UNIVERSITAT DE BARCELONA

Uso de líneas celulares como modelos *in vitro* para la evaluación de toxicidad y mecanismos de acción de contaminantes ambientales

Elisabet Pérez-Albaladejo

PROGRAMA DE DOCTORADO EN BIODIVERSIDAD

DEPARTAMENTO DE QUÍMICA AMBIENTAL

INSTITUTO DE DIAGNÓSTICO AMBIENTAL Y ESTUDIOS DEL AGUA

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

USO DE LÍNEAS CELULARES COMO MODELOS *IN VITRO* PARA LA EVALUACIÓN DE TOXICIDAD Y MECANISMOS DE ACCIÓN DE CONTAMINANTES AMBIENTALES

MEMORIA PRESENTADA POR

Elisabet Pérez-Albaladejo

PARA OPTAR AL GRADO DE DOCTORA POR LA

UNIVERSITAT DE BARCELONA

Doctoranda	**Directora**	**Tutora**
Elisabet Pérez-Albaladejo	Cinta Porte Visa	Mª Assumpció Borrell Thió

Fotografía de cubierta de lubina (*Dicentrarchus labrax*) en el Mar Mediterráneo cortesía de Josep Clotas Miquel, Estartit (España).

"Uno nunca se da cuenta de lo que ha hecho,

sólo ve lo que queda por descubrir".

Maria Skłodowska-Curie

AGRADECIMIENTOS

Para comenzar, quiero dar las gracias a Cinta Porte, mi directora de tesis, por su confianza y disponibilidad total durante todo este trabajo, por haberme transmitido su profesionalidad y rigurosidad científica, por sus apreciados y constructivos consejos, y por compartir conmigo parte de sus conocimientos y experiencia.

Extiendo mis agradecimientos a Anders Goksøyr, profesor del Departamento de Biología de la Universidad de Bergen, así como a los miembros de su equipo, por haberme dado la oportunidad de formar parte del mismo y enseñarme nuevas técnicas in vitro, y por hacerme sentir como en casa durante mi estancia en Noruega.

Igualmente quiero agradecer a los coautores de los artículos presentados en esta tesis y a todos los profesionales del CID-CSIC que han colaborado o ayudado de alguna manera en este trabajo, sus inestimables aportaciones y su buena disponibilidad. Entre ellos, me gustaría dar las gracias particularmente a Marta Casado, por su ayuda con el ensayo ER-RYA, a Fina Casas por su ayuda con el análisis de lípidos en Masslinx, a Jordi López y Mª Carmen Fernández-Pinos por la asistencia con el sistema de información geográfica ArcGis, y a Romà Tauler y Mireia Farrés por su ayuda con MatLab.

También quiero agradecer a Denise Fernandes su ayuda y amistad durante las primeras fases de esta tesis y durante nuestra estancia en Bergen, y a Silvia Pujol el haberme iniciado en el mundo de los cultivos celulares. Quiero dar las gracias también a mis compañeros de departamento, Melissa Faria, Marta Casado, Inma Fuertes, Laia Navarro, Rubén Martínez, Claudia Rivetti, Bruno Campos, a los jefes de los laboratorios vecinos Carlos Barata y

Benjamí Piña, y muy especialmente a María Blanco, Alejandra Gilabert y Anna Marqueño quienes han compartido conmigo el día a día en el laboratorio, así como a las que se fueron, Mª Carmen Fernández, Rita Jordaõ, Eva Oliveira, entre muchos otros compañeros que no cito porque me extendería demasiado, por compartir los momentos cafeteros de la mañana, por las interesantes y divertidas charlas de sobremesa, por su apoyo en la investigación y sus constructivos consejos, cada uno con su criterio y punto de vista personal aportando perspectivas desde diferentes ángulos, y sobre todo, por su amistad.

Especialmente quiero dar las gracias a Marta Gracia, amiga incondicional, por los divertidos vermuts terraceros, por soportar mis apasionadas charlas sobre contaminantes y sus efectos, y por apoyarme durante todo este tiempo en todos los aspectos. Igualmente, debo agradecer lo mismo a Vicente Vila, y su apoyo los fines de semana de trabajo con las células, así como los innumerables buceos en el Mar Rojo, y escapadas de desconexión total, que tanto se agradecían en momentos de "estrés experimental".

Finalmente, mis más sentidos agradecimientos, aunque nunca suficientes, van dirigidos a mi hermana Montse (Pollo), a mi hermano Carlitos (otro Pollo), a mis padres Teo y Encarna, a mi pequeño sobrino Alan (Pollito) y al resto de mi maravillosa y gran familia por ser una piña siempre, por su paciencia, comprensión, apoyo y cariño incondicional, a pesar de que los he tenido un poco abandonados durante estos últimos meses de escritura del manuscrito.

Muchas gracias a todos!

TABLA DE CONTENIDO

LISTA DE ABREVIATURAS **9**

I. INTRODUCCIÓN **13**

I.1. Ensayos *in vitro* como alternativa a la experimentación *in vivo* 18

I.1.1. Cultivos celulares 18

I.1.2. Fracciones subcelulares 22

I.2. Aplicaciones de los sistemas *in vitro* en toxicología ambiental 23

I.2.1. Citotoxicidad 23

I.2.2. Estrés Oxidativo 27

I.2.3. Toxicidad mediada por receptores 29

I.2.3.1. Receptor de hidrocarburos de arilo (AhR) 30

I.2.3.2. Receptor X de pregnano (PXR) 33

I.2.4. Disrupción endocrina 35

I.2.4.1. Esteroidogénesis en placenta 36

I.2.4.2. Síntesis de estrógenos en peces 39

I.2.4.3. Mecanismos de modulación de la actividad P450 aromatasa (CYP19) por agentes externos 40

I.2.5. Disrupción lipídica 42

I.2.5.1. Lípidos de membrana 47

I.3. Contaminantes ambientales 50

I.3.1. Sedimentos marinos como reservorio de contaminantes 50

I.3.2. Contaminantes emergentes 54

I.4. Modelos in vitro utilizados en esta tesis 62

II. OBJETIVOS **67**

III. INFORME DE FACTOR DE IMPACTO Y GRADO DE IMPLICACIÓN **73**

IV. PUBLICACIONES **79**

TEMA 1. EVALUACIÓN DE LA CALIDAD AMBIENTAL DE SEDIMENTOS MARINOS **81**

Artículo 1. Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays 83

Información complementaria al artículo 1 94

Artículo 2. Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell bioassays 97

Resultados adicionales del artículo 2 113

Artículo 3. Characterization of quality of sediments from Paranaguá Bay (Brazil) by combined in vitro bioassays and chemical analyses..... 115

Artículo 4. Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays 127

Resultados adicionales del Tema 1 139

TEMA 2. EVALUACIÓN DE LA TOXICIDAD DE CONTAMINANTES EMERGENTES 143

Artículo 5. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells 145

Información suplementaria al artículo 5 154

Artículo 6. Differental toxicity of alkylphenols in JEG-3 human placental cells: alteration of P450 aromatase and cell lipid composition..... 145

Información suplementaria al artículo 6 154

Artículo 7. Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells 171

V. DISCUSIÓN DE RESULTADOS 179

V.1. Evaluación de la calidad ambiental de sedimentos marinos 185

V.2. Evaluación de la toxicidad de contaminantes emergentes..... 199

VI. CONCLUSIONES..... 217

VII. BIBLIOGRAFÍA..... 221

LISTA DE ABREVIATURAS

2SBP	2-*sec*-Butilfenol
2TBP	2-*terc*-Butilfenol
4SBP	4-*sec*-Butilfenol
4TBP	4-*terc*-Butilfenol
AB	Alamar Blue
ABC	Transportadores de membrana dependientes de ATP
AhR	Receptor de hidrocarburos de arilo
ARNT	Traslocador nuclear de AhR
ATP	Adenosina trifosfato
BBP	Bencil butil ftalato
BNF	β-Naftoflavona
BPA	Bisfenol A
CFDA-AM	5-Carboxifluoresceina diacetato acetoximetil éster
CoA	Coenzima A
COP	Compuesto orgánico persistente
CP	4-Cumilfenol
CTP	Citidina trifosfato
DAG	Diacilglicérido
DBP	Dibutil ftalato
DDE	1,1-Dicloro-2,2-bis(p-clorofenil)etileno
DDT	Dicloro difenil tricloroetano
DEHP	Di(2-etilhexil) ftalato
DMP	Dimetil ftalato
DMSO	Dimetil sulfóxido
DNA	Ácido desoxirribonucleico
DP	4-dodecilfenol
DTBP	2,6-di-*terc*-Butilfenol
EC_{50}	Concentración a la cual hay un 50 % de efecto
EFSA	European Food Safety Authority
EROD	Etoxiresorufina-O-deetilasa
FADH	Flavín adenín dinucleótido
FBS	Suero fetal bovino
FMNH	Flavín mononucleótido
GL	Glucolípido
GP	Glucofosfolípido
GST	Glutation-S-transferasa
H_2DCF-DA	Diclorofluoresceína diacetato
HP	4-Heptilfenol
IC_{50}	Concentración a la cual hay un 50 % de inhibición
LBD	Dominio de unión a ligando
mRNA	Ácido ribonucleico mensajero
NADH	Nicotinamida adenina dinucleótido
NADPH	Nicotinamida adenina dinucleótido fosfato
NP	4-Nonilfenol
OP	4-*terc*-Octilfenol
PAH	Hidrocarburo aromático policíclico
PC	Fosfatidilcolina
PCB	Bifenilo policlorado
PFBA	Ácido perfluorobutanoico
PFBS	Perfluorobutanosulfonato
PFDoA	Ácido perfluorododecanoico
PFHxA	Ácido perfluorohexanoico
PFHxS	Perfluorohexanosulfonato
PFNA	Ácido perfluorononanoico
PFOA	Ácido perfluorooctanoico
PFOS	Perfluorooctanosulfonato
PVA	Alcohol de polivinilo
PVC	Policloruro de vinilo
PXR	Receptor X de pregnano
ROS	Especies reactivas de oxígeno
RXR	Receptor X retinoide
SL	Esfingolípido
SM	Esfingomielina
TAG	Triacilglicérido
TAP	4-*terc*-Amilfenol
TBBPA	Tetrabromobisfenol A
TBT	Tributil estaño
TCDD	2,3,7,8-Tetraclorodibenzo-p-dioxin
TPT	Trifenil estaño
TTBP	2,4,6-tri-*terc*-Butilfenol
UGT	Uridina difosfato glucuroniltransferasa
UPLC-MS/TOF	Cromatografía líquida de ultra-alta resolución conectado a espectrometría de masas con detector de tiempo de vuelo
WWTP	Planta de tratamiento de aguas residuales
zfPxr	Receptor X de pregnano de pez cebra

I. Introducción

El creciente desarrollo industrial representa evidentes ventajas para el estilo de vida actual, pero a su vez genera toneladas de sustancias químicas (hidrocarburos, plásticos, resinas, pesticidas, etc.) que provienen de actividades industriales, descargas urbanas y agrícolas, hospitales, tráfico marítimo y terrestre, entre otros. Estas sustancias se denominan xenobióticos (del griego *xeno-* "extraño" y *-biótico* "relativo a la vida"), y pueden ser definidos como aquellos compuestos químicos que pueden encontrarse en los organismos pero que no han sido sintetizados por ellos ni se encuentran de forma natural en la naturaleza. La resistencia a la degradación de algunos xenobióticos, junto con su condición de compuestos lipófilos, promueve su persistencia en el ambiente y su bioacumulación en el tejido adiposo, exponiendo a organismos de diferentes escalas tróficas. La toxicología ambiental surge de la necesidad de caracterizar el riesgo que los xenobióticos suponen para dichos organismos, y se define como "la ciencia que estudia los contaminantes en la biosfera y sus efectos en los constituyentes de la misma, incluyendo los seres humanos" (Newman, 2001).

El constante crecimiento demográfico mundial (de 1000 a más de 7000 millones de personas en 11 años; http://www.ine.es) apunta a que la demanda de artículos procedentes de la industria química seguirá aumentando durante los próximos años, y por ende la presencia de xenobióticos en el ambiente. La mayoría de productos químicos industriales están regulados por la normativa REACH (Registro, Evaluación, Autorización y Restricción de las sustancias químicas), implementada en el 2007 por la Comisión Europea. Actualmente hay más de 110000 sustancias químicas registradas en REACH (echa.europa.eu/documents/10162/13560/final_mb_09_2013_general_report_2012_en.pdf). A pesar de los esfuerzos de los

países más concienciados por controlar las emisiones de compuestos químicos al medio ambiente, otros territorios no comparten la misma preocupación. Muchos países emergentes, producen enormes cantidades de sustancias químicas con pocas o ninguna estrategia de gestión enfocada a minimizar los riesgos para la sociedad y el medio ambiente. Como resultado, los niveles más altos de contaminantes en tejidos humanos y animales se dan en estas áreas (Covaci et al., 2012). Además, dado que los contaminantes son transportados por la atmósfera y por las corrientes de agua, otras poblaciones del planeta se ven afectadas, incluidas las más remotas, como la fosa de las Marianas (> 6000 metros de profundidad; Jamieson et al., 2017), el Ártico (Villa et al., 2016) y zonas alpinas (Van Drooge et al., 2014).

La toxicología ambiental trabaja a distintas escalas biológicas, todas ellas interconectadas. Así, las alteraciones moleculares causadas por los xenobióticos afectan a la célula, al órgano y al individuo, y los daños individuales repercuten en la población y en la comunidad (Repetto and Repetto, 2009; Figura 1). La caracterización del riesgo, conocer los mecanismos de acción de los contaminantes, sus efectos y consecuencias a corto y a largo plazo, son labores de elevada complejidad dada la gran cantidad de compuestos y organismos implicados, interacciones entre contaminantes, ambiente, estado de salud del organismo, estado de desarrollo embrionario, edad y sexo (Bols et al., 2005).

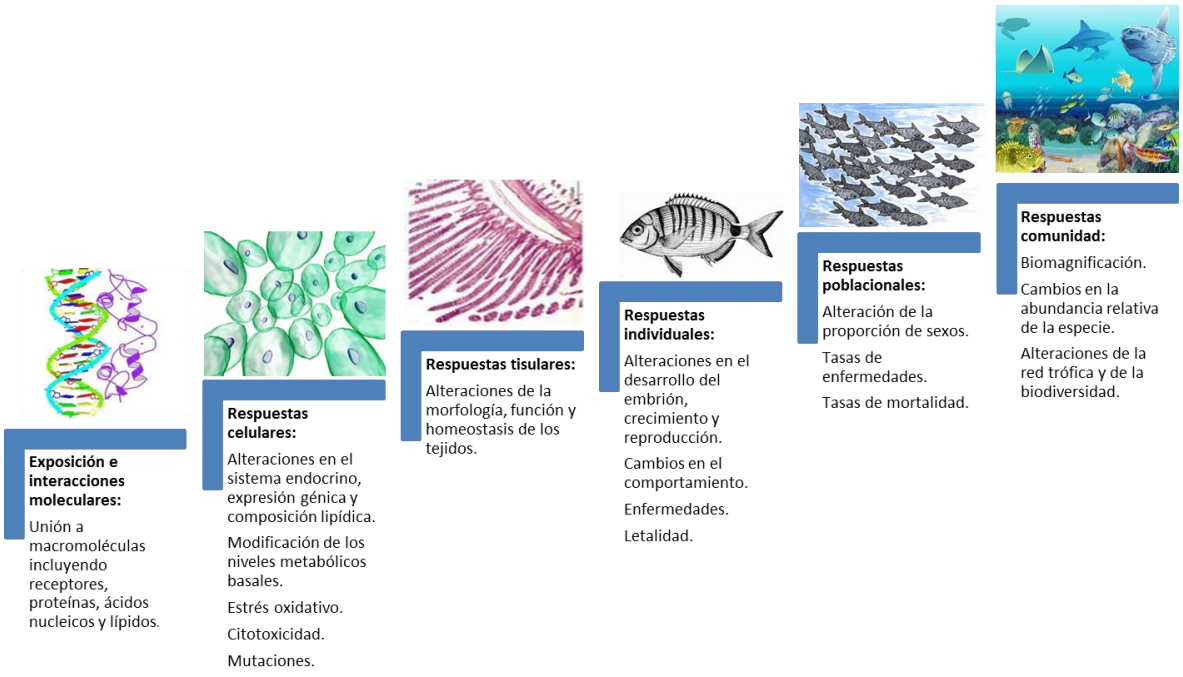


Figura 1: Respuestas a la presencia de contaminantes en el medio ambiente, a diferentes escalas biológicas.

I.1. ENSAYOS *IN VITRO* COMO ALTERNATIVA A LA EXPERIMENTACIÓN *IN VIVO*

Los ensayos *in vitro* son aquellos que se realizan fuera de un organismo vivo, e implican la utilización de células, órganos o tejidos aislados (echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/in-vitro-methods). En 2009, se utilizaron 1,4 millones de animales para experimentación sólo en España, ciento quince mil de los cuales se usaron en ensayos de toxicología. Sin embargo, la creciente preocupación por el bienestar animal junto con el elevado coste económico y temporal de los ensayos *in vivo* promueve que los científicos desarrollen continuamente nuevos métodos *in vitro*, lo cual ha conseguido reducir el uso de animales un 40 % desde 2009 (magrama.gob.es/es). Por su lado, la legislación europea también fomenta el uso de técnicas alternativas a los experimentos con animales: "...no deberá realizarse un experimento si se dispone de otro método científicamente satisfactorio y contrastado que permita obtener las mismas conclusiones, sin implicar la utilización de animales" (Directiva del Consejo 86/609/CEE; R.D. 1201/2005).

I.1.1. CULTIVOS CELULARES

Los cultivos celulares son sistemas formados por células aisladas de un tejido sano o tumoral, que se mantienen *in vitro* inmersas en un medio de cultivo nutritivo, en condiciones de temperatura, pH, atmósfera gaseosa y humedad controladas. Inicialmente, las aplicaciones de los cultivos celulares se enfocaban al campo de la medicina para la obtención de virus purificados para vacunas. Actualmente, se han encontrado aplicaciones en muchas otras disciplinas científicas que incluyen toxicología, biología celular, bioquímica,

inmunología, virología, reproducción asistida, investigación del cáncer, biotecnología, industria farmacéutica e industria agronómica. Además de reemplazar o reducir el número de animales utilizados en estudios toxicológicos, los ensayos *in vitro* ofrecen numerosas ventajas respecto a los experimentos *in vivo*. Dada la facilidad de aplicar dosis controladas en placas multipocillo que se analizan fácilmente (ej. en un fluorímetro de placas), los ensayos *in vitro* permiten estudiar los mecanismos de toxicidad aislados, de un gran número de sustancias químicas, rápida y económicamente. Posibilitan realizar numerosas réplicas, ya que necesitan muy poca cantidad de producto químico, lo cual minimiza la generación de residuos tóxicos. Los resultados obtenidos son reproducibles en otros laboratorios porque son sistemas notablemente homogéneos, lo cual reduce apreciablemente la variabilidad asociada a los experimentos *in vivo*, incluyendo precisión de la dosificación, influencias del estrés del animal, e interferencias con otros órganos que no son objeto de estudio. Es cierto que en ocasiones, los ensayos *in vitro* no pueden sustituir a la experimentación *in vivo*, ya que no tienen en cuenta la totalidad de las vías de toxicidad (algunas aún desconocidas) presentes en el organismo y su complejidad. Sin embargo, pueden sustituir perfectamente numerosas fases del estudio, incluyendo el ajuste de dosis de exposición, el cribado o screening, así como la obtención de una visión global de la toxicidad del compuesto (Repetto and Repetto, 2009).

Los cultivos celulares se clasifican principalmente en cultivos primarios y líneas celulares continuas. Los **cultivos primarios** proceden de un tejido sano del cual las células se disgregan por métodos enzimáticos o mecánicos, y se mantienen en medio de cultivo durante un periodo limitado de tiempo durante el cual se realizan los experimentos. Las células de un cultivo

primario normalmente no se reproducen y su viabilidad va mermando a lo largo del tiempo, por lo que tienen el inconveniente de que se deben obtener constantemente de un tejido vivo, lo cual limita el tiempo de experimentación. Otro inconveniente es la variabilidad entre cultivos, ya que proceden de individuos diferentes. La ventaja de los cultivos primarios es que conservan todas las funciones especializadas del tipo celular porque su genoma no está alterado.

Las **líneas celulares** se diferencian de los cultivos primarios en que las células se reproducen constantemente. También provienen de un tejido vivo, a menudo tumoral, por lo que su genoma suele ser heteroploide. Tienen la ventaja de que el cultivo puede dividirse en subcultivos o "pases" numerosas veces (entre 20 y 50 pases, dependiendo de la línea celular) antes de que entren en senescencia por acortamiento de los telómeros, lo cual les impide realizar la mitosis y empiezan a morir. Otra ventaja es que, durante los primeros pases, las células se pueden congelar en nitrógeno líquido hasta su nuevo uso, disponiendo así de un stock celular indefinidamente. En la actualidad, se comercializan más de cuatro mil líneas celulares que son utilizadas en la producción de virus, proteínas y ADN, y sirven como modelo de experimentación en toxicología y biomedicina. A pesar de las ventajas que ofrecen, las líneas celulares tienen la limitación de que han perdido algunas funciones y actividades enzimáticas, es decir, no contienen la dotación genómica completa. En este sentido, la selección de la línea celular dependerá del conocimiento disponible con respecto a la sensibilidad y características de las células, dado que se han descrito diferencias significativas entre líneas celulares. Por ejemplo, se han observado vías diferentes del metabolismo del benzo[a]pireno en dos líneas celulares de hígado de trucha (*Oncorhynchus mykiss*), RTL-W1 y R1

(Schirmer et al., 2000). Otro punto a tener en cuenta es que la selección del medio de cultivo y sus componentes tienen un impacto significativo en el ensayo *in vitro*. Por ejemplo, se ha demostrado que la presencia de suero fetal bobino (FBS) en el medio de cultivo aumenta la inducción de CYP1A por exposición a 2,3,7,8 tetraclorodibenzo-p-dioxina, pero no ocurre igual para el benzo[a]pireno. Similarmente, la presencia de FBS facilita la detección de agentes inductores de CYP1A en muestras ambientales (Schirmer et al., 2004). En algunos casos, se ha observado que la exposición en medio sin FBS, aumenta la sensibilidad de células de peces en ensayos de citotoxicidad (Dayeh et al, 2005). Además de alterar la concentración de FBS, se puede mejorar la sensibilidad del ensayo evitando otros componentes del medio. Por ejemplo, durante la determinación de la respuesta mediante la adición de colorantes fluorescentes (ej. Alamar Blue, CFDA-AM, Neutral Red), la mejor opción parece ser diluirlos en medios muy simples, como L-15/ex, que contiene únicamente sales, galactosa y piruvato. La ausencia de otros componentes (ej. antioxidantes) evita la interferencia y facilita la expresión de la citotoxicidad. Por otro lado, la concentración del solvente orgánico (ej. DMSO, etanol) en el cual están disueltos los químicos, podría afectar al ensayo. Actualmente este dato se tiene muy en cuenta y se añaden cantidades de solventes que no afectan a la funcionalidad celular. Sin embargo, en el pasado se observaron efectos tóxicos intensificados por la presencia de DMSO, incluyendo la proliferación de células MCF-7 o la fotocitotoxicidad de fluoranteno, mientras que otros se vieron amortiguados, como la sensibilidad de células de rata ante compuestos tipo dioxina, probablemente por la interacción del solvente con el compuesto (Schirmer, 2006).

Uno de los puntos clave en la investigación con cultivos celulares reside en conocer la **biodisponibilidad** del xenobiótico en los experimentos. Procesos como la acumulación del compuesto en las interfaces de la placa de cultivo (aire-líquido, líquido-plástico), baja solubilidad, evaporación, y unión a los componentes del FBS (proteínas, lípidos), pueden reducir considerablemente la fracción biodisponible del xenobiótico. Normalmente, las concentraciones de exposición en los ensayos *in vitro* no se comprueban, de manera que se asume que la concentración nominal está biodisponible en su totalidad para el sistema biológico modelo. Desgraciadamente, este hecho hace que en ocasiones se subestime el efecto tóxico de la sustancia. Conocer las concentraciones reales de exposición es primordial porque la respuesta observada podría ocurrir a concentraciones muy bajas, similares a las ambientalmente relevantes (Harris et al., 2014).

I.1.2. FRACCIONES SUBCELULARES

Las células intactas contienen la totalidad de moléculas y orgánulos necesarios para su ciclo celular y representan un modelo óptimo para estudios de toxicología ambiental porque integran las funciones y mecanismos celulares. Sin embargo, en ocasiones se necesita aislar una determinada fracción subcelular que contenga estructuras específicas (ej. un complejo enzimático concreto) y que conserven una función de interés para el estudio (ej. actividad enzimática). Las fracciones subcelulares se obtienen por lisis de las membranas celulares por choque osmótico, ultrasonidos u homogenización, en presencia de un tampón fisiológico que mantenga el pH. Las diferentes fracciones u orgánulos (mitocondrias, lisosomas, retículo endoplasmático, etc.) se separan por centrifugación en

función de su tamaño y densidad, conservando sus propiedades bioquímicas originales. Así, las fracciones subcelulares representan una herramienta complementaria a los ensayos con células intactas, que permiten investigar determinados procesos en vías metabólicas complejas, sin la interferencia de otras reacciones laterales que ocurren en la célula (Alberts et al., 1996).

I.2. APLICACIONES DE LOS SISTEMAS *IN VITRO* EN TOXICOLOGÍA AMBIENTAL

I.2.1. CITOTOXICIDAD

La primera interacción de los compuestos químicos con el organismo ocurre a nivel celular, por lo que los métodos *in vitro* con cultivos celulares como modelo biológico representan una herramienta adecuada para el estudio de los efectos y mecanismos de acción de los xenobióticos. La velocidad y facilidad con que el compuesto entra en la célula depende principalmente de la biodisponibilidad en el medio, de las propiedades físico-químicas de la molécula y de la permeabilidad de la membrana plasmática. Por ejemplo, una molécula hidrofóbica y menor de 1 μm atravesará la membrana prácticamente por difusión, gracias a las propiedades de la bicapa lipídica que forma la membrana celular. Una vez en la célula, absolutamente todos los compuestos químicos son, a determinadas concentraciones, capaces de reducir la viabilidad y causar la muerte celular. Sin embargo, los efectos subletales son los más preocupantes desde un punto de vista de caracterización del riesgo toxicológico ya que ocurren a concentraciones no citotóxicas y no son evidentes a simple vista, pero pueden generar situaciones irregulares en la célula, incluyendo estrés oxidativo, activación de receptores nucleares, disrupción endocrina, y cambios en la composición

lipídica, entre otros, que pueden llegar a causar daños irreparables en el individuo, alteraciones en el desarrollo del embrión, en la determinación del sexo, en el desarrollo de los caracteres sexuales secundarios, en el crecimiento y en la reproducción, llegando a provocar el desarrollo de enfermedades a largo plazo, incluso años después de la exposición.

Uno de los primeros métodos que se utilizaron para evaluar la citotoxicidad fue el ensayo de exclusión del colorante azul de tripano (Trypan Blue), que aún se utiliza actualmente. Se basa en el principio de que las células viables tienen la membrana intacta, y no incorporan el colorante en su citoplasma. Las células dañadas aparecen de color azul bajo el microscopio óptico. Actualmente, se utilizan más frecuentemente los métodos basados en la actividad metabólica celular, utilizando sustratos que son transformados por las células viables en productos que pueden ser detectados por fluorimetría o colorimetría. Tienen la ventaja de que permiten realizar múltiples ensayos de citotoxicidad en placas multipocillo, reduciendo el tiempo y el coste de la investigación. Entre ellos se encuentran: el ensayo basado en la acumulación de Neutral Red, un compuesto fluorescente, en los lisosomas de las células viables, indicando actividad lisosomal; otro ensayo basado en la actividad del enzima lactato deshidrogenasa en el medio extracelular, indicando muerte celular por daños irreversibles en la membrana plasmática; y el ensayo que utiliza bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT), uno de los más utilizados en pruebas de citotoxicidad. Este último se basa en la reducción de tetrazolio soluble, en cristales formazan insolubles e impermeables a la membrana plasmática, por NADPH, FADH, FMNH y NADH, aunque no por los citocromos. Tiene la desventaja de tener que redisolver los cristales de formazan en un solvente

(DMSO o HCl/isopropanol), y que es un método citotóxico, lo cual destruye las células en estudio.

En este sentido, el método del **Alamar Blue**, basado en la conversión de resazurina en resorufina (Figura 2), evita muchos de los inconvenientes citados y ofrece numerosas ventajas sobre otros ensayos. La resazurina ofrece gran sensibilidad, es soluble y estable en el medio de cultivo, permeable a la membrana celular y no es tóxica, de manera que permite realizar experimentos simultáneos con otros métodos y/o ensayos continuos, además de ser segura para el investigador y para el medio ambiente. La resazurina es de color azul no fluorescente, y es reducida a resorufina, un compuesto fluorescente y de color rosa, por los nucleótidos citados anteriormente, y también por los citocromos y enzimas reductasas mitocondriales. La fluorescencia se detecta en un fluorímetro de placas, a las longitudes de onda de excitación 530-560 nm, y de emisión 590 nm.

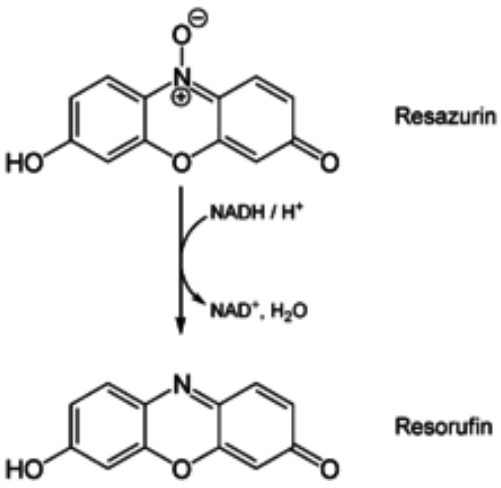


Figura 2: Principio del ensayo de citotoxicidad con Alamar Blue. Resazurina (azul, no fluorescente) es reducida por las células viables (en este ejemplo por NADH) a resorufina, compuesto rosa que emite fluorescencia.

La respuesta celular varía en función del modelo biológico, de las condiciones experimentales, incluyendo pH, temperatura y luz (resorufina es fotosensible), del tipo de compuesto (interacciones con la química del ensayo), y del tiempo de incubación ya que la resorufina puede reducirse a hidroresorufina (no fluorescente) en una reducción reversible. En este sentido, es interesante utilizar al menos dos métodos diferentes para evaluar la citotoxicidad, que permitan confirmar el resultado del estudio, además de valorar diferentes vías metabólicas implicadas en la toxicidad.

En esta tesis, se ha combinado el uso del Alamar Blue junto con otro colorante, el CFDA-AM, para cuantificar la citotoxicidad celular. Mientras que Alamar Blue ofrece información sobre la actividad metabólica, **CFDA-AM** es un indicador de la integridad de la membrana celular. Se trata de un sustrato apolar y no fluorescente, que entra en la célula por difusión y es convertido por las esterasas de la membrana plasmática en la molécula polar y fluorescente CF, que es secretada al exterior de la célula (Figura 3). Tiene la ventaja de que se puede usar conjuntamente con Alamar Blue, lo que reduce la cantidad de compuestos tóxicos (residuos) y el número de células utilizadas, además de economizar recursos y tiempo invertido.

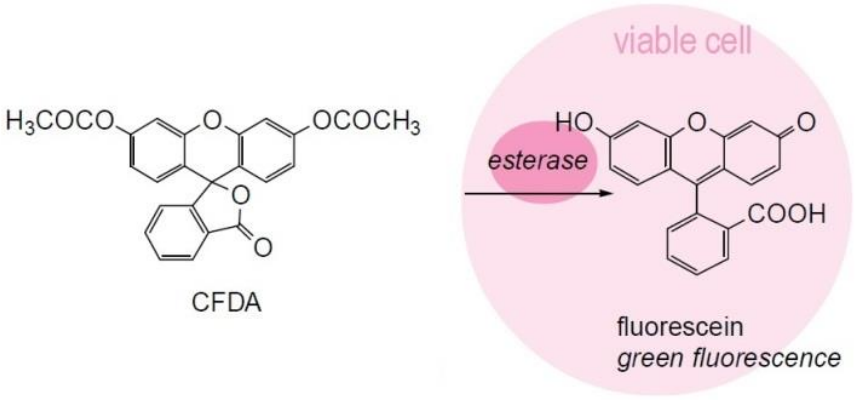


Figura 3: Principio básico del ensayo de citotoxicidad celular con CFDA. Las esterasas de las membranas viables transforman CFDA en fluoresceína, que emite fluorescencia.

I.2.2. ESTRÉS OXIDATIVO

Todos los animales necesitan oxígeno para producir energía en las mitocondrias, lo cual enmascara el hecho de que se trata de un gas tóxico. En la célula se generan, de manera natural, moléculas con un oxígeno reactivo, incluyendo el radical superóxido, peróxido de hidrógeno y radical hidroxilo, entre otros, denominados comúnmente especies reactivas de oxígeno (ROS), necesarias para el metabolismo basal. El radical hidroxilo es el más perjudicial, por ser comparativamente más reactivo que el resto. Es capaz de extraer electrones y protones de macromoléculas (ácidos nucleicos, proteínas, lípidos y carbohidratos), generando a su vez más ROS (Halliwell and Gutteridge, 2015). Los animales sobreviven al ROS generado porque han desarrollado defensas antioxidantes (ej. superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPX), ácido ascórbico (vitamina C), α-tocoferol (vitamina E), glutatión y carotenoides). Las especies reactivas de oxígeno no son todas perjudiciales, ni todos los antioxidantes son beneficiosos. La vida es un equilibrio entre los dos. Los antioxidantes sirven para mantener los niveles adecuados de ROS, permitiendo que realicen sus funciones biológicas sin dañar demasiado al organismo (Halliwell, 2006). Hace seis décadas que se conoce la existencia de ROS en los sistemas biológicos, y fue inmediatamente asociado a enfermedades y envejecimiento (Harman, 1956). Desde entonces, el daño oxidativo en biomoléculas, comúnmente denominado estrés oxidativo, producido por un desequilibrio entre ROS y los mecanismos antioxidantes ha sido ampliamente estudiado en diversas disciplinas: medicina, biología molecular, fisiología y ecología (Birnie-Gauvin et al., 2017).

Algunos compuestos (ej. el herbicida N,N'-dimethyl-4,4'-bipyridinium dichloride (paraquat)) son capaces de aumentar directamente los niveles de ROS, entrando en los ciclos redox (donando electrones al oxígeno molecular), mientras que otros (ej. aminotriazol y ditiocarbamatos), lo hacen indirectamente disminuyendo o inactivando las defensas antioxidantes. Se ha observado estrés oxidativo tras la exposición a compuestos del primer grupo (diquat) en la línea celular de carpa EPC, pez cebra (*Danio rerio*) y trucha (*Oncorhynchus mykiss*). En cuanto al segundo grupo, un ejemplo sería el metabolismo de algunos PAHs por CYP1A (ej. benzo[a]pireno), que consume glutatión, lo cual disminuye las defensas antioxidantes, y genera metabolitos cíclicos (ej. benzo[a]pireno diona) capaces de autooxidarse generando ROS (Wright et al., 2000; Livingstone, 2001; Stephensen et al., 2002; Bretaud et al., 2004; Hasselberg et al., 2004; Lushchak, 2016). En peces, se ha demostrado que la exposición a xenobióticos puede generar elevados niveles de peróxidos lipídicos por tener la capacidad antioxidante significativamente reducida (Ekambaram et al., 2014). En humanos, el estrés oxidativo es causante de enfermedades degenerativas como Parkinson, aterosclerosis, Alzheimer y cáncer (Sánchez-Valle and Méndez-Sánchez, 2013). Además, en mujeres embarazadas, elevados niveles de ROS en la placenta se asocian con resultados negativos como el aborto espontáneo, preeclampsia y restricciones de crecimiento intrauterino (Wu et al., 2016).

En esta tesis, se ha investigado el estrés oxidativo celular causado por contaminantes ambientales, así como por compuestos individuales, mediante un bioensayo basado en la oxidación de H_2DCF-DA por las moléculas ROS (Figura 4). El compuesto resultante (DCF) emite fluorescencia (528 nm) cuando es excitado a una longitud de onda de 485 nm, por lo que puede detectarse en un fluorímetro de placas.

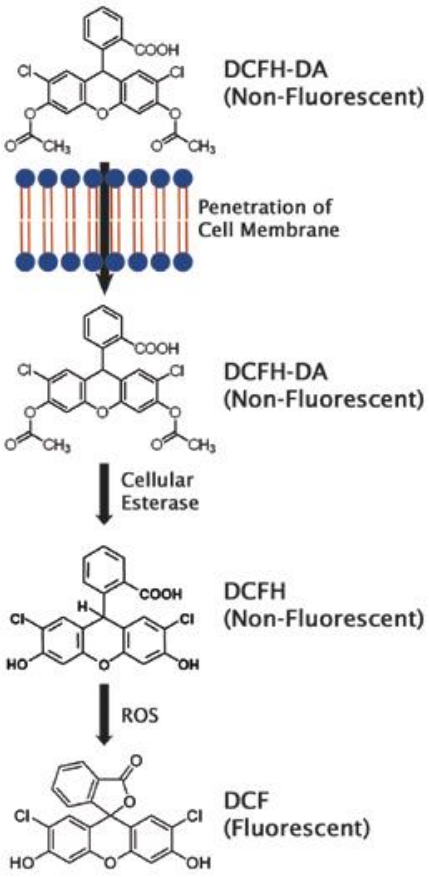


Figura 4: Bases del ensayo ROS. DCFH-DA entra en la célula atravesando la membrana plasmática, donde la actividad esterasa rompe los enlaces éster de los dos acetatos. En presencia de ROS, la molécula es oxidada resultando en DCF, sustancia fluorescente. A mayor fluorescencia, mayores niveles de ROS.

I.2.3. TOXICIDAD MEDIADA POR RECEPTORES

La presencia de contaminantes en el ambiente resulta en respuestas tóxicas en todos los niveles de organización biológica, pero el primer contacto ocurre a nivel celular. Una vez dentro de la célula, los contaminantes sufren procesos de biotransformación a moléculas más polares para facilitar su excreción. Así, las propiedades físicas que favorecen la absorción de los

xenobióticos (hidrofobicidad) se sustituyen por propiedades que facilitan su excreción en la orina o en las heces, es decir, aumentan su polaridad. Muchas vías metabólicas implicadas en el metabolismo de contaminantes se inician por la unión de un ligando a un receptor celular que, una vez activado, actúa como la señal que desencadena una cascada de reacciones metabólicas. La mayoría de enzimas que participan en la biotransformación tienen una actividad o concentración basal determinada necesaria para realizar las funciones metabólicas endógenas normales. Es decir, se trata de enzimas constitutivas cuya síntesis se realiza en ausencia de un estímulo externo discernible. Sin embargo, algunas de estas enzimas se sintetizan en respuesta a la presencia de xenobióticos a través de un proceso de inducción enzimática. La actividad o concentración de estas enzimas puede aumentar o disminuir, y pueden ser utilizadas como marcadores moleculares de exposición a contaminantes. Así mismo, el grado de respuesta de dichos biomarcadores indica también el grado de toxicidad de la muestra ambiental o compuesto químico objeto de estudio (Gibson et al., 2001). Los contaminantes pueden seguir diferentes rutas metabólicas, por lo que se hace necesario aplicar diferentes biomarcadores con el objetivo de identificar la presencia de diferentes clases de xenobióticos en muestras ambientales complejas (ej. sedimentos, agua y aire). En este sentido, el receptor de hidrocarburos de arilo es muy utilizado como biomarcador en estudios de toxicología por su gran sensibilidad ante la presencia de sustancias exógenas.

I.2.3.1. Receptor de hidrocarburos de arilo (AhR)

El receptor de hidrocarburos de arilo (AhR) es una proteína soluble presente en el citosol que actúa como factor de transcripción activado por ligando,

regulando la expresión del gen del citocromo P450 1A (*cyp1a*) (Figura 5). El gen *cyp1a* codifica enzimas encargadas de catalizar la oxidación de compuestos xenobióticos, normalmente hidrofóbicos, para obtener productos más polares y facilitar así su excreción. Los ligandos más conocidos de AhR son moléculas planares hidrofóbicas, incluyendo PAHs de elevado peso molecular, PCBs, furanos, pesticidas y compuestos tipo dioxina, entre otros. La respuesta transcripcional de *cyp1a* mediada por AhR es sensible a la concentración de dichos compuestos en la célula, e incrementa la actividad del enzima CYP1A.

El receptor AhR está altamente conservado en la evolución y se ha detectado en todos los grupos de vertebrados, incluyendo aves, mamíferos, reptiles, anfibios y peces (Fent, 2001). En los peces, la mayor actividad CYP1A se encuentra principalmente en el hígado, órgano altamente implicado en el metabolismo de xenobióticos, aunque también se ha detectado en otros tejidos como riñón, branquias, sistema nervioso y gónadas. Sin embargo, hay que tener en cuenta que, aunque la actividad CYP1A de los peces está, a menudo, correlacionada con la distribución ambiental de ligandos de AhR, algunos parámetros exógenos a los contaminantes, que incluyen temperatura ambiental, niveles hormonales y condición fisiológica de los peces, podrían afectar y modular la respuesta del sistema CYP1A (Sarasquete et al., 2000).

En este sentido, el uso de líneas celulares de peces (ej. PLHC-1 y ZFL) como modelo en ensayos de inducción CYP1A ofrece numerosas ventajas porque se cultivan en condiciones controladas y son un sistema homogéneo, minimizando así la posible variabilidad que a menudo se detecta en los peces obtenidos directamente del área de estudio, a la vez que se reduce el uso de animales para experimentación.

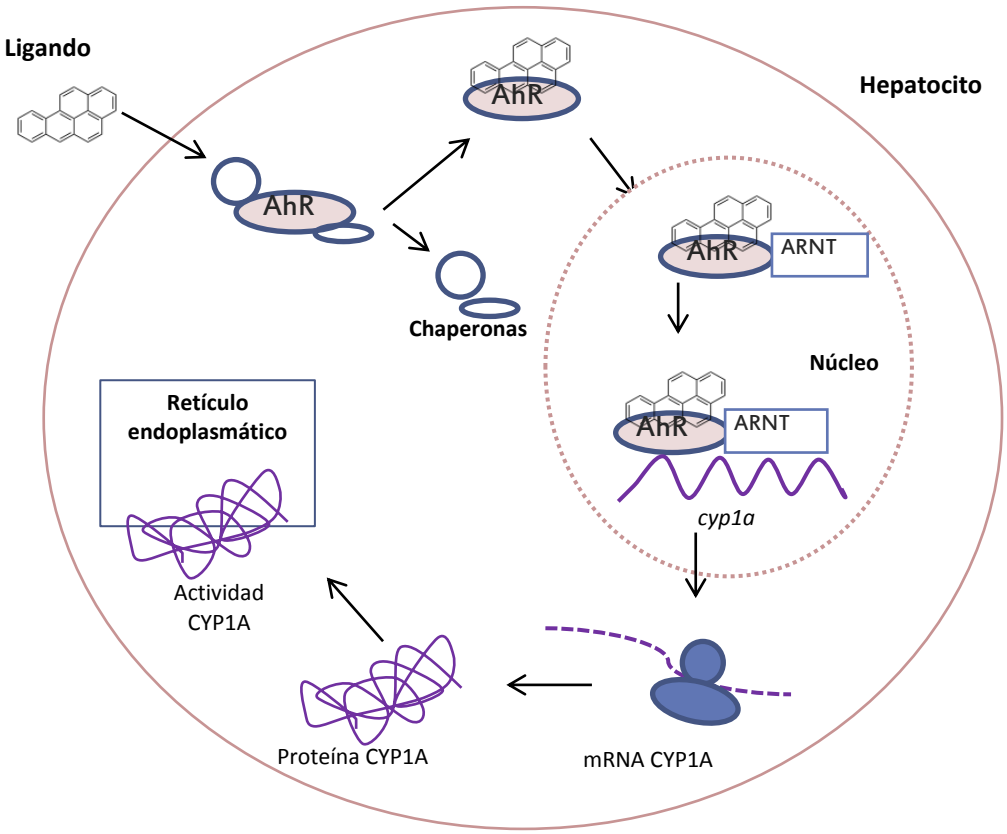


Figura 5: Mecanismo simplificado de activación AhR mediada por ligando, e inducción de CYP1A. AhR se encuentra inoperante en el citoplasma, unido a proteínas chaperonas hasta que es activado por un ligando. Una vez activado, AhR entra en el núcleo celular formando un heterodímero con una proteína translocadora (ARNT), se une al DNA cerca del promotor e induce la transcripción del gen *cyp1a*. El mRNA finalmente se traduce en la proteína CYP1A que se transporta al retículo endoplasmático, donde ejerce su actividad.

Los métodos de cuantificación de la inducción de CYP1A se realizan en los diversos pasos de la vía metabólica, incluyendo activación del receptor AhR, producción de mRNA y actividad catalítica (Sarasquete et al., 2000) (Figura 5). Entre ellos, la actividad 7-etoxiresorufina-O-deetilasa (EROD), característica de CYP1A, es un método reconocido por su alta sensibilidad ante la presencia de PAHs y otros ligandos. El **ensayo EROD**, utilizado en esta tesis, mide la desalquilación del sustrato no fluorescente 7-etoxiresoru-fina, en el producto fluorescente 7-hidroxiresorufina que puede ser detec-

tado fácilmente en un fluorímetro de placas (Sarasquete et al., 2000; Fent, 2001).

I.2.3.2. RECEPTOR X DE PREGNANO (PXR)

El PXR es un factor de transcripción residente en el núcleo celular, que protege a los organismos de los compuestos tóxicos, actuando como xenosensor y regulando genes implicados en el metabolismo de dichos compuestos. Una vez activado por un ligando, PXR forma un heterodímero con el receptor del ácido retinoico (RXR), el complejo se une al DNA e induce la expresión de genes del citocromo P450 (ej. *CYP3A, CYP2B*), enzimas de conjugación (sulfotransferasas, glutatión-S-transferasas, UDP-glucuronosiltransferasas, aldehído deshidrogenasas) y transportadores (ABC) (Figura 6). Así, la activación coordinada de PXR estimula la expresión de una larga gama de genes responsables de la solubilización y excreción de contaminantes (Kliewer et al., 2002 y 2003; Kretschmer et al., 2005). Este receptor ha sido caracterizado y denominado de distintas formas en primates (SXR: Steroid and Xenobiotic Receptor; Blumberg et al., 1998), mamíferos no primates (PXR; Kliewer et al., 1998), aves (CXR: Chicken X Receptor), peces (PXR; Moore, 2002) y anfibios (BXR: Benzoate X Receptor; Grün et al., 2002) (Milnes et al., 2008).

El receptor PXR es significativamente promiscuo por su divergencia evolutiva, de manera que los ligandos y el grado de activación pueden variar dependiendo de la especie. Esta tesis utiliza el receptor Pxr de pez cebra (zfPxr), que puede ser activado por una larga lista de xenobióticos estructuralmente variados, incluyendo fármacos (ej. clotrimazol), plastificantes (alquilfenoles, ftalatos, bisfenol A) y compuestos

organoclorados (clordano, o,p'-DDT, endosulfán, metoxicloro, toxafeno), entre otros (Milnes et al., 2008).

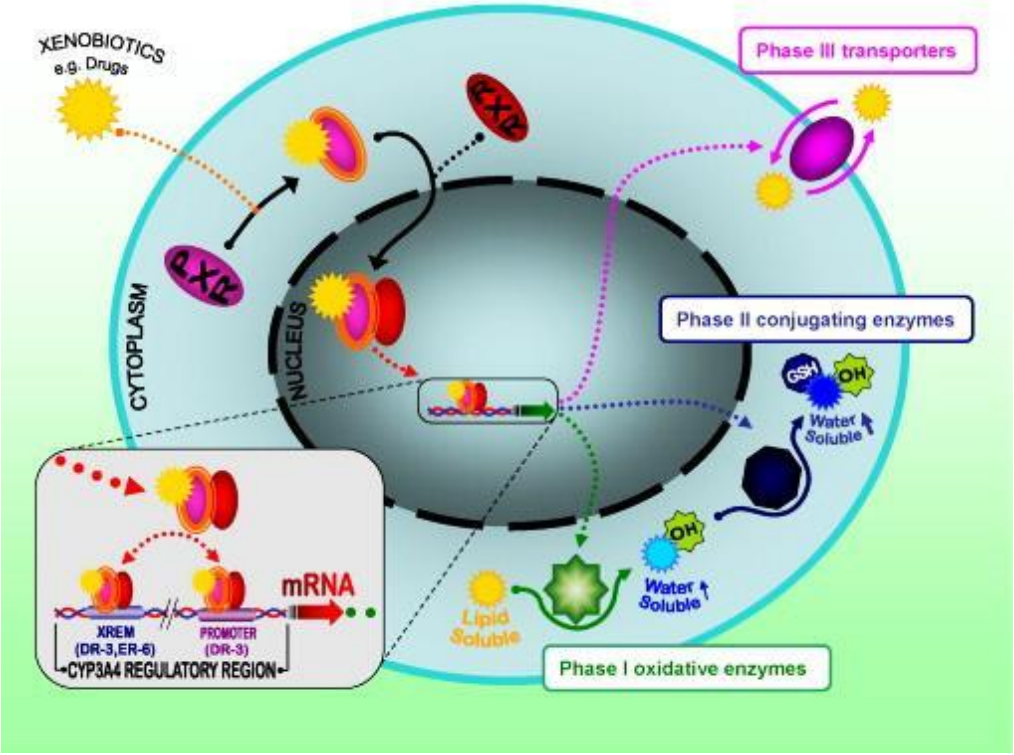


Figura 6: Mecanismo molecular de PXR. PXR se activa tras la unión de un xenobiótico o ligando endógeno, forma un heterodímero con RXR y se une a la región reguladora del gen diana, incluyendo CYP3A4, enzimas CYP, deshidrogenasas, carboxilesterasas, enzimas de fase II (GST, UGT, sulfotransferasas), y transportadores. Los citocromos catalizan reacciones de oxidación convirtiendo sustratos lipofílicos en sustancias más solubles añadiendo grupos hidroxilo, mientras que las enzimas de fase II catalizan reacciones de conjugación (ej. adición de glutatión) para incrementar solubilidad y reducir toxicidad. Finalmente, PXR incrementa la expresión de varios transportadores, facilitando su excreción (Matic et al., 2007).

La activación de zfPxr mediada por ligando puede detectarse y cuantificarse mediante **ensayos reporter *in vitro***, utilizando células transfectadas con plásmidos que contienen los vectores de interés, incluyendo el gen *zfpxr* (Lille-Langøy et al., 2015). Los ensayos de activación de zfPxr se han utilizado con éxito como herramienta para predecir el potencial tóxico de

diferentes compuestos químicos aunque no se tienen datos de muestras ambientales complejas.

I.2.4. DISRUPCIÓN ENDOCRINA

En las siguientes subsecciones se describe brevemente el sistema endocrino, la síntesis de hormonas esteroideas en la placenta humana y en el ovario de peces teleósteos, y se comentan los efectos y mecanismos de acción de los disruptores endocrinos capaces de modular la actividad catalítica de uno de los complejos enzimáticos más importantes de la esteroidogénesis, el citocromo P450 aromatasa (CYP19), objeto de estudio en esta tesis.

El sistema endocrino, junto con el sistema nervioso y el sistema inmunitario, integra información de todo el organismo y mantiene la homeostasis regulando numerosas funciones fisiológicas, incluyendo desarrollo, crecimiento, comportamiento y reproducción. En el sistema endocrino, la información se distribuye por todo el organismo mediante las hormonas. Las hormonas son las moléculas encargadas de transmitir las señales que desencadenan las reacciones bioquímicas necesarias para la vida, pudiendo tener efectos inductores de actividad metabólica, inhibidores, sinérgicos o antagónicos entre ellas (ej. insulina y glucagón). Así, el balance óptimo de los niveles hormonales es imprescindible para la salud del individuo y por ende de la población, de manera que cualquier alteración de este balance resultaría ciertamente en consecuencias negativas.

Desde hace décadas, está aumentando la frecuencia de detección de compuestos xenobióticos en el medio ambiente, capaces de interferir en el balance hormonal causando una desviación de las condiciones biológicas normales. Son los denominados disruptores endocrinos. Los disruptores

endocrinos pueden influir en los diferentes sistemas hormonales, incluyendo sistema hipotalámico, hipofisario, adrenal, tiroideo y gonadal, entre otros. Concretamente, la interferencia de los disruptores endocrinos con el metabolismo de las hormonas sexuales genera un interés particular debido a las numerosas consecuencias negativas observadas por esta causa en el desarrollo embrionario, diferenciación sexual y desarrollo reproductivo en especies de diversos grupos taxonómicos (Hoffman et al., 2002; Casals-Casas and Desvergne, 2011). Las reacciones implicadas en la síntesis de las hormonas sexuales o esteroidogénesis están catalizadas por enzimas del citocromo P450, reductasas e hidroxiesteroide-deshidrogenasas (HSDs), localizadas en las membranas mitocondriales y del retículo endoplasmático. La mayor parte de las reacciones implicadas en la síntesis de hormonas sexuales son irreversibles, y aquellas catalizadas por HSDs, aunque reversibles, generalmente ocurren en una misma dirección (Ghayee and Auchus, 2007).

I.2.4.1. ESTEROIDOGÉNESIS EN PLACENTA

En humanos, la placenta, junto con el feto y la madre (unidad materno-feto-placentaria), juega un papel clave en la esteroidogénesis regulando el balance de hormonas requerido para el mantenimiento del embarazo, la progresión del desarrollo del feto, y la preparación de la madre para la lactancia (Sanderson, 2009). El colesterol, precursor de las hormonas esteroideas, es aportado por la madre y transformado en pregnenolona por CYP11A en el sincitiotrofoblasto placentario. Debido a que la placenta carece de actividad CYP17 (esteroide 17α-hidroxilasa y 17,20-liasa), las vías dependientes de este enzima (conversión de pregnenolona en 17α-hidroxipregnenolona, y ésta en dehidroepiandrosterona (DHEA)) ocurren en

el feto, que sí está dotado de CYP17. Así, el feto suministra a la placenta los precursores necesarios para la síntesis de androstenediona y 16α-hidroxiandrostenediona. Finalmente, CYP19 (P450 aromatasa) cataliza la aromatización de andrógenos (19 carbonos) para obtener estrógenos (18 carbonos) (Figura 7).

Aunque aún no se conocen la totalidad de los mecanismos que regulan la esteroidogénesis en la placenta, se sabe que la expresión de *CYP19* es estimulada por el promotor I.1 y es controlada por cascadas de señales intracelulares dependientes de AMPc (adenosina monofosfato cíclica). AMPc activa una PKA (proteína quinasa) encargada de fosforilar determinadas proteínas y factores de transcripción implicados en la activación de genes que codifican enzimas esteroidogénicas y proteínas necesarias para la transferencia de electrones (Ghayee and Auchus, 2007).

Aunque existen otras vías de señalización, la vía mediada por PKA/AMPc, mediante fosforilación y desfosforilación de sustratos, es la principal cascada estimulada en la esteroidogénesis placentaria, y regula el balance hormonal necesario para el desarrollo del feto. Durante el embarazo humano, la placenta es el órgano con mayor expresión de *CYP19*. En los fetos varones, la actividad aromatasa placentaria se debe mantener baja durante las semanas 8-10 de la gestación, periodo crítico para la diferenciación sexual. De esta manera, se minimiza la conversión de andrógenos en estrógenos, lo cual alteraría el proceso de masculinización.

