highest positive correlated with *S. laietanus* from P areas. On the other hand, a correlation between samples collected in R sites and polyunsaturated PCs (36:5, 38:5, 38:6, 40:7, 40:8, 42:6) and PC-Ps 38:6 and 38:7 was shown (Fig. 21B).

A



B

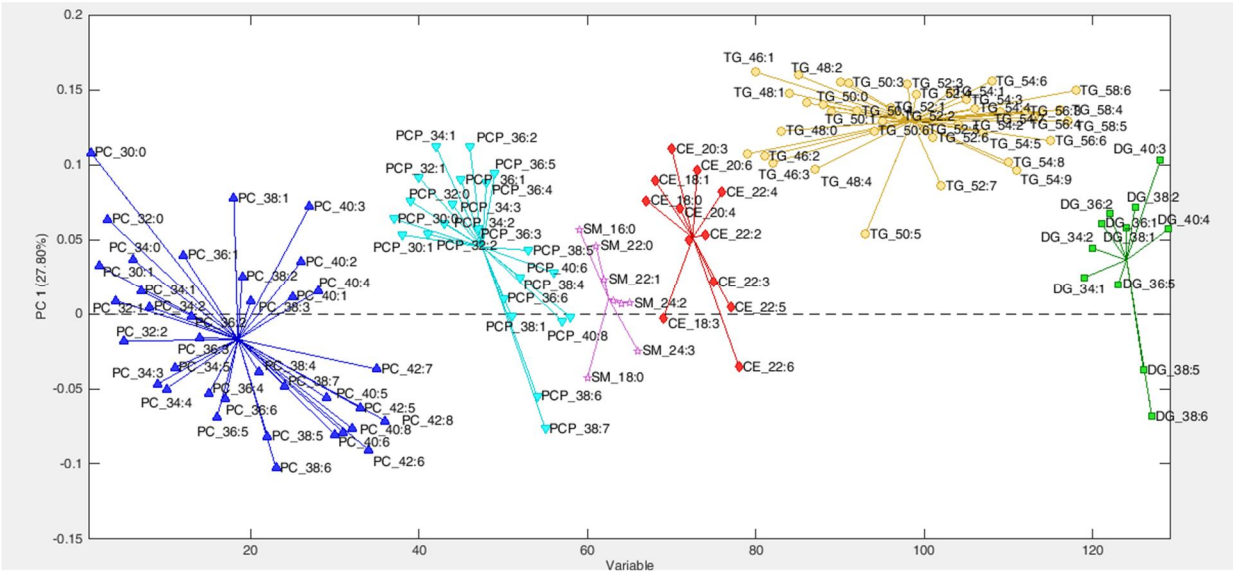


Figure 21. PCA analysis of muscle of *S. laietanus* from R and P sites (A) and loadings of variables (B) resulting from autoscaled pmol.

Volcano plots are shown in Figure 22. Setting a significance threshold of FC > 2 and $p < 0.05$, we found 15 lipids in muscles of *B.*

meridionalis and 14 in *S. laietanus* with significantly differences between fishes from P and R sites. PC-Ps 32:0, 36:4, 38:4, 38:6 were considerably accumulated in *B. meridionalis* from P sites, together with CEs 18:0, 20:4, 22:2, 22:4 and DG 38:5 in a lesser extent, whereas TGs with a high number of double bonds (TGs 50:5, 50:6, 52:6, 52:7, 54:9) and PC 40:5 were significantly reduced. Regarding *S. laietanus*, PC-Ps 36:4, 38:4 and TGs 46:0, 46:1 and 50:0 were at significant high proportion, whereas polyunsaturated PCs 34:4, 36:5, 36:6, 38:5, 40:5, 40:6, 40:7 and 42:6 were significant decreased in fish from P sites when compared with those from R sites.

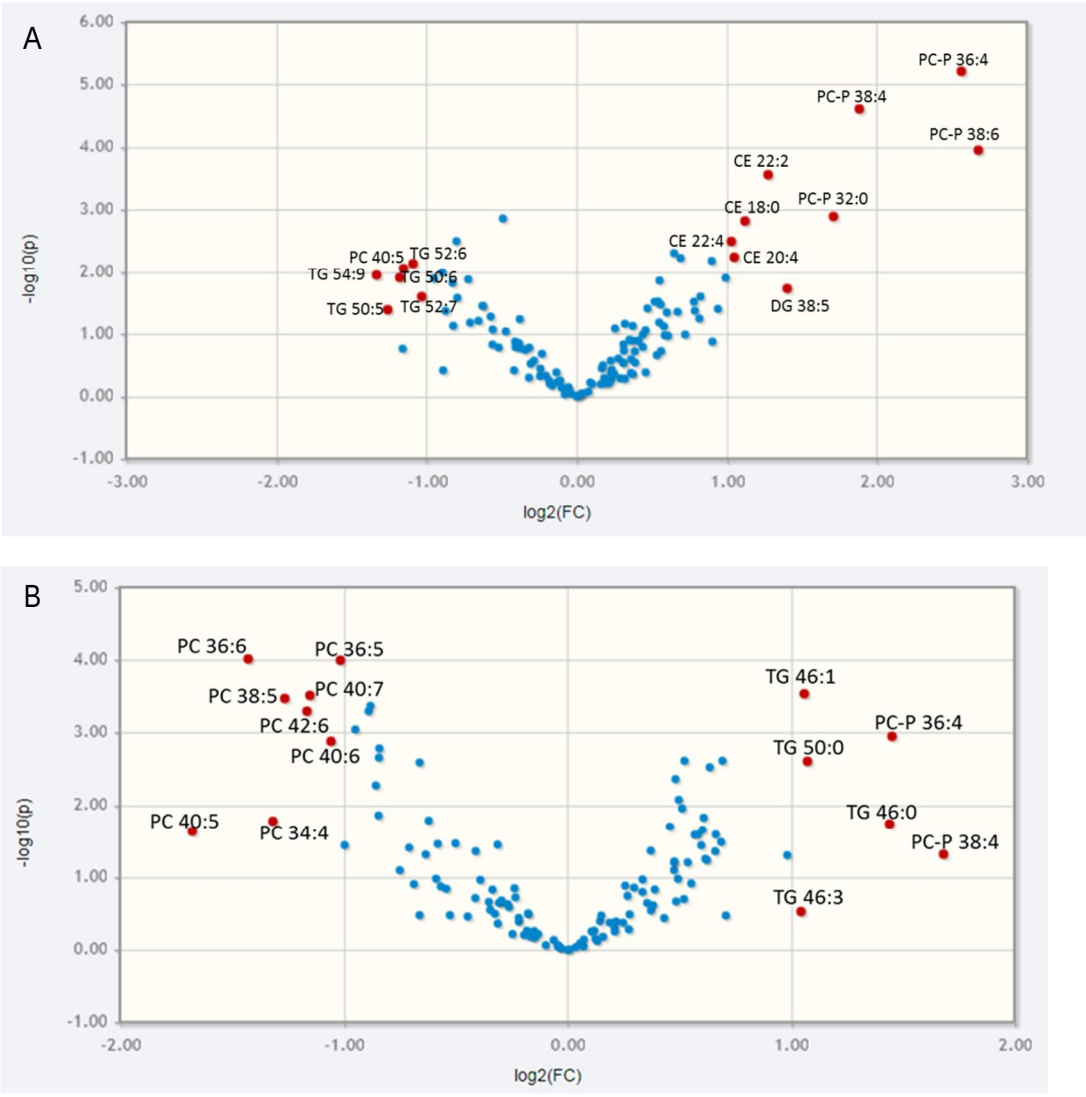


Figure 22. Important lipids selected by Volcano plot with fold change threshold (x) 2 and t-tests threshold ($p < 0.05$) in muscle of *B. meridionalis* (A) and *S. laietanus* (B). Red circles represent lipid molecules above the threshold and indicates representative lipids with highly significant fold changes. The further its position away from the (0,0), the more significant the feature is. Both fold changes and values are log transformed. The analysis was performed from pmol.

4. Discussion

The Ripoll River, as other Mediterranean rivers in semi-arid regions, is particularly sensitive to pollution loads, especially in the summer period, when the very low dilution ability of the river intensifies pollution stress and contaminants are concentrated in river waters. The analysis of bile evidenced that fish collected in upstream sites (R1-R3) were almost not exposed to HHCB, 2-naphtol and APs, which confirms this area as a good reference zone, only affected by diffuse contamination which may proceed from urbanised areas away (Prat and Rieradevall, 2006). In contrast, the analysis of bile from fish collected at downstream areas (P sites) showed recent and significant exposure to HHCB, PAHs and APs. These areas were mainly affected by industrial and urban effluents, which are the major source of organic pollution in Mediterranean rivers (Maceda-Veiga et al., 2010).

HHCB, a compound commonly used in a wide variety of personal care and consumer products, is one of the main polycyclic musks produced in the EU and US (Clara et al., 2011). WWTPs represent the main source of musks to the aquatic environment where it has been detected at concentrations of 20-4000 ng/L in WWTP effluents (Homem et al., 2015). In our study, the highest levels of HHCB (up to 2000 ng/g) in bile of *B. meridionalis* and *S. laietanus* were found in P3, corresponding to the entrance of an urban effluent into the river. Scarce information about

biliary levels of HHCB has been reported and, to our knowledge, this is the first report about bile levels of HHCB in fish from rivers. Concentrations in the range of 124-879 ng/g in the bile of grey mullets have been found in coasts of the North of Spain (Bizarro et al., 2014; Ros et al., 2015), lower than the values detected in the present study.

Regarding PAH metabolites, higher levels of 1-pyrenol and 2-naphtol (ranged from 20 to 225 ng/g bile) were found in bile of *B. meridionalis* and *S. laietanus* species from P sites than in fish collected in R sites. 1-pyrenol is the main metabolite of pyrene and has been regarded as the key PAH metabolite in fish (Ruddock et al., 2002). No significant differences in levels of 1-pyrenol were found among P sites, likely because of its pyrolytic origin (industrial, car traffic), and its widespread presence in the atmosphere of highly populated areas. In contrast, levels of 2-naphtol were significantly higher in P3 in the bile of both *B. meridionalis* and *S. laietanus* indicating a specific input in the site. Although 2-napthtol is often related to petrogenic origin, this compound is also used in dyes, pigments and synthetic fragrances, and may originate from textile industries located in the area (Colin et al., 216). Several studies reported biliary PAHs in fish detected by fluorimetric methods, but few data is avaiable using GS-MS techniques. Interestingly, similar levels of 1-pyrenol than those found in *B. meridionalis* and *S. laietanus*, were detected in the bile of other Mediterranean fish species; and also other PAHs were detected (1-naphtol: 14 ng/g; 2-phenylphenol: 40 ng/g; 9-fluorenol: 15 ng/g) (Escartín and Porte, 1999). However, higher levels of PAH metabolites (283-7600 ng/g) were found in the bile of marine fish inhabiting coasts affected by harbours, industries, waste treatment plants and high-populated cities, suggesting that Ripoll River is not specially affected by entries of pollutants from

petrogenic origin (Escartín and Porte, 1999; Puy-Azurmendi et al., 2010; Ruddock et al., 2003).

NP and OP are the breakdown products of AP polyethoxylates, that are commonly used in industrial products including detergents, emulsifiers, solubilizers, wetting agents and dispersants. There is a direct relationship between concentrations of NP and OP and the presence of urban or industrial activities near the sampling point and concentrations in the range of µg/L have been detected in urban and industrial wastewaters (Vitali et al., 2004; Céspedes et al., 2005; Brix et al., 2009). In the present study, the highest biliary levels of NP (1557 and 2157 ng/g bile for *B. meridionalis* and *S. laietanus*, respectively) were detected in station P2, which suggests a specific input of NP in the area. Regarding OP levels in bile, no significant differences were detected among P sites, suggesting not only urban and industrial STPs effluents located in P1 and P3 as sources of OP, but also some punctual entrance in P2. Higher concentrations of NP (from 12 to 112 fold) and OP (from 9 to 86-fold) have been previously reported in the bile of carp, barbel and chub collected from other Mediterranean rivers (Lavado et al., 2006; Viganò et al., 2015). Comparing with NP, lower levels of OP (up to 70 ng/g) were detected in the bile of *B. meridionalis* and *S. laietanus* from P areas, probably due to the lower commercial use of the latter.

Besides the analysis of pollutants in fish bile, the use of biochemical markers helped to detect biochemical disturbances that had occured as a consequence of exposure. EROD activity measured in liver microsomes of fish has been successfully used as biomarker of exposure to a wide variety of organic pollutants, including PAHs, dioxine-like compounds and organochlorine compounds, among others (Whyte et al., 2000). A significant EROD induction (5- and 8-fold) was detected in the livers of *B. meridionalis* and *S. laietanus* from P sites, respectively, in comparison to

those collected in R sites. These levels of EROD activity were higher than those detected in the liver of barbels (36-80 pmol/min/mg prot) collected at other Mediterranean rivers polluted with organochlorine compounds (PCB, DDTs, HCB and HCHγ) and PAHs after an oil spillage, indicating the high exposure of fish from P sites to AhR agonists which induce the enzymatic systems in fish (Lavado et al., 2006; Damásio et al., 2007). The presence of HHCB in the environment is not likely to induce this biomarker, as EROD was not significantly altered in sea bass following injection of 50 mg/kg of HHCB (Fernandes et al., 2013). In humans HHCB did not activate AhR either (Schreurs et al., 2005).

BFCOD activity has not been extensively used as a biomarker of pollution in fish as EROD activity. However, recent works are measuring this activity as a complementary biomarker in field studies (Della Torre et al., 2010; Quesada-García et al., 2013; Habila et al., 2017). *B. meriodionalis* and *S. laietanus* from P sites showed BFCOD activity 2- and 3-fold higher than fish collected in the reference areas (R), suggesting the exposure of these fish to different xenobiotics, including pesticides, and pharmaceuticals, among others (Burkina et al., 2015; Li et al., 2008, Crespo and Solé, 2017). Interestingly, the highest BFCOD activity was detected in fish from P3 station, located after an urban effluent. Recent studies reported up to 4 and 9-fold induction of BFCOD in grey mullets and barbels, respectively, collected in areas polluted by PAHs (ng/g) in comparison to reference areas, and suggested BFCOD as a possible general marker of CYP induction by PAHs (Della Torre et al., 2010). The lower hepatic levels in *S. laietanus* suggested a lower metabolism than in *B. meridionalis*.

The analysis of the gonads evidenced some endocrine alterations in *B. meridionalis* collected from P sites, namely delayed maturation in males and an increase in ovarian aromatase activity in females. These results

linked to the 2-fold higher VTG gene expression detected in the liver of *B. meridionalis* males from P sites with respect to males from R areas (data non published), are an indication of endocrine disruption in these species. Concentrations of NP and OP (856-7843 ng/g and 21-153 ng/g of bile, respectively), were detected in the bile of *B. meridionalis* in the range that promote endocrine disruption effects in grey mullet males from the Basque coast, including high prevalence of intersex gonads, detection of high levels of VTG protein and transcriptional levels of vtg mRNA (Puy-Azurmendi et al., 2013). P450-aromatase activity, a key step in the ovarian maturation and female reproduction in teleosts, was detected in all females of *B. meridionalis* and was tightly related to the maturation stage (Guiguen et al., 2010). In many teleosts, P450-aromatase increases during vitellogenesis, viz. the highest levels of aromatase expression were found in the vitellogenic stage in ovaries of rohu, *Labeo rohita* (Mills et al., 2014; Moulik et al., 2016). Females of *B. meridionalis* at late gametogenesis maturation stage, corresponding to oocytes filled with yolk, showed the highest P450-aromatase activity (12 pmol/h/min). Inhibition of aromatase activity has been reported in carps collected downstream an STP, in brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump and in carps exposed to high levels of NP (Noaksson et al., 2005; Lavado et al., 2006; Martin-Skilton et al., 2006). In contrast, a significant induction in CYP19 activity was observed in *B. meridionalis* from P sites suggesting exposure to a variety of domestic, agricultural, and industrial effluents containing estrogen-like compounds (e.g., estradiol, ethinylestradiol, APs). The presence of PAHs and their mixtures also would affect the development and the maturation process in fish (Le Bihanic et al., 2014a,b).

Recent advances in lipidomics have allowed investigating the effect of different xenobiotics in cells, tissues, plants and animals (Cajka and Fiehn, 2014). The UHPLC-ToF analysis allowed the detection about 130 lipids and the characterization of the lipid profile in the muscle of fish. PC was the main lipid subclass forming the muscle of *B. meridinalis* and *S. laietanus* (62 and 44%, respectively). This phospholipid was also the most abundant in eukaryotic cells (45-55%) and in other fish tissues as liver (Lie and Lambertsen, 1991; Vance and Steenbergen, 2005; Bohdanowicz and Grinstein, 2013). A relative decrease of PCs with a high number of double bonds (5-9) in fish collected in P sites was observed in both fish species. Reactive oxygen species (ROS) generated as subproducts of normal fish metabolism can be enhanced after exposure to xenobiotics, as PAHs. If ROS molecules are not detoxified, they may interact with cellular macromolecules inducing or suppressing the antioxidant defense system, causing oxidative tissue damage and lipid peroxidation (Damásio et al., 2007; Sheriff et al., 2014; Cífková et al., 2015). This oxidation process mainly affects to polyunsaturated fatty acids (PUFAs), which represent the major target for nonenzymatic oxidation by free radicals and ROS molecules (Bochkov et al., 2010). Among the cellular components, phospholipids containing PUFAs are the most susceptible to peroxidation (Luczaj et al., 2017). Tipical for fish phospholipids is the very high amount of PUFA, being highly susceptible to peroxidative deterioration (Mourente et al., 2007). PC-P is other lipid subclass highly vulnerable to oxidative stress; they serve as endogenous antioxidants and they represent the major pool for polyunsaturated fatty acids in cellular membranes (Engelmann, 2004; Braverman and Moser, 2012). Despite they do not represent one of the most abundant lipid subclass in muscle of *B. meridionalis* and *S. laietanus* (5 and 4%, respectively), statistical analysis point to some PC-P

molecules as one of the lipid species with the highest differences among fish from polluted and reference sites.

In teleost fish, TG composition could depend on nutritional status, temperature and physiological factors, as sex and reproduction (Bogevik et al., 2011; Maradonna et al., 2015). However, lastly, several studies reported a significant alteration of TGs in fish after exposure to single compounds including TBT, organophosphates, herbicides, bisphenol A and fibrates (Capitão et al., 2017; Pinto Persch et al., 2017). Most of the data reported was obtained from experiments of exposure performed in laboratory injecting or supplying compounds through the water, by using methods based on kits and dyings as Nile Red and Oil Red, but scarce studies were carried out in wild fish by using more sentitive techniques as ultra-high chromatography mass spectrometry. A 2.5-fold increase in TGs was reported in the ovaries of rare minnow after exposure to 0.015 mg/L of BPA (Zhang et al., 2016); TGs increased after exposure to 0.5 mg/L of difenoconazole in zebrafish embryos (Mu et al., 2016); and TGs increased in plasma of atlantic cod after exposure to 2 mg/kg of PCB-153 (Yadetie et al., 2017). Other aquatic organisms, as mussels collected from polluted sites, showed increased concentrations of TGs (Capuzzo and Leavitt, 1988; Chetty and Indira, 1994; Bergen et al., 2001). In P sites of Ripoll River, *S. laietanus* showed a significant increase of TGs with low number of double bounds, whereas in *B. meridionalis* polyunsaturated TGs suffered a depletion probably linked to oxidation of PUFAs.

An accumulation of CEs in the muscle of *B. meridionalis* from urban and industrial areas was also observed. Some individual chemicals affected CE levels in fish. For instance, an increase of CE levels in the liver of *Danio rerio* after exposure to 0.1 and 1 mg/L of PFNA has been reported (Zhang et al., 2012). In contrast, CEs detected in brain of *Gadus morhua*

suffered a reduction after exposure to 0.02 to 80 mg/kg to a mixture (1:1:1:1) of APs (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol) and after exposure to 17 β-estradiol (5 mg E2/kg) for 5 weeks (Meier et al., 2007). Some authors related the increase of TGs and CEs with an increase in the energy demand to mediate the effects of stress in fish.

Other lipid subclass as DGs and SMs were detected in the muscle of *B. meridionalis* and *S. laietanus*, although no significant differences were pointed out among fish from R and P sites. DGs are neutral lipids with functions in cell signalin and in the synthesis of PCs, via the condensation of CDP-choline, and in the synthesis TGs, via acylation of DGs (Oxley et al., 2007; Cole et al., 2012). Since DG and other lipid metabolism is closely relate, processes as hydrolysis of CEs, TGs or PCs could result in the production of DGs (Dissanayake et al., 2016). On the other hand, SMs have separate pathways for their synthesis and degradation than neutral and phospholipids, but their metabolism is close related: SM is produced by transferring the phosphocholine group of PC to ceramide, and in the process generates DGs (Deevska and Nikolova-Karakashian, 2017). SMs are important components of cell membranes and involved in regulation of cell function, membrane protein trafficking and inflammation, and also have function as transducers in eukaryotic cells, modulating cell growth, differentiation and cell death (Gault et al., 2011). Recently, their role in the regulation of lipid droplet biogenesis have been revealed and SMs have been shown to accumulate in parallel to TGs in liver, muscle and other tissues in animals and humans, evidencing that SM and neutral lipid accumulation could be interconnected (Potratz et al., 2016; Deevska and Nikolova-Karakashian, 2017). However, in our study no evidences of this relation were observed.

The presence of xenobiotics in bile and the biochemical and histological markers indicated higher exposure and higher metabolism response on *B. meridionalis* in comparison to *S. laietanus*. *B. meridionalis* is a benthic species, feeding mainly on benthic invertebrates, whereas *S. laietanus* is an omnivorous water-column dweller (Merciai et al., 2017). Being sediment a sink chemical, benthic invertebrates will be more enriched in pollutants than the pelagic ones, and thus, this fact may explain higher concentrations of pollutants in bile in *B. meridionalis* than in *S. laietanus*. Also lower responses in haematology endpoints, were found in *S. laietanus*, suggesting that *B. meridionalis* was the most sensitive to sewage discharge and a best sentinel species for metal monitoring due to its benthopelagic condition (Maceda-Veiga et al., 2013). *S. laietanus* is a fish species with great resistance and adaptability. The lower metabolism of detoxification enzymes in this fish specie, suggested that cells of these fish needed less energy to perform essential metabolic functions, such as detoxification, than *B. meridionalis.*

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Supporting Information

Table 9. Lipid molecules, theoretical mass, elemental composition, measured mass and retention time of PC, PC-P, CE, TG, DG and SM detected in muscle tissue of *B. meridionalis* and *S. laietanus*. All identified compounds in accordance to criteria of maximum permissible mass error of 5 ppm, with atom constraints and with $-0.5 \leq DBE \leq 15.0$. DBE: double-bond equivalent. Elemental composition of PC, PC-P and SM refers to the $[M+H]^+$ ions; TG, DG and CE refers to ammonium adducts $[M+NH_4]^+$.

Lipid subclass	Lipid specie	Theoretical mass (Da)	Elemental composition	Measured mass (Da)	Error (ppm)	DBE	RT (min)
PC	30:0	706.5387	C38H77NO8P	706.5386	-0.1	1.5	6.8
	30:1	704.5230	C38H75NO8P	704.5224	-1	2.5	6.1
	32:0	734.5700	C40H81NO8P	734.5714	1.9	1.5	8.85
	32:1	732.5543	C40H79NO8P	732.5538	-0.7	2.5	7.57
	32:2	730.5387	C40H77NO8P	730.5380	-1	3.5	7.0
	34:0	762.6013	C42H85NO8P	762.6016	0.4	1.5	9.84
	34:1	760.5856	C42H83NO8P	760.5842	-1.8	2.5	9.38
	34:2	758.5700	C42H81NO8P	758.5726	3.4	3.5	8.47
	34:3	756.5543	C42H79NO8P	756.5563	2.6	4.5	7.63
	34:4	754.5387	C42H77NO8P	754.5387	1.1	5.5	6.17
	34:5	752.5230	C42H75NO8P	752.5247	2.3	6.5	5.95
	36:1	788.6169	C44H87NO8P	788.6165	-0.5	2.5	9.8
	36:2	786.6013	C44H85NO8P	786.6018	0.6	3.5	8.9
	36:3	784.5856	C44H83NO8P	784.5863	0.9	4.5	8.3
	36:4	782.5700	C44H81NO8P	782.5706	0.8	5.5	7.7
	36:5	780.5543	C44H79NO8P	780.5538	-0.7	6.5	7.0
	36:6	778.5387	C44H77NO8P	778.5387	0.3	7.5	6.0
	38:1	816.6482	C46H91NO8P	816.6495	1.6	2.5	11.1
	38:2	814.6326	C46H89NO8P	814.6312	-1.7	3.5	10.2
	38:3	812.6169	C46H87NO8P	812.6166	-0.4	4.5	9.6
	38:4	810.6013	C46H85NO8P	810.6013	0	5.5	9.28
	38:5	808.5856	C46H83NO8P	808.5847	-1.1	6.5	8.13
	38:6	806.5700	C46H81NO8P	806.5703	0.4	7.5	7.63
	38:7	804.5543	C46H79NO8P	778.5387	3.7	8.5	6.3
	40:1	844.6795	C48H95NO8P	844.6792	-0.4	2.5	12.4
	40:2	842.6639	C48H93NO8P	842.6642	0.4	3.5	11.6
	40:3	840.6480	C48H91NO8P	840.6490	1	4.5	10.6
	40:4	838.6326	C48H89NO8P	838.6325	-0.1	5.5	9.7

	40:5	836.6169	C48H87NO8P	836.6167	-1.1	6.5	9.2
	40:6	834.6013	C48H85NO8P	834.6011	-0.2	7.5	8.8
	40:7	832.5856	C48H83NO8P	832.5875	2.3	8.5	7.8
	40:8	830.5700	C48H81NO8P	830.5695	-0.6	9.5	7.1
	42:5	864.6482	C50H91NO8P	864.6482	0	6.5	10.35
	42:6	862.6326	C50H89NO8P	862.6313	-1.5	7.5	9.87
	42:7	860.6169	C50H87NO8P	860.6171	0.2	8.5	9.22
	42:8	858.6013	C50H85NO8P	858.6013	-0.8	9.5	8.25
PC-P	30:0	692.5594	C38H79NO7P	692.5597	0.4	0.5	7.79
	30:1	690.5438	C38H77NO7P	690.5432	-0.9	1.5	6.77
	32:0	720.5907	C40H83NO7P	720.5905	-0.3	0.5	9.17
	32:1	718.5751	C40H81NO7P	718.5753	0.3	1.5	8.15
	32:2	716.5594	C40H79NO7P	716.5597	0.4	2.5	7.64
	34:1	746.6064	C42H85NO7P	746.6065	0.1	1.5	9.41
	34:2	744.5907	C42H83NO7P	744.5913	0.8	2.5	8.65
	34:3	742.5751	C42H81NO7P	742.575	2.4	3.5	7.57
	36:1	774.6377	C44H89NO7P	774.6381	0.5	1.5	11.24
	36:2	772.622	C44H87NO7P	772.6234	1.8	2.5	10.34
	36:3	770.6064	C44H85NO7P	770.607	0.8	3.5	9.41
	36:4	768.5907	C44H83NO7P	768.5911	0.5	4.5	8.95
	36:5	766.5751	C44H81NO7P	766.5746	-0.7	5.5	8.25
	36:6	764.5594	C44H79NO7P	764.5585	-1.2	6.5	7.24
	38:1	802.6690	C46H91NO7P	802.6697	0.7	1.5	11.98
	38:2	800.6533	C46H89NO7P	800.6533	0	2.5	10.95
	38:4	796.6220	C46H87NO7P	796.6218	-0.3	4.5	9.64
	38:5	794.6064	C46H85NO7P	794.6064	0	5.5	10.34
	38:6	792.5907	C46H83NO7P	792.5918	1.4	6.5	9.28
	38:7	790.5751	C46H81NO7P	790.5743	-1	7.5	8.35
	40:6	820.6220	C48H87NO7P	820.6229	1.1	6.5	9.93
	40:7	818.6064	C48H85NO7P	818.6065	0.1	7.5	8.84
	40:8	816.5907	C48H83NO7P	816.591	0.4	8.5	7.931
CE	18:0	670.6500	C45H84NO2	670.6502	0.1	4.5	17.2
	18:1	668.6337	C45H82NO2	668.6340	-0.4	5.5	16.3
	18:3	664.6057	C45H78NO2	664.6027	4.5	7.5	15.2
	20:3	692.6346	C47H82NO2	692.6346	0	7.5	17.02
	20:4	690.6190	C47H80NO2	690.6184	0.9	8.5	15.4
	20:5	688.6032	C47H78NO2	688.6027	0.7	9.5	14.89
	20:6	686.5876	C47H76NO2	686.5869	-0.7	10.5	14.26

	22:2	722.6815	C49H88NO2	722.6815	0	6.5	18.70
	22:3	720.6659	C49H86NO2	720.6659	0	7.5	18.02
	22:4	718.6502	C49H84NO2	718.6492	-1.4	8.5	16.98
	22:5	716.6346	C49H82NO2	716.6375	4	9.5	16.45
	22:6	714.6185	C49H80NO2	714.6184	0.1	10.5	15.93
TG	46:0	796.7394	C49H98NO6	796.7394	0	1.5	17.5
	46:1	794.7238	C49H96NO6	794.7236	-0.2	2.5	15.47
	46:2	792.7081	C49H94NO6	792.7087	0.6	3.5	14.75
	46:3	790.6925	C49H92NO6	790.6926	0.1	4.5	14.30
	48:0	824.7707	C51H102NO6	824.7715	1	1.5	17.05
	48:1	822.7551	C51H100NO6	822.7554	0.4	2.5	16.73
	48:2	820.7394	C51H98NO6	820.7408	1.7	3.5	16.22
	48:3	818.7238	C51H96NO6	818.7234	-0.5	4.5	15.19
	48:4	816.7081	C51H94NO6	816.7088	0.9	5.5	14.82
	50:0	852.8020	C53H106NO6	852.8027	0.8	1.5	17.30
	50:1	850.7864	C53H104NO6	850.7871	0.8	2.5	16.85
	50:2	848.7707	C53H102NO6	848.7704	-0.4	3.5	16.34
	50:3	846.7551	C53H100NO6	846.7540	-1.3	4.5	15.83
	50:4	844.7394	C53H98NO6	844.7401	0.8	5.5	15.17
	50:5	842.7238	C53H96NO6	842.7247	1.1	6.5	14.92
	50:6	840.7081	C53H94NO6	840.7078	-0.4	7.5	14.55
	52:0	880.8333	C55H110NO6	880.8326	-0.8	1.5	18.42
	52:1	878.8177	C55H108NO6	878.8193	1.8	2.5	17.47
	52:2	876.8020	C55H106NO6	876.8038	2.1	3.5	16.99
	52:3	874.7864	C55H104NO6	874.7878	1.6	4.5	16.40
	52:4	872.7707	C55H102NO6	872.7715	0.9	5.5	16.12
	52:5	870.7551	C55H100NO6	870.7552	0.1	6.5	15.44
	52:6	868.7394	C55H98NO6	868.7433	4.5	7.5	15.13
	52:7	866.7238	C55H96NO6	866.7249	1.3	8.5	14.59
	54:1	906.8490	C57H112NO6	906.8516	2.9	2.5	18.70
	54:2	904.8333	C57H110NO6	904.8334	0.1	3.5	17.71
	54:3	902.8177	C57H108NO6	902.819	1.4	4.5	17.18
	54:4	900.8020	C57H106NO6	900.8025	0.6	5.5	16.74
	54:5	898.7864	C57H104NO6	898.7875	1.2	6.5	16.78
	54:6	896.7707	C57H102NO6	896.7703	-0.4	7.5	15.81
	54:7	894.7551	C57H100NO6	894.7553	2.5	8.5	15.78
	54:8	892.7394	C57H98NO6	892.7416	2.5	9.5	15.32
	54:9	890.7238	C57H96NO6	890.727	3.6	10.5	14.35

	56:3	930.8490	C59H112NO6	930.8497	0.8	4.5	18.45
	56:4	928.8333	C59H110NO6	928.8348	1.6	5.5	17.71
	56:5	926.8177	C59H108NO6	926.8187	1.1	6.5	16.96
	56:6	924.8020	C59H106NO6	924.803	1.1	7.5	16.53
	58:4	956.8646	C59H114NO6	956.8643	-0.3	5.5	18.02
	58:5	954.849	C59H112NO6	954.8488	-0.2	6.5	17.49
	58:6	952.8333	C61H110NO6	952.8344	1.2	7.5	17.10
DG	34:1	612.5567	C37H74NO5	612.5566	-0.2	1.5	10.07
	34:2	610.5411	C37H72NO5	610.5411	0	2.5	9.49
	36:1	640.588	C39H78NO5	640.5878	-0.3	1.5	11.26
	36:2	638.5724	C39H76NO5	638.5723	-0.2	2.5	10.74
	36:5	632.5254	C39H70NO5	632.5246	-1.3	5.5	8.19
	38:1	668.6193	C41H82NO5	668.6199	0.9	1.5	17.05
	38:2	666.6193	C41H80NO5	666.6193	0	2.5	16.33
	38:5	660.5567	C41H74NO5	660.5567	0	5.5	9.65
	38:6	658.5411	C41H72NO5	658.5406	-0.8	6.5	8.95
	40:3	692.6193	C43H82NO5	692.618	-1.9	3.5	16.64
	40:4	690.6036	C43H80NO5	690.6035	-0.1	4.5	15.77
SM	16:0	703.5754	C39H80N2O6P	703.5739	-2.1	1.5	7.08
	18:0	731.6067	C41H84N2O6P	731.6077	1	1.5	8.54
	22:0	787.6693	C45H92N2O6P	787.6694	0.1	1.5	11.21
	22:1	785.6537	C45H90N2O6P	785.6539	0.2	2.5	10.34
	24:0	815.7006	C47H96N2O6P	815.7007	0.1	1.5	12.36
	24:1	813.6850	C47H94N2O6P	813.6853	0.3	2.5	11.59
	24:2	811.6690	C47H92N2O6P	811.6697	0.4	3.5	10.66
	24:3	809.6537	C47H90N2O6P	809.654	0.3	4.5	9.73

CAPÍTULO 2

Evaluación de los efectos del progestágeno sintético drospirenona en juveniles de lubina (*Dicentrarchus labrax*)

Artículo 3

'Drospirenone intake alters plasmatic steroid levels and cyp17a1 expression in gonads if juvenile sea bass (*Dicentrarchus labrax*)'

Maria Blanco[1], Denise Fernandes[1,2], Paula Medina[3,4], Mercedes Blázquez[3] and Cinta Porte[1]

[1]Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

[2]CIMA, University of Algarve, FCT, Campus de Gambelas 8005-139, Faro, Portugal.

[3]Instituto de Ciencias del Mar, ICM-CSIC, Passeig Maritim 37-49, 08003 Barcelona, Spain.

[4]University of Antofagasta, Av. Angamos 601, Antofagasta, Chile.

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'Drospirenone intake alters plasmatic steroid levels and *cyp17a1* expression in gonads if juvenile sea bass (*Dicentrarchus labrax*)'

Maria Blanco[1], Denise Fernandes[1,2], Paula Medina[3,4], Mercedes Blázquez[3] and Cinta Porte[1]

[1]Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

[2]CIMA, University of Algarve, FCT, Campus de Gambelas 8005-139, Faro, Portugal.

[3]Instituto de Ciencias del Mar, ICM-CSIC, Passeig Maritim 37-49, 08003 Barcelona, Spain.

[4]University of Antofagasta, Av. Angamos 601, Antofagasta, Chile.

ABSTRACT

Drospirenone (DRO) is one of the most widely used progestins in contraceptive treatments and hormone replacement therapies. The pharmacokinetics and potential toxicological effects of DRO were investigated in juvenile sea bass (*Dicentrarchus labrax*) exposed through the diet (0.01 to 10 µg DRO/g) for up to 31 days. DRO was detected in the blood (4 - 27 ng/mL) of fish exposed to the highest concentration, with no significant bioaccumulation over time and no alteration of hepatic metabolizing enzymes, namely, CYP1A and CYP3A-catalysed activities and UDP-glucuronyltransferase (UGT). Pregnenolone (P5), progesterone (P4), 17α-hydroxyprogesterone (17P4), 17α-hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T) were determined in plasma and gene expression of *cyp17a1*, *cyp19a1a* and *cyp11β* analyzed by qRT-PCR in gonads. The significant increase in plasmatic levels of 17P5, 17P4 and AD detected after 31 days exposure to 10 ng DRO/g together with the increased expression of *cyp17a1* in females evidence the ability of DRO to alter steroid synthesis at low intake concentrations (7 ng DRO/day). However, the potential consequences of this steroid shift for female reproduction remain to be investigated.

Key words: *progestins, Sea bass, sex steroids, metabolism, cyp17a1*.

1. Introduction

Drospirenone (DRO) (6β,7β,15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone) is a new generation progestin and a derivative of the synthetic mineralocorticoid 17α-spironolactone, with a pharmacologic profile similar to progesterone, and with anti-mineralocorticoid, anti-aldosterone and slight antiandrogenic activity (Krattenmacher, 2000; Rapkin et al., 2007). It is prescribed in contraceptive treatments and hormone replacement therapies in combination with estradiol or ethynilestradiol at doses up to 150-fold higher than estrogens, and it is one of the most widely used synthetic progestins in Europe (Fent, 2015). Thus, environmental concentrations of DRO are expected to be in the same range or higher than other synthetic estroges and progestins, which are detected at the low ng/L range in effluents (Besse and Garric, 2009). However, DRO and some other new generation progestins have not yet been extensively monitored in aquatic systems (Fent, 2015).

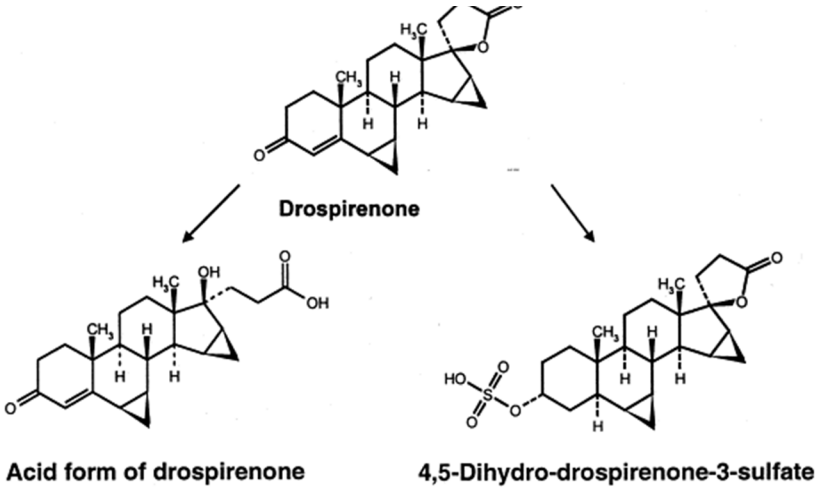

Figure 23. Molecular structure of DRO and their metabolites.

In teleost fish, natural progestins play an important role in the stimulation of oocyte growth and maturation as well as in spermatogenesis and sperm maturation, and they also act as sex pheromones (Nagahama and Yamashita, 2008; Scott et al., 2010). Moreover, fish possess similar drug targets as humans, and consequently, synthetic progestins can interact with those conserved targets and adversely affect reproduction (Runnalls et al., 2013). Thus, levonorgestrel or gestodene at concentrations of 100 ng/L stopped spawning almost completely in the fathead minnow; gestodene concentrations as low as 1 ng/L had significant effects on reproduction over 21 days, whereas desogestrel was less potent, but still reduced egg production at concentrations of or above 1 μg/L. Zeilinger et al. (2009) reported reduced fertility of fathead minnow at concentrations of levonorgestrel as low as 0.8 ng/L and DRO of 6.5 μg/L, while reproduction was not affected at lower concentrations of DRO (100 ng/L) (Runnalls et al., 2013). Additionally, norethindrone decreased fecundity in fathead minnow and medaka, and levonorgestrel disrupted the seasonal breeding cycle in male sticklebacks, both at concentrations in the low ng/L range (Paulos et al., 2010; Svensson et al., 2014).