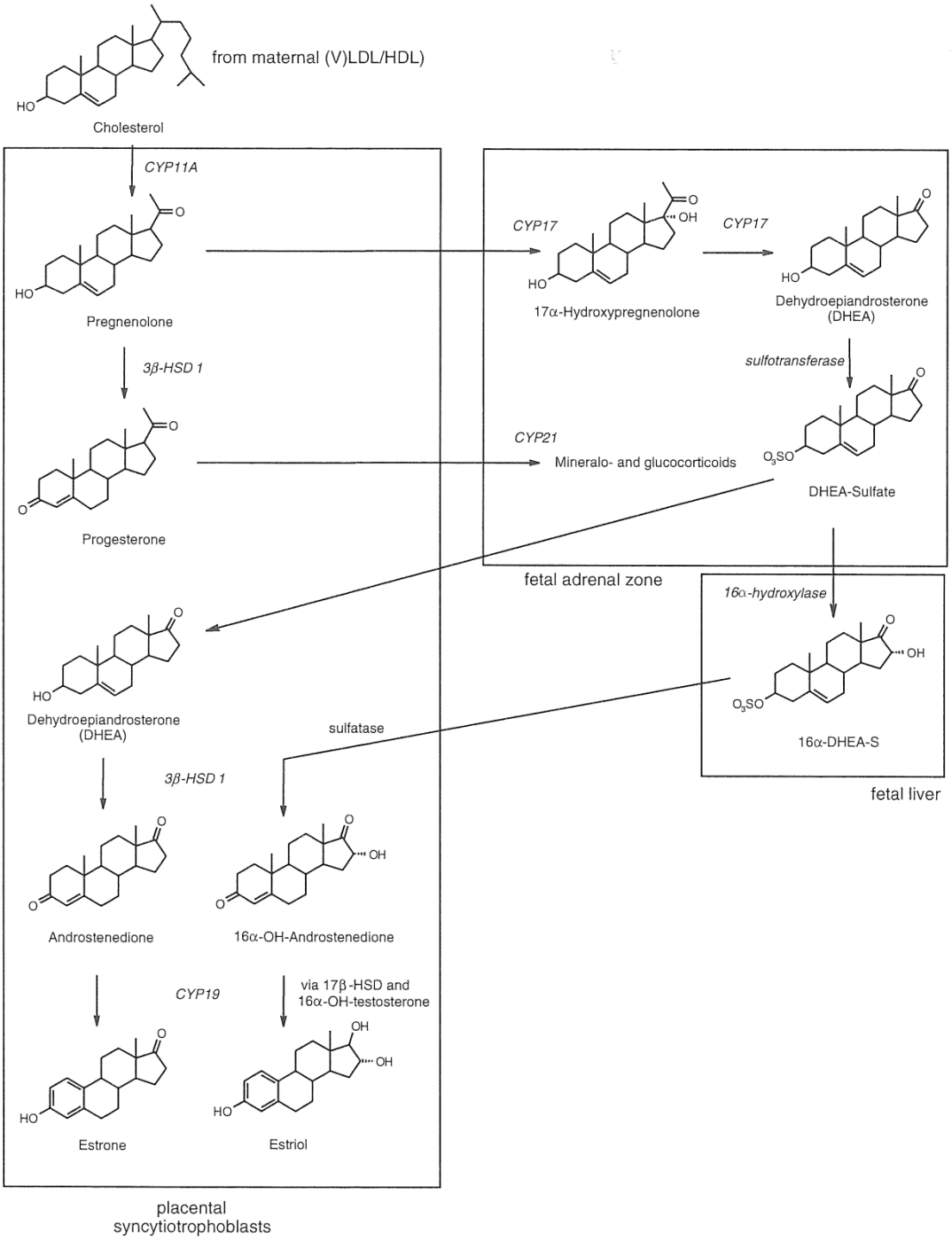


Figura 7: Esteroidogénesis en la unidad feto-placentaria (Sanderson, 2009).

Por el contrario, el feto femenino debe evitar la exposición a elevados niveles de andrógenos para evitar la masculinización. Después de la semana 12, se incrementa la actividad aromatasa en la placenta. Así, CYP19 es particularmente importante en mujeres embarazadas. Se ha demostrado que la deficiencia de actividad P450 aromatasa puede resultar en fenotipos maternal y fetal masculinizados debido a la acumulación de las hormonas masculinas androstenediona y testosterona (White, 2006; Ghayee and Auchus, 2007).

I.2.4.2. SÍNTESIS DE ESTRÓGENOS EN PECES

La implicación de la biosíntesis de estrógenos en la diferenciación ovárica está firmemente establecida en vertebrados, incluyendo pájaros, reptiles, anfibios y peces (Navarro-Martín et al., 2009). A diferencia de la mayoría de vertebrados, que disponen de una única forma CYP19, en peces se han caracterizado dos isoenzimas, Cyp19a y Cyp19b, cuya actividad se desarrolla principalmente en ovarios y en cerebro, respectivamente, y en menor medida en otros tejidos (pituitaria, retina, riñón, bazo, adiposo e hígado). Las dos formas derivan de genes separados y son regulados por mecanismos diferentes. Ambos genes aparecieron por una duplicación del genoma después de la divergencia de los peces sarcopterigios (ancestros de los tetrápodos), pero antes de la aparición de los teleósteos (pertenecientes al grupo de los actinopterigios). Curiosamente, la actividad P450 aromatasa en el cerebro de los peces es de cien a mil veces mayor que en mamíferos. En los adultos de lubina (*Dicentrarchus labrax*), el ratio de actividad Cyp19a:Cyp19b es 100:1 en ovarios y 1:1000 en cerebro (Piferrer et al., 2005).

En los ovarios de los peces teleósteos, P450 aromatasa controla los niveles de estrógenos en circulación. El estradiol generado se une y activa el receptor de estrógenos, y el complejo formado inicia la transcripción de determinados genes (Goksøyr, 2006). Como en la placenta humana, la señalización por AMPc/PKA es una vía importante de regulación de la expresión de la aromatasa ovárica en teleósteos. Además, se ha detectado la presencia de mRNA de *cyp19* en huevos inmediatamente después de la puesta, por lo que se deduce que existe transferencia materna al óvulo y que los embriones también poseen la capacidad de sintetizar estrógenos (Cheshenko et al., 2008). Así, la vía metabólica CYP19A es crítica para muchos aspectos relevantes de la biología de los peces, incluyendo diferenciación sexual, ciclo reproductivo y cambio de sexo en especies hermafroditas. La tasa de actividad Cyp19 depende de factores endógenos como el sexo del individuo (mayor en hembras que en machos), edad y estadio reproductivo, y de factores exógenos como la temperatura, estrés ambiental y presencia de agentes disruptores endocrinos en el medio acuático (Piferrer et al., 2005; Mills et al., 2014). Frecuentemente, el complejo P450 aromatasa es una diana para los disruptores endocrinos. Actualmente, la tecnología *in vitro* permite detectar los cambios en la actividad del enzima, permitiendo estimar el potencial disruptor endocrino de los contaminantes ambientales.

I.2.4.3. MECANISMOS DE MODULACIÓN DE LA ACTIVIDAD P450 AROMATASA (CYP19) POR AGENTES EXTERNOS

Los mecanismos de modulación de la esteroidogénesis por los disruptores endocrinos son múltiples e incluyen mimetización, agonismo y antagonismo del efecto de las hormonas endógenas (ej. competición por el receptor de

estrógenos), alteración de la síntesis y metabolismo de hormonas, cambios en los niveles de los receptores hormonales, regulación de la expresión del gen que codifica la proteína CYP19, alteración a nivel mRNA (degradación de mRNA), regulación post-traduccional (interacción con el complejo enzimático o degradación de proteínas) y alteración de la cascada de transducción de señales (AMPc/PKA), entre otros (Zhang et al., 2013). Sin embargo, el modo de acción de los disruptores endocrinos no implica necesariamente una única vía de las citadas sino que pueden actuar varios mecanismos conjuntamente. Algunos pesticidas (DDT, endosulfan, toxaphene, dieldrin, methiocarb, prochloraz, fenarimol, triadimefon y triadimenol) poseen la capacidad de unirse al receptor de estrógenos imitando los efectos del estradiol (Soto et al., 1994; Bonefeld-Jørgensen et al., 1997 & 2005; Andersen et al., 2002; Grünfeld and Bonefeld-Jørgensen, 2004; Kjeldsen et al., 2013b). Algunos disruptores endocrinos estrogénicos tienen, además, el potencial de alterar la expresión de *cyp19a* de peces durante la vitelogénesis mediante la modulación de la vía de señalización dependiente de CREB (cAMP-responsive element binding protein). Por otro lado, los fitoestrógenos apigenin, quercetin, chrysin, el fungicida imidazol y los compuestos organoestánnicos TBT y TPT, son capaces de inhibir la actividad P450 aromatasa en mamíferos y en peces, directamente a nivel de proteína (Cheshenko et al., 2008). Hay dos tipos de inhibidores de la actividad aromatasa a este nivel, los que compiten con el sustrato androstenediona para unirse al centro activo del enzima, y los que interactúan con el grupo prostético hemo del complejo P450 aromatasa (Brueggemeier et al., 2005). En carpas (*Cyprinus carpio*), la inhibición de la actividad P450 aromatasa ovárica observada por exposición a fenol, estaba asociada a una disminución de la expresión *cyp19* (Das et al., 2016). La inhibición del enzima implicaba además, una regulación a la baja de otros

genes dependientes de los niveles de estrógenos, incluyendo la expresión de vitelogenina y de la isoforma de aromatasa Cyp19b (Muth-Koehne et al., 2016). En experimentos *in vivo* con tilapia del Nilo (*Oreochromis niloticus*) y pez cebra (*Danio rerio*), la exposición a estrógenos aumentaba la actividad del enzima P450 aromatasa, mediante un incremento de los niveles de mRNA. En estos peces, cuando se inhibía la actividad P450 aromatasa, se observaba una masculinización (Uno et al., 2012). Por otro lado, aunque los estrógenos juegan un papel clave en el desarrollo de los caracteres sexuales femeninos, se ha demostrado que la regulación génica de *cyp19a* no está implicada en el desarrollo de ovarios en lubinas (*Dicentrarchus labrax*) macho expuestos a estrógenos exógenos (Navarro-Martín et al., 2009). Cabe mencionar que, dado que la actividad aromatasa puede ser regulada a nivel post-transcripcional, los cambios en la tasa de expresión génica y/o en los niveles de mRNA no pueden ser utilizados para predecir cambios en actividad P450 aromatasa (Villeneuve et al., 2006).

La actividad P450 aromatasa puede ser medida utilizando sustratos marcados con radioisótopos. En esta tesis, se ha utilizado un método basado en la detección del agua tritiada resultante de la reacción catalizada por el enzima aromatasa. Esta reacción aromatiza un sustrato marcado con tritio (^{3}H-androstenediona) liberando una molécula de agua que ha incorporado el ^{3}H, el cual puede detectarse en un contador de centelleo.

I.2.5. DISRUPCIÓN LIPÍDICA

En esta sección se describen brevemente las diferentes categorías de lípidos y sus funciones, se comentan las implicaciones de las diferentes especies lipídicas en las membranas celulares y sus mecanismos de síntesis, así como la importancia de las membranas celulares en la placenta humana, y se

mencionan las posibles alteraciones en la composición lipídica causadas por exposición a xenobióticos.

Los lípidos son componentes esenciales de los sistemas biológicos, asumiendo una notable variedad de funciones en la célula, algunas de ellas descubiertas recientemente. De hecho, los lípidos son tan importantes que las células invierten muchos recursos en generar cientos de lípidos diferentes usando para ello un 5 % de sus genes, lo cual es muy significativo (Van Meer et al., 2008). Los lípidos son moléculas hidrofóbicas o anfipáticas de estructuras variadas y complejas. Son la principal forma de almacenaje de energía celular y los principales componentes de las membranas celulares regulando su permeabilidad y fluidez, controlando el paso de sustancias y sirviendo de anclaje a las proteínas de membrana. Además, los lípidos son precursores y regulan la síntesis de otras biomoléculas (ej. esteroides, vitaminas y ácidos biliares), actúan como mensajeros (eicosanoides, derivados del fosfatidilinositol), y ayudan a mantener la temperatura corporal (triacilglicéridos). Dependiendo de los elementos que constituyen la molécula, los lípidos se clasifican en ocho categorías: acilos grasos, glicerolípidos, glicerofosfolípidos, esfingolípidos, esteroles, prenoles, sacarolípidos y poliquétidos (Brügger, 2014; Figura 8).

Los **acilos grasos** incluyen a los ácidos grasos y conjugados, octadecanoides, eicosanoides, docosanoides, alcoholes grasos, aldehídos grasos, ésteres grasos, amidas grasas, nitrilos grasos, éteres grasos, hidrocarburos e hidrocarburos oxigenados, siendo los ácidos grasos los más representativos. Los ácidos grasos son los principales bloques de construcción de los lípidos complejos (excepto algunos que no los contienen, como los esteroles). Son sintetizados por elongación de cadena a partir de acetil-CoA con sucesivos malonil-CoA, siendo aquellos de entre 14 y 22 carbonos los más

biológicamente relevantes. Algunos **ácidos grasos** insaturados, incluyendo el ácido linoleico (18:2) y el ácido linolénico (18:3), son imprescindibles para los mamíferos y deben ingerirlos en la dieta porque carecen de las enzimas necesarias para introducir dobles enlaces más allá del C9. Otros, como el ácido araquidónico, no se encuentra en plantas y sólo lo sintetizan los mamíferos a partir del ácido linoleico. Los glicerolípidos y glicerofosfolípidos se caracterizan por tener un glicerol con al menos un grupo hidroxilo sustituido. Basándose en el número de sustituciones del glicerol, el grupo de los glicerolípidos lo dominan los mono-, di- y tri-acilgliceroles, con uno, dos o tres ácidos grasos esterificados, respectivamente. Este grupo incluye además a los gliceroglicanos, que están unidos a un azúcar.

Por otro lado, los **glicerofosfolípidos**, principales componentes de la membrana celular, presentan dos ácidos grasos y además tienen el primer grupo hidroxilo del glicerol esterificado por un grupo fosfato, y éste a su vez esterificado por una base (colina, etanolamina, serina, inositol) que da nombre a las diferentes subclases (fosfatidilcolinas, fosfatidiletanolaminas, fosfatidilserinas y fosfatidilinositoles). En el caso especial de los plasmalógenos, el ácido graso del C1 del glicerol presenta un alquil (-O-CH2-) o O-alquenil éter (-O-CH=CH-). Los **esfingolípidos** tienen una base de cadena larga (comúnmente esfingosina) como estructura central, y son sintetizados a partir de serina y acetil-CoA. Dan lugar a las ceramidas, esfingomielinas y glicoesfingolípidos, entre otros, y participan activamente en la transducción de señales y reconocimiento celular. Los **esteroles**, otro grupo de lípidos, se sintetizan por la polimerización de pirofosfato de dimetilalilo, siendo el colesterol y sus derivados los más característicos.

Junto con los glicerofosfolípidos y las esfingomielinas, el colesterol constituye un componente muy importante de la membrana celular.

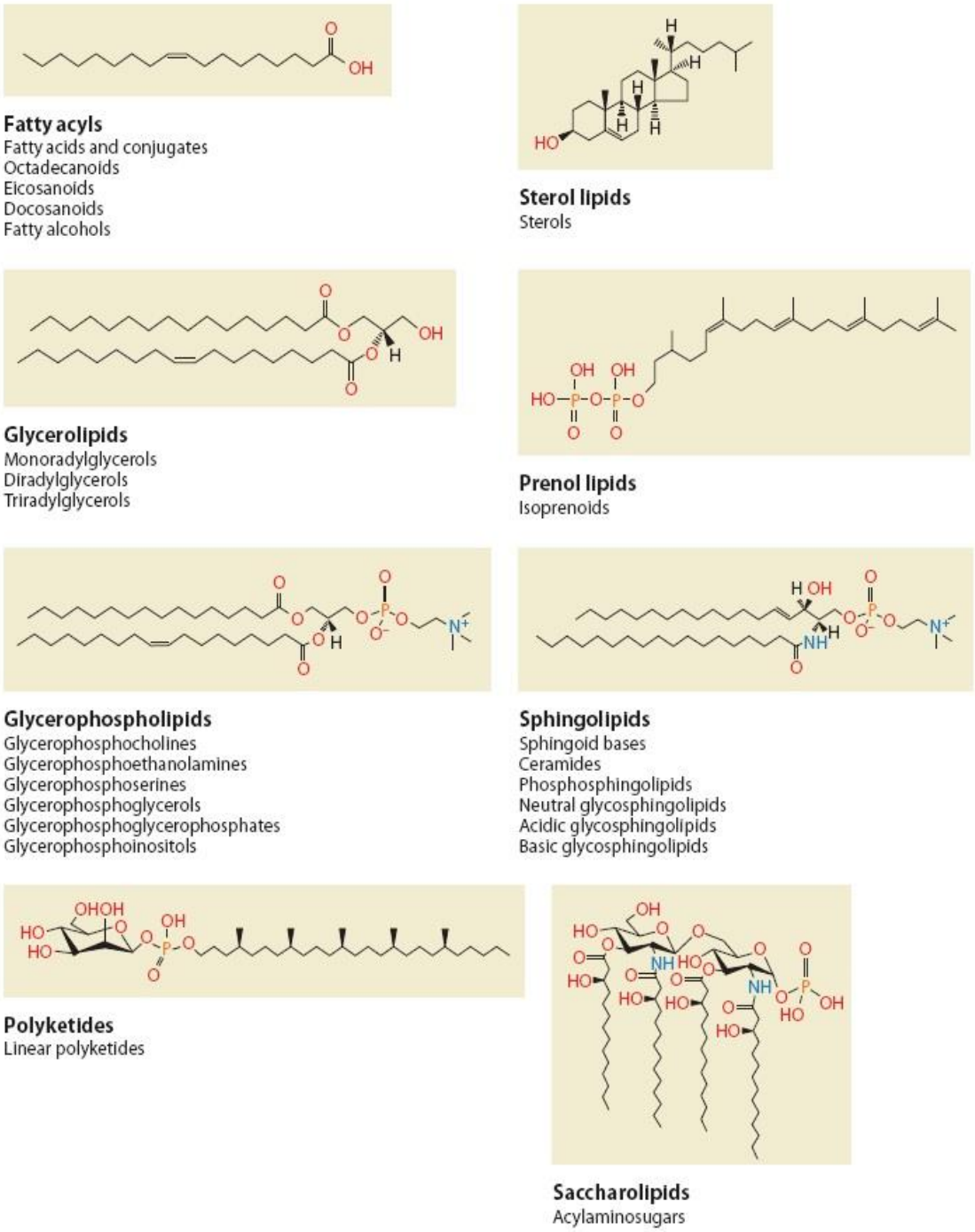


Figura 8: Se muestran las ocho categorías de lípidos, con ejemplos de cada clase (Brügger, 2014).

El grupo de los esteroles engloba además a los esteroides (hormonas sexuales), secosteroides (vitamina D) y ácidos biliares. El grupo de los **prenoles** comparte una vía de síntesis común con los esteroles, e incluye a los isoprenoides (carotenos, precursores de la vitamina A), quinonas e hidroquinonas, y vitaminas E y K. Los poliprenoles realizan funciones importantes, como el transporte de oligosacáridos a través de las membranas, reacciones de glicosilación y biosíntesis de polisacáridos. El siguiente grupo lo conforman los **sacarolípidos**, que se caracterizan por tener un azúcar unido al ácido graso, y forman estructuras compatibles con las bicapas de las membranas. Por último, los **poliquétidos** son sintetizados por actividad poliquétido-sintasa y luego modificados por glicosilación, metilación, hidroxilación y/u oxidación. Muchos compuestos antimicrobianos, antiparásitarios, y anticáncer son poliquétidos o derivados (ej. eritromicinas, tetraciclinas). Existen más de mil especies diferentes de lípidos. Aunque algunos no pueden sintetizarse y son aportados por la dieta, la gran mayoría se generan en la célula a partir de vías de biosíntesis complejas que usan principalmente ATP o CTP como fuente de energía y NADPH como reductor. Así, en el retículo endoplasmático se sintetizan fosfolípidos, colesterol, triacilgliceroles y ceramidas, estos últimos precursores de los esfingolípidos, y otros minoritarios o intermediarios de las vías de síntesis (diacilgliceroles, lisofosfolípidos). En el aparato de Golgi se sintetizan esfingolípidos, glicoesfingolípidos y glicoceramidas (Fahy et al., 2005).

Desde hace años, se sabe que existe una asociación entre las alteraciones en los niveles fisiológicos de lípidos y algunas enfermedades, incluyendo aterosclerosis, enfermedades infecciosas, obesidad, disfunciones cardiovasculares, cáncer y alteraciones neurodegenerativas (Van Meer et

al., 2008). En las últimas décadas, se ha sugerido la posibilidad de que muchas de esas alteraciones puedan estar causadas por la exposición cotidiana a contaminantes ambientales. De hecho, se ha demostrado que algunos xenobióticos son capaces de alterar el control celular sobre la adipogénesis y modificar el balance energético de la célula. Por ejemplo, tributiltin (TBT) promueve la adipogénesis uniéndose a los receptores RXR (retinoid X receptor) y PPAR (peroxisome proliferator-activated receptor), y consecuentemente induce la diferenciación de preadipocitos 3T3-L1 (Dimastrogiovanni et al., 2015).

I.2.5.1. LÍPIDOS DE MEMBRANA

Los principales componentes de las membranas celulares de los vertebrados son, junto con los esteroles, los glicerofosfolípidos y los esfingolípidos, que forman una bicapa con una parte hidrofóbica y una cabeza hidrofílica, esta última situada en la cara citoplasmática o extracelular (Figura 9). La abundancia relativa de cada especie lipídica confiere a la membrana la fluidez, permeabilidad y curvatura apropiadas para desarrollar las funciones propias de la bicapa. Estas propiedades están asociadas a los ácidos grasos que forman parte de los lípidos de membrana. Los ácidos grasos saturados tienen una temperatura de fusión más alta que aquellos insaturados, que aumenta con la longitud de la cadena de carbonos. Por ejemplo, el ácido palmítico (16:0) y el ácido palmitoleico (16:1) tan sólo se diferencian en un enlace doble. Sin embargo su temperatura de fusión varía en más de 60 ºC (63.1 ºC y -0.5 ºC, respectivamente). Evolutivamente, las células han adaptado su composición lipídica al ambiente externo para mantener su fluidez y permeabilidad adecuadas. Por ejemplo, a las bacterias de clima frío les conviene tener muchos ácidos grasos insaturados para bajar el punto de

fusión y evitar que las moléculas se empaqueten, lo que resultaría en una membrana demasiado rígida. En cambio, las arqueas hipertermófilas, que viven en hábitats con temperaturas de más de 100 ºC, presentan una monocapa de lípidos con una única cadena saturada de más de 30 carbonos, con ramificaciones (elevado punto de fusión) y con dos extremos polares para evitar que las altas temperaturas deshagan la estructura de la membrana. Así, los organismos pueden cambiar la cantidad de dobles enlaces en sus fosfolípidos y adaptarse así a vivir a distintas temperaturas.

Por otro lado, la curvatura de la membrana viene dada por la inclusión de fosfatidiletanolaminas en la bicapa de fosfatidilcolinas, así como por la distribución asimétrica de los lípidos de ambas capas. La inclusión de esfingomielinas aumenta el empaquetamiento de la membrana, debido a sus colas saturadas o trans-insaturadas que les permiten formar cilindros más altos y estrechos que las fosfatidilcolinas de la misma longitud de cadena. Los esteroles, principales lípidos no polares de las membranas, las hacen más fluidas (Van Meen et al., 2008). Las alteraciones en el balance de los lípidos de membrana desencadenan desórdenes en las funciones celulares. Por ejemplo, los cambios en el contenido de glicerofosfolípidos causan la coalescencia de las gotas lipídicas que contienen triacilgliceroles, contribuyendo a enfermedades relacionadas con el almacenaje de energía celular (Krahmer et al., 2013), mientras que cambios en la elongación o número de insaturaciones de los glicerofosfolípidos, alteran las propiedades físicas de la bicapa y consecuentemente afectan a la función de las proteínas que integran la membrana (Cook et al., 2002).

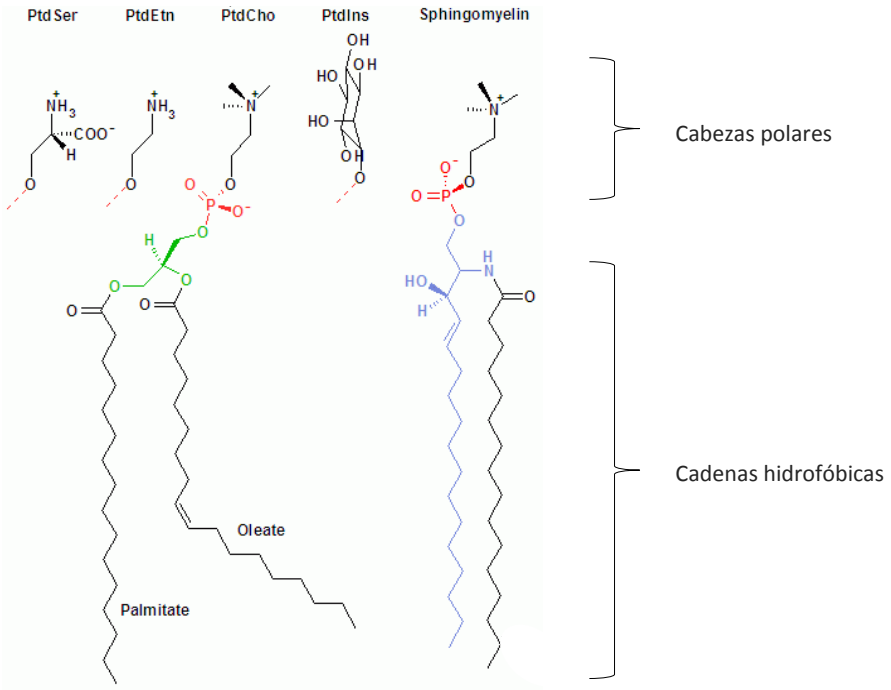


Figura 9: Estructura básica de los lípidos más abundantes de la membrana celular. PtdSer: fosfatidilserina, PtdEtn: fosfatidiletanolamina, PtdCho: fosfatidilcolina, PtdIns: fosfatidilinositol.

En mamíferos, la membrana celular cobra especial importancia en las células de la placenta, porque es el órgano encargado de transferir nutrientes y otras biomoléculas (ej. lípidos y hormonas) al compartimento fetal. La placenta humana presenta actividad lipoproteína lipasa, enzima encargada de liberar los ácidos grasos de los triacilgliceroles presentes en la sangre materna, y transferirlos al feto por difusión o por transporte activo (Coleman and Haynes, 1987). Los ácidos grasos de cadena larga y poliinsaturados son más propensos a cruzar la barrera placentaria, de forma que el compartimento fetal es rico en estos lípidos (Gude et al., 2004). Las células de placenta humana son capaces de sintetizar algunos ácidos grasos, incluyendo ácido oleico, palmítico y palmitoleico, y en menor cantidad, esteárico, mirístico y láurico (Herrera, 2002; Jones et al., 2014). A partir de estos ácidos grasos se sintetizan otros muchos mediante combinaciones de

elongaciones y desaturaciones, y se transfieren al feto o se utilizan en la síntesis de glicerofosfolípidos o glicerolípidos. Las elongaciones son catalizadas por enzimas situadas en la cara citoplasmática del retículo endoplasmático liso que utilizan la energía del CoA (ej. ácido palmítico (16:0) + malonil-CoA → ácido esteárico (18:0) + CoA + CO_2). Las desaturaciones, o adición de dobles enlaces, se realizan mediante reacciones de oxidación catalizadas por el complejo enzimático del citocromo P450 desaturasa, consumiendo O_2 y NADH (ej. ácido esteárico (18:0) + NADH + H^+ + O_2 → ácido oleico (18:1) + NAD^+ + 2 H_2O) (Cook et al., 2002; Haggarty, 2004; Innis, 2005).

Adicionalmente, la placenta juega un activo rol desde un punto de vista toxicológico. Debido a que el hígado fetal es inmaduro, la placenta presenta numerosos componentes característicos del tejido hepático adulto, incluyendo enzimas y proteínas transportadoras implicadas en el metabolismo de pigmentos, ácidos biliares y xenobióticos (Gude et al., 2004). Por motivos éticos, la información sobre los efectos de los xenobióticos sobre la composición lipídica de la membrana plasmática de las células de placenta humana es escasa. En este sentido, las líneas celulares ofrecen enormes posibilidades de estudio.

I.3. CONTAMINANTES AMBIENTALES

I.3.1. SEDIMENTOS MARINOS COMO RESERVORIO DE CONTAMINANTES

Las descargas urbanas, hospitalarias, industriales y agrícolas, así como el tráfico aéreo y naval son fuentes continuas que aportan contaminantes al

medio acuático, resultando en una mezcla de compuestos indefinida y desconocida. A pesar de los esfuerzos por depurar el agua en las plantas de tratamiento, muchos xenobióticos o sus metabolitos no se eliminan fácilmente y se vierten a los ríos. En época de lluvias o deshielo, el transporte de estas sustancias a través del río se incrementa debido al elevado caudal de agua, facilitando que los contaminantes viajen hasta el mar.

En el medio marino, los xenobióticos sufren procesos de transporte, sedimentación, adsorción, desorción, resuspensión y degradación, dependiendo su comportamiento de las propiedades físico-químicas del compuesto (ej. solubilidad en agua, K_{ow}, volatilidad) y del ambiente (ej. corrientes, viento, sustrato, temperatura). Uno de los destinos más comunes son los sedimentos marinos, que actúan como reservorios de los xenobióticos, quedando biodisponibles para los organismos bentónicos. A través de estos organismos, los contaminantes entran en la cadena trófica, y llegan a bioacumularse en las especies que ocupan los niveles superiores de la pirámide trófica (Chapman et al., 1998; Méndez-Fernández et al., 2014; Van Cauwenberghe and Janssen, 2014) (Figura 10). La mayoría de estos contaminantes causan serias amenazas para la salud de organismos marinos y seres humanos, y causan crisis ambientales en los ecosistemas marinos como pérdida de biodiversidad, proliferación de especies invasoras, eutrofización y anoxia (Tavakoly et al., 2014).

Las técnicas para la evaluación de la calidad de los sedimentos marinos se basan en organismos bioindicadores, análisis químicos y ensayos toxicológicos. Los xenobióticos más preocupantes se caracterizan por su elevada persistencia debido a su difícil degradación y a su hidrofobicidad. En puertos europeos (Barcelona (España), Port Vendres y Toulon (Francia), y

Constanza (Rumania)), se han llegado a detectar PAHs a concentraciones entre 1700 y 10000 ng/g (Baumard et al., 1998; Soclo et al., 2000; Coatu et al., 2013). Aunque los PAHs pueden provenir de procesos naturales como la quema de biomasa o erupciones volcánicas, la mayoría de estos compuestos tienen un origen antropogénico y provienen de combustión de carburante, vertidos y procesos industriales (Tobiszewski et al., 2012).

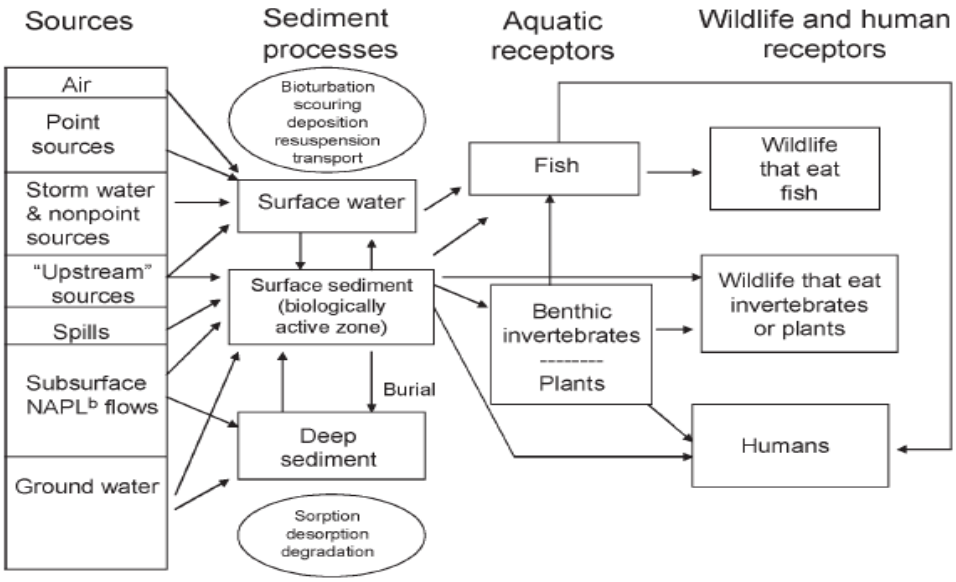


Figura 10: Modelo conceptual básico de contaminación de sedimentos (Wenning and Ingersoll, 2002).

Algunas sustancias (ej. PCBs y DDTs) que se detectan actualmente en ecosistemas acuáticos (en el rango de ng/g; Coatu et al., 2013), ya están en desuso desde hace más de sesenta años, pero su alta tasa de persistencia les hace perdurar de fuentes del pasado y representan un serio problema para el ecosistema. Ciertos fármacos, también pueden perdurar durante años en el medio ambiente. Por ejemplo, el ácido clofíbrico, metabolito del clofibrato que se receta habitualmente para regular los triacilglicéridos en sangre, puede persistir en el ambiente hasta veinte años, y se ha detectado en el Mar del Norte en concentraciones similares a los COPs, en el rango de

ng/L (Díaz-Cruz et al., 2003). Por otro lado, aunque otros contaminantes no son excesivamente persistentes, se detectan habitualmente debido a su continua entrada en el medio acuático (ej. 0.02 µg/L de bisfenol A en el río Ebro (España); Brossa et al., 2005).

Aunque los análisis químicos ofrecen una valiosa información sobre las concentraciones de contaminantes prioritarios en la matriz ambiental y su distribución geográfica, por sí solos son insuficientes para valorar el riesgo que conlleva la presencia de estos contaminantes para los seres vivos. Debido a los posibles efectos sinérgicos y antagónicos entre compuestos, las respuestas de los organismos acuáticos a la mezcla de contaminantes pueden resultar distintas a las que ejercerían esos mismos xenobióticos individualmente. Así mismo, los efectos biológicos pueden ocurrir a concentraciones por debajo del límite de detección del método analítico, y no necesariamente estar causados por el compuesto más abundante, sino por el más efectivo desde un punto de vista toxicológico. Por ejemplo, concentraciones de tan sólo 0.1 nM de TCDD inducen la expresión de *cyp1a* en la línea celular PLHC-1 (Villeneuve et al., 2000). En este sentido, aunque no ofrecen información de qué compuestos están implicados en las respuestas observadas, los ensayos toxicológicos tienen la ventaja de que permiten responder preguntas sobre biodisponibilidad, efectos y mecanismos de acción del conjunto de sustancias presentes en el sedimento. Dentro de este contexto, esta tesis investiga el uso de una selección de ensayos toxicológicos *in vitro* para la rápida detección de sustancias bioactivas en sedimentos marinos de diferentes áreas costeras.

I.3.2. CONTAMINANTES EMERGENTES

Se denominan "contaminantes emergentes" aquellos xenobióticos que, aunque no son necesariamente nuevos en el ambiente, son motivo de reciente preocupación debido a su frecuente detección en las plantas de tratamiento de aguas residuales, ecosistemas acuáticos y tejidos animales y humanos (Martínez-Bueno, 2012; Stuart et al., 2012). Actualmente, una gran variedad de contaminantes se consideran emergentes: bisfenol A, ftalatos, alquilfenoles, compuestos perfluorados, parabenos, triclosan, fragancias sintéticas, nanopartículas y compuestos bromados, entre otros. Los contaminantes emergentes están presentes en numerosos artículos de uso cotidiano y sanitario, incluyendo productos de cuidado personal, fármacos y material médico, detergentes, mobiliario, ropa, calzado y envases alimentarios. Aunque estos productos han mejorado en gran medida nuestro estilo de vida, al mismo tiempo representan una fuente constante de contaminación, principalmente por ingestión, pero también por absorción dérmica e inhalación de las sustancias químicas que contienen. En esta tesis se investigan determinados ftalatos, bisfenol A, alquilfenoles y compuestos perfluorados como modelos de contaminantes emergentes. En la Tabla 1 se muestran los usos más comunes de algunos de dichos compuestos de interés en esta tesis.

Los **plastificantes** son aditivos químicos añadidos a los polímeros plásticos para otorgarles propiedades de flexibilidad y durabilidad. Entre los países productores de plásticos, China encabeza el ranking (26 % de la producción global), seguida por Europa (20 %), Norte América (19 %) y resto de Asia (16 %). En Europa, el 40 % de los plastificantes se utiliza en la fabricación de envases, mientras que el resto se usa en material de construcción (20 %), automoción (9 %), material eléctrico (6 %), agricultura (3 %) y otros usos

(mobiliario, deportes y salud; 22 %) (www.plasticseurope.org). En 2014, la demanda de plásticos en Europa fue de 48 millones de toneladas, situando a España entre los países con mayor demanda (7 % del total) detrás de Alemania (25 %), Italia (14 %), Francia (10 %) y Reino Unido (8 %). Aunque Alemania es el país que más plásticos utiliza, su tasa de reciclaje/recuperación de energía es prácticamente 98 %, mientras que en España sólo se recupera el 50 %. Los ftalatos son los plastificantes más utilizados, seguidos por el bisfenol A y los alquilfenoles (Tabla 1) (Li et al, 2013; Russo et al., 2015; Tran et al., 2015).

Los **ftalatos**, ésteres de ácido ftálico derivados del petróleo, se empezaron a utilizar como plastificantes en 1920. El primer ftalato que se utilizó fue el DBP, aunque hoy en día, el DEHP es el más distribuido (51 % del total; Rahman et al., 2004) (Tabla 1). Actualmente, los ftalatos representan el 90 % de los plastificantes que se producen a nivel mundial. Actúan reduciendo las fuerzas de atracción entre las cadenas de los polímeros, permitiéndoles mayor movilidad y reduciendo su dureza. Se unen de forma no covalente a las cadenas, de manera que son susceptibles de migrar en circunstancias de elevadas temperaturas, manipulación y radiación (Fasano et al., 2012). Sus propiedades físico-químicas están condicionadas a la longitud de sus cadenas de alquilo. Por ejemplo, la solubilidad en agua disminuye desde los ftalatos de cadenas de alquilo cortas (ej. DMP) a los de cadenas largas (ej. DEHP). Este aumento en la hidrofobicidad proporciona a los ftalatos de mayor peso molecular, una fuerte capacidad de adsorción a la materia orgánica. En comparación con otras clases de productos químicos orgánicos, los ftalatos no son ambientalmente persistentes ni sujetos a un transporte a largo plazo. Se pueden bioacumular en invertebrados, peces y plantas, pero no existe biomagnificación porque los animales de escalas superiores

presentan eficientes mecanismos celulares para metabolizarlos y excretarlos (Shea, 2003). Sin embargo, debido a su presencia en el ambiente cotidiano, los ftalatos se detectan frecuentemente en tejidos humanos, los cuales sufren una exposición crónica a estos compuestos. En 1970, Jaeger y sus colaboradores observaron por primera vez la presencia de DEHP en sangre humana almacenada en bolsas de PVC destinada a transfusiones. A partir de entonces, numerosos estudios han demostrado el potencial de migración de los ftalatos al contenido del envase de alimentos y bebidas (Fasano et al., 2012), así como su transferencia a los pacientes que recibían tratamiento (diálisis y transfusiones) a través de tubos y bolsas de plástico en los hospitales (Testai et al., 2016). Las principales vías de exposición a los ftalatos son la ingesta de comida o bebida contaminada, vía parenteral, absorción dérmica e inhalación. Se han detectado ftalatos en diferentes tejidos humanos y animales, incluyendo sangre materna y fetal, lo cual implica que algunos de ellos pueden atravesar la barrera placentaria, quedando expuesto el embrión (Vandenberg et al., 2007).

Aunque los ftalatos no parecen tener una elevada toxicidad aguda (50 % mortalidad (LD_{50}) entre 1 y 30 g/kg en ratas), se conocen diversos efectos subletales adversos. Los estudios realizados con modelos animales y tejidos humanos señalan a los ftalatos como indiscutibles disruptores endocrinos. Sin embargo, existen diferencias interespecíficas ya que muchas veces los resultados en ratas no son extrapolables a los seres humanos. Por ejemplo, en cultivos primarios de células de granulosa ováricas humanas, la exposición a DBP altera la esteroidogénesis a través de la regulación a la baja de la expresión de *CYP11* y *CYP19* resultando en una menor producción de progesterona y estradiol, mientras que en ovarios de roedores se ha observado una inducción en la expresión de dichos genes

(Sen et al., 2016; Adir et al., 2017). Por otro lado, la exposición a DEHP resulta en una reducción de la producción de testosterona en explantes de gónadas masculinas humanas (Desdoits-Lethimonier et al., 2012), mientras que en ratones *in vivo* genera carcinoma hepatocelular, proliferación de peroxisomas y mitocondrias, aumento de actividad CYP4A1, proliferación de tejido hepático y supresión de la apoptosis, estando muchos de estos efectos asociados al receptor PPAR (Heudorf et al., 2007).

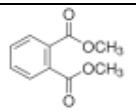
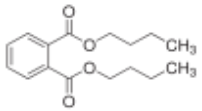
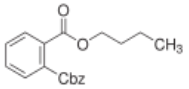
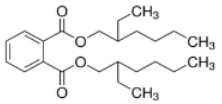
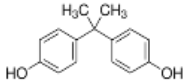
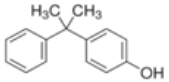
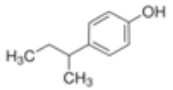
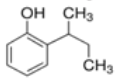
El **bisfenol A** (BPA), formado por dos grupos fenol, se utiliza en la fabricación de envases alimentarios, incluyendo botellas, latas, platos y vasos de plástico y fiambreras, y representa uno de los productos químicos de mayor producción mundial, alcanzando los cinco millones de toneladas anuales en 2014 (prweb.com/releases/2014/04/prweb11761146.htm). Al igual que para los ftalatos, varios estudios han confirmado la capacidad de BPA para atravesar la placenta humana, ya que se han detectado concentraciones de hasta 5.6 ng/mL (~25 nM) en sangre de cordón umbilical, en líquido amniótico y en sangre fetal (Yamada et al., 2002; Vandenberg et al., 2007). Por este motivo, se han invertido grandes esfuerzos en investigar los efectos tóxicos del BPA a nivel reproductivo. Por ejemplo, se sabe que concentraciones tan bajas como 0.1 y 1 µM de BPA, causan citotoxicidad en cultivos primarios de células de granulosa ováricas y de placenta humana, respectivamente (Xu et al., 2002; Benachour and Aris, 2009). Además, BPA tiene otros efectos tóxicos subletales multidireccionales: tiene potencial oxidativo, es capaz de unirse al receptor de hidrocarburos de arilo y a los receptores de estrógenos y andrógenos, perturba la función de varias hormonas (hormonas sexuales, leptina, insulina y tiroxina), causando efectos hepatotóxicos, inmunotóxicos, mutagénicos y carcinogénicos (Iso et al., 2006; Meeker et al., 2010, Clayton et al., 2011; Hassan et al., 2012; Ziv-Gal

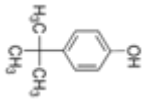
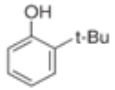
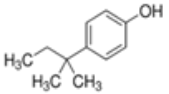
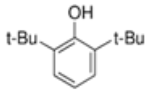
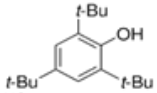
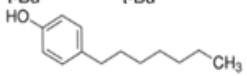
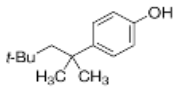
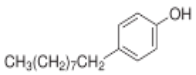
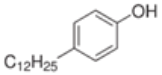
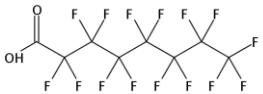
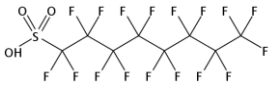
et al., 2013; Michałowicz, 2014). Se ha sugerido que la exposición a BPA y otros xenoestrógenos durante el desarrollo temprano podría ser la causa del incremento en la tasa de infertilidad, deformidades en el tracto genital y cáncer de mama observados en la población europea y estadounidense en los últimos 50 años (Vandenberg et al., 2007). Por su potencial de migración (hasta 0.8 µg/L; Fasano et al., 2012) junto a los datos toxicológicos generados, la Comisión Europea prohibió el uso de BPA en la fabricación de biberones (2010), y lo limitó en juguetes y artículos para bebés (2014). Así mismo, la EFSA (2015) redujo la ingesta diaria tolerable (TDI) de BPA de 50 a 4 µg/kg de peso al día y se comprometió a publicar una reevaluación en 2017 (www.eur-lex.europa.eu).

Los **alquilfenoles** (APs) son moléculas formadas por un anillo fenólico y una cadena alquilo, ramificada o no, con diferente número de carbonos, en posición *orto*- o *para*- (la posición *meta*- es muy inestable y no es común). La longitud de la cadena de carbonos, posición y grado de ramificación les otorgan diferentes propiedades físico-químicas, incluyendo persistencia e hidrofobicidad, que pueden influir en su comportamiento y toxicidad para los organismos expuestos. Se utilizan en la fabricación de detergentes industriales, productos de cuidado personal, pesticidas, lubricantes y envases de alimentos, entre otros (Tabla 1). Los APs con mayor distribución y por ende los más estudiados tienen ocho y nueve carbonos en su cadena, octilfenol (OP) y nonilfenol (NP), respectivamente. Estos dos compuestos se han detectado en alimentos, bebidas, tejidos humanos (incluyendo cordón umbilical y placenta) y animales en el rango de µg/kg (Fernández et al., 2007; Fasano et al. 2012; Geens et al., 2012; Zuo et al., 2015). En la actualidad, ambos compuestos, OP y NP, están considerados disruptores endocrinos.

Concentraciones de 1 nM aumentan la actividad caspasa-3 (mediador esencial de la apoptosis celular) y la secreción de gonadotropina coriónica en las células del trofoblasto en explantes de placenta humana (Bechi et al., 2006). Inducen la expresión del receptor de progesterona en la línea celular de carcinoma mamario humano MCF-7, y se unen al receptor de estrógenos, dificultando la unión específica del estradiol. Al interrumpir las funciones endocrinas normales, los APs generan fallos en la reproducción y carcinogénesis en los tejidos sensibles a los estrógenos (Soto et al., 1995; Sonnenschein et al., 1998; Blair et al., 2000). Debido a los efectos nocivos evidenciados para NP y OP, en la producción de algunos artículos se han sustituido por otros APs, como butilfenol y dodecilfenol. Sin embargo, la información actual disponible a nivel toxicológico de estos compuestos es muy escasa, por lo que se necesita invertir en estudios que generen nuevos datos en este sentido. Los **compuestos perfluorados** (PFCs) están formados por cadenas de carbono y flúor, aunque pueden contener también átomos de azufre. Son notablemente persistentes debido a la estabilidad que les confiere el enlace carbono-flúor, considerado el enlace simple más fuerte de la química orgánica. Estas características les confieren importantes propiedades, incluyendo impermeabilidad, antiadherencia y resistencia al fuego, que los hacen idóneos para su uso en numerosas ramas industriales desde 1950. Se aplican en recubrimientos de utensilios de cocina, ropa deportiva y militar de clima extremo, equipos de manipulación de alimentos, equipos médicos, aditivos para aceite de motor, espumas contra incendios, pintura y tinta, así como productos hidrófugos. Los compuestos perfluorados están ampliamente distribuidos en el medio ambiente y tienen un elevado potencial de bioacumulación debido a su resistencia a la degradación.

Tabla 1: Propiedades físico-químicas y usos más comunes de algunos de los contaminantes emergentes de interés en esta tesis. Fuentes: www.echa.europa.eu; www.sustainableproduction.org; www.cosmos.com.mx/wiki/cqfz/ftalato-de-dimetilo-dimetil-ftalato; www.nj.gov/health/eoh/rtkweb/documents/fs/1440.pdf ;pubchem.ncbi.nlm.nih.gov/compound/4-tert-Amylphenol#section=Formulations-Preparations; www.ec.europa.eu; www.scifinder.cas.org; www.sigmaaldrich.com; Brooke et al., 2005. Ps: ftalatos; APs: alquilfenoles; PFs: perfluorados; PM: peso molecular; Sol.: solubilidad en agua; PF: punto de inicio de fusión; n.d.: no disponible.

Grupo	Compuesto	Fórmula	PM	Log K_{ow}	Sol. (mmol/L)	PF (ºC)	Usos
Ps	Dimetil ftalato (DMP)		194.18	1.6	21	5.5	Repelente de insectos, perfumes, cepillos de dientes, herramientas, juguetes, envases de alimentos, aspirinas, y se añade al alcohol de farmacia para darle sabor amargo.
	Dibutil ftalato (DBP)		278.34	4.1	0.04	-35	Plastificante para PVC, PVA y caucho, solvente y fijador para pinturas y cosméticos. Adhesivos de látex, selladores, productos de limpieza automóvil, cosméticos, tintas, insecticidas, envases alimentarios, mobiliario, pintura y cápsulas para medicamentos.
	Butil bencil ftalato (BBP)		312.36	4.7	0.01	-35	Plastificante para PVC, poliuretano, polisulfido y polímeros acrílicos. Vinilos, selladores, adhesivos, cintas transportadoras de alimentos, envases de alimentos y cuero sintético.
	Di(2-etilhexil) ftalato (DEHP)		390.56	7.3	0.001	-55	Se añade al PVC para darle flexibilidad. Se usa en la fabricación de envases alimentarios, juguetes, cosméticos, calzado, impermeables, ropa, instrumental médico (bolsas de sangre, tubos intravenosos), mobiliario, material de construcción y artículos de automoción.
Pseudo-APs	Bisfenol A (BPA)		228.29	3.6	0.31	150	Aditivo para policarbonatos. Botellas de agua y leche, recubrimiento interior de latas de alimentos y refrescos, papel térmico, selladores dentales.
	4-Cumilfenol (CP)		212.29	4.2	0.17	74	Estabilizador de polímeros. Resinas, surfactantes no iónicos, aceites lubricantes.
APs	4-*sec*-Butilfenol (4SBP)		150.22	3.5	4.8	46	Plastificante, resinas, pesticidas.
	2-*sec*-Butilfenol (2SBP)		150.22	3.4	5.5	12	Reguladores de pH y tratamiento de agua, productos químicos de papel, tintes, desinfectantes, biocidas, yeso, cemento.

	4-*terc*-Butilfenol (4TBP)		150.22	3.4	6.3	96	Resinas solubles en aceite (adhesivos), lubricantes, plásticos, policarbonatos, colorantes mate, hules, estabilizador en jabones.
	2-*terc*- Butilfenol (2TBP)		150.22	3.3	7.2	-7	Estabilizador en plásticos. Intermediario en la obtención de resinas, perfumes, tensoactivos y plastificantes.
	4-*terc*-Amilfenol (TAP)		164.24	3.9	2.3	88	Biocidas, desinfectantes hospitalarios, fragancias, demulsificante, limpiadores, plásticos.
	2,6-di-*terc*-Butilfenol (DTBP)		206.32	4.5	0.7	37	Estabilizador en plásticos, resinas, tensoactivos, antioxidante para polímeros, aceites y ceras.
	2,4,6-tri-*terc*-Butilfenol (TTBP)		262.43	5.3	0.7	125	Estabilizador de UV y antioxidante para plásticos y gasolinas, aditivo en pinturas, barnices, aceites secantes y tintas de impresión.
	4-Heptilfenol (HP)		192.30	5.1	0.3	25	Intermediario para obtener aditivos de detergentes dispersantes y aceites lubricantes, estabilizadores y hules.
	4-*terc*-Octilfenol (OP)		206.32	5.2	0.3	84	Limpiadores industriales, detergentes, emulsionantes, pesticidas, material de construcción, cosméticos, espumante, medicamentos veterinarios.
	4-Nonilfenol (NP)		220.35	6.1	0.094	80	Piel sintética, papel, pinturas, textiles, detergentes industriales, agroquímicos, agentes dispersantes, resinas, recubrimiento de conductos/envases de gas y petróleo, acero, antiparásitos y antioxidantes.
	4-Dodecilfenol (DP)		262.43	7.7	0.04	117	Pesticidas, medicamentos veterinarios, tensoactivos, resinas, lubricantes, detergentes, agente secante y emulsionante.
PFs	Ácido perfluorooctanoico (PFOA)		414.07	6.4	32	52	Cubierta antiadherente en utensilios para cocinar y hornear. Emulsionante.
PFs	Ácido perfluorooctano sulfónico (PFOS)		500.13	4.5	15	n.d.	Tratamientos de resistencia al agua en pieles, alfombras, ropa deportiva, interiores del automóvil y envases de alimentos. Aditivos en limpiadores alcalinos, abrillantadores de suelos, películas fotográficas, limpiadores de prótesis dentales, champús y aditivos para revestimientos.

Los PFCs más utilizados son PFOS y PFOA. Se han detectado concentraciones de PFOS en cordón umbilical (11 ng/mL) y en sangre humana (50 ng/mL) de la población normal, mientras que en trabajadores expuestos se han llegado a encontrar concentraciones de hasta 2000 ng/mL (Olsen et al., 2009). Los datos disponibles sobre la toxicidad de los compuestos perfluorados los apuntan como disruptores endocrinos y obesógenos (Grün et al., 2009; Kjeldsen and Bonefeld-Jørgensen, 2013a). Por ejemplo, PFOA inhibe la actividad 3α-hidroxiesteroide deshidrogenasa y 17 α-hidroxiesteroide deshidrogenasa-3 por competición con el sustrato, disminuye los niveles de testosterona en las células de Leydig de ratones (Zhao et al., 2010), y reduce la expresión de *CYP11A* en la línea celular de córtex de glándula adrenal humana H295R, mientras que en estas células, PFOS altera la esteroidogénesis incrementando los niveles de estradiol (Kraugerud et al., 2011).

I.4. MODELOS *IN VITRO* UTILIZADOS EN ESTA TESIS

La elección del modelo biológico puede influir significativamente en la relevancia de los resultados en los estudios de toxicología ambiental. Cada sistema presenta características que aportan sensibilidad para determinados ensayos. A continuación se citan los diferentes modelos *in vitro* utilizados en esta tesis.

Para la evaluación del riesgo para la salud y reproducción humana se ha utilizado una línea celular de placenta humana. Debido a que la placenta tiene funciones de trasporte de productos entre la madre y el embrión, las sustancias tóxicas que la dañan pueden alterar posteriormente el desarrollo fetal (Mattison, 2010). Por lo tanto, los ensayos toxicológicos en células de

placenta pueden detectar compuestos susceptibles de poner en riesgo la salud del feto (Göhner et al., 2016). En esta tesis, la línea celular de placenta humana JEG-3 (Figura 11) se ha seleccionado como modelo *in vitro* porque posee muchas de las características biológicas y bioquímicas de los sincitiotrofoblastos. Las células JEG-3 tienen activas varias enzimas implicadas en la esteroidogénesis incluyendo CYP19, CYP11, 3α-HSD y 17α-HSD, mientras que carece de actividad CYP17 y 16α-hidroxilasa. Así, siempre que le sea suministrado el sustrato cuando el enzima que convierte a su precursor no está activo, las células JEG-3 son capaces de sintetizar niveles significativos de esteroides, incluyendo pregnenolona, progesterona y estradiol, y exhiben una elevada actividad basal P450 aromatasa comparado con otros modelos celulares, propiedades que la convierten en un modelo ideal para estudios de disrupción endocrina (Bahn et al., 1981; Sanderson, 2001). Además, se ha utilizado la fracción subcelular S9 aislada de JEG-3, como fuente del complejo enzimático P450 aromatasa. La fracción S9 consiste en fragmentos de retículo endoplasmático y enzimas citosólicos, de manera que permite observar efectos sobre la actividad del enzima en presencia de contaminantes sin el impedimento de tener que atravesar la barrera de la membrana celular. Así, la combinación del uso de las células JEG-3 intactas junto con la fracción S9 ofrecen la posibilidad de estudiar los mecanismos de acción de los disruptores endocrinos potenciales que modulan el enzima P450 aromatasa.

Por otro lado, se ha seleccionado la línea celular PLHC-1 (Figura 11), que proviene de un hepatocarcinoma del pez *Poeciliopsis lucida*. Esta línea celular conserva algunas funciones diferenciadas de los hepatocitos, incluyendo la capacidad de inducir la expresión de *cyp1a* tras la exposición a contaminantes ambientales como PCBs y PAHs, entre otros (Fent, 2001).

La respuesta de inducción de CYP1A en peces está considerada un valioso biomarcador en estudios de contaminación ambiental. Además, las células de peces PLHC-1 se cultivan a temperaturas más bajas (30 ºC) que las células de mamífero (37 ºC), acercándose más a las temperaturas del medio acuático. La línea celular PLHC-1 permite también realizar ensayos de generación de estrés oxidativo por la presencia de xenobióticos, así como de citotoxicidad. Sin embargo, dado que provienen de tejido hepático, las células PLHC-1 no presentan suficiente actividad P450 aromatasa. Por este motivo, se ha utilizado la fracción microsomal de ovario de lubina (*Dicentrarchus labrax*) como fuente de dicho complejo enzimático, para la detección de disruptores endocrinos en muestras ambientales, ya que la actividad de este enzima se ve a menudo alterada en presencia de estos xenobióticos.

Finalmente, se ha utilizado la línea celular COS-7 (Figura 11) proveniente de tejido renal de mono africano (*Cercopithecus aethiops*). Se trata de una línea celular tipo fibroblasto, apropiada para transfecciones por vectores que requieren la expresión del antígeno T del virus SV40 para su replicación (lgcstandards-atcc.org/Products/All/CRL-1651.aspx). En esta tesis, las células COS-7 se han transfectado con plásmidos que contienen el receptor zfPxr. Mediante un ensayo relativamente rápido y sencillo (ensayo de la luciferasa), las células COS-7 transfectadas permiten la detección de respuestas de activación de zfPxr por determinados xenobióticos (Lille-Langøy et al., 2015).

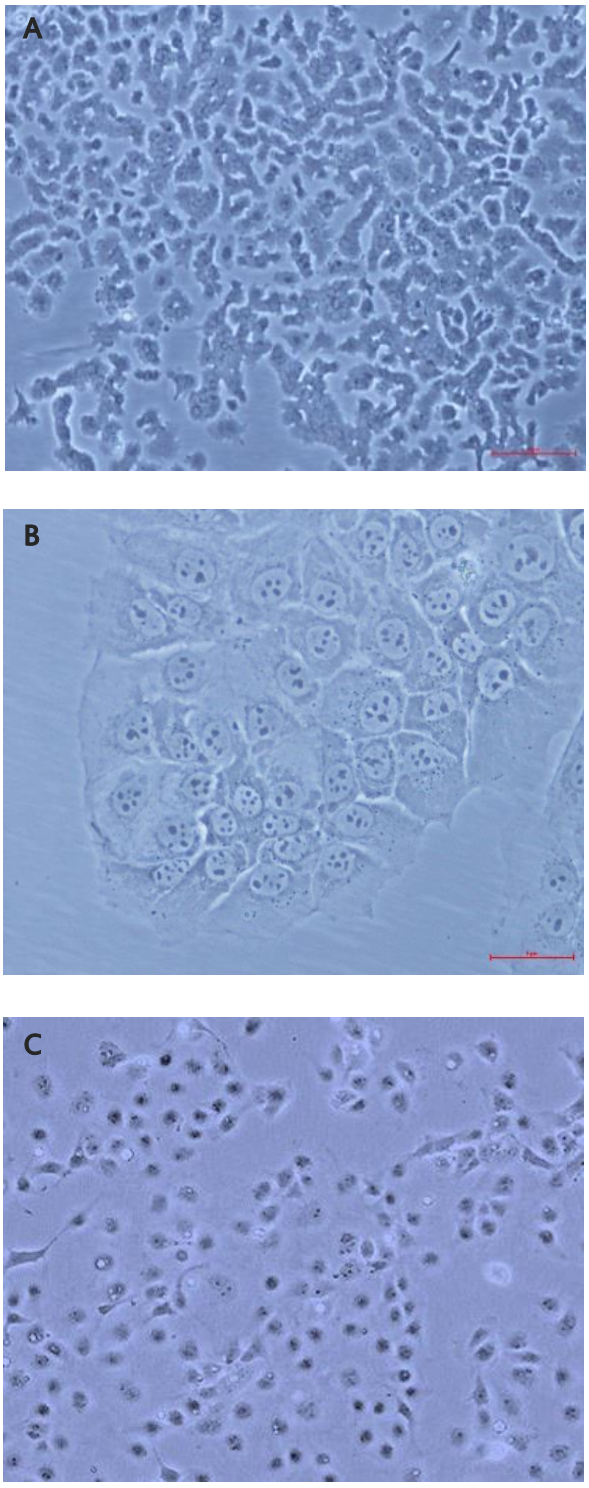


Figura 11: Microfotografías (10x) de cultivos de células PLHC-1 (A), JEG-3 (B) y COS-7 (C).

II. OBJETIVOS

El objetivo general de esta tesis es investigar la utilidad de diferentes bioensayos basados en el uso de líneas celulares (peces/humanas) y fracciones subcelulares, para la caracterización del riesgo ambiental que diversos contaminantes orgánicos comportan tanto para los ecosistemas acuáticos como para la salud humana, con especial énfasis en disruptores endocrinos y ligandos de receptores celulares implicados en la biotransformación de xenobióticos. Los objetivos específicos se definen a continuación.

OBJETIVO 1

Caracterizar la calidad ambiental de sedimentos marinos costeros de diferentes áreas del Mar Mediterráneo, Mar Negro, estuario del Algarve, Bahía de Paraguaná (Brasil) y Mar de Weddell (Antártida), mediante una batería de bioensayos *in vitro*, basada en el uso combinado de la línea celular PLHC-1, células COS-7 transfectadas con el receptor zfPxr, y fracciones microsomales de ovario de lubina (*Dicentrarchus labrax*). Los resultados de este objetivo se detallan en el Tema 1, en el cual se engloban los siguientes artículos:

1. Pérez-Albaladejo, E., Rizzi, J., Fernandes, D., Lille-Langøy, R., Karlsen, O. A., Goksøyr, A., Oros, A., Spagnoli, F., Porte, C. 2016. Assessment of the environmental quality of coastal sediments by using a combination of *in vitro* bioassays. Marine Pollution Bulletin, 108, 53-61.

2. Blanco, M., Pérez-Albaladejo, E., Barata, C., Piña, B., Kušpilic, G., Lille-Langøy, R., Karlsen, O.A., Goksøyr, A., Porte, C. Assessing the

environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays. Submitted to Science Total Environment.

3. Rizzi, J., Pérez-Albaladejo, E., Fernandes, D., Contreras, J., Froehner, S., Porte, C. 2016. Characterization of quality of sediments from Paranaguá Bay (Brazil) by combined *in vitro* bioassays and chemical analyses. Environmental Toxicology and Chemistry, 1-9.

4. Fernandes, D., Pujol, S., Pérez-Albaladejo, E., Tauler, R., Bebianno, M.J., Porte, C. 2014. Characterization of the environmental quality of sediments from two estuarine systems based on different *in vitro* bioassays. Marine Environmental Research, 96, 127-135.

Resultados adicionales: Sedimentos de la Antártida inducen CYP1A en células de peces PLHC-1.

OBJETIVO 2

Investigar la toxicidad de diferentes compuestos utilizados como plastificantes, surfactantes, retardantes de llama y recubrimientos antiadherentes, incluyendo ftalatos, bisfenol A, alquilfenoles y compuestos perfluorados, en la línea celular de placenta humana JEG-3. Se evalúa su capacidad de actuar como disruptores endocrinos y de alterar la composición lipídica de las células de placenta, prestando especial atención a la biodisponibilidad de los compuestos en el medio de cultivo. Los

resultados obtenidos se describen en el Tema 2, que abarca los siguientes artículos:

5. Pérez-Albaladejo, E., Fernandes, D., Lacorte, S., Porte, C. 2017. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells. Toxicology *in Vitro*, 38, 41-48.

6. Pérez-Albaladejo, E., Fajardo, K., Lacorte, S., Porte, C. 2017. Differental toxicity of alkylphenols in JEG-3 human placental cells: alteration of P450 aromatase and cell lipid composition. Submitted to Toxicology and Applied Pharmacology.

7. Gorrochategui, E., Pérez-Albaladejo, E., Casas, J., Lacorte, S., Porte, C. 2014. Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells. Toxicology and Applied Pharmacology, 277, 124-130.

III. Informe de factor de impacto y grado de implicación

Cinta Porte Visa, directora de la Tesis doctoral presentada por Elisabet Pérez Albaladejo, expone que la doctoranda ha participado activamente en la realización de todos los artículos presentados en la memoria, tal y como se refleja en la distribución de autores, siendo primera autora en tres de los siete artículos presentados, segunda autora en tres y tercera en uno de ellos. En general, ha participado en la discusión del diseño experimental, y ha contribuido de manera significativa al análisis de muestras, elaboración y redacción de los trabajos.

A continuación se detallan los artículos que forman parte de esta tesis, el grado de participación de la doctoranda y el factor de impacto (F.I.) de las revistas en las cuales los artículos han sido publicados o enviados para su publicación.

1. **Pérez-Albaladejo, E.**, Rizzi, J., Fernandes, D., Lille-Langøy, R., Karlsen, O. A., Goksøyr, A., Oros, A., Spagnoli, F., Porte, C. 2016. Assessment of the environmental quality of coastal sediments by using a combination of *in vitro* bioassays. *Marine Pollution Bulletin, 108, 53-61*. **F.I.: 3.099**

   Grado de participación: Realización de todo el trabajo experimental excepto ensayos de actividad aromatasa. Discusión de resultados. Elaboración del manuscrito.

2. Blanco, M., **Pérez-Albaladejo, E.**, Barata, C., Piña, B., Kušpilic, G., Lille-Langøy, Karlsen, O.A., Goksøyr, A., Porte, C. Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays. Submitted to *Science Total Environment.* **F.I.: 3.976**

Grado de participación: Realización del trabajo experimental junto con M. Blanco, excepto ensayos de activación de zfPxr. Participación en la elaboración del manuscrito.

3. Rizzi, J., **Pérez-Albaladejo, E.**, Fernandes, D., Contreras, J., Froehner, S., Porte, C. 2016. Characterization of quality of sediments from Paranaguá Bay (Brazil) by combined *in vitro* bioassays and chemical analyses. *Environmental Toxicology and Chemistry, 1-9*. **F.I.: 2.187**
   Grado de participación: Realización del trabajo experimental en el modelo celular PLHC-1. Discusión de resultados y participación de la elaboración del manuscrito.

4. Fernandes, D., Pujol, S., **Pérez-Albaladejo, E.**, Tauler, R., Bebianno, M.J., Porte, C. 2014. Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays. *Marine Environmental Research, 96, 127-135*. **F.I.: 2.769**
   Grado de participación: Realización de los ensayos de generación de especies reactivas de oxígeno.

5. **Pérez-Albaladejo, E.**, Fernandes, D., Lacorte, S., Porte, C. 2017. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells. *Toxicology in Vitro, 38, 41-48*. **F.I.: 3.338**
   Grado de participación: Realización de todo el trabajo experimental excepto análisis de concentraciones de compuestos en el medio de cultivo. Discusión de resultados. Elaboración del manuscrito.

6. **Pérez-Albaladejo,** E., Fajardo, K., Lacorte, S., Porte, C. 2017. Differential toxicity of alkylphenols in JEG-3 human placental cells: alteration of P450 aromatase and cell lipid composition. Submitted to *Toxicology and Applied Pharmacology*. **F.I.: 3.847**

   Grado de participación: Realización de todo el trabajo experimental. Identificación y cuantificación de lípidos en software Masslink junto con K. Fajardo. Discusión de resultados. Elaboración del manuscrito.

7. Gorrochategui, E., **Pérez-Albaladejo, E.**, Casas, J., Lacorte, S., Porte, C. 2014. Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells. *Toxicology and Applied Pharmacology, 277, 124-130*. **F.I.: 3.847**

   Grado de participación: Realización de los ensayos de citotoxicidad junto con E. Gorrochategui. Realización de los ensayos de actividad P450 aromatasa.

Barcelona, 6 de junio de 2017

Dra. Cinta Porte Visa

Directora de la tesis

IV. PUBLICACIONES

TEMA 1.

EVALUACIÓN DE LA CALIDAD AMBIENTAL DE SEDIMENTOS MARINOS

ARTÍCULO 1

Assessment of the environmental quality of coastal sediments by using a combination of *in vitro* bioassays

E. Pérez-Albaladejo[1], J. Rizzi[1,2], D. Fernandes[1], R. Lille-Langøy[3], O. A. Karlsen[3], A. Goksøyr[3], A. Oros[4], F. Spagnoli[5], C. Porte[1]

[1] Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain
[2] Department of Hydraulics and Sanitation, Federal University of Parana, Curitiba, Brazil
[3] Department of Biology, University of Bergen, Norway
[4] National Institute for Marine Research and Development (NIMRD) 'Grigore Antipa', Constanta, Romania
[5] Institute of Marine Sciences, National Research council, ISMAR-CNR, Italy

Marine Pollution Bulletin (2016), 108, 53-61

En este trabajo se ha evaluado la calidad ambiental de sedimentos marinos recolectados en diferentes áreas del Mar Adriático y Mar Negro, seleccionadas por estar influenciadas por fuentes potenciales de contaminación. Entre ellas, se encuentran las áreas marinas afectadas por las desembocaduras de los ríos Po y Danubio, efluentes urbanos y zonas de actividades portuarias, entre otros. La metodología incluye una batería de bioensayos basados en el uso de células PLHC-1, células COS-7 transfectadas con Pxr de pez cebra (zfPxr) y fracciones subcelulares de ovario de lubina. Los ensayos *in vitro* seleccionados estaban enfocados a la determinación de múltiples indicadores de actividad biológica o biomarcadores de exposición a contaminantes: citotoxicidad, estrés oxidativo, inducción de CYP1A, activación de zfPxr e inhibición de la actividad P450 aromatasa ovárica. Los extractos de sedimentos influenciados por los ríos Danubio (B2 y B3) y Po (A7), y aquellos recogidos

cerca de los puertos y descargas urbanas (B8, B10 y A2), mostraron una mayor citotoxicidad e inducción de CYP1A en la línea celular de peces PLHC-1, activación de zfPxr en las células COS-7 transfectadas, e inhibición de la actividad P450 aromatasa en microsomas de lubina (*Dicentrarchus labrax*). Se ha detectado una respuesta análoga de inducción de CYP1A y activación zfPxr, lo que sugiere la existencia de ligandos comunes de los receptores AhR y zfPxr, en los extractos de sedimentos. El estudio destaca la utilidad de los bioensayos seleccionados para identificar aquellos sedimentos que podrían poner en riesgo a los organismos acuáticos y que requieren acciones prioritarias con el fin de mejorar su calidad ambiental.

Marine Pollution Bulletin 108 (2016) 53–61

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays

Elisabet Pérez-Albaladejo [a], Juliane Rizzi [a,b], Denise Fernandes [a], Roger Lille-Langøy [c], Odd André Karlsen [c], Anders Goksøyr [c], Andra Oros [d], Federico Spagnoli [e], Cinta Porte [a,*]

[a] Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain
[b] Department of Hydraulics and Sanitation, Federal University of Parana, Curitiba, Brazil
[c] Department of Biology, University of Bergen, Norway
[d] National Institute for Marine Research and Development 'Grigore Antipa', Constanta, Romania
[e] Institute of Marine Sciences, National Research council, ISMAR-CNR, Italy

ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form 28 April 2016
Accepted 29 April 2016
Available online 17 May 2016

Keywords:
PLHC-1 cells
Sediments
Cytotoxicity
CYP1A induction
PXR agonists
Biomonitoring

ABSTRACT

The environmental quality of marine sediments collected in the area of influence of the Po and Danube Rivers was assessed by using a battery of bioassays based on the use of PLHC-1 cells, zebrafish-Pxr-transfected COS-7 cells, and sea bass ovarian subcellular fractions. This allowed the determination of multiple endpoints, namely, cytotoxicity, oxidative stress, induction of CYP1A, activation of zebrafish Pxr and inhibition of ovarian aromatase. Organic extracts of sediments influenced by the Danube River and collected near harbors and urban discharges showed significant cytotoxicity, CYP1A induction and inhibition of aromatase activity. An analogous response of CYP1A induction and zfPxr activation was observed, which suggests the existence of common ligands of AhR and PXR in the sediment extracts. The study highlights the usefulness of the selected bioassays to identify those sediments that could pose a risk to aquatic organisms and that require further action in order to improve their environmental quality.

1. Introduction

The Black Sea is an inland sea that receives industrial, agricultural, touristic and urban effluents along the shoreline, with the Romanian shelf as one of the most affected areas. The Danube River significantly influences the hydrography, chemistry and biology of the area, providing nutrients, but also urban, agricultural and industrial pollutants. The wind-driven water circulation and the Coriolis force lead to long residence time of the Danube's waters on the Romanian shelf (Cociasu et al., 1996). Moreover, marine navigation, which is considered extreme in the area (Constanta's harbor had 14,824 arrivals in 2014), coupled to harbor activities, such as boat building and maintenance of ships, and a gas station, constitute other entrances of contaminants (polycyclic aromatic hydrocarbons (PAHs), metals, petrol fuel additives and antifouling) that might have a strong impact on the quality of coastal ecosystems.

On the Mediterranean side, the Po River constitutes the most important Italian course of water, which flows 652 km from the Alps to Adriatic Sea. Its drainage basin is one of the most populated and industrialized areas of the European Union. It receives pollutants from agricultural, urban and industrial discharges, which are distributed along the western side of Adriatic Sea, with a decreasing southward trend due to mixing processes and the main anticyclonic circulation that causes the accumulation of fine sediments in a belt starting from the Po River mouths and parallel to the Italian coastline (Spagnoli et al., 2014). Ravenna harbor, one of the most important Italian cargo and passenger harbors, with an active petrochemical industry, is another source of contaminants in this area.

It is well known that sediments can act as long-term sinks for environmental pollutants, which can be further transferred into aquatic food chains via bottom-dwelling organisms and cause adverse effects in the ecosystem (Kosmehl et al., 2007). Several studies along the Rumanian coast have reported the presence of significant amounts of pollutants in sediments, namely metals (3–100 $\mu g \cdot g^{-1}$ copper; 0.01–10 $\mu g \cdot g^{-1}$ cadmium; 1.2–135 $\mu g \cdot g^{-1}$ lead; 1–212 $\mu g \cdot g^{-1}$ nickel; 5–170 $\mu g \cdot g^{-1}$ chromium), PAHs (0.093 to 4.5 $\mu g \cdot g^{-1}$) and organochlorinated pesticides (in the individual range 0.008–0.0335 $\mu g \cdot g^{-1}$) (Coatu et al., 2013; Ţigănuş et al., 2013). Similarly, concentrations of metals along the Adriatic coast were in the range of 22–46 $\mu g \cdot g^{-1}$ copper; 14–48 $\mu g \cdot g^{-1}$ lead; 56–152 $\mu g \cdot g^{-1}$ nickel (Spagnoli et al., 2014), while concentrations of PAHs ranged from 0.226 to 0.507 $\mu g \cdot g^{-1}$ (Magi et al., 2002).

However, analyzing and measuring the presence of chemicals in the sediments cannot be used as the only strategy to characterize sediment quality since it is not possible to identify every single chemical present,

* Corresponding author.
E-mail address: cinta.porte@cid.csic.es (C. Porte).

http://dx.doi.org/10.1016/j.marpolbul.2016.04.063
0025-326X/

nor the bioavailability of pollutants and the possible additive, synergistic or antagonistic interactions in bottom-dwelling organisms (Chapman, 2007). Moreover, the most abundant contaminants are not necessarily the ones with the highest biological impact in aquatic organisms (Brack et al., 2005).

Nowadays, different bioassays have been successfully developed in order to estimate the biological activity of pollutants present in environmental samples, integrating the interaction between them and covering endpoints such as acute and long-term toxicity, bioaccumulation and endocrine disrupting effects, among others (Schnell et al., 2013). This approach helps to monitor sediment quality and to characterize the health status of aquatic environments and, ultimately, to minimize threats and prevent adverse effects to aquatic wildlife (Fernandes et al., 2014).

As interactions of chemicals with biota initially take place at the molecular and cellular level, responses at these levels are considered the first manifestation of toxicity and they represent suitable tools for the early and sensitive detection of chemical exposure (Fent, 2001). Considering the key role that liver plays in xenobiotic metabolism/detoxification, fish hepatocytes and permanent liver cell lines are often chosen as cellular in vitro models in fish ecotoxicology (Bols et al., 2005). One well established permanent fish cell line is PLHC-1, derived from a topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma. This cell line express the aryl hydrocarbon receptor (AhR) and has the capacity to induce the expression of CYP1A after exposure to environmental pollutants such as dioxin-like compounds, pharmaceuticals and extracts of environmental matrices (i.e. sediments). PLHC-1 has also been successfully used to assess cytotoxicity, genotoxicity and oxidative stress (Huuskonen et al., 2000; Jung et al., 2001; Thibaut and Porte, 2008; Šrut et al., 2011; Schnell et al., 2013; Fernandes et al., 2014).

Apart from the AhR receptor, the pregnane X receptor (PXR) is a ligand-activated transcription factor that protects organisms from toxic chemicals by acting as a xenosensor and regulating genes involved in xenobiotic metabolism, including cytochrome P450 (CYP3A) genes and ABC transporters, among others (Kliewer et al., 2002; Kretschmer and Baldwin, 2005). Ligand activation of PXR can be detected and quantified by cell-based reporter assays (Lille-Langøy et al., 2015).

Contaminants may have different routes of phase I and phase II metabolism, and as sediments represent an indeterminate mixture of contaminants, it is necessary to apply different biomarkers in order to identify the presence of different classes of xenobiotics. Whereas induction of CYP1A-mediated ethoxyresorufin-O-deethylase (EROD) activity is an established biomarker used to assess exposure to aromatic hydrocarbons and planar halogenated hydrocarbons, zebrafish Pxr is a xenobiotic sensor activated by a variety of structurally diverse xenobiotics, including drugs (e.g. clotrimazole), plasticizers (octylphenol, phthalates, bisphenol A) and organochlorines (chlordane, *o,p′*-DDT, endosulfan, methoxychlor, toxaphene) (Milnes et al., 2008), and thus induces the expression of genes involved in phase I, II and III biotransformation.

Additionally, bioassays based on the use of fish gonad subcellular fractions have been successfully applied to detect endocrine disrupters in sediment extracts (Fernandes et al., 2014). Those bioassays detect compounds that interfere with the activity of key steroidogenic enzymes involved in the synthesis of active androgens (i.e. CYP17, CYP11β) or estrogens (CYP19) in fish gonads and that are therefore likely to alter endocrine processes, such as sexual differentiation, gamete growth and/or maturation (Schnell et al., 2009; Fernandes and Porte, 2013).

The aim of this study was to characterize the environmental quality of sediments collected from the Mediterranean and Black Seas coasts, including areas affected by the Po and the Danube Rivers, urban effluents, touristic places, harbor activities and potential reference sites, by using a selected set of in vitro bioassays: (a) the fish hepatoma cell line (PLHC-1) to determine the cytotoxicity, CYP1A and oxidative stress inducing agents in sediments, (b) ovarian microsomal fractions from sea bass (*Dicentrarchus labrax*) to detect those sediments that contain compounds that are likely to act as endocrine disrupters by inhibiting ovarian aromatase activity, and (c) zebrafish Pxr transfected COS-7 cells to study the potency of the sediments to activate zebrafish Pxr. This will help to discriminate those sediments that could pose a risk to aquatic organisms and to identify potentially impacted areas that require further attention and action to improve their environmental quality.

2. Materials and methods

2.1. Chemicals and reagents

Eagle's Minimum Essential Medium (MEM), Dubelco 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). 2′ 7′-Dichloro-dihydrofluorescein diacetate (H_2DCF-DA) and 3-morpholinosydnonimine (SIN-1) were obtained from Invitrogen (Molecular Probes, Barcelona, Spain).

2.2. Sediment collection and extraction

Sediments along the Italian coast were collected by a box-corer, from the mouths of Po River to the city of Ancona (Fig. 1A). The first centimeter was sub-sampled and immediately frozen. Sediments from the Black Sea were collected using a Van Veen grab sampler. The study areas were located around the mouth of the Danube River, close to main harbors, WWTP effluents, touristic resorts and city influenced areas (Fig. 1B). Geographic coordinates and main characteristics of the selected sites are given in Table 1.

All sediment samples were freeze-dried and stored at −20 °C until extraction. 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 into 500 μL of DMSO. The stock was equivalent to 12 g dry weight extract (eQsed)·mL^{-1}, and was serially diluted in DMSO to the desired concentrations.

2.3. Cell cultures

The PLHC-1 cell line (ATCC; CRL-2406), derived from topminnow (*P. lucida*) hepatocellular carcinoma, was routinely cultured in Eagle's Minimum Essential Medium supplemented with 5% FBS, 2 mmol·L^{-1} L-glutamine, 1 mmol·L^{-1} sodium pyruvate, 0.1 mmol·L^{-1} non-essential amino acids, 1.5 g·L^{-1} sodium bicarbonate, 50 U·mL^{-1} penicillin G and 50 μg·mL^{-1} streptomycin, and incubated at 30 °C.

The monkey (*Cercopithecus aethiops*) kidney COS-7 cell line was obtained from the ATCC CRL-1651. It was routinely grown in Eagle's Minimum Essential Medium supplemented with 10% FBS, 4 mmol·L^{-1} L-glutamine, 1 mmol·L^{-1} sodium pyruvate and 100 U·mL^{-1} penicillin/streptomycin.

Both PLHC-1 and COS-7 cells were cultured in a humidified incubator with 5% CO_2, at 30 and 37 °C, respectively. When 90% of confluence was reached, cells were dissociated with trypsin-EDTA for subculturing and experiments. Application of sediment extracts to cell cultures was done by diluting the extracts in culture medium, which was then added to culture plate wells. The final concentration of DMSO in culture wells never exceeded 0.5% (v/v), which was not toxic for cells. For each

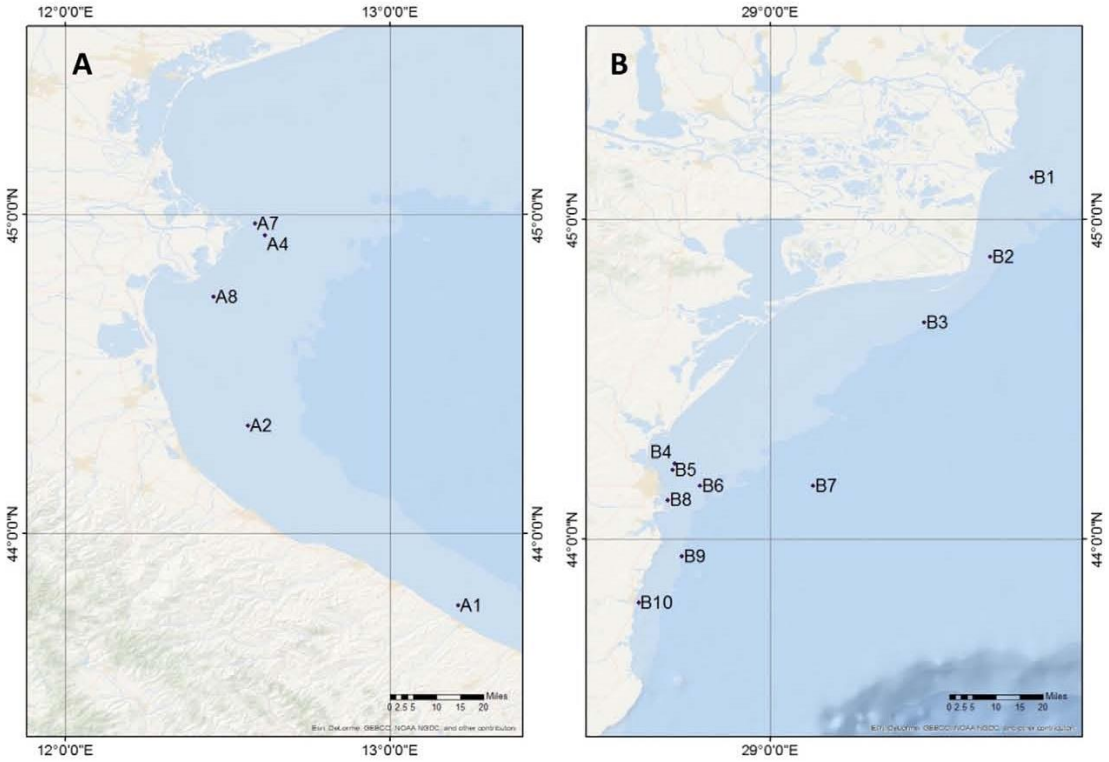


Fig. 1. Sampling points in the Adriatic and Black Seas, along the Italian (A) and the Romanian (B) coastline.

assay, non-exposed controls were included in which only the solvent (DMSO) was added to the cells.

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 7.5×10^4 cells in 200 μL of medium per well and were allowed to attach to the well for 24 h, prior exposure to sediment extracts for 24 h. 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). Toxicity tests were repeated at least three times.

2.5. Reactive oxygen species (ROS) generation

The generation of ROS in the presence of sediment extracts was determined by using the method of 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 treated with 20 μM 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) diluted in DPBS (1:10) supplemented with 10 mmol·L^{-1} glucose (DPBS-Glu). 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

Table 1
Geographic coordinates, sample depth and criteria for the selection of the sampling points.

Sample	Station name	Depth (m)	Lat (N)	Long (W)	Pressure/impacts
B1	Sulina	20	45,1333	29,8050	Danube mouth influenced area
B2	Sfantul Gheorghe	20	44,8836	29,6783	Danube mouth influenced area
B3	Portita	30	44,6767	29,4742	Danube mouth influenced area
B4	Cazino Mamaia	20	44,2349	28,7061	Touristic resort/upstream the city of Constanta
B5	Constanta North	20	44,2167	28,7003	Urban WWTP
B6	Constanta East	28	44,1667	28,7833	Roadstead of the Constanta harbor
B7	Constanta East	47	44,1667	29,1333	Increasing 20 nautical miles offshore (open sea)
B8	Constanta South	20	44,1218	28,6850	Constanta harbor, industrial and urban WWTP
B9	Costinesti	30	43,9450	28,7267	Touristic resort/downstream the city Constanta
B10	Mangalia	5	43,8002	28,5946	Mangalia harbor, urban WWTP
A7	Pila	18.5	44,9703	12,5850	Po mouth influenced area
A4	Pila South	25.5	44,9344	12,6143	Po mouth influenced area
A8	Goro	21.0	44,7414	12,4574	Po influenced area (35 nautical miles from Pila mouth)
A2	Ravenna	19.5	44,3394	12,5635	City harbor, petrochemical industry
A1	Marotta	12.0	43,7750	13,2094	Reference area

with a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths pairs of 485/528 nm. 3-Morpholinosydnonimine (SIN-1; 5 $\mu mol \cdot L^{-1}$) was used as a positive control (Spohn et al., 2009). Results were expressed as a percentage of the basal fluorescence in control wells (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 6.5×10^5 cells/well, in 48-well plates (Nunc; Roskilde, Denmark) and allowed to grow for 24 h. After the growth period, cells were exposed to sediment extracts, 1 $\mu mol \cdot L^{-1}$ β-naphthoflavone (BNF; positive control) or the carrier (0.5% DMSO). Samples were assayed in triplicate, in different cell passages. After 24 h exposure, the medium was aspirated, the cells rinsed with PBS and immediately incubated with 2 $\mu mol \cdot L^{-1}$ 7-ethoxyresorufin in 50 $mmol \cdot L^{-1}$ Na-phosphate buffer pH 8.0 at 30 °C. After 15 min of incubation, the fluorescence was read in the 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 as described by Lorenzen and Kennedy (1993), using bovine serum albumin (BSA) as standard. EROD activity was expressed as pmol of resorufin formed per minute and per milligram of protein (pmol/min/mg protein). The concentration required to induce an EROD response equivalent to 1 $\mu mol \cdot L^{-1}$ BNF (78 pmol/min/mg protein) (R_{BNF}) were determined from the fitted dose–response curves.

2.7. Ligand activation of zebrafish Pxr

Ligand activation of the zebrafish Pxr was monitored with the luciferase assay using the GAL4-DBD/Pxr-LBD fusion in COS-7 monkey kidney cells as described by Lille-Langøy et al. (2015) with some modifications. COS-7 cells were plated at $5 \cdot 10^3$ 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 was expressed as change in luciferase activity in test cells relative to non-exposed control (DMSO). 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.

2.8. Preparation of ovarian microsomal fractions and in vitro incubations

Sea bass (*D. labrax*) of marketable size were supplied by an aquaculture facility located in the Northeast of Spain. Immediately after collection, animals were sacrificed and gonads were dissected. A subsample of the central part of the gonad was fixed in 10% buffered formalin for histological examination to determine the sexual maturation stage (SMS). The rest of the gonad was deep-frozen in liquid nitrogen and stored at −80 °C. Only ovaries classified as early oogenic were selected for obtaining the microsomal fraction as described in Fernandes et al. (2014). Briefly, subsamples of 1 g of individual ovaries were flushed with ice cold 1.15% KCl and homogenized in 1:4 *w/v* of ice-cold 100 $mmol \cdot L^{-1}$ potassium phosphate buffer pH 7.4, containing 100 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ dithiothreitol (DTT), 0.1 $mmol \cdot L^{-1}$ phenanthroline and 0.1 $mg \cdot mL^{-1}$ trypsin inhibitor. Microsomal pellets were obtained by ultracentrifugation at 100,000-g for 60 min, and concentration of total proteins measured by the method of Bradford (1976), using bovine serum albumin as standard.

The effect of sediment extracts on ovarian aromatase activity was investigated in vitro by pre-incubating the microsomal fraction in the presence of the sediment extracts (2, 20, 60 mg eQsed $\cdot mL^{-1}$) for 10 min at 25 °C, prior to the addition of the reaction substrate and co-factor as indicated below. Control reactions consisted in the incubation of the microsomal fractions with carrier (0.5% DMSO) in the absence of sediment. Blanks that consisted of reactions stopped at time zero were also analyzed. Assays were carried out using individual gonads of 4–5 different individuals.

Aromatase activity was determined by the tritiated-water release method as described in Lavado et al. (2004). Microsomal protein (0.4 mg) was incubated at 25 °C for 1 h in the presence of 100 $mmol \cdot L^{-1}$ Tris–HCl, pH 7.6, 10 $\mu mol \cdot L^{-1}$ unlabeled androstenedione, 0.2 $mmol \cdot L^{-1}$ NADPH and [1β-^{3}H]-androstenedione (40 pmol, 1 μCi). The reaction was stopped by adding methylene chloride (3×3 mL). The possible remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (*w/v*) activated charcoal and 0.25% dextran in deionized water (4 mL). The solution was centrifuged and two aliquots of the supernatant (1 mL) were counted for ^{3}H radioactivity. The detection limit of the method was 45 fmol/min/mg protein.

2.9. Statistical analyses

The concentration of sediment extract resulting in 50% (EC_{50}) effect was calculated by using Sigmaplot 11.0 software. Normal distribution of data was verified with a Kolmogorov–Smirnov test. Statistical differences were analyzed by one-way ANOVA with Dunnett test, using SPSS 19.0. Level of significance was set at $p < 0.05$. To estimate the degree of anthropogenic impact in the different stations and the relative influence of variables on overall results, a principal component analysis (PCA) was carried out with PLS Toolbox 8.0/MatLab software.

3. Results

3.1. Cytotoxicity

Metabolic impairment and disruption of membrane integrity were determined in PLHC-1 cells after 24 h exposure to organic sediment extracts at concentrations ranging from 1 to 60 mg eQsed $\cdot mL^{-1}$. Notably, the sediment from B2 had an adverse effect on metabolic activity (AB), but only minor effect on membrane integrity (CFDA). The most potent sediments with regard to cytotoxicity were those from B8 (Constanta harbor; EC_{50}: 25 mg eQsed $\cdot mL^{-1}$), followed by B3 and B2 (Danube mouth) and B10 (EC_{50}: 50–60 mg eQsed $\cdot mL^{-1}$) (Table 2). Only low or no significant reduction in cell viability was observed following exposure to extracts from the other sediments collected in the Black Sea or the Adriatic Sea.

3.2. Induction of EROD activity

The dose response curves, maximum EROD activity and R_{BNF} values are shown in Fig. 2 and Table 2. The AhR agonist β-naphthoflavone (BNF) was included in the assays as a positive control and lead to an EROD activity of 78 ± 7 pmol/min/mg protein after 24 h of exposure at a concentration of 1 $\mu mol \cdot L^{-1}$ (0.2723 $\mu g \cdot mL^{-1}$). Sediments from Constanta harbor (B8) had the highest ability to induce EROD activity (R_{BNF}: 2.6 mg eQsed $\cdot mL^{-1}$), followed by those collected in the Danube

Table 2
EC_{50} expressed in mg eQsed·mL^{-1} in the cytotoxicity assay (AB, CFDA); maximum EROD activity (pmol/min/mg protein) and concentration of sediment required to induce a response equivalent to 1 μmol·L^{-1} β-naphthoflavone (R_{BNF}); maximum zfPXR induction and concentration of sediment required to induce 20% of the response induced by clotrimazole (REC_{20}); percentage of inhibition of P450 aromatase activity in ovarian microsomes incubated with to 2 mg eQsed·mL^{-1}, and ROS generation after 180 min of exposure to 60 mg eQsed·mL^{-1}. Values are mean ± SEM of at least three independent experiments. n.d.: not detected.

Sample site	Cytotoxicity EC_{50}		EROD		PXR		P450 aromatase	ROS
	AB	CFDA	Max. activity	R_{BFN}	Fold induction	REC_{20}	% of inhibition	Fold generation
B1	>60	>60	89.1 ± 9.2[a]	48.7	4.2 ± 0.5[a]	43.9	18.9 ± 1.5[a]	1.5 ± 0.03[a]
B2	56.2 ± 2.90	>60	137.4 ± 5.8[a]	14.0	7.57 ± 0.7[a]	7.3	53.6 ± 0.2[a]	2.8 ± 0.28[a]
B3	49.9 ± 0.75	58.3 ± 8.9	160.0 ± 7.5[a]	10.6	10.8 ± 2.0[a]	5.9	36.3 ± 0.2[a]	3.4 ± 0.50[a]
B4	>60	>60	34.9 ± 13.3[a]	>60	3.5 ± 0.5[a]	39.8	11.2 ± 1.6	2.6 ± 0.27[a]
B5	>60	>60	9.0 ± 1.6	>60	2.3 ± 0.4[a]	>60	5.5 ± 3.1	4.6 ± 0.12[a]
B6	>60	>60	114.9 ± 12.7[a]	52.8	2.3 ± 0.4[a]	>60	7.9 ± 2.4	3.6 ± 0.52[a]
B7	>60	>60	132.9 ± 12.7[a]	18.6	6.9 ± 0.3[a]	23.6	37.7 ± 0.8[a]	2.3 ± 0.20[a]
B8	25.4 ± 1.90	24.7 ± 1.7	130.8 ± 5.2[a]	2.6	14.2 ± 0.6[a]	<5	45.5 ± 1.2[a]	3.2 ± 0.81[a]
B9	>60	>60	33.1 ± 3.8[a]	>60	3.3 ± 0.5[a]	58.3	46.8 ± 0.5[a]	3.6 ± 0.49[a]
B10	58.7 ± 6.50	60.0 ± 4.4	135.5 ± 13.6[a]	31.6	4.9 ± 0.4[a]	30.7	20.9 ± 4.5[a]	2.9 ± 0.58[a]
A7	>60	>60	108.8 ± 7.1[a]	22.1	5.3 ± 0.8[a]	21.9	23.1 ± 3.8[a]	3.2 ± 0.03[a]
A4	>60	>60	117.5 ± 4.6[a]	21.5	5.2 ± 0.6[a]	18.7	20.6 ± 2.8[a]	2.4 ± 0.02[a]
A8	>60	>60	148.0 ± 3.3[a]	22.9	5.3 ± 0.7[a]	17.8	11.9 ± 3.0	1.1 ± 0.01
A2	>60	>60	168.6 ± 5.9[a]	19.5	5.9 ± 0.5[a]	22.1	16.0 ± 7.3	3.7 ± 0.04[a]
A1	>60	>60	11.9 ± 0.2	>60	1.7 ± 0.2	>60	11.9 ± 3.9	3.4 ± 0.03[a]

[a] Significant differences with respect to control.

mouth (B3 and B2; R_{BNF} : 10–14 mg eQsed·mL^{-1}). On the contrary, only minor EROD induction was recorded for sediments collected in B9, B4 and B5 (Black Sea), A1 (Adriatic Sea) (1–20 pmol/min/mg prot).

3.3. Oxidative stress

The oxidation of H_2DCF by ROS was monitored over 180 min exposure to sediment extracts. Most of the sediments significantly induced the generation of ROS in PLHC-1 cells after 180 min of exposure to 60 mg eQsed·mL^{-1} compared to solvent control cells, particularly B5 (5-fold induction), followed by A2 (Ravenna), B6 (Constanta East) and B9 (Costinesti) (Table 2). No significant generation of ROS was observed for A8 sediment extracts. Generation of ROS increased in a time dependent manner in most cases, except for B1. The only stations that significantly induced ROS after 15 min exposure to 20 mg eQsed·mL^{-1} (2-fold induction) were B2, B3 and B10 (Fig. 1 in the online version at http://dx.doi.org/10.1016/j.marpolbul.2016.04.063., Supplementary info). More than 9-fold increase of ROS was detected after 180 min of exposure to 5 μmol·L^{-1} SIN-1.

3.4. Ligand activation of zebrafish Pxr

The ability of the sediments to activate zebrafish Pxr was evaluated in vitro using a luciferase reporter assay. COS-7 cells were exposed to different concentrations (5–60 mg eQsed·mL^{-1}) of sediment extracts and nuclear receptor activation was reported as fold-induction relative to control (DMSO) (Fig. 3). All sediments activated Pxr from zebrafish and significantly induced expression of the luciferase reporter gene, with the exception of A1. Sediments from Constanta harbor (B8) induced the greatest response via zebrafish Pxr (14-fold; REC_{20} < 5 mg eQsed·mL^{-1}) followed by those collected in areas influenced by the Danube River, B3 and B2 (8 and 11-fold; REC_{20s} 6 and 7 mg eQsed·mL^{-1}, respectively) (Table 2; Fig. 3). Another group of sediments (A2, A4, A7, A8, B7 and B10) induced intermediate responses (5 to 7-fold induction, REC_{20}s 18–31 mg eQsed·mL^{-1}), while sediment extracts from B1, B4, B5, B6, B9 and A1 showed low or almost no Pxr activation (<4-fold induction, REC_{20} > 40 mg eQsed·mL^{-1}). The maximum increase of luciferase activity in cells exposed to clotrimazole -positive control- was 15.8 ± 0.23 fold, with an EC_{50} of 0.12 ± 0.01 μmol·L^{-1}.

3.5. Aromatase activity

Sediments from all stations inhibited P450 sea bass ovarian aromatase activity (CYP19), but particularly those from stations B2 and B3 (Danube mouth), B7 (open sea), B8 (Constanta harbor) and B9 (touristic resort) (36 and 54% of inhibition at 2 mg eQsed·mL^{-1}). Stations B4, B5 and B6 (Black Sea), and A8, A1 (Adriatic Sea) showed the lowest ability to inhibit aromatase activity as no significant inhibition was observed when tested at a concentration of 2 mg eQsed·mL^{-1} (Table 2).

3.6. Principal component analysis

A PCA was used to classify sediments according to the observed responses in the bioassays. The PCA rearranged the set of data in two factors, which together explained 88% of the total variance (Fig. 4). The predominant factor (PC1) accounted for 64% of the variance and showed a negative correlation between ROS generation and the other assays (P450aromatase inhibition (ARO), EROD activity (EROD) and zfPxr activation (PXR)). The second factor (PC2) explained 24% of the variance, with higher intensity for ROS generation. Additionally, the PCA closely associates PXR activation and EROD activity in its most positive end of PC1, indicating a similar response of these bioassays to sediment exposure. Overall, sediments from B8 (Constanta harbor), B3 and B2 (Danube mouth), A7 (Po mouth), B10 (Mangalia harbor) and A2 (Ravenna) were isolated on the positive side of PC1 and PC2, with high influence of EROD activity, zfPxr activation (PXR) and P450aromatase inhibition (ARO), whereas sediments from A1 (Marotta), B4 (Cazino Mamaia) and B6 (Constanta East) are isolated on the negative side, as the areas with less anthropogenic impact (Fig. 4).

4. Discussion

Toxicity screening of complex environmental samples such as sediments requires the use of multiple endpoints for the determination of specific modes of action of the chemical mixture bound to sediments. By using a battery of bioassays, this study examined the cytotoxicity, ability to generate reactive oxygen species (ROS), presence of endocrine disrupters (CYP19 inhibitors), and presence of AhR- and Pxr-agonists in sediments collected along the Romanian and Italian coast of the Black and the Adriatic Sea. Comparatively, higher anthropogenic impact was observed in sediments from the Black Sea than in those obtained from the Adriatic Sea. The highest cytotoxicity was detected in sediment extracts from harbors (B8, B10) and from the Danube mouth (B2, B3). Constanta harbor (B8) – average loss of 80% of cell viability when tested at 60 mg eQsed·mL^{-1} – is strongly affected by heavy boat traffic (more than 14,800 arrivals in 2014) and urban and industrial discharges; whereas Mangalia harbor (B10), a smaller port used mainly as shipyard, is impacted by a mixture of both boat traffic and WWTP effluents. The

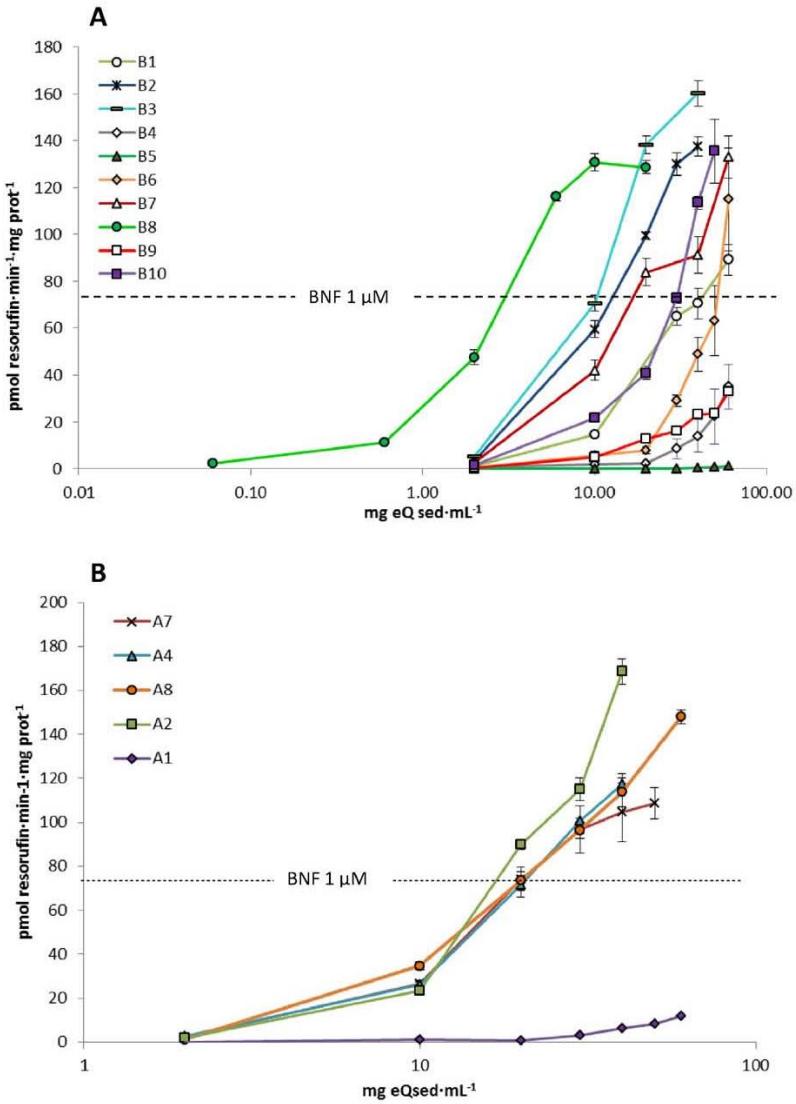


Fig. 2. CYP1A induction in PLHC-1 cells exposed for 24 h to different concentrations of sediments extracts from the Black Sea (A) and the Adriatic Sea (B). Values expressed as mean ± SEM of at least three independent experiments. Dotted line corresponds to the positive control.

areas influenced by the Danube mouth are associated with boat traffic and urban, industrial and agricultural discharges from several European cities. A North to South gradient of cytotoxicity, EROD induction, Pxr activation and ROS generation was observed in the Danube delta (B1 to B3), probably reflecting the fact that particulate matter and associated pollutants from the Danube River are dragged and redistributed in the direction of sea currents along the Black Sea. A North to South gradient (CYP19 inhibitors, EROD induction, Pxr activation and ROS generation) was also evidenced in the Adriatic, with the highest impact in sediments from the Po mouths (A4, A7), but also, Ravenna (A2), affected by its harbor and petrochemical industry (Lucialli et al., 2007).

Most of the sediment extracts stimulated the production of ROS in PLHC-1 cells. These harmful molecules, produced as byproducts from endogenous processes, have the potential to damage proteins, nucleic acids and lipids, resulting in functional loss and, consequently, in disease or death. Pollutants capable of enhancing ROS are mostly redox cycling compounds (e.g. quinines, nitroaromatics, nitroamines, herbicides), PAHs (e.g. benzene, PAH oxidation products), halogenated hydrocarbons (e.g. PCBs, lindane) dioxins and metals (Livingstone, 2001; Fernandes et al., 2014). Interestingly, sediments from coastal cities, namely Constanta (B5, B6), Ravenna (A2) and a touristic resort (B9), induced the highest generation of ROS (4.5-fold). In comparison, sediments from estuarine and coastal areas along Northern Spain produced less than 2-fold induction of ROS in PLHC-1 cells (Schnell et al., 2013).

CYP1A induction has often been related to the concentration of PAHs in marine sediments (Traven et al., 2008). Among PAHs, high molecular weight ones (e.g. dibenzo(a,i)pyrene, chrysene, benzo(a)pyrene and indeno(1,2,3-c,d)pyrene) have been shown to induce CYP1A activity in fish (PLHC-1, RTL-W1) and mice (H4IIE) hepatic cells (Behrens et al., 2001; Bosveld et al., 2002). Interestingly, the interaction of PAHs in mixtures of up to eight individual compounds was found to be additive based on the induction of EROD activity in PLHC-1 cells (Fent and Bätscher, 2000), but certainly additional unknown inducing/inhibiting agents might be present in the sediment extracts. Thus, dose–response curves of EROD activity for sediments from Constanta and Mangalia harbors and Ravenna (B8, B10, A2), and the Danube and Po mouths (B2, B3, A7, A4) were bell shaped (data not shown; Fig. 2

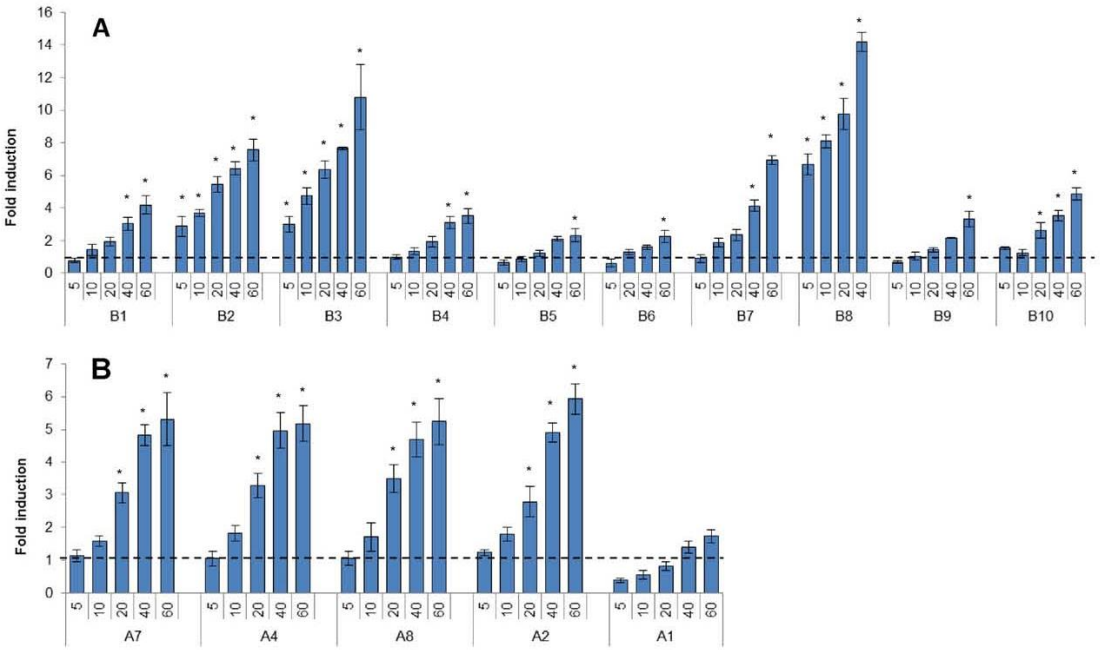


Fig. 3. In vitro activation of zebrafish Pxr measured in COS-7 cells using a luciferase reporter gene assay. Values expressed as mean ± SEM of at least three independent experiments, each assayed in triplicate. Activation of zfPxr presented as fold change of normalized luciferase activity. Dotted line is the control value. *Statistical differences from control. A: Black Sea; B: Adriatic Sea.

displays only the maximal EROD activity). This shape could be explained by the cytotoxicity associated to some samples (B8, B2, B3 and B10). However, such dose–response curves have also been reported for typical EROD inducers, like benzo[k]fluoanthene and for environmental samples, and have been attributed to overlaying effects of inducing and inhibiting compounds (Bols et al., 1999; Bosveld et al., 2002; Brack et al., 2002; Brack and Schirmer, 2003).

Zebrafish Pxr is activated by a variety of xenobiotics, including pesticides, plasticizers and industrial compounds, as well as by endogenous compounds, such are cholesterol precursors and metabolites and steroid hormones (Milnes et al., 2008; Ekins et al., 2008). In the present study, 1.5 $\mu mol \cdot L^{-1}$ clotrimazole induced a maximum of 16-fold increase in luciferase activity ($EC_{50} = 0.12\ \mu mol \cdot L^{-1}$), but also sediment extracts from Constanta harbor (B8) strongly induced luciferase activity (14-fold), followed by those collected in the Danube mouth (B3 and B2; 8 to 10-fold), and the Adriatic Sea (A7, A4, A8 and A2; 5–6-fold). This is in agreement with previous studies that demonstrated the presence of agonists of human PXR in water, sediments and passive samplers from French rivers (Creusot et al., 2010; Kinani et al., 2010).

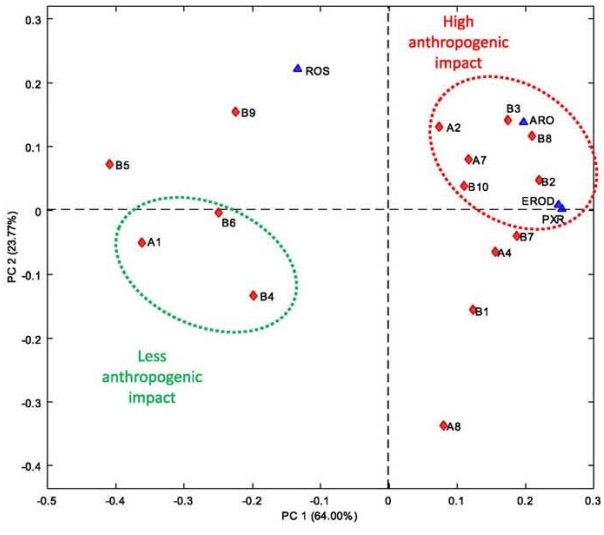


Fig. 4. Principal component analysis (PCA); PC1 versus PC2 biplot.). (▲) loadings for biological variables; (♦) site scores. Black Sea (B1 to B9), Adriatic Sea (A1 to A5).

The ligand specificity of Pxr is unusually wide for a nuclear receptor. Thus, very likely sediments contain several compounds that could contribute to the activation of zfPxr in an additive, synergistic or even inhibitory manner. The activation of Pxr by xenobiotics results in a coordinated expression of defensome genes in zebrafish liver and in rainbow trout hepatocytes, including *pxr*, *cyp3a* and *mdr1* (Bresolin et al., 2005; Wassmur et al., 2010). Thus, PXR functions as sensor for endogenous and exogenous compounds, and as a regulator of biotransformation. Although it has a protective function, it has also been suggested to be involved in toxicological pathways, e.g. enhancement of toxicity and disruption of metabolic homeostasis (Goksøyr, 2006; Zhai et al., 2007; Cheng et al., 2009). Thus, while activation of Pxr serve as a marker of exposure to xenobiotics, the absence of Pxr activation should not be considered as proof of absence of xenobiotics in the sample material.

It is worth highlighting the analogous response of CYP1A induction and zfPxr activation assays, which suggests the existence of common ligands in the sediment extracts. Both R_{BNF} and REC_{20}, allowed the classification of the sediments in three main groups regarding the presence of CYP1A inducers and activators of zfPxr: a first group corresponding to Constanta harbor and Danube mouth (B8, B2 and B3), with a high content of agonists, and both index below 14 mg eQsed·mL^{-1}; a second group constituted by sediments from the Po mouths (A7, A4 and A8), Ravenna (A2), Mangalia harbor (B10) and open sea (B7), with intermediate values, in the range of 18–32 mg eQsed·mL^{-1}; and a third group, corresponding to the touristic areas Cazino Mamaia and Costinesti (B4 and B9), North and East of Constanta (B5 and B6), Danube mouth (B1) and Marotta (A1; reference site), with relatively low presence of CYP1A-inducing and/or zfPxr-activating agents (Table 2, Figs. 2 & 3).

Likewise, Schnell et al. (2013) reported the presence of CYP1A inducers in estuarine and coastal sediments (N. Spain), the highest responses (IC_{50}: 0.1–1.5 mg eQsed·mL^{-1}) were observed in sediments collected in harbors or affected by industrial effluents and urban discharges.