Despite of these evidences, the mechanisms which mediate endocrine disruption by synthetic progestins are poorly understood. Modulated gonadotropin expression in the pituitary and changes of plasma sex steroid levels underlie many of the reproductive effects, as reduced fecundity or disturbed gonad development. However, the concentrations needed to induce changes in steroid levels are usually higher than those to reduce fecundity (Kumar et al., 2015). *In vitro* exposure of fathead minnow ovaries to progesterone resulted in increased synthesis of pregnenolone, 17α-hydroxyprogesterone, 17α,20β-dihydroxypregnenone and testosterone, while norethindrone had no significant effect, despite a non-significant

decrease of testosterone production (Petersen et al., 2015). Interestingly, DRO inhibited CYP17 activity (metabolism of 17α-hydroxyprogesterone to androstenedione) in carp testis mitochondrial fractions *in vitro* (IC50: 3.8μM). DRO was a stronger inhibitor than cyproterone acetate (IC50: 183μM), while other synthetic progestins (levonorgestrel and norethindrone) did not affect CYP17 activity (Fernandes et al., 2014).

Progestins are shown to alter the expression of steroidogenic enzymes in zebrafish and fathead minnows (Overturf et al., 2014; Fent, 2015). Transcriptional changes were generally more sensitive than changes on steroid levels and revealed a number of affected pathways, including steroid hormone receptor activities and steroid hormone mediated signaling pathways, cellular response to steroid hormone stimulus and thyroid hormone receptor activity (Zucchi et al., 2014). More recently, Zhao et al. (2015) reported significant and dose-dependent alterations of the circadian rhythm network in the brain of zebra fish exposed to progesterone and DRO.

Within this context, the present study was designed to investigate the effect of DRO exposure in the hepatic metabolism (CYP1A and CYP3A-catalysed activities and UDP-glucuronyltransferase (UGT)), circulating steroid levels and expression of key steroidogenic enzymes (*cyp17a1*, *cyp19a1a*, *cyp11β*) in gonads of juveniles of European sea bass (*Dicentrarchus labrax*), with the aim of gathering information on the dynamics and the mode of action of this synthetic progestin in juvenile fish, in a period particularly sensitive to the effect of exogenous steroids (Piferrer et al., 2005).

2. Material and methods

2.1. Chemicals

Drospirenone, *p*-nitrophenol (pNP), uridine 5'-diphosphoglucuronic acid (UDPGA), NADPH, 7-ethoxyresorufin (7-ER), bovine serum albumin (BSA; fatty acid free, ≥ 99% purity), methyl tert-butyl ether (MTBE) and hydroxylamine hydrochloride were purchased from Sigma-Aldrich (Steinheim, Germany). D8-17-hydroxyprogesterone (d8-17P4) was obtained from C/D/N Isotopes (Quebec, Canada); d9-progesterone (d9-P4), d4-pregnenolone (d4-P5), d3-testosterone (d3-T), pregnenolone (P5), progesterone (P4), 17-hydroxyprogesterone (17P4), 17-hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T) were obtained from Sigma-Aldrich (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin (7-BFC) was from Cypex (Dundee, Scotland, UK). Dulbecco's Phosphate Buffered Saline (DPBS) was obtained from Gibco (Life technologies). Primers of selected genes and SuperScript III Reverse Transcriptase were obtained from Invitrogen. All solvents were from Merck (Darmstadt, Germany).

2.2. Experimental design

Juvenile European sea bass - 243 days post-hatching (dph) (100-185 mm length) reared at the experimental animal facility of the Institute of Marine Science (Barcelona, Spain), were randomly distributed into six 50 L tanks (30 individuals per tank) for acclimatization at a flow rate of 1.2 L water/min with 80% oxygen saturation, natural temperature (14.5-15ºC) and photoperiod (light:dark, 9:15). After the acclimatization period (4 weeks), fish were fed with commercial pellets spiked with DRO at a

concentration of 0.01, 0.1, 1 and 10 µg/g, with a daily average intake of 0.7 g of pellet feed per fish. The experimental diets were prepared following the alcohol evaporation method adapted for the sea bass (Blázquez et al., 1995). Briefly, food pellets were sprayed with the different concentrations of DRO dissolved in ethanol, being the solvent completely evaporated afterwards. A solvent control (SC) group – pellets only sprayed with ethanol – and a control group –untreated pellets- were also included in the study. The highest concentrations (1 and 10 µg/g of DRO) corresponded to typical doses of estrogens or androgens used in experiments of sex reversal in this species (Blázquez et al., 1998; 2001), while concentrations of 0.01 and 0.1 µg/g are close to the human therapeutical dose (0.05 □g/g of DRO).

Fish were sampled after 2, 4, 8, 16 and 31 days of exposure. They were anesthetized with 0.2% phenoxyethanol and the individual weights and lengths measured. Blood (approx. 1 mL) was taken from the caudal vein, transferred into heparinised tubes, centrifuged (1,000 x *g*; 15 min), and the plasma separated and stored at -80°C. Immediately after, fish were sacrificed by quickly severing the spinal cord and the liver and the gonads were dissected. A fragment of the central part of the left gonad from each fish was separated and fixed in 4% PAF (buffered paraformaldehyde) for further histological analysis. The rest of the gonads and the liver were immediately frozen in liquid nitrogen and stored at -80°C. All fish were treated in accordance with the Spanish regulations (Royal Decree Act 53/2013) and the European legislation (2010/63 EU) concerning the protection of vertebrates used for experimental and other scientific purposes. All the steps were taken to reduce suffering of the animals.

2.3. Analysis of drospirenone in plasma

Circulating levels of DRO in plasma were determined after 2, 4, 8, 16 and 31 days of exposure to the highest concentration (10 μg/g). Acetonitrile (400 μL) was added to 100 μL of plasma, centrifuged at 4,000 x *g* for 10 min and the resulting supernatant (10 μL) injected in an UPLC-MS/MS system (Ultra Acquity LC System, TQ Detector, Waters, USA). To determine extraction efficiency a known concentration of DRO was added to plasma of non-exposed individuals and extracted as mentioned above. DRO was detected under positive electrospray ionization (ES+) and multiple reaction mode (MRM) measuring the transition of precursor ion fragmentation (367 m/z) to product ions (97/91 m/z) under a collision energy of 41/75 Ev and with a capillary voltage of 3.50 Kv and cone voltage of 40 V. The analysis was performed using a Zorbax Eclipse Plus C-18 column (2.1 mm x 50 mm, 1.8 μm) (Agilent, Loveland, U.S) connected to a pre-column Zorbax Eclipse Plus C-18 (2.1 mm x 5 mm, 1.8 μm) (Agilent, Loveland, U.S) with a mobile phase composed of acetonitrile (A) and Milli-Q water containing 0.1% (v/v) of formic acid (B). The run consisted of 0.5 min at 25% A, a 8 min linear gradient from 25% A to 90% A, 2 min at 90% A and back over to initial state at 1 min, allowing 1 min for column re-equilibration. The total run-time was 12 minutes at a flow rate of 0.3 mL/min. The column was maintained at 50ºC. The retention time of DRO, under our assay conditions, was of 4 min and the limit of detection was < 1 μg/L. The efficiency of the extraction procedure was of 98 ± 0.5% (n = 6).

2.4. Plasma steroid analysis

Plasmatic steroid levels (i.e. pregnenolone (P5), progesterone (P4), 17-ydroxyprogesterone (17P4), 17-hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T)) were analysed after 4 and 31 days of exposure. Plasma samples were extracted according to the procedure described by Keski-Rahkonen et al. (2011). 400 µL of plasma together with 10 µL of internal standard (IS; mix solution of deuterated steroids) at 10 ppb was placed into glass screw top tubes, vigorously mixed and extracted twice with 1 mL of MTBE after centrifugation at 2,000 rpm for 10 min. The resulting organic layer was transferred into and HPLC vial, evaporated under nitrogen current and reconstituted in 100 µL of hydroxylamine solution (100 mM in 50% methanol (v/v)) and heated at 60ºC for 1 hour before being injected (10 µL) into the UPLC-MS/MS system.

Calibration curve was prepared by substituting the plasma with 4% of BSA solution (2 g of BSA in 50 mL of DPBS). Thus, working solutions of mix steroids (P5, d4-P5, P4, d9-P4, 17P4, d8-17P4, 17P5, AD, T, d3-T) dissolved in methanol were added to the BSA solution, before being extracted twice with 1 mL of MTBE, evaporated under nitrogen and reconstituted in hydroxylamine solution (heated at 60ºC for 1 h). The calibration curve consisted of ten concentration levels: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 ppb, a zero sample (only IS added, to calculate extraction efficiency), and a blank (no standards added).

Samples were analysed by an UPLC-MS/MS system (Ultra Acquity LC System, TQ Detector, Waters, USA), with a Zorbax Eclipse Plus C-18 column (2.1 mm x 50 mm, 1.8 µm) (Agilent, Loveland, U.S) connected to a pre-column Zorbax Eclipse Plus C-18 (2.1 mm x 5 mm, 1.8 µm) (Agilent,

Loveland, U.S). Steroids (T, d3-T, AD, P4, d9-P4, P5, d4-P5, 17P4, d8-17P4 and 17P5) were detected under positive electrospray ionization (ES+) and multiple reaction mode (MRM), measuring the transition of precursor ion fragmentation to product ions under a capillary voltage of 3.00 Kv and cone voltage of 34 V (Table 10). The mobile phase consisted of methanol (A) and Milli-Q water (B) both containing 0.1% (v/v) of formic acid. The run was as follows: 0.1 min at 10% A, a 2 min linear gradient from 10% A to 70% A, a 3 min linear gradient from 70% A to 90% A, 2 min at 90% A and back to initial conditions at 1 min, allowing 2 min for column re-equilibration. The total run-time was 10 min at a flow rate of 0.2 mL/min. The column was maintained at 48ºC. The limit of detection was < 50 ng/L for all steroids, with the exception of P5 < 10 ng/L. Deuterated steroids added to the samples in order to compensate for matrix effects and to evaluate the efficiency of the extraction procedure, showed recoveries of 79 ± 9% for d3-T, 68 ± 14% for d4-P5, 97 ± 11% for d8-17P4 and 84 ± 22% for d9-P4 (n = 20).

Table 10. Masses of precursor and product ions of steroids detected in plasma by UPLC-MS/MS, obtained under positive electrospray ionization (ES+) subsequent to collision induced dissociation with multiple reactions monitoring (MRM).

Steroids	Precursor ion (m/z)	Product ion (m/z)	Fragmentor voltaje (V)	Collision energy
Androstenedione	317	112;124	40	30;30
Testosterone	304	112;124	50	30;30
D3-testosterone	307	112;124	40	30;30
Progesterone	345	112;124	40	30;30
D9-progesterone	354	115;128	50	30;30
Pregnenolone	332	86;60	40	25;25
D4-pregnenolone	336	90	40	30
17α-hydroxyprogesterone	361	112;124	40	30;30
D8-17α-hydroxyprogesterone	369	115;128	50	35;35
17α-hydroxypregnenolone	348	330;312	10	10;20

2.5. Histological analysis

Histological analyses were performed in order to unequivocally assess the sex of the fish used in the study since visual classification was not possible due to the low degree of sexual development of the gonads. With this purpose, portions of gonadal tissues were fixed in 4% PAF at 4ºC overnight. Fixed gonads were dehydrated using a graded series of ethanol concentrations (70-100%) and embedded in paraplast (Leica, Germany). Sections for histological examination were cut at 5 μm on a retracting microtome and stained with haematoxylin and eosin.

2.6. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA from gonads of control and DRO-exposed groups after 2 and 31 days of treatment was isolated with TRIZOL (Invitrogen, Paisley, UK) following the manufacturer's instructions. The concentration and quality of

the RNA was assessed by spectrophotometry and gel electrophoresis. Total RNA (2 µg) was reverse transcribed to cDNA with Superscript III (200 units; Invitrogen) and random hexamer primer (50 mM) following the manufacturer's instructions. The resulting cDNAs were used as a template to amplify and quantify the expression levels of *cyp17a1*, *cyp19a* and *cyp11β* genes. Previously, A TBLASTN search (Altschul et al., 1997) was conducted to identify the *cyp17a1* sequence in the European sea bass genome database (Tine et al., 2014), using as queries various known vertebrate sequences. Once identified, specific primers were designed for its complete amplification. Full CDS sequence was cloned and sequenced in order to verify its identity when compared with the sea bass genome. The verified and cloned sequence was finally deposited in the GenBank database under the accession number KT932710. Full sequences for sea bass *cyp19a1a* and *cyp11beta* were available in the GenBank under accession numbers AJ318516 and AF449173, respectively. Specific primers for detecting housekeeping and target genes were designed using primer 3 software (http://www.bioinformatics.nl//cgi-bin/primer3plus/primer3plus.cgi) and examined for their specificity and amplification efficiency using serial dilutions of template (Table 11). The melting curve analysis (60ºC for 15 s and 95ºC for 15 s) after the amplification phase displayed a single peak for every gene, ensuring primers specificity. Moreover, PCR efficiencies (E) of all primers ranged between 90% and 110%. All qRT-PCR reactions were analysed on 7300 Real Time PCR System (Applied Biosystems) using SYBR Green (Select Master Mix, Applied Biosystems). Samples were run in triplicate in 96-well plates in a 15 µL reaction, containing 7.5 µL of SYBR Green, 0.75 µL of each of forward and reverse primer, 1 µL of cDNA sample and 5 µL nuclease-free water. The qRT-PCR amplification was carried out using the following program: an activation step for 10 min at 95ºC, followed by 40

cycles of 15 s denaturation at 95ºC and a 1 min annealing/extension step at 60ºC. Finally, a dissociation step of 15 s at 95ºC followed by 15 s at 60ºC was added. The 18s rRNA was selected as reference gene for normalization due to its stable expression among the solvent control and treated groups.

Data were collected and compiled using SDS 2.3 software (Applied Biosystems) that was used to calculate gene expression levels. For each gene, a non-template control was included to confirm the absence of DNA contamination. In addition, the same control sample was used in all runs to calculate the intra- and inter-assay variations. Control values were adjusted for differences in E of each primer set using Q-gene (Müller et al., 2002). Values were normalized (normalized expression; NE) to the reference gene following the equation $NE = (Eref)^{Ctref}/(Etarget)^{Cttarget}$. Replicates were averaged and shown as mean normalized expression (MNE) ± SEM.

Table 11. Primer sequences and variables used for the amplification and calculation of the efficiency (E) of the real-time qPCR reactions.

Gene	Primer sequence (5'→3')	Slope	E	R^2	Amplicon Size (bp)
cyp17a1	Sense: TTGCAGGAAGACCCAGAACT Antisense: TCTCCAAACATGCACAGAGC	-2.92	2.20	0.97	133
cyp19a1a	Sense: AGACAGCAGCCCAGGAGTTG Antisense: TGCAGTGAAGTTGATGTCCAGTT	-3.33	1.99	0.99	106
cyp11β	Sense: CTGGAAGCCAGTTGCCATGT Antisense: TCCTCCACTGCCCAAATAA	-3.33	1.99	0.99	98
18S	Sense: GCATGCCGGAGTCTCGTT Antisense: TGCATGGCCGTTCTTAGTTG	-3.33	1.99	0.99	70

2.7. Biochemical determinations

Hepatic microsomal fractions were prepared as described in Fernandes et al. (2013). Livers (0.5 g) were flushed with ice-cold 1.15% KCl and homogenized in 1:4 w/v cold homogenization buffer containing 100 mM

KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 x *g* for 15 min, and the obtained supernatant centrifuged at 12,000 x *g* for 45 min. The resulting supernatant was further centrifuged at 100,000 x *g* for 60 min to obtain the microsomal fraction. Microsomal pellets were resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Proteins were measured according to the method of Bradford (1976), using BSA as standard.

EROD activity was determined in the microsomal fraction of the liver and was assayed by incubating 0.1 mg of protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 (final volume of 250 μL) at 30ºC for 10 min. The reaction was stopped by adding 400 μL of ice-cold acetonitrile and after centrifugation (2,000 x *g*; 10 min) an aliquot of the supernatant (200 μL) was transferred into a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelength pairs of 537/583 using a Varioskan microplate reader (Thermo Electron Corporation). Quantification was performed using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute.

BFCOD activity was analysed according to the procedure described by Thibaut et al. (2006). The assay consisted in incubating 25 μg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume of 250 μL) at 30ºC for 10 min. The reaction

was stopped by addition of 75 µL of acetonitrile (20:80, v/v), the fluorescence was read in a 200 µL aliquot transferred into a 96-multiwell plate at the excitation/emission wavelength pairs of 409 and 530 nm, using a Varioskan microplate reader (Thermo Electron Corporation), and the activity calculated as the amount of 7-hydroxy-4-(trifluoromethyl)-coumarin (pmol) generated per milligram of protein per minute.

UDP-glucuronosyltransferase activity (UGT) was determined in hepatic microsomal fraction and was assayed by incubating 0.25 mg of proteins (pre-treated for 15 min with 0.2% Triton X-100 on ice) with 3.0 mM of UDPGA in 50 mM Tris-HCl buffer pH 7.4, containing 10 mM $MgCl_2$ (final volume of 260 µL). The reaction was initiated by the addition of 81 □M *p*-nitrophenol (*p*NP) and run for 30 min at 30ºC in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid (TCA), centrifuged (1,500 x *g*; 15 min), alkalinized with 0.1 mL of 10 N KOH and the remaining *p*NP was measured spectrophotometrically at 405 nm. Activity was calculated as the amount of *p*NP (nmol) consumed per milligram of protein per minute.

2.8. Statistical analysis

Plasma steroid levels were determined in pooled samples of two individuals. Enzymatic activities were determined in duplicate in six individual fish per tank. Comparisons between treatment groups were made using one-way ANOVA followed by multiple independent group comparison (Dunnett and Tukey's test). DRO levels in plasma were analysed by non-parametric Kruskal-Wallis test. RNA gene expression was determined in six individual fish per treatment and results were expressed as mean normalised expression (MNE) ± SEM and analysed by non-

parametric Mann-Whitney test comparing results between control and exposed groups.

In all instances transformations of the data were performed when the assumption of normality of residuals was not met. All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and *p* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Biological parameters and histological examination of the gonads

The morphometric parameters of the fish are presented in Table 12. Individuals were rather similar in body weight, length and condition factor (CF) and no significant differences were observed among the different groups. In addition, histological examination of the gonads revealed no effects of DRO in treated fish at any concentration and exposure length.

Table 12. Biological parameters (mean ± SEM, n = 5-9) of sea bass (*Dicentrarchus labrax*).

Exposure time	Concentration	Length (cm)	Weight (g)	CF (g/cm^3)*
2 days	Control (C)	13.7 ± 1.1	35.8 ± 3.6	1.5 ± 0.5
	Solvent control (SC)	13.4 ± 0.5	32.0 ± 5.2	1.4 ± 0.7
	0.01µg/g	12.5 ± 0.6	36.3 ± 7.8	1.7 ± 0.2
	0.1 µg/g	12.4 ± 0.7	32.0 ± 5.2	1.6 ± 0.1
	1 µg/g	12.0 ± 0.3	32.7 ± 2.5	1.9 ± 0.2
	10 µg/g	10.8 ± 1	30.5 ± 3.6	1.8 ± 0.3
4 days	Control (C)	12.9 ± 0.4	34.5 ± 2.4	1.6 ± 0.04
	Solvent control (SC)	12.8 ± 0.5	34.4 ± 4.4	1.6 ± 0.05
	0.01 µg/g	12.9 ± 0.4	38.3 ± 4.0	1.7 ± 0.03
	0.1 µg/g	12.7 ± 0.3	35.4 ± 2.2	1.7 ± 0.04
	1 µg/g	12.0 ± 0.4	29.3 ± 2.9	1.7 ± 0.08
	10 µg/g	12.6 ± 0.6	36.4 ± 5.0	1.8 ± 0.03
8 days	Control (C)	12.2 ± 0.2	29.1 ± 1.9	1.6 ± 0.04
	Solvent control (SC)	12.7 ± 0.4	34.0 ± 3.7	1.6 ± 0.05
	0.01 µg/g	12.5 ± 0.5	36.6 ± 3.6	1.9 ± 0.09
	0.1 µg/g	13.0 ± 0.2	38.4 ± 1.4	1.8 ± 0.06
	1 µg/g	12.9 ± 0.2	36.5 ± 1.6	1.7 ± 0.02
	10 µg/g	12.9 ± 0.4	36.4 ± 3.6	1.7 ± 0.04
16 days	Control (C)	12.5 ± 0.6	34.9 ± 5.3	1.7 ± 0.02
	Solvent control (SC)	12.4 ± 0.4	32.4 ± 3.7	1.7 ± 0.05
	0.01 µg/g	15.6 ± 0.5	33.0 ± 3.4	1.6 ± 0.04
	0.1 µg/g	12.0 ± 0.6	30.9 ± 4.2	1.8 ± 0.04
	1 µg/g	12.8 ± 0.5	38.4 ± 5.1	1.8 ± 0.07
	10 µg/g	13.8 ± 0.6	45.5 ± 6.2	1.7 ± 0.07
31 days	Control (C)	12.4 ± 0.5	31.6 ± 3.7	1.6 ± 0.15
	Solvent control (SC)	12.1 ± 0.5	31.2 ± 5.2	1.7 ± 0.2
	0.01 µg/g	13.3 ± 0.3	38.7 ± 2.7	1.6 ± 0.03
	0.1 µg/g	13.3 ± 0.6	40.4 ± 4.7	1.7 ± 0.08
	1 µg/g	13.0 ± 0.5	35.4 ± 3.9	1.6 ± 0.1
	10 µg/g	13.4 ± 0.5	40.8 ± 5.1	1.6 ± 0.15

*Condition factor (CF) was calculated as (weight/$length^3$) x 100.

3.2. Drospirenone in plasma

DRO, in the range of 4-27 ng/mL, was detected circulating in plasma of individuals exposed to 10 µg DRO/g food (Fig. 24). The highest concentration was detected after four days of exposure and the lowest at 31 days. Levels of DRO in plasma of individuals exposed to 0.01 to 1 µg DRO/g were below the detection limit of the method (1 ng/mL).

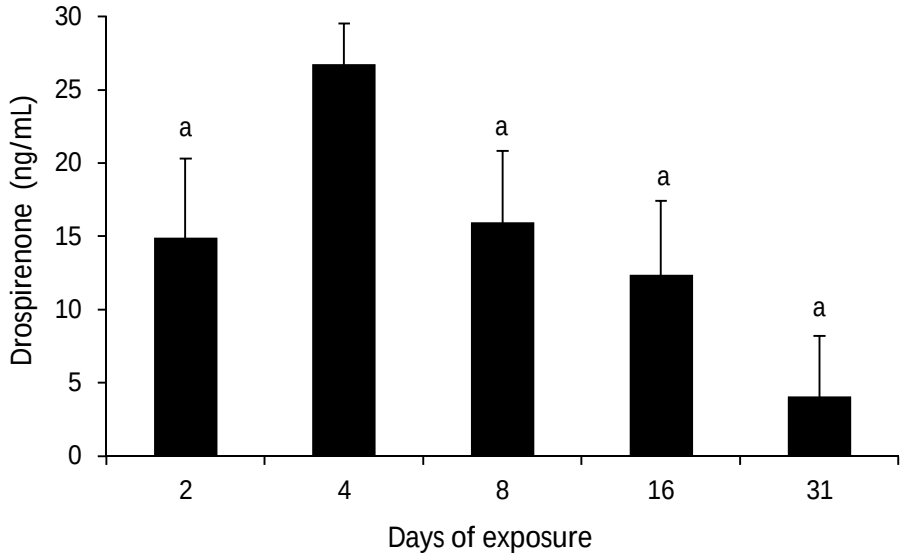

Figure 24. Concentrations of DRO in plasma of sea bass (*Dicentrarchus labrax*) after exposure to pellet feed contaminated with 10 µg/g of DRO. Values are mean ± SEM (n = 5-6). Distinct letters indicate significant differences between exposure days.

3.3. Steroids in plasma

Steroids were analysed in the plasma of individuals exposed to DRO for 4 and 31 days (Fig. 25). No significant differences in plasma steroid levels were observed between exposed and control groups after 4 days of treatment. However, levels of 17P5 (up to 7-fold), 17P4 (up to 3-fold) and AD (up to 2-fold) were significantly increased in individuals exposed to the lowest concentration of DRO (0.01 µg DRO/g food) for 31 days, while no

changes were observed for P5, P4 and T. Exposure to higher concentrations of DRO (0.1, 1 and 10 µg DRO/g food) did not significantly alter circulating steroid levels in juvenile sea bass.

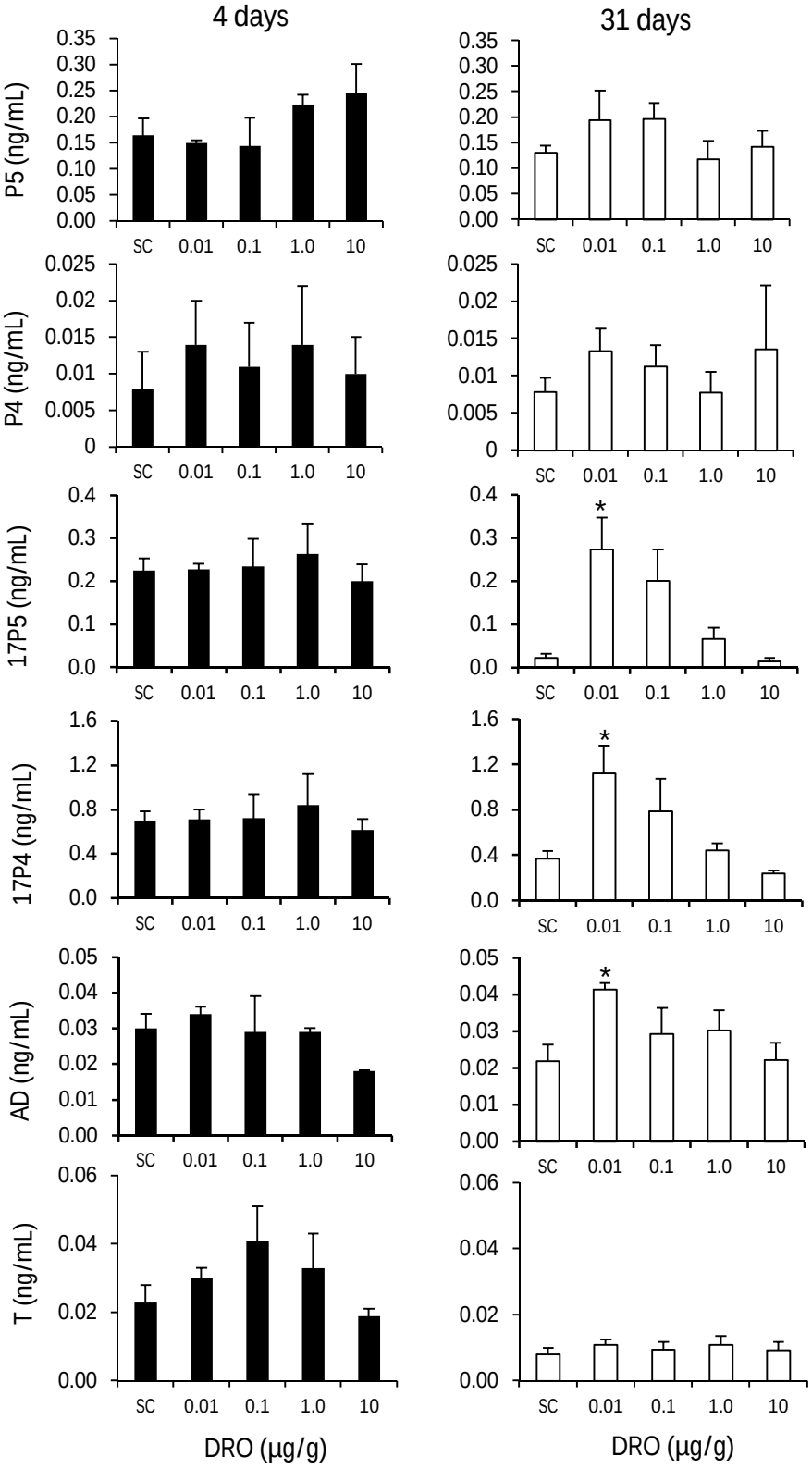

Figure 25. Plasma steroid levels (mean ± SEM (n = pools of 2 individuals) after 4 and 31 days of exposure to DRO. Results of control samples (SC) are shown as mean of control and solvent control. *Significant differences relative to the control ($p < 0.05$).

3.4. RNA gene expression

Data from control and exposed fish, regardless of exposure concentration, were pooled for the analysis in order to obtain a statistically robust sample size that could take into consideration males and females as separate variables. This is particularly important considering that the expression levels of the genes *cyp19a1a* and *cyp11β* exhibit sex-related differences (Blázquez et al 2009). After 2 days of DRO administration, a significant 2-fold induction of *cyp17a1* was detected in the ovaries of exposed females (Fig. 26). Expression of *cyp17a1* was one order of magnitude higher in male gonads, and it was not altered by exposure. Neither *cyp19a1a* nor *cyp11β* expression were altered by DRO treatment. After 31 days of dietary exposure to DRO, no differences in the expression of *cyp17a1*, *cyp19a1a* or *cyp11β* were observed between control and treated fish (Fig. 26).

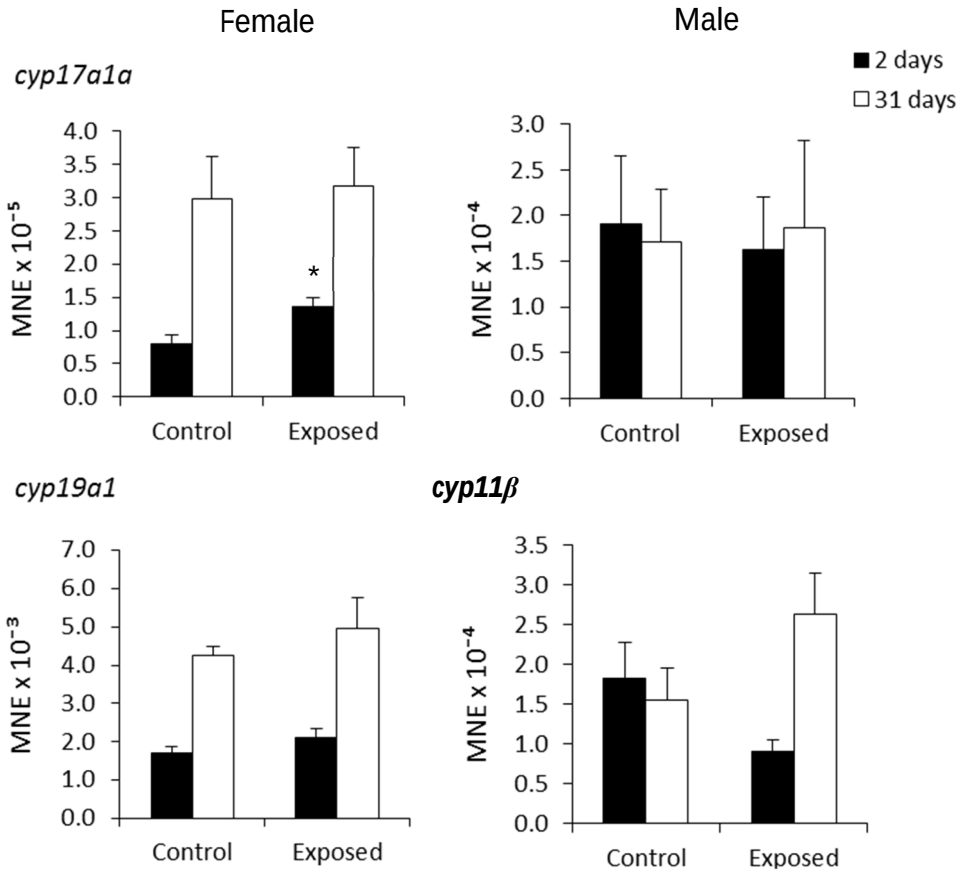

Figure 26. Mean normalised expression (MNE ± SEM) of *cyp17a1*, *cyp19a1a* and *cyp11β* genes in gonads of sea bass (*Dicentrarchus labrax*) exposed for 2 and 31 days to DRO. *Significant differences relative to the control ($p < 0.05$). Data from the different exposure concentrations (n = 13 males, 7 females) and from control groups (n= 4 males, 6 females) were pooled.

3.5. Hepatic metabolism

DRO had no significant effect in the activity of EROD, BFCOD or UGT after 2 or 31 days of treatment (Fig. 27). Nonetheless, a tendency towards lower EROD and UGT activities was detected after 2 days of exposure to 0.1 µg DRO/g food. EROD activity ranged from 32 to 59 pmol/min/mg protein at 2 days of exposure, while after 31 days of exposure the variability among tanks was smaller (31 to 43 pmol/min/mg protein). A similar tendency was observed for UGT. EROD, BFCOD and UGT

activities were also determined after 8 days of exposure, and no statistically significant differences were detected among treatments (data not shown).

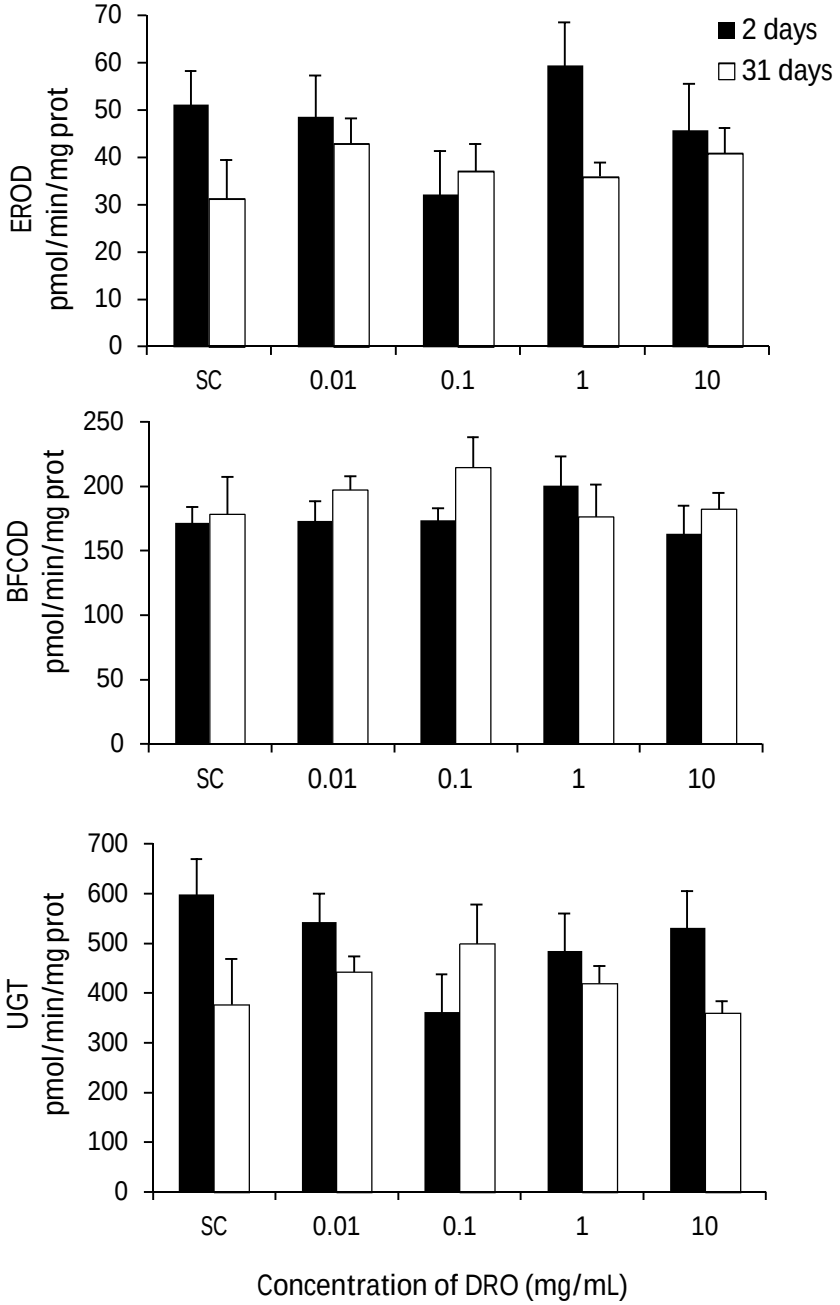

Figure 27. EROD (CYP1A), 7-BFCOD (CYP3A) and UDPGT (UGT) activities (mean ± SEM, n = 6), determined in liver microsomal fractions of juvenile sea bass exposed for 2 and 31 days to different concentrations of DRO (0.01, 0.1, 1 and 10 µg/g of pellet feed). SC = solvent control.

4. Discussion

The detection of DRO in plasma of sea bass after 2, 4, 8, 16 and 31 days of exposure confirms the uptake of the compound and its distribution in the body. Considering a daily intake of 0.7 g food pellets/fish, the concentration of DRO detected in plasma represents only 0.06 to 0.4% of the amount theoretically ingested daily. If we assume that, as in humans, the absolute bioavailability of DRO after oral administration is of 76% and that approximately 95-97% of the compound binds to serum proteins (Krattenmacher, 2000), a maximum of 160 ng DRO/mL would be expected in the plasma of sea bass exposed to 10 µg/g. However, the maximum plasmatic concentration detected was 27 ng/mL after four days of exposure, and decreased to 5 ng/mL after 31 days. Certainly, some desorption of DRO from food might occur while in water, prior to being ingested, although food was eaten within 5 min. Altogether, the results indicate no significant bioconcentration of DRO in plasma over time and suggest that the compound is metabolized by juvenile sea bass. Similarly, DRO was reported to have a relatively low bioconcentration factor (BCF: 36) in mussels after 2 weeks of exposure to 100 mg DRO/L; concentrations were below detection limit for those mussels exposed to 0.01 mg/L (Gilroy et al., 2014). However, despite the evidence of a metabolism of DRO in juvenile sea bass, CYP1A, CYP3A and UGT activities determined in liver microsomal fractions were not significantly altered by exposure, suggesting that the metabolism of DRO was probably CYP-independent. In humans, DRO is mainly metabolized in the liver to 4,5-dihydrodrospirenone-3-sulfate and to the acid form that is generated by opening of the lactone ring, both metabolites are reported not to be pharmacologically active and are CYP-independent (Krattenmacher, 2000). Nonetheless, it has been reported that DRO is metabolized to a minor extent (4-7%) by CYP3A4 in human

liver microsomes, and an inhibition of CYP3A4 can moderately increase DRO exposure in individuals treated with DRO, suggesting a potential involvement of the enzyme in DRO metabolism (FDA, 2006; Wiesinger et al., 2015).

No significant changes in length, weight and condition factor were observed in sea bass regardless of the concentration or exposure length. Similarly, Zucchi et al. (2014) reported no significant morphometric changes in zebrafish (*Danio rerio*) exposed up to 5 µg/L DRO for 14 days, despite a significant decrease in the gonadosomatic index (GSI), strong inhibition of VTG mRNA and altered transcription of *cyp19a1a*. In the present work, the expression of *cyp19a1a* and *cyp11β* in gonads of juvenile sea bass was not altered by exposure, but a 2-fold up-regulation of *cyp17a1* was detected in ovaries of exposed females. *Cyp17a1* encodes for a protein that has both 17α-hydroxylase and 17,20-lyase activities and catalyzes the conversion of pregnenolone and progesterone to their 17α-hydroxylated products, and subsequently to dehydroepiandrosterone (DHEA) and AD. Although our findings should be interpreted with caution, as females from all exposure concentrations were pooled, it is interesting to notice that in agreement with an activation of *cyp17a1*, significantly increased plasmatic levels of 17P4, 17P5 and AD were detected in sea bass exposed to 0.01 µg DRO/g food for 31 days, while T levels were not altered. Similarly, Runnalls et al. (2013) reported no effect of DRO in plasmatic concentrations of T and 11-ketotestosterone after 31 days of exposure in males of fathead minnow. Neither Zeilinger et al. (2009) observed masculinization of female fathead minnow exposed to DRO (70 µg/L). Altogether, these findings are in agreement with the fact that DRO is not an agonist of fish androgen receptors (ARα and ARβ), but a weak agonist of the progesterone receptor (Ellestad et al., 2014; Bain et al., 2015), and as

such, it might have progesterone-like action at relatively low concentrations.