Special attention should be paid to the inhibition of ovarian CYP19 activity by sediments from the Danube influenced areas (B2, B3), Constanta harbor (B8), touristic area Costinesti (B9) and open sea in front of Constanta East (B7) that indicates the presence of potential endocrine disrupting chemicals in those extracts. Similar effects (up to 59% inhibition by 1.5 mg eQsed·mL^{-1}) were reported by Fernandes et al. (2014) in sediments from the Arade estuary, and attributed to the presence of endocrine disrupter compounds (EDs), such as pesticides and their metabolites, PAHs, nonylphenol, bisphenol A, organotin compounds and herbicides. Field studies are scarce, but an inhibition of aromatase activity has been reported in carps and roach from the Ebro and Seine Rivers exposed to urban effluents and pesticides (Lavado et al., 2004; Gerbron et al., 2014), but also in red mullets collected in an area affected by oil refineries and oil-transport (Martin-Skilton et al., 2006).

Overall, the combination of in vitro bioassays used in this study highlighted harbors (B8, B10 and to some extent A2), affected by urban effluents, but also oil/chemical spills from vessels, as the most impacted areas. Interestingly, a treatment plant for sewage and another one for oil residues were built in the Constanta harbor in 2007 (http://www.ecoport8.eu/page_files/WP6_Ecoguide-Final.pdf) in order to solve the constant problem of sewage and spills. In this sense, monitoring periodically the sediments by using a battery of bioassays similar to the ones used in this study would be useful in order to assess the effectiveness of the implemented technologies and/or future actions taken to reduce sources of toxic chemicals in sediments.

Comparatively, sediment extracts from the mouth of the Danube River were more toxic and responsive in the different bioassays than those from the Po, which agrees with the fact that the size and flow of the Danube (2900 km; 6500 m^3/s) far surpasses that of the Po River (650 km; 1500 m^3/s). Recently, Lechner et al. (2014) reported a gradient of microplastics in Danube water that was of 4 g/s in Vienne (Austria), 17 g/s in Pančevo (Serbia), and reached 48 g/s in Danube mouth. This may well reflect the behavior of other contaminants that end up in the mouth of the Danube and affect the Rumanian coast.

In summary, the combination of different bioassays, focusing on cytotoxicity, induction of CYP1A activity, Pxr activation, ROS generation and inhibition of ovarian P450 aromatase provided useful information to discriminate between polluted and less impacted areas in the Adriatic and Black Sea, and may serve as a first screening tool for the assessment of the quality of benthic ecosystems. Furthermore, this in vitro approach may allow a significant reduction in the number of bioindicator organisms to be used in environmental monitoring studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.04.063.

Acknowledgments

This work has been supported by project "Policy-oriented marine Environmental Research in the Southern European Seas" (PERSEUS), grant agreement No. 287600, and "NILS Science and Sustainability" (ES07; ABEL-CM-01-2013).

References

Behrens, A., Schirmer, K., Bols, N., Segner, H., 2001. Polycyclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1, and in primary cultures of rainbow trout hepatocytes. Environ. Toxicol. Chem. 20, 632–643.

Bols, N.C., Schirmer, K., Joyce, E.M., Dixon, D.G., Greenberg, B.M., Whyte, J.J., 1999. Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-o-deethylase activity in a trout liver cell line. Ecotoxicol. Environ. Saf. 44, 118–128.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. In: Mommsen, T.P., Mohn, T.W. (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier B.V, Amsterdam, pp. 43–84.

Bosveld, A.T.C., de Bie, P.A.F., van den Brink, N.W., Jongepier, H., Klomp, A.V., 2002. In vitro EROD induction equivalency factors for the 10 PAHs generally monitored in risk assessment studies in The Netherlands. Chemosphere 49, 75–83.

Brack, W., Schirmer, K., 2003. Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P4501A-inducers in a contaminated sediment. Environ. Sci. Technol. 37, 3062–3070.

Brack, W., Schirmer, K., Kind, T., Schrader, S., Schüürmann, G., 2002. Effect-directed fractionation and identification of cytochrome p4501A-inducing halogenated aromatic hydrocarbons in a contaminated sediment. Environ. Toxicol. Chem. 21, 2654–2662.

Brack, W., Schirmer, K., Erdinger, L., Hollert, H., 2005. Effect-directed analysis of mutagens and ethoxyresorufin-O-deethylase inducers in aquatic sediments. Environ. Toxicol. Chem. 24, 2445–2458.

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

Bresolin, T., de Freitas Rebelo, M., Celso Dias Bainy, A., 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comp. Biochem. Physiol. 140, 403–407.

Chapman, P.M., 2007. Determining when contamination is pollution — weight of evidence determinations for sediments and effluents. Environ. Int. 33, 492–501.

Cheng, J., Ma, X., Krausz, K.W., Idle, J.R., González, F.J., 2009. Rifampicin-activated human pregnane X receptor and CYP3A4 induction enhance acetaminophen-induced toxicity. Drug Metab. Dispos. 37, 1611–1621.

Coatu, V., Ţigănuş, D., Oros, A., Lazăr, L., 2013. Analysis of hazardous substance contamination of the marine ecosystem in the Romanian Black Sea coast, part of the marine strategy framework directive (2008/56/EEC) implementation. Recherches Marines 43, 144–186.

Cociasu, A., Dorogan, L., Humborg, C., Popa, L., 1996. Long-term ecological changes in Romanian coastal waters of the Black Sea. Mar. Pollut. Bull. 32, 32–38.

Creusot, N., Kinani, S., Balaguer, P., Tapie, N., LeMenach, K., Maillot-Maréchal, E., Porcher, J.M., Budzinski, H., Aït-Aïssa, S., 2010. Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples. Anal. Bioanal. Chem. 396, 569–583.

Dayeh, V., Bols, N., Schirmer, K., Lee, L., 2003. The use of fish-derived cell lines for investigation of environmental contaminants. In: Hodgson, E. (Ed.), Current Protocols in Toxicology. Wiley & Sons Inc., New York, pp. 1–17.

Ekins, S., Reschly, E.J., Hagey, L.R., Krasowski, M.D., 2008. Evolution of pharmacologic specificity in the pregnane X receptor. BMC Evol. Biol. 8, 103.

Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. in Vitro 15, 477–488.

Fent, K., Bätscher, R., 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: demonstration of additive interactions. Environ. Toxicol. Chem. 19, 2047–2058.

Fernandes, D., Porte, C., 2013. Hydroxylated PAHs alter the synthesis of androgens and estrogens in subcellular fractions of carp gonads. Sci. Total Environ. 447, 152–159.

Fernandes, D., Pujol, S., Pérez-Albaladejo, E., Tauler, R., Bebianno, M.J., Porte, C., 2014. Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays. Mar. Environ. Res. 96, 127–135.

Gerbron, M., Geraudie, P., Fernandes, D., Rotchell, J.M., Porte, C., Minier, C., 2014. Evidence of altered fertility in female roach (*Rutilus rutilus*) form the River Seine (France). Environ. Pollut. 191, 58–62.

Goksøyr, A., 2006. Endocrine disruptors in the marine environment: mechanisms of toxicity and their influence on reproductive processes in fish. J. Toxicol. Environ. Health 69A, 175–184.

Huuskonen, S.E., Tuvikene, A., Trapido, M., Fent, K., Hahn, M.E., 2000. Cytochrome P4501A induction and porphyrin accumulation in PLHC-1 fish cells exposed to sediment and oil shale extracts. Arch. Environ. Contam. Toxicol. 38, 59–69.

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20, 149–159.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.M., Aït-Aïssa, S., 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut. 158, 74–83.

Kliewer, S.A., Goodwin, B., Willson, T.M., 2002. The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. Endocr. Rev. 23, 687–702.

Kosmehl, T., Krebs, F., Manz, W., Braunbeck, T., Hollert, H., 2007. Differentiation between bioavailable and total hazard potential of sediment-induced DNA fragmentation as measured by the comet assay with zebrafish embryos. J. Soils Sediments 7, 377–387.

Kretschmer, X.C., Baldwin, W.S., 2005. CAR and PXR: xenosensors of endocrine disrupters? Chem. Biol. Interact. 155, 111–128.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol. Appl. Pharmacol. 196, 247–257.

Lebel, C.P., Ischiropoulos, H., Bondys, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Lechner, A., Keckeis, H., Lumesberger-Loisl, F., Zens, B., Krusch, R., Tritthart, M., Glas, M., Schludermann, E., 2014. The Danube so colourful: a potpourri of plastic litter outnumbers fish larvae in Europe's second largest river. Environ. Pollut. 188, 177–181.

Lille-Langøy, R., Goldstone, J.V., Rusten, M., Milnes, M.R., Male, R., Stegeman, J.J., Blumberg, B., Goksøyr, A., 2015. Environmental contaminants activate human and polar bear (*Ursus maritimus*) pregnane X receptors (PXR, NR1I2) differently. Toxicol. Appl. Pharmacol. 284, 54–64.

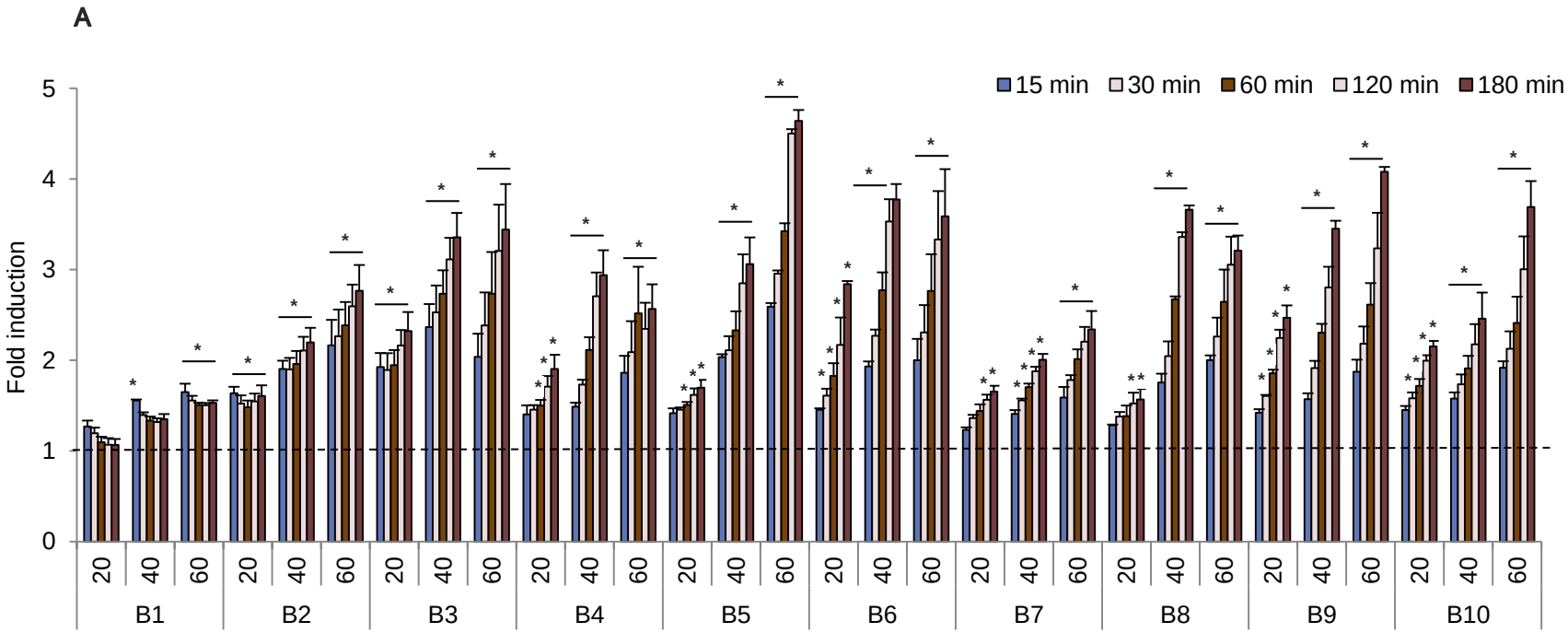
Livingstone, D., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Lorenzen, A., Kennedy, S.W., 1993. A fluorescence-based protein assay for use with a microplate reader. Anal. Biochem. 214, 346–348.

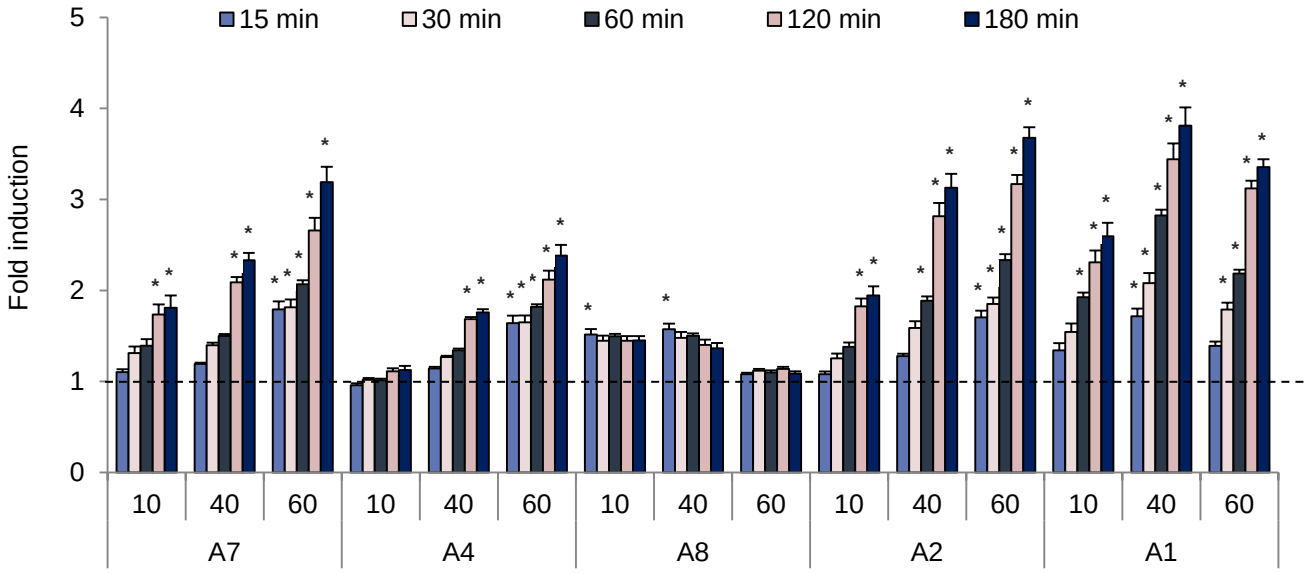
Lucialli, P., Ugolini, P., Pollini, E., 2007. Harbour of Ravenna: the contribution of harbour traffic to air quality. Atmos. Environ. 41, 6421–6431.
Magi, E., Bianco, R., Ianni, C., Di Carro, M., 2002. Distribution of polycyclic aromatic hydrocarbons in the sediments of the Adriatic Sea. Environ. Pollut. 119, 91–98.
Martin-Skilton, R., Lavado, R., Thibaut, R., Minier, C., Porte, C., 2006. Evidence of endocrine alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. Environ. Pollut. 141, 60–68.
Milnes, M.R., Garcia, A., Grossman, E., Grün, F., Shiotsugu, J., Tabb, M.M., Kawashima, Y., Katsu, Y., Watanabe, H., Blumberg, B., 2008. Activation of steroid and xenobiotic receptor (SXR, NR1/2) and its orthologs in laboratory, toxicologic, and genome model species. Environ. Health Perspect. 116, 880–885.
Schnell, S., Martin-Skilton, R., Fernandes, D., Porte, C., 2009. The interference of nitro- and polycyclic musks with endogenous and xenobiotic metabolizing enzymes in carp: an in vitro study. Environ. Sci. Technol. 43, 9458–9464.
Schnell, S., Olivares, A., Piña, B., Echavarri-Erasun, B., Lacorte, S., Porte, C., 2013. The combined use of the PLHC-1 cell line and the recombinant yeast assay to assess the environmental quality of estuarine and coastal sediments. Mar. Pollut. Bull. 77, 282–289.
Spagnoli, F., Dinelli, E., Giordano, P., Marcaccio, M., Zaffagnini, F., Frascari, F., 2014. Sedimentological, biogeochemical and mineralogical facies of Northern and Central Western Adriatic Sea. J. Mar. Syst. 139, 183–203.
Spohn, P., Hirsch, C., Hasler, F., Bruinink, A., Krug, H.F., Wick, P., 2009. C60 fullerene: a powerful antioxidant or a damaging agent? The importance of an in-depth material characterization prior to toxicity assays. Environ. Pollut. 157, 1134–1139.
Šrut, M., Traven, L., Štambuk, A., Kralj, S., Žaja, R., Mičovič, V., Klobučar, G.I.V., 2011. Genotoxicity of marine sediments in the fish hepatoma cell line PLHC-1 as assessed by the Comet assay. Toxicol. in Vitro 25, 308–314.
Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A. Toxicol. in Vitro 22, 1128–1135.
Ţigănuş, D., Coatu, V., Lazăr, L., Oros, A., Spînu, A.D., 2013. Identification of the sources of polycyclic aromatic hydrocarbons in sediments from the Romanian Black Sea sector. Recherches Marines 43, 187–196.
Traven, L., Žaja, R., Lončar, J., Smital, T., Mičovič, V., 2008. CYP1A induction potential and the concentration of priority pollutants in marine sediment samples — in vitro evaluation using the PLHC-1 fish hepatoma cell line. Toxicol. in Vitro 22, 1648–1656.
Wassmur, B., Grāns, J., Kling, P., Celander, M.C., 2010. Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 100, 91–100.
Zhai, Y., Pai, H.V., Zhou, J., Amico, J.A., Vollmer, R.R., Xie, W., 2007. Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. Mol. Endocrinol. 21, 138–147.

Información complementaria al artículo 1

Figure S1. ROS production in PLHC-1 cells after 15 and 180 minutes of exposure to different concentrations of sediments extracts from the Black Sea (A) and the Adriatic Sea (B). Concentrations are showed as mg eQsed/mL. Values are expressed as fold-induction with respect to control cells as mean ± SEM of at least three different plates assayed. Dotted line is control value. *Significant differences respect to control. * All of them were significantly different from control.



B


15 min
30 min
60 min
120 min
180 min
Fold induction
5
4
3
2
1
0
10
40
60
A7
A4
A8
A2
A1

ARTÍCULO 2

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

M. Blanco[1], E. Pérez-Albaladejo[1], C. Barata[1], B. Piña[1], G. Kušpilic[2], R. Lille-Langøy[3], O.A. Karlsen[3], Goksøyr[3], C. 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

Submitted to the Science of the Total Environment Journal

RESUMEN

La ciudad de Split, situada en el Mar Adriático croata, se extiende a lo largo de la Bahía de Kaštela y el Canal de Brač, ambos bajo la influencia de actividades antropogénicas. El objetivo de este estudio era caracterizar la calidad ambiental de los sedimentos marinos costeros de estas áreas, mediante una batería de bioensayos complementarios utilizando células PLHC-1, células COS-7 transfectadas con el receptor Pxr de pez cebra (zfPxr), y levaduras recombinantes (ER-RYA), como modelos biológicos, para detectar la presencia de contaminantes bioactivos en dichos sedimentos. Los extractos de sedimentos procedentes de la Bahía de Kaštela, cerca de la zona industrial y el puerto mercante de Split, mostraban una mayor citotoxicidad, y un mayor potencial para activar zfPxr e inducir actividad CYP1A. Por el contrario, los sedimentos procedentes del Canal de Brač, una zona impactada por tráfico de barcos y descargas urbanas y agrícolas, no mostraban citotoxicidad significativa en las células de peces, sugiriendo que

existe una dilución de los contaminantes desde la parte más oriental de la Bahía de Kaštela hacia el Canal de Brač y aguas abiertas del Mar Adriático. No se han encontrado evidencias de estrogenicidad en ninguna de las muestras investigadas mediante el ensayo ER-RYA, aunque no se descarta la presencia de compuestos disruptores endocrinos en los sedimentos, dada la baja sensibilidad del ensayo observada. En general, la batería de ensayos *in vitro*, señalan a la Bahía de Kaštela, particularmente la estación S1, como el área con mayor impacto antropogénico, mientras que las respuestas observadas para los sedimentos del Canal de Brač indicaban una calidad ambiental satisfactoria en términos de ausencia de compuestos citotóxicos y muy bajos niveles de inductores CYP1A y activadores de zfPxr.

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], 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*

Abstract

A battery of cell-based bioassays using PLHC-1 cells, zebrafish-Pxr-transfected COS-7 cells and recombinant yeast assay (ER-RYA), were applied to detect the presence of bioactive pollutants in sediment extracts 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. Striking response activating zfPxr was observed. However, no evidence of estrogenicity was detected for any of the tested sediments extracts with the ER-RYA assay. The battery of *in vitro* assays identified Kaštela Bay as the area with the higher anthropogenic impact, where sediment-bound pollutants could pose risk to aquatic organisms, while sediments from the Brač Channel showed rather low response in the different bioassays. Moreover, the ability of single compounds (pharmaceuticals, alkylphenols, PCBs, pesticides) and their mixture to activate zebrafish Pxr (zfPxr) was assessed in the COS-7 cell-line. 4-Nonylphenol, carbamazepine, chlordane, dieldrin and 4-*tert*-octylphenol significantly activated zfPxr receptor.

Keywords: Sediment; EROD; PLHC-1; zebrafish Pxr; ER-RYA; Kaštela Bay

1. Introduction

Nowadays, over half of the world's population resides in urban areas, the majority of them located in coastal zones, and 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 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. 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, and 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, bioaccumulation

and endocrine disrupting effects, among others (Creusot et al., 2010; Schnell et al., 2013; Fernandes et al., 2014). Due to the key role that 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. Among them, the PLHC-1 cell line, derived from topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma, expresses the aryl hydrocarbon receptor (AhR) and shows CYP1A induction after exposure to environmental pollutants, e.g. PAHs, pharmaceuticals and sediment extracts (Thibaut and Porte, 2008; Traven et al., 2008; Pérez-Albaladejo et al., 2016).

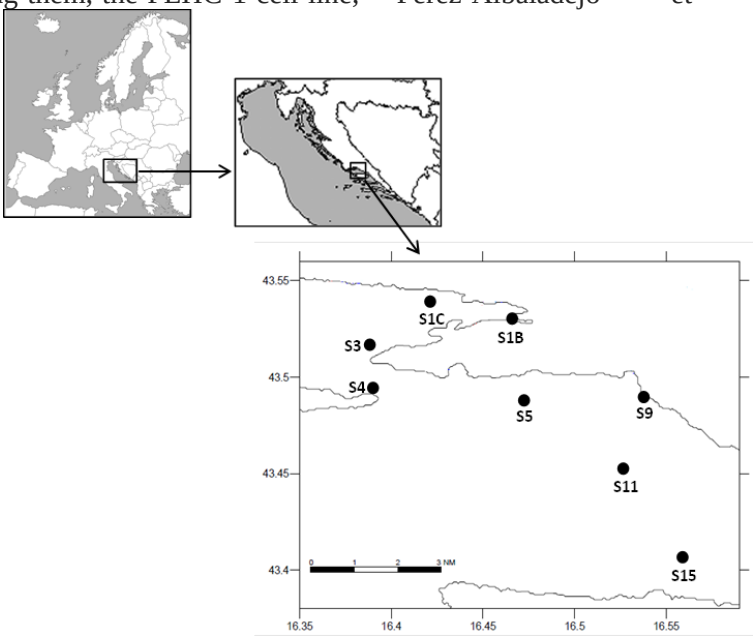


Figure 1. Map of the sampling sites in Kaštela Bay (S1B, S1C, S3, S4) and the Brač Channel (S5, S9, S11, S15) in Croatia.

Other adverse responses to chemicals, such as oxidative stress and genotoxicity have been successfully determined in PLHC-1 cells (Puerto et al., 2009; Šrut et al., 2011; Schnell et al., 2013).

Beside the AhR, the pregnane X receptor (Pxr) is a 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 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*); the most common variant was activated by known Pxr agonists, clotrimazole and pregnenolone 16α-carbnonitrile (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 estradiol-equivalent (EEQ) loads that integrate the contribution of all bioactive compounds present in the environmental extract.

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 metals, particularly 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 kidney COS-7 cell line to detect 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 where compared to those previously obtained in other areas of the Mediterranean in order to evidence the usefulness of the selected bioassays to assess the quality of sediments in costal monitoring programs. Additionally, the ability of a number of model compounds representative of different pollution sources, namely 1,2,3-trichlorobenzene, 4-nonylphenol (4-NP), 4-tert-octylphenol (4-OP), bisphenol A (BPA), carbamazepine, chlordane, dieldrin, omeprazol, PCB-153, perfluorononanoic acid (PFNA), and perfluorooctanoic acid (PFOA) to activate zfPxr was also investigated to further understand which pollutants could be responsible for the Pxr responses observed in the organic extracts of sediments.

Table 1. Geographic coordinates, sample depth and criteria for the selection of the sampling points.

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 29’08.04’’	16º 28’08.64’’	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. 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) and 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. 1). Geographic coordinates and main characteristics of the selected sites are given in Table 1. A subsample was transferred to a stainless steel container, mixed and the composite sediment 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.

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 /streptomycin.

Both PLHC-1 and COS-7 cells were cultured in a humidified incubator with 5% CO_2, at 30 and 37 ºC, respectively. When 90% of confluence was reached, cells were dissociated with trypsin-EDTA for subculturing and experiments. Application of sediment extracts to cell cultures was done by diluting the extracts in culture medium, so that the final concentration of DMSO in culture wells was of 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. Results 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). Values were expressed as percentage of viability compared with control cells, as mean ± SD of at least six replicates in three different plates assayed.

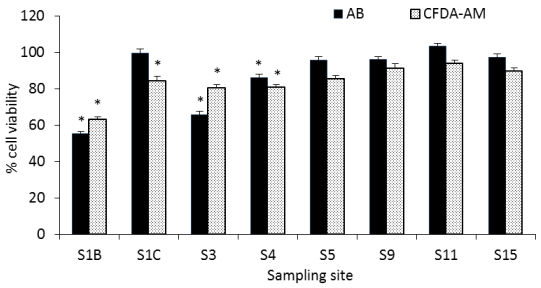


Figure 2. Cell viability of PLHC-1 cells after 24 h exposure to sediment extracts from Kaštela Bay and Brač Channel tested at 60 mg eQsed/mL. Values are mean ± SEM of 4 independent assays. *Statistically significant decrease in cell viability relative to control cells ($p < 0.05$).

2.5. 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 carrier (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 and per milligram of protein (pmol/min/mg protein). R_{BNF} value was calculated as the concentration of sediment required to induce an EROD response equivalent to 1µM BNF.

2.6. Ligand activation of zebrafish Pxr

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 10^3 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 was expressed as change in luciferase activity in test cells relative to non-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 chemicals, including 1,2,3-trichlorobenzene, 4-NP, 4-OP, BPA, carbamazepine, chlordane, dieldrin, omeprazol, PCB-153, 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). Nuclear receptor activation was referred as fold change of normalized luciferase activity with respect to DMSO control cells. 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.

2.7. Recombinant yeast assay (ER-RYA)

ER-RYA assays were performed using the yeast strain BY4741 as described in Schnell et al. (2013). Samples were tested in triplicate and added to the yeast cells in DMSO (5% v/v). After 6 h of incubation at 30 ºC with serial dilutions of each sample, β-galactosidase activity was determined on-plate using the fluorogenic substrate 4-methylumbelliferone β-D-galactopyranoside. Fluorescence was recorded in a Synergy 2 spectrofluorometer (BioTek, USA) at 355/460 nm excitation/emission wavelengths. 17β-Estradiol (10 nM) was used as positive control. ER ligand activity 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.

Table 2. Maximum zfPxr induction and concentration of sediment required to induce 20% of the response induced by 60 nM 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}); and concentration of organochlorinated compounds in the sediment extracts. Values are mean ± SEM of at least three independent experiments. *Statistically significant differences from control ($p < 0.05$). Sediments expressed as mg eQsed/mL.

Sample point	zfPxr Fold induction	REC_{20}	EROD Max. activity	R_{BNF}	Organochlorines (ng/g dw) ∑PCB	∑DDT	HCB	HCHγ
Kaštela Bay								
S1B	19 ± 1*	0.9	110 ± 9	4.9	35.4	1.4	0.67	0.09
S1C	13 ± 1*	2.1	126 ± 5	16.6	28.0	1.0	3.94	< 0.05
S3	10 ± 1*	7.4	114 ± 6	22.7	5.8	0.8	0.45	< 0.05
S4	10 ± 2*	11.5	128 ± 1	31.8	3.1	0.4	0.18	< 0.05
Brač Channel								
S5	4 ± 1*	18.7	64 ± 16	> 60	0.3	0.09	< 0.05	< 0.05
S9	4 ± 1*	14.5	56 ± 11	> 60	0.1	0.07	< 0.05	< 0.05
S11	2 ± 0.3*	53.2	53 ± 10	> 60	< 0.12	< 0.05	< 0.05	< 0.05
S15	4 ± 1*	24.1	67 ± 3	> 60	0.3	0.14	< 0.05	< 0.05

2.8. 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 hours. 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, p,p'-DDE and PCBs, whereas the second fraction, eluted with 45 mL hexane/dichloromethane (70:30 v/v), contained lindane, p,p'-DDD and p,p'-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 Ni63. 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^{-1} 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.

2.9. 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.

Quantitative prediction of combined effects was performed by adapting previously established approaches (Faust et al., 2003) that have been modify to fit responses having different Emax (Faria et al., 2016). First, concentration-response relationships for the individual components of the mixture were established (see eq. 1), calculating expected combined effects according to either the

model of concentration addition and/or independent action. Subsequently, predicted values were compared with experimentally observed effects. Predicted values of studied individual components were estimated from the obtained concentration – response data, considering fluorescence changes relative to control treatments and by fitting observed responses to the modified non-linear Hill model.

$$R(c_i) = \frac{E\max}{100 + \left(\frac{EC50}{c_i}\right)^p} \quad (1)$$

Where, $R(c_i)$ is the Percentage fluorescent change (%) at concentration ci relative to controls, which was fixed to 0; Emax is the maximal fluorescence effect in %; c_i is the concentration of compound (i); *p* is the Hill index; and EC_{50} is the concentration of compound that corresponds to 50% of the maximal effect.

3. Results

3.1. 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 2. Significant cytotoxicity was detected in sediments collected in Kaštela Bay (S1B, S3, S4), while low (Kaštela Bay: S1C) or no cytotoxicity (Brač Channel: S5, S9, S11, S15) was detected for the other sediment's extracts.

Dose-response curves for EROD activity for the positive control (BNF) and all the sediment extracts are shown in Figure 3. Maximum EROD activity and concentration of sediment required to induce a response equivalent to 1 µM BNF (R_{BNF}) are given in Table 2. Sediments from S1B had the highest ability to induce EROD activity in PLHC-1 cells (110 ± 9 pmol/min/mg protein; R_{BNF}: 4.9 mg eQsed/mL). The other sediments collected in Kaštela Bay (S1C, S3, S4) also induced EROD activity (114-128 pmol/min/mg protein; R_{BNF}: 17-32 mg eQsed/mL). On the contrary, minor EROD induction (53-67 pmol/min/mg protein) was observed for sediments collected along the Brač Channel (S5, S9, S11, S15), with R_{BNF} > 60 mg eQsed/mL. The AhR agonist BNF at a concentration of 1 µM lead to an EROD activity of 66 ± 1.7 pmol/min/mg protein after 24 h of exposure.

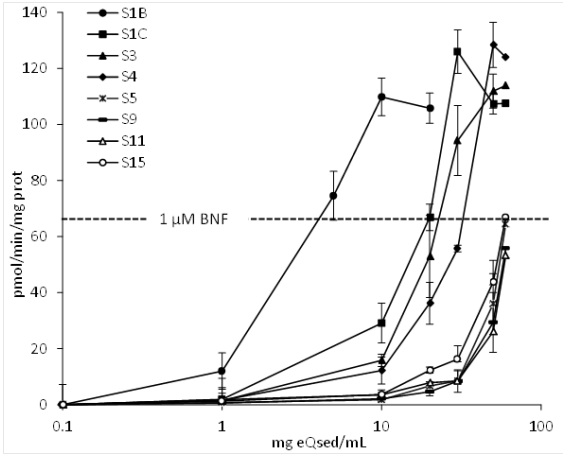


Figure 3. EROD activity in PLHC-1 cells exposed for 24 h to different concentrations of sediments extracts from Kaštela Bay and Brač Channel. Values are expressed in pmol/min/mg of protein, as mean ± SEM of at least three independent experiments. Dotted line corresponds to the positive control.

3.2. Ligand activation of zebrafish Pxr

All sediments significantly activated Pxr from zebrafish and induced the expression of the luciferase reporter gene (Fig. 4). 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 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.

Moreover, the ability of model compounds, likely to be present in the sediments, to activate zfPxr was evaluated in single and mixture exposures (Fig. 5A). 4-NP, 4-OP, carbamazepine, chlordane and dieldrin showed a significant ability to activate zfPxr (2.7 to 9-fold), whereas BPA, 1,2,3-trichlorobenzene, omeprazol, PCB-153, PFNA and PFOA did not significantly activate the receptor (< 1.7-fold). The maximum induction was observed at the

highest concentration tested (100 μM), with the exception of chlordane (9.0 ± 0.9) and 4-NP (3.6 ± 0.4), for which the maximum induction was detected at 30 and 50 μM, respectively (Fig. 5A). Figure 5B shows the results of an equimolar mix of the single compounds together with predicted combined effects of the assayed mixture according to the independent and concentration addition model and those of individual mixture constituents (grey lines). Results indicate that chlordane contributes most to the tested mixture. Observed mixture effects are antagonistic due to the contribution of the other weak agonists that ameliorated the effect of chlordane alone.

3.3. Estrogen receptor- recombinant yeast assay

Estrogenic activity was below the quantification limit 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.

3.4. Organochlorine content in sediments

Concentrations of HCB, lindane (γ-HCH), Σ DDTs (pp'DDE + pp'DDD + pp'DDT) and 7 PCBs congeners (sum of PCB 28, 52, 101, 118, 138, 153 and 180) in the analysed sediments are shown in Table 2. 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, pp'DDE and pp'-DDD were more abundant than pp'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 isomer detected in sediments, the highest concentration was found in S1B (0.1 ng/g d.w).

4. Discussion

Toxicity screening of complex environmental samples, such as sediments, require the use of multiple endpoints that allow the determination of different modes of action of the chemical mixture trapped in the matrix. By using the PLHC-1 cell line, it was possible to detect significant cytotoxicity in sediment extracts from station S1B, an industrial zone including the Port of Split and the mouth of Jadro River. High concentrations of PCBs, DDTs, HCBs, (35.4, 1.4, and 0.7 ng/g d.w.), but also PAHs (2.21 μg/g d.w.) and metals, such as Pb and Hg (6.5 and 2.3 μg/g d.w., respectively) have been detected in sediments from this station in Kaštela Bay, as a result of industrial and domestic wastewater discharges to this semi-enclosed and relatively shallow area (Kušpilić, unpublished results). 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 cytotoxicity in PLHC-1 cells and had levels of organochlorinated compounds up to 100-fold lower. The observed decreases in cell viability do not necessarily imply organ or organism-level effects, but they are an indicator of adverse effects at the cellular level and a prerequisite for further toxic responses at the organism level. 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 or Pxr (1 to 10 mg eQsed/mL). The determination of cytotoxicity was also used as a quality control measure for the induction assays to verify that cell vitality was not adversely affected.

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 inducers (analogs of 2,3,7,8-tetracholordibenzo-p-dioxin, 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 development of dose-response relationships

and has both, practical and economical value, allowing the detection of highly toxic persistent and non-persistent AhR inducers (Schnell et al., 2013). In the present work, EROD assay indicated the presence of significant amounts of AhR agonists in sediments 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, indicate the dilution of pollutants from the easternmost part of Kaštela bay to the Brač Channel and the open waters of the Adriatic Sea, and evidence reduced inputs of pollutants in the Brač Channel, which is mainly affected by ship traffic, freshwater and wastewater discharges and agricultural activities.

Many studies have evidenced the ability of environmental chemicals to activate the PXR receptor, but information regarding its activation by mixtures of pollutants and environmental samples is scarce. The activation of human PXR by river water samples, moderately impacted by agricultural and urban inputs, containing alkylphenols, hormones, pharmaceuticals, pesticides, PCBs, BPA and effluents from sewage treatment works (STW) was reported by Kinani et al. (2010) and Creusot et al. (2010). Recently, Pérez-Albaladejo et al. (2016) reported the activation of zfPXR by chemicals bound to sediments from Constanta harbour (14-fold; REC_{20}: < 5) and the Danube mouth (11-fold; REC_{20}: 5.9). However, the activation of zfPxr by sediment extracts from Kaštela Bay was even higher (19-fold; REC_{20}: 0.9 mg eQsed/mL).

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, as well as agricultural drainage wastewater originating from various agricultural activities along the shore. Among the single compounds tested for their ability to activate zfPxr, alkylphenlos (4-NP, 4-OP) and persistent organochlorines (triclorobenzene, chlordane, dieldrin) significantly activated zfPxr (2.7 to 9-fold), while the abundant PCB-153 isomer did not activate the receptor. When assayed in an equimolar mixture, the ability of those compounds to activate the zfPxr was lower than predicted, probably due to the contribution of weak agonists that acted ameliorating the effect of chlordane. These results reinforce the importance of evaluating the synergistic and antagonistic effects that occur in a mixture, but also give special significance to the high zfPxr activation response detected in sediment extracts from Kaštela Bay (19-fold, S1B) and the need to further investigate which the causing agents are.

The recombinant yeast assay did not evidence 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).

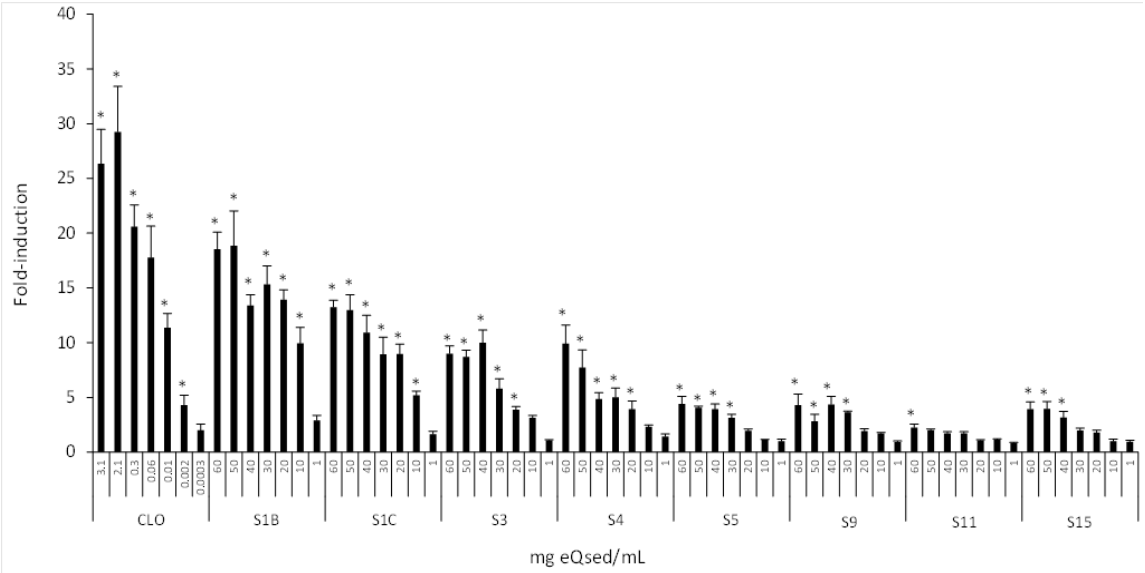


Figure 4. In vitro ligand activation of zfPxr by sediments collected from Kaštela Bay presented as fold change of normalized luciferase activity respect to DMSO control cells (mean ± SEM). Dotted line corresponds to DMSO control. *Statistically significant differences from control ($p < 0.05$).

Thus, although the concentration of estrogenic compounds in sediment extracts from the studied area is below the detection limit of the ER-RYA assay (Noguerol et al., 2006a; Gago-Ferrero et al., 2012; Puy-Azurmendi et al., 2014), we cannot discard the presence of compounds that by modulating the activity of enzymes involved in steroid synthesis/metabolism, might act as endocrine disrupters (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) under the influence of the Port of Split, the mouth of Jadro River and historical contamination from a former PVC chlor-alkali plant, as the most impacted area. The work has also evidenced a dilution of pollutants from the Eastern part of Kaštela Bay towards the open waters of the Adriatic Sea, and has showed 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, EROD and zfPxr induction) using internal calibrators may allow the comparison of sediments from different coastal areas. Thus, the responses (percentage of cell viability, R_{BNF}, REC_{20}) obtained in the present work for Kaštela Bay, particularly from station S1C are analogous to the values recorded in Constanta harbour, the mouth of the Danube River and other areas of the Adriatic Sea under the influence of the Po River (Pérez-Albaladejo et al., 2016).

Responses for the Brač Channel indicate good environmental status in terms of absence of cytotoxic compounds and very low levels of CYP1A inducers.

In conclusion, this approach may facilitate a first characterization of the environmental quality of coastal sediments as well as the identification of hot-spots that require further action to improve their environmental quality, and may contribute in the future to a significant reduction in the number of bioindicator organisms to be used in environmental monitoring studies.

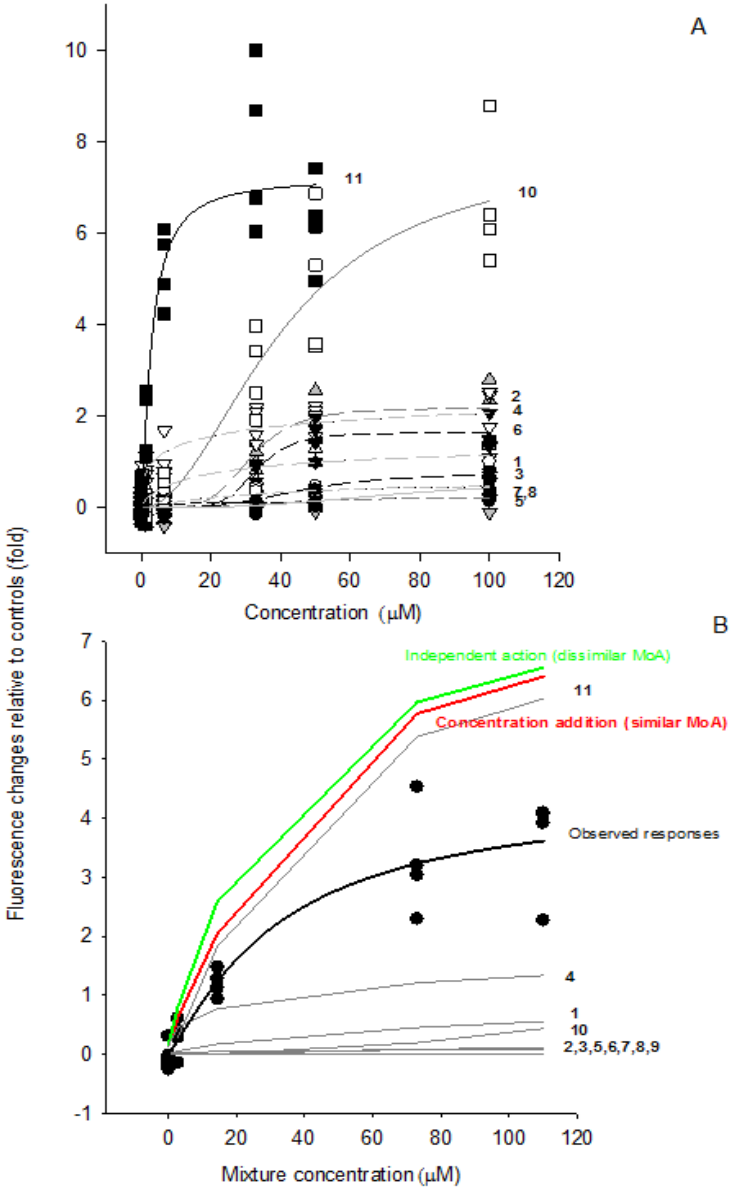


Figure 5. Single (A) and mixture (B) effects on the 11 tested compounds on zfPXR. In graph A fitted regression lines are depicted. Graph B includes observed responses of individual mixture constituents and their mixture (grey and black lines) and the fitted regression curve, plus predicted joint additive effects following and independent action and concentration addition models (green and red lines). Numbers 1 to 11 correspond to 1: 1,2,3, triclorobenzene, 2: 4-NP, 3: omeprazole, 4: dieldrin, 5: PFOA, 6: OP, 7: bisphenol A, 8: PCB 153, 9: PFNA, 10: carbamazepine, 11: chlordane. Each symbol is a single observation.

Acknowledgements

This work has been supported by the project "Policy-oriented marine Environmental Research in the Southern European Seas" (PERSEUS), grant agreement No. 287600, and "NILS Science and Sustainability" (ES07; ABEL-CM-01-2013). Maria Blanco acknowledges a pre-doctoral fellowship (FPI, BES-2012-054438) from the Spanish Government.

References

Bainy, A.C., Kubota, A., Goldstone, J.V., Lille-Langøy, R., Karchner, S.I., Celander, M.C., Hahn, M.E., Goksøyr, A., Stegeman, J.J. 2013. Functional characterization of a full length pregnane X receptor, expression *in vivo*, and identification of PXR alleles, in Zebrafish (*Danio rerio*). Aquatic Toxicology 142–143, 447–457.

Bogdanović, T., Ujević, I., Sedak, M., Listeš, E., Šimat, V., Petričević, S., Poljak, V. 2014. As, Cd, Hg and Pb in four edible shellfish species from breeding and harvesting areas along the eastern Adriatic Coast, Croatia. Food Chemistry 146, 197–203.

Brack, W., Schirmer, K., Erdinger, L., Hollert, H. 2005. Effect-directed analysis of mutagens and ethoxyresofurin-*O*-deethylase inducers in aquatic sediments. Environmental Toxicology and Chemistry 24, 2445–2458.

Chapman, P.M. 2007. Determining when contamination is pollution - Weight of evidence determinations for sediments and effluents. Environment International 33, 492–501.

Creusot, N. Kinani, S., Balaguer, P., Tapie, N., LeMenach, K., Maillot-Maréchal, E., Porcher, J-M., Budzinski, H., Aït-Aïssa, S. 2010. Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples. Analytical and Bioanalytical Chemistry 396, 569–583.

Dayeh, V.R., Bols, N.C., Schirmer, K., Lee, L.E.J. 2003. The use of fish-derived cell lines for investigation of environmental contaminants: An update following OECD's fish toxicity testing framework no. 171. Current Protocols in Toxicology 1, 1–20.

Ekins, S., Reschly, E.J., Hagey, L.R., Krasowski, M.D. 2008. Evolution of pharmacologic specificity in the pregnane X receptor. BMC Evolutionary Biology 8, 103.

Faria, M., Pavlichenko, V., Burkhardt-Medicke, K., Soares, A.M.V.M., Altenburger, R., Barata, C., Luckenbach, T. 2016. Use of a combined effect model approach for discriminating between ABCB1- and ABCC1-type efflux activities in native bivalve gill tissue. Toxicology and Applied Pharmacology 297, 56-67.

Fent, K. 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicology in Vitro 15, 477–488.

Fernandes, D., Pujol, S., Pérez-Albaladejo, E., Tauler, R., Bebianno, M.J., Porte, C. 2014. Characterization of the environmental quality of sediments from two estuarine systems based on different *in vitro* bioassays. Marine Environmental Research 96, 127–135.

Gago-Ferrero, P., Badia-Fabregat, M., Olivares, A., Piña, B., Blánquez, P., Vicent, T., Caminal, G., Díaz-Cruz, M.S., Barceló, D. 2012. Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1. Science of the Total Environment 427-428, 355-363.

Jakšić, Ž. Batel, R., Bihari, N., Mičić, M., Zahn, R.K. 2005. Adriatic coast as a microcosm for global genotoxic marine contamination - A long-term field study. Marine Pollution Bulletin 50, 1314–1327.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J-M., Aït-Aïssa, S. 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environmental Pollution 158, 74–83.

Kliewer, S.A., Goodwin, B., Willson, T.M. 2002. The nuclear pregnane X receptor: A key regulator of xenobiotic metabolism. Endocrine Reviews 23, 687–702.

Kljaković-Gašpić, Z., Herceg-Romanić, S., Kožul, D., Veža, J. 2010. Biomonitoring of organochlorine compounds and trace metals along the Eastern Adriatic coast (Croatia) using *Mytilus galloprovincialis*. Marine Pollution Bulletin 60, 1879–1889.

Kwokal, Ž., Frančišković-Bilinski, S., Bilinski, H., Branica, M. 2002. A comparison of anthropogenic mercury pollution in Kaštela Bay (Croatia) with pristine estuaries in Öre (Sweden) and

Krka (Croatia). Marine Pollution Bulletin 44, 1152–1169.

Lille-Langøy, R., Goldstone, J.V., Rusten, M., Milnes, M.R., Male, R., Stegeman, J.J., Blumberg, B., Goksøyr, A. 2015. Environmental contaminants activate human and polar bear (*Ursus maritimus*) pregnane X receptors (PXR, NR1I2) differently. Toxicology and Applied Pharmacology 284, 54–64.

Lorenzen, A., Kennedy, S.W., 1993. A fluorescence-based protein assay for use with a microplate reader. Analytical Biochemistry 214, 346–348.

Lovrenčić Mikelić, I., Oreščanin, V., Barišić, D. 2013. Distribution and origin of major, minor, and trace elements in sediments and sedimentary rocks of the Kaštela Bay (Croatia) coastal area. Journal of Geochemical Exploration 128, 1–13.

Milnes, M.R., Garcia, A., Grossman, E., Grün, F., Shiotsugu, J., Tabb, M.M., Kawashima, Y., Katsu, Y., Watanabe, H., Iguchi, T., Blumberg, B. 2008. Activation of steroid and xenobiotic receptor (SXR, NR1/2) and its orthologs in laboratory, toxicologic, and genome model species. Environmental Health Perspectives 116, 880–885.

Milun, V., Grgas, D., Landeka Dragičević, T. 2016. Assessment of PCB and chlorinated pesticide accumulation in mussels at Kaštela Bay (Eastern Adriatic). Science of The Total Environment 562, 115-127.

Noguerol, T-N., Boronat, S., Casado, M., Raldúa, D., Barceló, D., Piña, B. 2006a. Evaluating the interactions of vertebrate receptors with persistent pollutants and antifouling pesticides using recombinant yeast assays. Analytical and Bioanalytical Chemistry 385, 1012–1019.

Noguerol, T-N., Boronat, S., Jarque, S., Barceló, D., Piña, B. 2006b. Detection of hormone receptor ligands in yeast by fluorogenic methods. Talanta 69, 351–358.

Pérez-Albaladejo, E., Rizzi, J., Fernandes, D., Lille-Langøy, R., Karlsen, O.A., Goksøyr, A., Oros, A., Spagnoli, F., Porte, C. 2016. Assessment of the environmental quality of coastal sediments by using a combination of *in vitro* bioassays. Marine Pollution Bulletin 108, 53–61.

Puerto, M., Pichardo, S., Jos, A., Cameán, A.M. 2009. Oxidative stress induced by microcystin-LR on PLHC-1 fish cell line. Toxicology in Vitro 23, 1445–1449.

Puy-Azurmendi, E., Olivares, A., Vallejo, A., Ortiz-Zarragoitia, M., Piña, B., Zuloaga, O., Cajaraville, M.P. 2014. Estrogenic effects of nonylphenol and octylphenol isomers *in vitro* by recombinant yeast assay (RYA) and *in vivo* with early life stages of zebrafish. Science of the Total Environment 466–467, 1–10.

Quirós, L., Céspedes, R., Lacorte, S., Viana, P., Raldúa, D., Barceló, D., Piña, B. 2005. Detection and evaluation of endocrine-disruption activity in water samples from portuguese rivers. Environmental Toxicology and Chemistry 24, 389–395.

Schnell, S., Olivares, A., Piña, B., Echavarri-Erasun, B., Lacorte, S., Porte, C. 2013. The combined use of the PLHC-1 cell line and the recombinant yeast assay to assess the environmental quality of estuarine and coastal sediments. Marine Pollution Bulletin 77, 282–289.

Šrut, M., Traven, L., Štambuk, A., Kralj, S., Žaja, R., Mićović, V., Klobučar, G.I.V. 2011. Genotoxicity of marine sediments in the fish hepatoma cell line PLHC-1 as assessed by the Comet assay. Toxicology in Vitro 25, 308–314.

Thibaut, R., Porte, C. 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: Cytotoxicity and interactions with cytochrome P450 1A. Toxicology in Vitro 22, 1128–1135.

Traven, L., Žaja, R., Lončar, J., Smital, T., Mićović, V. 2008. CYP1A induction potential and the concentration of priority pollutants in marine sediment samples - *In vitro* evaluation using the PLHC-1 fish hepatoma cell line. Toxicology in Vitro 22, 1648–1656.

UNEP/IAEA/IOC (2011) Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods For Marine Pollution studies 71, p. 48.

von Glasow, R., Jickells, T.D., Baklanov, A., Carmichael, R., Church, T.M., Gallardo, L., Hughes, C., Kanakidou, M., Liss, P.S., Mee, L., Raine, R., Ramachandran, P.,

Ramesh, R., Sundseth, K., Tsunogai, U., Uematsu, M., Zhu, T. 2013. Megacities and large urban agglomerations in the coastal zone: interactions between atmosphere, land and marine ecosystems. AMBIO 42, 13–28.

Wada, T., Gao, J., Xie, W. 2009. PXR and CAR in energy metabolism. Trends in Endocrinology and Metabolism 20, 273–279.

Whyte, J.J., Jung, R.E., Schmitt, C.J., Tillitt, D.E. 2000. Ethoxyresorufin-*O*-deethylase (EROD) Activity in Fish as a Biomarker of Chemical Exposure. Critical Reviews in Toxicology 30, 347–570.

Generación de ROS en células PLHC-1 expuestas a sedimentos de la costa de Croacia.

Complementariamente a los bioensayos detallados en el artículo 2, se han realizado ensayos para determinar el estrés oxidativo en la línea celular de peces PLHC-1 durante la exposición a los extractos de sedimentos de la Bahía de Kaštela y el Canal de Brač, en la costa de Croacia. La técnica empleada se basa en la oxidación de H_2DCF por el ROS generado en las células a lo largo de 120 minutos de exposición a los extractos de sedimentos. Las células PLHC-1 mostraban niveles significativos de ROS (50 % más que las células control) en presencia de 60 mg eQsed/mL de extractos de las estaciones S11 y S15 del Canal de Brač mientras que el resto de estaciones generaban entre un máximo de 30 % de ROS. La inducción máxima de ROS se observó a los 15 minutos de exposición a los extractos, mientras que en los minutos posteriores se recuperaban los niveles basales de ROS (Figura 11).

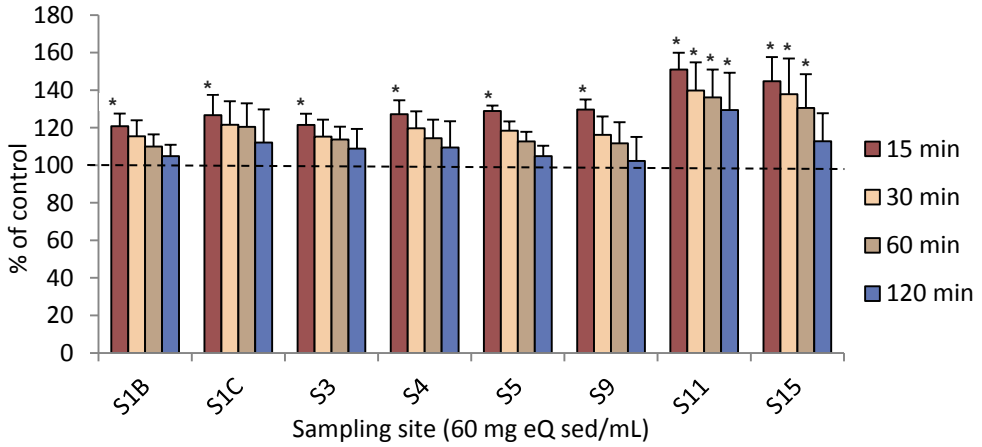


Figura 11: Porcentaje de generación de ROS en células PLHC-1, en presencia extractos de sedimentos de la costa de Croacia a una concentración de 60 mg eQ sed/mL, durante 15, 30, 60 y 120 minutos. La línea punteada representa las células control (100 %). *Estadísticamente significativo.

ARTÍCULO 3

Characterization of quality of sediments from Paranaguá Bay (Brazil) by combined *in vitro* bioassays and chemical analyses

J. Rizzi[1,2], E. Pérez-Albaladejo[1], D . Fernandes[1], J. Contreras[1], S. Froehner[2], C. Porte[1]

[1]Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain
[2]Department of Hydraulics and Sanitation, Federal University of Parana, Curitiba, Brazil

Environmental Toxicology and Chemistry (2016) 1-9

RESUMEN

Los estuarios son ecosistemas altamente vulnerables que requieren especial atención por su elevado valor ecológico. El Complejo Estuárico de Paranaguá (Sur de Brasil) se caracteriza por la presencia de manglares, islas e islotes en una extensión de 612 km^2. El eje Este-Oeste está influenciado por un elevado tráfico naval debido a la presencia del puerto de Paranaguá, el más extenso para la exportación de grano de Sudamérica, mientras que el eje Norte-Sur es un área protegida sin actividades antropogénicas ni descargas industriales. En el presente estudio se caracteriza la calidad de los sedimentos del Estuario de Paranaguá combinando análisis químicos para determinar las concentraciones de hidrocarburos aromáticos policíclicos (PAHs), bifenilos policlorados (PBCs) y pesticidas organoclorados (OCPs), y ensayos *in vitro* para detectar la presencia de compuestos citotóxicos, presencia de agentes inductores de CYP1A y agentes pro-oxidantes generadores de estrés oxidativo en células PLHC-1, y disruptores de la actividad P450 aromatasa en la fracción microsomal de ovario de lubina

(*Dicentrarchus labrax*). Los resultados mostraban que, a pesar de los relativamente bajos niveles de contaminantes detectados mediante los análisis químicos, los sedimentos obtenidos en los puertos (P1 y P4) mostraban elevada citotoxicidad y contenían niveles significativos de agentes inductores de CYP1A en las células PLHC-1. Así mismo, todos los extractos (60 mg eQsed/mL) inducían la generación de ROS (hasta el doble de los niveles de las células control) en dichas células. Los extractos de sedimentos a concentraciones 2 mg eQsed/mL, inhibían la actividad CYP19 (hasta un 30 %) en la fracción microsomal de ovario de lubina, siendo las estaciones P5 y P7 aquellas que menor potencial de inhibición mostraban (30 % a 10 mg eQsed/mL) respecto al resto. Este resultado indica la presencia de agentes disruptores endocrinos en todas las estaciones investigadas. En general, la combinación de análisis químicos y ensayos *in vitro* ha permitido agrupar las áreas marinas estudiadas según su impacto antropogénico, apuntando a las estaciones P1 y P4 como las más impactadas por la contaminación antropogénica, y englobando al resto de estaciones (P7, P12, P16, P10 y P17) en un nivel de calidad ambiental intermedio, con un impacto antropogénico moderado.

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 9999, No. 9999, pp. 1–9, 2016

Printed in the USA

SETAC Latin America

CHARACTERIZATION OF QUALITY OF SEDIMENTS FROM PARANAGUÁ BAY (BRAZIL) BY COMBINED IN VITRO BIOASSAYS AND CHEMICAL ANALYSES

JULIANE RIZZI,*†‡ ELISABET PÉREZ-ALBALADEJO,‡ DENISE FERNANDES,‡ JAVIER CONTRERAS,‡ SANDRO FROEHNER,†§ and CINTA PORTE‡

†Water Resources and Environmental Engineering Graduate Program, Federal University of Paraná, Curitiba, Paraná, Brazil
‡Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research, Spanish Council for Scientific Research, Barcelona, Catalunya, Spain
§Department of Environmental Engineering, Federal University of Paraná, Curitiba, Paraná, Brazil

(Submitted 29 February 2016; Returned for Revision 4 April 2016; Accepted 7 July 2016)

Abstract: The present study characterizes the quality of sediments from the Paranaguá Estuarine Complex (South Brazil). Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs) were determined in sediment samples together with a series of different in vitro bioassays. The fish hepatoma cell line (PLHC-1) was used to determine the presence of cytotoxic compounds and CYP1A- and oxidative stress-inducing agents in sediment extracts. Ovarian microsomal fractions from sea bass (*Dicentrarchus labrax*) were used to detect the presence of endocrine disrupters that interfered with the synthesis of estrogens (ovarian CYP19). Despite the relatively low levels of pollutants and no evidence of negative effects based on guideline levels, sediments collected close to harbors were enriched with CYP1A-inducing agents and they showed higher cytotoxicity. In contrast, sediments from internal areas inhibited CYP19 activity, which suggests the presence of endocrine disrupters at these sites. Overall, the selected bioassays and the chemistry data led to the identification of potentially impacted areas along the Paranaguá Estuarine Complex that would require further action to improve their environmental quality. *Environ Toxicol Chem* 2016;9999:1–9. © 2016 SETAC

Keywords: Sediment contamination Organic pollutants In vitro bioassays Endocrine disruption Biomonitoring

INTRODUCTION

Estuaries are highly vulnerable ecosystems that deserve special attention because of their high ecological significance. Because they also provide excellent opportunities for development of socioeconomic activities, estuaries are among the aquatic ecosystems most susceptible to the continuous influx of heterogeneous contaminant mixtures [1]. The distribution and fate of pollutants is often associated with the proximity of anthropogenic activities. However, transport processes, physicochemical properties of the compounds, and environmental conditions will also significantly affect the environmental behavior of pollutants [2].

Sediments are widely recognized as environmental sinks and reservoirs capable of long-term storing of pollutants. Certain characteristics, such as grain size and total content of carbon (TOC), may affect the accumulation of those pollutants: for instance, organic carbon acts as an accumulator of chemical compounds and constitutes a measure of sorption capacity of the sediment, while fine sediments have greater cohesive properties [3]. Therefore, the health of aquatic ecosystems is often assessed by measuring sediment characteristics and the pollutants trapped in sediments. It is not feasible, however, to identify and measure the concentrations of all potential toxicants in the sediment and the synergistic and antagonistic interactions among them by using chemical analysis alone [4]. Indeed, there are many challenges in establishing a safety limit of pollutant levels [5].

* Address correspondence to juliane.rizzi@gmail.com
Published online 8 July 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3553

Endocrine disrupters are exogenous agents that interfere with the production, transport, metabolism, action, or secretion of natural hormones or hormone receptors that are responsible for maintaining homeostasis and the development of regulatory processes [6,7]. Estrogenic activity can be detected by various methods such as ligand-binding assays or reporter-gene or yeast assays, among others, based on the molecular and cellular mechanisms of estrogen action [7].

In vitro mechanism-based cellular bioassays have proved to be valuable tools for the detection and quantification of biologically active chemicals in environmental mixtures [8]. The use of in vitro tests reduces the need for whole-animal testing and provides powerful and predictive tools for risk assessment [9]. Such tests allow the evaluation of alterations in key toxicological pathways in response to chemicals. However, they have a limited value for risk assessment, because they do not take into account all toxicity pathways (which are not completely known) and do not consider the complexity of humans and animals [8,9]. Therefore, because sediments have to be processed to provide a form suitable for in vitro exposure (e.g., concentrated organic sediment extracts), mechanisms such as bioavailability, bioaccessibility, and water solubility of chemicals contained in the sediment are not considered.

The use of fish cells offers some advantages and disadvantages. For instance, the loss of realism and biological relevance is compensated for by other aspects, such as reduced experimental noise, reduced costs, and the relative ease of assaying a wide range of compounds and concentrations, as well as ethical issues [10]. Among them, PLHC-1 is a permanent fish cell line that retains the capability to induce cytochrome P450 (CYP)1A through activation of the aryl hydrocarbon receptor by numerous pollutants such as pharmaceuticals, polycyclic aromatic hydrocarbons (PAHs), and dioxins. The PLHC-1

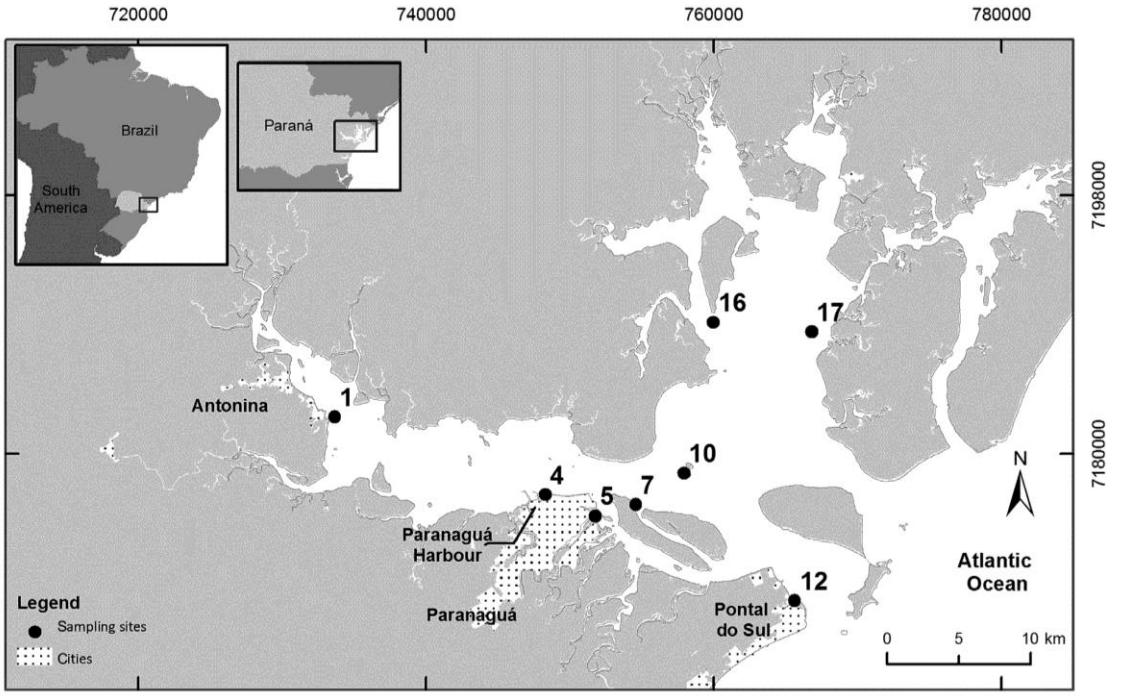


Figure 1. Map of the sampling sites.

line has also been used successfully to assess cytotoxicity and oxidative stress of different compounds, including sediments extracts [11].

The CYP enzymes also play a key role in the synthesis of sex steroids in fish gonads. In fish ovaries, androgens are converted into estrogens (estrone and estradiol) by cytochrome P450 aromatase (CYP19). The interference of pollutants with this enzymatic pathway may alter endogenous levels of active hormones within the organism. It may become a potential mechanism of endocrine disruption by affecting sexual differentiation and gamete growth in fish [6,10]. Bioassays based on the use of fish gonad subcellular fractions have been successfully applied to detect endocrine disrupters in sediment extracts [4,12].

The purpose of the present study was to characterize the quality of sediments from the Paranaguá Estuarine Complex by integrating results from the analysis of priority pollutants and different in vitro bioassays to detect cytotoxic compounds and endocrine disrupters trapped in the sediments. The PLHC-1 cell line was used to evaluate the cytotoxicity, generation of reactive oxygen species (ROS), and CYP1A-inducing agents (activity of ethoxyresorufin-O-deethylase [EROD]) in sediment-trapped pollutants. Ovarian microsomal fractions from female sea bass (*Dicentrarchus labrax*) were used to detect the presence of bioactive chemicals acting as endocrine disrupters by altering the synthesis of estrogens (ovarian CYP19).

MATERIALS AND METHODS

Study area

The Paranaguá Estuarine Complex has an extension of approximately 612 km^2 characterized by the presence of mangroves, islands, and inlets. The largest port for grain export in South America, the Paranaguá Port, is located on the east–west axis, where fertilizers, minerals, and petroleum derivatives are also handled [13]; the navigation channel of the Port (Galheta Channel) and a channel for discharging and small boat traffic (DNOS) are also located there. The urban population of the city of Paranaguá is 150 000 [14]. In contrast, the North–South axis is an Environmental Protected Area with no anthropogenic activities or industrial pressures in the vicinity.

Organic compounds such as PAHs, polychlorinated biphenyls (PCBs), and organochlorinated pesticides (OCPs) have already been reported at this estuary [15–17] and may represent an environmental problem, considering the possible negative effects on aquatic organisms even at low concentrations.

Sediment sampling

Sediments were collected with a Van Veen grab sampler at 8 stations, including areas influenced by harbor activities, navigation, urban effluents, and potential reference sites (Figure 1). The contents of multiple grabs were composited at each station and homogenized. Physical and chemical parameters (coordinates, depth, temperature, salinity, sigma-t, pH, and turbidity) from each site were obtained with a Multi-Parameter Water Quality Meter Probe, U-50 series from Horiba (Table 1). In the laboratory, samples were lyophilized and stored at 20 °C.

Chemical analysis

Total organic carbon. The TOC content was measured in all samples after acid digestion to remove the carbonate fraction with hydrochloric acid 16% (v/v). The organic carbon was converted to CO_2 and quantified (TOC-VCPH; Shimadzu).

Extraction, cleanup, and chromatographic analysis of PAHs. To quantify 16 PAHs listed as priority by the US Environmental Protection Agency, the procedure described by Machado et al. [18] was followed with minor modifications. Sediment (10 g) was extracted using an accelerated solvent extractor (ASE; Dionex) in 5-min static cycles (100 °C; 4 MPa)

Table 1. Geographic coordinates and physicochemical parameters of sampling points (temperature 26.4–27.2 °C and pH 7.6–8)

Sample	Longitude	Latitude	Depth (m)	Salinity (%)	Turbidity (NTU)	Description of the area	Pressure/impact
P1	733642	7182601	4.75	22.9	25.1	Antonina Bay	Agricultural activities upstream/harbor activities
P4	748298	7177168	5.6	27.3	8.6	Paranaguá Harbor	Industrial/harbor activities
P5	751753	7175715	3.3	25.5	4.8	Paranaguá City/Itibere River	Urban/industrial activities/river discharge
P9	754571	7176497	5.7	29.2	5.7	Cotinga Island	Runoff from urban effluents
P10	757924	7178675	7.95	27.6	6.6	Cobras Island/Galheta Channel	Intense ship navigation
P12	765632	7169797	9.2	29.5	10.8	Pontal do Paraná/DNOS channel	Intense traffic of tourist boats
P16	759967	7189170	3.85	27.9	22.0	Rasa Island/Laranjeiras Bay	Agricultural activities upstream/protected area
P17	766806	7188513	4.45	28.0	170.8	Pecas Island/Laranjeiras Bay	Low traffic of tourist boats/preserved area

with a mixture of dichloromethane/acetone (1:1, v/v). Sulfur was removed from the extracts using activated copper. The PAHs were isolated from other compounds using columns (7-mm inner diameter) filled with silica gel (~1.3 g) and sodium sulfate (~0.2 g) and eluted with toluene:hexane (1:3, v/v) (3 mL × 4 mL). This fraction was dried under gentle nitrogen flow and redissolved with hexane. A solution with the 16 priority PAHs (PAH Mix Z-014G; AccuStandard) was used as a standard solution to obtain a calibration curve for quantification. Deuterated PAHs (fluorine-d10 and acenaftilene-d8) were added as internal standards to monitor the efficiency of the analytical procedure. The recovery rates were higher than 75% for most of the PAHs. Quantification was conducted by gas chromatography–mass spectrometry (GC-MS) coupled to a Varian 430 Quadrupole mass spectrometer with electron-impact ionization. The GC was equipped with a factor 4 VF 5MS fused silica capillary column (60 m, 0.25-mm inner diameter, 0.25-μm film thickness) and helium as a carrier gas with a constant flow of 1.2 mL min^{-1}. Both full scan and selected ion monitoring modes were applied.

Extraction, cleanup, and chromatographic analysis of PCBs and OCPs. The analytical procedure for PCBs and OCPs was based on the methodology described in Combi et al. [16] with minor modifications. The sediment (10 g) was extracted using ASE (Dionex) in 7-min static cycles (130 °C; 1500 psi) with a mixture of n-hexane/acetone (1:1, v/v). Activated copper was added to remove sulfur. The extracts were concentrated and then purified using a flash column (7-mm inner diameter) filled with alumina (~1.5 g). Elution was performed with n-hexane/dichloromethane (70:30) and the eluate was concentrated under nitrogen. Tetrachloro-m-xylene (M-8082-SS-10X; AccuStandard) was added as internal standard. The determination of PCBs (sum of 51 congeners) and OCPs (sum of 24 compounds including DDT, hexachlorocyclohexane isomers [HCHs], and chlordane-related compounds [CHLs]) was performed by using an Agilent 6890 GC with electron capture detection. The GC column was an HP-5 fused silica column (length, 30-m; inner diameter, 250 μm; film thickness, 0.25 μm). The quantification of the analytes was based on a mixture of surrogate standards of PCB 103 (C-103N) and PCB 198 (C-198N) (both from AccuStandard), which was added before sample extraction.

Toxicological analysis

For toxicological tests, 3 g of sediment (125 μm size) were extracted with a mixture of dichloromethane/hexane (1:1, v/v) and dichloromethane/acetone (1:1, v/v), as described in Fernandes et al. [4]. The extracts were combined, evaporated, and reconstituted in dimethyl sulfoxide (DMSO) and serially diluted to the desired concentrations. The DMSO never exceeded 0.5% in the exposure medium.

PLHC-1 bioassays. The PLHC-1 cells were obtained from ATCC (CRL-2406) and routinely cultured in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin G, and 50 μg/mL streptomycin, and incubated at 30 °C, 5% CO_2. When 90% of confluence was reached, cells were dissociated with trypsin–ethylenediamine tetraacetic acid and resuspended in culture medium for further bioassays.

Cell viability was evaluated with Alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) [19,20]. Cells, seeded at a density of 75 000 cells at 200 μL per well in a 96-well plate, were incubated for 24 h and then exposed to sediment extracts at concentrations ranging from 2 mg dry weight sediment equivalent extract (eQsed)/mL to 60 mg eQsed/mL for 24 h. The medium was removed, and cells were washed with 200 μL phosphate-buffered saline (PBS; pH 7.4) before addition of a solution containing Alamar Blue and CFDA. Fluorescence was measured after 1 h of incubation at the excitation/emission (ex/em) wavelengths pairs of 530/590 nm for Alamar Blue, and 485/530 nm for CFDA-AM in a fluorescent plate reader (Varioskan, Thermo Electron). Results are expressed as percentage of relative fluorescent units (RFUs) of control cells.

Generation of ROS was measured in PLHC-1 cells exposed to sediment extracts as described in LeBel et al. [21], with slight modifications. Cells were seeded at a density of 10^5 cells at 200 μL per well; after 24 h of incubation, the culture medium was removed, cells were washed with PBS, and a solution of 20 μM 2′,7′-dichlorofluorescein (H_2DCF-DA) was added. After 30 min of incubation, cells were washed with PBS and exposed to sediment extracts to stimulate ROS generation. The fluorescence emitted by the oxidized H_2DCF-DA was measured at the ex/em wavelength pairs of 485/528 nm in a fluorescent plate reader (Varioskan; Thermo Electron) after 15 min, 30 min, and 60 min. As positive control, 5 μM of SIN-1 was used. Results are expressed as fold induction of the basal fluorescence in control cells (% DMSO). Treatments were carried out in triplicate.

Activity of EROD was measured according to a procedure previously described [4]. Cells were seeded in a 48-well plate at a density of 6.5×10^5 cells at 500 μL per well and incubated for 24 h. Then the culture medium was replaced by a culture medium containing the sediment extracts, 0.5% DMSO (solvent control), or 1 μM of β-naphtholflavone (BNF; positive control), and incubated for 24 h. After the exposure period, the medium was aspirated and the cells were rinsed with 500 μL 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 in the microplate reader (Varioskan; Thermo Electron), the fluorescence was directly read at the ex/em wavelength pairs of 537/583 nm. Quantification was made by calibration with 7-hydroxyresorufin. Fluorescamine was used to measure the protein content of cells, using bovine serum albumin (BSA) as standard [22]. Results are expressed as mean of pmol of resorufin generated per minute per milligram of protein (pmol/min/mg prot) for at least 3 independent replicates.

Ovarian P450 aromatase activity (CYP19): Microsomal fraction and in vitro incubation. Sea bass (*Dicentrarchus labrax*) were supplied by an aquaculture facility located in the Northeast of Spain. Immediately after collection, animals were euthanized and gonads were dissected. A subsample of the central part of the gonad was fixed in 10% buffered formalin for histological examination to determine the sexual maturation stage (SMS). The rest of the gonad was deep-frozen in liquid nitrogen and stored at 80 °C until preparation of subcellular fractions as described by Fernandes et al. [4].

The interaction with sea bass steroidogenic enzymes was investigated in vitro by preincubating the microsomes in the presence of the sediment extracts (at concentrations of 60 mg eQsed/mL, 20 mg eQsed/mL, 10 mg eQsed/mL, and 2 mg eQsed/mL) for 10 min at 25 °C, prior to the addition of the reaction substrate and cofactor. The solvent was removed from the assay by evaporation under a gentle flow of nitrogen before starting the preincubation. Control reactions consisted of incubation of the corresponding subcellular fraction in the absence of sediments. Blanks that consisted of reactions stopped at time 0 were also analyzed. Assays were carried out using individual gonads from 4 to 5 different individuals.

Aromatase activity was determined by the tritiated-water release method as previously described in Lavado et al. [23]. For the assay, 0.1 mg of ovarian microsomal proteins were incubated for 1 h at 25 °C in the presence of 100 mM Tris–HCl, pH 7.6, 0.2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), and [1β-3H] androstenedione (40 pmol, 1 μCi). The reaction was stopped using 3 mL of cold methylene chloride, and the excess substrate was further eliminated from the aqueous phase by extraction with methylene chloride (×3). The remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in Milli-Q water. The solution was centrifuged for 60 min at 3600 rpm, and the amount of titrated water formed was counted in 2 aliquots of the aqueous phase (Tri-Carb 2100TR, Packard).

Statistical analysis

Statistical differences were detected by analysis of variance (ANOVA) with 1 factor with Dunnett's test, using the software SPSS 19.0. Dose–response curves and concentrations which caused 50% of effect and inhibition (EC50 and IC50) were calculated by using Sigmaplot 13.0. A p value of <0.05 was considered statistically significant. To summarize the obtained information and to estimate the degree of anthropogenic impact in the different stations, a principal component analysis was carried out with PLS Toolbox 8.0 and MatLab software.

RESULTS

Chemical concentrations and composition of sediments

Sediments from inner parts of estuary P1 (east–west axis) and P17 and P16 (north–south axis) had the highest TOC contents (85 mg C/g sediment, 70 mg C/g sediment, and 52.3 mg C/g sediment, respectively; Table 2). In terms of sediment grain size distribution, P1 was mostly muddy, with high silt (<62 μm) and fine sand (250–62 μm) contents, 46% and 56%, respectively. In general, fine and medium sand (63–500 μm) and silt (4–63 μm) fractions dominated most of the sample compositions, whereas clay particles (<4 μm) were absent.

Total concentration of PAHs in sediments ranged from 36.9 ng/g to 89.1 ng/g dry weight. The site near Paranaguá city (P5) had the highest concentration, and sites P16 (36.9 ng/g) and P17 (37.3 ng/g), both located in the north–south axis of the estuary, had the lowest.

The values of PCBs ranged from 0.1 ng/g to 7.6 ng/g dry weight and those of HCHs from 0.2 ng/g to 13.0 ng/g dry weight. Site P5 had the highest concentration of both groups of compounds (Table 2), whereas sediments from internal parts of the estuary had the highest concentrations of DDT (P1 = 1.89 ng/g and P17 = 1.15 ng/g).

Cytotoxicity

Sediments collected from P1, P4, and P10 had the highest cytotoxicity (EC50 between 53 mg eQsed/mL and 88 mg eQsed/mL), whereas no significant cytotoxicity was recorded in sediments collected from other areas. The highest concentration tested was 60 mg eQsed/mL (Table 3).

Table 2. Concentrations of organic compounds in sediments (ng/g dry sediment) and total organic carbon (mg C/g sed)

Sample	∑PAHs[a]	∑PCBs[b]	∑7 PCBs[c]	∑HCHs[d]	∑DDTs[e]	∑CHLs[f]	∑OCPs[g]	TOC
P1	73.98	0.34	0.31	0.64	1.89	0.16	2.81	84.95
P4	69.71	2.31	1.47	0.76	1.29	0.35	2.66	52.28
P5	89.14	7.59	5.92	12.99	nd	0.74	13.73	23.66
P7	83.11	0.13	0.12	0.45	0.62	0.30	1.38	25.62
P10	73.88	1.26	0.08	0.42	0.92	0.02	2.27	47.69
P12	50.47	2.95	nd	0.24	0.57	0.06	1.06	16.15
P16	36.92	0.87	nd	0.34	0.71	0.22	1.93	46.49
P17	37.27	0.37	nd	0.40	1.15	0.36	2.07	70.03

[a]Sum of 16 priority polycyclic aromatic hydrocarbons (PAHs).
[b]Sum of 51 congeners of polychlorinated biphenyls (PCBs).
[c]Sum of PCBs 28, 52, 101, 118, 138, 153, and 180.
[d]Sum of α-, β-, δ-, and γ-hexachlorocyclohexane isomers (HCHs).
[e]Sum of dichlorodiphenyldichloroethylenes, dichlorodiphenyldichlorethanes, and dichlorodiphenyltrichloroethanes.
[f]Sum of chlordane-related compounds (CHLs).
[g]Sum of organochlorinated pesticides (OCPs).
TOC = total organic carbon; nd = not detected.

Table 3. Contaminants measured in the sediments[a] against classical sediment quality guidelines[b]

	Average	Maximum	ERL	ERM	TEL	PEL
∑7 PCBs	0.99 ± 1.4	1.47	22.7	180	21.6	189
∑ DDT	0.89 ± 0.4	1.89	1.58	46.1	3.89	51.7
∑ CHLs	0.28 ± 0.2	0.74	0.50	6.0	2.26	4.79
Dieldrin	nd	nd	0.02	8.0	0.72	4.30
γ-HCH	0.11 ± 0.1	0.23	nd	nd	0.32	0.99
Acenafthene	0.36 ± 0.1	0.63	16	500	6.71	88.9
Acenaphthylene	0.21 ± 0.1	0.68	44	640	5.87	128
Anthracene	0.37 ± 0.2	1.08	85.3	1100	46.9	245
Fluorene	0.15 ± 0.0	0.29	19	540	21.2	144
Naphthalene	2.60 ± 2.2	3.80	160	2100	34.5	391
Phenathrene	0.82 ± 0.3	1.46	240	1500	86.7	544
Benzo[*a*]anthracene	0.48 ± 0.3	1.22	261	1600	74.8	693
Benzo[*a*]pyrene	2.30 ± 2.5	12.42	430	1600	88.8	763
Chrysene	1.60 ± 1.4	6.56	384	2800	108	846
Dibenzo[*a, h*]anthracene	0.11 ± 0.2	0.76	63.4	260	6.22	135
Fluoranthene	0.51 ± 0.2	0.96	600	5100	113	1494
Pyrene	0.50 ± 0.4	1.22	665	2600	153	1398

[a]Averaged ± relative standard deviation and maximum values in ng/g dry sediment.
[b]Values described by Macdonald et al. [24] and National Oceanic and Atmospheric Administration [25] in ng/g dry sediment.
ERL= effects range-low; ERM = effects range-median; TEL = threshold-effect level; PEL = probable effects level; PCBs = polychlorinated biphenyls; CHLs = chlordane-related compounds; HCH = hexachlorocyclohexane isomer; nd = not detected.

Generation of ROS

Production of ROS was recorded over 60-min exposure to sediment extracts. Sediments from all stations were able to generate ROS in PLHC-1 cells in the range of 1.5-fold to 2-fold induction (Figure 2). Cells exposed to the positive control (SIN-1) induced ROS up to 1.9-fold, 2.4-fold, and 4.2-fold, after 15 min, 30 min, and 60 min, respectively (data not shown).

EROD activity

Sediments from P4 had the highest ability to induce EROD activity (IC50, 12 mg eQsed/mL), followed by those from P10 and P1 (IC50 equal to 55 mg eQsed/mL and 61 mg eQsed/mL, respectively; Table 2 and Figure 3). In contrast, low EROD activity was recorded in cells exposed to sediments from P7, P12, P16, and P17 (<11 pmol/min/mg prot), which indicates the presence of very low concentrations of CYP1A inducers.

Ovarian CYP19 activity

All organic extracts strongly inhibited P450 aromatase enzyme activity (by 68–97%) when tested at a concentration of 60 mg eQsed/mL (Figure 4). The sites P17, P1, P16, P12, P7, and P4 had to the highest inhibition of enzyme activity (IC50s ranged from 4.7 mg eQsed/mL to 7 mg eQsed/mL), whereas P10 and P5 had IC50s of 16 mg eQsed/mL and 24 mg eQsed/mL, respectively.

Factorial analysis

The principal component analysis rearranged the set of data into 2 factors, which together explained 79% of the total variance. The predominant factor accounted for 50% of the variance and grouped the sediments from P1 and P4 on the positive end, whereas sediments from P5 were located on the extreme negative side of the axis. The second factor explained 29% of the variance and grouped the sediments from P7, P10, P12, P16, and P17 on the negative side, whereas it isolated P5 on the positive end (Figure 5). Overall, among the sediments tested, those collected from P1 and P4 (Antonina and Paranaguá harbors) showed the highest anthropogenic impact. An association among CYP1A, CYP19, and CFDA assays, as well as the presence of PAHs and CHLs in samples, was detected (Figure 5).

DISCUSSION

The chemical concentration data were compared with values indicated by sediment quality guidelines, which define critical

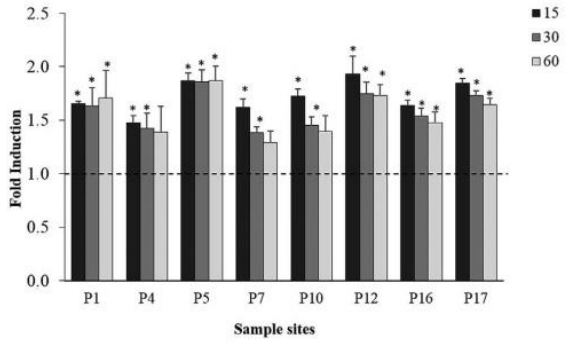


Figure 2. Generation of ROS after 15 min, 30 min, and 60 min of exposure to extracts of sediments at a concentration of 60 mg dry weight sediment equivalent extract/mL. Results are expressed as fold induction with respect to control cells (mean ± standard error of the mean, $n = 3$ assays). An asterisk (*) indicates significant difference from control cells.

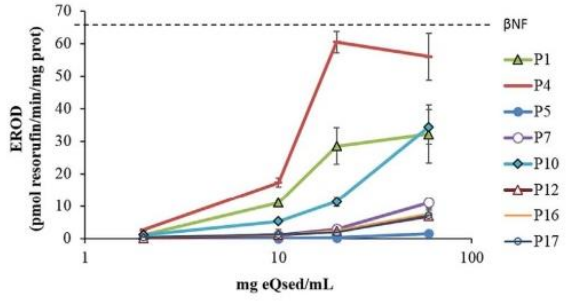


Figure 3. Ethoxyresorufin-O-deethylase (EROD) activity in PLHC-1 cells after 24 h of exposure to sediment extracts. Results are expressed in pmol of resorufin/min/mg protein as mean ± standard error of the mean of at least 3 assays. Dotted line represents positive control (β-naphtholflavone [BNF]).

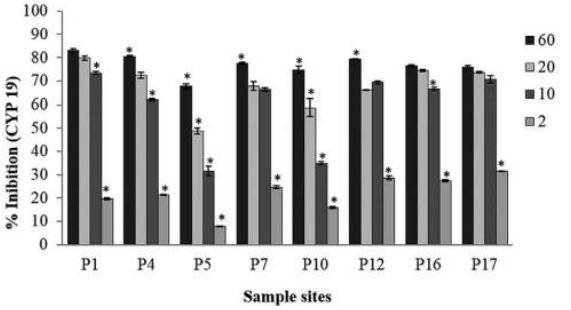


Figure 4. In vitro interference of sediment extracts from the Paranaguá Estuary on microsomal ovarian P450 aromatase activity (2 dry weight sediment equivalent extrac [eQsed]/mL, 10 mg eQsed/mL, 20 mg eQsed/mL, and 60 mg eQsed/mL) in sea bass tests. Values are mean ± SEM ($n = 3$). An asterisk (*) indicates significant difference relative to control ($p < 0.05$).

levels of organic compounds and classify them according to expected effects on organisms such as effects range-low, effects range-median, threshold-effects level, and probable-effects level [24,25]. All values were below the limits, and therefore, there was no indication of any effect for biota according to the limits defined by the sediment quality guidelines for PAHs, PCBs (sum of 7 congeners: 28, 52, 101, 118, 138, 153, and 180), DDTs, CHLs, and dieldrin (Table 3). In terms of organic carbon concentration, sediments from P1 were the only ones close to the alert value of 10% (TOC = 8.5%).

Some compounds were positively correlated, indicating the same origin or source in the environment. The HCHs were positively correlated with PCBs (0.91, $p < 0.05$) and CHLs (0.84, $p < 0.05$). Site P5, located near the Itibere River, had the highest levels of these compounds, demonstrating its negative influence on sediment quality. Nevertheless, DDTs did not show any correlation with the other pollutants. They were mainly detected at the inner parts of the estuary (Antonina and Laranjeras Bay), where it might receive the influence of upstream agricultural activities.

The bioassays selected allowed assessment of sediment quality from another perspective: whether the mixture of organic contaminants trapped in the toxic sediments contained significant amounts of cytotoxic and/or endocrine disrupter compounds. The highest cytotoxicity was associated with areas influenced by harbor activities, namely, sediments next to Antonina and Paranaguá harbors (P1 and P4, respectively), followed by sediment extracts from P10, an area that is affected by commercial shipping. Interestingly, sediments from P10 showed higher cytotoxicity with Alamar Blue than with CFDA-AM, suggesting a higher ability of the toxicants to impair mitochondrial activity than membrane stability of PLHC-1 cells (Table 4).

Extracts from P1, P4, and P10 also had the highest content of CYP1A inducers, with EC50s in the range of 12 mg to 61 mg eQsed/mL. In contrast, P5, P7, P12, P16, and P17 contained a rather small amount of CYP1A inducers. Chemicals such as PAHs and PCBs can induce EROD activity in fish [26]. However, in the natural environment, fish are exposed to complex mixtures of pollutants that can act as both inducers and inhibitors [23,27]. Estrogens such as estradiol and octylphenol, for example, have a modulating effect on the expression of CYP1A in fish, inhibiting EROD activity [28].

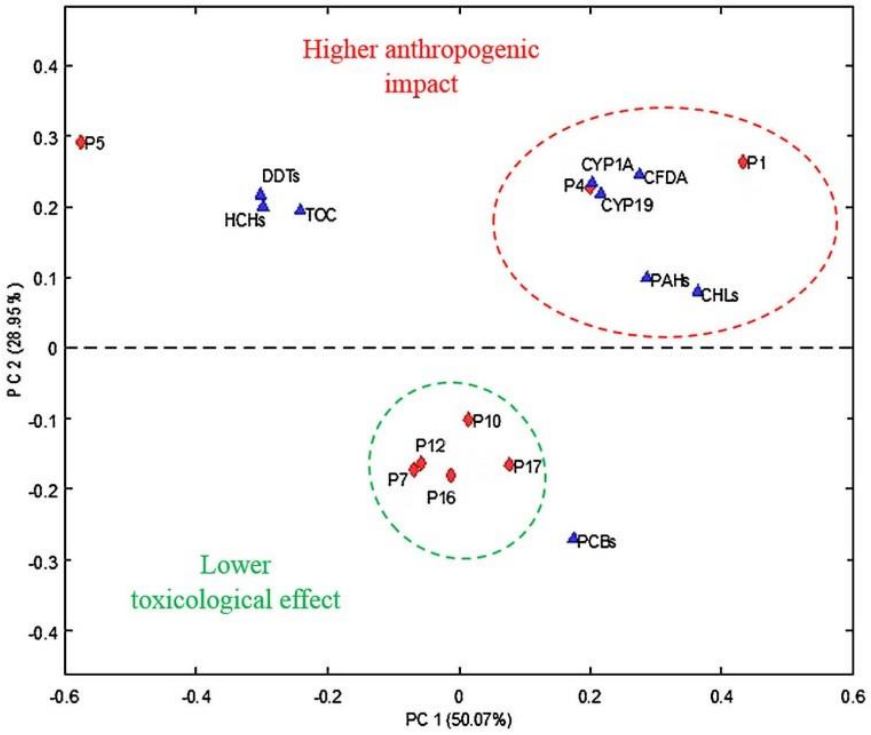


Figure 5. Factor analysis with principal component extraction: pricnipal component 1 (PC1) versus principal component 2 (PC2) biplot. Blue triangles represent loadings for biological and chemimcal variables; red diamonds represent site scores: Paranaguá Estuary (P1, P4, P5, P7, P10, P12, P16, and P17). HCHs = hexachlorocyclohexane isomers; TOC = total organic carbon; PAHs = polycyclic aromatic hydrocarbons; CHLs = chlordane-related compounds; PCBs = polychlorinated biphenyls.

Table 4. Summarized toxicological data for sediments collected from Paranaguá Estuarine Complex[a]

Site	AB[b]	CFDA[b]	EROD[c]	CYP 19[d]	ROS[e]
P1	53.0 ± 5.4	74.5 ± 4.4	61.1 ± 1.4	5.5 ± 1.5	1.66 ± 0.0
P4	64.6 ± 3.8	87.0 ± 2.0	12.0 ± 1.3	7.10 ± 1.3	1.47 ± 0.1
P5	nd	nd	nd	23.66 ± 1.8	1.87 ± 0.1
P7	nd	nd	nd	6.58 ± 1.9	1.62 ± 0.1
P10	87.5 ± 6.2	nd	54.6 ± 1.4	16.30 ± 2.2	1.93 ± 0.2
P12	nd	nd	nd	5.65 ± 1.9	1.73 ± 0.1
P16	nd	nd	nd	5.62 ± 1.7	1.64 ± 0.1
P17	nd	nd	nd	4.69 ± 1.8	1.85 ± 0.0

[a]Values are mean ± standard error of the mean of at least 3 independent experiments.
[b]Median effective concentration (EC50) expressed in mg eQsed/mL in the cytotoxicity assay (AB, CFDA).
[c]Concentration of sediment required to induce a response equivalent to 1 μM β-naphthoflavone.
[d]Median inhibitory concentration (IC50) for ovarian CYP19 assay.
[e]Reactive oxygen species (ROS) generation after 15-min exposure to 60 mg eQsed/mL.
AB = Alamar Blue; CFDA = 5-carboxyfluorescein diacetate acetoxymethyl ester; nd = not detected.

The production of ROS may be increased in the presence of natural or synthetic compounds derived from urban, industrial, or agricultural activities [29]. The potential anthropogenic compounds capable of promoting ROS generation are those that undergo redox cycles (quinones, aromatic compounds, nitroamines, bipyridyls herbicides), PAHs (benzene, PAH oxidation products), halogenated hydrocarbons (bromobenzene, dibromomethane, PCBs, lindane), dioxin, pentachlorophenol, and metal pollutants [30]. In general, ROS generation in exposed cells was similar in all other sediments (<2-fold induction), suggesting similar exposure to ROS-generating compounds. Nonetheless, sites P1, P5, and P12 showed slightly higher values. These sites are located near Antonina, Paranaguá, and Pontal do Paraná cities, respectively. Sediments from P5 were collected near the mouth of the Itibere River, which flows through Paranaguá City. In turn, P12 sediments were collected next to the discharge of an artificial drainage channel (DNOS), which received sewage entering from Pontal do Sul City. The external sector of the channel is dominated by marinas and boat traffic. It has been determined that both places suffer from the impact of untreated urban sewage [31,32].

Various anthropogenic pollutants act as endocrine disruptors (e.g., PCBs, pesticides, pharmaceuticals, personal care products) by altering the expression and biosynthesis of steroids. Bioassays using microsomal fractions of ovarian sea bass have been previously used to detect the presence of endocrine disrupters in marine sediments [4,12]. This test detects agents that modulate aromatase activity (CYP19), which is involved in processes of sexual differentiation, embryo growth, and maturation. Inhibition of CYP19 activity detected in sediments from this estuary indicates the presence of potential endocrine-disrupting chemicals (68–83% at 60 mg eQsed/mL). A similar range was reported for sediments from the Arade (65–92% at 60 mg eQsed/mL) and Guardiana (22–91% at 60 mg eQsed/ mL) estuaries [4]. Pérez-Albadalejo et al. [12] also reported inhibition of CYP19 activity by sediment extracts from the Adriatic and Black Sea, with inhibition slightly greater in most places (up to 54% by 2 mg eQsed/ mL).

Sediments from internal areas of the Paranaguá Estuary sites P1 and P17 inhibited ovarian CYP19 activity more efficiently (up to 31% inhibition by 2 mg eQsed/ mL), suggesting the presence of potential endocrine disrupters at these sites. In regard to the compounds analyzed, P1 and P17 showed the highest concentrations of DDTs (o,p′-DDT and p,p′-DDE) among all sediment samples. Site P1 also had the highest concentration of the PAH benzo[*a*]pyrene BaP (12.4 ng/g). The compounds DDT and BaP are estrogenic chemicals, which may interfere with the endocrine system of fish. The presence of DDT has led to reduced fecundity or larval survival. It induces vitellogenesis in male fish and adversely affects the endocrine system [10]. Also, fish exposed to BaP showed decreased androstenedione and estradiol synthesis through inhibition of cytochrome P450 17,20-lyase, 17b-hydroxysteroid dehydrogenase, and cytochrome P450 aromatase (CYP19) in different in vitro ovarian steroidogenic enzyme systems [33].

Sites P5 and P10 had the lowest ability to inhibit the activity of CYP19, even though P5 showed high concentrations of β-HCHs (12.99 ng/g), which are recognized endocrine disruptors [10]. Site P5 is greatly influenced by urban discharges. It showed the highest concentration of PCBs, although concentrations of the toxic planar congeners 77, 126, and 169 were low.

In sum, the toxicological results showed that P1 and P4 seem to be the most degraded areas at the estuary. The least contaminated area was P7. Sediments from P16 and P17, situated north of Laranjeiras Bay, were slightly contaminated according to the results from cytotoxicity, ROS generation, and EROD induction tests. In terms of CYP19 inhibition, both sites, especially P17, were highly affected by endocrine disruptor substances.

A few studies have used toxicological assays in the Paranaguá Estuary, but none included evaluation of endocrine disrupters. Morphological (liver and gill histopathology) and genetic (piscine micronucleus and DNA strand breaks) biomarkers measured in the demersal catfish *Cathorops spixii* revealed the presence of damage in fish from both reference and contaminated sites inside Paranaguá Bay [34]. Fish (*Atherinella brasiliensis*) from areas influenced by port and urban activities of the Bay showed levels of PAHs in bile, somatic indexes, activity of catalase, and genetic parameters characteristics of areas impacted by hazardous pollutants, although on a minor scale [35].

With regard to sediment toxicity, another study also found a gradient of increased toxicity toward the inner region of this estuary [13]. The area next to Antonina (equivalent to P1) was classified as highly toxic, mainly through a high concentration of metals. Also, the inner part of the north–south axis (where low toxicity was expected because of its location away from the shipping channel and ports) was only moderately impacted toxicologically, similar to what was observed in the present study (P16, P17). At sites where circulation is limited, such as internal areas, the higher residence time may promote accumulation of toxic compounds in sediments, as was reported for tributyltin (TBT) [36].

Principal component analysis was used to group sediments according to the response in the different bioassays and the levels of pollutants. The ROS assay was not included in the principal component analysis because the response was very similar at all sites (<2-fold induction). The first component further explains the variables isolating P5 on the negative side and grouping P1 and P4 on the positive side (more impacted). The analysis groups P7, P12, P16, P10, and P17 are in a central zone, suggesting that these sites are not as impacted as P1 and P4, even though they are not clean.

In addition, according to the principal component analysis, the effects shown in CYP1A, CYP19, and CFDA assays were associated with the presence of PAHs and CHLs in sediments.

This is in agreement with the fact that PAHs, mainly those of higher molecular weight, can activate CYP1A (EROD activity) by binding with the aryl hydrocarbon receptor (AhR) [26,37,38]. Moreover, other chemicals could be also responsible for EROD induction (i.e., planar chemicals such as halogenated dioxins and biphenyls) [38]. Inhibition of P450 aromatase activity by HCHs, fungicides, and PAHs has been reported in rainbow trout ovarian microsomes, human placental JEG-3 cells, and transfected human embryonic kidney 293 cells [10,39,40]. Overall, among all the sediments tested, those collected near harbors (P1 and P4) showed the highest anthropogenic impact. Sites P7, P10, P12, P16, and P17 showed less anthropogenic impact, in agreement with the fact that those sediments had fewer chemical pollutants, causing a minor response in the biological systems assayed.

CONCLUSIONS

Although chemical analysis of the sediments indicated low levels of organic compounds, and there was no evidence of negative effects based on guideline levels, the information provided by in vitro bioassays allowed the detection of significant amounts of cytotoxic compounds and endocrine disrupters in sediments from the estuary. The integrated use of both tools can be extremely useful for recognizing areas with different degrees of impact and sediment quality.

Acknowledgment—J. Rizzi thanks the Coordination of Improvement of Higher Education Personnel of Brazil (CAPES; PDSE Program: Process 3887-13-8) for the doctoral fellowship. We thank C. Martins from the Center of Marine Science of the Federal University of Paraná (CEM/UFPR) for assistance with organic carbon analyses and M. Lamour from CEM/UFPR for assistance with the grain size analyses.

Data availability—Data used for the present study are available on request to the corresponding author (juliane.rizzi@gmail.com).

REFERENCES

1. Kennish MJ. 2002. Environmental threats and environmental future of estuaries. *Environ Conserv* 29:78–107.
2. Eggleton J, Thomas KV. 2004. A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. *Environ Int* 30:973–80.
3. Ehlers LJ, Luthy RG. 2003. Contaminant bioavailability in soil and sediment. *Environ Sci Technol* 37:295–302.
4. Fernandes D, Pujol S, Pérez-Albaladejo E, Tauler R, Bebianno MJ, Porte C. 2014. Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays. *Mar Environ Res* 96:127–135.
5. Diamond ML, Wit CA, Molander S, Scheringer M, Backhaus T, Lohmann R, Arvidsson R, Bergman A, Hauschild M, Holoubek I, Persson L, Suzuki N, Vighi M, Zetzsch C. 2015. Exploring the planetary boundary for chemical pollution. *Environ Int* 78:8–15.
6. Porte C, Janer G, Lorusso LC, Ortiz-Zarragoitia M, Fossi MC, Canesi L. 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comp Biochem Physiol C* 143:303–315.
7. Kiyama R, Wada-Kiyama Y. 2015. Estrogenic endocrine disruptors: Molecular mechanisms of action. *Environ Int* 83:11–40.
8. Louiz I, Kinani S, Gouze ME, Ben-Attia M, Menif D, Bouchonnet S, Porcher JM, Ben-Hassine OK, Aït-Aïssa S. 2008. Monitoring of dioxin-like, estrogenic and anti-androgenic activities in sediments of the Bizerta Lagoon (Tunisia) by means of in vitro cell-based bioassays: Contribution of low concentrations of polynuclear aromatic hydrocarbons (PAHs). *Sci Total Environ* 402:318–329.
9. Andersen ME; Krewski, D. 2009. Forum series, Part I—Toxicity testing in the 21st century: Bringing the vision to life. *Toxicol Sci* 107:324–330.
10. Scholz S, Mayer I. 2008. Molecular biomarkers of endocrine disruption in small model fish. *Mol Cell Endocrinol* 293:57–70.
11. Schnell S, Olivares A, Piña B, Echavarri-Erasun B, Lacorte S, Porte C. 2013. The combined use of the PLHC-1 cell line and the recombinant yeast assay to assess the environmental quality of estuarine and coastal sediments. *Mar Pollut Bull* 77:282–289.
12. Pérez-Albadalejo E, Rizzi J, Fernandes D, Lille-Langøy R, Karlsen OA, Goksøyr A, Oros A, Spagnoli F, Porte C. 2016. Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays. *Mar Pollut Bull* 108:53–61.
13. Choueri RB, Cesar A, Torres RJ, Abessa DMS, Morais RD, Pereira CDS, Nascimento MRL, Mozeto AA, Riba I, DelValls TA. 2009. Integrated sediment quality assessment in Paranaguá Estuarine System, Southern Brazil. *Ecotox Environ Safe* 72:1824–1831.
14. Brazilian Institute of Geography and Statistics. 2016. Systematic survey of agricultural production. [cited 2016 June 29]. Available from: http://cod.ibge.gov.br/5HT
15. Froehner S, Maceno M, Machado KS, Da Luz EC, Botelho D. 2010. Distribution of polycyclic aromatic hydrocarbons in marine sediments and their potential toxic effects. *Environ Monit Assess* 168:205–213.
16. Combi T, Taniguchi S, Ferreira PA, Mansur AV, Figueira RCL, Mahiques MM, Montone RC, Martins CC. 2013. Sources and temporal patterns of polychlorinated biphenyls around a large South American grain shipping port (Paranaguá Estuarine System, Brazil). *Arch Environ Contam Toxicol* 64:573–582.
17. Abreu-Mota MA, Moura Barboza CA, Bícego MC, Martins CC. 2014. Sedimentary biomarkers along a contamination gradient in a human-impacted sub-estuary in Southern Brazil: A multi-parameter approach based on spatial and seasonal variability. *Chemosphere* 103:156–163.
18. Machado KS, Figueira RCL, Cõcco LC, Froehner S, Fernandes CVS, Ferreira PAL. 2014. Sedimentary record of PAHs in the Barigui River and its relation to the socioeconomic development of Curitiba, Brazil. *Sci Total Environ* 482-483:42–52.
19. Schirmer K, Chan AGJ, Greenberg BM, Dixon DG, Bols NC. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol In Vitro* 11:107–119.
20. Dayeh V, Bols N, Schirmer K, Lee L. 2003. The use of fish-derived cell lines for investigation of environmental contaminants. *Curr Protoc Toxicol* 1:1.5.
21. LeBel CP, Ischiropoulos H, Bondy SC. 1992. Evaluation of the probe 2070- dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* 5:227–231.
22. Lorenzen A, Kennedy SW. 1993. A fluorescence-based protein assay for use with a microplate reader. *Anal Biochem* 214:346–348.
23. Lavado R, Thibaut R, Raldúa D, Martín R, Porte C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol Appl Pharm* 196:247–57.
24. Macdonald DD, Carr RS, Calder FD, Long ER, Ingersoll CG. 1996. Development and evaluation of sediment quality guidelines. *Ecotoxicology* 5:253–278.
25. National Oceanic and Atmospheric Administration. 2008. Screening quick reference tables—SQuiRT. [cited 2016 June 29]. Available from: http://www.gesamp.org/data/gesamp/files/file_element/4a2a322c8acb2c26cc0234685eac71fa/SQuiRTs.pdf
26. Fent K, Bätscher R. 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: Demonstration of additive interactions. *Environ Toxicol Chem* 19:2047–2058.
27. Fernandes D, Andreu-Sanchez O, Bebianno MJ, Porte C. 2008. Assessment of pollution along the Northern Iberian shelf by the combined use of chemical and biochemical markers in two representative fish species. *Environ Pollut* 155:327–335.
28. Navas JM, Segner H. 2000. Modulation of trout 7-ethoxyresorufin-O-dee-thylase (EROD) activity by estradiol and octylphenol. *Mar Environ Res* 50:157–162.
29. Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101:13–30.
30. Livingstone DR. 2001. Contaminant-stimulated reactive oxygen species and oxidative damage in aquatic organisms. *Mar Pollut Bull* 42:656–666.
31. Martins CC, Braun JAF, Seyffert BH, Machado EC, Fillmann G. 2010. Anthropogenic organic matter inputs indicated by sedimentary fecal steroids in a large South American tropical estuary (Paranaguá estuarine system, Brazil). *Mar Pollut Bull* 60:2137–2143.
32. Kolm HE, Andretta L. 2003. Bacterioplancton in different tides of the Perequê tidal creek, Pontal do Sul, Paraná, Brasil. *Braz J Microbiol* 34:97–103.
33. Kennedy CJ, Smyth KR. 2015. Disruption of the rainbow trout reproductive endocrine axis by the polycyclic aromatic hydrocarbon benzo[a]pyrene. *Gen Comp Endocrinol* 219:102–111.
34. Katsumiti A, Valdez Domingos FX, Azevedo M, da Silva MD, Damian RC, Almeida MIM, Silva de Assis HC, Cestari MM, Randi MAF, Oliveira Ribeiro CA, Freire CA. 2009. An assessment of acute biomarker responses in the demersal catfish *Cathorops spixii* after the

Vicuña Oil Spill in a harbour estuarine area in Southern Brazil. *Environ Monit Assess* 152:209–222.
35. Ribeiro CAO, Katsumiti A, França P, Maschio J, Zandoná E, Cestari MM, Vicari T, Roche H, Assis HCS de, Filipak Neto F. 2013. Biomarkers responses in fish (*Atherinella brasiliensis*) of Paranaguá Bay, Southern Brazil, for assessment of pollutant effects. *Braz J Oceanogr* 61:1–11.
36. Santos DM, Araújo IP, Machado EC, Carvalho-Filho MAS, Fernandez MA, Marchi MRR, Godoi AFL. 2009. Organotin compounds in the Paranaguá Estuarine Complex, Paraná, Brazil: Evaluation of biological effects, surface sediment, and suspended particulate matter. *Mar Pollut Bull* 58:1926–1931.
37. Bosveld ATC, Bie AAF de, Brink NW van den, Jongepier H, Klomp AV. 2002. In vitro EROD induction equivalency factors for the 10 PAHs generally monitored in risk assessment studies in the Netherlands. *Chemosphere* 49:75–83.
38. Damásio JB, Barata C, Munné A, Ginebreda A, Guasch H, Sabater S, Caixach J, Porte C. 2007. Comparing the response of biochemical indicators (biomarkers) and biological indices to diagnose the ecological impact of an oil spillage in a Mediterranean river (NE Catalunya, Spain). *Chemosphere* 66:1206–1216.
39. Hinfray N, Porcher JM, Brion F. 2006. Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. *Comp Biochem Physiol* 144C:252–262.
40. Patel MR, Scheffler BE, Wang L, Willett KL. 2006. Effects of benzo(a) pyrene exposure on killifish (*Fundulus heteroclitus*) aromatase activities and mRNA. *Aquat Toxicol* 77:267–278.

ARTÍCULO 4

Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays

D. Fernandes[1], S. Pujol[2], E. Pérez-Albaladejo[2], R. Tauler[2], M. J. Bebianno[1], C. Porte[2]

[1] FCT, CIMA, University of Algarve, Faro, Portugal
[2] Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

Marine Environmental Research 96 (2014) 127-135

RESUMEN

En este artículo se investiga la calidad ambiental de sedimentos de los estuarios de los ríos Arade y Guadiana (sur de Portugal), mediante diferentes bioensayos *in vitro* enfocados a determinar la citotoxicidad y presencia de agentes capaces de inducir CYP1A y generar estrés oxidativo en la línea celular de peces PLHC-1, así como detectar compuestos capaces de actuar como disruptores endocrinos interfiriendo con la síntesis de andrógenos (CYP17, CYP11β) y estrógenos (CYP19), en la fracción subcelular de gónadas de lubina (*Dicentrarchus labrax*). Aproximadamente el 60 % de los extractos de sedimento de las estaciones del estuario del río Arade eran citotóxicos a 60 mg eQsed/mL, mientras que únicamente una muestra del estuario del Guadiana mostraba citotoxicidad. Los sedimentos del estuario del Arade obtenidos cerca de los puertos y de efluentes de aguas residuales contenían concentraciones elevadas de agentes inductores de CYP1A, mientras que aquellos recolectados en el alto Guadiana inducían estrés oxidativo en las células PLHC-1. Por otro lado, varios extractos de ambos estuarios eran capaces de inhibir significativamente las actividades

de los complejos enzimáticos CYP17, CYP11β y CYP19 en las fracciones subcelulares de lubina, lo que indica la presencia de disruptores endocrinos, particularmente en el estuario del río Arade. En general, los resultados indican una mayor presión antropogénica en el estuario del río Arade, especialmente en las áreas A2, A3, A5, y en la zona G7 del estuario del Guadiana, afectada por minería. Por el contrario, las zonas G1 y G2 conservan una calidad ambiental óptima, con reducidos o nulos niveles de contaminantes orgánicos.