The analysis of pharmaceuticals in fish plasma can be an excellent tool to assess the risk for pharmacological effects, as plasma concentrations can be easily compared with human therapeutic levels (Fick et al., 2010). Thus, concentrations of levonorgestrel of 2.4 ng/L were enough to bioaccumulate in plasma of rainbow trout at levels exceeding up to 4 times the human therapeutic dose. Nevertheless, in the present study, only sea bass from the high exposure group had plasmatic concentrations of DRO (5-27 ng/mL) relatively close to human therapeutic concentrations (20-25 ng/mL; maximum 60-87 ng/mL). Nonetheless, fish from the low exposure group, with plasmatic concentrations well below human therapeutic doses, showed increased plasmatic levels of 17P4, 17P5 and AD after 31 days exposure. These results, together with the increased expression of *cyp17a1* in females, evidence the ability of DRO to alter steroid synthesis in juvenile sea bass with a mean uptake of 7 ng DRO/day (10 ng/g x 0.7g food/day). However, the consequences in terms of impaired gonad development and/or reproduction in adult sea bass remain unknown.

Overall, the work contributes to the better understanding of the impact of DRO in fish. The question on whether environmental concentrations of DRO are likely to have an endocrine effect in fish still remain open, as so far, concentrations of DRO in water are unknown and it is uncertain whether the uptake of DRO through the water will have the same endocrine disruptive effect as through the food. However, this work (a) evidences the ability of DRO to increase plasmatic levels of 17P5, 17P4 and AD in juvenile sea bass and to increase the expression of *cyp17a1* in ovaries after a daily intake of 7 ng DRO for up to 31 days, and (b) stresses the need of performing further exposure experiments at the low ng/L

concentration range to ascertain whether the same endocrine disruptive effect is likely to occur at environmentally relevant concentrations of DRO.

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CAPÍTULO 3

Uso de sistemas in vitro para la evaluación toxicológica de sedimentos y disruptores lipídicos

Artículo 4

'Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays'

Maria Blanco[1], Elisabet Pérez-Albaladejo[1], Carlos Barata[1], Benjamí Piña[1], Grozdan Kušpilić[2], Vesna Milun[2], Roger Lille-Langøy[3], Odd André Karlsen[3], Anders Goksøyr[3], Cinta Porte[1]

[1]Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain.
[2]Institute of Oceanography and Fisheries, Split, Croatia.
[3]Department of Biology, University of Bergen, Norway.

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[1]Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain.

[2]Institute of Oceanography and Fisheries, Split, Croatia.

[3]Department of Biology, University of Bergen, Norway.

ABSTRACT

A battery of cell-based bioassays, including PLHC-1 cells, zebrafish-Pxr-transfected COS-7 cells and estrogen receptor-recombinant yeast assay (ER-RYA), were applied to detect the presence of bioactive pollutants in sediments collected from Kaštela Bay and Brač Channel (Croatia). Exposure of PLHC-1 cells to the sediment extracts evidenced significant cytotoxicity and presence of CYP1A inducers in sediments collected in Kaštela Bay, near the industrial zone and cargo port of Split. Sediments from this area, which is highly contaminated with PCBs, HCB, DDTs and γ-HCH, also activated the zebrafish Pxr (zfPxr) reporter system. No evidence of estrogenicity was detected for any of the sediments extracts in the ER-RYA assay. Importantly, the battery of in vitro assays identified Kaštela Bay as the area with the higher anthropogenic impact, where sediment-bound pollutants could pose a risk to aquatic organisms. In contrast, sediments from the Brač Channel showed rather low response in the different bioassays.

Key words: *Sediment; EROD; PLHC-1; zebrafish Pxr; estrogenic activity; Kaštela Bay.*

1. Introduction

More than half of the world's population resides in urban areas, many of them located in coastal zones. This tendency is expected to increase in the next decades (von Glasow et al., 2013). The Mediterranean area is not an exception; highly urbanized and densely populated areas are located along the coast together with important harbours, industry and tourism activities. This produces severe pollution loads that can impact coastal ecosystems and potentially compromise the many ecosystem services they provide, and consequently, human health.

Coastal sediments can act as both, sinks and sources of pollutants. Thus, approaches to biomonitor sediment quality are essential in order to characterize the health status of the aquatic environments and, ultimately, minimize threats and prevent the adverse effects to aquatic wildlife. However, the assessment of the environmental quality of sediments remains a challenge as sediments contain complex mixtures of toxicants. It has become evident that traditional chemical analyses cannot be used as the only strategy to characterize sediment quality since it is not feasible to identify every single chemical present. Importantly, this type of analysis does not provide information on the bioavailability of pollutants or their combined (synergistic/antagonistic) effects in benthic organisms (Chapman, 2007). Besides, the most abundant or most often analyzed contaminants are not necessarily the ones with the highest biological impact in aquatic organisms (Brack et al., 2005).

As the interaction of chemicals with biota initially takes place at the molecular and cellular level, responses at these levels are considered the first manifestation of toxicity and they are used as suitable tools for the early and sensitive detection of chemical exposure (Fent, 2001). Currently,

different cell-based bioassays including fish and human cell models have been successfully applied to estimate the biological activity of sediment-bound pollutants, integrating the interaction between them and covering endpoints such as acute and long-term toxicity, oxidative stress, bioaccumulation and endocrine disrupting effects (Creusot et al., 2010; Schnell et al., 2013; Fernandes et al., 2014). Due to the key role that the liver plays in the metabolism of xenobiotics, fish hepatocytes and fish liver cell lines are often used to study the toxicological impact of pollutants and their mixtures. The PLHC-1 cell line, derived from topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma, expresses the aryl hydrocarbon receptor (AhR) and shows elevated *cyp1a* transcript levels and CYP1A enzymatic activity after exposure to environmental pollutants, e.g. polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals and sediment extracts (Thibaut and Porte, 2008; Traven et al., 2008; Pérez-Albaladejo et al., 2016). Other adverse responses to chemicals, such as genotoxicity or contaminant-stimulated reactive oxygen species (ROS) production have also been successfully assessed with PLHC-1 cells (Puerto et al., 2009; Šrut et al., 2011; Schnell et al., 2013). Besides AhR, the pregnane X receptor (PXR) is another ligand-activated transcription factor that acts as xenosensor and regulates the expression of genes involved in the metabolism of xenobiotics, including cytochrome P450 (CYP3A), phase II enzymes and ABC transporters (Kliewer et al., 2002). PXR plays also a complex role in energy and lipid homeostasis, as it promotes lipogenesis and suppresses fatty acid β-oxidation and gluconeogenesis (Wada et al., 2009). A variety of ligands, including pharmaceuticals, pesticides, emerging contaminants, steroids, vitamins and environmental samples have been shown to activate this receptor (Milnes et al., 2008; Ekins et al., 2008; Kinani et al., 2010; Pérez-Albaladejo et al., 2016). Recently, Pxr has been successfully cloned and functionally characterized in zebrafish (*Danio*

rerio). It was shown to be activated by PXR agonists known from other species, including clotrimazole and pregnenolone 16α-carbonitrile (PCN), but to a lesser extent than the human PXR (Bainy et al., 2013).

Additionally, the estrogen receptor-recombinant yeast assay (ER-RYA) has been used to assess the estrogenic activity of different contaminants and environmental samples (Quirós et al., 2005; Noguerol et al., 2006a; Schnell et al., 2013). ER-RYA is based on a yeast strain that is genetically engineered (human estrogen receptor and β-galactosidase) to elicit an easy-to-read response following exposure to estrogens or estrogen-like compounds. This assay allows quantifying of estradiol-equivalent (EEQ) loads that integrate the contribution of all bioactive compounds present in environmental extracts.

Split is the second largest Croatian city, located in the eastern Adriatic Sea. The city stretches along the coastline of Kaštela Bay and Brač Channel, both under a strong influence of anthropogenic activities. Due to the rapid urbanization and industrialization during the second half of the 20th century, Kaštela Bay became one of the most polluted areas along the eastern Adriatic coast (Jakšić et al., 2005). Although wastewater discharges to the bay have been considerably reduced in the past decade, a large amount of chemicals has accumulated in sediments over the years, viz. polychlorinated biphenyls (PCBs), dichlorodiphenyl-trichloroethanes (DDTs), lindane, chlordane, and trace metals, particularly mercury (Hg) (Kwokal et al., 2002; Lovrenčić Mikelić et al., 2013). Many of these pollutants have also been detected in aquatic fauna (Kljaković-Gašpić et al., 2010; Bogdanović et al., 2014; Milun et al., 2016). The bay is connected to the Brač Channel, which is the most direct route from international waters to the Port of Split, one of the leading passenger ports in the Mediterranean.

The aim of this study was to characterize the environmental quality of sediments collected from Kaštela Bay and the Brač Channel (Split area) by applying different cell-based bioassays targeting complementary endpoints, namely: a) the fish hepatoma cell line (PLHC-1) to determine the presence of cytotoxic compounds and AhR agonists (Cyp1a inducers), b) the zebrafish Pxr transfected COS-7 cell line for detection of zfPxr agonists, and c) the ER-RYA to detect the presence of estrogenic compounds in sediment organic extracts. The biological activities detected by the bioassays were compared to those previously obtained in other areas of the Mediterranean in order to weigh the usefulness of the selected bioassays to assess the quality of sediments in costal monitoring programs.

2. Materials and methods

2.1. Chemicals and reagents

Eagle's Minimum Essential Medium (MEM), Dulbecco Eagle's Minimum Essential Medium (DMEM), Opti-MEM, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin and trypsin-EDTA were from Gibco BRL Life Technologies (Paisley, Scotland, UK). 7-Ethoxyresorufin, 7-hydroxyresorufin, β-naphthoflavone (BNF), dimethyl sulfoxide (DMSO), clotrimazole and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Steinheim, Germany), while 4-methylumbelliferone β-D-galactopyranoside was obtain from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Sediment collection and extraction

Sediments were collected in triplicate with a Van Veen grab sampler along Kaštela Bay and Brač Channel (Fig. 28). Geographic coordinates and main characteristics of the selected sites are given in Table 13. A subsample was transferred to a stainless steel container, mixed, and the composite sediments were stored at -20°C and freeze-dried. Six grams of dried and homogenized sediment (<125 μm) were extracted twice with 20 mL dichloromethane/hexane (1:1), followed by dichloromethane/acetone (1:1). For each extraction step, the sample was sonicated at room temperature and centrifuged. The extracts were combined, evaporated, and reconstituted in 500 μL of DMSO. The stock was equivalent to 12 g dry weight extract (eQsed)/mL, and was serially diluted in DMSO to the desired concentrations.

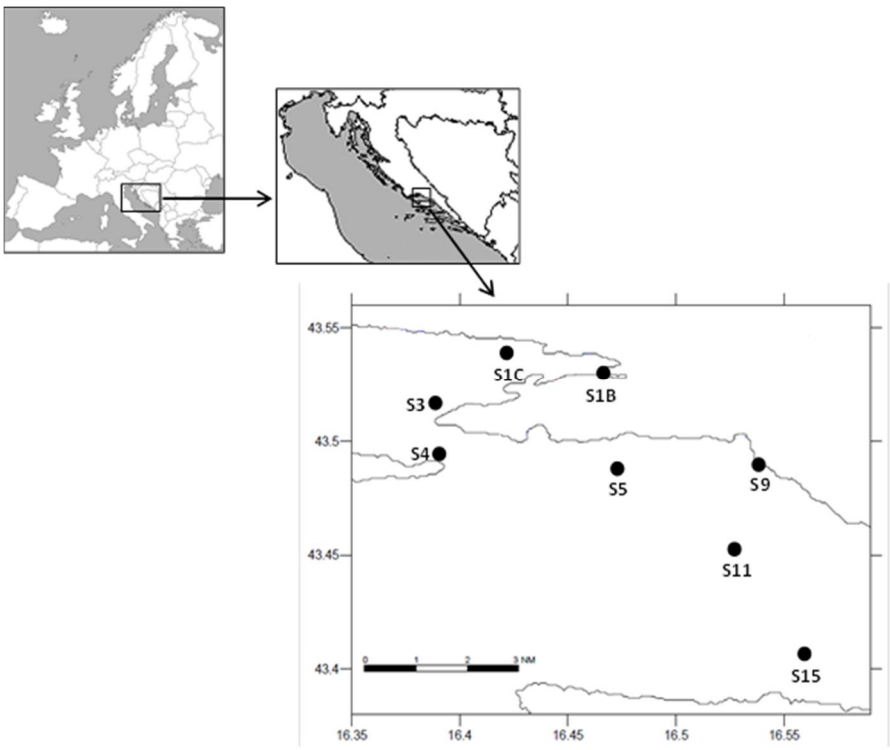

Figure 28. Map of the sampling sites in Kaštela Bay (S1B, S1C, S3, S4) and the Brač Channel (S5, S9, S11, S15) in Croatia.

Table 13. Geographic coordinates, sample depth and criteria for the selection of the sampling points in Kaštela Bay and the Brač Channel.

Sample	Depth (m)	Latitude (N)	Longitude(E)	Pollution sources
Kaštela Bay				
S1B	18	43º 31’ 48’’	16º 27’ 12’’	Industrial, Jadro River discharges
S1C	28	43º 32’ 28’’	16º 24’ 39’’	Former chloralkali plant, marine traffic (Port of Split)
S3	38	43º 31’ 06’’	16º 22’ 54’’	Marine traffic
S4	44	43º 29’ 44’’	16º 23’ 11’’	Marine traffic
Brač Channel				
S5	23	43º 29’ 08’’	16º 28’ 08’’	Marine traffic (Split Ferry port)
S9	25	43º 29’ 20’’	16º 32’ 02’’	Žrnovnica River discharge
S11	48	43º 27’ 00’’	16º 31’ 36’’	Split wastewater treatment plant
S15	56	43º 24’ 48’’	16º 33’ 18’’	Cetina River discharge

2.3. Cell cultures

The PLHC-1 cell line (ATCC; CRL-2406) was cultured in Eagle's Minimum Essential Medium supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin G and 50 μg/mL streptomycin.

COS-7 cells (ATCC CRL-1651) were grown in Eagle’s Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate and 100 U/mL penicillin G and 100 μg/mL streptomycin.

Both PLHC-1 and COS-7 cells were cultured in a humidified incubator with 5% CO_2, at 30 and 37ºC, respectively. When 90% confluence was reached, cells were dissociated with trypsin-EDTA for subculturing and experiments (splitting factor was 1:3 to 1:4). Exposure to the sediment extracts was carried out by diluting the extracts in culture

medium, so that the final concentration of DMSO in culture wells was 0.5% (v/v).

2.4. Cell viability

Cytotoxicity was evaluated using two fluorescent dyes, Alamar Blue (AB; Biosource International, Invitrogen, Spain) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Molecular Probes, Invitrogen, Spain), which monitor metabolic activity and membrane integrity, respectively (Dayeh et al., 2003). PLHC-1 cells were seeded in 96-well plates (Nunc; Roskilde, Denmark) at a density of 7.5 x 10^4 cells per well and were allowed to attach for 24 h prior to exposure to sediment extracts at 60 mg eQsed/mL for 24 h. Responses were obtained as fluorescent units at the excitation/emission wavelengths of 530/590 nm for AB, and 485/530 nm for CFDA-AM in a fluorescent plate reader (Varioskan, Thermo Electron Corporation). Results were expressed as percentage of viability compared to solvent control cells, as mean ± SD of at least six replicates in three different plates assayed.

2.5. Reactive oxygen species (ROS) generation

The generation of ROS in the presence of sediment extracts was determined as described in Lebel et al. (1992), with slight modifications. PLHC-1 cells were seeded in 96 well plates at a density of 10^5 cells per well, and allowed to attach overnight. Then, cells were washed with PBS and 20 μM 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) diluted in DPBS (1:10) supplemented with 10 mmol/L glucose (DPBS-Glu) added. After 30 min incubation at 30°C, the cell monolayers were washed with PBS and exposed to sediment extracts diluted in DPBS-Glu in order to stimulate ROS production. After 60 min of exposure, the fluorescence of

oxidized H_2DCF was measured in a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths pairs of 485/528 nm. 3-Morpholinosydnonimine (SIN-1; 5 μmol/L) was used as a positive control (Spohn et al., 2009). Results were expressed as a percentage of the basal fluorescence in control wells (0.5% DMSO). Experiments were carried out in triplicate.

2.6. Induction of EROD activity

The assay was performed as indicated in Fernandes et al. (2014) with some modifications. PLHC-1 cells were seeded at 6.5 x 10^5 cells/well in 48-well plates and allowed to grow for 24 h. Thereafter, cells were exposed to different concentrations of sediment extracts, 1 μM β-naphthoflavone (BNF; positive control) or the solvent (0.5% DMSO). Samples were assayed in triplicate. After 24 h exposure, the medium was aspirated, the cells were rinsed with PBS, and immediately incubated with 2 μM 7-ethoxyresorufin in 50 mM Na-phosphate buffer pH 8.0 at 30°C. After 15 min of incubation, the fluorescence was read in a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelength pairs of 537/583 nm. Quantification was made by calibration with 7-hydroxyresorufin. Subsequently, cells were washed with PBS and total cellular proteins were measured with fluorescamine, using bovine serum albumin (BSA) as standard (Lorenzen and Kennedy, 1993). EROD activity was expressed as pmol of resorufin formed per minute per milligram of protein (pmol/min/mg protein). R_{BNF} value was obtained from the dose-response curves, as the concentration of sediment required to induce an EROD response equivalent to 1 μM BNF. Experiments were carried out in triplicate.

2.7. Ligand activation of zebrafish Pxr

Ligand activation of zebrafish Pxr was monitored with the luciferase assay expressing the GAL4-DBD/Pxr-LBD fusion protein in COS-7 monkey kidney cells as previously described in Bainy et al. (2013). COS-7 cells were seeded at cell density of 5 x 10^3 cells/well in 96-well plates and cultivated as described in Section 2.3. After 24 hours, COS-7 cells were transiently transfected with a thymidine kinase promoter reporter plasmid, (MH100)x4_tk_luc, with 4 GAL4 upstream activation sequences (Harmon et al., 1995), a receptor plasmid, pCMX-GAL4-zfPxr, encoding a fusion protein of the yeast GAL4 DNA-binding domain and the hinge and ligand binding domain of zfPxr AA111-430), and the control plasmid, pCMV-β-GAL, a CMV promoter plasmid encoding β-galactosidase. The amount of plasmids was 0.5 µg/plate of receptor and 10 µg/plate of luciferase reporter and β-galactosidase transfection control plasmid. After 24 h incubation, transfected cells were exposed for 24 h to different concentrations of sediment extracts (1, 10, 20, 30, 40, 50, 60 mg eQsed/mL) and clotrimazole (positive control). Cells exposed to 0.5% v/v DMSO were used as blank. Following exposure, cells were lysed and luciferase activity was measured as emitted light from the conversion of luciferin to oxyluciferin in a Perkin Elmer EnSpire plate reader. Luciferase activities were normalized for variations in transfection efficiencies by dividing the luciferase activity by β-galactosidase activity. β-galactosidase activity was measured as the amount of ortho-nitrophenol formed from the hydrolyzation of ortho-nitrophenyl-β-galactoside (ONPG) by reading absorbance at a wavelength of 405 nm in a plate reader (Perkin Elmer EnSpire). Activation of PXR was expressed as fold induction of luciferase activity in cells exposed to sediment extracts relative to cells exposed to the carrier (0.5% v/v DMSO). Dose-response curves were fitted by non-linear regression using Sigma Plot

12.0 software, and REC_{20} values were estimated from the dose-response curves as the concentration of sediment extract necessary to produce a response equal to 20% of the response induced by the positive control (1 µM clotrimazole) (Lille-Langøy et al., 2015; Pérez-Albaladejo et al., 2016).

2.8. Recombinant yeast assay (ER-RYA)

ER-RYA assays were performed using the yeast strain BY4741 as described in Schnell et al. (2013). Yeast strains were grown overnight in minimal medium (6.7 g/L yeast nitrogen base without amino acids (DIFCO, Basel, Switzerland) and ammonium sulfate) supplemented with 0.1 g/L of prototrophic markers and glucose as a carbon source. Cells were grown to early exponential phase (OD∼0.1). Samples were tested in triplicate and added to the yeast cells in DMSO (0.5% v/v) at different dilutions (1, 10, 20, 30, 40, 50 and 60 mg eQsed/mL). Positive (10 nM estradiol) and negative (0.5% DMSO) controls were included in each plate. Inhibitory activities, either by toxicity or by antagonistic effects, were tested by simultaneously adding 10 nM estradiol (final concentration) and each compound at the second highest dilution tested in the assay and comparing the obtained activity to those of the positive controls. Plates were incubated for 6 h at 30°C under mild shaking. For the β-galactosidase assay, 50 µL of the incubated cell cultures was mixed with 50 µl Y-PER (PIERCE, Rockford, IL, USA) and incubated at 30°C for 30 min. Then, 50 µL of assay buffer supplemented with 0.1% 2-mercaptoethanol and 0.5% of the 4-methylumbelliferone β-D-galactopyranoside (MuGal) solution (FluorAce β-galactosidase Reporter Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA) were added to cells. β-galactosidase activity was determined on-plate using the fluorogenic substrate 4-methylumbelliferone β-D-galactopyranoside. Fluorescence was recorded for 15–20 min (one

measurement per minute) in a Synergy 2 spectrofluorometer (BioTek, USA) at 355/460 nm excitation/emission wavelengths. ER ligand activation values were calculated as estradiol equivalents (EEQ), defined as the concentration of 17β-estradiol required to elicit the same response as the sample in the assay. These values were calculated from dose–response curves by adjusting β-galactosidase values to a first-order Hill equation, as described in Noguerol et al. (2006b), using at least nine determinations for each value. The detection limit of the assay was 0.14 ng EEQ/g sediment d.w.

2.9. Chemical analysis

Analysis of organochlorines (OCs) in sediment was based on the procedure recommended by UNEP/IAEA/IOC (2011). Briefly, 10 g of sieved sample with addition of internal standards (PCB-29, PCB-198, ε-HCH) was extracted with a mixture of n-hexane/dichloromethane (50:50 v/v) in a Soxhlet apparatus for 8 h. The extract was evaporated and concentrated to 6 mL. The removal of sulphur and sulphur compounds was performed using activated copper. Extracts were evaporated and concentrated to a volume of 1 mL. Fractionation of OC compounds into classes was performed using adsorption chromatography with a florisil column. The first fraction, eluted with 65 mL hexane, contained HCB, 4,4'-DDE and PCBs, whereas the second fraction, eluted with 45 mL hexane/dichloromethane (70:30 v/v), contained lindane, 4,4'-DDD and 4,4'-DDT. All fractions were rotary evaporated and concentrated to a volume of 1 mL with a gentle nitrogen stream.

The final extracts were analysed on a gas chromatograph (Agilent Technologies, model 6890N) equipped with a μ-ECD Ni^{63}. A fused silica HP-5 capillary column (J&W Scientific: 30 m length, 0.32 mm i.d. and

0.25 µm film thickness) was used for OCs separation. Splitless mode of injection (~1 µL) with splitter closing time (0.8 min) was applied to all samples. The oven temperature was programmed from an initial temperature of 70°C (2 min hold) to 260°C at a rate of 3°C/min and then maintained for 25 min. The injector and detector temperature were 250°C and 300°C, respectively. Nitrogen was used as carrier (1 mL/min) and make-up (60 mL/min) gas. Qualitative and quantitative analysis of OCs were performed by comparison with external standards. For quality assurance and quality control of the chemical analysis internal standards, procedural blanks and reference materials were used.

2.10. Statistical analysis

Comparisons between sediments and control groups were made using one-way ANOVA followed by multiple independent group comparison (Dunnett and Tukey's test). Log transformation of the data was performed when the assumption of normality of residuals was not met. All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and STATA SE 12.0, and p-values lower than 0.05 were considered statistically significant. The concentration of sediment extract resulting in 50% effect (EC_{50}) was calculated by using SigmaPlot 11.0 software.

Ligand activation of the zebrafish Pxr was monitored with the luciferase assay expressing the GAL4-DBD/Pxr-LBD fusion protein in COS-7 monkey kidney cells as described by Lille-Langøy et al. (2015) with some modifications. COS-7 cells were plated at 5 x 103 cells/well in 96-well plates and transfected with (MH100)x4_tk_luc (reporter), pCMX-GAL4-zfPxrAB/Tu (effector) and pCMV-β-galactosidase (transfection control) plasmids 24 h after plating. After one day incubation, transfected

cells were exposed for 24 h to different concentrations of sediment extracts. Clotrimazole and DMSO (0.5% v/v) were used as positive and solvent controls, respectively. Following exposure, cells were lysed and luciferase activity was measured as emitted light from the conversion of luciferin to oxyluciferin in a Perkin Elmer EnSpire plate reader. Luciferase activities were normalized for variations in transfection efficiencies by dividing the luciferase activity by β-galactosidase activity. β-galactosidase activity was measured as the amount of ortho-nitrophenol formed from the hydrolyzation of ortho-nitrophenyl-β-galactoside (ONPG) by reading absorbance at a wavelength of 405 nm in a plate reader (Perkin Elmer EnSpire). Ligand activation of zfPxr was expressed as change in luciferase activity in exposed cells relative to solvent-exposed control (DMSO). COS-7 cells were exposed to different concentrations of sediment extracts (1, 10, 20, 30, 40, 50 and 60 mg eQsed/mL) and a number of model compounds, including organochlorines (1,2,3-trichlorobenzene, chlordane, dieldrin, PCB-153), alkylphenols (4-NP, 4-OP), BPA, pharmaceuticals (omeprazole, carbamazepine), and perfluorinated chemicals (PFNA and PFOA) in the range of 0.05-100 μM. An equimolar mix formed by all the single compounds was tested at different concentrations (0.1, 0.6, 3, 15, 73, 110 μM) and joint effects on the activation of zfPxr were assessed and confronted with existing concentration addition and independent action additive models using the modified non-linear Hill model (Faria et al., 2016). The concentrations that resulted in luciferase activity corresponding to 20% of the maximum zfPxr response to clotrimazole (REC_{20}) were determined from dose-response curves.

3. Results

3.1. Viability and EROD activity in PLHC-1 cells

The cytotoxicity of organic sediment extracts in PLHC-1 cells after 24 h exposure to 60 mg eQsed/mL is shown in Figure 29. Significant cytotoxicity was detected in sediments collected in Kaštela Bay (S1B, S3, S4), while low (Kaštela Bay: S1C) or no cytotoxicity was observed for the other sediment's extracts (Brač Channel: S5, S9, S11, S15).

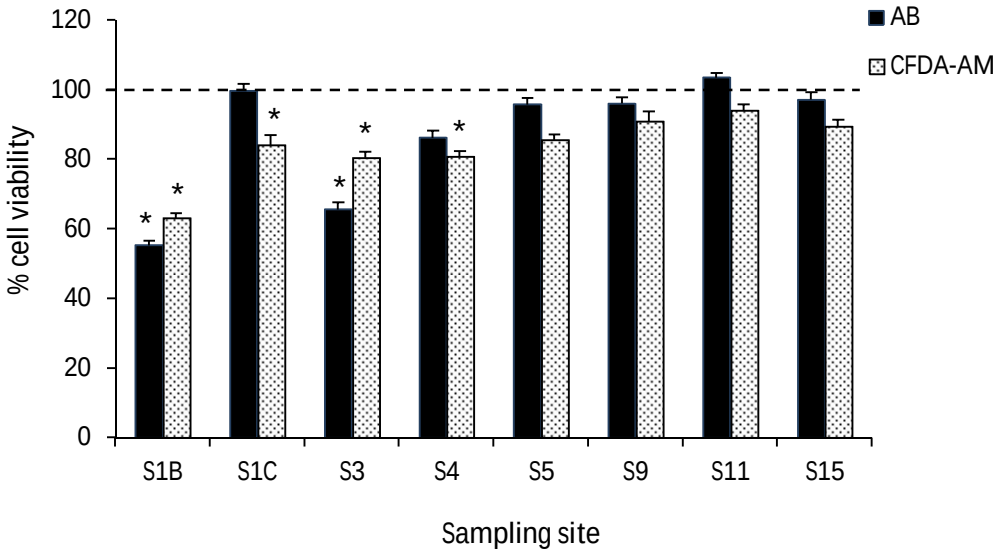

Figure 29. Cell viability (mean ± SEM, n = 4) of PLHC-1 cells after 24 h exposure to sediment extracts from Kaštela Bay and Brač Channel tested at 60 mg eQsed/mL. *Statistically significant decrease in cell viability relative to control cells (dotted line) ($p < 0.05$).

Dose-response curves for EROD activity for the positive control (BNF) and all the sediment extracts are shown in Figure 29. The AhR agonist BNF at a concentration of 1 µM led to an EROD activity of 66 ± 1.7 pmol/min/mg protein, which was used as reference to obtain R_{BNF} values from the dose-response curves.

Maximum EROD activity and R_{BNF} are given in Table 14. Sediments from S1B had the highest ability to induce EROD activity in PLHC-1 cells at 10 mg eQsed/mL (110 ± 9 pmol/min/mg protein; R_{BNF}: 4.9 mg eQsed/mL), whereas decreasing induction was detected at higher concentrations (≥30 mg eQsed/mL), likely associated to cytotoxicity. The other sediments collected in Kaštela Bay (S1C, S3, S4) did induce EROD activity (114-128 pmol/min/mg protein), but higher concentration of sediment was needed to reach the maximal induction (R_{BNF}: 17-32 mg eQsed/mL). On the contrary, minor EROD induction (53-67 pmol/min/mg protein) and high R_{BNF} > 60 mg eQsed/mL were observed for sediments collected along the Brač Channel (S5, S9, S11, S15).

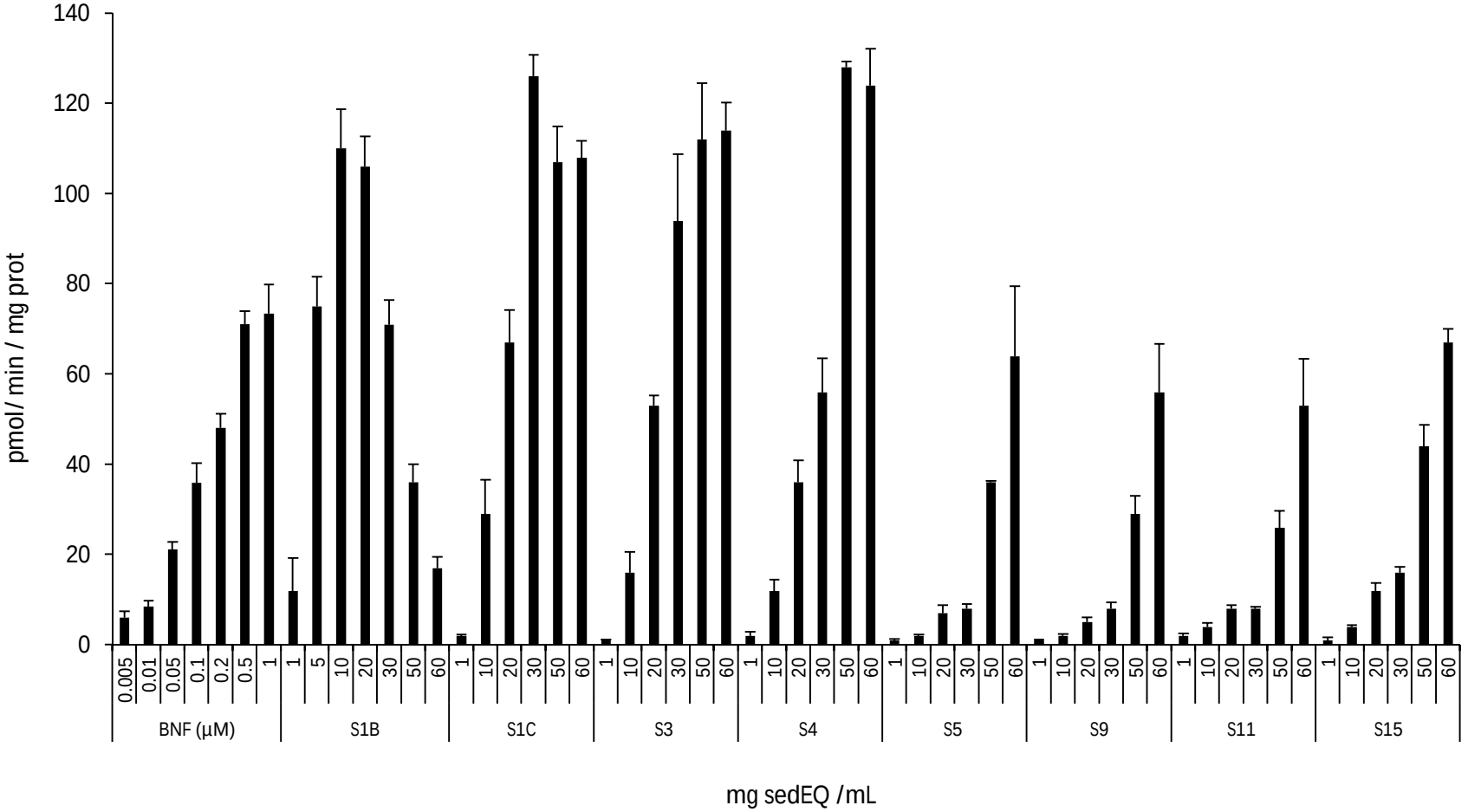

Figure 30. EROD activity in PLHC-1 cells exposed for 24 h to different concentrations of sediments extracts from Kaštela Bay and Brač Channel. Values are expressed in pmol/min/mg of protein, as mean ± SEM of at least three experiments. β-naphthoflavone (BNF; positive control) expressed in µM.

3.2. Oxidative stress

The oxidation of H_2DCF by ROS was monitored in PLHC-1 cells, over 120 min of exposure to sediment extracts at 60 mg eQsed/mL. The maximum ROS production was detected after 15 min for all of the sediment extracts, particularly for S11 and S15 (1.51 and 1.45 fold, respectively) (Table 14; Fig. 31). ROS levels decreased over time; after 120 min incubation, no significant generation of ROS was observed. The positive control (5 µM SIN-1) induced the generation of ROS over time, from 2.8-fold at 15 min to 11-fold after 180 min exposure.

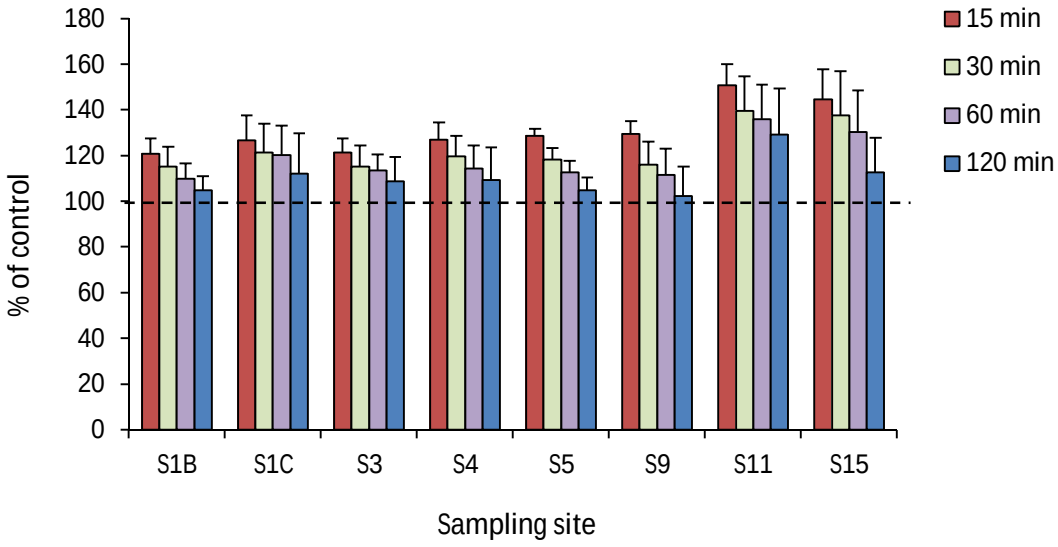

Figure 31. ROS production in PLHC-1 cells after 15, 30, 60 and 120 min of exposure to different concentrations of sediments extracts from the Kaštela Bay and Brač Channel. Concentrations are showed as 60 mg eQsed/mL. Values are expressed as fold-induction with respect to control cells (mean ± desvest, n = 3 different plates assayed). Dotted line is control value. *Significant differences respect to control ($p < 0.05$).

3.3. Ligand activation of zebrafish Pxr

All sediments significantly induced the transcriptional activity of Pxr from zebrafish and promoted the expression of the luciferase reporter gene (Table 14, Fig. 32). Viability of COS-7 cells after 24 h exposure to

sediment extracts was previously assessed in order to confirm that the observed effects were not due to cytotoxicity (Fig. 33). Sediments from Kaštela Bay (S1B, S1C) induced the highest activation of zebrafish Pxr (10- and 5-fold at 10 mg eQsed/mL, respectively) followed by sediments from S3 and S4 (4-fold at 20 mg eQsed/mL). In contrast, those sediments sampled along the Brač Channel (S5, S9, S11, S15) induced luciferase activity less than 4-fold at the highest concentration tested (60 mg eQsed/mL). The increase of luciferase activity in cells exposed to the positive control (1 μM clotrimazole) was 20.6 ± 2.0 fold.

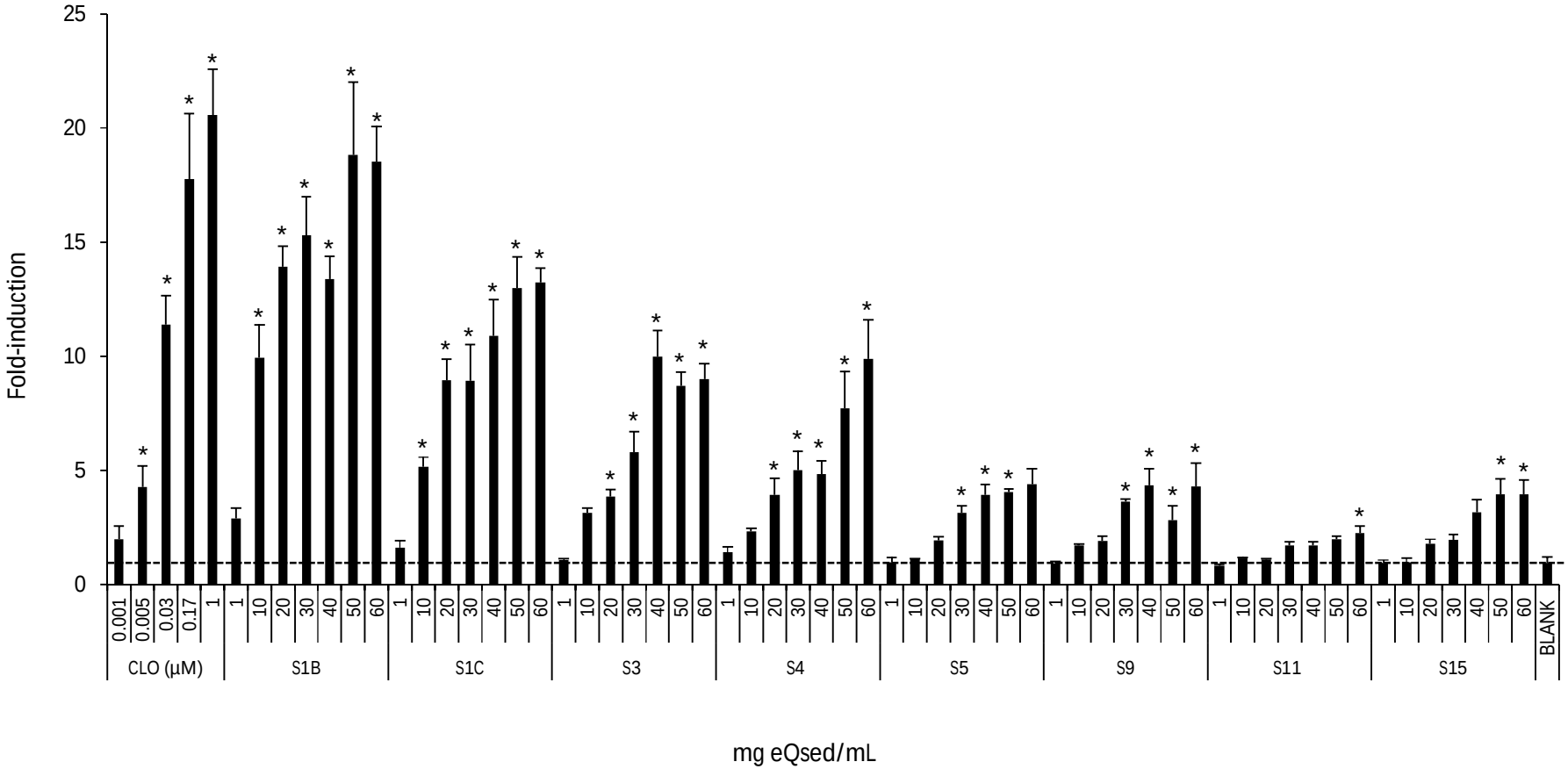

Figure 32. *In vitro* ligand activation of zfPxr by sediments collected from Kaštela Bay and Brač Channel presented as fold change of normalized luciferase activity respect to blank (0.5% v/v DMSO). Values are mean ± SEM of at least three independent experiments. CLO: clotrimazole (positive control) expressed in µM. *Statistically significant differences from control ($p < 0.05$).