Marine Environmental Research 96 (2014) 127–135

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Characterization of the environmental quality of sediments from two estuarine systems based on different *in-vitro* bioassays

Denise Fernandes [a,*], Silvia Pujol [b], Elisabet Pérez-Albaladejo [b], Romà Tauler [b], Maria João Bebianno [a], Cinta Porte [b,*]

[a] FCT, CIMA, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[b] Environmental Chemistry Department, IDAEA-CSIC, C/Jordi Girona 18, 08034 Barcelona, Spain

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form
27 September 2013
Accepted 27 September 2013

Keywords:
Estuaries
Sediments
PLHC-1
EROD
Reactive oxygen species
Ovarian CYP19
CYP17
CYP11β

ABSTRACT

This study characterizes the environmental quality of sediments from the Arade and Guadiana estuaries using different in-vitro bioassays: a) fish hepatoma cell line (PLHC-1) to determine cytotoxicity and presence of CYP1A and oxidative stress inducing agents; b) gonad subcellular fractions from sea bass (*Dicentrarchus labrax*) to detect compounds that are likely to act as endocrine disrupters by interfering with the synthesis of androgens (CYP17, CYP11β) and estrogens (CYP19). Approximately 60% of extracts from the Arade estuary were cytotoxic when tested at 60 mg eQsed/mL, while only one sample from Guadiana showed cytotoxicity. Sediments from Arade collected close to harbours and waste water effluents were enriched with CYP1A inducing agents, while those from the upper Guadiana induced oxidative stress in PLHC-1 cells. On the other hand, several extracts from both estuaries were able to significantly inhibit CYP17, CYP11β and CYP19 activities in gonad subcellular fractions of sea bass, which indicates the presence of endocrine disrupters, particularly in several sites from the Arade estuary. Overall, the study highlights the usefulness of in-vitro bioassays to identify those sediments that could pose risk to aquatic organisms and that require further action to improve their environmental quality.

1. Introduction

Sediments represent a reservoir for anthropogenic pollutants, which may potentially cause adverse effects in resident benthos and other aquatic organisms. Thereby, approaches to biomonitor sediment quality are essential in order to characterize the health status of aquatic environments and, ultimately minimize the threats and prevent the adverse effects to aquatic wildlife. However, surveying the effects and responses of aquatic organisms to sediment-bound pollutants remains a challenge, due to the complex geochemical nature of sediments and to the presence of multiple classes of pollutants. Besides, the toxicity and risk assessment of contaminated sediments cannot be solely based on chemical analysis as a strategy to characterize sediment quality, since chemical analysis alone does not provide information about potential hazards to the organisms and it is not feasible to identify and measure the concentration of all the pollutants present in sediments. Moreover, the bioavailability of pollutants and the possible additive, synergistic or antagonistic interactions in bottom-dwelling organisms are not taken into account (Chapman, 2007). On the other hand, in-vitro bioassays reflect an estimation of the biological activity of the pollutants present in the environmental sample, integrating the interaction between them and covering endpoints such are acute and long-term toxicity, bioaccumulation and endocrine effects, among others.

Interactions of chemicals with biota take place at the cellular level, making cellular responses not only the first manifestation of toxicity, but also suitable tools for the early and sensitive detection of chemical exposure (Fent, 2001). Considering the involvement of the liver in detoxification, fish hepatocytes are often chosen as in-vitro model systems and, the use of permanent cell lines have advantages over the use of primary cell culture as they can eliminate the need for animals as well as their easy culture, availability and reproducibility (Bols et al., 2005). One well established permanent fish cell line is PLHC-1, derived from topminnow (*Poeciliopsis lucida*) hepatocellular carcinoma, which has been demonstrated to bear metabolic activity and to contain an active aryl hydrocarbon receptor (AhR) (Babich et al., 1991). This cell line has the capacity to induce the expression of CYP1A after exposure to environmental pollutants such as dioxin-like compounds, pharmaceuticals and extracts of environmental matrices i.e. sediments (Hahn et al., 1993, 1996; Huuskonen et al., 2000; Jung et al., 2001; Thibaut and Porte, 2008).

* Corresponding authors. Tel.: +34 93 4006175; fax: +34 93 2045904.
E-mail addresses: deniseferna@gmail.com, drfqam@cid.csic.es (D. Fernandes), cpvqam@cid.csic.es (C. Porte).

0141-1136/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marenvres.2013.09.019

In recent years, special attention has been given to the disruption of fish reproduction by pollutants (Monteiro et al., 2000; Fernandes et al., 2007a; Cheshenko et al., 2008). Several steroidogenic cytochrome P450 enzymes (CYPs) play a key role in the synthesis of sex steroids in fish gonads. Among them, CYP17 catalyzes the conversion of 17α-hydroxyprogesterone (17P4) to androstenedione (AD), a precursor of testosterone (T); both T and AD can be transformed in testes to their respective 11β-hydroxlated metabolites (βT, βAD), a reaction catalyzed by CYP11β (Kime, 1993; Liu et al., 2000; Devlin and Nagahama, 2002). 11β-hydroxlated metabolites can be further metabolized in testes to 11-ketoandrogens, where 11-keto-testosterone (11-KT) is the most potent androgen in fish being more effective than testosterone in stimulating secondary sexual characters, influencing spermatogenesis and stimulating reproductive behaviour in male teleost fish (Borg, 1994; Kusakabe et al., 2003). On the other hand, androgens (AD and T) are converted into estrogens (estrone and estradiol) through aromatization in female gonads; this reaction is catalyzed by cytochrome P450 aromatase (CYP19), a key enzyme regulating local and systemic levels of estrogens in the body (Cheshenko et al., 2008). The interference of pollutants with the synthesis of all these steroids may alter bioavailable amounts of active hormones within the organism, and be a potential mechanism of endocrine disruption by affecting sexual differentiation, gamete growth and/or maturation of fish. Previous studies have shown the ability of a wide range of anthropogenic chemicals (e.g. surfactants, pharmaceuticals, personal care products, polycyclic aromatic hydrocarbon metabolites) to interfere with the activity of key steroidogenic enzymes (i.e. CYP17, CYP11β, CYP19 and 11β-HSD) in fish gonads (Fernandes et al., 2007a, 2011; Schnell et al., 2009; Fernandes and Porte, 2013).

Estuaries are key habitats for a diverse group of ecologically relevant species, and in particular the benthic organisms found in sediments form a large part of the species diversity, biomass and productivity of estuaries. However, besides their ecological significance, estuaries also provide excellent opportunities for the development of socio-economic activities (e.g. fisheries, industry, agriculture and tourism) due to the proximity of urban and industrial settlements and therefore, are among the aquatic ecosystems most susceptible to the continuous influx of heterogeneous contaminant mixtures (Kennish, 2002). Consequently, studies on the environmental conditions and ecological health of these important ecosystems are required, in order to encourage sustainable development.

The aim of this study was to characterize the environmental quality of sediments collected from different sites of the Arade and Guadiana estuaries (South of Portugal) – including areas affected by urban effluents, harbour activities and potential reference sites – by using different in-vitro bioassays: (a) the fish hepatoma cell line (PLHC-1) to determine the cytotoxicity and the presence of CYP1A and oxidative stress inducing agents in sediments and, (b) gonad subcellular fractions from the sea bass (*Dicentrarchus labrax*) to detect the presence in sediments of compounds that are likely to act as endocrine disrupters by interfering with the synthesis of androgens (CYP17, CYP11β) and estrogens (ovarian CYP19). This will help to discriminate those sediments that could pose risk to aquatic organisms and to identify potentially impacted areas that require further action to improve their environmental quality.

2. Materials and methods

2.1. Chemicals and reagents

Eagle's Minimum Essential Medium (MEM), foetal bovine serum, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin G, streptomycin, phosphate buffered saline (PBS), Dulbecco's phosphate buffered saline (DPBS) and trypsin-EDTA were from Gibco BRL Life Technologies (Paisley, Scotland, UK). 7-Ethoxyresorufin, 7-hydroxyresorufin, β-naphtoflavone (bNF), dimethyl sulfoxide (DMSO), nicotinamide adenine dinucleotide phosphate (NADPH) and unlabelled steroids were purchased from Sigma–Aldrich (Steinheim, Germany). 2′7′-dichloro-dihydrofluorescein diacetate (H_2DCF-DA) and 3-morpholinosydnonimine (SIN-1) were obtained from Invitrogen (Molecular Probes, Barcelona, Spain). [^{3}H]17α-Hydroxyprogesterone (60–100 Ci/mmol) and [1β-^{3}H]androstenedione (15–30 Ci/mmol) were purchased from Perkin–Elmer Life Sciences. Copper (fine powder particle <63 μm), hydrochloric acid (HCl) and all the solvents used of analytical grade were from Merck (Darmstadt, Germany).

2.2. Sediment collection and extraction

Surface sediment samples were collected in February 2011 using a Van Veen grab sampler from several sites along the Arade and Guadiana estuaries (S Portugal), considering different potential pollutant inputs (Fig. 1). Sample location (grid references), environmental parameters and main characteristics of the selected sites are given in Table 1. Sediment samples were kept in ice cold decontaminated glass containers and immediately transported to the laboratory where they were stored at −20 °C until freeze-drying.

Three grams of freeze-dried and homogenized sediment (125 μm) were extracted twice with 20 mL dichloromethane/hexane (1:1, v/v), followed by dichloromethane/acetone (1:1, v/v) (x2). For each extraction step, the sample was sonicated 10 min at room temperature and centrifuged 10 min at 2500 rpm. The extracts were combined, evaporated to about 5 mL, transferred to a 10 mL vial containing 0.5 g of HCl-activated copper (to eliminate elemental sulphur from the organic extracts) and stored in the fridge overnight. The supernatant was then collected, evaporated to complete dryness under a gentle nitrogen stream and reconstituted into 250 μL of DMSO or ethanol, depending on the bioassay. Extracts were serially diluted in DMSO or ethanol to desired concentrations, expressed as equivalent mg of dry sediment per mL (eQsed/mL).

2.3. Cell culture

The PLHC-1 cell line (ATCC; CRL-2406), derived from topminnow (*P. lucida*) hepatocellular carcinoma was routinely cultured in Eagle's Minimum Essential Medium supplemented with 5% foetal bovine serum, 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, in a humidified incubator with 5% CO_2 at 30 °C. When 90% of confluence was reached, cells were dissociated with 0.05% (w/v) trypsin and 0.5 mM EDTA for subculturing and experiments. Application of sediment extracts to cell cultures was done by diluting the extracts in culture medium which was then added to culture wells. The final concentration of DMSO in culture wells never exceeded 0.5% (v/v). For each assay a control was performed by adding only the solvent (DMSO) to the cells.

2.4. Cell viability

For exposure to sediment extracts, flasks at 90% of confluence were used to seed 96-well tissue culture plates (Nunc; Roskilde, Denmark) at a cell density of 75,000 cells per well in 200 μL of medium. Cells were allowed to attach for 24 h prior to 24 h exposure to sediment extracts. Two fluorescent dyes were used to monitor the effect of sediment extracts on cell viability (Schirmer

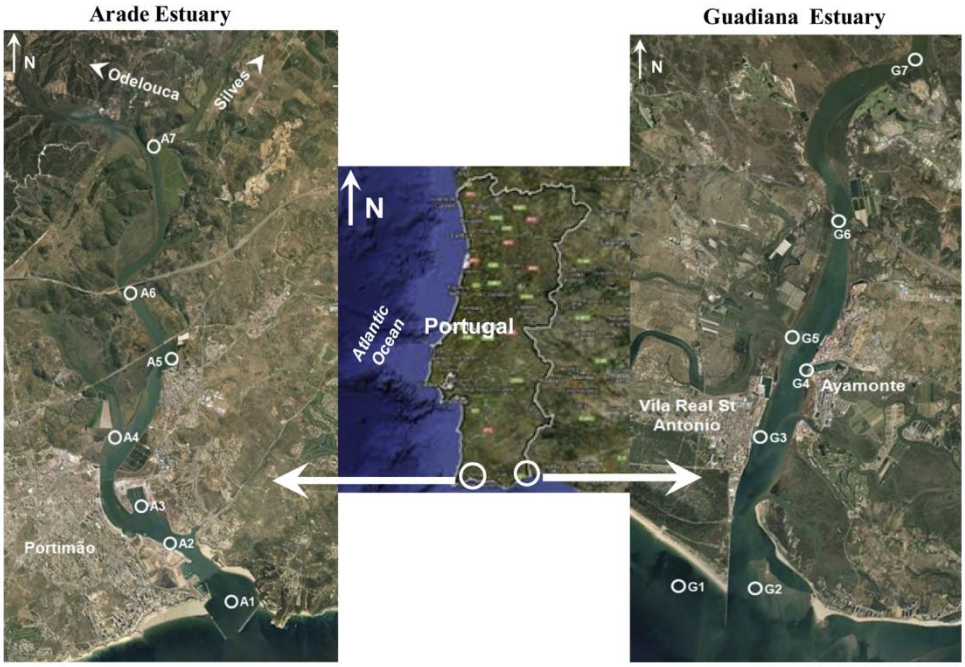


Fig. 1. Map of the sampling sites.

et al., 1997; Dayeh et al., 2003). Metabolic activity was monitored with Alamar Blue (AB, reazurin; Biosource International, Invitrogen, Barcelona, Spain). Cell membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Invitrogen, Barcelona, Spain). The assays were performed as described previously in detailed step-by-step protocols on the use of these dyes and on the analysis and interpretation of the results (Dayeh et al., 2003). Results were recorded 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 fluorescence plate reader (Varioskan, Thermo Electron Corporation).

2.5. Reactive oxygen species (ROS) generation

The generation of ROS in the presence of sediment extracts was determined by using the method of LeBel et al. (1992) with slight modifications. Cells were seeded at a density of 100,000 cells per well and allowed to attach overnight in a humidified incubator with

Table 1
Geographic coordinates, water environmental parameters and general characteristics of the sampling sites along the studied estuaries.

Estuary sites	Longitude (W)	Latitude (N)	Distance[a] (km)	T °C	pH	S‰	O_2 (mg/L)	Main pressures/impacts
Arade								
A1	8°31′22.86″	37°06′48.9″	0.73	14.8	8.08	27.6	7.81	Near the estuary mouth (intensive boat traffic)
A2	8°31′43.26″	37°07′31.5″	1.89	14.6	8.03	21.3	7.49	Commercial and touristic harbour of Portimão (boat traffic and harbour activities)
A3	8°31′44.70″	37°08′3.66″	2.88	14.9	8.04	22.1	7.12	Shipyard and fishing harbour; socioeconomic surface of 20,000 m^2 (manoeuvres, boat traffic and fishing activities)
A4	8°31′35.70″	37°08′35.2″	3.86	12.9	7.65	5.17	8.82	Waste water effluents of the city of Portimão (~60,000 inhabitants)
A5	8°30′22.68″	37°08′54.8″	4.90	13.7	7.77	12.7	8.19	Marina resort (recreational activities and waste water effluents)
A6	8°30′17.76″	37°09′50.7″	6.55	14.5	7.99	21.8	7.27	Site under the highway bridge (car traffic)
A7	8°29′6.72″	37°10′29.6″	8.42	13.5	7.50	5.84	8.12	Animal husbandry, agriculture and the tributary of the city of Silves (~10,000 inhabitants)
Guadiana								
G1	7°24′8.28″	37°09′51.0″	1.25	16.0	8.11	35.7	7.15	Coastal area outside the estuary, beach zone (reference site)
G2	7°24′9.48″	37°10′31.5″	0.00	13.8	8.15	15.9	9.07	Estuary mouth (intensive boat traffic)
G3	7°24′46.32″	37°11′34.8″	2.19	13.6	8.14	16.0	9.16	Commercial and touristic harbour of Vila Real de St. António (boat traffic and harbour activities)
G4	7°24′36.96″	37°12′33.8″	3.85	13.3	8.12	14.5	9.27	Commercial and touristic harbour of Ayamonte (boat traffic and harbour activities)
G5	7°24′57.36″	37°12′42.1″	4.24	12.5	8.05	8.90	9.80	Waste water effluents of the city of Vila Real de St. Antonio (~20,000 inhabitants)
G6	7°25′19.68″	37°14′19.0″	7.28	13.1	8.21	12.1	9.44	Site under the highway bridge (intensive car traffic)
G7	7°25′49.74″	37°15′25.1″	9.47	12.4	7.95	7.28	9.88	Abandoned mines, cattle breeding and intensive agriculture

[a] From de estuary mouth.

5% CO_2 at 30 °C. Then, cells were washed with PBS and loaded with 20 μM 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) diluted in 1:10 of DPBS supplemented with 10 mM glucose (DPBS-Glu). After 30 min incubation at 30 °C, the cell monolayers were washed twice with PBS and exposed to sediment extracts at 60 mg eQsed/mL in DPBS-Glu in order to stimulate ROS production. The fluorescence of oxidized H_2DCF was measured 15 min after with a microplate reader (Varioskan, Thermo Electron Corporation), at the excitation and emission wavelengths pairs of 485/528 nm. 3-Morpholinosydnonimine (SIN-1; 5 μM) was used as a positive control (Spohn et al., 2009). Results were expressed as a percentage of the basal fluorescence in control wells (% DMSO).

2.6. Induction of EROD activity

Cells were exposed to various concentrations of sediment extracts (1–60 mg eQsed/mL) in order to establish dose–response curves. PLHC-1 cells were seeded at 650,000 cells per well (500 μL) in 48-well plates (Nunc; Roskilde, Denmark) and allowed to grow for 24 h. After the growth period, the culture medium was changed and replaced by medium containing β-naphthoflavone (BNF; 1 μM) that was used as a positive control, sediment extracts or the carrier (0.5% DMSO). Treatments were carried out in triplicate. After 24 h exposure the medium was aspirated and the cells were rinsed with 500 μL 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 in the microplate reader (Varioskan, Thermo Electron Corporation), the fluorescence was directly read at the excitation/emission wavelength pairs of 537/583 nm. Quantification was made by calibration with 7-hydroxyresorufin.

After quantification of 7-hydroxyresorufin, cells were washed with PBS and the amount of total cellular protein was determined with fluorescamine as described by Lorenzen and Kennedy (1993), using bovine serum albumin (BSA) as standard. EROD activity was expressed as pmol of resorufin formed per minute and per milligram of protein.

2.7. Gonad subcellular fractions and in-vitro incubations

Sea bass (*D. labrax*) of marketable size were supplied by an aquaculture facility located in the Northeast of Spain. Immediately after collection, animals were sacrificed and gonads were dissected. A subsample of the central part of the gonad was fixed in 10% buffered formalin for histological examination to determine the sexual maturation stage (SMS). The rest of the gonad was deep-frozen in liquid nitrogen and stored at −80 °C until preparation of subcellular fractions.

Subsamples of ~1 g of individual gonads of both males and females were flushed with ice cold 1.15% KCl and homogenized in 1:4 w/v of ice-cold 100 mM potassium–phosphate buffer pH 7.4, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline and 0.1 mg/mL trypsin inhibitor. Homogenates were centrifuged at 500 × *g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000 × *g* for 20 min. The resulting pellet, termed mitochondrial fraction, was resuspended in 100 mM potassium–phosphate buffer pH 7.4, containing 1 mM EDTA, 20% w/v glycerol and supplemented with 1 mM DTT, 0.1 mM phenanthroline and 0.1 mg/mL trypsin inhibitor in a ratio of 0.4 mL buffer/g of gonad tissue. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min to obtain the microsomal fraction. The pellet (microsomal fraction) was resuspended in the same buffer as the mitochondrial fraction and both, mitochondrial and microsomal proteins were measured by the method of Bradford (1976), using bovine serum albumin (BSA) as standard. Mitochondrial fraction was only extracted from early-spermatogenic testes, while microsomes were prepared from early-oogenic ovaries, since, the highest CYP17, CYP11β and CYP19 specific activities occurs at this stage of sexual maturation (Lavado et al., 2004; Fernandes et al., 2007a).

The interaction with sea bass steroidogenic enzymes was investigated in-vitro by pre-incubating the corresponding subcellular fraction (mitochondria or microsomes) in the presence of the sediment extracts (60 mg eQsed/mL and 1.5 mg eQsed/mL) for 10 min at 25 °C, prior to the addition of the reaction substrate and co-factor as indicated below. Sediment extracts were prepared in ethanol because 0.5% of DMSO showed to inhibit up to 20% the activity of the studied enzymes in gonads. The solvent was removed from the assay by evaporation under a gentle nitrogen stream before starting the pre-incubation. Control reactions consisted in the incubation of the corresponding subcellular fraction in the absence of sediments. Blanks that consisted of reactions stopped at time zero were also analyzed. Assays were carried out using individual gonads of 4–5 different individuals.

2.7.1. Ovarian P450 aromatase activity (CYP19)

Aromatase activity was determined by the tritiated-water release method as described in Lavado et al. (2004). Ovarian microsomal proteins (0.4 mg) were incubated at 25 °C for 1 h in the presence of 100 mM Tris–HCl, pH 7.6, [1β-^{3}H]-androstenedione (42.5 pmol, 1 μCi) and 0.2 mM NADPH. The reaction was stopped by adding 3 mL of ice cold dichloromethane and the organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction (×3). The remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 mL). The solution was centrifuged (1500 × *g*; 60 min) and two aliquots of the supernatant (1 mL) were counted for ^{3}H radioactivity with a liquid scintillation counter (Tri-Carb 2100 TR, Packard).

2.7.2. Testes CYP17 and CYP11β activities

CYP17 activity was assessed in testes by incubating mitochondrial fraction (0.2 mg protein) in 50 mM Tris–HCl 10 mM MgCl2 buffer pH 7.4 with 0.2 μM [^{3}H]17α-hydroxyprogesterone and 1 mM NADPH in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30 °C. Similarly, CYP11β was assessed by incubating mitochondrial proteins (0.5 mg) in 50 mM Tris–HCl 10 mM MgCl2 buffer pH 7.4 with 0.2 μM [^{3}H]androstenedione, and 1 mM NADPH in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30 °C. Both incubations were stopped by adding 250 μL of acetonitrile and after centrifugation (1500 × *g*; 10 min), 100 μL of supernatant was injected into a reversed-phase HPLC system coupled with a radiometric detector. HPLC analyses were performed on a Agilent Technologies 1200 series system equipped with a 250 × 4 mm LiChrospher 100 RP-18 (5 μM) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 μM). Separation of metabolites was performed at 1 mL/min with a mobile phase composed of A) 80% water, 10% acetonitrile and 10% methanol, and B) 50% acetonitrile and 50% methanol. The run consisted on a 28 min linear gradient from 40% B to 100% B, 28–33 min at 100% B (isocratic mode), 33–38 min linear gradient from 100% B to 40% B and 38–48 min at 40% B. Radioactive metabolite peaks were monitored by on-line radioactivity detection with a Flow Scintillation Analyzer Radiomatic 610 TR (PerkinElmer) using Ultima-Flow M (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. CYP17 activity was determined by measuring the formation of androstenedione (AD), while CYP11β by measuring the formation of 11β-hydroxyandrostenedione (βAD).

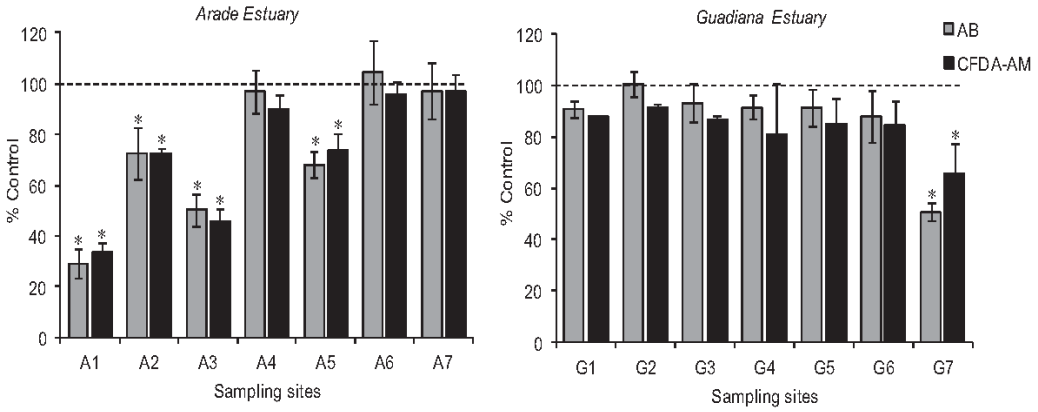


Fig. 2. Cell viability of PLHC-1 cells upon 24 h exposure to sediment extracts from the Arade and Guadiana estuaries tested at 60 mg eQsed/mL. Values are mean ± SEM of 5 independent assays. *Statistically significant decreases in cell viability relative to the control ($p < 0.05$). No significant differences were detected between AB and CFDA-AM assays.

Both metabolites were quantified by integrating the area under the radioactive peaks.

2.8. Statistical analysis

Results are reported as mean ± SEM. Statistical significances were assessed by using one-way ANOVA analysis of variance with Dunnett's test for differences from control and Tukey's test for differences between AB and CFDA-AM assays. All statistics were analyzed using the software package SPSS version 19.0 (SPSS Inc., Chicago, IL). Level of significance was set at $P < 0.05$. The concentration of sediment extract resulting in 50% (EC50) effect was calculated using Sigma-Plot version 8.02. In order to summarize the information and estimate the degree of anthropogenic impact, a principal component analysis (PCA) was carried out using the PLS Toolbox (Eigenvector INC, Manson WA, USA, 2013) and MATLAB (Mathworks, MA, USA, 2013) computer and visualisation software.

3. Results

3.1. Cell viability

The cytotoxicity of the sediment extracts is presented in Fig. 2. All sediment extracts were tested at a concentration of 60 mg eQsed/mL. Significant cytotoxic effects were observed for four out of seven extracts from the Arade estuary and for one out of seven extracts from the Guadiana estuary, tested after 24 h incubation with PLHC-1 cells. The most cytotoxic extract was from site A1 from the Arade estuary (near the estuary mouth), with an average cell viability of 30.0 ± 5.0%. A dose–response curve was performed with A1 revealing EC50s of 26.1 ± 3.7 and 26.7 ± 4.6 mg eQsed/mL for AB and CFDA-AM, respectively. The sediment extract from A3 (shipyard and fishing harbour) produced an average cell viability of 48.0 ± 5.7%, followed by A5 (71.1 ± 5.9%; marina resort) and A2 (72.5 ± 6.1%; commercial harbour of Portimão city). In the Guadiana estuary the upper-stream sediment extract G7 (58.1 ± 7.7%; area influenced by abandoned mines, intensive agriculture and cattle breeding) was also significantly cytotoxic ($p < 0.05$).

The metabolic activity, measured with Alamar Blue, and cell membrane integrity, evaluated with CFDA-AM, gave similar responses after exposure to sediment extracts.

3.2. Reactive oxygen species (ROS) generation

H_2DCF-DA is a cell permeable molecule that is de-acetylated to cell impermeable H_2DCF by cytoplasmic esterases. H_2DCF can be oxidized by hydrogen peroxide or hydroxyl radical to the fluorescent molecule DCF. The oxidation of H_2DCF was monitored over 15 min. The exposure to 5 μM of SIN-1 (positive control) led to a 3-fold increase of ROS production (Fig. 3). Four to five out of seven sediment extracts tested at 60 mg eQsed/mL significantly induced ROS generation in PLHC-1 cells (Fig. 3). The upper sites G7 and G6 from the Guadiana estuary and A7 from the Arade were the most efficient in oxidizing H_2DCF: ROS production increased 1.52-, 1.44- and 1.43-fold after exposure, respectively. Meanwhile, extracts from sites G4, G5, A3, A4, A5 and A6 also increased ROS production (31–38%) in PLHC-1 cells but to a lesser extent than those from upstream sites. No ROS generation could be detected for the sediment extracts collected from the downstream sites of both estuaries (i.e. A1, A2, G2 and G3) and from the reference site (G1; coastal area) using the H_2DCF probe.

3.3. Induction of EROD activity

The aryl-hydrocarbon receptor agonist β-naphthoflavone (βNF) at a concentration of 1 μM was included in the assay as a positive control and lead to an EROD activity of 102.5 ± 7.5 pmol/min/mg protein after 24 h of exposure. Dose response curves for EROD activity following 24 h exposure to the sediment organic extracts are shown in Fig. 4. Extracts from the Arade estuary, apart from A1 and A6, led to higher EROD activity in PLHC-1 cells than those from the Guadiana estuary, indicating a higher content of CYP1A inducing agents in sediments from the former. In fact, four out of seven extracts from the Arade estuary tested at concentrations above 10 mg eQsed/mL led to an induction of EROD activity 2-fold higher than βNF (positive control) (Fig. 4A). Among the EROD inducing extracts, A2 (commercial harbour of Portimão city) was the most potent one with a maximum EROD activity of 188.0 ± 17.6 pmol/min/mg protein when tested at 60 mg eQsed/mL and a EC50 of 8.3 ± 1.0 mg eQsed/mL; followed by A5 (marina resort; EC50 14.9 ± 1.9 mg eQsed/mL), A3 (shipyard and fishing harbour; EC50: 18.1 ± 2.7 mg eQsed/mL) and A4 (area of waste water effluents of Portimão city; EC50: 23.5 ± 3.1 mg eQsed/mL), whereas A6 and A7 showed a maximum EROD activity of 40.4 and 83.0 pmol/min/mg protein respectively, when tested at 60 mg eQsed/mL and no EC50s were

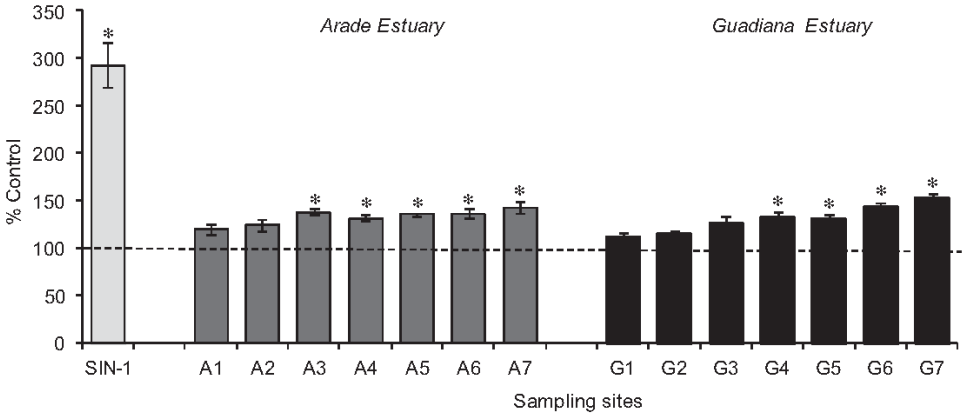


Fig. 3. ROS production of PLHC-1 cells after 24 h of exposure to sediment extracts from de Arade and Guadiana estuaries at 60 mg eQsed/mL and after exposure to 5 μM SIN-1 (positive control) monitored over 15 min. Values are mean ± SEM of 3 different assays. *Statistically significant differences from control ($p < 0.05$).

determined. In contrast, A1 revealed rather low EROD activities (1.0–2.8 pmol/min/mg protein) when tested from 1 to 40 mg eQsed/mL, being lower than the average activity of the solvent controls 6.3 ± 0.4 pmol/min/mg protein.

As for the Guadiana estuary, the highest EROD induction was observed in extracts from G3 (commercial and touristic harbour of Vila Real St Antonio city) with a maximum activity of 69.3 ± 1.1 pmol/min/mg protein tested at 60 mg eQsed/mL (Fig. 4B). Meanwhile, for the majority of sediments from the Guadiana estuary EROD activity ranged between 30.5 and 41.5 pmol/min/mg protein after 24 h of exposure to 60 mg eQsed/mL (Fig. 3B), with exception of G1 (5.6 ± 0.3 pmol/min/mg protein) and G2 (6.9 ± 0.7 pmol/min/mg protein) that indicated similar EROD activities as solvent controls.

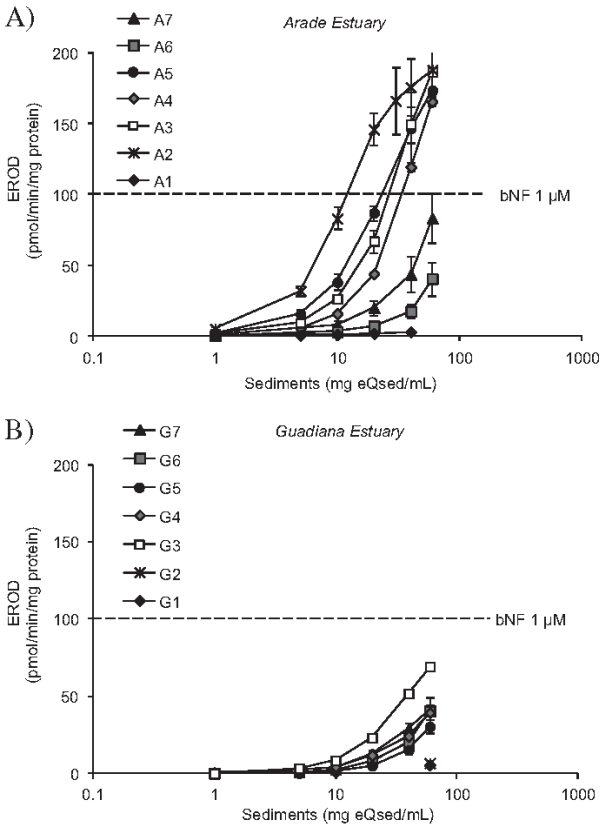


Fig. 4. EROD activity in PLHC-1 cells following 24 h exposure to different concentrations of sediment extracts. A) Arade estuary and B) Guadiana Estuary. Values are mean ± SEM of at least five different assays. Results are expressed as pmol resorufin/min/mg protein. EROD activity of solvent controls (0.5% DMSO): 6.31 ± 0.43 pmol/min/mg protein.; and of bNF (positive control) at 1 μM: 102.5 ± 7.5 pmol/min/mg protein.

3.4. Ovarian P450 aromatase activity (CYP19)

All organic extracts from the Arade estuary strongly inhibited P450 aromatase activity (65–92%) in ovarian microsomes when tested at 60 mg eQsed/mL (Fig. 5A). As for the Guadiana estuary, aromatase inhibition was observed in five out of seven extracts (Fig. 5A). Sites G7 (91%) and G3 (84%) were the strongest inhibitors of CYP19 in the Guadiana when tested at 60 mg eQsed/mL, followed by G1 (34%), G6 (24%) and G4 (22%). When sediment extracts were tested at a concentration 40-fold lower (1.5 mg eQsed/mL), four out of seven extracts in the Arade estuary were still able to significantly inhibit aromatase activity (27–59%; A2, A3, A5, A6), whereas for the Guadiana estuary, inhibition was only observed in site G7 (58%; upper-stream site) (Fig. 5A).

3.5. Testes CYP17 and CYP11β activities

All samples from the Arade estuary, apart from A1, significantly inhibited the synthesis of androgens in testes mitochondria when tested at 60 mg eQsed/mL. Three out of six extracts had an inhibitory effect on both CYP17 and CYP11β activities (Fig. 5B): A3 was the strongest inhibitor (41 & 43%), followed by A7 (31 & 20%) and A2 (22 & 21%). Meanwhile, extracts A4, A5 and A6 elicited a 22–32% inhibition on CYP17 activity. In contrast, for the Guadiana estuary only G4 (commercial and touristic harbour of Ayamonte) had an inhibitory effect on both CYP17 (48%) and CYP11β (26%) activities (Fig. 5B), whereas G5, G6 and G7 indicated a 22–44% inhibition of CYP17. Generally, extracts tested in the present work showed a higher ability to interfere with CYP17 than with CYP11β (Fig. 5B).

3.6. Principal component analysis

A PCA was used to classify sediments according to the level of anthropogenic impact (Fig. 6). The PCA with two components explained 65% of the total variance in the results. All variables got

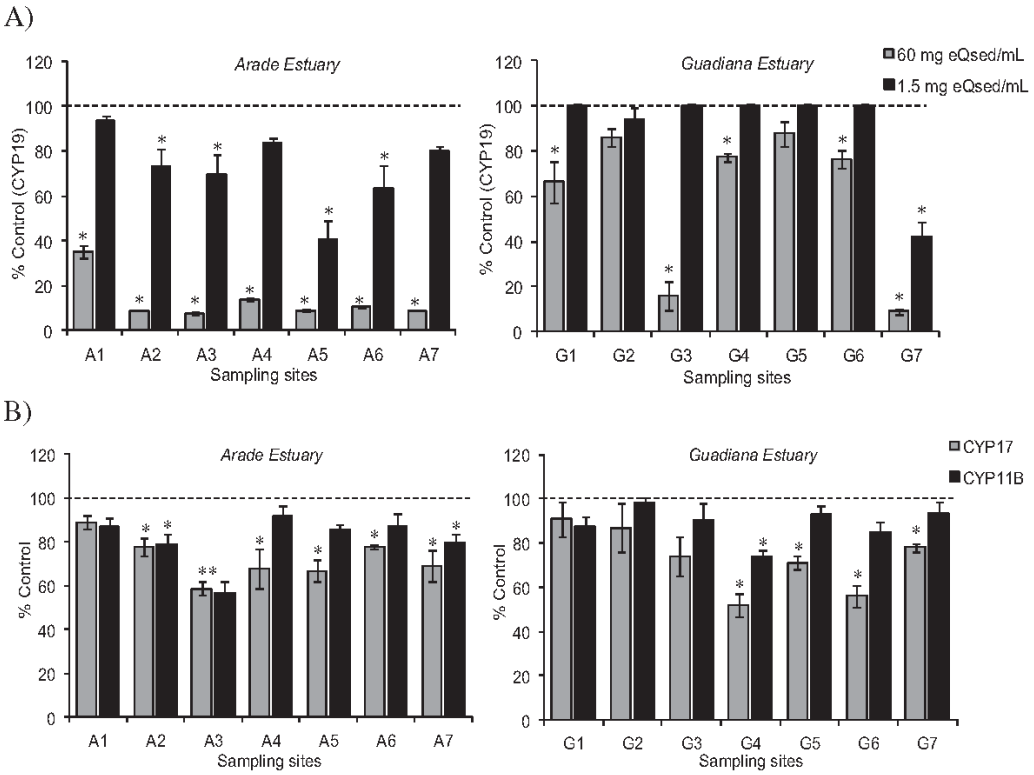


Fig. 5. In-vitro interference of sediment extracts from the Arade and Guadiana estuaries on A) microsomal ovarian P450 aromatase activity (1.5 and 60 mg eQsed/mL) and B) mitochondrial CYP17 and CYP11β activity (60 mg eQsed/mL) in sea bass testes. Values are mean ± SEM ($n = 4$–5). *Significant differences relative to the control ($p < 0.05$). Specific activities: CYP19: 1.23 ± 0.19 pmol/h/mg protein; CYP17: 260.1 ± 27.9 pmol/h/mg protein; CYP11β: 42.3 ± 4.0 pmol/h/mg protein.

positive loadings on the first principal component (PC1, explained variance = 40.4%), with higher intensity for EROD and CYP19 activities and lower for CYP17 activity. The second (PC2, explained variance = 24.3%) was characterized by a negative correlation between sediment toxicity (AB and CFDA-AM) and CYP17 activity.

When PC1 and PC2 factor scores were plotted, sediments A2, A3, A5 and G7 were isolated to the positive end of PC1, whereas sediments G1 and G2 were located in the extreme negative side of this axis, according to their level of anthropogenic impact (Fig. 6). Sediment A1 with high cytotoxicity and EROD inhibition was clustered on the positive side of PC2. The other sediments differ little among them on this axis.

4. Discussion

Toxicity screening of complex environmental samples such as sediments warrants the use of multiple endpoint measurements that can address specific modes of action of different chemical constituents. By means of an array of in-vitro bioassays, this study examined the potential cytotoxicity and occurrence of AhR-inducing compounds, generation of reactive oxygen species (ROS) and presence of endocrine disruptors in organic extracts of sediments from two estuary systems. The highest cytotoxicity was detected in sediment extracts from site A1 (Arade estuary mouth) followed by extracts from sites A3, A5 and A2, all of them affected by boat traffic and recreational activities. Comparatively, only the upper Guadiana extract G7 indicated cytotoxicty –with an average loss of 42% of cell viability-. Overall, the cytotoxicity probes evidenced a higher anthropogenic impact in the Arade estuary.

Regarding CYP1A induction in PLHC-1 cells after 24 h exposure to sediments extracts, all samples, apart from A1, G1 and G2, indicated the presence of CYP1A inducing agents. The highest EROD activities were observed in extracts collected from the lower-middle part of the Arade estuary (i.e. A2, A3, A4, A5), all showing an induction 2-fold higher than 1 μM of βNF (positive control), when tested at concentrations above 10 mg eQsed/mL. The highest induction of EROD activity was detected in site A2 (commercial and touristic harbour of Portimão), suggesting a higher content of pollutants capable of binding to the AhR in sediments from this area. AhR ligands are hydrophobic aromatic compounds with planar structure of a particular size, which fit the binding site (Fent, 2001). Although, not all PAHs are capable of CYP1A induction, Fent and Bätscher (2000) have shown that pyrene, chrysene, benzo[*a*]pyrene, phenanthrene and fluoranthene among other PAHs, induce CYP1A activity in PLHC-1 cells, although with different potencies. High levels of PAHs (1690 ng/g d.w., as sum of 16 PAHs) have been reported in sediments from A2, in particular phenanthrene (379 ng/g d.w.), fluoranthene (376 ng/g d.w.), pyrene (264 ng/g d.w.) and chrysene (137 ng/g d.w.), when compared to the other sites of the Arade estuary (sum of 16 PAHs < 160–368 ng/g d.w.) (Bebianno et al., 2012). Moreover, high levels of 1-pyrenol equivalents, which is regarded as the best general indicator of PAH exposure in fish (Ruddock et al., 2002), have been detected in fish bile collected in site A2, together with significant induction of hepatic EROD activity (Fernandes et al., 2007b). Meanwhile, the induction of EROD activity by sediment extracts of the Guadiana estuary was in general low in comparison to the activities observed with extracts from the Arade; the highest EROD activity was detected in G3 (a

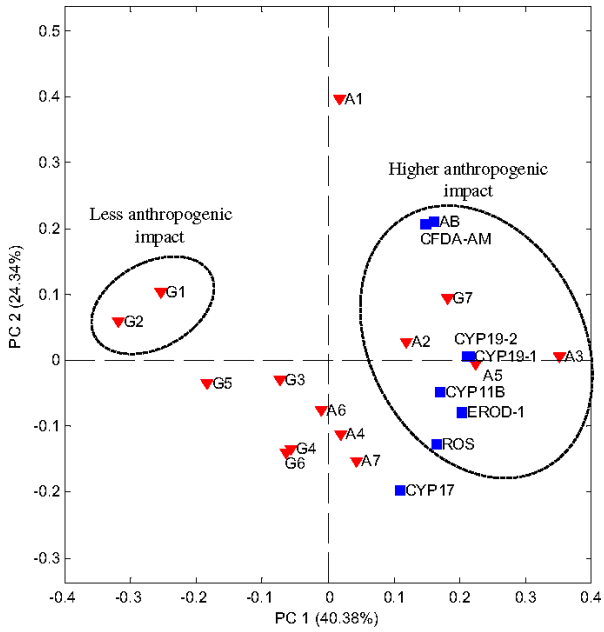


Fig. 6. Principal component analysis (PCA); PC1 versus PC2 biplot. (■) loadings for biological variables; (▼) site scores: Guadiana estuary (G1 to G7), Arade estuary (A1 to A7). EROD-1: 60 mg eQsed/mL; CYP19-1: 60 mg eQsed/mL; CYP19-2: 1.5 mg eQsed/mL.

commercial and touristic harbour). Accordingly, levels of PAHs (19.8–38.1 ng/g d.w. as sum of 16 PAHs) up to 10-fold lower are reported in sediments from the Guadiana estuary (Serafim et al., 2013). Thus, EROD activity in PLHC-1 cells provides a fingerprint of the presence of CYP1A inducing agents (i.e. PAHs) in sediment extracts, although it also represents the cumulative impact of all potential chemicals extracted from the sediment.

It is worth mentioning that sediment extracts from A1 did not induce EROD activity despite the highest cytotoxicity detected in this sample. It has been reported that the presence of metals in environmental samples can inhibit EROD activity in PLHC-1 cells and mask real concentrations of CYP1A inducers (Brüschweiler et al., 1996). Although the presence of metals in the dichlormethane-hexane/acetone sediment extracts is very unlikely, other pollutants have been reported to inhibit hepatic CYP1A activity in fish (Fent and Bucheli, 1994; Morcillo and Porte, 1997; Willett et al., 2001), and both synergistic and antagonistic effects may occur in a test system that contains complex mixtures of compounds.

ROS production in cells can increase due the presence of natural and/or anthropogenic compounds released by urban, industrial and agricultural discharges. These undesired molecules, produced as by-products from endogenous processes like the mitochondrial electron transport or by oxido-reductase enzymes and metal catalyzed oxidations, have the potential to cause a number of deleterious effects (Livingstone, 2001). The sensitivity of H2DCF-DA as a probe to detect ROS generation in PLHC-1 cells was demonstrated using SIN-1 as a positive control. A 3-fold increase of ROS was detected after 15 min of exposure compared to control (Fig. 3). As for samples, results were quite similar between estuaries; a 1.5-fold increase in ROS production was observed in cells exposed to sediment extracts from the middle- and upper-sites of both estuaries. Pollutants capable of enhancing ROS are mostly redox cycling compounds (e.g. quinines, nitroaromatics, nitroamines, herbicides), PAHs (e.g. benzene, PAH oxidation products), halogenated hydrocarbons (e.g. PCBs, lindane) dioxins and metals (Livingstone, 2001). Such contaminants can stimulate ROS production by a variety of mechanisms, both direct and indirect, such as biotransformation of PAHs to redox cycling quinones, induction of CYPs, disruption of membrane systems by lipophilic pollutants and metal redox reactions with O_2 and ROS (Schlezinger et al., 1999; Strolin-Benedetti et al., 1999; Livingstone, 2001). The positive relationship detected between ROS and EROD activity, as indicated in the PCA (Fig. 6), could partially explain the enhancement of ROS generation by some of the mechanisms mentioned above. Interestingly, a significant induction of ROS (1.7-fold) was observed in PLHC-1 cells exposed to estuarine sediment extracts from the Northern coast of Spain, those sediment extracts significantly induced EROD activity (EC50: 0.10 ± 0.03 mg eQsed/mL) and had very high concentrations of PAHs (58,713 ng/g d.w.) (Schnell et al., 2013).

Together with the PLHC-1 cell line, fish gonadal subcellular fractions were a complementary tool to assess the presence of endocrine disruptors in sediment extracts. Ovarian CYP19 activity was inhibited up to 90% by sediment extracts from both estuaries when tested at 60 mg eQsed/mL; whereas the inhibition of the synthesis of androgens (i.e. CYP17 and CYP11β) in testis was less pronounced (up to 48%). When sediments were tested at a lower concentration (1.5 mg eQsed/mL) inhibition of CYP19 activity in ovaries was still detected for the Arade extracts (i.e. A2, A3, A5 and A6) and for extract G7 (Guadiana). Endocrine disruptor compounds, such as pesticides and their metabolites, PAHs, bisphenol A, organotin compounds and herbicides such as diuron, have been reported in water and/or sediment samples of the Guadiana estuary (Almeida et al., 2007), together with a high incidence of intersex (feminisation) in male clams *Scrobicularia plana* (Gomes et al., 2009). Moreover, significant high levels of nonylphenol (2000–8000 ng/g of bile) were shown in bile of fish from the Arade estuary (Fernandes et al., 2009). Interestingly, NP was reported to lower ovarian P450 aromatase activity in exposed turbot (Martin-Skilton et al., 2006) and to inhibit in-vitro CYP17 activity in testes of male sea bass (Fernandes et al., 2007a). Several other environmental pollutants have shown the ability to interfere with the synthesis of active androgens in male fish gonads: (a) antidepressive drugs, fluoxetine and fluvoxamine inhibited CYP17 and CYP11β enzymes in carp, with IC50s in the range of 321–550 μM (Fernandes et al., 2011); (b) fungicides such as, ketoconazole and clotrimazole with IC50s of 38 μM for CYP17 and 1.0–4.4 μM for CYP11β (Fernandes and Porte, 2013); (c) polycyclic musks, galaxolide and tonalide with IC50s in the range of 200 μM for CYP17 (Schnell et al., 2009); (d) polycyclic aromatic hydrocarbon metabolites, such as 9-phenanthrol with IC50s of 11 and 31 μM for CYP17 and CYP11β, respectively (Fernandes and Porte, 2013).

This study highlights the higher anthropogenic impact on sediments of the Arade when compared to the Guadiana estuary. The PCA clearly discriminates site G1 (Reference site; beach area) and G2 (estuary mouth) from the Guadiana estuary (G2) as the less impacted areas (Fig. 6). In contrast, the most impacted sites were those located at the lower-middle part of the Arade estuary (i.e. A2, A3 and A5), namely the areas affected by harbour/marina, shipyard and fishing activities; and the upper-stream site (G7) from the Guadiana estuary, an area potentially influenced by the intensive agriculture activities (e.g. olive and vineyard crops) and cattle breeding that is carried out along the hydrological basin of the river (Cravo et al., 2006; Almeida et al., 2007) (Fig. 6).

Overall, the combination of different bioassays, focussing on cytotoxicty, CYP1A1 induction, ROS generation and inhibition of steroidogenic enzymes in fish gonad subcellular fractions, provided useful information to discriminate between polluted and less impacted areas and may serve as a first screening tool for the

assessment of the quality of benthic ecosystems. Furthermore, this in-vitro approach may allow a significant reduction in the number of bioindicator organisms to be used in environmental monitoring studies.

Acknowledgement

Denise Fernandes acknowledges a postdoctoral fellowship (SFRH/BPD/34289/2006) from the Portuguese Fundação para a Ciência e Tecnologia (FCT). Dr. Rui Company and Catarina Pereira are acknowledged for their assistance in the sediment sampling.

References

Almeida, C., Serôdio, P., Florêncio, M.H., Nogueira, J.M.F., 2007. New strategies to screen for endocrine-disrupting chemicals in the Portuguese marine environment utilizing large volume injection-capillary gas chromatography-mass spectrometry combined with retention time locking libraries (LVI-GC-MS-RTL). Anal. Bioanal. Chem. 387, 2569–2583.

Babich, H., Rosenberg, D.W., Borenfreund, E., 1991. In vitro cytotoxicity studies with the fish hepatoma cell line PLHC-1 (*Poeciliopsis lucida*). Ecotoxicol. Environ. Saf. 21, 327–336.

Bebianno, M.J., Cravo, A., Duarte, D., Gonzalez-Rey, M., Pereira, C.G., Rey, F., 2012. PORTONOVO Project: Water Quality in Harbours. INTERREG IVB, Atlantic Area Programme, Ref: 2009-1/119. Final Report, Partner 6. Technical Report, p. 73 http://www.portonovoproject.org/.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schrimer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. In: Mohn, T.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fish, vol. 6. Elsevier Science, Amsterdam, Netherlands.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. 109C, 219–245.

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

Brüschweiler, B.J., Würgler, F.E., Fent, K., 1996. Inhibitory effects of heavy metals on cytochrome P4501A induction in permanent fish hepatoma cells. Arch. Environ. Contam. Toxicol. 31, 475–482.

Chapman, P.M., 2007. Do not disregard the benthos in sediment quality assessments! Mar. Pollut. Bull. 54, 633–635.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31–62.

Cravo, A., Madureira, M., Felícia, H., Rita, F., Bebianno, M.J., 2006. Impact of outflow from the Guadiana River on the distribution of suspended particulate matter and nutrients in the adjacent coastal zone. Estuar. Coast. Shelf Sci. 70, 63–75.

Dayeh, V.R., Schirmer, K., Lee, L.E.J., Bols, N.C., 2003. The use of fish-derived cell lines for investigation of environmental contaminants. In: Hodgson, E. (Ed.), Current Protocols in Toxicology. Wiley, New York, pp. 1–17.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological and environmental influences. Aquaculture 208, 191–364.

Fent, K., Bätscher, R., 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: demonstration of additive interactions. Environ. Toxicol. Chem. 19, 2047–2058.

Fent, K., Bucheli, T.D., 1994. Inhibition of hepatic microsomal monooxygenase system by organotins in vitro in freshwater fish. Aquat. Toxicol. 28, 107–126.

Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. Vitro 15, 477–488.

Fernandes, D., Bebianno, M.J., Porte, C., 2007a. Mitochondrial metabolism of 17α-hydroxyprogesterone in male sea bass (*Dicentrarchus labrax*): a potential target for endocrine disruptors. Aquat. Toxicol. 85, 258–266.

Fernandes, D., Porte, C., Bebianno, M.J., 2007b. Chemical residues and biochemical responses in wild and cultured European sea bass (*Dicentrarchus labrax* L.). Environ. Res. 103, 247–256.

Fernandes, D., Bebianno, M.J., Porte, C., 2009. Assessing pollutant exposure in cultured and wild sea bass (*Dicenthrachus labrax*) from the Iberian Peninsula. Ecotoxicology 18, 1043–1050.

Fernandes, D., Schnell, S., Porte, C., 2011. Can pharmaceuticals interfere with the synthesis of active androgens in male fish? An in vitro study. Mar. Pollut. Bull. 62, 2250–2253.

Fernandes, D., Porte, C., 2013. Hydroxylated PAHs alter the synthesis of androgens and estrogens in subcellular fractions of carp gonads. Sci. Total Environ. 447, 152–159.

Gomes, T., Gonzalez-Rey, Bebianno, M.J., 2009. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana estuary (Portugal). Ecotoxicology 18, 1104–1109.

Hahn, M.E., Lamb, T.M., Schultz, M.E., Smolowitz, R.M., Stegeman, J.J., 1993. Cytochrome P4501A induction and inhibition by 3,3′,4,4′-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). Aquat. Toxicol. 26, 185–208.

Hahn, M.E., Woodward, B.L., Stegeman, J.J., Kennedy, S.W., 1996. Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: response to TCDD, TCDF and two planar PCBs. Environ. Toxicol. Chem. 15, 582–591.

Huuskonen, S.E., Tuvikene, A., Trapido, M., Fent, K., Hahn, M.E., 2000. Cytochrome P4501A induction and porphyrin accumulation in PLHC-1 fish cells exposed to sediment and oil shale extracts. Arch. Environ. Contam. Toxicol. 38, 59–69.

Jung, D.K.J., Klaus, F., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20, 149–159.

Kennish, M.J., 2002. Environmental threats and environmental future of estuaries. Environ. Conserv 29, 78–107.

Kime, D., 1993. Classical and non-classical reproductive steroids in fish. Rev. Fish Biol. Fish 3, 160–180.

Kusakabe, M., Nakamura, I., Young, G., 2003. 11β-Hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in Rainbow trout: cloning, sites of expression, and seasonal changes in gonads. Endocrinology 144, 2534–2545.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol. Appl. Pharm. 196, 247–257.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Liu, S., Govoroun, M., D'Cotta, H., Ricordel, M.J., Lareyre, J.-J., McMeel, O.M., Smith, T., Nagahama, Y., Guiguen, Y., 2000. Expression of cytochrome P45011β (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. J. Steroid. Biochem. Mol. Biol. 75, 291–298.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Lorenzen, A., Kennedy, S.W., 1993. A fluorescence-based protein assay for use with a microplate reader. Anal. Biochem. 214, 346–348.

Martin-Skilton, R., Thibaut, R., Porte, C., 2006. Endocrine alteration in juvenile cod and turbot exposed to dispersed crude oil and alkylphenols. Aquat. Toxicol. 78S, S57–S64.

Monteiro, P.R.R., Reis-Henriques, M.A., Coimbra, J., 2000. Polycyclic aromatic hydrocarbons inhibit *in vitro* ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). Aquat. Toxicol. 48, 549–559.

Morcillo, Y., Porte, C., 1997. Interaction of tributyl- and tri-phenyltin with the microsomal monooxygenase system of molluscs and fish from the Western Mediterranean. Aquat. Toxicol. 38, 35–46.

Ruddock, P.J., Bird, D.J., McCalley, D.V., 2002. Bile metabolites of polycyclic aromatic hydrocarbons in three species of fish from the Severn estuary. Ecotoxicol. Environ. Saf. 51, 97–105.

Schirmer, K., Chan, A.G.J., Greenberg, G.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. Vitro 11, 107–119.

Schlezinger, J.J., White, R.D., Stegeman, J.J., 1999. Oxidative inactivation of cytochrome P-450 1A (CYP1A) stimulated by 3,3′,4,4′-tetrachlorobiphenyl: production of reactive oxygen by vertebrate CYP1As. Mol. Pharm. 56, 588–597.

Schnell, S., Martin-Skilton, R., Fernandes, D., Porte, C., 2009. The interference of nitro- and polycyclic musks with endogenous and xenobiotic metabolizing enzymes in carp: an in vitro study. Environ. Sci. Technol. 43, 9458–9464.

Schnell, S., Olivares, A., Piña, B., Lacorte, S., Porte, C., 2013. Characterization of the environmental quality of coastal sediments from the Northern coast of Spain based on different in-vitro bioassays. Mar. Pollut. Bull. http://dx.doi.org/10.1016/j.marpolbul.2013.09.047 (in press).

Serafim, A., Company, R., Lopes, B., Pereira, C., Cravo, A., Fonseca, V.F., França, S., Bebianno, M.J., Cabral, H.N., 2013. Evaluation of sediment toxicity in different Portuguese estuaries: ecological impact of metals and polycyclic aromatic hydrocarbons. Estuar. Coast. Shelf Sci. 130, 30–41.

Spohn, P., Hirsch, C., Hasler, F., Bruinink, A., Krug, H.F., Wick, P., 2009. C60 fullerene: a powerful antioxidant or a damaging agent? The importance of an in-depth material characterization prior to toxicity assays. Environ. Pollut. 157, 1134–1139.