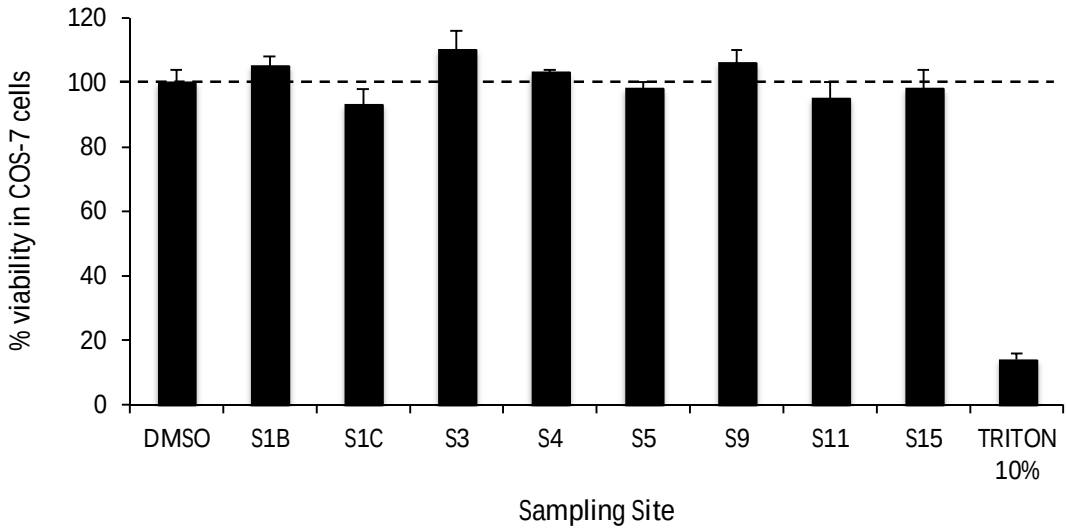

Figure 33. Cell viability (Alamar Blue) of COS-7 cells after 24 h of exposure to sediment extracts from Kaštela Bay and Brač Channel tested at 60 mg eQsed/mL. Values are expressed as mean ± SEM of 3 replicates. DMSO (0.5% v/v): negative control, 10% Triton: positive control.

3.4. Estrogen receptor- recombinant yeast assay

Estrogenic activity was below the quantification limit (0.14 ng EEQ/g sediment d.w.) of the assay in all samples; thus, no relevant concentrations of estrogenic compounds were detected in the sediment extracts with the ER-RYA assay. 17β-Estradiol (10 nM) was used as positive control and lead to an activity 14 ± 2.7 fold with respect to unexposed yeast cells.

3.5. Organochlorine content in sediments

Concentrations of HCB, lindane (γ-HCH), Σ DDTs (4,4'-DDE + 4,4'-DDD + 4,4'-DDT) and 7 PCBs congeners (sum of PCB 28, 52, 101, 118, 138, 153 and 180) in the analysed sediments are shown in Table 14. PCBs were the most abundant organochlorines detected in sediments, followed by HCB,

DDTs and γ-HCH. Among the seven PCB congeners determined, PCB-153 (33%), PCB-138 (26%) and PCB-180 (22%) were the most prevalent. Among DDT metabolites, 4,4'-DDE and 4,4'-DDD were more abundant than 4,4'-DDT. Quantitative results point to a decreasing concentration gradient from the inner part of Kaštela Bay towards the Brač Channel. Sediments from S1C had the highest concentration of HCB (3.9 ng/g d.w.), while those from S1B showed high concentrations of DDTs and PCBs (1.4 and 35.4 ng/g d.w.). γ-HCH was the only HCH-isomer detected in sediments, the highest concentration was found in S1B (0.1 ng/g d.w.).

Table 14. Maximum induction of zfPxr reporter activity and concentration of sediment required to induce 20% of the maximum response induced by clotrimazole (REC_{20}); maximum EROD activity (pmol/min/mg protein) and concentration of sediment required to induce a response equivalent to 1 μM β-naphthoflavone (R_{BNF}); ROS generation after 15 min of exposure to 60 mg eQsed/mL and concentration of organochlorinated compounds in the sediment extracts. Values are mean ± SEM of at least three independent experiments.

	zfPxr		**EROD**		**ROS**	**Organochlorines (ng/g d.w.)**			
Sample site	**Max. induction**	**REC_{20}**	**Max. Activity**	**R_{BNF}**	**Fold generation**	**∑PCB**	**∑DDT**	**HCB**	**γ-HCH**
Kaštela Bay									
S1B	19 ± 1*	1.7 ± 0.05	110 ± 9	4.9 ± 1.1	121 ± 2	35.4	1.4	0.67	0.09
S1C	13 ± 1*	5.8 ± 0.9	126 ± 5	16.6 ± 0.3	127 ± 4	28.0	1.0	3.94	< 0.05
S3	10 ± 1*	16.2 ± 0.7	114 ± 6	22.7 ± 1.2	122 ± 2	5.8	0.8	0.45	< 0.05
S4	10 ± 2*	23.9 ± 1.7	128 ± 1	31.8 ± 0.9	129 ± 2	3.1	0.4	0.18	< 0.05
Brač Channel									
S5	4 ± 1*	50.4 ± 0.4	64 ± 16	> 60	130 ± 1	0.3	0.09	< 0.05	< 0.05
59	4 ± 1*	60.0 ± 1.8	56 ± 11	> 60	130 ± 2	0.1	0.07	< 0.05	< 0.05
S11	2 ± 0.3*	> 60	53 ± 10	> 60	151 ± 3	< 0.12	< 0.09	< 0.05	< 0.05
S15	4 ± 1*	> 60	67 ± 3	> 60	145 ± 4	0.3	0.14	< 0.05	< 0.05

*Statistically significant differences from control ($p < 0.05$). Sediment concentrations are expressed as mg eQsed/mL.

4. Discussion

Toxicity screening of complex environmental samples, such as sediments, require the use of multiple endpoints in order to determine the different modes of action of the chemical mixture trapped in the matrix. In this study, we have applied a battery of bioassays based on three different cell types to explore the presence of bioactive pollutants in sediments from Split coastal area. By using the PLHC-1 cell line, significant cytotoxicity was detected in sediment extracts from station S1B, an industrial zone affected by the Port of Split and the mouth of Jadro River. Sediment extracts from the other stations in Kaštela Bay also lead to a decrease of cell viability (S1C, S3, S4), while those collected along the Brač Channel showed no significant cytotoxicity in PLHC-1 cells. It should be noted that cytotoxicity in sediments from Kaštela Bay was observed at higher concentrations (60 mg eQsed/mL) than induction of xenobiotic metabolic pathways, such as EROD activity (5 to 30 mg eQsed/mL) or activation of zfPxr (1 to 10 mg eQsed/mL). This endpoint was mainly used as a quality control measure for the other assays to verify that cell viability was not adversely affected (Escher et al., 2013).

EROD activity, a catalytic measure of CYP1A induction, is one of the most widely applied biomarkers in fish-based biomonitoring studies. It is a marker of exposure to AhR agonists (such as analogs of 2,3,7,8-tetracholordibenzo-p-dioxin (TCDD), dioxin-like PCBs, PAHs and many others), but also a biochemical response that might precede adverse effects at various levels of biological organization (Whyte et al., 2000). The determination of EROD activity in cell-based bioassays facilitates the determination of dose-response relationships and has both, practical and economic advantages, allowing the detection of highly toxic persistent and

non-persistent CYP1A inducers (Schnell et al., 2013). In the present work, EROD assays indicated the presence of significant amounts of AhR agonists in sediments collected from S1B (R_{BNF}: 4.9 mg eQsed/mL) in comparison to other sediments from Kaštela Bay (S1C, S3 and S4; R_{BNF}: 17-32 mg eQsed/mL) or the Brač Channel (R_{BNF} > 60 mg eQsed/mL). These findings, together with cytotoxicity data and chemical analysis, indicate a gradient of pollutants from the easternmost part of Kaštela bay to the Brač Channel and the open waters of the Adriatic Sea, and reflect reduced inputs of pollutants into the Brač Channel, which is mainly affected by ship traffic, freshwater and wastewater discharges and agricultural activities.

The detection of AhR agonists and cytotoxic compounds in sediments collected in S1B and other stations in Kaštela bay is well in agreement with chemical analysis; up to 100-fold higher concentrations of PCBs, DDTs and HCB (35.4, 1.4, and 0.7 ng/g d.w.) were detected in station S1B in comparison to other stations in the Brač Channel. Certainly, information provided by chemical analysis in this work is limited and many other compounds may have contributed to bioassay responses, including PAHs that have been detected at rather high concentrations in sediments from stations SIB (2.3 µg/g d.w.) and S1C (0.5 µg/g d.w.) in Kaštela Bay, as a result of naval traffic and industrial wastewater discharges to this semi-enclosed and relatively shallow area (Mandić and Vrančić, 2017).

Regarding the use of zfPxr assay as environmental biosensor, little information is available on its relevance to assess the quality of environmental samples, as only few studies have reported its induction by environmental chemicals (Ekins et al., 2008; Milnes et al., 2008). In contrast, the activation of hPXR by environmental pollutants (Kliewer et al., 2002; Lemaire et al., 2007; Mnif et al., 2007; Creusot et al., 2010; Lille-

Langøy et al., 2015), and environmental matrices, e.g. river water and sediment samples, moderately impacted by agricultural and urban inputs, containing phthalates, alkylphenols, hormones, pharmaceuticals, pesticides, PCBs, BPA and/or affected by effluents from sewage treatment plants, has been reported (Kinani et al., 2010; Creusot et al., 2010; Mnif et al., 2012; Neale et al., 2015). Actually, information regarding zfPxr activation by mixtures of pollutants and environmental samples is still scarce (Milnes et al., 2008). Recently, Pérez-Albaladejo et al. (2016) reported the activation of zfPxr by chemicals bound to sediments from Constanta harbour (E_{max}: 14-fold; REC_{20}: < 5 mg eQsed/mL) and the Danube River mouth (E_{max}: 11-fold; REC_{20}: 5.9 mg eQsed/mL). Interestingly, sediment extracts from Kaštela Bay (E_{max}: 19-fold; REC_{20}: 0.9 mg eQsed/mL) had even higher potency and higher content of zfPxr agonists than those from Constanta harbour. The compounds responsible for the high zfPxr induction in sediments from Kaštela Bay remain unknown. Sediments from this area, and particularly stations S1B and S1C, showed high concentrations of organochlorinated compounds, particularly PCBs and HCB. Most of the industry (steel and cement plant, brewery, food and beverages), ports, shipyards and a former PVC chlor-alkali plant are located in the eastern part of the bay and might significantly contribute to the continuous entrance of pollutants.

Certainly, sediment extracts could contain, as well as xenobiotics, natural organic substances which may bind to AhR and zfPxr. Therefore, a possible contribution of these organic substances to the observed responses in EROD and zfPxr assays cannot be discarded. However, form our previous experience, we believe that this contribution is generally very low, as the activity induced by sediment extracts collected in clean areas is close to blank (i.e. A1 from Italian Adriatic Sea; Pérez-Albaladejo et al., 2016).

Moreover, exposure of PLHC-1 cells to sediment extracts led to an increase of ROS levels, reaching a maximum after 15 min (1.5-fold) incubation. ROS generation decreased at longer exposure times, probably associated to the low oxidative potential of compounds presents in the extracts. ROS generation was rather low in comparison to the levels generated by sediment extracts from other areas in the Mediterranean (Pérez-Albaladejo et al., 2016).

The recombinant yeast assay did not indicate the presence of estrogenic compounds in sediment extracts from Kaštela Bay nor the Brač Channel, not even on those collected in S11, near the Split waste water treatment plant outlet. Similarly, Schnell et al. (2013) reported no estrogenicity in estuarine and coastal sediments from North Spain, with the exception of those directly impacted by a non-depurated urban effluent, which showed weak estrogenic activity. Also coastal sediments from the Adriatic and Black Sea did not show estrogenic response in the ER-RYA assay (unpublished results), despite the ability of those sediment extracts to inhibit ovarian aromatase activity in sea bass microsomal fractions (Pérez-Albaladejo et al., 2016). Thus, although the concentration of estrogenic compounds in sediment extracts from the studied area is below the detection limit of the ER-RYA assay (0.14 ng EEQ/g sediment d.w.), we cannot discard the presence of compounds that may act as endocrine disrupters by modulating the activity of enzymes involved in steroid synthesis/metabolism (Fernandes et al., 2014).

Overall, the combination of *in vitro* bioassays focusing on cytotoxicity and detection of AhR and Pxr agonists highlighted station S1B (Kaštela Bay) as the most impacted area. This station is under the influence of the Port of Split, the mouth of Jadro River, and historical contamination from a former PVC chlor-alkali plant. This work also shows a dilution

gradient of pollutants from the Eastern part of Kaštela Bay towards the open waters of the Adriatic Sea, and a comparatively reduced impact of pollutants in the Brač Channel, despite of the presence in the area of significant pollution sources, e.g. marine traffic (Split Ferry port), Žrnovnica and Cetina River discharges, and the outlet of Split wastewater treatment plant.

The use of cell-based bioassays and multiple endpoints (cytotoxicity, ROS, EROD, ER and zfPxr induction) combined with internal calibrators may allow the comparison of sediments from different coastal areas. Table 15 summarizes the measurable responses (percentage of cell viability, ROS, R_{BNF}, REC_{20}) obtained in the present work in comparison to those detected in sediment extracts from the Adriatic (Italy) and the Black Sea (Pérez-Albaladejo et al., 2016), by using a color code that facilitates the visualization of the magnitude of the effect. Green color indicates effect at high concentrations (low potency), and transition to red indicates increased presence of active contaminants (high potency). The code is based on a medium value (yellow) corresponding to: 50% cytotoxicity (AB assay), 30 mg eQsed/mL (R_{BNF} and REC_{20}) and 2.5-fold generation of ROS.

Table 15. Summary of toxic responses (cell viability, CYP1A induction, zfPxr activation and ROS generation) observed for sediment extracts from Split coastal area in comparison to the responses detected in other marine areas from the Adriatic and the Black Seas. Color scale goes from green to red. Yellow corresponds to: 50% cell viability at 60 mg eQsed/mL (AB), 30 mg eQsed/mL (R_{BNF} and REC_{20}), and 2.5-fold induction (ROS).

Area	Criteria of selection	Sampling Site	Viability AB	CYP1A R_{BNF}	zfPxr REC_{20}	ROS
Black Sea[1]	Danube mouth north	B1				
	Danube mouth	B2				
	Danube mouth south	B3				
	Touristic resort/upstream Constanta	B4				
	Urban WWTP	B5				
	Roadstead of the Constanta harbor	B6				
	Open sea (20 nautical miles offshore)	B7				
	Constanta harbor, industrial and urban WWTP	B8				
	Touristic resort, downstream Constanta	B9				
	Mangalia harbor, urban WWTP	B10				
Adriatic Sea-*Italy*[1]	Po mouth	A7				
	Po mouth	A4				
	Po influenced area (35 nautical miles from Pila mouth)	A8				
	City harbor, petrochemical industry	A2				
	Diffuse pollution	A1				
Kaštela Bay	Industrial, Jadro River discharges	S1B				
	Former chloralkali plant, marine traffic (Port of Split)	S1C				
	Marine traffic	S3				
	Marine traffic	S4				
Brač Channel	Marine traffic (Split Ferry port)	S5				
	Zrnovnica River discharge	S9				
	Center of the channel	S11				
	Cetina River discharge	S15				

[1]Pérez-Albaladejo et al. (2016).

Full color scale: 

Sediment samples from the Brač Channel are depicted in green, indicating rather good quality in terms of absence of cytotoxic compounds, very low levels of CYP1A inducers, and intermediate/low levels of zfPxr agonists. In contrast, sediments from Kaštela Bay, particularly those from station S1B, show the highest content of CYP1A inducers and zfPxr agonists, analogous to the values recorded in Constanta harbour and the mouth of the Danube River.

In conclusion, the selected cell-based bioassays are suitable to benchmark the environmental quality of coastal sediments as well as to identify those areas that require further action to improve their environmental quality. The detection of ROS species, zfPxr and Ahr agonists, as well as estrogen receptor ligands in sediment extracts, can be further complemented with other endpoints (e.g. genotoxicity) and hopefully lead to a significant reduction of the costs and the number of bioindicator organisms to be used in environmental monitoring studies.

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CAPÍTULO 3

Uso de sistemas *in vitro* para la evaluación toxicológica de sedimentos y disruptores lipídicos

Artículo 5

'Lipidomic analysis of PLHC-1 cells: the effects of culture medium, Retinoic Acid, and Rosiglitazone'

Maria Blanco and Cinta Porte

Environmental Chemistry Department, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

In preparation

In preparation

‘Lipidomic analysis of PLHC-1 cells: the effects of culture medium, Retinoic Acid, and Rosiglitazone’

Maria Blanco and Cinta Porte

Environmental Chemistry Department., IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

ABSTRACT

Ultrahigh resolution mass spectrometry was applied to characterize changes in PLHC-1 cell lipidome after 24 h incubation in different culture mediums, namely (a) complete growth medium (SMEM) supplemented with 0, 1, 5% FBS, and (b) Dulbecco's phosphate buffered saline with 0.2% glucose (DPBS-GLU). Moreover, PLHC-1 cells were exposed to 9-cis-retinoic acid (9-*cis*-RA) and rosiglitazone (ROSI), RXR and PPARγ agonists, respectively; in SMEM-5%FBS control to asses changes in cell lipidome. Flow injection analysis coupled to high resolution mass spectrometry allowed the analysis of more than 200 individual lipid species. The lipid profile of PLHC-1 cells cultured in SMEM-5%FBS was enriched in phosphatidylcholines (PC) (41%), followed by phosphatidylethanolamines (PE) (20%), PE-plasmalogens (PE-P) (13%), phosphatidylinositol (PI) (13%), and triacylglycerols (TG) (5%). Multivariate analysis Heatmaps and univariate analysis Volcano plots, were set up to target the most significant lipids affected in each treatment. When FBS was absent or restricted to 1%, cell growth was reduced up to 20% and a quantitative depletion of all lipid metabolites was observed, being TG and PI the lipids that greater were reduced. In contrast, although cells grown in DPBS-GLU showed a reduction in their viability up to 50% and a semi quantitative decrease of all membrane lipids, a relative increase in TGs (up to 32% of total lipids) was observed. Specifically, cells cultured in DPBS-GLU were able to synthesize FA (18:2, 20:0, 22:0) from glucose and to store them in specific TGs (46:1, 48:1, 50:1 and 52:1). Furthermore, cells treated with of 9-*cis*-RA suffered a decreased in the relative abundance of

TG, suggesting the existence of RXR receptor; whereas cells exposed to ROSI did not alter the lipid profile, suggesting the absence of PPAR receptor in PLHC-1 cells. Key words: *Rutilus rutilus*, Ebro River, organochlorine compounds, biomarkers, bile.

Key words: *PLHC-1, cells, lipids, viability, Orbitrap, mass spectrometry, culture mediums, FBS, glucose, ROSI, 9-cis-Retinoic Acid.*

1. Introduction

Lipids are essential metabolites that participate in a wide range of biological processes as compartmentalization, energy production, energy storage, cell-signalling processes, protein trafficking and membrane organizing tasks (Van Meer, 2005; Wenk, 2005; Oresic et al., 2008). Alterations in lipid metabolism may reveal variations in several enzyme levels, activities and/or gene expression patterns, and consequently induce disorders and diseases associated to obesity, diabetes, cardiovascular dysfunctions, inflammation, cancer and neurological affections as Alzheimer (Li et al., 2006; Bergheanu et al., 2008; Cífková et al., 2015). Ecologic factors including temperature, nutrition, maturation stage and reproduction may alter lipid homeostasis in organisms. Some biocides (organotin), plasticizers (BPA and BPA analogues, phthalates), detergents (alkylphenols), organophosphates, pharmaceuticals (fibrates, naproxen, diclofenac) and pesticides/herbicides (DDT, atrazine) are reported to induce triacylglycerol accumulation, adipogenesis, and modify body weight in mammals, amphibians, teleost fish, molluscs, and arthropods (Capitão et al., 2017).

Cell cultures provide the best experimental system for studying responses at the molecular and cellular level. Since responses in biota initially take place at a cellular level, cells are valuable tools for an early

and sensitive detection of lipid alterations and may be used as models in lipid metabolism studies. Several studies focused on the analysis of lipids have been carried out in cells (Cajka and Fiehn, 2014). Culture medium is one of the most important single factors in cell viability (Lindl and Gdtraunthaler, 2008). The role of culture components in either promoting or masking the effects of particular agents on cell cultures can be delineated. Serum provides hormone factors for cell growth and proliferation; promoting cell differentiation; supplying transport proteins, essential nutrients, trace elements, minerals, lipids, adherence and extension factors; and stabilizing and detoxifying factors needed for maintaining a favourable growth atmosphere. One of the most usual complements used in cell culture is fetal bovine serum (FBS), an ubiquitously essential supplement used in the culture of most types of human and animal cells, required for attachment, growth and proliferation of cells (Brunner et al., 2010). However, due to the large quantity of serum derived from animals (ranged from a few hundred litters to several thousand litters), the increase of FBS demands, high price, variability of FBS between batches and risk of contaminations, the need to reduce or replace the use of FBS is claimed (Brindley et al., 2012). Because of the sensitivity of cells to their microenvironment, any change in culture conditions can affect cellular physiology, thereby altering physical and biological characteristics, such as cell lipidome.

Receptor activated by peroxisome proliferator factor (PPAR) and the retinoid X receptor (RXR) are nuclear receptors critically involved in the control of lipid metabolism (Feige et al., 2006). PPAR consists in three isoforms (PPARα, PPARβ, PPARγ) (Liss and Finck, 2017). Among them, PPARγ, mainly expressed in adipose tissue, plays an essential role in the regulation of adipocyte differentiation, adipogenesis, and lipid metabolism

(Lefterova et al., 2009). Thiazolidinediones such as rosiglitazone (ROSI) targets and activates PPARγ. Although the FDA (Food and Drug Administration) has restricted the use of ROSI because an increase in the risk of cardiovascular events, this drug has been widely used for the treatment of type 2 diabetes and nonalcoholic fatty liver disease (NAFLD). On the other hand, RXR receptors have been among the most studied nuclear receptor subfamilies, until the identification of their first endogenous ligand, the vitamin A derivative, 9-*cis*-retinoic acid (Mangelsdorf and Evans, 1995). Retinoic acid (RA) is an essential metabolite which controls physiological processes including development, nervous system function, immune response, cell proliferation and differentiation and reproduction (Kane et al., 2012). Its role in the control of lipid and energy metabolism and the potential implications for chronic disorders, including obesity, diabetes, nonalcoholic fatty liver disease and atherosclerosis, has been recognized (Bonet et al., 2003). The activity of 9-*cis*-RA is mediated mainly by activating RXR and retinoic acid receptors (RAR) (Boglino et al., 2017).

In this context, the aim of this study was to characterize changes in the lipidome of PLHC-1 cells cultured 24 h in different culture mediums namely a) Complete Eagle's Minimum Essential Medium (CGM) with 5% FBS (5%FBS) as control, CGM with 1% FBS (1%FBS) or with no FBS (0%FBS), and Dulbecco's phosphate buffered saline with 0.2% glucose (DPBS-GLU); and b) evaluating the effects of ROSI and 9-*cis*-RA in PLHC-1 cells lipidome by using flow injection analysis coupled to a high resolution mass spectrometer (FIA-MS/HR). PLHC-1, derived from topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma, is an established fish cell line used to evaluate the induction of enzymes involved in metabolism of xenobiotics (CYP1A), lipid peroxidation and

alterations in membrane properties (Choi and Oris, 2000; Traven et al., 2008; Fernandes et al., 2014). The presence of PPARs in PLHC-1 was reported, although no further studies confirmed the results (Caminada and Fent, 2008). PLHC-1 was selected in this study as an *in vitro* model to assess the interference of different cell culture mediums and nuclear receptor agonists on cell lipidome.

2. Materials and methods

2.1. Chemicals

Eagle's Minimum Essential Medium (MEM), Opti-MEM, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), Trypan Blue, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin and trypsin-EDTA, were purchased from Gibco BRL Life Technologies (Paisley, Scotland, UK). Butylated hydroxytoluene (BHT), Lipid Mixture 1000X, Trypan Blue, Rosiglitazone (ROSI) and 9-*cis*-Retinoic Acid (9-*cis*-RA) were purchased from Sigma-Aldrich (Steinheim, Germany). Lipid standards were obtained in Avanti Polar Lipids (Alabaster, AL, USA). All solvents were from Merch (Darmstadt, Germany).

2.2. Cell culture and lipid experimental conditions

The PLHC-1 cell line (ATCC; CRL-2406), derived from topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma, was routinely cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin G and 50 μg/mL streptomycin (CGM) with 5% FBS, and incubated at 30ºC with 5% of CO_2.

CGM with 5% of FBS was considered as control growth medium conditions in the experiments (5%FBS). When 90% of confluence was reached, cells were dissociated with 0.05% (w/v) trypsin. Experiments were carried out on confluent cell monolayers from passages 4 to 20.

PLHC-1 cells cultured in different mediums

Cells were seeded at a density of 60 x 10^4 cells in 500 µL of CGM with 5% FBS per well in 24-well plates (Nunc; Roskilde, Denmark), and then transferred for 24 h to (a) CGM with 5% FBS (5%FBS), with 1% FBS (1%FBS) or with no FBS (0%FBS), and (b) DPBS supplemented with 0.2% of glucose (DPBS-GLU).

PLHC-1 cells exposed to ROSI and 9-cis RA

Additionally, in a second experiment, cells were seeded in 24-well plates at a density of 60 x 10^4 cells in 500 µL of CGM with 5% FBS per well, and allowed them to attach overnight. Cells were exposed to ROSI and 9-*cis*-RA (10 µM), using DMSO as solvent control (0.5% v/v), in CGM with 5% FBS for 24 h. All treatments were assayed in triplicate.

2.3. Cell growth and number of cells

Cell viability was evaluated using two fluorescent dyes, Alamar Blue (AB; Biosource International, Invitrogen, Spain) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM; Molecular Probes, Invitrogen, Spain), which monitor metabolic activity and membrane integrity, respectively (Dayeh et al., 2003).

PLHC-1 cells cultured in different culture mediums

PLHC-1 cells were seeded in 96-well plates (Nunc; Roskilde, Denmark) at a density of 7.5 x 10^4 cells in 200 μL of CGM with 5% FBS per well. After 24 h of attach, medium was replaced by 100 μL of different culture mediums (5%FBS, 1%FBS, 0%FBS and DBPS-GLU).

PLHC-1 cells exposed to ROSI and 9-cis RA

Cells were seeded in a 96-well plate at a rate of 7.5 x 10^4 cells in 200 μL of CGM with 5% FBS per well, and allowed them to attach overnight. Medium was replaced and cells were exposed to 10 μM of 9-*cis*-RA or ROSI (final DMSO concentration was of 0.5% v/v).

For both experiments, cells were rinsed with PBS after 24 h exposure, and incubated for 1 h with the dye solution (5% v/v AB and 4 mM CFDA-AM). Results were obtained as relative fluorescent units (RFUs) at the excitation/emission wavelengths pairs of 530/590 nm for AB, and 485/530 nm for CFDA-AM in a fluorescent plate reader (Varioskan, Thermo Electron Corporation) and cell viability was expressed as percentage of control cells (CGM with 5% FBS in experiment of culture mediums and DMSO in exposure experiment).

To count the number of cells, after incubation in the different culture mediums, cells were washed with PBS, trypsinized and resuspended in 300 μL of CGM-5% FBS and transferred to an eppendorf where they were diluted in Trypan Blue. The counting chamber was filled up, viable cells were counted and the total number of cells calculated. Experiments were carried out in triplicate.

2.4. Lipid analysis

After exposure, the medium was removed and cells were trypsinised, resuspended in PBS, centrifuged at 10000 *g* for 15 min and the pellet collected. Lipids were extracted with a solution of methanol:chloroform (1:2, v/v) containing 0.01% of BHT (twice), vortexed for 1 min, and after 1 h incubation at room temperature, extracted in an ultrasonic bath for 2 min and centrifuged at 10,000 x g for 15 min (x2). The supernant was evaporated to dryness under a nitrogen stream and stored at -80ºC in an argon atmosphere. Prior analysis, an aliquot of internal standar mixture containing equal amounts (~200 pmol) of different lipids covering each lipid family namely, 16:0 D31-18:1 PC; 16:0 D31-18:1 PE; 16:0 D31-18:1 PS; 16:0 D31-18:1 PI, 16:0 D31-18:1 PG; 1,2,3-17:0 TG, 1,3-17:0 D5 DG; 17:0 MG and 17:0 CE (Avanti Polar Lipids, Inc. Alabama, USA) was added.

The analysis of lipids was performed as described in Vichi et al. (2012) with some modifications. FIA of 5 µL reconstituted lipid extracts were analysed in an Orbitrap-Exactive (Thermo Fisher Scientific) equipped with an electrospray source (H- ESI II), a Surveyor MS Plus pump and an Accela Open AS autosampler kept at 10ºC (Thermo Fisher Scientific, San Jose, California). The mobile phase was methanol:dichlorometane 80:20 at 50 µL/min. Mass spectra were acquired in full scan both in ESI+ and ESI- ionization mode produced by a spray voltage of 3 kV, capillary voltage 37 V, tube lens 150V and skimmer voltage 40V. The sheath gas flow rate was set at 35 a.u. (arbitrary units), and the aux gas flow rate was 5 a.u. Capillary and heater temperature were set to 400°C and 30ºC. The acquisition mass range was set to *m/z* 200-2000 and the total analysis time was 2 min.

Ultrahigh resolving power defined as R: 100,000 (m/z 200, FWHM) was set.

The mass peaks considered were single positive charged sodium molecular ions, [M+Na^+] (PC, LPC, PC-P, SM, TG, DG) and single negative charged [M-H^+] (PE, LPE, PE-P, PG, PS, PI, FA). MG and CE were not able to be identified. Data from LIPID MAPS®, exact mass, isotopic distributions, charge, adducts formed, number of ring plus double bond (RDB = 0.5-15) and elements in formula, C≤ 100, H≤ 150, O≤ 8, Na≤ 1, P≤ 1, N≤ 1, were used for the identification of the lipid molecules with a maximum permitted mass error fixed at < 5 ppm. Mass spectra were processed with Xcalibur (v2.1, Thermo Fisher Scientific, Bremen, Germany) and the lipid species were quantified with internal standards.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences among samples respect to the control (5%FBS). All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and *p* values lower than 0.05 were considered statistically significant. In all instances, transformations of the data were performed when the assumption of normality of residuals was not met.

Based on the screened molecules, lipid profile was analyzed by using Metaboanalyst 3.0 Software (McGill University). Principal Component Analysis (PCA) was used as model to compare the lipid profile of cells cultured in different mediums to control (5%FBS) and cells treated with ROSI and 9-*cis*-RA to control (0.5% DMSO). Prior to PCA analysis, data

were scaled by autoscaling (scaled by mean-centering and dividing by standard deviation of each variable).

Volcano plot, via univariate statistical analysis, were further created to determine alterations in the relative abundance of lipids. Volcano plot sorts the lipids along two dimensions, the biological, represented by the log fold change (FC), and the statistical, represented by the negative logarithm of the *p* value (0.05). Red circles represent features above the threshold. The further its position away from the (0,0), the more significant the feature is. Further information about lipids affected after different treatments in PLHC-1 was obtained using Heatmaps via multivariate statistical analysis. Heatmaps allow visualizing changing patterns in lipid concentrations across experimental conditions using fully color gradients. Prior to Volcano plot and Heatmaps analysis, data were normalized by sum and scaled by mean-centering and dividing by standard deviation of each variable.

3. Results

3.1. Viability and number of cells

The viability of PLHC-1 cells for 24 h cultured with reduced percentage of FBS (1 and 0%) and DPBS-GLU is shown in Figure 34A. Viability significantly decreased up to 70% in those cells cultured with DPBS-GLU, in comparison to control cells (5%FBS), while cells cultured in 1 and 0%FBS restriction mediums showed a decrease of 10 and 25%, respectively. The number of cells was significant lower in DPBS-GLU cells (5 times) (Fig. 34B). Also, the number of cells after 24 h cells cultured in 1 and 0%FBS, decreased 1.2 and 1.4 times, respectively, in comparison to those cultured in 5%FBS.

No significant toxicity was detected in PLHC-1 cells after 24 h of exposure to 10 µM of 9-*cis*-RA and 10 µM ROSI.

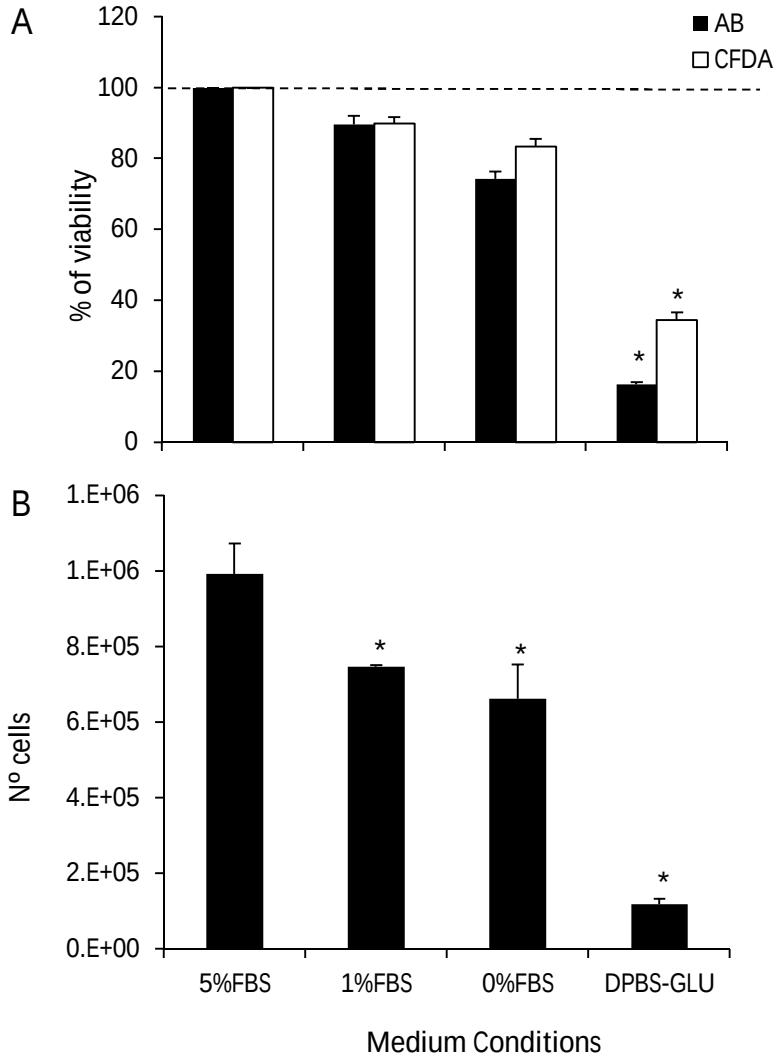


Figure 34. Cell viability (A) and number of cells (B) of PLHC-1 after 24 h growing in different culture mediums (5%FBS, 1%FBS, 0%FBS and DBPS-GLU). Values are mean ± SEM of 3 independent assays. *Statistically significant differences relative to 5%FBS control cells (dotted line) ($p < 0.05$).

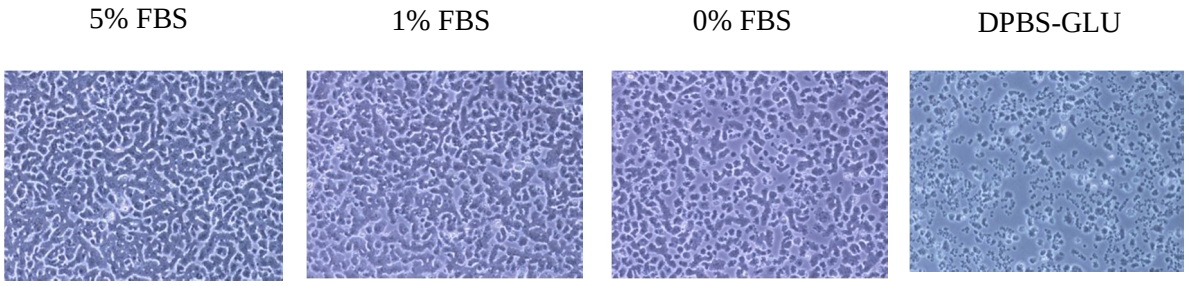


Figure 35. Microscopic images of PLHC-1 cells cultured for 24 h in 5, 1 and 0%FBS and DBPS-GLU mediums.

3.2. Lipid detection

The analysis of PLHC-1 cells by FIA-MS/HR Orbitrap equipped with electrospray source ionization (ESI), in positive and negative modes, allowed the detection of 212 lipid species belonging to different lipid classes and subclasses, including 20 phosphatidylcholines (PC), 21 plasmalogen-phosphatidylcholines (PC-P), 3 lyso-phosphatidylcholines (LPC), 21 diacylglicerols (DG), 43 triacylglicerols (TG) and 9 sphingomielins (SM) under positive ESI; and 20 phosphatidylethanolamines (PE), 19 plasmalogen-phosphatidylethanolamines (PE-P), 3 lyso-phosphatidylethanolamines (LPE), 12 phosphatidylserines (PS), 4 phosphatidylglicerols, (PG), 19 phosphatidylinositols (PI), and 18 fatty acids (FA) under negative ESI (Table 17).

The use of internal standard allowed the semi-quantification of all lipid molecules (pmol), with the exception of FA, as no internal standard was added. To calculate the pmol of SM and PC-P, the PC standard was used as its standard was not available. The lipidome of PLHC-1 was enriched in PC 32:1, 34:1/2, 36:1/2, 38:4; PC-P 32:1, 34:1; PE 34:1, 36:1/2,

38:4; PE-P 34:2, 36:4, 38:5/6/7; PS 36:1, 40:4/5/6; PI 38:3/4; SM 24:4; TG 50:2, 52:2/3, 54:3 and DG 34:1, 38:3/6 (Table 17). The main lipid subclass detected in PLHC-1 cells were PC (41%) followed by PE (20%), PE-P and PI (13%) and TG (5%) (Table 16; Fig. 36A,B), whereas the most abundant FA identified were 16:0, 18:0, 18:1 and 18:2 (Fig. 37A,B).

Table 16. Relative abundance of lipid subclasses (percentage of pmol ± SEM) and PC/PE, TG/DG, TG/GP lipid ratios after 24 h of PLHC-1 cells cultured in different medium (5, 1 and 0%FBS and DBPS-GLU) and in cells exposed to 10 µM of 9-*cis*-RA and ROSI in CGM-5%FBS.

Lipid Subclass	Eagle's Minimum Essential Medium (CGM)			DPBS-GLU	Eagle's Minimum Essential Medium (CGM-5%FBS)		
	Control 5% FBS	1%FBS	0% FBS	0.2% glucose	Control 0.5% DMSO	9-*cis*-RA (10 µM)	ROSI (10 µM)
PC	41±1	43±0.5	44±1	39±1	44±2	45±1	42±0.5
LPC	0.1±0.01	0.1±0.02	0.02±0.01	0.02±0.01*	0.2±0.01	0.1±0.02	0.1±0.03
PC-P	2±0.2	3±0.1	3±0.2	3±0.2	3±0.3	3±0.4	2±0.1
PE	20±0.4	22±0.4	22±1	16±1*	20±0.5	22±2	24±1*
LPE	1±0.1	1±0.2	0.3±0.1*	1±0.2	1±0.2	1±0.1	2±0.1
PE-P	13±1	14±0.3	14±1	11±1	13±1	13±2	13±1
PS	2±0.1	3±0.1	3±0.1	2±0.1	2±0.1	2±0.3	2±0.1
PG	0.2±0.0.2	0.2±0.01	0.2±0.01	0.3±0.02	0.2±0.02	0.3±0.01	0.2±0.01
PI	13±0.3	10±0.2*	9±0.3*	8±0.2**	10±2	12±1	9±0.5
SM	1±0.2	2±0.1	2±0.1	1±0.1	2±0.2	2±0.3	2±0.3
TG	5±1	2±0.3*	2±0.3*	17±1*	4±0.3	1±0.2*	4±1
DG	1±0.1	1±0.04	1±0.1	1±0.2	1±0.1	1±0.05	1±0.1
Ratios							
PC/PE	2.1±0.1	2.0±0.04	2.0±0.1	2.4±0.1*	2.2±0.03	2.1±0.1	1.7±0.03**
TG/DG	6.6±0.6	2.0±0.3*	2.7±0.3*	17±2.3*	5.1±0.3	1.1±0.1**	5.0±0.6
TG/GP	0.05±0.003	0.02±0.003*	0.02±0.00	0.21±0.01*	0.05±0.001	0.01±0.001**	0.04±0.004

*Statistically differences to CGM-5%FBS.

**Statistically differences to DMSO.

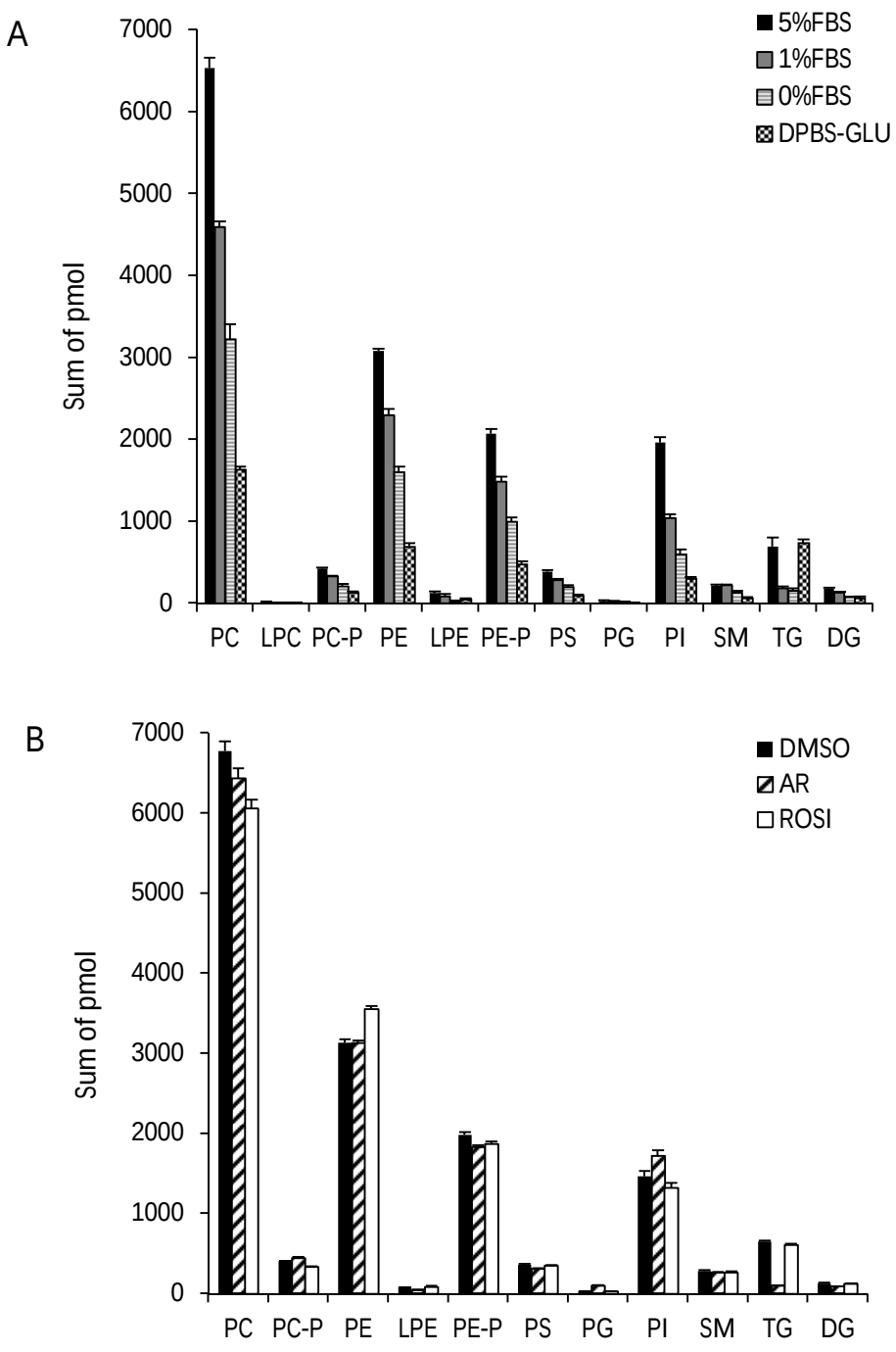
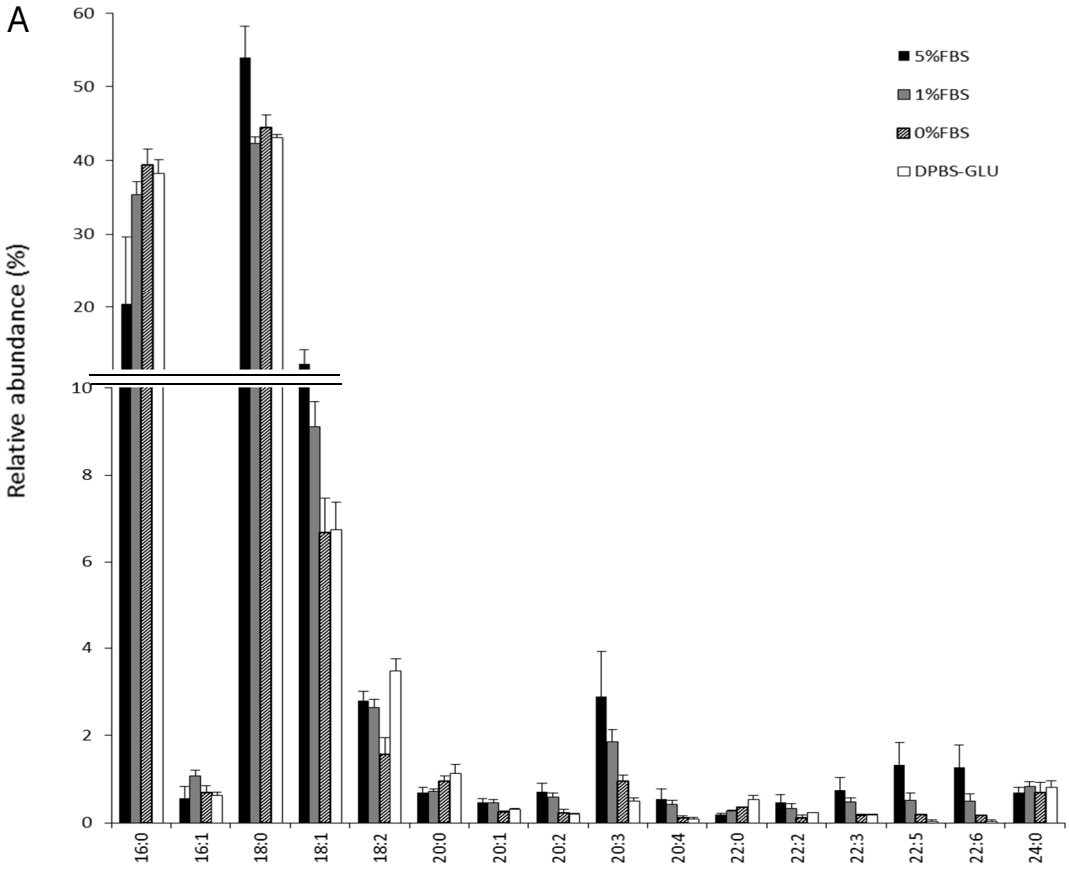


Figure 36. Total pmol of lipid families in PLHC-1 cells cultured in different medium conditions for 24h in different mediums (5%FBS, 1%FBS, 0%FBS, DPBS-GLU) (A) and in PLHC-1 cells exposed to 10 µM of 9-*cis*-RA, ROSI and 0.5% DMSO (negative control) (B).


A
Relative abundance (%)
5%FBS
1%FBS
0%FBS
DPBS-GLU
16:0
16:1
18:0
18:1
18:2
20:0
20:1
20:2
20:3
20:4
22:0
22:2
22:3
22:5
22:6
24:0

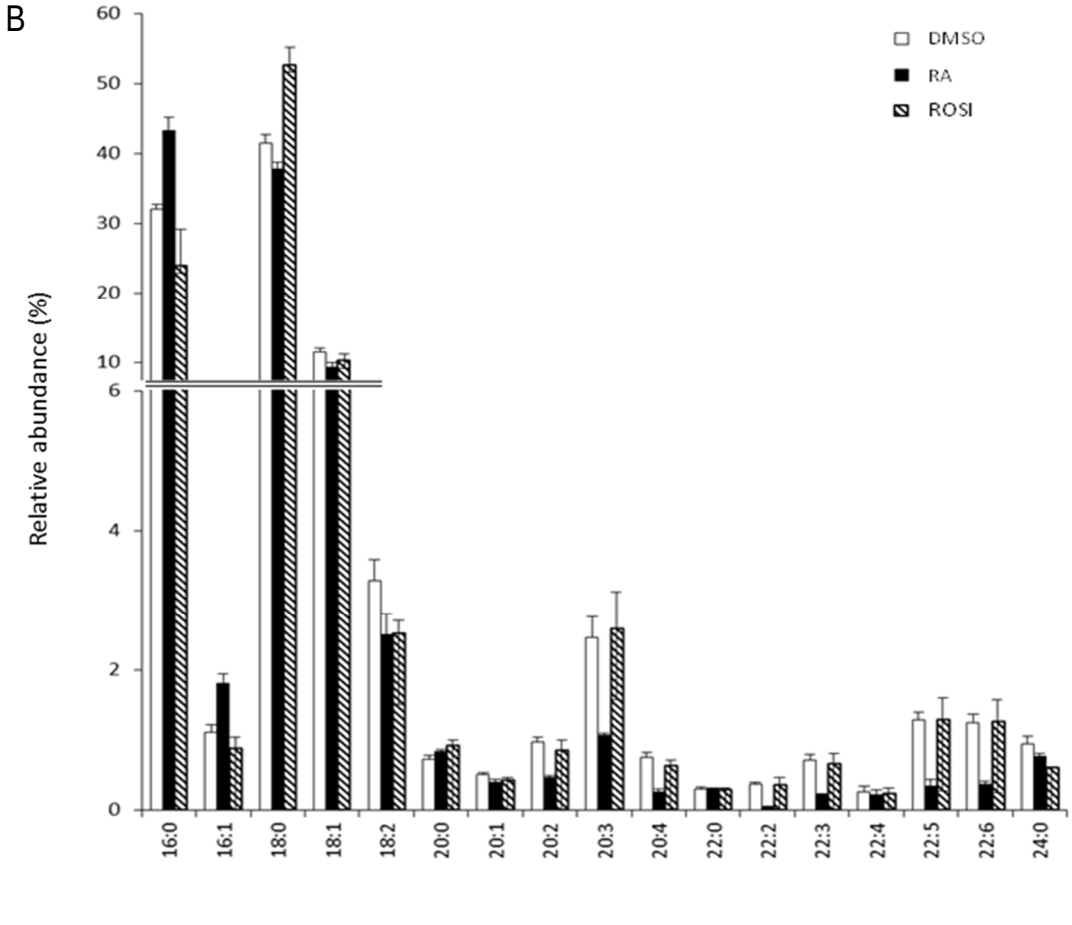

B
Relative abundance (%)
DMSO
RA
ROSI
16:0
16:1
18:0
18:1
18:2
20:0
20:1
20:2
20:3
20:4
22:0
22:2
22:3
22:4
22:5
22:6
24:0

Figure 37. Relative abundance of FA lipids detected in PLHC-1 cells under different medium conditions (5%FBS, 1%FBS, 0%FBS, DPBS-GLU) (A) and in PLHC-1 cells exposed to 10 µM of 9-*cis*-RA and ROSI (B). *Statistically significant differences relative to 5%FBS cells ($p < 0.05$).

PLHC-1 cells cultured in different mediums

PCA scores plot allowed the separation of the lipidome of cells cultured in different mediums (Fig. 38). These differences were explained by two non-dependent principal components that assumed the 90% of the variable variance. Principal component 1 (PC1) (75%) described the variance between 5%FBS, from DPBS-GLU and 1%FBS, whereas PC2 (14.7%) separated 5%FBS from 1%FBS and 0%FBS.

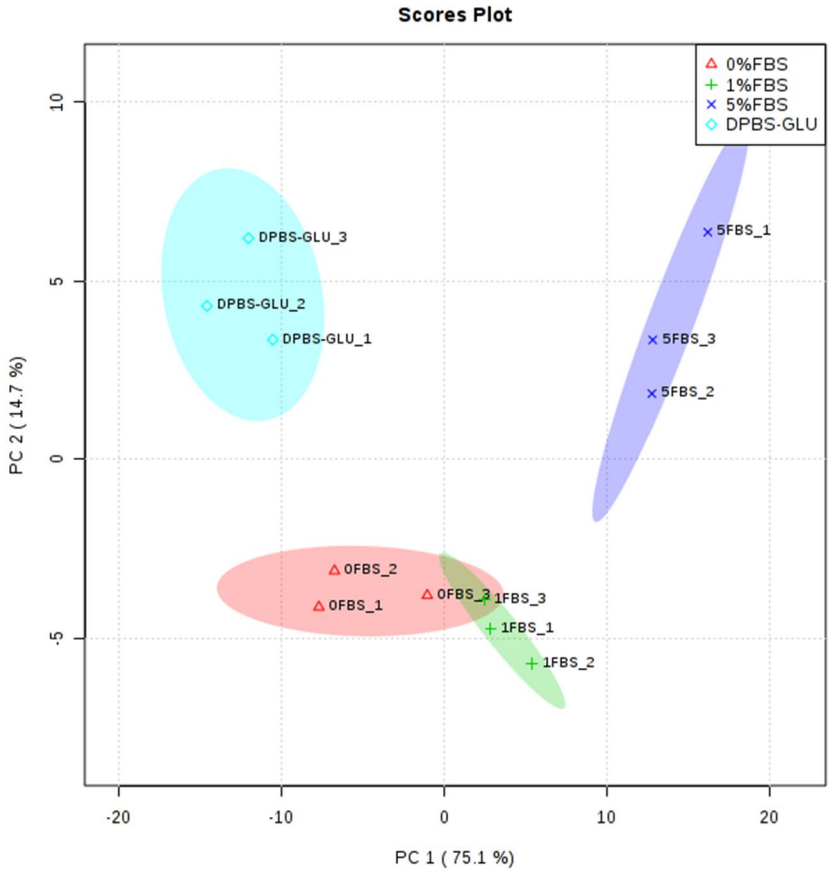
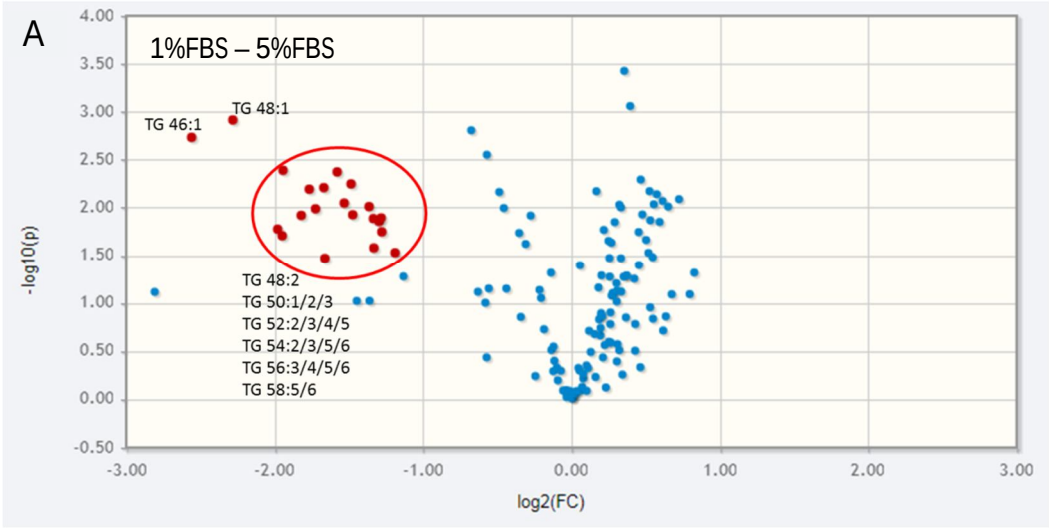
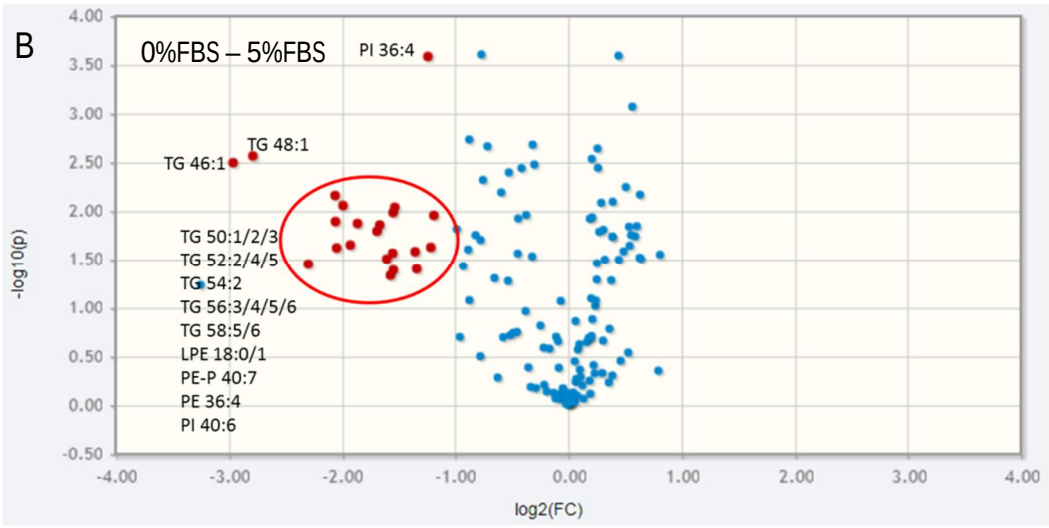


Figure 38. PCA analysis of PLHC-1 cells cultured in 5%, 1% and 0%FBS and DBPS-GLU.

The semi-quantitative analysis performed in cells cultured in reduced FBS conditions (1 and 0%FBS) shown a general decrease of all the lipids (Table 16; Fig. 36A). However, the relative distribution pointed out significant differences among cultured mediums. Volcano plot showed a relative reduction of TGs in these cells (Fig. 39A). Regarding cells cultured in without FBS (0%FBS), Volcano plot also showed a significant reduction of TG, PI (36:4, 40:6) and lipids formed by ethanolamines (LPE 18:0/1; PE-P 40:7; PE 36:4) (Fig. 39B). Cells growing in DPBS-GLU suffered a significant dismiss of all the lipids, except TG, which increase a 3.4% (Table 16; Fig. 36A). Volcano plot show that TG (specifically 46:1, 48:1, 50:1 and 52:1) was the main lipid subclass that relatively increased in cells cultured in DPBS-GLU medium (Fig. 39C). The multivariant analysis performed by Heatmaps showed further and detailed information about the main 50 lipids that suffered alterations in the different cell treatments (Fig. 42).

Regarding FA, a relative increase in the abundance of FA 16:0/1 together with a significant decrease of 18:0 was observed in cells cultured in 1%FBS, whereas in cells cultured in 0%FBS an increase of 16:0 together with a decrease of 18:0, 18:1, 18:2 and all 20- and 22-carbon FA, except those saturated (20:0, 22:0, 24:0) which their relative abundance did not change (Fig. 37A). An increase in the relative abundance of FA 16:0, 18:2, 20:0, 22:0 together with a relative decrease of 18:0, 18:1 and 20- and 22-carbon FA detected, except those saturated, were observed in PLHC-1 cells cultured in DPBS-GLU medium (Fig. 37A).


A
1%FBS – 5%FBS
TG 46:1
TG 48:1
TG 48:2
TG 50:1/2/3
TG 52:2/3/4/5
TG 54:2/3/5/6
TG 56:3/4/5/6
TG 58:5/6
-log10(p)
log2(FC)


B
0%FBS – 5%FBS
PI 36:4
TG 46:1
TG 48:1
TG 50:1/2/3
TG 52:2/4/5
TG 54:2
TG 56:3/4/5/6
TG 58:5/6
LPE 18:0/1
PE-P 40:7
PE 36:4
PI 40:6
-log10(p)
log2(FC)

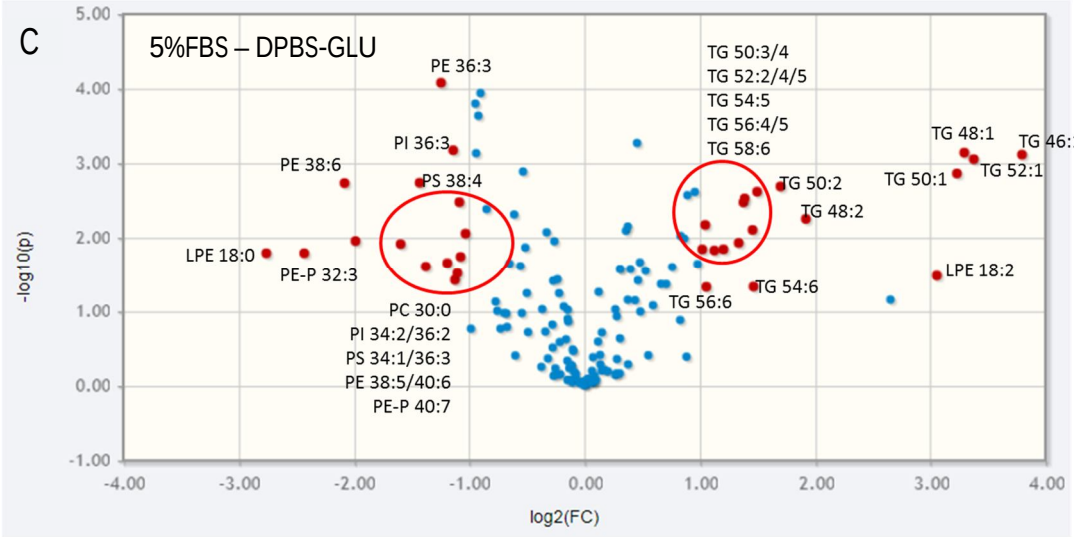

C
5%FBS – DPBS-GLU
PE 36:3
PI 36:3
PE 38:6
PS 38:4
LPE 18:0
PE-P 32:3
PC 30:0
PI 34:2/36:2
PS 34:1/36:3
PE 38:5/40:6
PE-P 40:7
TG 50:3/4
TG 52:2/4/5
TG 54:5
TG 56:4/5
TG 58:6
TG 56:6
TG 54:6
TG 50:2
TG 48:2
TG 48:1
TG 46:1
TG 50:1
TG 52:1
LPE 18:2
-log10(p)
log2(FC)

Figure 39. Volcano plots (fold change > 2; *p* value < 0.05) representing lipid profiles of PLHC-1 cells cultured in 1%FBS (A), 0%FBS (B), DPBS-GLU (C), compared to 5%FBS control.

PLHC-1 cells exposed to ROSI and 9-cis RA

PCA analysis was also applied to determine changes in the lipidome of PLHC-1 after exposure to ROSI and 9-*cis*-RA. According to results, control cells were clearly differentiated from cells treated with 9-*cis*-RA by two non-dependent principal components that assumed the 86.6% of the explained variance (Fig. 40A) and from cells exposed to ROSI with the 65.5% of the total explained variance (Fig. 40B).

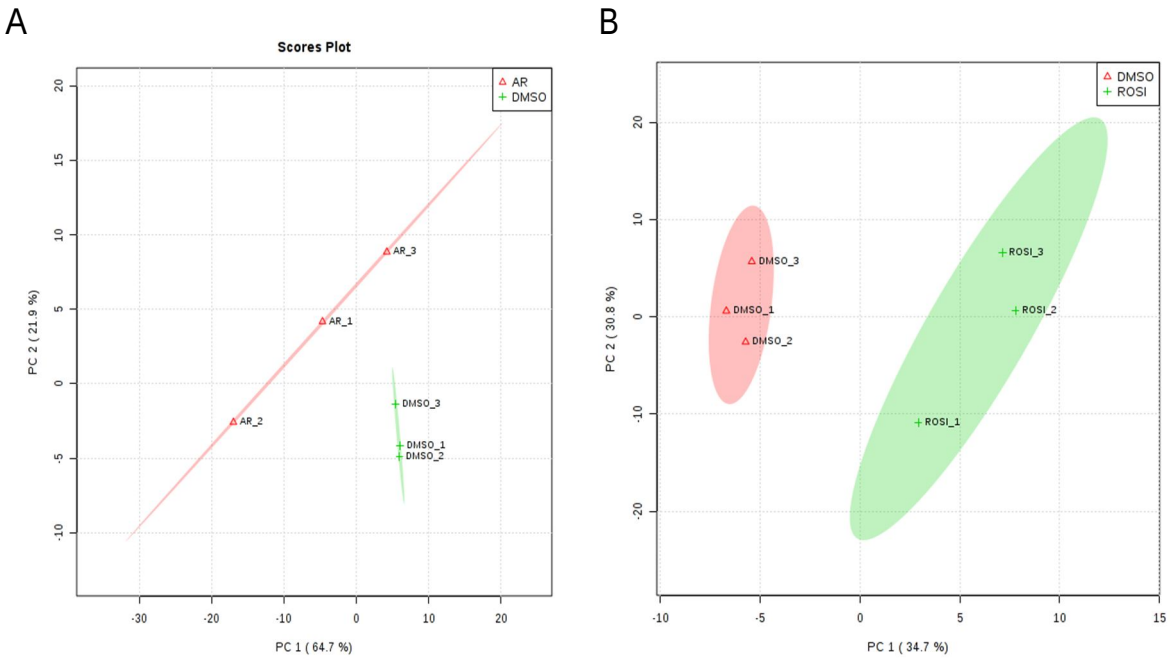


Figure 40. PCA analysis of PLHC-1 cells exposed to 10µM of 9-*cis*-RA (A), and 10µM of ROSI (B) respect to DMSO.

The semi-quantitative analysis performed in cells exposed to 10 µM of 9-*cis*-RA determined a significant decrease of TG, together with an increase of PI (Table 16; Fig. 36B). The relative distribution analyzed by Volcano plot shown a significant decrease of all the TGs in cells exposed to

RA (Fig. 41A). On the other hand, PLHC-1 cells exposed to ROSI quantitatively suffered a decrease of PC, whereas PE increased (Table 16; Fig. 36B). However, relatively, no significant differences were observed (Fig. 41B). The multivariant analysis performed by Heatmaps cluster showed further and detailed information about the main 50 lipids that suffered alterations in the different cell treatments (Fig. 43).

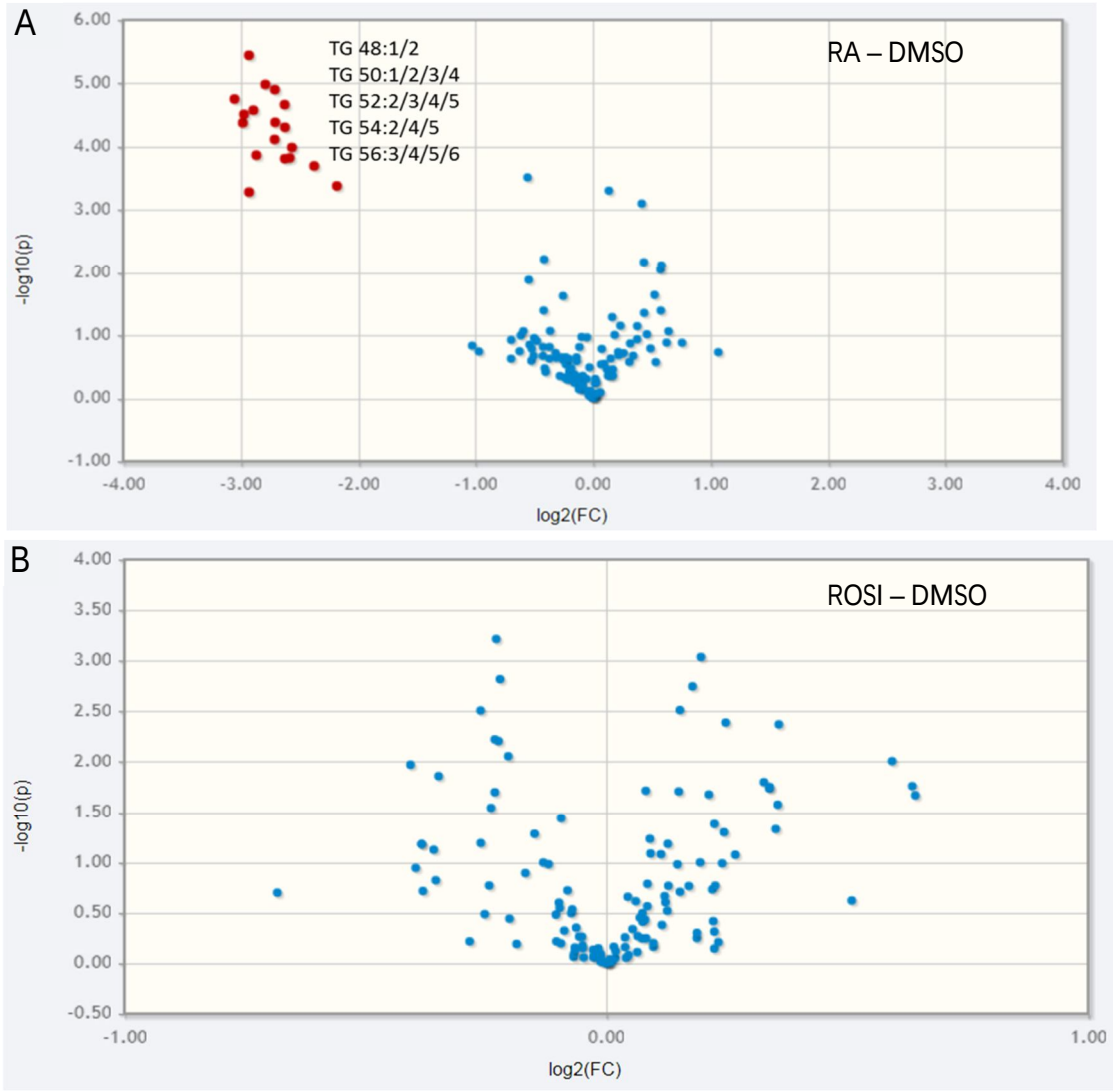


Figure 41. Volcano plots (fold change > 2; *p* value < 0.05) representing lipid profiles of PLHC-1 cells cultured in 9-*cis*-RA (A), ROSI (B), compared to 5%FBS control.

Regarding FA, cells exposed to 9-*cis*-RA showed a relative increase in the abundance of 16:0/1, and a concomitant decrease in FA 18:2, 20:2/3/4, 22:2/:3/5/6. A

significant decrease in relative abundance of FA 18:2 and 24:0 was found in cells treated with 10 µM of ROSI together with an increase in FA 18:0 and 20:0 (Fig. 37B).

4. Discussion

In this study, a comprehensive and a comparative assessment of lipid profile of PLHC-1 cells a) cultured with different mediums and b) exposed to agonists of PPARγ and RXR lipid receptors, was performed. Recent advances in metabolomics technologies, including the development of mass spectrometry systems, have made possible to analyze a wide range of lipids. The analysis by FIA coupled to an Orbitrap analyzer allowed the detection of more than 200 individual lipids in PLHC-1 cells. Lipid profile of cells cultured under optimal conditions of 5%FBS was mainly enriched in PC followed by PE, PI, PE-P and TG. PC subclass is the most abundant phospholipid in eukaryotic cells, comprising 45-55% of the total lipids (Vance and Steenbergen, 2005; Bohdanowicz and Grinstein, 2013). PC and PE lipids are physiologically important as the principal components of eukaryotic cellular membranes, although their distribution in the plasma membrane is asymmetric: the majority of PCs are localized to the outer leaflet, whereas PEs are enriched in the inner leaflet (Devaux, 1991). PI, restricted to the cell membrane inner leaflet, are involved in biological functions including induction of cell proliferation in thyroid cells, modulation of actin, cytoskeleton organisation in fibroblasts, reduction of the invasive potential of tumour cell lines and effects in inflammatory and immune responses (Corda et al., 2009). Plasmalogens constitute a special class of phospholipids characterized by the presence of a vinyl - ether bond at the sn-1 position membranes. PE- and PC-plasmalogens, like the corresponding diacylphospholipids PE and PC, follow an asymmetric distribution, being PE-plasmalogens predominantly found in the inner leaflet (Devaux, 1991). Although their physiological functions is still not

fully resolved, biological roles including mediators of membrane dynamics and intracellular transport, signal transduction, antioxidants and source of second messengers have been attributed (Brites et al., 2004).

FBS is considered an indispensable supplement in cell culture. The concentration of FBS in media ranges from approximately 2 to 20%, and 10% FBS is the most common used in experiments (Karnieli et al., 2017). PLHC-1 cells are usually cultured in medium supplemented with 5%FBS, considered the optimal growing conditions in these cells. Guidelines for good cell cultures practice (GCCP) recommends the use of serum free substitutes for current and new *in vitro* methods or replace FBS with synthetic alternatives (Jochems et al., 2002; Karnieli et al., 2017). Although any proposal has still been established as serum replacement, in the last 10 years serum alternatives including plant constituents, hormones, growth factors, lipids, anti-oxidants, vitamins, or human platelet lysates have been considered (van der Valk et al., 2010; Rauch et al., 2011). The reduction or absence of FBS in the culture media may affect the induction of enzymes involved in the metabolism of xenobiotics (CYP1A) in cells and the sensitivity of cells in cytotoxicity assays (Schirmer et al., 2004; Dayeh et al., 2003). Since FBS is formed by lipids including FA, TG, phospholipids and CE, their reduction on cell culture also may alter lipid metabolism (Brunner et al., 2010; Gregory et al., 2011). PLHC-1 cells cultured under conditions of FBS restriction (1 and 0%FBS) suffered a progressive decrease of all the lipid species detected. The reduction of total lipids could be linked to the decrease in the number of cells cultured in mediums with low (1%) or no FBS (up to 1.7-fold lower in 0%FBS), which entails a decrease in cell membranes and the lipids that formed them. Also a decrease in the absolute number of cell nuclei in bovine adipose-derived stem cells from subcutaneous tissue incubated for 14 days was observed in

absence of FBS (Sandhu et al., 2017). Heatmaps and Volcano plot of normalized lipids allowed visualizing relative changes in the lipid profile of cells cultured in FBS restricted mediums. Thus, a relative decrease of TG and some PI were observed in cells growing in both 1 and 0%FBS mediums. A relative decrease of some ethanolamines was additionally detected in cells cultured without FBS. These results evidence suggest that when reducing FBS concentrations, the main lipid subclass affected was TG, followed by PI and some ethanolamine-lipids. Several studies report TGs as being one of the most susceptible lipid subclass to external factors. Cells cultured in conditions of FBS restriction, may be under a stress, as the lack of serum affecting on cell attachment, growth and proliferation. Consequently, cells could redistribute energy and try to adapt to these conditions. TG, as the main lipid subclass involved in the energy storage, are the first to be mobilized in many fish species when environmental conditions change, being their metabolism and synthesis dynamically balanced depending on cellular energy requirements (Sargent et al., 1989; Dulloo et al., 2004; Van Dijk et al., 2005).

The composition of lipids accumulated in the liver could provide potential insights into the pathophysiology of cells and lipid ratios can serve as indexes for assessing cell's physiological status. A high TG/DG ratio have been reported in livers with NAFLD (nonalcoholic fatty liver disease) (Puri et al., 2007). The decrease of TG/DG in cells cultured under FBS restricted conditions was related to the high depletion of TGs in these cells. TG/GP ratio indicates the relative importance of the storage lipids (TG) and the structural lipids (GP) and tends to be a little higher for nutrient-limited cells (Kilham et al., 1997). According to that, this ratio was decreased in cells cultured under FBS restricted conditions.

PLHC-1 cells cultured in DPBS supplemented with 0.2% of glucose suffered a general depletion of all lipids, with the exception of TGs. The viability of PLHC-1 cells and the number of cells was significantly reduced under these conditions. Due to the lack of FBS but also of other biomolecules and salts included in traditional culture mediums. PE and PI were the lipids that showed a highest depletion. However, cells had the ability to form TG (46:1, 48:1, 50:1, 52:1), from glucose (0.2%). Although the pathways that regulate hepatic TG metabolism are not yet completely understood, it is known the lipids that are formed in the liver, come mostly from excess of glucose via lipogenesis that are led to the synthesis of FA then esterified to form TGs (Quiroga and Lehner, 2012). PC/PE ratio is a key regulator of cell membrane integrity and plays a role in the progression of steatosis into teatohepatitis (Li et al., 2017). Increasing PC/PE together with a 3-fold increase in the TG/DG ratio was observed in cells cultured in DPBS-GLU, related to the high decrease of membrane lipids and the accumulation of TG in these cells.

Regarding FA, many pathways can result in their production, being palmitic acid (FA 16:0) the first FA formed from malonyl and acetyl-CoA (Han et al., 2016). Desaturation and elongation of these FA chains result in different FA isomers. The FA considered the most physiologically relevant (16:0 (palmitic acid), 18:0 (estearic acid) and 18:1 (oleic acid)), together with 16:1 (palmitoleic acid), 18:2 (linoleic acid) and 20:0 (araquidonic acid) were the most abundant in PLHC-1 cells cultured in control conditions of 5%FBS. Specifically, 16:0, 18:0 and 18:1 were the most abundant FA in these cells (20, 53 and 12%, respectively). Some studies reflect that the main FA lipid source in the culture medium provides from FBS, including FA 16:0, 18:0/1/2, 20:3/4, 22:5/6 (Tocher et al., 1998; Brunner et al., 2010; Gregory et al., 2011). The absence of FBS in cell

culture promoted a decrease of FA 18:0/1/2, 20:2/3/4, 22:2/3/5/6, reducing elongation on unsaturation FA. However, the supplement of glucose in the medium stimulated the synthesis of FA 18:2, 20:0 and 22:0 and its subsequent storage in TG, specifically TG 46:1, 48:1, 50:1 and 52:1.