Strolin-Benedetti, M., Brogin, G., Bani, M., Oesch, F., Hengstler, J.G., 1999. Association of cytochrome P450 induction with oxidative stress in vivo as evidenced by 3-hydroxylation of salicylate. Xenobiotica 29, 1171–1180.

Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P4501A. Toxicol. Vitro 22, 1128–1135.

Willett, K.L., Wassenberg, D., Lienesch, L., Reichert, W., Di Giulio, R.T., 2001. In vivo and in vitro inhibition of CYP1A-dependent activity in *Fundulus heteroclitus* by the polynuclear aromatic hydrocarbon fluoranthene. Toxicol. Appl. Pharm. 177, 264–271.

RESULTADOS ADICIONALES DEL TEMA 1

Sedimentos de la Antártida inducen CYP1A en las células de peces PLHC-1.

Se ha investigado la citotoxicidad y la presencia de agonistas de CYP1A en los sedimentos de la plataforma continental Antártica, con el objetivo de estimar el impacto antropogénico en este área remota y presumiblemente prístina de los océanos mundiales.

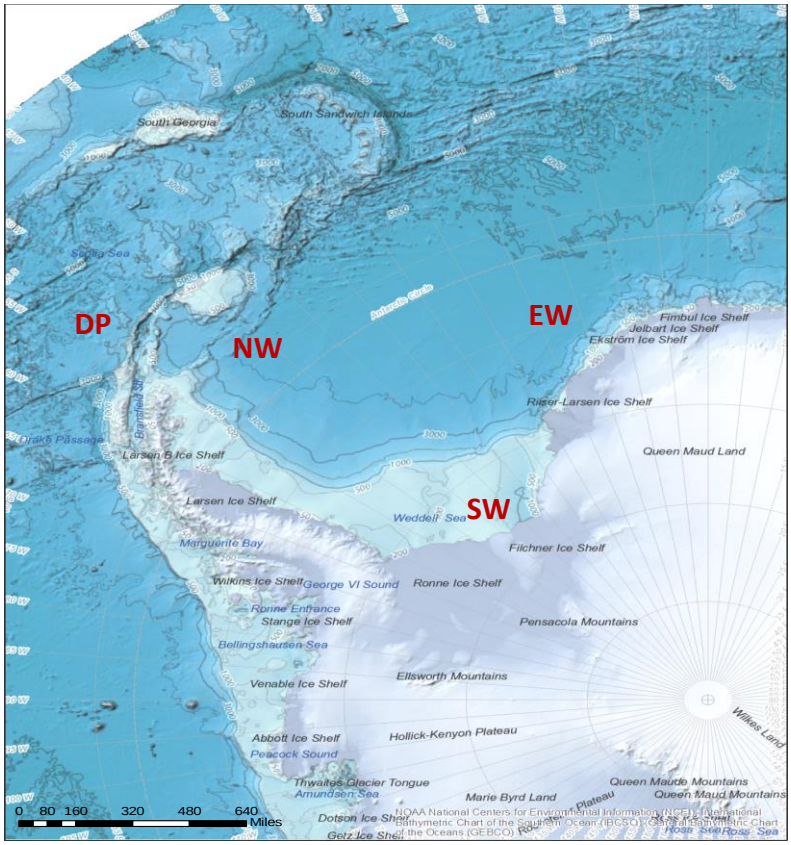


Figura 12: Mapa de las áreas de muestreo en el Mar de Wedell (Antártida). A la zona DP (Drake Passage) corresponden las muestras 202 y 238; a NW (Northwestern Weddell Sea), 120 y 163; a EW (Eastern Weddell Sea), 260, 276, 279, 283); y a SW (Southern Weddell Sea), 108, 145, 153, 198).

Las áreas de estudio están situadas en el Paso de Drake de las islas Shetland del Sur y el Estrecho de Bransfield (DP), Noroeste del Mar de Weddell (NW), plataforma de Filchner en el Atlántico más austral (SW), y plataforma

de Austasen en el mar de Weddell (EW) (Figura 12). Se recolectaron tres cores de sedimento de cada zona, y se utilizaron los primeros milímetros de cada core (sedimento superficial) para la extracción (ver metodología en artículo 1). La línea celular de peces PLHC-1 se utilizó como modelo *in vitro* para determinar la viabilidad celular y actividad EROD tras 24 horas de exposición a los extractos de sedimentos (metodología en artículo 1). Los resultados, mostrados en la Tabla 2 y en la Figura 13, revelaron la citotoxicidad de los extractos de sedimento de las áreas NW y DP, así como su capacidad para inducir significativamente la actividad 7-etoxiresorufina-O-deetilasa (EROD). No se observó citotoxicidad y se detectó baja o ninguna presencia de inductores de CYP1A en los sedimentos de las zonas SW y EW.

Tabla 2: Porcentaje de viabilidad (AB y CFDA) a una concentración de 60 mg eQsed/mL, R_{BNF} (concentración de extracto de sedimento requerida para inducir la misma respuesta que el control positivo β-naftoflavona 1 µM (34 ± 0.67 mg eQsed/mL)), e inducción máxima EROD (pmol resorufina/min/mg proteína), en células PLHC-1 expuestas 24 h a sedimentos de la plataforma continental Antártica.

		Viability (%)		EROD	
Sample areas and criteria	Station number	AB	CFDA	R_{BNF}	Max
Eastern Weddell Sea (EW), Austasen shelf (scientific bases)	260	86 ± 7.5	86 ± 5.9	n.d.	16 ± 1.3
	276	82 ± 8.9	81 ± 7.2	n.d.	30 ± 3.1
	279	61 ± 5.2	71 ± 3.0	85	44 ± 11
	283	88 ± 6.1	89 ± 6.0	111	38 ± 11
Southern Weddell Sea (SW), Filchner Depression (remote area)	108	108 ± 3.9	101 ± 3.2	n.d.	24 ± 3.8
	145	98 ± 5.2	96 ± 3.0	n.d.	8.4 ± 2.1
	153	102 ± 7.5	99 ± 8.9	n.d.	7.7 ± 1.4
	198	100 ± 5.6	93 ± 6.0	n.d.	9.7 ± 0.9
Northwestern Weddell Sea (NW) (boat traffic)	120	43 ± 6.7	55 ± 9.0	16	96 ± 9.8
	163	76 ± 5.1	79 ± 7.1	15	122 ± 16
Drake Passage / Bransfield Strait (DP) (boat traffic)	202	45 ± 4.6	56 ± 11	21	93 ± 5.5
	238	67 ± 7.5	91 ± 8.9	47	68 ± 7.6

Estas importantes revelaciones demuestran que la entrada de sustancias nocivas afecta significativamente al ecosistema Antártico. El caso de la región más septentrional del área de estudio es el más dramático porque coincide con la zona de paso de la mayoría de barcos que viajan a la Antártida, cuya frecuencia ha crecido exponencialmente durante los últimos 30 años. Los barcos y las estaciones de investigación liberan contaminantes al medio ambiente principalmente a través de sus canales de combustión escape, aguas grises, aguas de lastre y capa anti-incrustante de sus quillas. Aunque es probable que estas sustancias se liberen directamente al mar, no hay que olvidar que la nieve también es capaz de incorporar contaminantes orgánicos atmosféricos eficientemente, de manera que transfiere su contenido al hielo marino y glaciares que actúan como reservorios de substancias perjudiciales, en escalas de tiempo en el rango de días a milenios (Nazarenko et al., 2016; Isla et al., en preparación). Esta gran escala de tiempo se debe a que la persistencia de los xenobióticos en la región Antártica es mayor que en bajas latitudes, dado que los contaminantes están protegidos de la fotodegradación por los pocos niveles lumínicos, y de la degradación microbiana por las bajas temperaturas. Estos hallazgos sugieren claramente que las actividades antropogénicas generan un notable impacto en la plataforma continental antártica amenazando a las ricas y diversas comunidades bentónicas, anteriormente consideradas como relativamente aisladas de la contaminación. La investigación sigue abierta para identificar las fuentes potenciales de estos contaminantes y sus registros históricos.

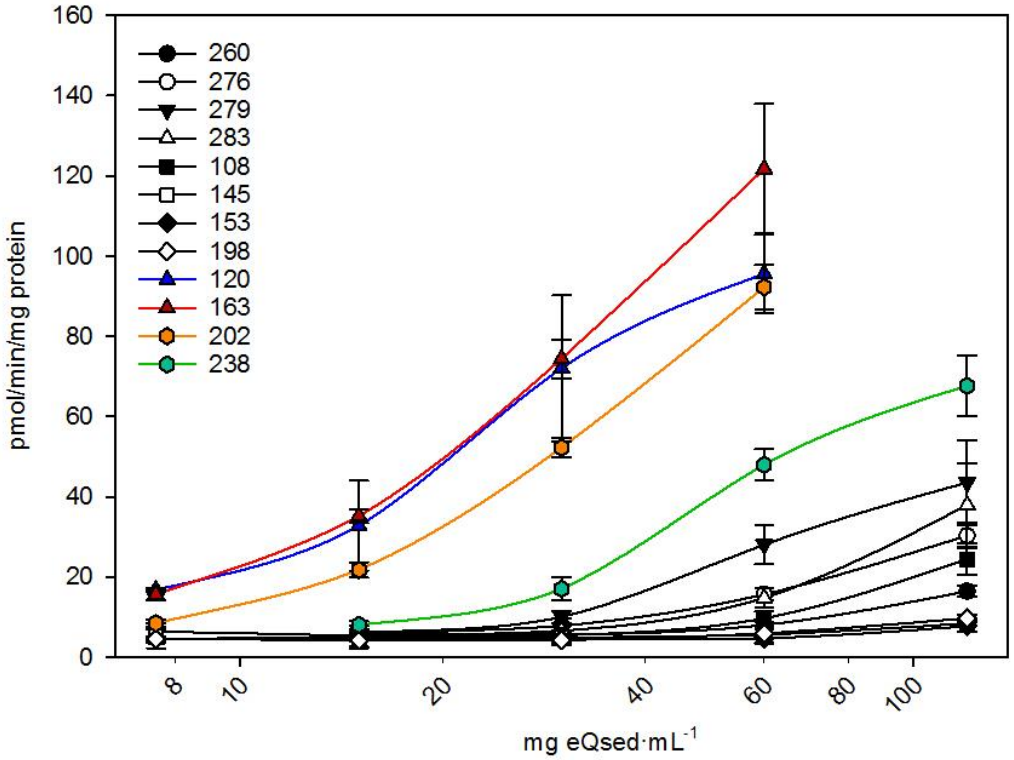


Figura 13: Inducción de actividad EROD en la línea celular PLHC-1, por los sedimentos de la Antártida. El agente inductor β-naftoflavona se incluyó en los ensayos como control positivo, mostrando una inducción de actividad EROD de 34.4 ± 0.67 pmol/min/mg proteína.

TEMA 2.

EVALUACIÓN DE LA TOXICIDAD DE CONTAMINANTES EMERGENTES

ARTÍCULO 5

Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells

E. Pérez-Albaladejo, D. Fernandes, S. Lacorte, C. Porte

Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

Toxicology in Vitro (2017), 38, 41-48

RESUMEN

Los plastificantes son aditivos químicos que confieren elasticidad y flexibilidad a los polímeros utilizados en la fabricación de numerosos productos de consumo incluyendo envases de alimentos y bebidas. Son compuestos potencialmente tóxicos, que pueden interferir con el sistema endocrino humano y cruzar la barrera placentaria, de manera que el período embrionario es particularmente vulnerable. Este trabajo investiga la toxicidad comparada y habilidad para interferir con la síntesis de esteroides y generar especies reactivas de oxígeno (ROS), de un grupo de plastificantes ampliamente distribuidos, incluyendo bisfenol A (BPA), nonilfenol (NP), octilfenol (OP), bencil butil ftalato (BBP), dibutil ftalato (DBP), di(2-etilhexil)ftalato (DEHP) y dimetil ftalato (DMP), en células de placenta humana JEG-3. Además, se ha investigado la biodisponibilidad de estos compuestos en el medio de cultivo inmediatamente después de añadirlo al pocillo de la placa (T0) y tras 24 horas de exposición (T24). Después de 24 h de exposición, OP y NP mostraban la más alta toxicidad (EC_{50} entre 36 y 40 μM), seguidos por BPA (138-219 μM), mientras que no se observó

citotoxicidad significativa para los ftalatos. A pesar de ello, BBP y DBP disminuían la actividad P450 aromatasa (IC_{50} experimental entre 14 y 15 μM), mientras NP y OP (20 μM) incrementaban la actividad del enzima. En conjunto, este estudio evidencia la toxicidad diferencial y la habilidad para modular la actividad P450 aromatasa en las células de placenta, de algunos compuestos ampliamente utilizados como plastificantes, y destaca la necesidad de determinar la biodisponibilidad de los ftalatos para mejorar la sensibilidad de los ensayos *in vitro*.

Toxicology in Vitro 38 (2017) 41–48

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells

Elisabet Pérez-Albaladejo *, Denise Fernandes, Silvia Lacorte, Cinta Porte *

Environmental Chemistry Department, IDAEA–CSIC, C/ Jordi Girona 18-26, 08034 Barcelona, Spain

ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 22 September 2016
Accepted 2 November 2016
Available online 04 November 2016

Keywords:
P450 aromatase
ROS generation
JEG-3 cells
Bioavailability
Plasticizers

ABSTRACT

Plasticizers are suspected to be toxic and/or to modulate or disrupt the endocrine system of humans and to cross the placental barrier, being embryonic and fetal development a particularly vulnerable period. This work investigates the comparative toxicity and ability to interfere with the synthesis of steroids and to generate reactive oxygen species (ROS) of a selected number of plasticizers, including bisphenol A (BPA), nonyl- (NP) and octylphenol (OP), benzyl butyl phthalate (BBP), dibutyl phthalate (DBP), di(2-ethylhexyl)phthalate (DEHP) and dimethyl phthalate (DMP), in the human placenta JEG-3 cells. Moreover, the bioavailability of chemicals in culture medium has been investigated. After 24 h exposure, OP and NP showed the highest cytotoxicity (EC_{50}: 36–40 μM) followed by BPA (138–219 μM), whereas no significant toxicity was observed for phthalates. Notwithstanding, BBP and DBP significantly decreased P450 aromatase activity (experimental IC_{50}: 14–15 μM), while NP and OP (20 μM) increased the activity. Overall, this study evidences the differential toxicity and ability to modulate placental aromatase activity of some of the compounds nowadays used as plasticizers, and highlights the need of an accurate determination of the bioavailability of chemicals to improve the sensitivity of in-vitro tests.

1. Introduction

Plasticizers are man-made chemicals mainly used to improve the elasticity, flexibility, color, resistance and/or durability of polymers. As they are not chemically bound, they can leach, migrate into food and liquids or evaporate, and they have become ubiquitous environmental contaminants. Humans are continuously exposed to these compounds during their lifetime through oral, inhalation and dermal exposure. Phthalates, bisphenol A (BPA) and alkylphenols are among the most widely used plasticizers.

Phthalates are used in different consumer products such are cosmetics, pharmaceuticals, insecticides, adhesives, toys and food/beverage packaging, being food a major source of exposure to humans (Fasano et al., 2012). Some of the most commonly used phthalates are di-(2-ethylhexyl) phthalate (DEHP), butyl benzyl phthalate (BBP), dibutyl phthalate (DBP) and dimethyl phthalate (DMP) (Table 1). Tolerable daily intakes (TDIs) have been specified by the European Food Safety Authority (EFSA) for some of these compounds: viz. 10 μg/kg body weight/day for DBP, 50 for DEHP and 500 for BBP. They have been detected in human blood and umbilical cord at concentrations up to 0.09 μg/mL BBP (~0.3 μM), 0.26 μg/mL DBP (~0.9 μM), 4.7 μg/mL DEHP (~12 μM), and 0.01 μg/mL DMP (~0.06 μM) (Latini et al., 2003; Huang et al., 2014).

BPA is used in combination with other chemicals in polycarbonate based plastics bottles, tableware and storage containers. Residues of BPA are also present in epoxy resins used to make protective coatings and linings for food and beverage cans and vats. In the last years, the European Commission banned BPA for the manufacture of infant feeding bottles, and the European Food Safety Authority reduced the specific TDI from 50 to 4 μg/kg/day (EFSA, 2015). BPA has been detected in umbilical cord blood, amniotic fluid and maternal and fetal blood (Vandenberg et al., 2007).

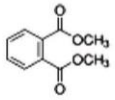
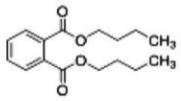
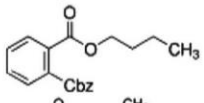
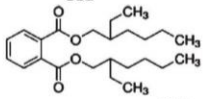
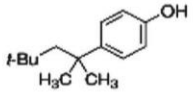
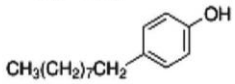
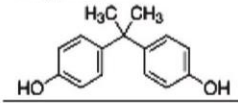
On the other hand, alkylphenols (APs) are degradation products of alkylphenol ethoxylates widely used in lubricants, detergents, resins, herbicides, cosmetics and as additives in plastics. Nonylphenol (NP) represents around 80% of APs production, while octylphenol (OP) make up most of the remaining 20%. In humans, the main sources of exposure to APs are inhalation, ingestion of contaminated food and dermal absorption. APs have been detected in human blood and in umbilical cord at concentrations reaching 210 ng/g NP (~1 μM) and 1.15 ng/mL OP (~0.01 μM) (Tan and Mohd, 2003; Chen et al., 2008). No TDI has been established for OP or NP in Europe. However, the European Commission has restricted to <0.1% the use of NP in cleaning products, textiles, paper and personal care products.

Plasticizers are suspected to be toxic and/or to modulate or disrupt the endocrine system of humans and wild-living animals that are daily exposed to those environmental pollutants. Thus, BPA decreased

* Corresponding authors.
E-mail addresses: ealqam@cid.csic.es (E. Pérez-Albaladejo), cinta.porte@cid.csic.es (C. Porte).

http://dx.doi.org/10.1016/j.tiv.2016.11.003
0887-2333/

Table 1
Physicochemical properties of the studied compounds. Sources: Cousins and Mackay (2000); https://scifinder.cas.org, 2015. MW: molecular weight.

Molecule	Compound	CAS no	MW	K_{ow}	Water solubility (μM)
	Dimethyl phthalate	131-11-3	194.18	1.6	21,000
	Dibutyl phthalate	84-74-2	278.34	4.1	40
	Butyl benzyl phthalate	85-68-7	312.36	4.7	10
	Di(2-ethylhexyl) phthalate	117-81-7	390.56	7.3	1
	4-*tert*-octylphenol	140-66-9	206.32	5.2	300
	4-*tert*-nonylphenol	84852-15-3	220.35	6.1	94
	Bisphenol A	80-05-7	228.29	3.6	310

cell viability in primary human endometrial endothelial cells (>100 μM) and in BeWo cells (EC_{50} = 100–125 μM) (Bredhult et al., 2007; Mørk et al., 2010), suppressed CYP19 activity in human fetal osteoblastic (SV-HFO) and ovarian granulosa-like (KGN) cell lines (>5 μM), and it is associated with recurrent miscarriage in women (Sugiura-Ogasawara et al., 2005; Watanabe et al., 2012), among other effects. Regarding phthalates, DEHP inhibited testosterone production in human adrenocortical cells NCI-H295R and suppressed steroidogenesis in human testis explants (Desdoits-Lethimonier et al., 2012). Some phthalates, such as BBP, DBP and DEHP, may bind to the estrogen receptor and have a weak estrogenic activity, enhancing the proliferation of MCF-7 cells (Harris et al., 1997; Okubo et al., 2003), while such estrogenic effects was not detect in-vivo (Milligan et al., 1998). Moreover, exposure to plasticizers leads to the activation of peroxisome proliferator-activated receptors, the increase of fatty acid oxidation, and the reduction in the ability to cope with the augmented oxidative stress. Oxidative stress, a state characterized by an imbalance between pro-oxidant molecules, including reactive oxygen species, and antioxidant defenses, has been identified to be associated to reproductive organ malformations, reproductive defects, and decreased fertility (Mathieu-Denoncourt et al., 2015).

Plasticizers have been reported to cross human placenta and to reach the fetus, being embryonic and fetal development a particularly vulnerable period (Ikezuki et al., 2002; Latini et al., 2003; Tan and Mohd, 2003; Vandenberg et al., 2007; Chen et al., 2008; Wittassek et al., 2009; Balakrishnan et al., 2010). However, studies in humans are limited due to obvious ethical considerations and the difficulty in identifying individuals not exposed to these compounds. Therefore, alternative methods based in the use of different cell lines have been used. Among them, the human placental choriocarcinoma JEG-3 cell line is a good model, since it expresses the specific steroidogenic enzymes found in placenta and has a very high aromatase activity (Samson et al., 2009). Placental aromatase, encoded by CYP19, catalyzes the aromatization of fetal and maternal androgens into estrogens, which are essential for a healthy pregnancy. It is a key enzyme in the biosynthesis of estrogens and a critical end-point often targeted by endocrine disrupters (Vinggaard et al., 2000). Moreover, exposure to plasticizers at early life stages is suspected to cause irreversible damage and developmental abnormalities, which may not be manifested until later in life. It is assumed that fetuses (i.e., pregnant women) and children are especially vulnerable groups. Thus, reduction of gestational age, low birth weight and dysmorphic disorders of the genital tract detected in male infants have been significantly associated with prenatal exposure to phthalates and/or alkylphenols (Swan et al., 2005; Huang et al., 2014).

Thus, the aim of this work was to assess the cytotoxicity and ability to disrupt P450 aromatase activity and to produce oxidative stress of a group of widely used plasticizers, including BBP, DBP, DEHP, DMP, BPA, NP and OP, in the human placental choriocarcinoma cell line JEG-3. Moreover, concentration of the plasticizers in culture medium was monitored in order to assess their bioavailability and to relate observed effects to experimental rather than nominal concentrations.

2. Material and methods

2.1. Chemicals and solutions

Eagle's Minimum Essential Medium (MEM), fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin and streptomycin, phosphate buffered saline (PBS), phosphate buffered saline with Ca and Mg (DPBS) and trypsin-EDTA 0.25% were from Gibco BRL Life Technologies (Paisley, Scotland, UK). 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCF-DA), 3-morphoolinosydnonime (SIN-1) and plasticizers (Table 1) were purchased from Sigma (Steinheim, Germany). Alamar Blue (AB) was from Biosource (Solingen, Germany) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) from Molecular Probes (Eugene, OR, USA). 1β ^{3}H-androst-4-ene-3,17-dione (^{3}H-AD) was from PerkinElmer, Ink (Boston MA, USA). Stock solutions of plasticizers were prepared in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany).

2.2. Cell culture

JEG-3 cells, derived from a human placental carcinoma, were obtained from the American Type Culture Collection (ATCC-HTB-36). They were routinely grown in Eagle's MEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL of penicillin and 50 μg/mL streptomycin. Cells were cultured in 75 cm^2 polystyrene flasks (Corning; NY, USA) in a humidified incubator with 5% CO_2 at 37 °C. When 90% confluence was reached, cells were dissociated with 0.25% (w/v) trypsin and 0.9 mM EDTA for subculturing and experiments.

2.3. Cytotoxicity

Cytotoxic effects of plasticizers in JEG-3 cells after 24 h exposure were measured using two fluorescent dyes. Alamar Blue (AB) to monitor metabolic activity, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) to evaluate cell membrane integrity as described in Schirmer et al. (1997). Cells were seeded in 96-well plates at a rate of 2.5×10^4 cells per well and left to grow 24 h before adding the selected plasticizers dissolved in DMSO. Control wells received culture medium containing 0.5% DMSO. After 24 h exposure to plasticizers, cells were rinsed with PBS and incubated for 1 h with the dye solution (5% *v/v* AB and 4 mM CFDA-AM) in modified Leibovitz L-15 medium without vitamins neither amino acids. Cell viability was measured using a fluorescence plate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths of 530/590 nm for AB, and 485/530 nm for CFDA-AM. Cell viability was expressed as a percentage of control cells.

2.4. Reactive oxygen species (ROS) generation

The production of ROS in JEG-3 cells in the presence of the different plasticizers was determined by using the method of LeBel et al. (1992) with slight modifications. Cells were seeded into 96-well plate (4×10^4 cells per well). On the day of the experiment, cells were rinsed with PBS and incubated with 20 μM H_2DCF-DA in 1:10 DPBS solution supplemented with 10 mM glucose. After 30 min incubation, cells were washed with PBS and exposed to plasticizers diluted in DPBS-Glucose in order to stimulate ROS production. 3-Morpholinosydnonimine (SIN-1; 5 μM) was used as a positive control. The fluorescence of oxidized DCF was measured 15, 30, 60 and 120 min after in a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelength pairs of 485/528 nm. Results were expressed as a percentage of the basal fluorescence in control wells.

2.5. P450 aromatase activity

Aromatase activity in human placental cells JEG-3 was determined by measuring the amount of ^3H_2O formed during the aromatization of [1β-3H]androstenedione (^{3}H-AD) as described in Lephart and Simpson (1991), with some modifications. Cells were seeded in 24 well-plates at a density of 10^5 cells per well and allowed to attach overnight before exposure to the plasticizers or 0.5% DMSO (carrier) in culture medium. After 24 h exposure (37 °C, 5% CO_2), cells were rinsed with PBS and incubated for 60 min in the presence of 40 nM ^{3}H-AD in DPBS-Glucose. The reaction was stopped by placing the plate on ice and aspirating 1 mL of medium that was extracted with 3 mL of dichloromethane (×3). The amount of titrated water formed was counted in an aliquot of the aqueous phase (Tri-Carb 2100TR, Packard). Aromatase activity was expressed in fmol/min/mg protein or as percentage of activity in solvent control cells.

Additionally, P450 aromatase activity was determined in S9 fractions isolated from JEG-3 cells as follows. Cells were rinsed twice with PBS before being detached from culture flasks with trypsin-EDTA. After centrifugation, trypsin-EDTA was removed and cells were resuspended in cold potassium phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 20% glycerol. Then, cells were homogenized by sonication in ice, centrifuged at 9000*g* for 20 min and the supernatant immediately stored at −80 °C until use. Proteins were measured according to Bradford (1976), using bovine serum albumin as standard. S9 fractions (3–5 mg protein/mL) were incubated with the different plasticizers (nM to μM range) dissolved in DMSO (0.5% DMSO in the assay) at 37 °C for 30 min in a final volume of 1 mL, in the presence of 100 mM Tris-HCl pH 7.6, 40 nM ^{3}H-AD and 200 μM NADPH. The reaction was stopped by adding 3 mL of ice-cold dichloromethane, and organic metabolites and the excess of substrate eliminated from the aqueous phase by extraction. The possible remaining titrated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 mL). The solution was centrifuged (10 min × 3600 rpm), and two aliquots of the supernatant (1 mL) were counted for ^{3}H-radioactivity with a liquid scintillation counter (Tri-Carb 2100TR, Packard).

2.6. Analysis of plasticizers in exposure medium

Cells were seeded in 96-well plates at a rate of 2.5×10^4 cells per well and left to grow 24 h before adding the selected plasticizers in the range of 5–500 μM. An aliquot of the exposure medium (90 μL) was taken right after dosing (T0) and 24 h after (T24). Each sample consisted in a pool of three wells, and three samples were analyzed per treatment and dose. Samples were extracted with ethyl acetate (×3) and the obtained extracts evaporated to dryness.

For the analysis of OP, NP and BPA, dry residues were resuspended in acetonitrile:water (1:1) and analyzed by HPLC coupled with a diode array detector (DAD) in a Agilent Technologies 1200 series system equipped with an analytical Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm column (Agilent, USA) protected by a guard column C18, 4.0 × 3.0 mm (Phenomenex, USA). Separation of target compounds was performed using a mobile phase composed of A) water and B) acetonitrile. The run consisted on a 32 min gradient starting at 20% B (2 min), to 50% B in 11 min, condition that was maintained for 2 min, and to 100% B in 15 min, and this condition was maintained for 2 min and initial conditions were regained in 3 min. The flow rate was set at 1 mL/min. The elution of compounds was monitored at 210 nm (BPA) and 222 nm (NP and OP) using a G1315D DAD model (Agilent 1200 series). Compounds were quantified by integrating the area under the peaks and using an external calibration curve.

For the analysis of phthalates, the dry extract was dissolved in isooctane and analyzed by gas chromatography–mass spectrometry (GC–MS) in electron ionization mode (70 eV) using an Agilent 6890 series GC system coupled with an Agilent 5973 Network mass selective detector. The column, a HP-5MS 30 m × 0.25 mm i.d., film thickness 0.25 μm (Agilent J&W), was programmed from 90 °C to 140 °C at 10 °C/min and from 140 to 300 °C at 4 °C/min. The carrier gas was Helium at 80 kPa. The injector temperature was 280 °C and the ion source and the analyzer were maintained at 230 °C and 150 °C, respectively. Calibration curves were performed with pure standards over a concentration range from 0.05 to 1.0 μg/mL. Acquisition was performed in selected ion monitoring mode (SIM). The ions used for monitoring and quantification were: *m/z* 163 for DMP; *m/z* 149 and 223 for DBP; *m/z* 206, 149 and 91 for BBP; and *m/z* 279, 167 and 149 for DEHP. Identification of phthalates was performed by comparison of retention times and the mass spectra with those of reference compounds. To ensure the reliability of the results, instrumental and method blanks were analyzed.

2.7. Statistical analysis

Dose-response curves and concentrations which caused a 50% decline of cell viability (EC_{50}) and enzymatic activity (IC_{50}) were calculated by using Sigmaplot 11.0 software. Statistical differences between

each test and the respective control were analyzed by one-way ANOVA with Dunnett test, using SPSS 19.0, except in the cases of inequality of variance and no normal data, which were analyzed with non-parametric signed rank Mann-Whitney. Level of significance was set at $p < 0.05$.

3. Results

3.1. Cytotoxicity

OP and NP had the strongest cytotoxic effect in JEG-3 cells with estimated nominal EC_{50} values in the range of 35–40 μM, followed by BPA (138–218 μM) (Fig. 1; Table 2). Comparatively, DBP was less cytotoxic (466 ± 63 μM). No significant cytotoxicity was observed for the other phthalates - BBP, DEHP and DMP - (Fig. 1; Table 2).

3.2. Reactive oxygen species (ROS)

The oxidation of H2DCF was monitored after 15, 30, 60 and 120 min exposure to plasticizers. Phthalates (5 to 500 μM) did not induce the generation of ROS in JEG-3 cells, while BPA, NP and OP did. Exposure to 40 μM OP lead to 3.3-fold induction of ROS after 15 min exposure; ROS generation decreased over time when OP was tested at a concentration of 100 μM. Exposure of JEG-3 cells to 40 to 100 μM NP for 15 min lead to a 2.9-fold increase in ROS production (Fig. 2), and similarly to OP, a decrease in ROS production along time was observed

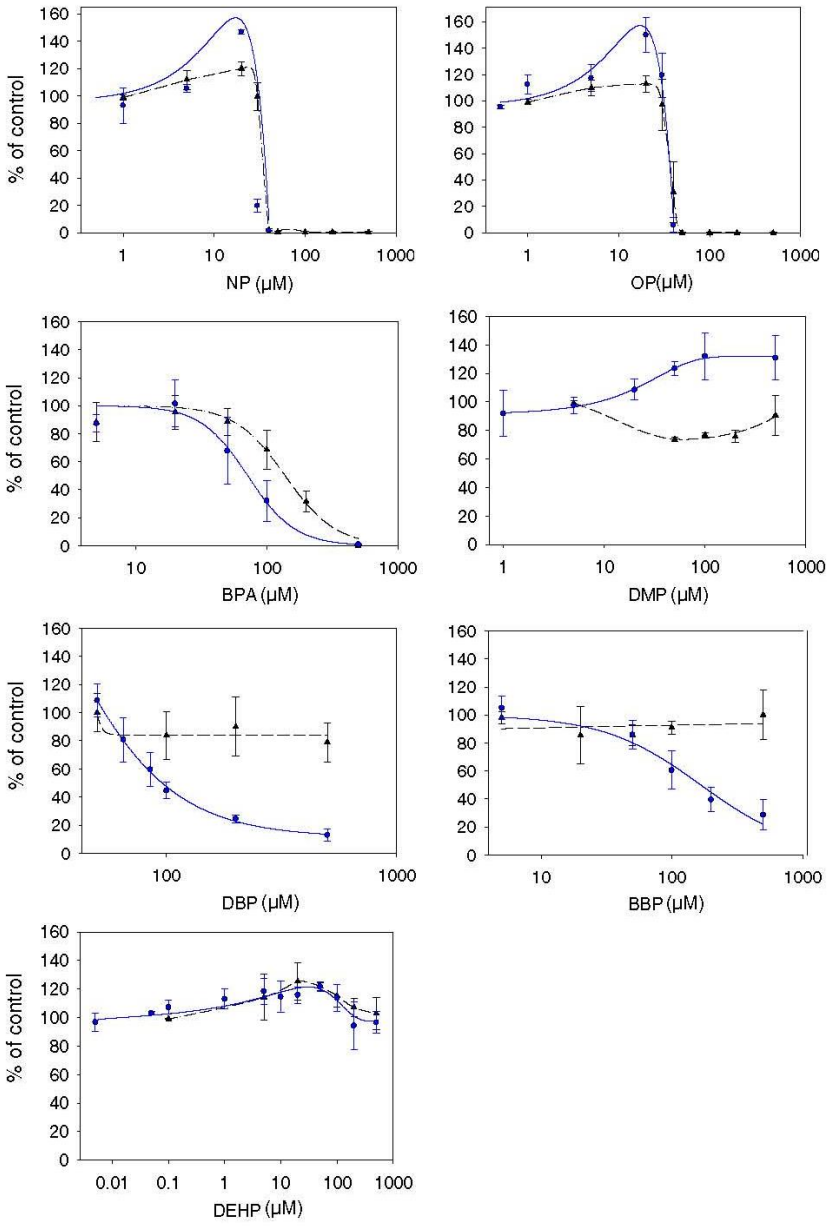


Fig. 1. Cytotoxicity measured with Alamar Blue (dotted line) and P450 aromatase activity (solid line) in JEG-3 cells exposed to plasticizers in the range of 0.01 to 500 μM (nominal concentration) for 24 h. Results are expressed as percentage of control cells as mean ± SD of at least three different assays. Specific activity of P450 aromatase in control cells was 280 ± 38 fmol/min/mg of protein.

Table 2
Cytotoxicity of plasticizers in JEG-3 cells after 24 h exposure (EC_{50}); inhibition of P450 aromatase activity (IC_{50}) determined in either JEG-3 cells or S9 fraction isolated from JEG-3 cells. n.d.: not detected.

Compound	Cytotoxicity EC_{50}		P450 aromatase IC_{50}	
	AB	CFDA	S9	JEG-3
DMP	n.d.	n.d.	n.d.	n.d.
DBP	n.d.	466 ± 63	63.5 ± 6.2	104 ± 9
BBP	n.d.	n.d.	38.6 ± 7.8	166 ± 31
DEHP	n.d.	n.d.	n.d.	n.d.
OP	38.0 ± 0.1	40.0 ± 0.01	74.2 ± 7.5	n.d.
NP	35.5 ± 0.5	36.3 ± 3.6	39.8 ± 4.1	n.d.
BPA	138 ± 13	218 ± 14	93.7 ± 1.8	71 ± 7

when tested at a concentration of 100 μM, probably due to cytotoxicity. BPA was less efficient in oxidizing H2DCF; ROS generation increased from 1.7 to 2-fold after 15 min exposure to 50–100 μM BPA (Fig. 2). A 2.6-fold increase of ROS production was observed after 15 min exposure to 5 μM 3-Morpholinosydnonimine (SIN-1) that was used as positive control; a maximum of 28-fold induction was observed after 120 min.

3.3. P450 aromatase activity

JEG-3 cells were exposed to the selected plasticizers in a wide range of nominal concentrations, from 1 nM to 500 μM. No significant effects on aromatase activity were observed in cells exposed to the plasticizers at concentrations below 1 μM. BPA and the phthalates, BBP and DBP, inhibited aromatase activity in JEG-3 cells after 24 h exposure, with IC_{50}s of 71, 167 and 104 μM, respectively (Fig. 1; Table 2). In contrast, exposure of JEG-3 cells to OP and NP for 24 h led to an increase of aromatase activity, reaching a maximum at 20 μM (50% increase); the activity decreased drastically at higher concentrations, probably associated to the toxicity of both compounds (aromatase IC_{50} ~ cytotoxicity EC_{50} ~ 36–40 μM; Fig. 1, Table 2). Aromatase activity was significantly increased in cells exposed to DMP (32%) at concentrations above 50 μM. DEHP had no significant effect (Fig. 1).

S9 subcellular fractions isolated from JEG-3 were used as a source of aromatase enzyme and the interference of plasticizers with the catalytic activity investigated after 30 min incubation. Among the tested compounds, BBP and NP (IC_{50} 40 μM) were the strongest inhibitors of aromatase activity in S9 assays, followed by DBP, OP and BPA (IC_{50} 64, 74 and 94 μM, respectively). DEHP and DMP (1–500 μM) had no inhibitory effect (Table 2).

3.4. Analysis of plasticizers in exposure medium

The concentration of plasticizers was determined in culture medium immediately after dosing (T0) and after 24 h exposure (T24). Interestingly, the analysis of blank samples allowed the detection of significant amounts of DEHP (0.62 ± 0.07 μM) and DBP (0.15 ± 0.01 μM) in the culture medium, the origin of which was probably migration from polyethylene terephthalate plastic bottles into the stored culture medium. The other plasticizers investigated were below detection limit.

Among the tested plasticizers, BPA was the only compound for which nominal and experimental concentrations were not significantly different (Fig. 3). Experimental concentrations of NP and OP were close to nominal when tested at low concentrations (<100 μM); however, they progressively decreased to 60% of nominal when tested at 500 μM. For the phthalates (DEHP, BBP and DBP) experimental concentrations were far below nominal doses, both at T0 and T24. Measured concentrations of DEHP gradually decreased from 103% of nominal dose at 5 μM to 10% of nominal dose at 500 μM when measured at T0, and from 80% to 2% of nominal dose, when measured at T24 (Fig. 3). Similarly, the experimental concentrations of DBP and BBP were below nominal dose (30% to 60%), the major loses detected at high concentrations after 24 h of exposure (T24). In contrast, the experimental concentration of DMP was close to nominal at T0, and 60–70% of nominal at T24 (Fig. 3).

When measured concentrations were used to re-calculate EC_{50} cytotoxicity values, significant differences were detected for DBP (experimental EC_{50} = 38.8; Table 3), but not for OP, NP and BPA. Regarding aromatase activity, re-calculated IC_{50} values based on experimental concentrations for DBP and BBP were significantly lower (13.8 and 14.7 μM; Table 3) than those based on nominal concentrations (104 and 166 μM, respectively; Table 2).

4. Discussion

Cytotoxic effects following exposure to alkylphenols and BPA have often been detected in animal and human cells involved in reproductive functions. Thus, Bonefeld-Jørgensen et al. (2007) reported decreased viability on JEG-3 cells exposed for 18 h to 1 mM NP or OP. Gong and Han (2006) showed that 20 μM NP significantly increased ROS production in rat Sertoli cells, leading to a loss of mitochondrial membrane potential and lipid peroxidation, and subsequently, a decrease in cell viability. Similarly, 1 μM BPA was cytotoxic to primary human placental cells after 24 h exposure (Benachour and Aris, 2009), and concentrations as low as 0.1 nM BPA were cytotoxic for primary murine ovarian granulosa cells exposed for 72 h (Xu et al., 2002). In the present work, NP, OP and

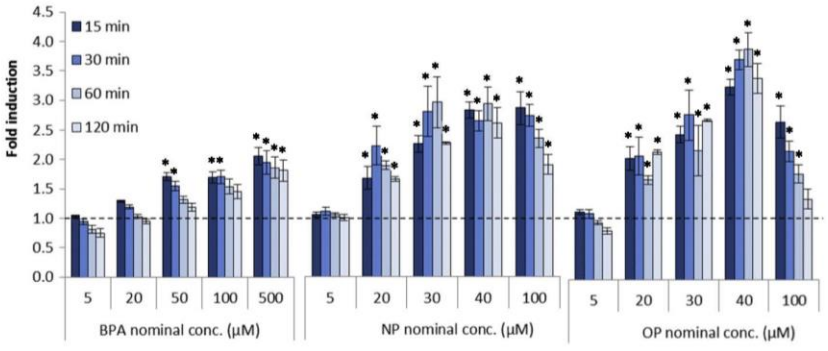


Fig. 2. ROS production in JEG-3 cells after 15, 30, 60 and 120 min exposure to BPA, OP and NP. Values are expressed as fold induction as mean ± SD of at least six replicates in three different plates assayed. Dotted line represents ROS production in control cells. *Statistically significant differences from control ($p < 0.05$).

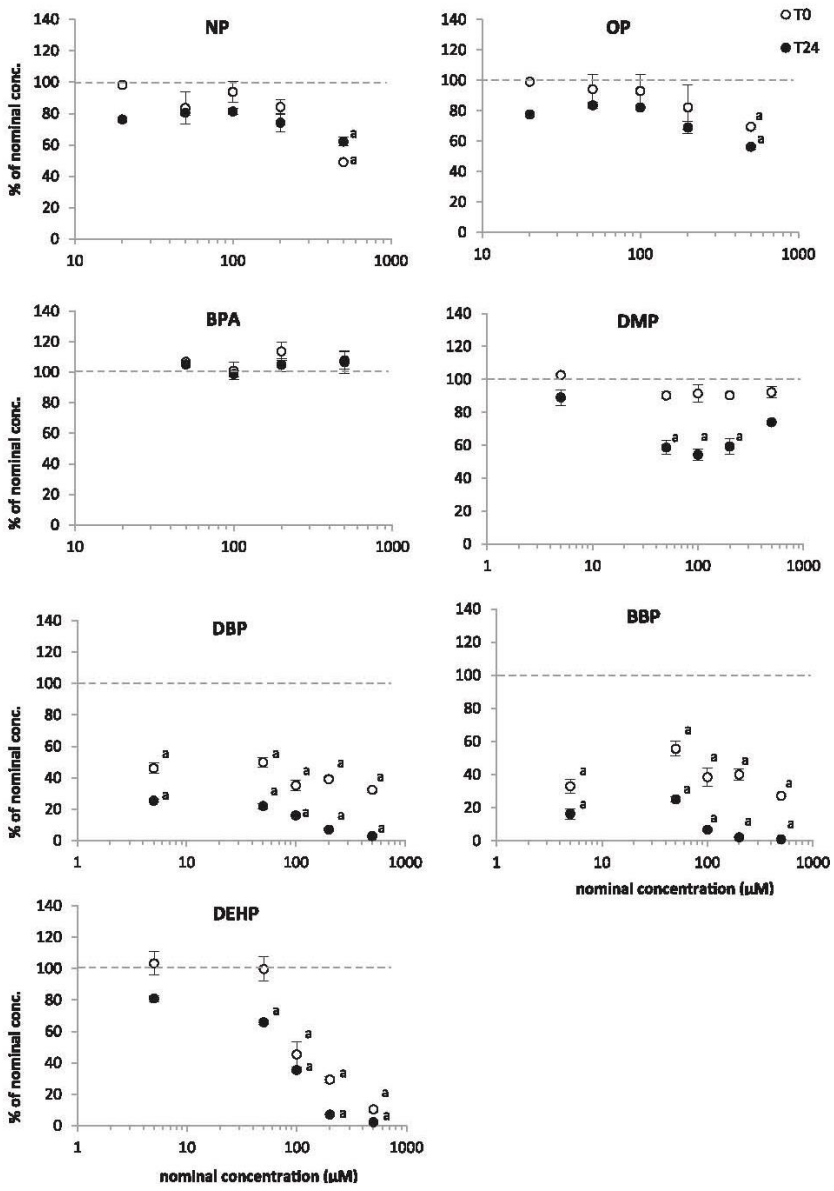


Fig. 3. Nominal concentrations of tested plasticizers versus percentage of nominal concentrations detected in culture medium right after dosing (white circles) and 24 h after (black circles). Values below 100% (dotted line) indicate a reduced presence of the respective chemical in culture medium. Concentrations are expressed in µM, mean ± SD of three replicates. [a]Statistically significant differences from nominal concentration ($p < 0.05$).

Table 3
Cytotoxicity (EC_{50}) and P450 aromatase activity (IC_{50}) calculated respect to measured concentrations at T24. Results are expressed as µM ± SEM. n.d.: not detected.

Compound	AB	CFDA	P450 aromatase JEG-3
DBP	n.d.	38.8 ± 6.2*	13.8 ± 1.1*
BBP	n.d.	n.d.	14.7 ± 1.5*
OP	28.7 ± 0.9	30.0 ± 1.89	n.d.
NP	27.9 ± 7.2	28.7 ± 5.3	n.d.
BPA	141 ± 14	230 ± 31	72.0 ± 6.8

* Significant differences with the homologous values calculated with nominal concentrations (Table 2).

BPA, generated cytotoxicity and induced intracellular ROS production in JEG-3 cells in a dose dependent manner, suggesting a role of ROS in decreasing cell viability. Interestingly, when culture medium was supplemented with 1 to 40 mM vitamin E (D-α-tocopherol succinate), a significant increase in the cytotoxicity of NP (25 µM), OP (30 µM) and BPA (100 µM) was observed (Fig. 1, supplementary info). Similarly, Aydogan et al. (2008) reported oxidative damage in the brain of male rats exposed to BPA, NP and OP, and co-administration of vitamin C along with these compounds aggravated the oxidative damage. ROS generation was also detected in mouse hippocampal HT-22 cells exposed to 100 µM BPA, resulting in decreased cell viability (Lee et al., 2008).

Endocrine disrupting chemicals (ED) can affect aromatase activity by directly reacting with the enzyme or through indirect mechanisms that can result in up- or down- regulation. In the present study, the S9 assay measured interaction of the test chemicals with the P450 aromatase enzyme complex without the problems of reduced bioavailability of the compounds in culture medium (viz. phthalates) and the need to penetrate through the cell membrane. The S9 assay allowed the detection of BBP and NP (IC_{50} ~ 40 μM) as the strongest inhibitors of P450 aromatase activity, followed by DBP, OP and BPA (IC_{50} ~ 64–94 μM), while DEHP and DMP did not disturb the activity of the enzyme. On the other hand, the aromatase assay performed in intact cells, integrates uptake and metabolism of the compounds as well as up −/down-regulation of the enzyme or modulation of signal transduction pathways, among others. This allowed the detection of an estrogenic effect of NP, OP (50% induction of the activity), and DMP (30%); whereas, BPA and the phthalates DBP and BBP inhibited the enzyme (IC_{50} 71, 104 and 167 μM, respectively). The IC_{50} for BBP was 4-fold higher in intact cells, probably associated to the low bioavailability of the compound in culture medium and therefore the need for higher exposure concentrations to reach the target (Fig. 3).

NP and OP increased aromatase activity in JEG-3 cells in a very narrow range of concentrations (20–30 μM), but the activity drastically decreased at higher concentrations due to cytotoxicity, but also to inhibition of the enzyme, as shown in the S9 assay (IC_{50} for NP: 40 ± 4 μM). The detection of increased aromatase activity in JEG-3 cells after exposure to NP and OP is of particular relevance since classical toxicological studies tend to work with large dilution factors (i.e. 1:10) to cover a wide range of concentrations and might often fail to see those effects that occur in a narrow range of concentrations. Bonefeld-Jørgensen et al. (2007) reported that NP, and to lesser extent OP, caused a dose dependent inhibition of aromatase activity in the range of 0.001 to 10 μM in JEG-3 cells after 18 h exposure. Interestingly, the experimental set-up was different from ours, as they exposed cells at ~50% confluence in serum-free medium and this might have slightly changed the sensitivity of the assay. To our knowledge, this is the first report showing that NP and OP increase aromatase activity in human placental cells at concentrations close to their EC_{50} for cytotoxicity. The mechanisms through which OP and NP induce aromatase activity may be multiple, including, impairment of detoxification mechanisms, up-regulation of CYP19, regulation at the post-translational level and/or changes in signaling pathways, among others.

In contrast, BPA inhibited CYP19 both, in S9 incubations (IC_{50} = 94 ± 2 μM) and JEG-3 cells (IC_{50} = 71 ± 7 μM), with a slightly lower IC_{50} in the latter, which might be in agreement with a down-regulation of the enzyme in JEG-3 cells. Interestingly, Nativelle-Serpentini et al. (2003) reported a dose-related inhibition of aromatase activity in JEG-3 cells by BPA (25–100 μM) after 18 h exposure, while short exposure times (2–6 h) enhanced the activity. The authors did not observe alteration of CYP19 expression and concluded that BPA directly interacted with the enzyme. However, several years after, decreased CYP19 mRNA expression in JEG-3 cells after 24 h exposure to BPA (5–100 μM) was described (Huang and Leung, 2009; Quignot et al., 2012). Also a down-regulation of CYP19 gene transcription was detected in human osteoblastic SV-HFO and ovarian KGN cell lines after exposure to BPA (Watanabe et al., 2012). Moreover, the observed disruption of P450 aromatase following exposure to BPA, but also, NP and OP (>20 μM), might be related to the generation of ROS, as pointed out by Milczareka et al. (2008) that revealed that NADPH- and iron-dependent lipid peroxidation in human placental microsomes (HPM) inhibited placental aromatase, possibly due to degradation of the enzyme induced by lipid radicals.

The analysis of free concentrations of the selected plasticizers in culture medium allowed the detection of significant differences in terms of bioavailability, but also the detection of minor amounts of DEHP and DBP (0.15–0.60 μM) in the blanks. The origin of these compounds is unknown, but certainly, the leaching from the polyethylene terephthalate (PET) plastic container during storage and/or sterilization of the medium, should be considered, as DEHP and DBP are reported to migrate from PET bottles into water (Keresztes et al., 2013).

Some discrepancies between nominal and experimental concentrations were observed for NP and OP. Most likely, their condition of surfactants confers them the ability to accumulate at the interfaces of the system (air-water, plastic-water) and this favors their volatility. Thus, when JEG-3 cells were exposed to 100 to 500 μM OP for 24 h, concentrations of OP in the range of 3.6 to 17.9 μg/mL (18 to 83 μM) were detected in the culture medium of adjacent wells (Pérez-Albaladejo, unpublished results). Moreover, Heringa et al. (2004) showed losses of OP due to protein binding and partitioning to the cell membranes, which are inherent to the complexity of cell culture mediums (Kramer et al., 2007; Smith et al., 2010). Consequently, EC_{50}/IC_{50} values for OP and NP based in measured concentrations tended to be lower than those calculated from nominal doses (Tables 2 & 3), although the observed differences were still acceptable at concentrations below 20–30 μM.

On the contrary, major differences between measured and nominal concentrations were evidenced for phthalates, particularly for BBP and DBP, even at low nominal concentrations (Fig. 3). Interestingly, when serum was removed from culture medium, the measured concentrations of phthalates were similar or even lower than those detected in serum supplemented medium, suggesting that the binding of these compounds to serum components was not significant (Fig. 2, supplementary info). Indeed, microscopic examination evidenced the accumulation of droplets of DBP, BBP and DEHP, both in the bottom and the surface of plastic/glass wells, when tested at concentrations above 20 μM in serum free medium. Precipitation of BBP and DBP was also observed in serum supplemented medium, but at higher concentrations (≥100 μM). Therefore, the apparent insensitivity of JEG-3 cells to phthalate exposure in comparison to other plasticizers might be associated to the high hydrophobicity and consequently, low bioavailability of phthalates in culture medium. Notwithstanding, BBP and DBP significantly decreased aromatase activity in JEG-3 cells at experimental concentrations in the range of 5 to 40 μM (experimental IC_{50}s ~ 14 μM; Table 3). These concentrations are well below the IC_{50}s calculated based on nominal concentrations (104 to 167 μM). On the other hand, DMP and DEHP had no inhibitory effect on placental aromatase activity.

Regarding the significance of the obtained results, it is of particular concern the alteration of P450 aromatase activity by DBP, BBP and alkylphenols at experimental concentrations in the range of 5 to 40 μM, which are close to concentrations reported in human cord blood (DBP ~ 21 μM; OP ~ 6 μM; NP ~ 5 μM) of low-birth-weight neonates in China (Lin et al., 2008). Certainly, pregnant women are particularly vulnerable since P450 aromatase is responsible for estrogen production and the crucial sexual steroid balance necessary for normal embryonic and fetal development, and plasticizers have been reported to cross human placenta and to reach the fetus. However, studies of the effects on pregnancy outcomes of in utero exposure to phthalates yielded conflicting results. Premature birth and decreased anogenital distance were the most commonly reported outcomes resulting from a moderate level of exposure to phthalates (e.g. <11 μg/L in maternal urine for MEHP). The principal metabolites detected and involved were primary metabolites of di-2(ethylhexyl)-phthalate (DEHP) and dibutyl-phthalate (DBP) (Marie et al., 2015).

Overall, this study highlights the need of an accurate determination of the bioavailability of chemicals, particularly phthalates, to improve the sensitivity of in-vitro tests, but also the interpretation of the results, and evidences the ability of the phthalates, BBP and DBP, and the alkylphenols, NP and OP, to modulate placental aromatase activity.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

This work was supported by the project MIGRAPLAST, funded by the Spanish Ministry of Economy and Competitiveness (IPT-2011-0709-060000).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2016.11.003.

References

Aydogan, M., Korkmaz, A., Barlas, N., Kolankaya, D., 2008. The effect of vitamin C on bisphenol A, nonylphenol and octylphenol induced brain damages of male rats. Toxicology 249, 35–39.

Balakrishnan, B., Henare, K., Thorstensen, E.B., Ponnampalam, A.P., Mitchell, M.D., 2010. Transfer of bisphenol A across the human placenta. Am. J. Obstet. Gynecol. 202, 393.e1–393.e7.

Benachour, N., Aris, A., 2009. Toxic effects of low doses of Bisphenol-A on human placental cells. Toxicol. Appl. Pharmacol. 241, 322–328.

Bonefeld-Jørgensen, E.C., Long, M., Hofmeister, M.V., Vinggaard, A.M., 2007. Endocrine-disrupting potential of bisphenol-A, bisphenol-A dimethacrylate, 4-n-nonylphenol and 4-n-octylphenol in vitro: new data and a brief review. Environ. Health Perspect. 115, 69–76.

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

Bredhult, C., Bäcklin, B.M., Olovsson, M., 2007. Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro. Reprod. Toxicol. 23, 550–559.

Chen, M.L., Chang, C.C., Shen, Y.J., Hung, J.H., Guo, B.R., Chuang, H.Y., Mao, I.F., 2008. Quantification of prenatal exposure and maternal-fetal transfer of nonylphenol. Chemosphere 73, S239–S245.

Cousins, I., Mackay, D., 2000. Correlating the physical-chemical properties of phthalate esters using the 'three solubility' approach. Chemosphere 41, 1389–1399.

Desdoits-Lethimonier, C.A., Albert, O., Le Bizec, B., Perdu, E., Zalko, D., Courant, F., Lesné, L., Guillé, F., Dejucq-Rainsford, N., Jégou, B., 2012. Human testis steroidogenesis is inhibited by phthalates. Hum. Reprod. 27, 1451–1459.

EFSA, 2015. Report on the two-phase public consultation on the draft EFSA scientific opinion on bisphenol A. EFSA supporting publication 2015:EN-740. http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/740e (last accessed 13.03.16).

Fasano, E., Bono-Blay, F., Cirillo, T., Montuori, P., Lacorte, S., 2012. Migration of phthalates, alkylphenols, bisphenol A and di(2-ethylhexyl) adipate from food packaging. Food Control 27, 132–138.

Gong, Y., Han, X.D., 2006. Nonylphenol-induced oxidative stress and cytotoxicity in testicular Sertoli cells. Reprod. Toxicol. 22, 623–630.

Harris, C., Henttu, P., Parker, M.G., Sumpter, J., 1997. The estrogenic activity of phthalate esters in vitro. Environ. Health Perspect. 105, 802–811.

Heringa, M.B., Schreurs, R.H.M.M., Busser, F., van der Saag, P.T., van der Burg, B., Hermens, J.L.M., 2004. Toward more useful in vitro toxicity data with measured free concentrations. Environ. Sci. Technol. 38, 6263–6270.

Huang, H., Leung, L.K., 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol. Lett. 189, 248–252.

Huang, Y., Li, J., Garcia, J.M., Lin, H., Wang, Y., Yan, P., Wang, L., Tan, Y., Luo, J., Qiu, Z., Chen, J.A., Shu, W., 2014. Phthalate levels in cord blood are associated with preterm delivery and fetal growth parameters in Chinese women. PLoS One 9 (2), e87430.

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y., Taketani, Y., 2002. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum. Reprod. 17, 2839–2841.

Keresztes, S., Tatár, E., Czégény, Z., Záray, G., Mihucz, V.G., 2013. Study on the leaching of phthalates from polyethylene terephthalate bottles into mineral water. Sci. Total Environ. 458–460, 451–458.