Liver plays a central role in the maintenance of systemic lipid homeostasis. Disturbances in lipogenesis, fat oxidation and/or lipoprotein metabolism in the liver can favor obesity, dyslipidemia and the accumulation of lipid droplets in the hepatocyte (hepatic steatosis). RA, the main bioactive metabolite of vitamin A, is stored and metabolized mainly in the liver (O'Byrne and Blaner, 2013). The mechanisms of action of 9-*cis*-RA are mediated by ligand-activated RAR and RXR as homodimer or heterodimer with other nuclear receptors (He et al., 2013). Specifically, 9-*cis*-RA has been demonstrated to exhibit high-affinity for RXR (Levin et al., 1992). Consequently, retinoid might modulate lipid metabolism. However, 9-*cis*-RA effects on hepatic lipid and lipoprotein metabolism are controversial (Bonet et al., 2012). Some studies reported an accumulation of TG in human preadipocyte cells (AML-I cells) and mice treated with 9-*cis*-RA (Vu-Dac et al. 1998; Sedova et al., 2004; Morikawa et al., 2013). Other studies specify that the increase in TG levels occurred in response to high doses of 9-*cis* RA in rodents (Sofonova, 1994; Reddonet et al., 2008). However, the most common effects reported after 9-*cis*-RA treatment is a reduction in lipid and TG content. 9-*cis*-RA (100 nM) suppressed lipid accumulation and inhibited adipogenesis in mouse preadipocyte 3T3-L1 cells (Sagara et al., 2013). Accordingly, Amengual et al. (2010) showed enhance lipid oxidation, inhibition of lipid biosynthesis, reduction of body fat and reduction of TG content in the liver of rodents. These findings were also in line with the increased accumulation of TG and hepatic lipids due to a deficiency of vitamin A (Kang et al., 2007). In our study, PLHC-1 cells

treated for 24 h with 10 µM of 9-*cis*-RA, highly and significantly decreased the content of TGs. Consequently, the TG/DG ratio was lower than in control conditions. The exposure to ROSI also promoted the synthesis of FA 16:0 and 16:1 (palmitic and palmitoleic FA, respectively) and the reduction in the elongation on unsaturation FA The results suggested that the response of PLHC-1 cells to the 9-*cis*-RA might occur through activation of RXR receptor involved in lipid metabolism.

ROSI is commonly clinically used to improve insulin sensitivity in the treatment of diabetes type II. ROSI treatment for 25 d led to a significant reduction in serum levels of free FAs and TGs in both wild type and Lcn2$^{-/-}$ mice (Jin et al., 2011). In adipose tissue, ROSI stimulates adipogenesis and the expression of genes which favor storage of TG, thus lowering circulating free FA concentrations in Lcn2$^{-/-}$ mice (Berger and Moller, 2002; Guo et al., 2013). However, ROSI did not affect liver weight, lipid accumulation and lipogenic gene expression in lactating C57B1/6J in liver of mice (Vyas et al., 2014). Neither hepatic cholesterol or TG contents were affected in 3T3-L1 after exposure to ROSI 1nM for 7 days (Wang et al., 2017). No lipid alterations in PLHC-1 cells exposed to 10 µM of ROSI were observed in our study. ROSI acts mostly by activating PPARγ, which is mainly expressed in the adipose tissue and is implicated in adipose tissue lipid metabolism (Liss and Finck, 2017). Since no alterations on lipid levels were observed in PLHC-1 cells, these results suggest that PPARγ is not expressed in these cells. Caminada and Fent (2008) reported in a conference abstract the presence of PPARγ in PLHC-1 cells but no further data has been published in scientific journals, suggesting that this information was not completely confirmed. Cells exposed to ROSI decreased hepatic PC/PE ratio, which is strongly associated with decreased rates of survival in mouse (Ling et al., 2012). In fact, ROSI induced

apoptosis of cultured macrophages (Chinetti et al., 1998). In PLHC-1 cells the decrease in PC/PE was related to the increase on PE after the exposure to 24h.

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Supporting Information

Table 17. Lipid molecules detected in PLHC-1 cells cultured for 24h in different mediums (5%FBS, 1%FBS, SFM, DPBS-GLU) and in PLHC-1 cells exposed to 10 µM of 9-*cis*-RA, ROSI and 0.5% DMSO (control). All identified compounds were in accordance to criteria of maximum permissible mass error of 5 ppm, with atom constraints and with -0.5 ≤ DBE ≤ 15.0. (DBE: double-bond equivalent in Orbitrap). Results are shown as pmol/well (mean ± SEM, n = 3).

Lipid family	C/db	5%FBS	1%FBS	0%FBS	DPBS-GLU	DMSO	RA	ROSI
	28:0	6 ± 0.2	2 ± 0.4	1 ± 0.3	0.5 ± 0.1	7 ± 0.1	15 ± 1	7 ± 1
	30:0	38 ± 1	18 ± 0.4	11 ± 1	4 ± 1	40 ± 2	55 ± 2	39 ± 1
	30:1	65 ± 1	49 ± 0.4	34 ± 2	9 ± 0.5	71 ± 3	101 ±	75 ± 2
	32:0	52 ± 10	32 ± 2	21 ± 2	10 ± 1	69 ± 3	48 ± 8	55 ± 1
	32:1	660 ± 9	445 ± 7	316 ± 14	141 ± 3	678 ± 26	858 ± 28	638 ± 16
	32:2	234 ± 5	191 ± 2	146 ± 7	54 ± 0.7	241 ± 9	218 ± 18	242 ± 8
	34:0	97 ± 30	70 ± 2	48 ± 4	24 ± 2	137 ± 9	119 ± 5	84 ± 2
	34:1	1555 ± 21	1020 ± 19	722 ± 36	377 ± 7	1629 ± 54	1676 ± 60	1463 ± 45
	34:2	747 ± 8	577 ± 11	414 ± 22	177 ± 2	782 ± 26	679 ± 40	723 ± 24
	34:3	136 ± 3	116 ± 1	92 ± 3	38 ± 1	141 ± 5	113 ± 8	137 ± 6
	34:4	13 ± 0.3	10 ± 1	6 ± 1	2 ± 1	14 ± 1	10 ± 1	14 ± 2
	36:0	86 ± 24	73 ± 3	50 ± 2	24 ± 1.1	125 ± 4	120 ± 5	79 ± 4
	36:1	604 ± 19	406 ± 5	276 ± 18	159 ± 4	634 ± 19	559 ± 20	528 ± 18
PC	36:2	644 ± 8	431 ± 8	284 ± 19	144 ± 3	665 ± 21	599 ± 15	571 ± 19
	36:3	438 ± 7	327 ± 6	233 ± 11	119 ± 2	429 ± 13	361 ± 24	392 ± 13
	36:4	126 ± 1	92 ± 2	70 ± 2	46 ± 1	122 ± 3	129 ± 3	116 ± 5
	36:5	24 ± 2	17 ± 1	11 ± 1	8 ± 1	23 ± 1	19 ± 1	29 ± 3
	36:6	4 ± 1	2 ± 0.3	n.d	0.6 ± 0.01	5 ± 0.05	2 ± 0.4	5 ± 2
	38:1	23 ± 3	18 ± 0.4	10 ± 2	5 ± 1	27 ± 1	16 ± 3	20 ± 1
	38:2	80 ± 3	58 ± 1	39 ± 2	19 ± 2	78 ± 2	59 ± 5	66 ± 2
	38:3	323 ± 4	256 ± 5	178 ± 11	94 ± 2	302 ± 8	249 ± 16	270 ± 8
	38:4	306 ± 5	215 ± 4	147 ± 8	91 ± 2	287 ± 9	228 ± 15	256 ± 7
	38:5	115 ± 1	69 ± 2	49 ± 2	37 ± 0.4	111 ± 4	89 ± 7	103 ± 4
	38:6	56 ± 0.2	29 ± 1	21 ± 1	20 ± 1	56 ± 2	44 ± 3	55 ± 3
	40:3	19 ± 0.3	17 ± 1	10 ± 2	5 ± 1	18 ± 1	15 ± 1	16 ± 1
	40:4	44 ± 1	37 ± 0.2	27 ± 2	16 ± 0.4	42 ± 2	31 ± 3	37 ± 1
	40:6	53 ± 1	28 ± 0.3	18 ± 2	16 ± 2	52 ± 2	36 ± 2	47 ± 1
	16:0	6 ± 1	3 ± 1	0.03 ± 0.01	n.d	11 ± 2	2 ± 0.02	8 ± 2
LPC	18:0	3 ± 0.1	3 ± 0.3	0.2 ± 0.08	n.d	6 ± 1	1 ± 0.02	5 ± 1
	18:1	5 ± 1	4 ± 1	1.2 ± 0.5	n.d	10 ± 1	4 ± 0.01	10 ± 2
	30:0	3 ± 0.4	1.2 ± 0.2	1 ± 0.1	n.d	4 ± 1	3 ± 1	1 ± 0.4
	32:1	55 ± 4	52 ± 1.3	38 ± 2	20 ± 1	65 ± 3	81 ± 1	53 ± 1
PC-P	32:2	27 ± 2	25 ± 1	18 ± 1	8 ± 1	31 ± 1	42 ± 0.3	27 ± 1
	34:1	62 ± 5	56 ± 1	41 ± 2	24 ± 1	77 ± 2	108 ± 7	62 ± 2
	34:2	35 ± 3	34 ± 1	21 ± 2	11 ± 2	42 ± 1	52 ± 3	37 ± 1

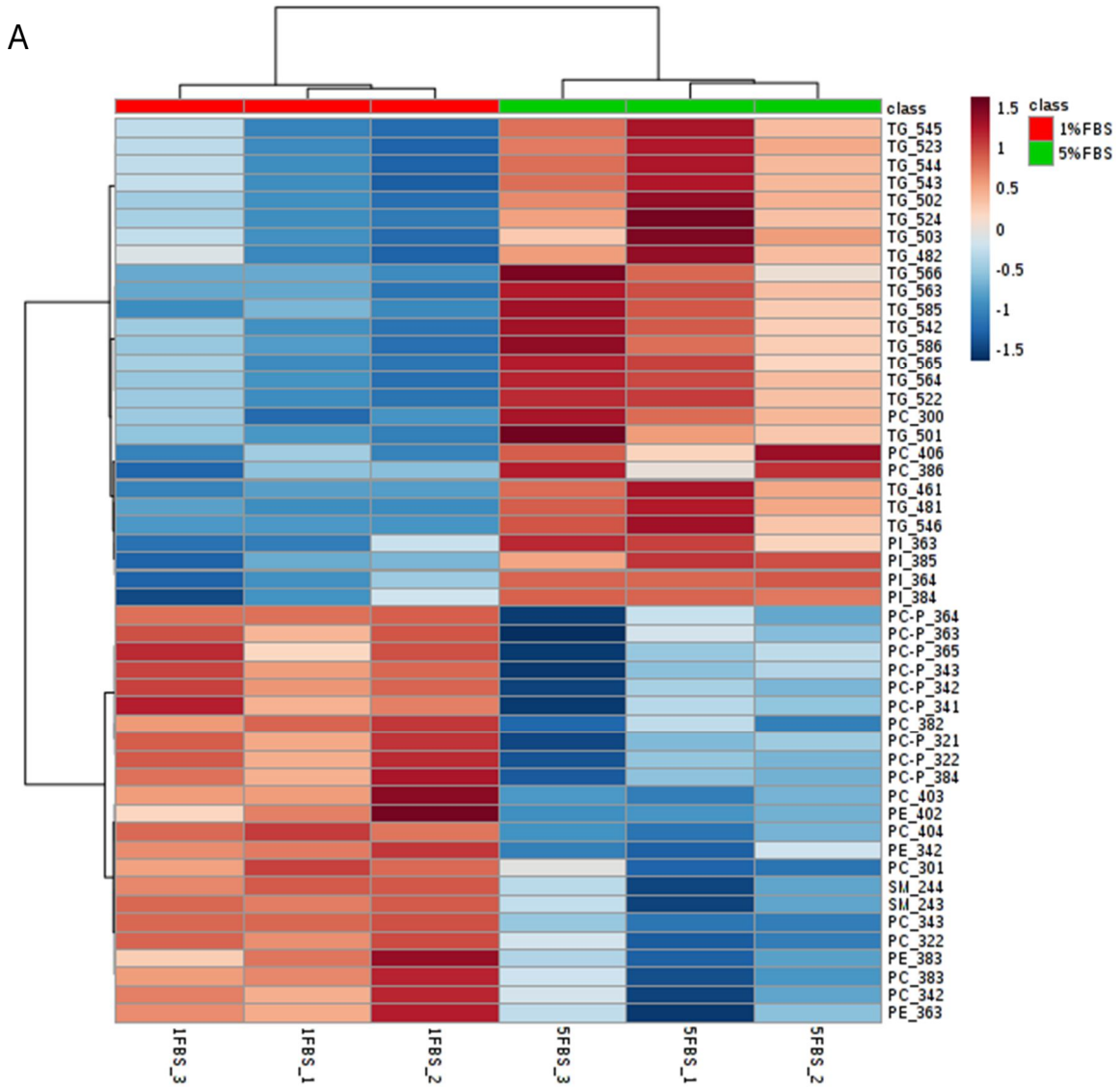
	34:3	11 ± 1	11 ± 0.2	6 ± 1	3 ± 0.7	15 ± 0.4	10 ± 1	12 ± 1
	34:4	4 ± 0.6	1 ± 0.1	2 ± 0.3	n.d	3 ± 1	3 ± 0.4	1 ± 0.1
	36:1	9 ± 2	7 ± 0.1	3 ± 1	3 ± 0.1	11 ± 0.4	7 ± 1	7 ± 1
	36:2	16 ± 2	14 ± 0.3	9 ± 1	4 ± 1	18 ± 1	15 ± 2	14 ±1
	36:3	36 ± 3	29 ± 1	23 ± 2	15 ± 1	36 ± 1	37 ± 1	29 ±1
	36:4	22 ± 2	19 ± 0.1	14 ± 1	11 ± 1	25 ± 1	20 ± 2	20 ± 1
	36:5	11 ± 1	10 ± 0.3	5 ± 1	4 ± 1	12 ± 0.4	8 ±1	12 ± 1
	36:6	6 ± 1	5 ± 1	2 ± 1	1.3 ± 0.4	6 ± 0.2	4 ± 0.2	7 ± 2
	38:3	6 ± 1	6 ± 0.1	2 ± 1	1.4 ± 0.1	7 ± 1	6 ± 1	4 ± 1
	38:4	16 ± 1	15 ± 1	8 ± 1	5 ± 1	17 ± 1	16 ± 1	14 ± 0.4
	38:5	16 ± 1	12 ± 0.2	6 ± 1	5 ± 1	17 ± 0.4	14 ± 1	14 ± 1
	38:6	21 ± 2	17 ± 1	10 ± 1	11 ± 0.5	23 ± 1	20 ± 1	17 ± 0.3
	38:7	14 ± 1	10 ± 0.4	5 ± 1	5 ± 1.2	15 ± 1	14 ± 1	14 ± 1
	40:6	3 ± 0.2	n.d	n.d	n.d	n.d	n.d	n.d
	40:7	4 ± 0.3	1.3 ± 0.1	1.3 ± 0.4	n.d	3 ± 0.2	3 ± 0.3	2 ± 0.4
	40:8	3 ± 0.2	2 ± 0.3	1 ± 0.4	1.2 ± 0.3	3 ± 0.3	5 ± 0.2	3 ± 1
PE	32:1	46 ± 2	30 ± 2	22 ± 2	11 ± 1	52 ± 2	66 ± 4	63 ± 2
	34:1	216 ± 8	160 ± 6	111 ± 5	63 ± 4	239 ± 7	179 ± 15	274 ± 12
	34:2	126 ± 5	102 ± 3	72 ± 10	28 ± 6	137 ± 4	99 ± 11	155 ± 7
	34:3	14 ± 4	8 ± 1	6 ± 1	n.d	n.d	n.d	n.d
	36:1	321 ± 11	232 ± 14	158 ± 7	91 ± 43	348 ± 9	406 ± 36	371 ± 20
	36:2	258 ± 3	200 ± 11	136 ± 8	48 ± 2	279 ± 8	202 ± 13	310 ± 13
	36:3	131 ± 1	103 ± 4	70 ± 07	15 ± 2	133 ± 4	138 ± 6	148 ± 5
	36:4	60 ± 3	26 ± 5	9 ± 5	n.d	48 ± 2	46 ± 9	59 ± 4
	38:1	58 ± 4	46 ± 5	29 ± 4	15 ± 3	67 ± 1	64 ± 2	68 ± 5
	38:2	113 ± 5	72 ± 5	50 ± 2	22 ± 4	114 ± 4	105 ± 6	115 ± 5
	38:3	350 ± 8	311 ± 20	243 ± 15	94 ± 7	342 ± 10	373 ± 19	383 ± 15
	38:4	446 ± 5	334 ± 21	238 ± 21	109 ± 8	438 ± 16	293 ± 32	481 ± 1
	38:5	143 ± 1	81 ± 4	53 ± 4	19 ± 5	133 ± 8	175 ± 17	149 ± 3
	38:6	80 ± 1	38 ± 5	21 ± 6	5 ± 2	79 ± 2	67 ± 2	96 ± 20
	40:1	127 ± 4	106 ± 5	76 ± 3	32 ± 3	123 ± 5	244 ± 45	155± 13
	40:2	56 ± 2	53 ± 2	37 ± 1	17 ± 2	53 ± 0.6	56 ± 3	66 ± 7
	40:3	127 ± 4	106 ± 5	76 ± 3	32 ± 3	123 ± 5	126 ± 5	155 ± 13
	40:4	134 ± 2	107 ± 4	77 ± 3	33 ± 1	139 ± 7	190 ± 26	160 ± 8
	40:5	201 ± 3	132 ± 5	92 ± 1	49 ± 3	201 ± 6	213 ± 16	257 ± 16
	40:6	83 ± 3	50 ± 2	30 ± 3	10 ± 2	85 ± 5	79 ± 6	86 ± 2
LPE	18:0	44 ± 8	31 ± 7	7 ± 2	2 ± 1	49 ± 6	29 ± 1	60 ± 9
	18:1	20 ± 1	16 ± 5	2 ± 1.6	3 ± 2	28 ± 4	14 ± 2	24 ± 4
	18:2	48 ± 31	37 ± 32	16 ± 7	46 ± 13	12 ± 3	5 ± 1	78 ± 31
PE-P	32:1	87 ± 23	45 ± 9	43 ± 9	37 ± 5	54 ± 6	31 ± 3	59 ± 8
	32:2	53 ± 6	44 ± 4	25 ± 7	9 ± 3	65 ± 1	60 ± 1	59 ± 3
	32:3	17 ± 3	18 ± 2	8 ± 3	n.d	24 ± 1	22 ± 0.3	20 ± 3
	34:1	26 ± 2	18 ± 2	13 ± 5	4 ± 1	25 ± 1	24 ± 2	17 ± 3
	34:2	194 ± 9	144 ± 11	87 ± 13	49 ± 5	206 ± 8	197 ± 2	186 ± 7
	34:3	60 ± 3	47 ± 4	30 ± 6	11 ± 3	65 ± 2	60 ± 0.8	62 ± 2
	34:5	49 ± 13	22 ± 5	20 ± 5	15 ± 3	33 ± 5	15 ± 3	27 ± 3

	36:2	61 ± 3	48 ± 0.8	28 ± 7	14 ± 2	63 ± 3	55 ± 1	57 ± 3
	36:3	86 ± 2	64 ± 4	42 ± 7	19 ± 4	83 ± 2	77 ± 1	72 ± 2
	36:4	215 ± 6	176 ± 7	120 ± 12	49 ± 4	219 ± 5	208 ± 7	211 ± 5
	36:5	147 ± 7	97 ± 5	63 ± 5	25 ± 8	137 ± 5	120 ± 3	124 ± 2
	38:4	133 ± 7	110 ± 6	76 ± 7	34 ± 5	128 ± 5	116 ± 1	123 ± 4
	38:5	228 ± 9	180 ± 11	123 ± 11	51 ± 3	223 ± 9	219 ± 2	212 ± 5
	38:6	171 ± 8	120 ± 7	90 ± 6	43 ± 7	171 ± 5	160 ± 1	154 ± 2
	38:7	352 ± 16	223 ± 11	153 ± 14	88 ± 3	342 ± 13	324 ± 3	335 ± 2
	40:4	34 ± 5	28 ± 4	17 ± 4	3 ± 0.3	32 ± 3	26 ± 0.3	36 ± 1
	40:5	38 ± 1	30 ± 2	18 ± 4	6 ± 2	38 ± 1	31 ± 1	24 ± 1
	40:6	78 ± 4	51 ± 2	30 ± 5	17 ± 5	77 ± 3	76 ± 1	75 ± 4
	40:7	38 ± 1	22 ± 1	8 ± 3	3 ± 2	38 ±1	35 ± 2	75 ± 7
PS	34:1	16 ± 1	12 ± 1	7 ± 1	2 ± 0.3	18 ± 1	16 ± 2	19 ± 0.4
	36:1	36 ± 1	28 ± 1	19 ± 1	8 ± 1	38 ± 1	32 ± 2	37 ± 2
	36:2	21 ± 1	15 ± 1	10 ± 1	4 ± 0.7	21 ± 1	18 ± 2	19 ± 1
	36:3	4 ± 0.4	3 ± 0.2	1.5 ± 1	0.2 ± 0.1	4 ± 0.2	3 ± 1	4 ± 0.2
	38:2	12 ± 0.4	10 ± 0.4	6 ± 1	3 ± 0.4	13 ± 1	10 ± 2	12 ± 1
	38:3	29 ± 1	23 ± 2	16 ± 2	4 ± 0.6	28 ± 1	24 ± 2	24 ± 1
	38:4	26 ± 1	18 ± 1	11 ± 2	3 ± 0.6	22 ± 1	20 ± 2	22 ± 1
	40:3	28 ± 2	25 ± 2	18 ± 2	10 ± 1	26 ± 1	24 ± 2	25 ± 1
	40:4	67 ± 3	55 ± 3	40 ± 4	22 ± 1	60 ± 3	56 ± 5	58 ± 1
	40:5	54 ± 2	39 ± 2	30 ± 3	16 ± 1	52 ± 3	42 ± 4	50 ± 1
	40:6	87 ± 6	53 ± 3	40 ± 5	24 ± 1	79 ± 4	66 ± 6	81 ± 1
	40:7	8 ± 1	5 ± 0.2	3 ± 1	1 ± 0.1	7 ± 0.1	6 ± 1	7 ± 0.3
PG	34:0	7 ± 0.1	4 ± 0.6	4 ± 0.2	2 ± 0.1	6 ± 0.4	19 ± 0.02	5 ± 0.2
	34:1	17 ± 2	11 ± 1	9 ± 1	6 ± 1	18 ± 1	51 ± 0.1	19 ± 1
	36:1	8 ± 1	5 ± 0.4	4 ± 0.3	2 ± 0.1	8 ± 0.3	24 ± 0.2	8 ± 0.3
	36:2	3 ± 0.1	2 ± 0.1	1 ± 0.2	2 ± 0.1	3 ± 0.1	7 ± 0.02	3 ± 0.1
PI	32:0	2 ± 0.2	1 ± 0.2	n.d	n.d	n.d	n.d	n.d
	32:1	6 ± 0.2	3 ± 0.4	1 ± 0.7	n.d	5 ± 0.1	5 ± 0.3	6 ± 0.4
	34:0	33 ± 2	23 ± 1	18 ± 1	10 ± 0.4	27 ± 2	35 ± 2	26 ± 0.5
	34:1	64 ± 1	40 ± 2	27 ± 3	20 ± 1	53 ± 2	77 ± 7	54 ± 1
	34:2	24 ± 2	15 ± 1	8 ± 1	3 ± 0.9	18 ± 0.4	22 ± 1	16 ± 1
	34:3	4 ± 0.2	3 ± 0.3	1 ± 0.4	n.d	n.d	n.d	n.d
	36:1	13 ± 1	9 ± 0.4	5 ± 1	4 ± 1	11 ± 1	11 ± 0.5	8 ± 0.4
	36:2	52 ± 3	30 ± 1	18 ± 2	6 ± 1	39 ± 1	57 ± 6	35 ± 2
	36:3	119 ± 9	60 ± 4	33 ± 5	13 ± 2	86 ± 4	81 ± 5	86 ± 4
	36:4	40 ± 2	17 ± 2	8 ± 2	5 ± 0.6	31 ± 1	49 ± 6	34 ± 1
	38:3	944 ± 52	526 ± 37	305 ± 48	122 ± 11	639 ± 34	759 ± 62	549 ± 32
	38:4	610 ± 32	287 ± 26	165 ± 20	81 ± 8	422 ± 20	474 ± 29	381 ± 19
	38:5	76 ± 6	33 ± 2	19 ± 1	13 ± 2	56 ± 2	65 ± 4	57 ± 2
	38:6	6 ± 1	2 ± 0.2	0.5 ± 0.2	1 ± 0.4	4 ± 0.3	3 ± 0.3	4 ± 0.4
	40:3	58 ± 5	33 ± 2	18 ± 2	10 ± 1	37 ± 1	36 ± 1	33 ± 2
	40:4	46 ± 3	29 ± 2	17 ± 2	7 ± 1	31 ± 1	33 ± 2	31 ± 2
	40:5	19 ± 2	11 ± 1	6 ± 1	2 ± 1	14 ± 0.4	15 ± 1	12 ± 1

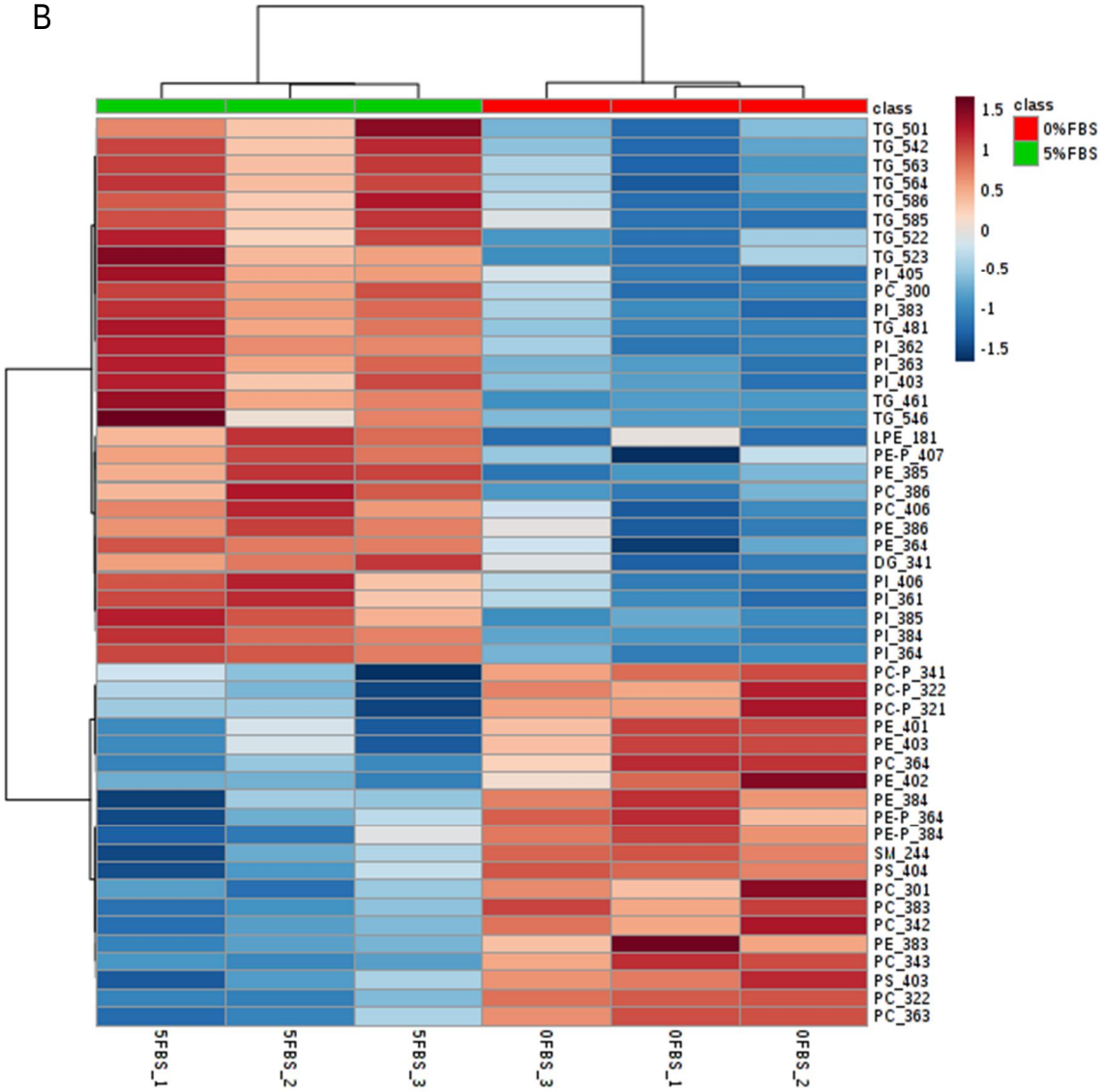
	40:6	10 ± 1	4 ± 1	2 ± 1	2 ± 0.8	7 ± 0.2	5 ±0.2	6 ± 1
	40:7	3 ± 1	1.3 ± 0.1	1.3 ± 0.2	n.d	2 ± 0.3	2 ± 0.1	2 ± 0.1
SM	16:0	26 ± 9	26 ± 1	17 ± 4	8 ± 2	44 ± 1	37 ± 0.3	33 ± 2
	18:0	12 ± 4	12 ± 1	7 ± 2	4 ± 1	20 ± 0.4	14 ± 0.4	15 ± 1
	22:0	8 ± 3	8 ± 1	4 ± 1	2 ± 1	15 ± 1	10 ± 0.3	12 ± 1
	22:1	14 ± 4	16 ± 1	9 ± 2	3 ± 1	23 ± 1	14 ± 1	18 ± 1
	24:0	5 ± 1	5 ± 0.4	4 ± 0.8	4 ± 1	9 ± 0.3	8 ± 0.1	7 ± 0.4
	24:1	34 ± 10	31 ± 1	19 ± 3	8 ± 2	53 ± 1	42 ± 1	45 ± 4
	24:2	n.d	n.d	n.d	n.d	3 ± 0.1	2 ± 0.1	3 ± 1
	24:3	39 ± 3	40 ± 1	25 ± 3	11 ± 4	48 ± 2	53 ± 2	54 ± 3
	24:4	71 ± 6	77 ± 2	51 ± 5	24 ± 2	83 ± 3	85 ± 5	89 ± 5
	24:5	7 ± 1	6 ± 0.9	n.d	n.d	6 ± 1	4 ± 0.1	7 ± 1
TG	46:1	6 ± 2	1 ± 0.3	n.d	28 ± 3	6 ± 1	2 ± 0.2	5 ± 1
	46:2	4 ± 1	1 ± 0.4	n.d	7 ± 2	5 ± 0.4	1 ± 0.1	3 ± 1
	48:1	20 ± 6	3 ± 0.3	2 ± 1.4	66 ± 7	24 ± 1	5 ± 1	20 ± 2
	48:2	30 ± 9	10 ± 3	14 ± 3	35 ± 4	36 ± 2	6 ± 1	36 ± 2
	48:3	5 ± 3	n.d	n.d	n.d	n.d	n.d	n.d
	48:4	n.d	n.d	n.d	1 ± 0.2	n.d	n.d	n.d
	50:1	27 ± 7	7 ± 2	5 ± 2	102 ± 11	34 ± 1	6 ± 1	30 ± 2
	50:2	61 ± 18	15 ± 4	13 ± 4	60 ± 4	68 ± 3	11 ± 1	64 ± 7
	50:3	43 ± 12	11 ± 3	8 ± 3	24 ± 2	44 ± 2	7 ± 1	43 ± 4
	50:4	15 ± 5	4 ± 0.9	6 ± 2	9 ± 0.3	14 ± 1	2 ± 0.3	14 ± 1
	50:5	2 ± 0.4	n.d	n.d	2 ± 0.5	n.d	n.d	n.d
	52:1	13 ± 4	3 ± 0.4	2 ± 1	70 ± 6	n.d	n.d	n.d
	52:2	71 ± 21	19 ± 4	18 ± 3	63 ± 4	79 ± 4	12 ± 1	71 ± 7
	52:3	68 ± 19	19 ± 5	18 ± 3	41 ± 3	72 ± 4	10 ± 1	67 ± 5
	52:4	32 ± 10	9 ± 2	6 ± 3	20 ± 2	33 ± 1	5 ± 1	31 ± 3
	52:5	10 ± 3	2 ± 0.1	2 ± 0.4	7 ± 0.3	8 ± 0.3	1 ± 0.02	8 ± 1
	52:6	1 ± 0.3	n.d	n.d	1 ± 0.6	n.d	n.d	n.d
	54:1	4 ± 2	n.d	n.d	5 ± 4	n.d	n.d	n.d
	54:2	26 ± 8	7 ± 2	4 ± 2	16 ± 2	28 ± 1	4 ± 0.1	23 ± 3
	54:3	65 ± 19	20 ± 5	25 ± 4	33 ± 3	69 ± 3	8 ± 1	62 ± 5
	54:4	47 ± 14	14 ± 4	13 ± 3	36 ± 3	48 ± 2	6 ± 1	46 ± 3
	54:5	26 ± 8	7 ± 2	7 ± 2	20 ± 1	24 ± 1	3 ± 0.3	26 ± 2
	54:6	9 ± 3	2 ± 0.2	2 ± 0.3	7 ± 2	8 ± 0.2	1 ± 0.2	11 ± 3
	56:2	5 ± 1	1 ± 0.1	n.d	3 ± 1	4 ± 0.2	1 ± 0.4	2 ± 1
	56:3	15 ± 5	3 ± 0.5	2 ± 1	6 ± 1	13 ± 1	2 ± 0.1	11 ± 1
	56:4	26 ± 7	8 ± 2	5 ± 2	18 ± 2	25 ± 1	3 ± 0.06	20 ± 2
	56:5	25 ± 7	7 ± 2	6 ± 2	22 ± 2	21 ± 1	3 ± 0.1	22 ±3
	56:6	13 ± 4	3 ± 0.3	2 ± 1	9 ± 2	11 ± 0.4	1 ± 0.2	17 ± 3
	56:7	6 ± 2	n.d	n.d	6 ± 1	3 ± 0.3	0.6 ± 0.1	14 ± 4
	56:8	2 ± 0.4	n.d	n.d	2 ± 0.1	n.d	n.d	n.d
	58:3	1 ± 0.1	n.d	n.d	1 ± 0.3	n.d	n.d	n.d
	58:4	6 ± 1	1.3 ± 0.2	1 ± 0.6	3 ± 1	4 ± 0.3	1 ± 0.02	3 ± 1
	58:5	9 ± 3	2 ± 0.3	4 ± 0.4	6 ± 2	9 ± 0.1	1 ± 0.2	7 ± 1

	58:6	8 ± 2	2 ± 0.6	1.2 ± 1	8 ± 1	6 ± 0.02	1± 0.4	7 ± 2
	58:7	6 ± 2	1 ± 0.3	1.2 ± 0.2	6 ± 1	5 ± 0.4	0.3 ± 0.1	11 ± 3
	58:8	3 ± 1	n.d	n.d	4 ± 0.03	n.d	2 ± 1	16 ± 3
	32:0	6 ± 1	3 ± 1	3 ± 0.2	2 ± 0.4	5 ± 0.3	6 ± 0.3	5 ± 1
	32:1	11 ± 1	7 ± 1	3 ± 1	4 ± 1	12 ± 0.3	9 ± 1	12 ± 1
	32:2	5 ± 0.1	4 ± 0.1	1.3 ± 0.8	1.3 ± 1	5 ± 0.1	4 ± 0.02	5 ± 0.6
	34:0	1.5 ± 0.1	3 ± 0.4	1.5 ± 0.3	2 ± 0.6	n.d	n.d	n.d
	34:1	23 ± 1	12 ± 1	4 ± 2	7 ± 3	23 ± 1	16 ± 2	20 ± 1
	34:2	15 ± 1	10 ± 1	5 ± 1	5 ± 1	16 ± 1	10 ± 1	16 ± 1
	34:3	4 ± 0.1	2 ± 0.3	1.2 ± 0.3	1 ± 0.7	3 ± 0.1	2 ± 0.1	3 ± 1
	36:1	8 ± 1	6 ± 1	3 ± 1	4 ± 1	9 ± 0.1	5 ± 0.2	8 ± 1
	36:2	15 ± 1	10 ± 1	7 ± 1	6 ± 1	16 ± 1	11 ± 0.4	16 ± 1
	36:3	12 ± 1	8 ± 0.4	5 ± 1	4 ± 1	11 ± 1	7 ± 0.1	11 ± 1
DG	36:4	4 ± 0.3	3 ± 0.2	2 ± 0.2	2 ± 0.7	4 ± 0.3	3 ± 0.3	4 ± 0.3
	38:2	4 ± 0.6	3 ± 0.2	1.4 ± 1	1.3 ± 1	3 ± 0.2	2 ± 0.1	3 ± 0.4
	38:3	25 ± 5	24 ± 1	20 ± 1	11 ± 1	26 ± 1	18 ± 2	26 ± 1
	38:4	21 ± 2	19 ± 1	15 ± 0.2	10 ± 1	24 ± 1	15 ± 1	24 ± 1
	38:5	7 ± 0.2	4 ± 1	2 ± 1	3 ± 0.3	7 ± 1	4 ± 0.2	7 ± 1
	38:6	3 ± 0.5	1 ± 0.3	n.d	n.d	3 ± 0.3	2 ± 0.03	3 ± 0.3
	40:3	0.9 ± 0.1	2 ± 0.3	2 ± 0.4	1.1 ± 0.4	n.d	n.d	n.d
	40:4	2 ± 0.8	4 ± 0.3	2 ± 1	2 ± 1	n.d	n.d	n.d
	40:5	5 ± 0.4	4 ± 0.4	2 ± 0.5	3 ± 0.9	4 ± 0.3	1 ± 0.4	4 ± 1
	40:6	6 ± 0.1	4 ± 0.1	1.5 ± 1	2 ± 1	6 ± 1	3 ± 0.2	6 ± 1
	40:7	3 ± 0.6	0.4 ± 0.1	n.d	1 ± 0.3	2 ± 0.4	1 ± 0.2	3 ± 1

Figure 42. Heatmaps representing lipid profiles of PLHC-1 cells cultured in 1%FBS (A), 0%FBS (B), DPBS-GLU (C), compared to 5%FBS control. Each line corresponds to one lipid, and each column corresponds to each individual cell medium. Each lipid was illustrated through different colors, allowing a visual assessment of the relative abundance of each one: Dark blue and dark red colors correspond to the fewer and larger amounts, respectively.



B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TG_481
PI_362
PI_363
PI_403
TG_461
TG_546
LPE_181
PE-P_407
PE_385
PC_386
PC_406
PE_386
PE_364
DG_341
PI_406
PI_361
PI_385
PI_384
PI_364
PC-P_341
PC-P_322
PC-P_321
PE_401
PE_403
PC_364
PE_402
PE_384
PE-P_364
PE-P_384
SM_244
PS_404
PC_301
PC_383
PC_342
PE_383
PC_343
PS_403
PC_322
PC_363
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5FBS_2
5FBS_3
0FBS_3
0FBS_1
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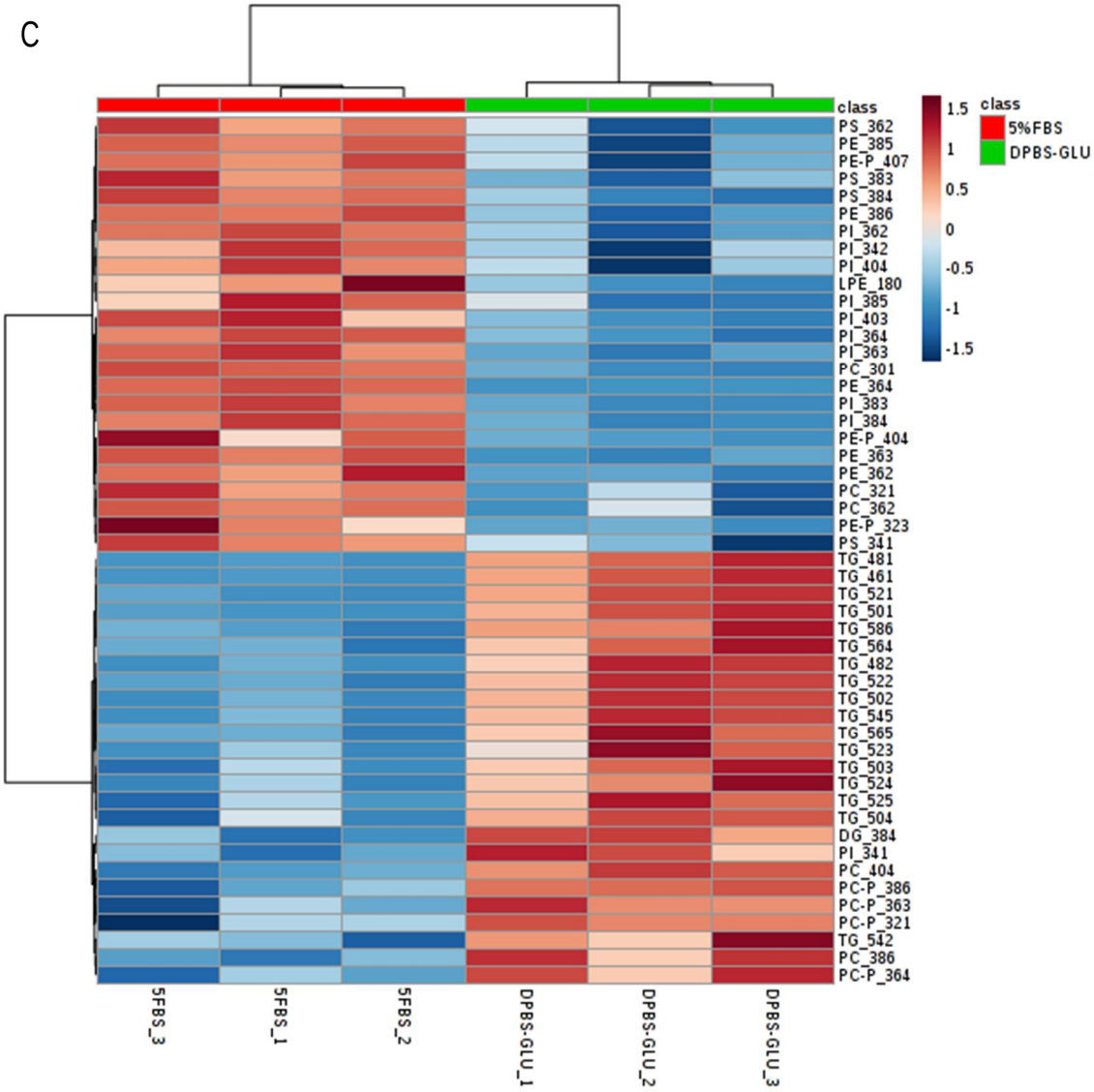
-1
-1.5
class
0%FBS
5%FBS

C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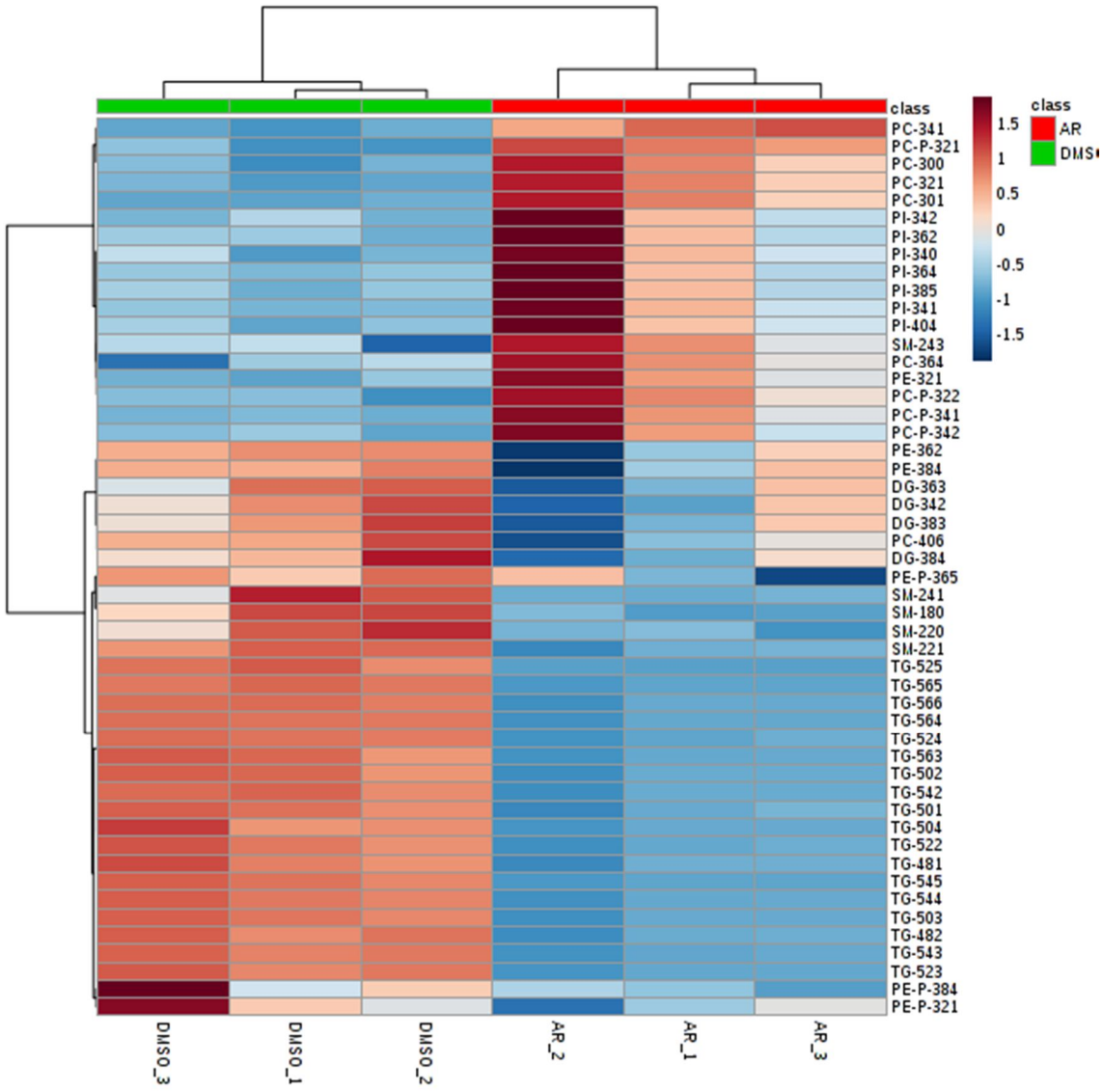
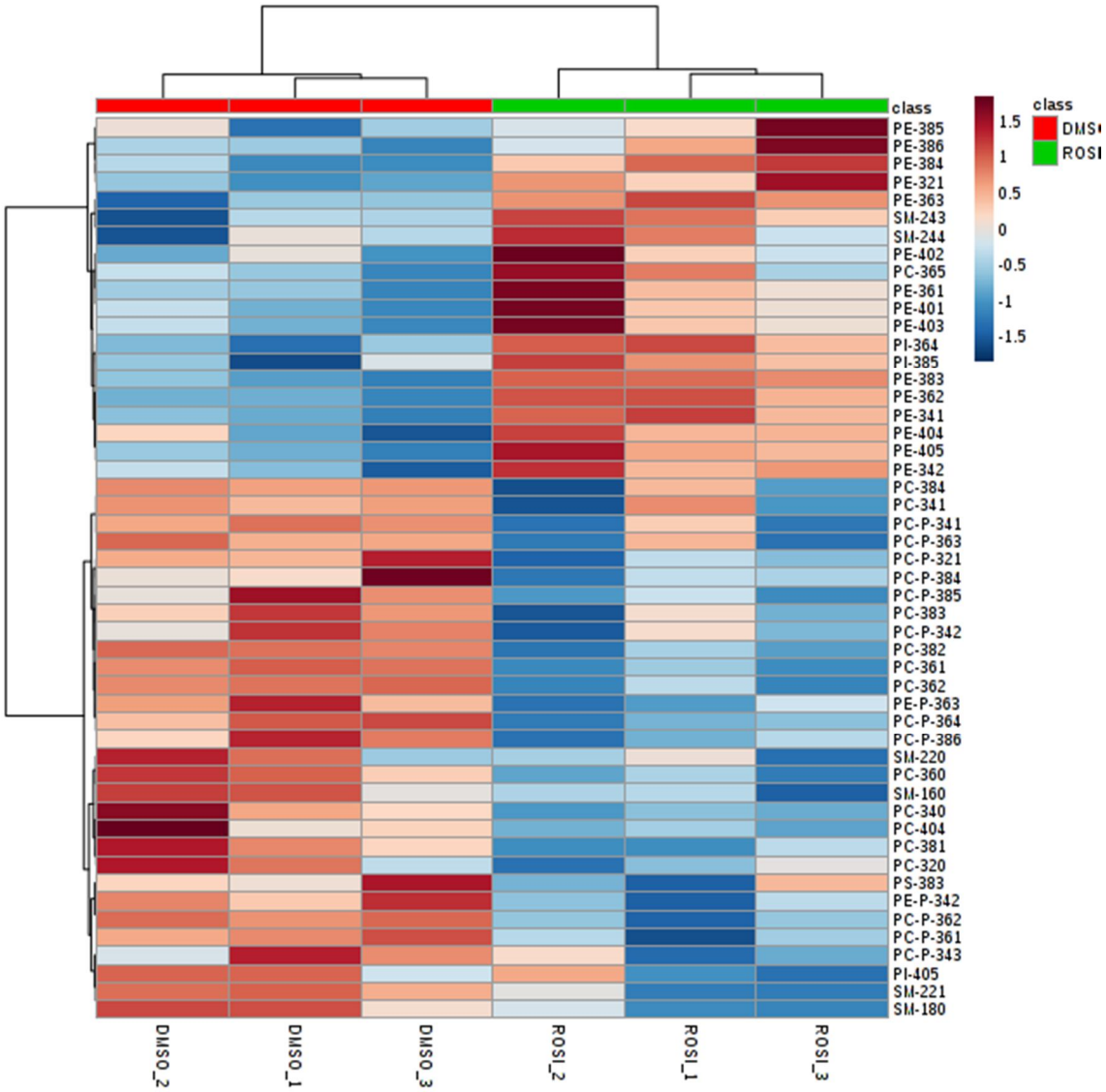
PC-P_364
5FBS_3
5FBS_1
5FBS_2
DPBS-GLU_1
DPBS-GLU_2
DPBS-GLU_3
1.5
1
0.5
0
-0.5
-1
-1.5
class
5%FBS
DPBS-GLU

Figure 43. Heatmaps representing lipid profiles of PLHC-1 cells exposed to 9-*cis*-RA (A), and ROSI (B), compared to 5%FBS control. Each line corresponds to one lipid, and each column corresponds to each individual cell medium. Each lipid was illustrated through different colours, allowing a visual assessment of the relative abundance of each one: Dark blue and dark red colours correspond to the fewer and larger amounts, respectively.

A



B


class
PE-385
PE-386
PE-384
PE-321
PE-363
SM-243
SM-244
PE-402
PC-365
PE-361
PE-401
PE-403
PI-364
PI-385
PE-383
PE-362
PE-341
PE-404
PE-405
PE-342
PC-384
PC-341
PC-P-341
PC-P-363
PC-P-321
PC-P-384
PC-P-385
PC-383
PC-P-342
PC-382
PC-361
PC-362
PE-P-363
PC-P-364
PC-P-386
SM-220
PC-360
SM-160
PC-340
PC-404
PC-381
PC-320
PS-383
PE-P-342
PC-P-362
PC-P-361
PC-P-343
PI-405
SM-221
SM-180
DMSO_2
DMSO_1
DMSO_3
ROSI_2
ROSI_1
ROSI_3
1.5
1
0.5
0
-0.5
-1
-1.5
class
DMSO
ROSI

4. DISCUSIÓN GENERAL

Discusión General

El uso de biomarcadores en organismos acuáticos ha contribuido a la compresión de los mecanismos de acción de los compuestos químicos, poniendo de relieve cómo los contaminantes pueden afectar a los sistemas biológicos a distintos niveles incluyendo molecular, bioquímico, histológico y conductual, entre otros. Por ello, la integración de múltiples medidas incluyendo biotransformación, genotoxicidad, neurotoxicidad o disrupción endocrina es necesaria en la evaluación del riesgo ambiental. Desde el año 2003, los avances en tecnologías como espectrometría de masas han permitido detectar y profundizar en el análisis de lípidos en matrices ambientales y cada vez más estudios están poniendo de manifiesto efectos en la síntesis y acumulación de lípidos tras la exposición a compuestos químicos.

En esta Tesis el impacto de xenobióticos, incluyendo disruptores endocrinos y lipídicos, se ha estudiado mediante el uso combinado de biomarcadores en peces (*in vivo*) y en líneas celulares (*in vitro*) (Tabla 18).

Tabla 18. Biomarcadores y técnicas analíticas utilizadas en esta Tesis para determinar efectos biológicos de contaminantes en organismos acuáticos *in vivo* y en células *in vitro.*

	Biomarcador	**Tejido**	**Indicador**	**Técnica analítica**
In vivo				
Peces	Citocromo P4501A	Hígado	Exposición a xenobióticos (PAHs, PCBs, furanos, dioxinas)	Fluorimetría (EROD)
	Citocromo P4503A	Hígado	Exposición a xenobióticos (farmacéuticos, pesticidas)	Fluorimetría (BFCOD)
	UDPGT	Hígado	Exposición reciente a xenobióticos	Espectrofotometría (UGT)
	Niveles de DRO	Plasma	Exposición reciente/efecto de bioacumulación	UPLC-MS/MS
	Niveles de esteroides	Plasma	Disrupción endocrina	UPLC-MS/MS
	APs/PAHs/HHCB	Bilis	Exposición reciente a xenobióticos	GC-MS-EI
	OCs	Músculo	Exposición a xenobióticos	GC-ECD
	Genes *cyp19a1, cyp11β, cyp17a1*	Gónada	Disrupción endocrina	qPCR-RT
	Actividad aromatasa	Gónada	Disrupción endocrina	Contador de centelleo líquido
	Composición lipídica	Músculo	Disrupción lipídica	LC-MS-ToF
	Histología	Gónada	Disrupción endocrina	Tinción,observación al microscopio
In vitro				
Células PLHC-1	Citocromo P4501A	Hígado	Exposición a xenobióticos (PAHs, PCBs, furanos, dioxinas)	Fluorimetría (EROD)
	Formación de ROS	Hígado	Exposición a compuestos oxidantes	Fluorimetría (H_2DCF-DA)
	Composición lipídica	Hígado	Disrupción lipídica	FIA-Orbitrap
Células COS7	Activación receptor zfPxr	Transfectadas con plásmidos de pez cebra	Inducción CYP3A	Luminiscencia
Yeast	Activación ER	Modificadas genéticamente	Disrupción endocrina	Fluorimetría (ER-RYA)

GC-ECD: Cromatografía de gases con detector de captura de electrones.
H_2DCF-DA: 2′7′-diclorodihidrofluorescein diacetato.

4.1. Utilización de biomarcadores para la evaluación de la calidad ambiental de ríos mediterráneos.

Los peces han demostrado ser buenos organismos centinela para evaluar la condición y el funcionamiento de los ecosistemas acuáticos (Birk et al., 2012). La primera parte de esta Tesis correspondiente al capítulo III, se centra en el estudio de indicadores químicos y respuestas bioquímicas en peces capturados en dos ríos mediterráneos (Ebro y Ripoll) sometidos a fuertes presiones antropogénicas. En el estudio del río Ripoll se incluyen análisis de efectos en la acumulación de lípidos. En ambos ríos se han utilizado especies autóctonas de ciprínidos como organismos centinela: *B. meridionalis*, un pez bentónico que habita en cursos altos de ríos, *S. laietanus*, un pez pelágico presente en el curso medio y bajo de ríos, y *R. rutilus*, un pez bentónico tolerante a aguas dulces y salobres, presente en cursos bajos de ríos, y utilizado con éxito para evaluar la calidad del agua en ríos de toda Europa (Mas-Martí et al., 2010; Bjerregaard et al., 2006; Gerbron et al., 2014; Huertas et al., 2016).

En el río Ebro a la altura de Flix, durante 1 siglo se estuvieron vertiendo de manera incontrolada compuestos radioactivos, metales y OCs procedentes de una factoría química (Erquimia-Ercros, S.A), que ha producido la acumulación de 700.000 - 800.000 m^3 de residuos en los sedimentos. Para su retirada, en el año 2010 se aprobó un proyecto europeo que incluía 2 fases: 1- la construcción de un muro de contención para evitar el transporte de contaminantes aguas abajo del río, cuyas obras comenzaron en el año 2010, y 2- la retirada de los sedimentos contaminados mediante una draga una vez finalizada la construcción del muro, que empezó en abril de 2013. Procesos tales como perturbaciones físicas inducidas por corrientes de agua, dragado u otras actividades, pueden provocar la resuspensión de contaminantes a la columna de agua, volviéndolos

disponibles para los organismos acuáticos (Latimer et al., 1999). En base a la hipótesis de que la construcción del muro y/o las posteriores actividades de dragado podían potenciar la movilidad de contaminantes desde Flix hacia el curso inferior del río Ebro, se realizaron 2 muestreos: el primero en octubre de 2012, 2 años después del inicio de la construcción del muro; y el segundo en junio de 2013, coincidiendo con la extracción de sedimentos.

Flix se considera un punto caliente de contaminación. En el músculo de carpas capturadas en esta área, en el año 2000 se encontraron concentraciones de OCs 90 veces superiores que las detectadas en los peces procedentes de la zona de referencia (RR) (Lavado et al, 2006). Sin embargo, en el año 2012, las concentraciones más elevadas de estos compuestos se detectaron en el músculo de *R. rutilus* en la estación de BE, situada a 50 km de Flix, siendo de hasta 38, 45 y 17 veces superiores los niveles de PCBs, DDTs y HCBs, respectivamente, que los detectados en los peces de la estación RR. Estos resultados sugieren que las actividades de dragado produjeron una liberación, resuspensión y transporte aguas abajo de OCs que estaban retenidos en los sedimentos.

En junio de 2013, las concentraciones de OCs en el músculo de *R. rutilus* capturados en FL y BE fueron 5 y 10 veces inferiores a las reportadas en 2012, sugiriendo que los contaminantes resuspendidos como consecuencia del dragado, se mantuvieron retenidos dentro de la barrera. De hecho, las concentraciones de OCs en 2013 son las más bajas reportadas en peces en FL desde los primeros análisis llevados a cabo en la zona, lo que resalta la eficacia del muro de contención y las obras de dragado (Tabla 19).

Tabla 19. Concentraciones de OCs (ng/g d.w.) en peces capturados en Flix.

Especie	***Cyprinus carpio**[a]* (músculo)	***Cyprinus carpio**[b]* (todo el pez)	***Alburnus alburnus**[b]* (todo el pez)	***Silurus glanis**[c]* (músculo)	***Rutilus rutilus**[c]* (músculo)	***Rutilus rutilus**[d]* (músculo)	***Rutilus rutilus**[d]* (músculo)
Año muestreo	**2000**	**2003**	**2003**	**2005**	**2006**	**2012**	**2013**
∑DDT	147	983	487	140-340	95	111	19
∑HCH	0.5	n.a	n.a	n.d-3	1	3	0.5
∑PCB	477	n.a	n.a	80-190	82	114	11
HCB	7.8	n.a	n.a	23-90	258	20	4

*Concentraciones reportadas por peso fresco de tejido. Se transforman en concentración por peso seco multiplicando por un factor de corrección = 5, considerando un contenido de agua en tejido del 80%.

n.a: no analizado / n.d: no detectado.

[a]Lavado et al., 2006 / [b]Lacorte et al., 2006 / [c]Huertas et al., 2016 / [d]Esta Tesis.

La Figura 44 muestra una extensa revisión de concentraciones de DDTs y PCBs reportadas en músculo de *Rutilus* en ríos europeos desde 1981 hasta la actualidad (los datos representados en ríos en España corresponden a datos del río Ebro). El valor más alto de DDTs corresponde con las concentraciones detectadas en BE en el año 2012 (966 ng/g), pero en general, los niveles de DDTs son similares entre ríos europeos, estando la mayoría de ellos por encima de los 14 µg/kg w.w., establecidos por los estándares de calidad ambiental en fauna silvestre acuática de la Canadian Tissue Residue Guideline (2001) (en la UE actualmente no existe directriz equivalente). Estos datos resultan inquietantes teniendo en cuenta la prohibición del uso del DDT en muchos países desde los años 80. De hecho, algunos de los estudios publican datos de 4,4'-DDE, cuya presencia es indicativo de antiguos residuos de DDT que se han degradado progresivamente en este metabolito más persistente, en lugar del total de DDTs (Shaw et al., 2005). De hecho, el 4,4'-DDE ha sido el isómero dominante en todos los peces de Flix analizados en esta Tesis. Concentraciones de PCBs reportadas en ríos de República Checa, Polonia,

y Reino Unido se sitúan por debajo de los valores establecidos por el Reglamento de la Unión Europea nº 1259/2011 del 2 de diciembre de 2011, relativo a niveles máximos en productos alimenticios (125 µg/kg w.w. para *Rutilus*) (Comisión Europea, 2011). Sin embargo, ríos en Alemania, Francia y Finlandia junto con el río Ebro muestreado en 2012, superan los niveles del Reglamento.

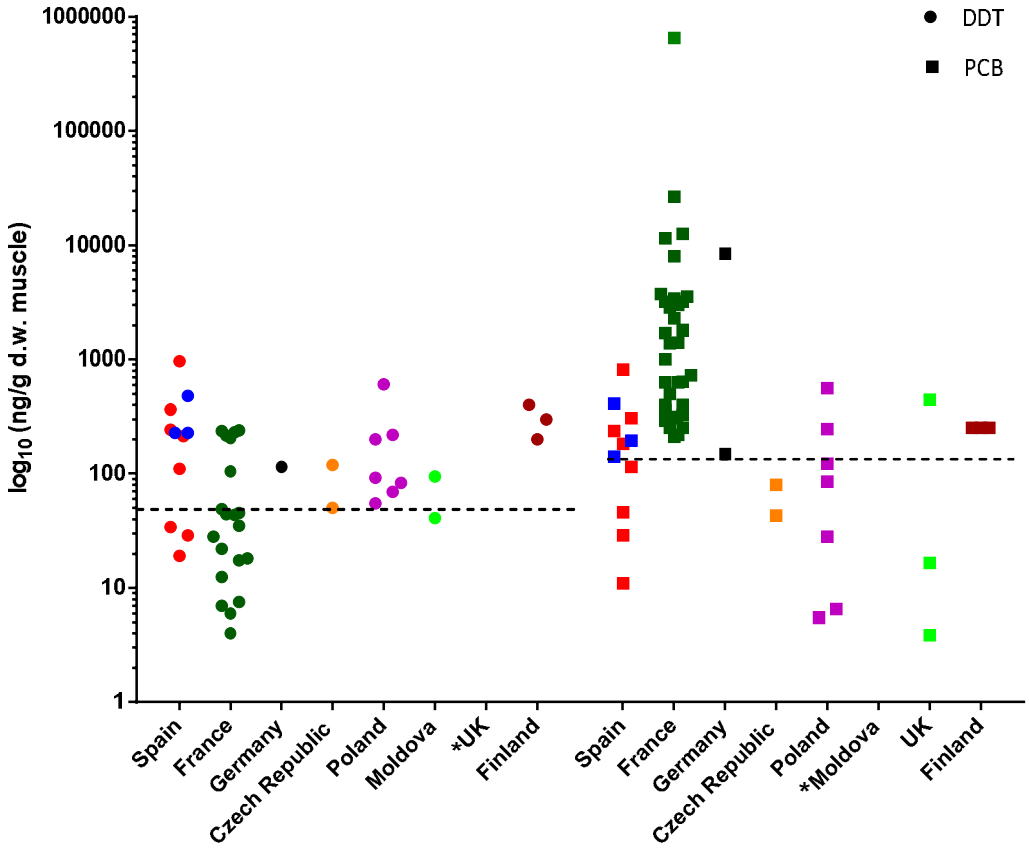


Figura 44. Concentraciones de DDTs y PCBs (ng/g d.w.) reportadas en músculo de *Rutilus* en ríos europeos.

Los símbolos en rojo corresponden a datos reportados en el estudio del Río Ebro en esta Tesis.

Referencias: Azimi and Rocher (2016); Niewiadowska et al., (2015)[a]; Huertas (2015)[a]; Niewiadowska et al., (2014)[a]; Hrádková et al., (2012)[a]; Teil et al., (2012); Tomza-Marciniak and Witczak, (2010)[a]; Sapozhnikova et al., (2005)[a]; Yamaguchi et al., (2003); Chevreuil et al., (1995)[o a]; Gazzard and Yorke (1993)[a]; Haiber and Schöler, (1994)[a]; Monod et al., (1988)[a]; Cemagref, (1985)[o a]; Schüler et al., (1985)[o a]; AFBSN, (1984)[a]; Pyssalo et al., 1983[a].

Líneas punteadas hacen referencia a los valores establecidos para DDTs por la Canadian Council of Ministers of the Environment (2001), y para PCBs por la Comisión Europea (2011)[a].

[o]Reportan datos de 4,4'-DDE.

*Datos no disponibles.

[a]Concentraciones reportadas por peso fresco de tejido. Se transforman en concentración por peso seco multiplicando por un factor de corrección = 5, considerando un contenido de agua en músculo del 80%.

Los compuestos químicos absorbidos por los peces, además de ser acumulados en tejidos como músculo, pueden ser metabolizados y posteriormente excretados a través de la bilis como metabolitos hidroxilados y/o conjugados (Koenig et al., 2013). El análisis de los metabolitos biliares ha sido utilizado con éxito como marcador de exposición reciente a contaminantes en peces, dado que la bilis representa la principal vía de excreción para muchos xenobióticos (Beyer et al., 2011).

En el río Ebro, pese a las diferencias significativas espaciales y temporales detectadas en los niveles de OCs en el músculo de *R. rutilus*, el análisis biliar no ha evidenciado diferencias sustanciales de APs y PAHs entre los puntos de muestreo, debido principalmente a la alta variabilidad interindividual. Sin embargo, el análisis realizado en la bilis de *B. meridionalis* y *S. laietanus* del río Ripoll, sí ha reflejado una exposición reciente a PAHs (2-naftol y 1-pirenol), APs (NP y OP) y HHCB de los peces procedentes de zonas urbanizadas (P). La contaminación en esta área principalmente se ha asociado a efluentes industriales y urbanos (Maceda-Veiga et al., 2010). De hecho, las descargas de efluentes son la principal fuente de contaminación orgánica en los ríos mediterráneos (Teixidó et al., 2001). Existe una relación directa entre las concentraciones de NP y OP y la presencia de actividades urbanas o industriales, detectándose concentraciones de hasta μg/L en aguas residuales urbanas e industriales (Vitali et al., 2004; Céspedes et al., 2005, Brix et al., 2009). En el río Ripoll, las concentraciones más altas de NP detectadas (1557 y 2157 ng/g en *B. meridionalis* y *S. laietanus*, respectivamente), sugieren un aporte específico de NP en la zona, ya que el punto en el que se detectan (P2), no coincide con la salida de ningún efluente de plantas de tratamiento de aguas residuales. También los niveles de OP sugieren una entrada puntual en P2 de APs, ya que los valores detectados en la bilis de estos peces fueron

similares a los hallados en efluentes de plantas de tratamientos de aguas urbanas e industriales, ubicadas en P1 y P3.

Las concentraciones más elevadas de los PAHS 1-pirenol y 2-naftol (20-225 ng/g) también se han encontrado en la bilis de peces procedentes de sitios urbanizados. 1-pirenol es el principal metabolito del pireno, es uno de los compuestos más abundantes presentes en la bilis de los peces y se considera el mejor indicador de exposición a PAH en peces (Ruddock et al., 2002). Sin embargo, los niveles de 1-pirenol en los sitios P no han mostrado diferencias significativas, probablemente debido a su origen pirolítico (industrial, tráfico de automóviles) y a su presencia generalizada en la atmósfera de las zonas con alta densidad poblacional, lo que también se refleja en la alta variabilidad de los valores detectados. Por el contrario, las concentraciones más altas de 2-naftol se detectaron a la salida de un efluente urbano tanto en la bilis de *B. meridionalis* como de *S. laietanus*, sugiriendo la cercanía a una fuente de PAHs. Aunque 2-naftol está frecuentemente relacionado con un origen petrogénico, este compuesto también se utiliza en tintes, pigmentos y fragancias sintéticas, que podrían proceder de las industrias textiles presentes en la zona (Colin et al., 2016a).

HHCB es uno de los principales almizcles policíclicos producidos en la UE y Estados Unidos. Los niveles más elevados de este compuesto se han detectado en la bilis de peces capturados a la salida de un efluente urbano (Clara et al., 2011). Los efluentes de plantas de tratamiento de aguas residuales representan la principal fuente de contaminación de HHCB en el medio acuático, donde se ha detectado en concentraciones de 20-4000 ng/L de agua (Homem et al., 2015). En el río Ebro, pese a no encontrar diferencias en PAHs y APs, sí se encontró un incremento significativo de la concentración de HHCB en las estaciones RR y BE, lo cual podría atribuirse a una mayor captación debida a los sedimentos

resuspendidos aguas abajo de FL y/o a la presencia de alguna fuente puntual de contaminación en la zona de referencia (RR).

En la Figura 45 se observa una comparativa de las concentraciones de metabolitos biliares en peces de agua dulce capturados en las cuencas de ríos catalanes de los que se dispone de información: Llobregat, Ebro y Ripoll. En general, las concentraciones más bajas de compuestos, especialmente de naftol, OP y HHCB, se han encuentrado en los puntos de referencia del río Ripoll, confirmando la óptima calidad ambiental de esta zona (Prat and Rieradevall, 2006). Las concentraciones de naftol han sido similares en los peces de los 3 ríos (2-226 ng/g), mientras que carpas capturadas en el río Llobregat han estado expuestas a los niveles más elevados de 1-pirenol (5566 ng/g). Respecto a los APs, las concentraciones más elevadas de NP (de 12 a 112 veces) y OP (de 9 a 86 veces) se detectaron en la bilis de carpas, barbos y bagres procedentes de Flix en el río Ebro en el año 2000 (Lavado et al., 2006). Los niveles de HHCB son similares tanto en el río Ebro como el Ripoll, y sólo a la salida de una planta de tratamiento de aguas residuales en el río Ripoll se han reportado valores ligeramente más elevados (1277 ng/g).

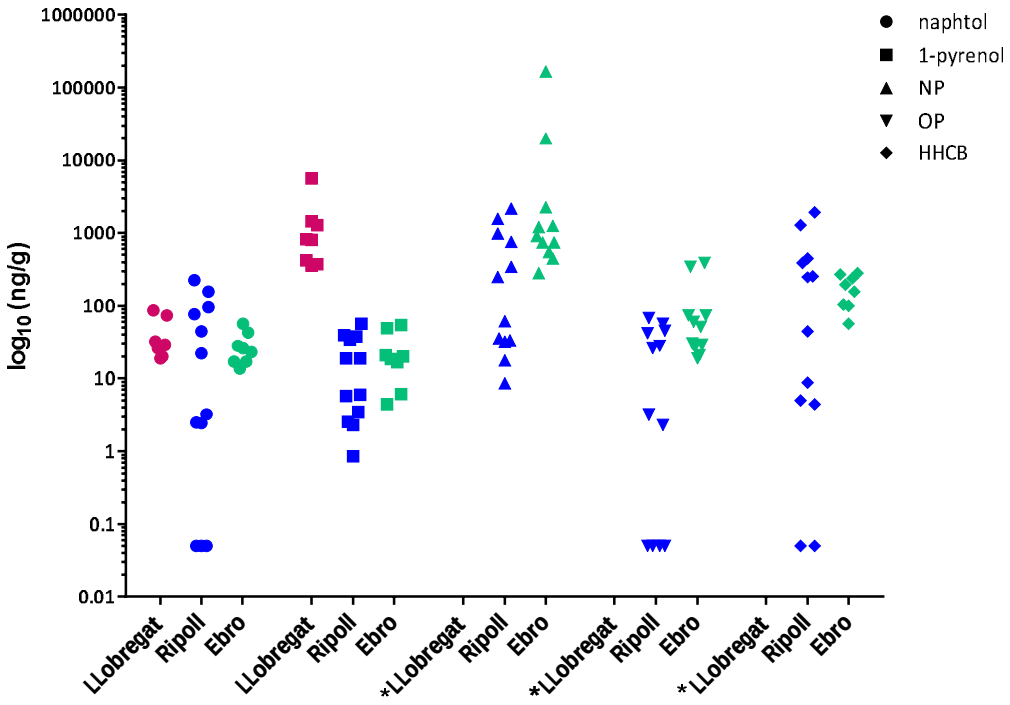


Figura 45. Niveles de metabolitos biliares (PAHs, NP, OP y HHCB) analizados por GC-MS-EI en peces capturados en ríos mediterráneos en Cataluña.
Datos reportados en Fernandes et al., (2002); Lavado et al. (2006); esta Tesis.
*Datos no disponibles.

Como consecuencia de la exposición a xenobióticos, los peces pueden ver afectados sus sistemas a través de varios mecanismos de acción. Por ejemplo, la exposición a xenobióticos no-polares conduce a la inducción de enzimas implicadas en metabolismo de Fase I (CYP1A y CYP3A) y de Fase II (UDPGT). El biomarcador EROD ha sido aplicado con éxito en peces en ríos mediterráneos para detectar alteraciones bioquímicas en CYP1A como consecuencia a la exposición de PCDDs, PCBs o PAHs (Whyte et al., 2000; Colin et al., 2016b). En el hígado de *R. rutilus* capturados aguas abajo de FL en el río Ebro durante la construcción del muro (octubre 2012), y de *B. meridionalis* y *S. laietanus* capturados en las zonas urbanizadas, se ha obervado una inducción de EROD de 2.5, 5 y 8 veces en comparación con los peces procedentes de las estaciones de referencia, respectivamente. Los valores de EROD fueron 9 y 5 veces inferiores en *R. rutilus* que en *B. meridionalis* y *S. laietanus*,

respectivamente, poniendo de manifiesto una menor exposición de estos peces a xenobióticos como PAHs y PCBs. De hecho, los niveles de EROD en *B. meridionalis* y *S. laietanus* del río Ripoll son más elevados que los detectados en hígado de barbos (36-80 pmol/min/mg prot) capturados en otros ríos mediterráneos contaminados por OCs (PCB, DDT, HCB y γ-HCH) y PAHs después de un derrame de hidrocarburos, lo que indica la alta exposición de los peces que habitan en las zonas urbanizadas del río Ripoll a agonistas de AhR que inducen los sistemas enzimáticos en los peces (Lavado et al., 2006; Damásio et al., 2007). Es importante destacar que la relación contaminante-efecto no es siempre detectable en estudios de campo, ya que existen muchos factores ambientales que pueden influir en las respuestas bioquímicas de los peces, incluyendo la presencia de metales que inhiben la actividad EROD y/o la temperatura. Es poco probable que la presencia de HHCB en el medio acuático produzca una inducción de la actividad EROD, ya que este biomarcador no se ha visto alterado significativamente después de la inyección de 50 mg/kg de HHCB en lubinas (Fernandes et al., 2013). En humanos, HHCB tampoco activa AhR (Schreurs et al., 2005).

BFCOD es una medida de la actividad del CYP3A inducida por esteroides, ácidos biliares y xenobióticos como fármacos. Los estudios de la actividad BFCOD en peces son escasos, aunque en los últimos años se está tomando en consideración como biomarcador de desintoxicación en peces en estudios ecotoxicológicos en campo. Por ejemplo, una significativa inducción de BFCOD (9,5 veces) se detectó en el hígado de barbos (*Barbus callensis*) como resultado del derrame de aguas residuales domésticas e industriales en la presa más importante de Argelia (Habila et al., 2017). En los análisis en el hígado de *R. rutilus* capturados en el río Ebro, CYP3A no ha mostrado diferencias entre las diferentes zonas

muestreadas ni durante la construcción del muro ni durante la extracción de sedimentos. Hay que tener en cuenta que los contaminantes presentes en los tejidos de un organismo no tienen porque ser necesariamente biológicamente activos. Por lo tanto, no es sorprendente que los residuos de tejidos no se correlacionen bien con las respuestas biológicas. Esto significa que no siempre es posible convertir directamente concentraciones en efectos o viceversa (Hylland et al., 2017). En el río Ripoll, BFCOD se ha inducido hasta 2 y 4 veces más en el hígado de *B. meridionalis* y *S. laietanus* procedentes de las zonas urbanizadas que en los peces de las zonas de referencia. Estudios recientes detectaron una inducción de BFCOD de hasta 4 y 9 veces en *Mullus barbatus* y barbos, respectivamente, muestreados en áreas contaminadas por PAH (ng/g) en comparación con áreas de referencia. En machos de *Oryzias latipes* se ha visto que HHCB modula los niveles de expresión de mRNA de *cyp3A40* (Yamauchi et al., 2008), mientras que en microsomas de hígado de carpas (*Cyprinus carpio*) la actividad CYP3A se ha visto inhibida por la exposición a HHCB (IC50 = 68 ± 12 µM) (Schnell et al., 2009). Estos resultados apuntan que HHCB y PAHs podrían alterar la actividad CYP3A, coincidiendo con los altos niveles de PAHs y de HHCB detectados en el río Ripoll en las zonas contaminadas. De hecho algunos autores sugieren BFCOD como un posible marcador general de inducción de CYP por PAH (Della Torre et al., 2010). La mayor inducción de BFCOD en el río Ripoll se detectó en los peces capturados a la salida de un efluente urbano, origen de fármacos y de HHCB en ríos. CYP3A está implicado en el metabolismo del 60% de fármacos en humanos, por lo que la inducción de BFCOD en este punto podría relacionarse con la presencia de estos compuestos.

En referencia a la actividad UGT, se ha observado un aumento significativo en *R. rutilus* capturados en el punto más cercano a la

desembocadura del río Ebro en 2013, de acuerdo con la mayor actividad de EROD observada en esta estación en 2012. AhR se une de manera promiscua a muchos compuestos xenobióticos y se activa por compuestos como dibenzo-*p*-dioxinas tetracloradas (TCDDs), algunos PCBs, PAHs y algunos pesticidas organoclorados (OCs), entre otros. En general, la desintoxicación de xenobióticos a través de la vía de señalización AhR incluye el metabolismo de desintoxicación de Fase I (CYP1A) y Fase II (UGT). En consecuencia, la respuesta de ambas actividades suele seguir la misma tendencia, aunque la respuesta de UDPGT a los contaminantes ambientales, incluidos los PAHs, parece menos pronunciada que la de Fase I, lo que convierte este bioenzima en un biomarcador menos adecuado de la exposición en peces (Van der Oost et al., 2003; Martínez-Gómez et al., 2009). En *B. meridionalis* y *S. laietanus*, este marcador no fue analizado, debido la baja actividad que se observó en las pruebas realizadas, principalmente en *S. laietanus*.

Complementariamente a los estudios en los enzimas de detoxificación, se analizaron indicadores de disrupción endocrina para evaluar la posible exposición de los peces a este tipo de compuestos. El análisis en gónadas de *B. meridionalis* muestreados en puntos urbanizados del río Ripoll, mostraron indicios de alteración endocrina, incluyendo un retraso en la maduración de machos y un aumento en la actividad aromatasa en los ovarios de las hembras. Estos resultados junto con un incremento en la expresión génica de VTG, 2 veces mayor detectada en el hígado de los machos de *B. meridionalis* de los sitios P con respecto a los machos procedentes de las áreas R (datos no publicados), indican efectos de alteración endocrina en esta especie. Acorde con estos resultados, concentraciones de NP y OP (856-7843 ng/g y 21-153 ng/g, respectivamente) se detectaron en la bilis de *B. meridionalis* en el rango en

que estos compuestos promueven efectos de disrupción endocrina en machos de *Chelon labrosus* en costas del Norte de la Península Ibérica, incluyendo una alta prevalencia de intersex en gónadas y niveles elevados de VTG y de mRNA *vtg* (Puy-Azurmendi et al., 2013). La actividad aromatasa se detectó en todas las hembras de *B. meridionalis*, mostrando una estrecha relación con el desarrollo y maduración de las hembras (Guiguen et al., 2010). En muchos teleósteos, la P450-aromatasa aumenta durante la vitelogénesis, como por ejemplo en ovarios de *Labeo rohita* donde los niveles más altos de expresión de aromatasa se encontraron en la etapa vitelogénica (Mills et al., 2014; Moulik et al., 2016). De acuerdo con estos datos, las hembras de *B. meridionalis* en etapas de maduración avanzadas con ovocitos llenos de VTG, han mostrado la mayor actividad de P450-aromatasa (12 pmol/h/min). Algunos estudios han mostrado inhibición de la actividad aromatasa en carpas muestreadas aguas abajo de una planta de tratamiento de aguas residuales, en *Salvelinus fontinalis* expuestos a lixiviados de un vertedero de basura, y en carpas expuestas a altos niveles de NP (Noaksson et al., 2005; Lavado et al., 2006; Martin-Skilton et al., 2006). Sin embargo, en hembras de *B. meridionalis* capturadas en sitios urbanizados se ha observado una inducción significativa en la actividad CYP19, sugiriendo estar expuestas a efluentes domésticos, agrícolas e industriales que contenían compuestos similares a los estrógenos (por ejemplo: E2, EE2, APs). En *R. rutilus* del río Ebro no se han observado alteraciones en gónadas, ni evidencias de retraso en la maduración de los peces. En estudios previos en este río, se observó la presencia de células inmaduras en el lumen, agregados de macrófagos en los testículos y reducción en los niveles de T y E2 en el plasma de carpas macho, mientras que en hembras se reportó un retraso en la maduración (Lavado et al., 2006). Además, altas concentraciones de PCBs (95 ng/g w.w.), DDTs (29 ng/g w.w.) y HCB (1.65 ng/g w.w.) se detectaron en el

músculo de estas carpas, por lo que los autores sugirieron que no sólo los APs, sino también los OCs podrían ser responsables de las alteraciones endocrinas significativas detectadas (Lavado et al., 2004). La presencia de PAHs y sus mezclas también puede afectar al desarrollo y al proceso de maduración en peces (Le Bihanic et al., 2014a,b). Ciertamente, los niveles biliares de NP y OP han sido 3 y 5 veces más bajos en *R. rutilus* que en carpas, respectivamente, mientras que los residuos de OCs en el músculo, incluyendo PCBs, HCBs y DDTs fueron hasta 43, 2 y 8 veces más bajos en *R. rutilus* que en carpas, respectivamente, lo que indica una exposición más reducida a PAHs y OCs en *R. rutilus* en comparación con las carpas.

Tabla 20. Respuestas de los biomarcadores químicos y bioquímicos en las especies de peces analizados en esta Tesis, en zonas de referencia y en zonas impactadas por actividades humanas.

Tejido	Biomarcador	*R. rutilus* 2012	*R. rutilus* 2013	*B. meridionalis*	*S. laietanus*
Zonas de referencia					
Músculo	∑DDT	29 ± 3	34 ± 4	n.a	n.a
	∑PCBs	29 ± 2	46 ± 4	n.a	n.a
	∑HCH	2 ± 1	4 ± 1	n.a	n.a
Bilis	PAHs	9 ± 7	10 ± 6	7 ± 2	3 ± 0.4
	APs	391 ± 97	2702 ± 1572	21 ± 4	44 ± 16
	HHCB	99 ± 13	117 ± 44	17 ± 2	3 ± 0.4
Hígado	EROD	6 ± 1	5 ± 0.7	29 ± 2	10 ± 1
	BFCOD	81 ± 6	80 ± 6	161 ± 10	54 ± 2
	UDPGT	311 ± 40	341 ± 22	n.a	n.a
Gónada	Aromatasa	3.4 ± 1	0.8 ± 0.3	1.7 ± 0.13	n.a
Zonas impactadas					
Músculo	∑DDT	598 ± 65	158 ± 27	n.a	n.a
	∑PCBs	469 ± 44	184 ± 30	n.a	n.a
	∑HCH	5 ± 1	1 ± 1	n.a	n.a
Bilis	PAHs	41 ± 21	26 ± 12	177 ± 33	98 ± 25
	APs	5894 ± 4811	3318 ± 1942	1013 ± 180	1088 ± 176
	HHCB	281 ± 44	222 ± 30	1230 ± 2	98 ± 0.4
Hígado	EROD	12 ± 1	7 ± 0.3	153 ± 8	71 ± 6
	BFCOD	91 ± 4	97 ± 4	391 ± 13	201 ± 9
	UDPGT	357 ± 21	404 ± 15	n.a	n.a
Gónada	Aromatasa	1.7 ± 0.3	1.6 ± 0.2	5.5 ± 0.7	n.a

n.a: no analizado
PAHs = ∑naftol+1pirenol. APs = ∑NP+OP.
Río Ebro (*R. rutilus*): zona de referencia = RR; zonas impactadas = promedio (FL, BE, A).
Unidades: DDTs,PCBs, HCH, PAHs, APs, HHCB: ng/g tejido.
EROD, BFCOD, UDPGT, aromatasa: pmol/min/mg proteína

Los avances recientes en lipidómica han permitido detectar un nuevo grupo de compuestos xenobióticos conocidos como disruptores lipídicos, capaces de producir efectos en el metabolismo lipídico de células, tejidos, plantas y animales (Cajka and Fiehn, 2014). El estudio de lípidos se incorporó en los análisis en peces muestreados en el río Ripoll, para determinar efectos en el perfil lipídico de los peces que habitan zonas contaminadas. Este análisis no se llevó a cabo en el músculo *R. rutilus* en el

río Ebro, puesto que al ser un método experimental en proceso de puesta a punto, se prefirió trabajar con las especies de *B. meridionalis* y *S. laietanus* que habían mostrado importantes diferencias significativas en los biomarcadores de exposición y de disrupción endocrina.

El análisis UHPLC-ToF ha permitido la detección de aproximadamente 130 lípidos y la caracterización del perfil lipídico en el músculo de *B. meridionalis* y *S. laietanus*. La principal subclase de lípidos presente en el músculo de los peces son PCs (62 y 44%, en *B. meridionalis* y *S. laietanus*, respectivamente). Este fosfolípido es el más abundante en células eucariotas (45-55%) y en otros tejidos de peces como el hígado (Lie and Lambertsen, 1991; Vance and Steenbergen, 2005; Bohdanowicz and Grinstein, 2013). Los peces de ambas especies muestreados en los sitios contaminados mostraron una reducción en las PCs con un alto número de dobles enlaces (5-9). Esta disminución se relaciona con procesos de oxidación, llevadas a cabo por especies reactivas de oxígeno (ROS) generadas como subproductos del metabolismo de xenobióticos, como PAHs (Damásio et al., 2007; Sheriff et al., 2014; Cífková et al., 2015). Los ácidos grasos poliinsaturados (PUFAs) representan la diana principal de la oxidación no enzimática por radicales libres y moléculas ROS, siendo los fosfolípidos que contienen PUFAs los más susceptibles a la oxidación (Bochkov et al., 2010; Luczaj et al., 2017). Los fosfolípidos de los peces están formados por altas cantidades PUFAs y por ello son altamente susceptibles al deterioro oxidativo (Mourente et al., 2007). PC-P es otra subclase de lípidos altamente vulnerable al estrés oxidativo; sirviendo como antioxidantes endógenos y representando el grupo principal de PUFAs en las membranas celulares (Engelmann, 2004; Braverman and Moser, 2012). A pesar de no representar una de las subclases de lípidos más abundantes en el músculo de *B. meridionalis* y *S. laietanus* (5 y 4%,

respectivamente), el análisis estadístico univariante apunta a algunas moléculas de PC-P como unas de las especies lipídicas con mayores diferencias entre peces de sitios contaminados y de referencia.

La segunda subclase de lípidos más abundante en el músculo de los peces son TGs (23 y 37% en *B. meridionalis* y *S. laietanus*, respectivamente). En peces teleósteos, la composición de TGs puede depender del estado nutricional, temperatura y factores fisiológicos, como el sexo y la reproducción (Bogevik et al., 2011; Maradonna et al., 2015). Alteraciones significativas de TGs se han observado en peces después de la exposición a compuestos químicos como TBT, organofosfatos, herbicidas, BPA y fibratos (Capitão et al., 2017; Pinto Persch et al., 2017). La cantidad de TGs ha aumentado 2,5 veces en ovarios de *Gobiocypris rarus* después de la exposición a 0.015 mg/L de BPA (Zhang et al., 2016); tras la exposición a 0.5 mg/L de difenoconazol en embriones de pez cebra (Mu et al., 2016); y en plasma de bacalao atlántico después de la exposición a 2 mg/kg de PCB-153 (Yadetie et al., 2017). Otros organismos acuáticos, como mejillones capturados en sitios contaminados, también han mostrado un aumento en concentraciones de TGs (Capuzzo and Leavitt, 1988; Chetty and Indira, 1994; Bergen et al., 2001). *S. laietanus* capturados en zonas urbanizadas del río Ripoll han mostrado un aumento significativo de TGs formados por un bajo número de dobles enlaces, mientras que en *B. meridionalis* los TGs poliinsaturados han sufrido una disminución, probablemente relacionada con la oxidación de los PUFAs.

La tercera subclase más abundante en el músculo de *B. meridionalis* y *S. laietanus* son CEs (6 y 9%, respectivamente). *B. meridionalis* procedentes de áreas urbanizadas del río Ripoll acumularon CEs en el músculo. Xenobióticos incluyendo PFNA, APs y E2 han alterado los niveles de CE en hígado de pez cebra y cerebro de *Gadus morhua*

(Meier et al., 2007; Zhang et al., 2012). Algunos autores han relacionado el aumento de TGs y CEs con un aumento en la demanda de energía para mediar los efectos del estrés en los peces.

4.2. Combinación de biomarcadores para la evaluación de los efectos de drospirenona en juveniles de lubina (*Dicentrarchus labrax*).

Continuamente están apareciendo nuevos compuestos emergentes de los que se desconocen sus potenciales efectos en organismos. Los experimentos *in vivo* en laboratorio mediante ensayos de exposición en condiciones controladas, son una buena herramienta para validar estudios preliminares realizados *in vitro*. En el capítulo IV de esta Tesis se combina el uso de biomarcadores implicados en el metabolismo de xenobióticos y biomarcadores de disrupción endocrina para evaluar los efectos de DRO, un progestágeno sintético ampliamente utilizado en tratamientos hormonales en países europeos, en juveniles de lubina que se encontraban en un período sensible de la diferenciación sexual (Besse and Garric, 2009; Liu et al., 2011), para validar resultados previos realizados *in vitro* en fracciones mitocondriales de gónadas de carpa (*Cyprinus carpio*) donde DRO inhibió las actividades CYP17 y CYP11B implicadas en la síntesis de esteroides (Fernandes et al., 2014a). Pese a su alto consumo, sólo un estudio (lago Balatón, Hungría) ha reportado concentraciones de DRO en el medio acuático (0.66 ng/L) y escasos estudios existen sobre sus efectos en organismos acuáticos (Avar et al., 2016).

La lubina es la especie de teleósteo marino con más importancia comercial cultivada en acuicultura en las regiones mediterráneas (FAO, 2017). Su selección en este estudio como especie centinela se basa en el

conocimiento que se tiene de su morfología, fisiología y reproducción, así como de los niveles de esteroides sexuales a lo largo del ciclo de maduración y de la expresión de genes implicados en la síntesis de esteroides (Blázquez et al., 2004; Blázquez et al 2005; Navarro-Martín et al., 2009).

Los resultados obtenidos en plasma de lubina después de 2, 4, 8, 16 y 31 días de exposición han confirmado la ingesta de DRO a través de la dieta y su posterior distribución en el organismo. Sin embargo, no se han evidenciado efectos de bioconcentración en el plasma, sugiriendo que DRO se metaboliza rápidamente, acorde con lo observado previamente en mejillones después de 2 semanas de exposición a 100 mg de DRO/L, donde se estableció un factor de bioconcentración de DRO relativamente bajo (BCF: 36), y en humanos (Gilroy et al., 2014). Sin embargo, a pesar de la evidencia de un metabolismo de DRO en juveniles de lubina, las actividades CYP1A, CYP3A y UGT determinadas en las fracciones microsomales del hígado no se han visto significativamente alteradas, lo que sugiere que el metabolismo de DRO se produce independientemente del sistema CYP. De hecho, en humanos DRO se metaboliza principalmente en el hígado a 4,5-dihidrodrospirenona-3-sulfato y a la forma ácida que se genera por la apertura del anillo de lactona; estos metabolitos no son farmacológicamente activos y se forman independientemente del sistema CYP (Krattenmacher, 2000). Sin embargo, algunos estudios indican que el isoenzima CYP3A4 metaboliza DRO en microsomas hepáticos humanos, aunque en un porcentaje bajo (4-7%), y la inhibición de CYP3A4 puede aumentar moderadamente la concentración de DRO (Wiesinger et al., 2015).

La exposición a DRO no afectó al peso, talla y factor de condición de los peces, independientemente de la concentración o tiempo de

exposición. Del mismo modo, tampoco se observaron cambios morfométricos significativos en pez cebra (*Danio rerio*) expuestos hasta 5 µg/L de DRO durante 14 días, a pesar de producir una disminución significativa del índice gonadosomático (GSI), una fuerte inhibición del ARNm VTG y una la alteración en la transcripción del gen *cyp19a1a* (Zucchi et al., 2014). No se observaron alteraciones en la expresión de los genes *cyp19a1a* y *cyp11β* en gónadas de juveniles de lubina en nuestro estudio, aunque sí se detectó una expresión 2 veces superior de *cyp17a1* en ovarios de hembras expuestas. *Cyp17a1* codifica para una proteína que tiene las actividades 17α-hidroxilasa y 17,20-liasa y cataliza la conversión de P4 y P5 en sus productos 17α-hidroxilados y posteriormente en DHEA y AD. Aunque nuestros hallazgos deben interpretarse con precaución, ya que las hembras de todas las concentraciones de exposición han sido agrupadas debido a la falta de n muestral, es interesante observar que de acuerdo con una activación de *cyp17a1*, los niveles plasmáticos de 17P4, 17P5 y AD fueron significativamente más altos en lubinas expuestas a 0,01 µg DRO/g de pienso durante 31 días. Por el contrario, los niveles de T, P4 y P5, hormonas en cuya síntesis el isoenzima CYP17a1 no está involucrado, no se vieron alterados (Fig. 46).

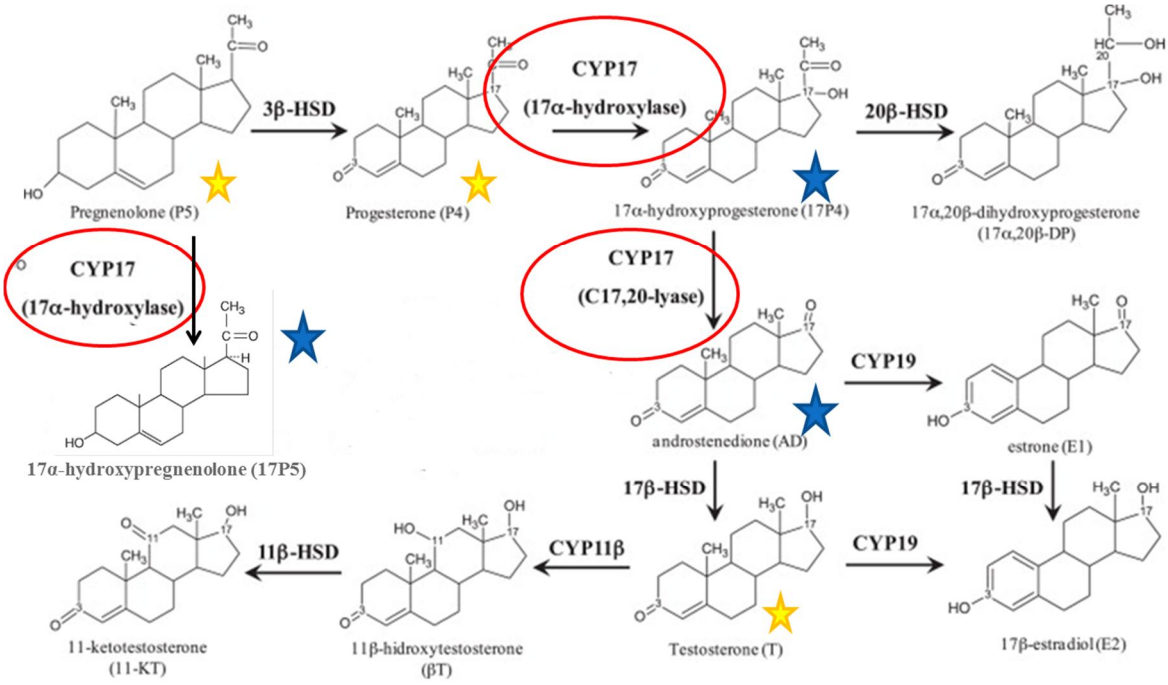


Figura 46. Vías metabólicas involucradas en la biosíntesis de esteroides y afectadas por exposición a DRO en juveniles de lubinas. Círculos rojos: vías metabólicas estudiadas mediante la expresión de genes. Estrellas azules: esteroides alterados por la exposición a DRO. Estrellas amarillas: esteroides no afectados por la exposición a DRO.

Otros estudios tampoco han detectado efectos de DRO en los niveles de T y 11-ketotestosterona en plasma después de 31 días de exposición en machos de *Pimephales promelas* (Runnalls et al., 2013). Zeilinger et al. (2009) tampoco observaron masculinización en *P. promelas* expuestos a 70 µg/L de DRO. En conjunto, estos hallazgos están de acuerdo con el hecho de que el DRO no es un agonista de receptores de andrógenos de peces (ARα y ARβ), sino un agonista débil del receptor de P4 (Ellestad et al., 2014; Bain et al., 2015). Como tal, DRO podría tener una acción similar a la P4 a concentraciones relativamente bajas.

Con este estudio también se ratifica que el análisis de fármacos en plasma de peces puede ser una excelente herramienta para evaluar el riesgo de efectos farmacológicos, ya que las concentraciones plasmáticas pueden

compararse con dosis terapéuticas humanas (Fick et al., 2010). En el presente estudio, sólo las lubinas del grupo de alta exposición (10 mg/kg DRO) presentaron dosis plasmáticas de DRO (5-27 ng/mL) relativamente próximas a las concentraciones terapéuticas humanas (20-25 ng/mL, máximo 60-87 ng/mL). Los peces del grupo de baja exposición (0.01 mg/kg DRO), con concentraciones plasmáticas de DRO muy por debajo de las dosis terapéuticas humanas, mostraron aumento de 17P4, 17P5 y AD en plasma después de 31 días de exposición. Estos resultados, junto con la inducción en la expresión de *cyp17a1* en hembras, evidencian la capacidad del DRO para alterar la síntesis de esteroides en juveniles de lubinas tras una exposición de 7 ng de DRO/día (10 ng/g x 0,7 g de alimento/día). Sin embargo, las consecuencias en términos de alteración del desarrollo y/o reproducción en adultos, todavía se desconocen.

4.3. Uso de sistemas *in vitro* como herramientas para la evaluación toxicológica de sedimentos y de alteraciones lipídicas en líneas celulares.

Leyes como la prohibición del uso de animales en experimentación en la industria cosmética en el año 2009 han contribuido a la implantación de nuevas prácticas en el laboratorio y la consecuente reducción del número de animales utilizados en estos años desde 1,5 millones a 800.000. Con esta premisa, las líneas celulares se han convertido en herramientas útiles en estudios de toxicología ambiental. Aunque con ciertas limitaciones en la extrapolación de resultados, las líneas celulares nos permiten evaluar de una forma rápida, sencilla, barata y con menor generación de residuos, diferentes baterías de ensayos para evaluar efectos y mecanismos de acción de contaminantes aislados y/o sus mezclas (Bols et al., 2005).

Los sedimentos son matrices interesantes des del punto de vista ambiental, debido a que actúan como reservorio de los contaminantes que llegan a los sistemas acuáticos. La caracterización del riesgo de muestras complejas, (ej. extractos de sedimentos), requiere del uso de múltiples bioensayos para determinar diferentes modos de acción de la mezcla de contaminantes presentes en la matriz. En esta Tesis se ha investigado el impacto de la contaminación en la zona costera de Split (Croacia) a través del análisis de los sedimentos marinos mediante el uso de múltiples bioensayos en 3 tipos de sistemas celulares: células PLHC-1, células COS-7 transfectadas con plásmidos PXR de pez cebra y levaduras de la cepa BY4741 modificadas genéticamente con el receptor de estrógenos humano.

El uso de la línea celular PLHC-1, permitió detectar citotoxicidad significativa en extractos de sedimentos de una estación situada en una zona industrial afectada por el Puerto de Split y la desembocadura del río Jadro (S1B). Los extractos de sedimentos de otras estaciones de la bahía de Kaštela (S1C, S3, S4) también afectaron a la viabilidad celular, mientras que los extractos de sedimentos muestreados a lo largo del canal de Brač no mostraron una citotoxicidad significativa en las células PLHC-1. Cabe señalar que la citotoxicidad en los sedimentos procedentes de la bahía de Kaštela se ha observado a la concentración más altas (60 mg eQsed/mL) que las utilizada en el resto de análisis (inducción de EROD y zfPxr y ROS), ya que este parámetro se implementa más como una medida de control de calidad para verificar que la mortalidad celular no afecta a las respuestas de los otros bioensayos, que como un marcador en sí (Escher et al., 2013). También los sedimentos de la estación S1B han mostrado la mayor actividad EROD (R_{BNF}: 4,9 mg eQsed/mL) en las células PLHC-1 en comparación con el resto de sedimentos de la bahía de Kaštela (R_{BNF}: 17-32 mg eQsed/mL) o el Canal de Brač (R_{BNF} > 60 mg eQsed/mL). Los

extractos de sedimentos de la bahía de Kaštela también han mostrado mayor potencia de activaciónde zfPxr y mayor contenido de agonistas de este receptor (Emax: 19 veces, REC_{20}: 0.9 mg eQsed/mL) que los del canal de Brač. Estos biomarcadores muestran un gradiente de dilución de contaminantes desde la parte oriental de la bahía de Kaštela, caracterizada por un mayor impacto de actividades humanas, aguas más confinadas, con menor tasa de renovación, hacia las aguas del canal del Brac, que son más abiertas al mar Adriático y están influenciadas por las corrientes marinas. Una respuesta gradual también se ha observado en ensayos de CYP1A, activación de zfPxr y generación de estrés oxidativo en el Delta del Danubio a favor de las corrientes marinas en el Mar Negro (Pérez-Albaladejo et al., 2016).

La detección de agonistas de AhR, zfPxr y compuestos citotóxicos en los sedimentos de la bahía de Kaštela está relacionado con las concentraciones de hasta 100 veces mayores de PCB, DDT y HCB (35,4, 1,4 y 0,7 ng/g d.w.) detectadas en la estación S1B en comparación con las cuantificadas en el canal de Brač. La información proporcionada por el análisis químico en este trabajo es limitada y otros compuestos podrían haber contribuido a las respuestas observadas en los bioensayos, como por ejemplo PAHs, que se han detectado en concentraciones bastante elevadas en los sedimentos de las estaciones S1B (2,3 μg/g d.w.) y S1C (0.5 μg/g d.w.) en la bahía de Kaštela, como consecuencia del tráfico naval y las descargas de aguas residuales industriales a esta zona más confinada y relativamente poco profunda (Mandić and Vrančić, 2017). Además de los xenobióticos, las sustancias orgánicas naturales también pueden unirse a AhR y zfPxr, por lo que su posible contribución a las respuestas observadas en los ensayos de EROD y zfPxr no puede descartarse. Sin embargo, a partir de nuestra experiencia previa y dado que la actividad inducida por los

extractos de sedimentos recogidos en áreas limpias es cercana al blanco, se sugiere que esta contribución es generalmente muy baja.

Por el contrario, el ensayo de levadura recombinante no permitió detectar la presencia de compuestos estrogénicos en los extractos de sedimentos de esta zona. De forma similar, Schnell et al. (2013) no encontró estrogenicidad en sedimentos de estuarios y zonas costeras del norte de España, con excepción de los impactados directamente por un efluente urbano no depurado, que mostró una actividad estrogénica débil. Sedimentos de las costas del mar Adriático y del mar Negro tampoco mostraron respuesta estrogénica en el ensayo ER-RYA (resultados no publicados), a pesar de la capacidad de estos extractos de sedimentos para inhibir la actividad de la aromatasa ovárica en la fracción microsomal de lubina (Pérez-Albaladejo et al. 2016). Por lo tanto, aunque la concentración de compuestos estrogénicos en los extractos de sedimentos del área estudiada están por debajo del límite de detección del ensayo ER-RYA (0,14 ng EEQ/g de sedimento d.w.), no se puede descartar la presencia de compuestos que puedan actuar como disruptores endocrinos que modulen la actividad de enzimas implicadas en la síntesis o metabolismo de esteroides (Fernandes et al., 2014b).

El uso de bioensayos basados en células (citotoxicidad, ROS, EROD, ER e inducción de zfPxr) combinados con calibradores internos permite la comparación de sedimentos de diferentes áreas de la costa mediterránea (Tabla 15, Capítulo V.I). Las muestras de sedimentos del canal de Brač presentan un estado ambiental óptimo en términos de ausencia de compuestos citotóxicos y niveles muy bajos de inductores de CYP1A y de agonistas de zfPxr (R_{BNF} y REC_{20} > 60 mg eQsed/mL), siendo una de las zonas más limpias en comparación con el mar Negro y el mar Adriático (Pérez-Albaladejo et al., 2016). Por el contrario, los sedimentos

de la bahía de Kaštela, en particular de la estación S1B, muestran el mayor contenido de inductores CYP1A y agonistas zfPxr, análogos a los valores registrados en el puerto de Constanta y en la desembocadura del río Danubio (R_{BNF} =3-14 mg eQsed/mL; REC_{20} = 2-7 mg eQsed/mL) (Pérez-Albaladejo et al., 2016). Aunque se necesitan más estudios, la activación de PXR se plantea como un potencial biosensor ambiental.

Los avances en nuevas metodologías de análisis como la cromatografía y la bioinformática, están permitiendo actualmente grandes progresos en la detección de disruptores lipídicos, para conocer sus mecanismos de acción y llegar a consolidar biomarcadores de alteración de lípidos. En el apartado V.II de esta Tesis se analiza el perfil lipídico de las células PLHC-1 derivadas de hepatocarcinoma de la especie de pez *Poeciliopsis lucida* cultivadas con diferentes medios y expuestas a agonistas de los receptores lipídicos PPARγ y RXR para caracterizar el lipidoma de esta línea celular y comprobar su eficacia como modelo *in vitro* en la alteración lipídica y plantear posibles biomarcadores lipídicos.

El análisis por FIA acoplado a un analizador Orbitrap permitió la detección de aproximadamente 130 lípidos y la caracterización del perfil lipídico de las células. Según organizaciones como LIPID Maps se estima que existen unos 10.000-100.000 lípidos. Aunque nuestros métodos nos han permitido cuantificar de una forma fiable ya unos 200 lípidos, aún queda mucho recorrido para llegar a poder cuantificar todo el lipidoma de los organismos o matrices acuáticas. Las células PLHC-1 están formadas principalmente por PC, fosfolípido más abundante en células eucariotas (45-55%), seguido de PE, PI, PE-P y TG (Bohdanowicz and Grinstein, 2013). Los FA más abundantes fueron los FA considerados fisiológicamente más relevantes e incluyen el 16:0 (ácido palmítico), 18:0 (ácido esteárico) y 18:1 (ácido oleico) (20, 53 y 12%, respectivamente).

El FBS se considera un suplemento indispensable en el cultivo celular, sin embargo, en los últimos años organizaciones internacionales recomiendan sustituir o reemplazar el uso de FBS en cultivos celulares. El FBS está formado por FA, TG, fosfolípidosy CE, entre otros, y su reducción en el cultivo celular puede alterar el metabolismo de los lípidos (Brunner et al., 2010; Gregory et al., 2011). La concentración de FBS en los medios de cultivo celular varía de 2 a 20%, (Karnieli et al., 2017) y las células PLHC-1 se cultivan en medio suplementado con FBS al 5%, consideradas las condiciones de crecimiento óptimas en estas células. Las células PLHC-1 cultivadas en condiciones de restricción de FBS (1 y 0% de FBS) sufriron una disminución progresiva de todas las especies de lípidos detectadas, posiblemente relacionada con la disminución del número de células cultivadas en estos medios (reducción de hasta 1.7 veces en 0% FBS), lo que implica una disminución de las membranas celulares y de los lípidos que las forman. En células madre derivadas de tejido adiposo bovino del tejido subcutáneo incubado durante 14 días en ausencia de FBS, también se ha observado una disminución en el número absoluto de núcleos celulares (Sandhu et al., 2017). Sin embargo, TG y PI fueron las familias que relativamente más diminuyeron en las células cultivadas en 1 y en 0% de FBS, mientras que además, algunos lípidos formados por etanolaminas, también sufrieron una reducción relativa en cultivos sin suero. Esto sugiere que al reducir las concentraciones de FBS, la principal subclase de lípidos afectada es TG, seguido de PI y de algunos lípidos formados por etanolaminas. Varios estudios han informado que los TGs son una de las subclases de lípidos más susceptibles a factores externos. Las células cultivadas en condiciones de restricción con FBS pueden estar bajo estrés, ya que la falta de suero afecta la unión, el crecimiento y la proliferación de las células. En consecuencia, las células podrían redistribuir la energía e intentar adaptarse a estas condiciones. Los TG, como la principal subclase

lipídica involucrada en el almacenamiento de energía, son los primeros en movilizarse en muchas especies de peces cuando cambian las condiciones ambientales, siendo su metabolismo y síntesis equilibrados dinámicamente en función de los requerimientos de energía celular (Sargent et al., 1989; Dulloo et al., 2004; Van Dijk et al., 2005). Algunos estudios reflejan que la principal fuente de FA en el medio de cultivo viene proporcionada por el FBS, incluyendo FA 16:0, 18:0/1/2, 20:3/4, 22:5/6 (Tocher et al., 1998; Brunner et al., 2010; Gregory et al., 2011). La ausencia de FBS en el cultivo de PLHC-1 produjo la disminución de FA 18:0/1/2, 20:2/3/4, 22:2/3/5/6.

Las células PLHC-1 cultivadas en DPBS suplementado con 0.2% glucosa sufrieron una disminución general de todos los lípidos, con la excepción de TGs, relacionado con la falta de viabilidad celular y la significativa disminución en el número de las células PLHC-1. Estos resultados sugieren que la viabilidad de las células está relacionada no sólo con la presencia de FBS, sinó también de otras biomoléculas y sales incluidas en los medios de cultivo tradicionales. Sin embargo, pese a su cultivo en condiciones no óptimas, las células mostraron la capacidad de formar TG (46: 1, 48: 1, 50: 1, 52: 1), a partir de aporte de 0.2% glucosa. Aunque las vías que regulan el metabolismo de TG hepáticos todavía no se conocen por completo, se sabe que los lípidos que se forman en el hígado provienen principalmente del exceso de glucosa vía lipogénesis que conducen a la síntesis *de novo* de FA y luego se esterifican para formar TG (Quiroga and Lehner, 2012). Los FA 18:2, 20:0 y 22:0 se acumularon significativamente en células cultivadas en DPBS-GLU., lo que sugiere la capacidad de las células PLHC-1 para sintetizar estos FA a partir de glucosa y almacenarlos en TG, específicamente TG 46:1, 48:1, 50:1 y 52:1.

El hígado juega un papel central en el mantenimiento de la homeostasis lipídica sistémica. Alteraciones en la lipogénesis, oxidación de lípidos y/o el metabolismo de lipoproteínas en hígado pueden favorecer la obesidad, la dislipidemia y la acumulación de gotas de lípidos en el hepatocito (esteatosis hepática). El AR, principal metabolito bioactivo de la vitamina A, se almacena y metaboliza principalmente en el hígado (O'Byrne and Blaner, 2013). Los mecanismos de acción de 9-*cis*-RA están mediados por RAR y RXR activados por ligando, como homodímero o heterodímero con otros receptores nucleares (He et al., 2013). Específicamente, se ha demostrado que 9-*cis*-RA muestra alta afinidad por RXR (Levin et al., 1992). En consecuencia, el retinoide podría modular el metabolismo de los lípidos. Sin embargo, los efectos del 9-*cis*-RA sobre el metabolismo de los lípidos y las lipoproteínas hepáticas son controvertidos (Bonet et al., 2012). Algunos estudios han reportado acumulación de TG en células de preadipocitos humanos (células AML-I) y ratones tratados con 9-*cis*-RA (Vu-Dac et al., 1998; Sedova et al., 2004; Morikawa et al., 2013). Otros estudios especifican que el aumento en los niveles de TG se produce en respuesta a altas dosis de 9-*cis* RA en roedores (Sofonova, 1994; Reddonet et al., 2008). Sin embargo, los efectos más comunes reportados después del tratamiento con 9-*cis*-RA son la reducción del contenido de lípidos y de TG. 9-*cis*-RA (100 nM) reduce la acumulación de lípidos e inhibe la adipogénesis en células de preadipocitos de ratón 3T3-L1 (Sagara et al., 2013). Amengual et al. (2010) mostraron oxidación de lípidos, inhibición de la biosíntesis, reducción de la grasa corporal y reducción del contenido de TG en el hígado de roedores. En nuestro estudio, las células PLHC-1 tratadas durante 24 h con 10 μM de 9-*cis*-RA disminuyeron de forma significativa el contenido de TG. Los resultados sugieren que la respuesta de las células PLHC-1 a 9-*cis*-RA podría suceder a través de la activación del receptor RXR implicado en el metabolismo de los lípidos.

ROSI se utiliza clínicamente para mejorar la sensibilidad a la insulina en el tratamiento de la diabetes tipo II y cambios en los niveles FAs y TG se ha observado en ratones en plasma, tejido adiposo (Berger and Moller, 2002; Jin et al., 2011; Guo et al., 2013). Sin embargo, ROSI no afecta a la acumulación de lípidos, como TG yCE, y a la expresión de genes lipogénicos en el hígado de ratones ((Vyas et al., 2014; Wang et al., 2017). Del mismo modo, no se han observado alteraciones lipídicas en células PLHC-1 expuestas a 10 μM de ROSI en nuestro estudio. ROSI actúa principalmente activando PPARγ, que se expresa principalmente en el tejido adiposo y está implicado en el metabolismo lipídico del tejido adiposo (Liss and Finck, 2017). La no alteración en los niveles de lípidos en las células PLHC-1 sugiere que PPARγ no se expresa en estas células. Aunque, Caminada and Fent (2008) informaron en un resumen de conferencia la presencia de PPARγ en células PLHC-1, no se han publicado más datos en revistas científicas, lo que sugiere que esta información no está completamente confirmada.

Este trabajo proporciona información sobre el lipidoma de las células PLHC-1 en diferentes condiciones de cultivo, y plantea el uso de esta línea celular como una herramienta para detectar alteración en el metabolismo de lípidos por exposición a contaminantes. Sin embargo, todavía no se han podido establecer biomarcadores que ayuden a relacionar cambios en el perfil lipídico de PLHC-1 con efectos en el sistema celular. El estudio de biomarcadores lipídicos se está aplicando con gran énfasis en el campo de la biomedicina para establecer relaciones con enfermedades relacionadas con alteraciones lipídicas como Alzheimer o cáncer. Así por ejemplo la oxidación de las PCs PC(16:0,9:0(COOH)), PC(18:0,5:0(COOH)) y PC(16:0,8:0(COOH)) se sugiere como biomarcador en pacientes con

enfermedades de tejidos grasos, por actuar como uno de los mecanismos fisiopatológicos (Najdekr et al., 2015).

Los resultados de esta Tesis reflejan que la combinación de biomarcadores de exposición a xenobióticos y biomarcadores de disrupción endocrina, junto con nuevos estudios en lípidos es una herramienta óptima y necesaria en la evaluación del riesgo ambiental en sistemas acuáticos, en experimentos de exposición y estudios de campo. Además, pone de manifiesto la efectiva aplicación de bioensayos celulares en estas evaluaciones que permitirá en el futuro reducir considerablemente el uso de organismos en Toxicología Ambiental.

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5. CONCLUSIONES

Conclusiones

I. El uso integrado de biomarcadores químicos, incluyendo compuestos organoclorados en músculo y metabolitos en bilis, y de biomarcadores bioquímicos, incluyendo enzimas de biotransformación, activación de receptores, expresión de genes, cambios histológicos y análisis de esteroides en plasma, constituyen una herramienta útil para la evaluación del impacto de compuestos xenobióticos en peces expuestos en laboratorio y en estudios de campo.

II. El uso de estos biomarcadores en el río Ebro ha permitido detectar la liberación de contaminantes organoclorados atrapados en los sedimentos de Flix y su acumulación en el músculo de *Rutilus rutilus* aguas abajo durante la construcción del muro de contención; mientras que, durante el dragado de sedimentos, los niveles de compuestos organoclorados fueron los más bajos reportados desde los primeros estudios en la zona.

III. El análisis de metabolitos biliares, la inducción de las enzimas CYP1A y CYP3A en *B. meridionalis* y *S. laietanus* y el incremento de actividad aromatasa junto con el retraso en la maduración de las gónadas en *B. meridionalis*, indican que los peces del río Ripoll están altamente expuestos a contaminantes (PAHs, APs y HHCB) y que los peces están sometidos a efectos de disrupción endocrina.

IV. El análisis por UHPLC-TOF del músculo de *B. meridionalis* y *S. laietanus* ha discriminar los perfiles lipídicos de los peces procedentes de áreas contaminadas y áreas de referencia. Los resultados indican una oxidación de las PCs más insaturadas, junto con un aumento de lípidos neutros (TGs y CEs) en peces de áreas contaminadas.

V. La exposición de juveniles de lubina a drospirenona a través de la dieta ha producido inducción de la expresión del gen *cyp17a1* en ovarios, y el aumento de los niveles plasmáticos de 17-hidroxipregnenolona, 17-hidroxiprogesterona y androstenediona, sugiriendo que este compuesto produce efectos de disrupción endocrina.

VI. La combinación de bioensayos *in vitro*, incluyendo la detección de agonistas de AhR y PXR, pone de relieve que la bahía de Kaštela es el área más contaminada de Split (Croacia), detectándose un claro gradiente de dilución de contaminantes hacia aguas abiertas del mar Adriático, y permite identificar áreas que requieren actuaciones para mejorar su calidad ambiental, con una reducción significativa de costes y del número de organismos utilizados en programas de vigilancia ambiental.

VII. El análisis de lípidos en la línea celular PLHC-1 mediante FIA acoplado a espectrometría de masas de alta resolución ha permitido el análisis de 200 especies de lípidos, cuyos perfiles lipídicos cambian en función de las condiciones del medio de cultivo y tras la exposición a 9-*cis*-RA.