Kramer, N.I., van Eijkeren, J.C., Hermens, J.L., 2007. Influence of albumin on sorption kinetics in solid-phase microextraction: consequences for chemical analyses and uptake processes. Anal. Chem. 79, 6941–6948.

Latini, G., De Felice, C., Presta, G., Del Vecchio, A., Paris, I., Ruggieri, F., Mazzeo, P., 2003. In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. Environ. Health Perspect. 111, 1783–1785.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe of 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Lee, S., Suk, K., Kim, I.K., Jang, I.S., Park, J.W., Johnson, V.J., Kwon, T.K., Choi, B.J., Kim, S.H., 2008. Signaling pathways of bisphenol A-induced apoptosis in hippocampal neuronal cells: role of calcium-induced reactive oxygen species, mitogen-activated protein kinases, and nuclear factor-kB. J. Neurosci. Res. 86, 2932–2942.

Lephart, E., Simpson, E., 1991. Assay of aromatase activity. Methods Enzymol. 206, 477–483.

Lin, L., Zheng, L.X., Gu, Y.P., Wang, J.Y., Zhang, Y.H., Song, W.M., 2008. Levels of environmental endocrine disruptors in umbilical cord blood and maternal blood of low-birth-weight infants. Chin. J. Prev. Med. 42 (3), 177–180.

Marie, C., Vendittelli, F., Sauvant-Rochat, M.P., 2015. Obstetrical outcomes and biomarkers to assess exposure to phthalates: a review. Environ. Int. 83, 116–136.

Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R., Langlois, V.S., 2015. Plasticizer endocrine disruption: highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. Gen. Comp. Endocrinol. 219, 74–88.

Milczareka, R., Sokołowskaa, E., Hallmanna, A., Kalethab, K., Klimeka, J., 2008. NADPH- and iron-dependent lipid peroxidation inhibit aromatase activity in human placental microsomes. J. Steroid Biochem. Mol. Biol. 110, 230–235.

Milligan, S.R., Balasubramanian, A.V., Kalita, J.C., 1998. Relative potency of xenobiotic estrogens in an acute in vivo mammalian assay. Environ. Health Perspect. 106, 23–26.

Mørk, T.J., Sorda, G., Bechi, N., Rasmussen, B.S., Nielsen, J.B., Ietta, F., Rytting, E., Mathiesen, L., Paulesu, L., Knudsen, L.E., 2010. Placental transport and in vitro effects of Bisphenol A. Reprod. Toxicol. 30, 131–137.

Nativelle-Serpentini, C., Richard, S., Séralini, G., Sourdaine, P., 2003. Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells. Toxicol. in Vitro 17, 413–422.

Okubo, T., Suzuki, T., Yokoyama, Y., Kano, K., Kano, I., 2003. Estimation of estrogenic and anti-estrogenic activities of some phthalate diesters and monoesters by MCF-7 cell proliferation assay in vitro. Biol. Pharm. Bull. 26, 1219–1224.

Quignot, N., Desmots, S., Barouki, R., Lemazurier, E., 2012. A comparison of two human cell lines and two rat gonadal cell primary cultures as 'in vitro' screening tools for aromatase modulation. Toxicol. in Vitro 26, 107–118.

Samson, M., Labrie, F., Luu-The, V., 2009. Specific estradiol biosynthetic pathway in choriocarcinoma (JEG-3) cell line. J. Steroid Biochem. Mol. Biol. 116, 154–159.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. in Vitro 11, 107–119.

Smith, K.E., Oostingh, G.J., Mayer, P., 2010. Passive dosing for producing defined and constant exposure of hydrophobic organic compounds during in vitro toxicity tests. Chem. Res. Toxicol. 23, 55–65.

Sugiura-Ogasawara, M., Ozaki, Y., Sonta, S., Makino, T., Suzumori, K., 2005. Exposure to bisphenol A is associated with recurrent miscarriage. Hum. Reprod. 20, 2325–2329.

Swan, S., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S., Lynn Teague, J., 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113, 1056–1061.

Tan, B.L.L., Mohd, M.A., 2003. Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. Talanta 61, 385–391.

Vandenberg, L., Hauser, R., Marcus, M., Olea, N., Welshons, W., 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24, 139–177.

Vinggaard, A.M., Hnida, C., Breinholt, V., Larsen, J.C., 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol. in Vitro 14, 227–234.

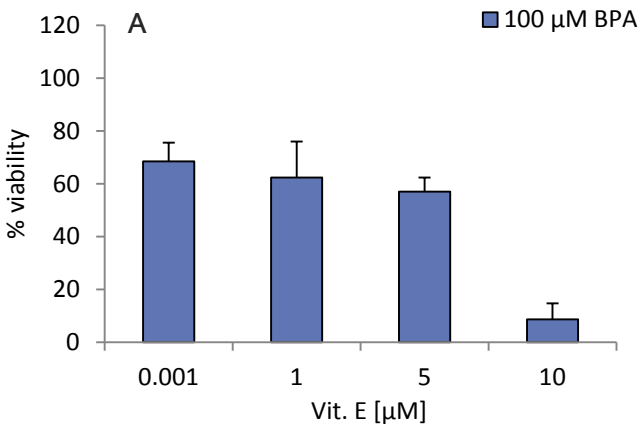
Watanabe, M., Ohno, S., Nakajin, S., 2012. Effects of bisphenol A on the expression of cytochrome P450 aromatase (CYP19) in human fetal osteoblastic and granulosa cell-like cell lines. Toxicol. Lett. 210, 95–99.

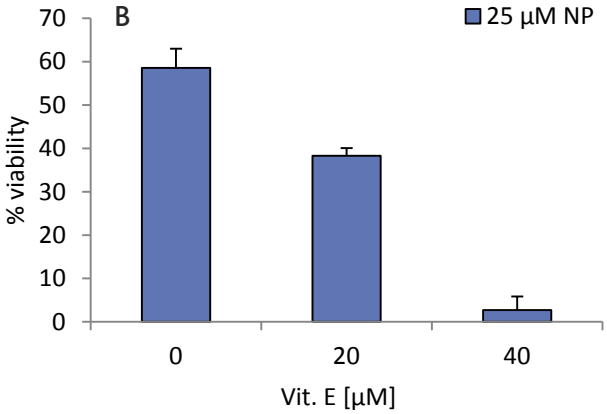
Wittassek, M., Angerer, J., Kolossa-Gehring, M., Schäfer, S.D., Klockenbusch, W., Dobler, L., Günsel, A., Müller, A., Wiesmüller, G.A., 2009. Fetal exposure to phthalates—a pilot study. Int. J. Hyg. Environ. Health 212, 492–498.

Xu, J., Osuga, Y., Yano, T., Morita, Y., Tang, X., Fujiwara, T., Takai, Y., Matsumi, H., Koga, K., Taketani, Y., Tsutsumi, O., 2002. Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells. Biochem. Biophys. Res. Commun. 292, 456–462.

INFORMACIÓN SUPLEMENTARIA AL ARTÍCULO 5

Figure 1. Increasing cytotoxicity (Alamar Blue assay) of (A) 100 µM BPA, (B) 25 µM NP, and (C) 30 µM OP in JEG-3 cells co-exposed with different concentrations of vitamin E. *Statistical significant differences respect to cells incubated without vitamin E. No significant effect on cell viability was observed after exposure of control cells (non-exposed) at concentrations of vitamin E as high as 40 µM.





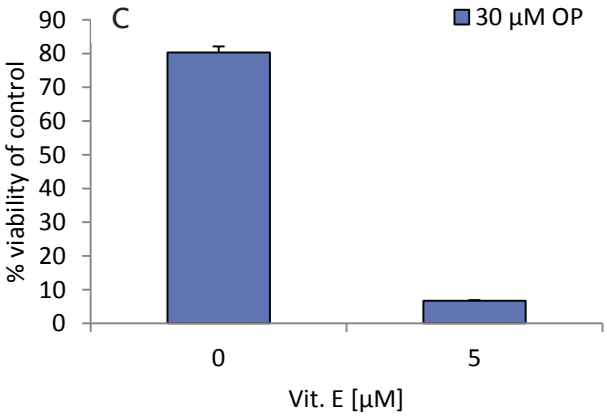
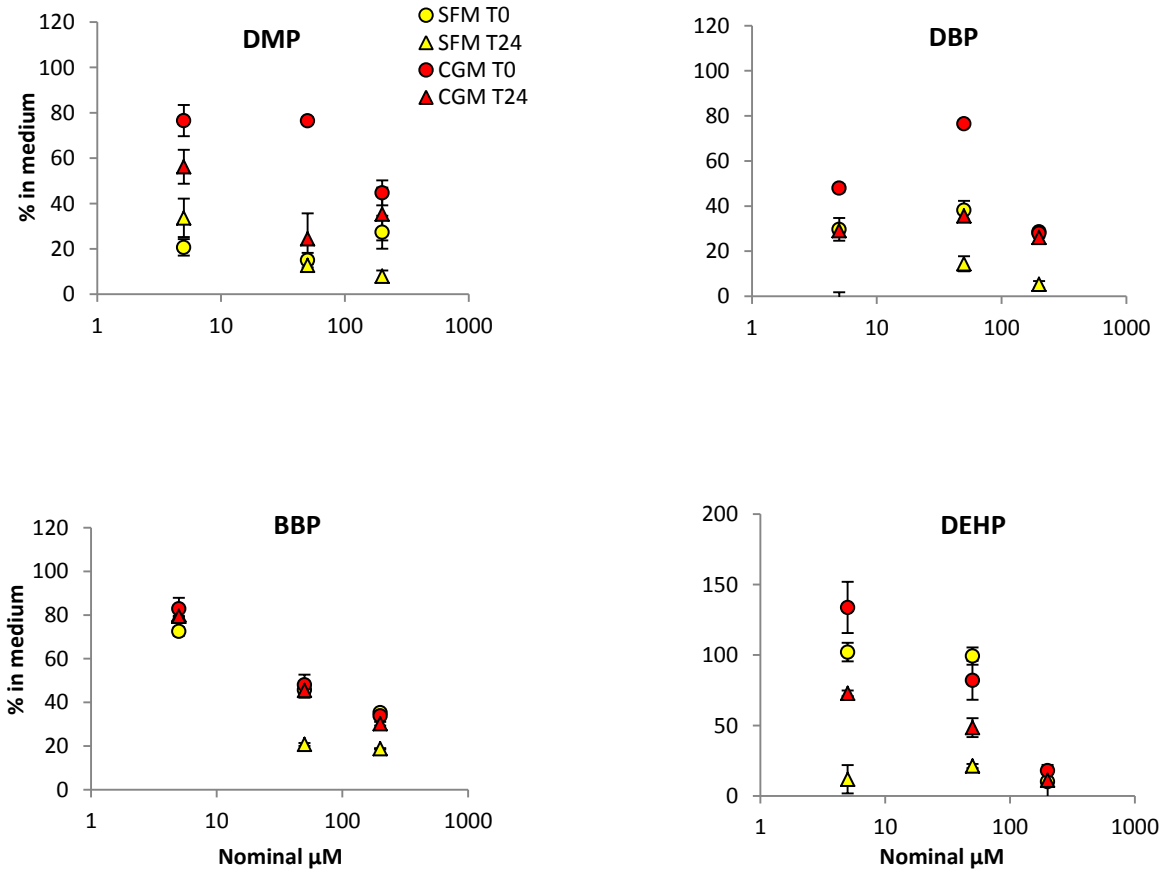


Figure 2. Experimental concentrations of phthalates determined in serum free medium (SFM) or complete medium (CGM) right after dosing (T0) and after 24 h (T24), expressed as mean ± SEM of three independent experiments. Experiment carried out in glass wells.



ARTÍCULO 6

Differental toxicity of alkylphenols in JEG-3 human placental cells: alteration of P450 aromatase and cell lipid composition

E. Pérez-Albaladejo, K. Fajardo, S. Lacorte, C. Porte

Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

Submitted to Toxicology and Applied Pharmacology.

RESUMEN

Este trabajo, complementario al artículo 5, investiga la toxicidad comparada, habilidad para generar especies reactivas de oxígeno (ROS) y modular la actividad P450 aromatasa, así como alterar el perfil lipídico, de varios alquilfenoles en la línea celular de placenta humana JEG-3. La citotoxicidad de los alquilfenoles estaba estrechamente relacionada con su hidrofobicidad. Después de 24 h de exposición, 4-dodecilfenol (DP), 4-heptilfenol (HP) y 4-cumilfenol (CP) mostraban la más alta citotoxicidad (EC_{50} 18-65 μM). A pesar de no ser citotóxico, 2,4,6-tri-*tert*-butilfenol (TTBP) inhibía significativamente la actividad aromatasa (IC_{50} 58 μM), mientras que DP y CP inducían la actividad hasta 1.4 veces a 10-20 μM. Sorprendentemente, HP inducía la generación de ROS hasta 43 veces más que el control, y probablemente como consecuencia de ello, se observó una reducción generalizada de las especies lipídicas poliinsaturadas en las células tras 24 h de exposición a 20 μM del compuesto. Por el contrario, las células expuestas a dosis no tóxicas de DP (10 μM) aumentaban significativamente la abundancia de fosfatidilcolinas y triacilglicéridos a costa de reducir los niveles de diacilglicéridos. Por su lado, la presencia de

20 µM de TTBP inducía una acumulación generalizada de triacilglicéridos, evidenciando las propiedades obesogénicas del compuesto. En general, este estudio revela la toxicidad diferencial de los alquilfenoles, así como su habilidad para modular la actividad aromatasa y/o alterar la composición lipídica, y destaca a DP, HP, CP y TTBP como aquellos que causan los efectos más perjudiciales en las células de placenta JEG-3.

Differential toxicity of alkylphenols in JEG-3 human placental cells: alteration of P450 aromatase and cell lipid composition

Elisabet Pérez-Albaladejo, Kristina Fajardo, Silvia Lacorte, Cinta Porte

Environmental Chemistry Department, IDAEA –CSIC-, C/ Jordi Girona 18-26, 08034 Barcelona, Spain

Abstract

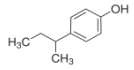
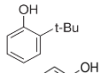
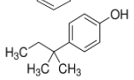
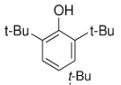
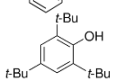
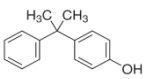
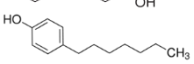
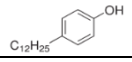
Alkylphenols (APs) are suspected endocrine disrupters that can cross the placental barrier and interfere with embryonic and foetal development. This work investigates their comparative toxicity, ability to generate reactive oxygen species (ROS) and inhibit aromatase activity of several alkylphenols in the human placenta choriocarcinoma cell line JEG-3. Cytotoxicity was closely related to the hydrophobicity of APs. 4-Dodecylphenol (DP), 4-heptylphenol (HP) and 4-cumylphenol (CP) showed the highest cytotoxicity (EC_{50}: 18-65 µM) after 24 h exposure. 2,4,6-tri-*tert*-Butylphenol (TTBP) significantly inhibited aromatase activity (IC_{50}: 58 µM), whereas DP and CP at concentrations of 10-20 µM increased the activity up to 1.4 fold. HP highly induced the generation of ROS (43-fold at 40 µM), far followed by 4-*tert*-amylphenol (TAP), 4-sec-butylphenol (4SBP) and 4-*tert*-butylphenol (4TBP) (3.4 to 6.5-fold induction at 100-200 µM). Significant changes in cellular lipids were observed after exposure to HP, DP and TTBP (10 - 20 µM). Overall, this study evidences the differential toxicity and ability to modulate aromatase activity and/or disrupt lipid composition of several alkylphenols, and highlights DP, HP, CP and TTBP as the ones that caused the most harmful effects on placental JEG-3 cells.

Keywords: P450 aromatase; ROS generation; JEG-3 cells; alkylphenols; lipid composition

2. Introduction

Alkylphenols (APs) are high-production volume chemicals used as nonionic surfactants in a variety of products, including industrial detergents, synthetic leathers, lubricants, polycarbonate and epoxy resins, herbicides, cosmetics and others (Brook et al., 2005). The chemical structure of APs is a phenol ring with an alkyl chain tail of 1 to 12 carbon atoms at ortho-, meta- or para- position, branched (tert- and sec-) or lineal. This confers upon APs different physico-chemical and toxicological characteristics (e.g. water solubility, persistence) that vary with the length of the carbon chain and the position of the substituent (Tollefsen et al., 2008; Xie et al., 2013).

Table 1. . Properties of tested alkylphenols (https://scifinder.cas.org). MW: molecular weight; MS: molar solubility.

Name	Compound	No CAS	MW	Log K_{ow}	MS (mmol/L)	Formula
4SBP	4-sec-butylphenol	99-71-8	150.22	3.50	4.80	
2SBP	2-sec-butylphenol	89-72-5	150.22	3.37	5.50	
4TBP	4-*tert*-butylphenol	98-54-4	150.22	3.40	6.30	
2TBP	2-*tert*-butylphenol	88-18-6	150.22	3.27	7.20	
TAP	4-*tert*-amylphenol	80-46-6	164.24	3.91	2.30	
DTBP	2,6-di-*tert*-butylphenol	128-39-2	206.32	4.48	0.66	
TTBP	2,4,6-tri-*tert*-butylphenol	732-26-3	262.43	5.30	0.70	
CP	4-cumylphenol	599-64-4	212.29	4.24	0.17	
HP	4-heptylphenol	1987-50-4	192.30	5.12	0.30	
DP	4-dodecylphenol	104-43-8	262.43	7.67	0.04	

Octylphenol and nonylphenol (OP and NP), the most widely studied and distributed APs, have been thoroughly investigated. Humans are exposed to these compounds through inhalation, ingestion of contaminated food and dermal absorption and concentrations up to 200 ng/g NP (~1 μM) and 1.15 ng/mL OP (~0.006 μM) have been reported in blood, umbilical cord and placenta (Tan and Mohd, 2003; Chen et al., 2008; Huang et al., 2014; Azzouz et al., 2016). Both compounds can accumulate in lipids, bind to proteins, cause acute cytotoxicity, oxidative stress and act as endocrine disrupters (Soto et al., 1991; Bonefeld-Jørgensen et al., 2007; Xie et al., 2013; Kim et al., 2015; Lepretti et al., 2015; Pérez-Albaladejo et al., 2017).

4-Dodecylphenol (DP), some isomers of butylphenol (2-tert-, 4-tert, 2,4-di-tert-), and 4-cumylphenol (CP) are nowadays used as substitutes of NP (Brooke et al., 2005). Among them, CP increased cell growth in MCF-7 cells, whereas 4-*tert*-butylphenol (4TBP) has been classified as estrogenic and has been reported to cause vitiligo, a cutaneous disease which causes depigmentation in melanocytes, in exposed workers (Soto et al., 1991; Yang et al., 2000; Hashimoto et al., 2001; Toosi et al., 2012). In 2000, the OECD calculated the daily intake of 4TBP as 9 μg/kg body weight/day. However, despite the increasing production and distribution of short chain APs, information about their potential toxicological effects is still scarce.

The endocrine disruptive properties of APs are of particular concern. Several studies have demonstrated the estrogenic activity of APs by mimicking 17β-estradiol in PNT1A human prostate cells, inducing cell proliferation and binding to progesterone receptor in human estrogen-sensitive MCF7 cells, triggering mitotic activity in the endometrium of rats, and inducing P450 aromatase activity in JEG-3 human placental cells (Soto et al., 1991; Forte et al., 2016; Pérez-Albaladejo et al., 2017). The human placental choriocarcinoma JEG-3 cell line expresses high levels of P450 aromatase, a key enzyme in steroidogenesis. It has often been used as a model to detect endocrine disrupter compounds, as an alteration of aromatase function may result in disorders of the reproductive system and trigger some types of cancer (Vingaard et al., 2000; Huang and Leung, 2009; Samson et al., 2009).

Additionally, alkylphenols, as amphipathic molecules, are likely to alter the lipid bilayer of cell membranes, but also act as obesogenic and this might have negative consequences for cell function (Masuno et al., 2003). This is of particular concern since the human placenta plays an active role in the release of free fatty acids from maternal serum triacylglycerols and the preferential transfer of long chain polyunsaturated fatty acids to the fetus (Jones et al., 2014), as well as the conversion of a certain proportion of arachidonic acid to prostaglandins, the incorporation of some fatty acids into phospholipids and the synthesis of specific fatty acids (Herrera, 2002). Thus, any disruption on the lipid homeostasis in placental cells will probably have harmful consequences for embryo development.

In the present study, the comparative toxicity of several alkylphenols of different chain length and different isomers, namely, 2-sec-butylphenol (2SBP), 2-tert-butylphenol (2TBP), 4-tert-butylphenol (4TBP), 4-sec-butylphenol (4SBP), 4-tert-amylphenol (TAP), 4-heptylphenol (HP), 4-dodecylphenol (DP), 4-cumylphenol (CP), 2,6-di-tert-butylphenol (DTBP) and 2,4,6-tri-tert-butylphenol (TTBP) was assessed in human placental JEG-3 cells targeting different end-points, namely cytotoxicity, generation of reactive oxygen species and disruption of aromatase activity. In addition, the ability of TTBP, HP and DP to alter the lipid composition of cell membranes was assessed by ultra-high performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC/TOF-MS).

2 Material and methods

2.1. Chemicals and solutions

Eagle's Minimum Essential Medium, fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin and streptomycin, phosphate buffered saline (PBS), phosphate buffered saline with Ca, Mg (DPBS) and trypsin-EDTA 0.25% were from Gibco BRL Life Technologies (Paisley, Scotland, UK). 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) and 3-morphoolinosydnonime (SIN1) were from Sigma (Steinheim, Germany). Alamar Blue (AB) was from Biosource (Solingen, Germany) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) from Molecular Probes (Eugene, OR, USA). 1β 3H-androst-4-ene-3,17-dione (3H-AD) was purchased from PerkinElmer, Inc. (Boston MA, USA). Stock solutions of APs were prepared in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany). The analytical standards 4SBP, 2SBP, 4TBP, 2TBP, TAP, DTBP, TTBP, CP and DP were from Sigma-Aldrich (St. Louis, MO, USA). HP was from Dr. Ehrenstorfer (LGC Standards). The properties and chemical

structure of studied compounds are indicated in Table 1.

2.2 Cell culture

The JEG-3 cell line, derived from a human placental carcinoma, was obtained from the American Type Culture Collection (ATCC-HTB-36). Cells were routinely grown in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 2 mM *L*-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL of penicillin and 50 µg/mL of streptomycin. JEG-3 cells were cultured in 75 cm^2 polystyrene flasks (Corning; NY, USA) in a humidified incubator with 5% CO_2 at 37 ºC. When 90% confluence was reached, cells were dissociated with 0.25% (*w/v*) trypsin and 0.9 mM EDTA (trypsin-EDTA) for subculturing and experiments.

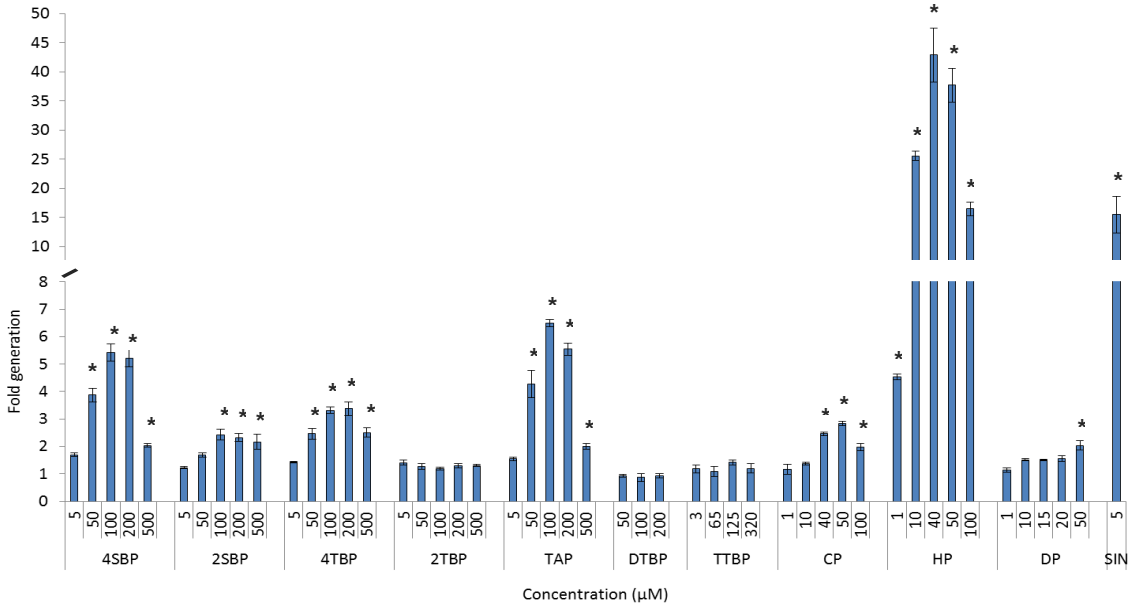


Figure 1. Generation of ROS in JEG-3 cells after 60 minutes exposure to alkylphenols (µM) and 5 µM SIN-1 (positive control). Values are expressed as fold induction as mean ± SD of at least six replicates in three different plates assayed.*Statistically significant differences from control ($p < 0.05$).

2.3 Cytotoxicity

Cytotoxic effects of APs in JEG-3 cells after 24 h exposure were measured using two fluorescent dyes, namely Alamar Blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), to monitor metabolic impairment and membrane integrity, respectively (Schirmer et al., 1997). Cells were seeded in 96-well plates at a density of 2.5 x 10^4 cells per well in 100 µL of medium and left to grow for 24 h. APs were dissolved in DMSO and were added to the medium so that the final solvent concentration was 0.5% (v/v), which was not cytotoxic for cells. Control wells received the culture medium containing 0.5% DMSO. To assess the effect of vitamin E on cytotoxicity, cells were exposed to APs in medium supplemented with 40 µM α-tocopherol (Sigma, Steinheim, Germany).

After 24 h exposure, cells were rinsed with PBS and incubated for 1 h with the dye solution (5% v/v AB and 4 mM CFDA-AM) in modified Leibovitz L-15 medium without vitamins or amino acids. Cell viability was measured using a fluorescence plate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths of 530/590 nm for AB, and 485/530 nm for CFDA-AM. Cell viability was expressed as a percentage of control cells.

2.4 Reactive oxygen species (ROS) generation

The production of ROS in JEG-3 cells exposed to APs was measured as described in LeBel et al. (1992) with slight modifications. Cells were seeded into 96-well plates 24 h before the assay (4 x 10^4 cells per well; 100 µL of medium). On the day of the experiment, cells were rinsed with PBS and incubated with 20 µM H_2DCF-DA in a 1:10 DPBS solution supplemented with 10 mM glucose. After 30 minutes incubation, cells were washed with PBS and exposed to APs dissolved in DMSO

and further diluted in DPBS-glucose (100 µL/well). The final concentration of DMSO in the assay was 0.5%. 3-Morpholinosydnonimine (SIN-1) at a concentration of 5 µM was used as a positive control. DCF fluorescence was measured after 60 min in a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelength pairs of 485/528 nm. Results were expressed as the ratio of fluorescence in exposed cells to control cells as fold induction.

2.5 P450 aromatase activity

Aromatase activity in JEG-3 human placental cells was determined by measuring the amount of ^3H_2O formed during the aromatization of [1β-3H]androstenedione (^{3}H-AD) as described in Lephart and Simpson (1991), with some modifications. Cells were seeded in 24 well-plates at a density of 10^5 cells per well and allowed to attach overnight before exposure to the APs or 0.5% DMSO (solvent control) in culture medium (500 µL). After 24 h exposure (37 ºC, 5% CO_2), cells were rinsed with PBS and incubated for 30 min in the presence of 40 nM ^{3}H-AD in 1 ml of DPBS-glucose. The reaction was stopped by placing the plate on ice and immediately aspirating 0.8 mL of medium that was extracted in triplicate with 3 mL of dichloromethane. The amount of tritiated water formed was counted in an aliquot of the aqueous phase (Tri-Carb 2100TR, Packard). Aromatase activity was expressed in fmol/min/mg protein or as percentage of activity in solvent control cells.

2.6 Lipid analysis

JEG-3 cells were exposed for 24 h to 10 µM DP, 10 and 20 µM HP, and 20 µM TTBP in 24-well plates, as indicated above. Control cells were exposed to culture medium containing 0.1% DMSO. After exposure, the medium was removed and cells were trypsinized, resuspended in PBS, centrifuged at 10000-g for 10 min and the pellet collected. An aliquot of internal standard mixture containing equal amounts (~200 pmol) of different lipids, namely, 16:0 D31-18:1 PC, 1,3-17:0 D5 DG and 1,2,3-17:0 TG (Avanti Polar Lipids, Inc., Alabama, USA), was added prior to lipid extraction with a solution of methanol:chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT). The mixture was vortexed for 1 min, and after 30 min incubation at room temperature, extracted in an ultrasonic bath for 5 min (x2). The solvent was evaporated and the extracts stored at -80 ºC under an argon atmosphere.

The lipid composition was analyzed using ultra-high performance liquid chromatography (Waters ACQUITY UHPLC) with a C8 silica column (Waters ACQUITY UPLC BEH, 100 x 2.1 mm, 1.7 µm particle size), coupled with positive electrospray ionization mass spectrometry (Waters/LCT Premier XE TOF) (UPLC-MS/ToF). Mobile phases were (A) methanol with 1 mM ammonium formate, and (B) water with 2 mM ammonium formate, and both phases supplemented with 0.2% formic acid. Initial conditions were 80% of A, gradually increasing to 99% in 15 min, maintained for 3 min, and returned to 80% in 2 min, plus 2 min for stabilization of the system. The flow rate was 0.3 mL/min and the column temperature was 30ºC. Identification of lipids was carried under the criteria of exact mass with an error of ±5 mDa using MassLinx 4.1 software. The amount of lipid species (pmol·10^6 cells) was calculated relative to internal standards. Lipid species were annotated as <number of carbons of fatty acids>:<number of double bonds in fatty acid chains>.

2.7 Statistical methods

Dose-response curves and concentrations causing 50% decline on cell viability (EC_{50}) and inhibition of aromatase activity (IC_{50}) were performed with Sigmaplot 11.0 software. Significant differences from controls were determined using one way ANOVA with Dunnett's or Turkey's post hoc test, with with SPSS Statistics v.23 software. Level of significance was set at $p < 0.05$. Lipid profile was analysed by using Metaboanalyst software (version 3.0; Xia et al., 2016). The missing values were replaced by the half of the minimum value in the original data. Data were normalized by mean-centering and dividing by standard deviation of each variable. Partial least square - discriminant analyses (PLS-DA) have been used to compare the lipid profile of the control with the treated cells, and to generate a ranking of lipid variables of importance (VIP) > 1.

3. Results

3.1 Cytotoxicity and generation of reactive oxygen species

Low or no significant cytotoxicity was detected for butylphenols 4TBP, 2TBP, 2SBP and TTBP (EC_{50} > 385 µM), as well as for 4SBP, DTBP and TAP (EC_{50}: 120 - 280 µM) after 24 h. of exposure.

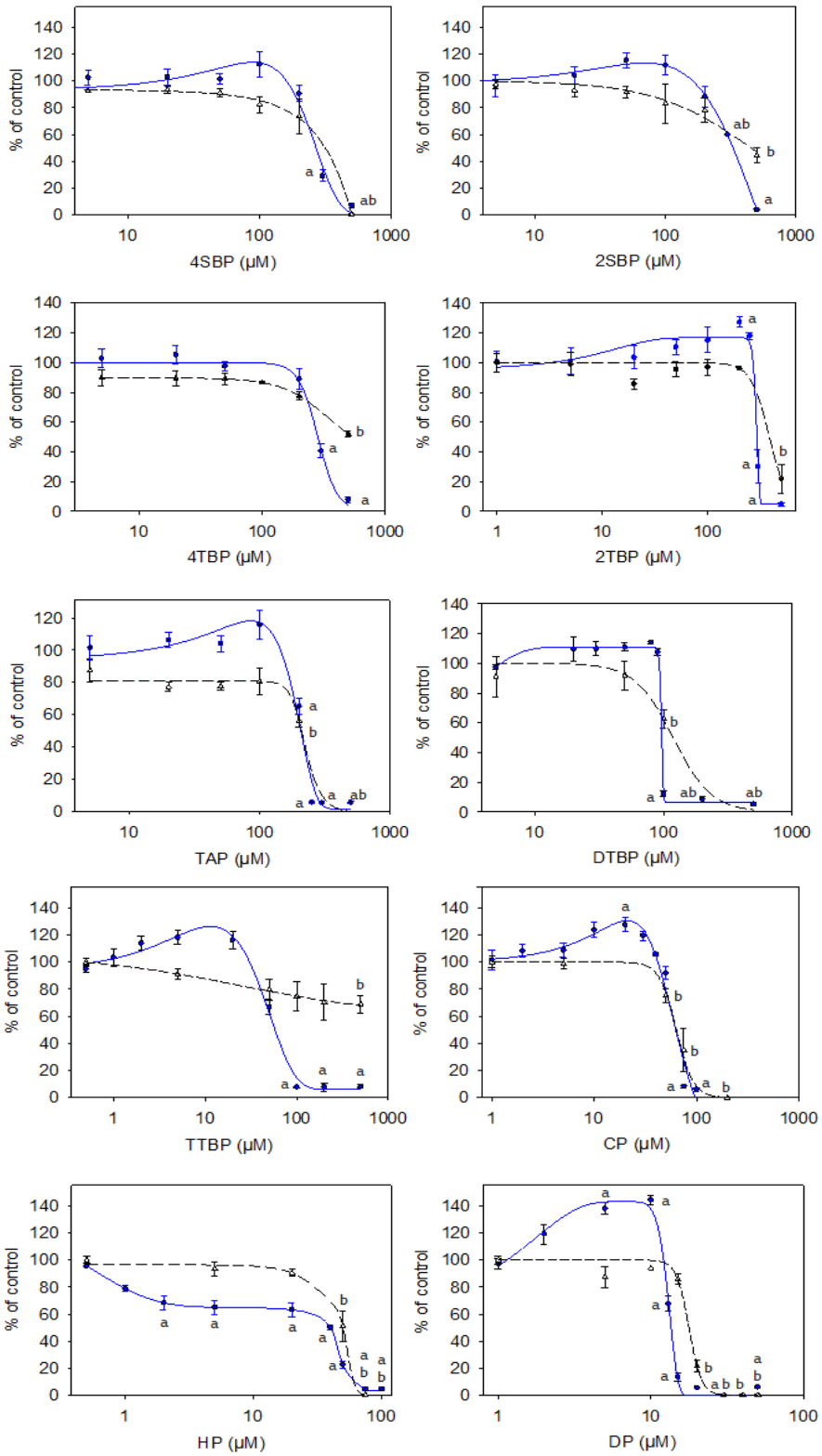


Figure 2. Aromatase activity (solid line) and cytotoxicity-Alamar Blue assay (dotted line) in JEG-3 cells after 24 h exposure to alkylphenols. Aromatase activity in control cells was 336 ± 22 fmol/min/mg protein. Values expressed as percentage of activity in control cells (mean ± SD) of at least three assays. [a,b]Statistically significant differences from control ($p < 0.05$) for aromatase activity and cytotoxicity.

On the contrary, the long chain DP showed the highest cytotoxicity (EC_{50}: 18 µM), followed by HP and CP (EC_{50}: 50 and 65 µM, respectively) (Table 2). No significant differences between AB and CFDA probes were observed.

Regarding generation of ROS, the short chain APs substituted in position 4, namely TAP, 4SBP and 4TBP, significantly induced ROS in JEG-3 cells (3.4 to 6.5-fold induction at 100-200 µM), whereas butylphenol isomers substituted in position 2, and those di- and tri- substituted, generated lower (2.5-fold; 2SBP) or no ROS (2TBP, DTBP, TTBP). The long chain DP and CP slightly induced the production of ROS (2 to 3-fold at 50 µM) (Fig. 1). Surprisingly, HP had the highest response in the H_2DCF-DA assay (43-fold induction at 40 µM). SIN-1, which was used as positive control, generated an induction of ROS of 15.4 ± 3.3 fold over control cells.

Given the high ability of most APs to generate ROS in JEG-3 cells, cytotoxicity assays (AB) were performed in the presence of 40 µM vitamin E, in order to assess the protective effect of this antioxidant on cytotoxicity. Nonetheless, the cytotoxicity of TBP, 2SBP, TAP, CP and HP increased significantly with vitamin E (EC_{50}: 193, 110, 77, 24 and 19 µM, respectively) (Table 2).

3.2 P450 aromatase activity

The tri-substituted TTBP, which showed no cytotoxicity at the highest concentration tested (IC_{50} > 500 µM), and HP, significantly inhibited aromatase activity, with IC_{50} of 58 and 41 µM, respectively. 4TBP also inhibited aromatase activity, but at higher concentrations (IC_{50}: 283 µM). For the other compounds (4SBP, 2TBP, TAP, DTBP, CP and DP), dose response curves for inhibition of aromatase activity and cytotoxicity were closely related, and the apparent inhibition was mostly attributed to cytotoxicity (Fig. 2, Table 2).

Additionally, P450 aromatase activity was significantly increased in cells exposed to the long chain DP (up to 1.4 fold at 10 µM), the 2-benzene rings CP (1.3-fold at 20 µM) and 2TBP (1.3-fold at 200 µM) (Fig. 2).

3.3 Analysis of lipids

A total of 69 lipid species were identified and quantified by UPLC-ToF in JEG-3 cells with PCs, PL-PCs and TGs as most abundant classes (Fig. 3). DAGs and TAGs were detected as $[M+NH_4]^+$ adducts, whereas $[M+H]^+$ were formed for PCs and PL-PCs. Among the lipid classes, PC 34:1, PL-PC 34:1 and DG 34:1 were the more abundant, followed by PCs 36:2, PC 32:1 and PC 34:2. Regarding TAGs, TAG 50:1, TAG 52:2, TAG 52:3 and TAG 54:3 were the predominant species (Fig. 3).

PLS-DA scores plot revealed a clear separation of the lipid profile between control cells and cells exposed to HP, DP and TTBP. The ranking VIP scores plot shows the lipid species that are contributing to these differences (Fig. 4). Although no changes in lipid profile of cells was observed in presence of 10 µM HP, at higher concentration (20 µM), HP exposure triggered a general decline (between 0.4 to 0.7 fold, data not showed) of lipids; the decrease was more evident for polyunsaturated PL-PCs (36:5, 36:4, 38:6, 38:4, 38:5 and 40:4), polyunsaturated TAGs (52:4, 54:4, 56:4, 56:5, 58:5) and DAGs (32:2, 34:2, 34:3, 36:2, 36:3, 38:4). At the same time, an increase (1.3-2.1 fold) of saturated lipids (DAG 32:0, TAG 50:0 and PCs 32:0) and monounsaturated lipids (PC 32:1, PC 34:1 and PC 36:1) was observed in HP-exposed cells (Fig 4A). In contrast, DP exposed cells were characterized by a significant increase of PL-PC 32:0 (1.6 fold), several PCs: PC 32:0 (2.9 fold), PC 32:1, PC 32:2, PC 34:1, PC 34:2, PC 34:3, PC 36:4, PC 36:5, PC 38:5 and PC 40:7 (between 1.3 and 1.6 fold), and TAGs: TAG 50:3, TAG 54:5, TAG 54:6 and TAG 56:6 (1.3-1.7 fold), and a significant depletion of PL-PC 38:3, PL-PC 38:4 and most DAGs (between 0.4 and 0.8 fold) (Fig. 4B). Interestingly, cell exposure to 20 µM of TTBP was associated to a significant increase of most TAGs (1.3 to 2.0 fold), whereas PL-PC 36:1, PL-PC 38:3 and DAG 36:1 decreased 0.7 fold (Fig. 4C).

4. Discussion

The most hydrophobic and long chain APs (DP and HP), the 2-benzene rings (CP), and the tri-substituted butylphenol (TTBP) induced the highest toxicological response in JEG-3 cells in terms of cytotoxicity and/or alteration of aromatase activity. Thus, the twelve-carbon chain DP was the most cytotoxic compound (EC_{50}: 18 µM), followed by HP (~ 50 µM) and CP (~ 65 µM). On the contrary, the short chain APs (2SBP, 2TBP, 4SBP, 4TBP, TAP, DTBP and TTBP) showed lower or no significant cytotoxicity (EC_{50} > 119 µM) nor ability to alter aromatase activity in JEG-3 cells.

Generally, cytotoxicity (log EC_{50}) increased with the hydrophobicity of the selected alkylphenols regardless of the length, structure and position of the alkyl chain, except for TTBP that was not cytotoxic despite having a log K_{ow} > 5; and CP and HP that showed an EC_{50} lower than expected (Fig. 5). This contrasts with the results obtained in rainbow trout primary hepatocytes,

where no clear relationship between cytotoxicity and hydrophobicity for the most hydrophobic APs was observed (Tollefsen et al., 2008). Interestingly, both rainbow trout hepatocytes and placenta cells, responded similarly to HP, DP and TTBP in terms of cytotoxicity, but higher cytotoxicity was reported for the short chain 2TBP, 4TBP, 4SBP and DTBP in rainbow trout hepatocytes (EC_{50} between 28 and 217 µM) in comparison to placenta cells (EC_{50}: 120 to >500 µM), which suggest stronger metabolism and detoxification mechanisms in the latter.

Among the tested compounds, DP was the most lipophilic and the strongest inhibitor of P450 aromatase (IC_{50}: 13 µM), followed by HP, TTBP and CP. Moreover, exposure to DP leads to a significant increase of aromatase activity (up to 44% at 10-20 µM) at concentrations close to cytotoxicity. To our knowledge, this is the first report showing that DP increases aromatase activity in human placental cells. Similarly, NP and OP increased aromatase activity in JEG-3 cells in a very narrow range of concentrations (20-30 µM), but the activity drastically decreased at higher concentrations (Pérez-Albaladejo et al., 2017).

The mechanisms through which DP and other long chain alkylphenols induce aromatase activity in JEG-3 cells may be multiple, including up-regulation of *CYP19*, regulation at the post-translational level and/or changes in signalling pathways, among others. The observed effects on placental P450 aromatase are of particular concern, since this activity is responsible for estrogen production and the sexual steroid balance necessary for normal embryonic and fetal development in pregnant women.

Despite the low cytotoxicity and ability to disrupt aromatase activity, the short-chain APs substituted in position 4, namely, TAP, 4SBP and 4TBP, significantly induced the generation of ROS (up to 7-fold). Surprisingly, HP caused up to a 43-fold ROS increase in exposed cells. H_2DCF-DA, the probe used to assess intracellular ROS generation, is deacetylated by cellular esterases into a non-fluorescent compound, which is subsequently oxidized by ROS into 2′,7′-dichlorofluorescein (DCF).

It has been reported that some compounds (viz. tetrabromobisphenol A) can stimulate the conversion of H_2DCF to DCF even in cell-free models (Tetz et al., 2013; Szychowski et al., 2016). Nevertheless, this was not the case for HP, as no fluorescence was detected in the absence of cells.

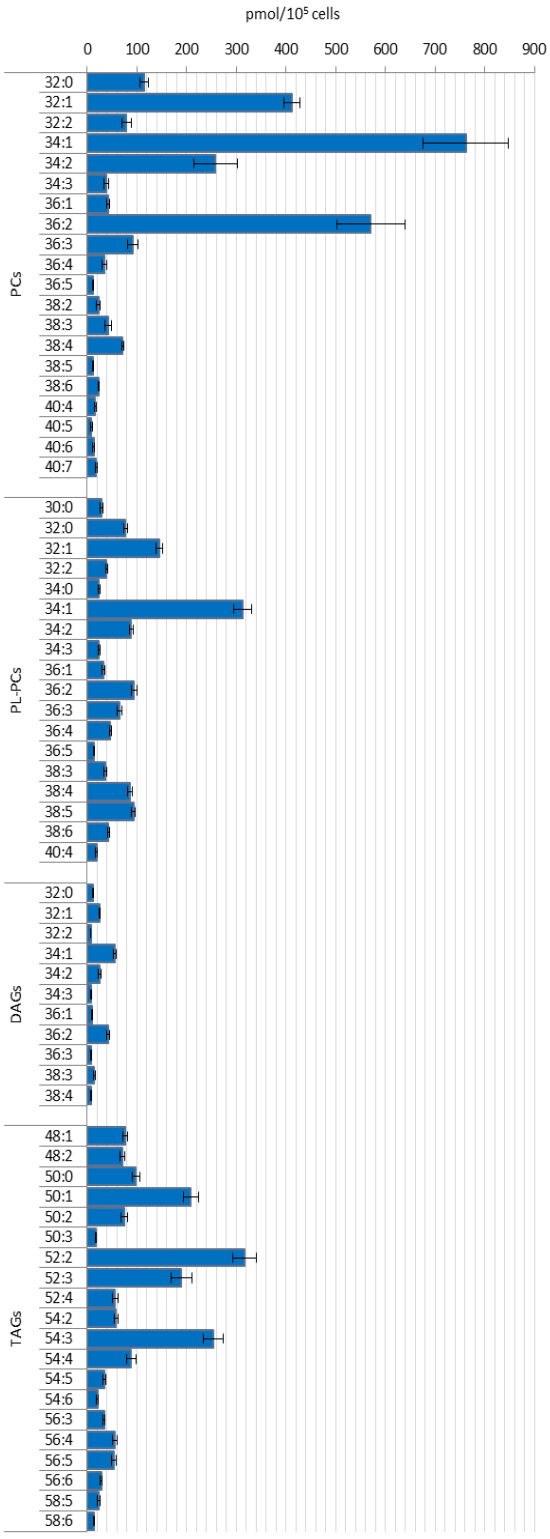
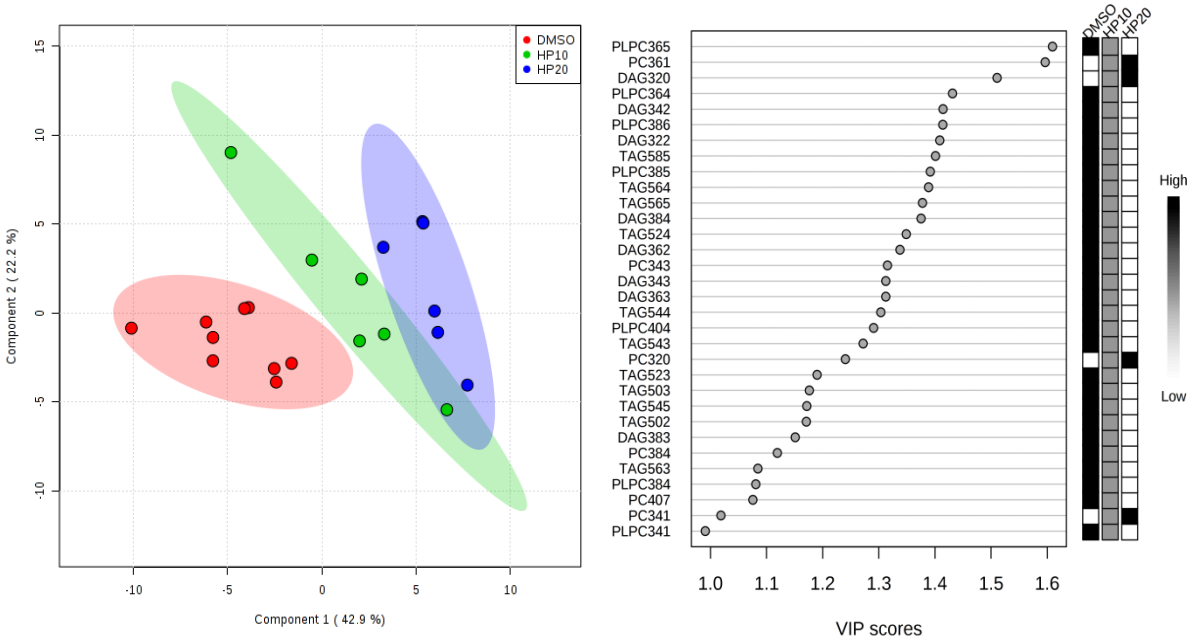
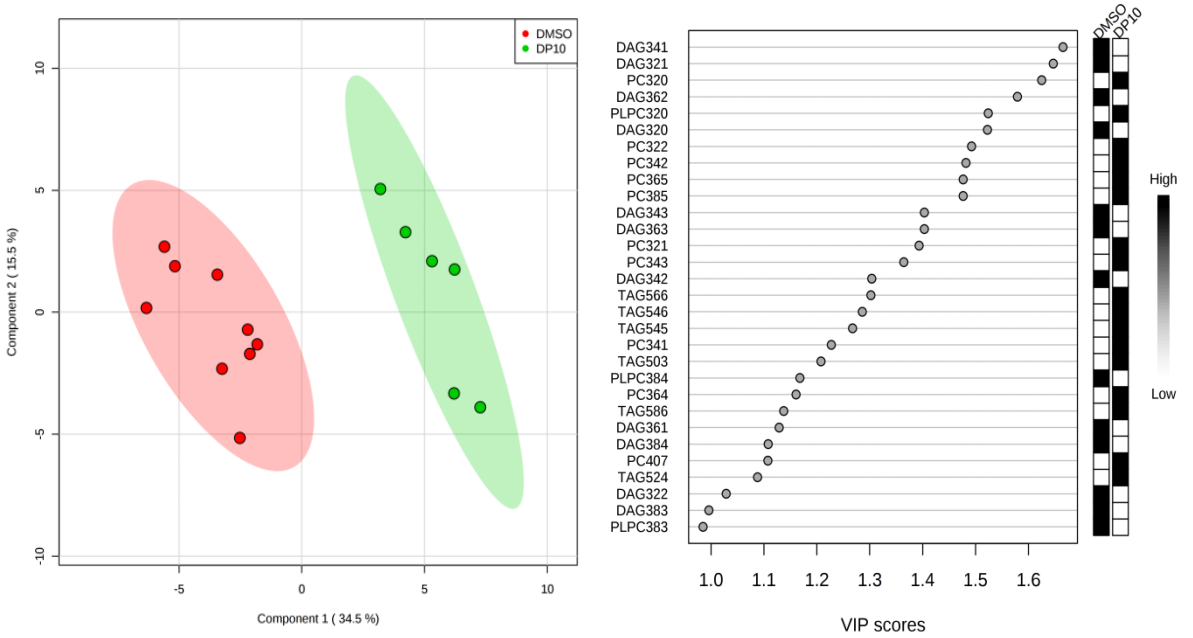


Figure 3. Lipid species (PCs, PL-PCs, DAGs, TAGs) detected in JEG-3 cells. Values are expressed as mean ± SD of three independent samples.

A


DMSO
HP10
HP20
Component 2 (22.2 %)
Component 1 (42.9 %)
PLPC365
PC361
DAG320
PLPC364
DAG342
PLPC386
DAG322
TAG585
PLPC385
TAG564
TAG565
DAG384
TAG524
DAG362
PC343
DAG343
DAG363
TAG544
PLPC404
TAG543
PC320
TAG523
TAG503
TAG545
TAG502
DAG383
PC384
TAG563
PLPC384
PC407
PC341
PLPC341
DMSO
HP10
HP20
High
Low
VIP scores

B


DMSO
DP10
Component 2 (15.5 %)
Component 1 (34.5 %)
DAG341
DAG321
PC320
DAG362
PLPC320
DAG320
PC322
PC342
PC365
PC385
DAG343
DAG363
PC321
PC343
DAG342
TAG566
TAG546
TAG545
PC341
TAG503
PLPC384
PC364
TAG586
DAG361
DAG384
PC407
TAG524
DAG322
DAG383
PLPC383
DMSO
DP10
High
Low
VIP scores

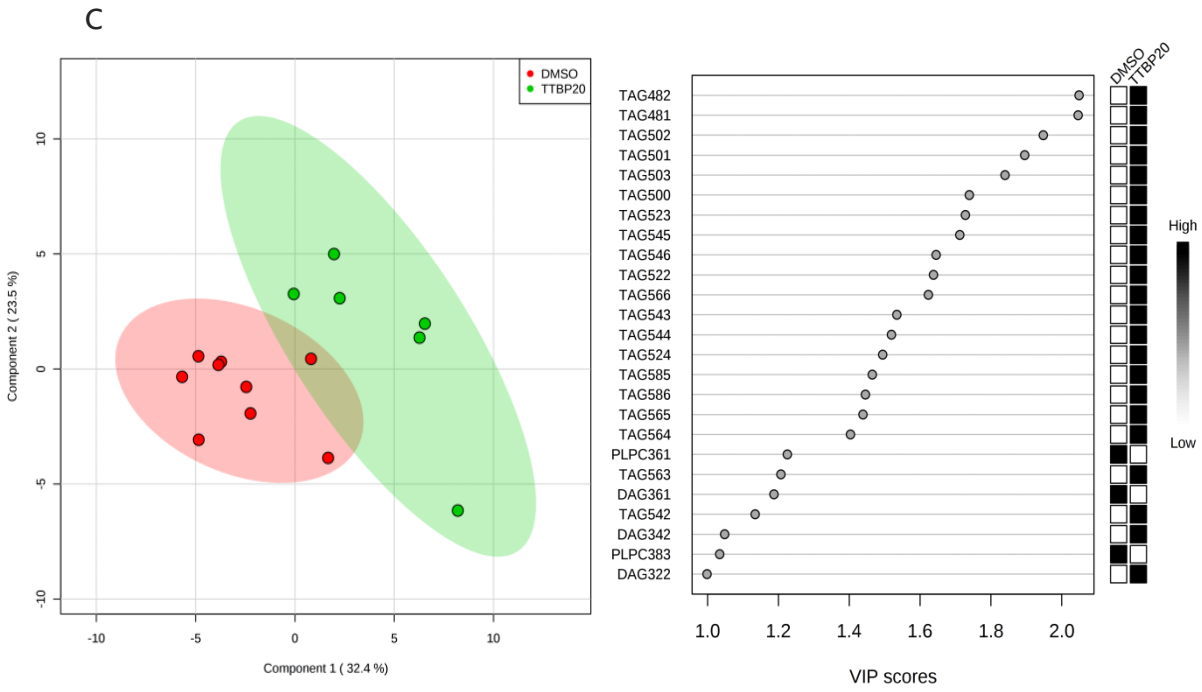


Figure 4. In the previous page and above it is presented the important features identified by PLS-DA, which shows significantly changed levels of lipids with the variable importance in projection (VIP) value >1, in cells exposed to APs for 24 h. On the left is showed score plots of 69 lipid species detected in JEG-3 cells exposed to 0.1% DMSO and: (A) 10 µM DP, (B) 10 and 20 µM HP, and (C) 20 µM TTBP. On the right, it is shown the most important metabolites ranked by the PLS-DA VIP score. The mini heatmap on the right indicate their concentration variations respect to control (DMSO). The lipid species are annotated as <lipid class><total fatty acyl chain (2 digits)><total number of unsaturated bonds (1 digit)>.

On the other hand, the intracellular redox chemistry of H_2DCF is complex, e.g. peroxidases are also capable of inducing H_2DCF oxidation that may generate DCF semiquinone free radical, DCF phenoxyl radical and $O_2\cdot^-$, which would then cause H_2DCF oxidation and an amplification of the fluorescence (Tarpey and Fridovich, 2001). Thus, due to the multiple pathways that can lead to DCF fluorescence, these results should be interpreted with caution.

Interestingly, when culture medium was supplemented with no toxic dosis of an antioxidant, 40 µM of vitamin E, a significant increase in the cytotoxicity of most APs was detected (2SBP, 4TBP, 2TBP, TAP, CP and HP). Increased cytotoxicity was previously reported in JEG-3 cells co-exposed to octyl- and nonylphenol and vitamin E (Pérez-Albaladejo et al., 2017). Similarly, Kontek et al. (2014) reported a significant increase in apoptotic cell death in A549 and HT29 cancer cell lines when irinotecan was co-exposed with 25 µM vitamin E. Certainly, vitamin E efficiently scavenges peroxyl radicals and protects cells against oxidative stress, but it may act as a pro-oxidant at high concentrations (Niki, 2014).

Due to the surfactant nature of the studied compounds, one might hypothesize that they can impair the functioning of cell membranes and other lipid components, and consequently induce toxicity. Our work was focused on identifying alterations in key lipids, namely PCs, PL-PCs, DAGs and TAGs. The synthesis of these lipids is tightly regulated. For example, alterations of membrane composition may result in imperfect packing of the lipid bilayer, changes in fluidity or dynamism of the membrane, transport, signal transduction and loss of cell integrity (Wolfgang and Lane, 2006). The most abundant lipid species detected in JEG-3 cells contained acyl chains saturated, di- or mono- unsaturated (e.g.: PC 32:1, PC 34:1, PC 34:2, PC 36:2, PL-PC 32:1, PL-PC 34:1, DAG 34:1, DAG 36:2, TAG 52:2 and TAG 50:1). This agrees with the fact that free fatty acids C16:0 (palmitic acid) and C18:0 (stearic acid), usually located in cytoplasm, are rapidly consumed to generate membrane lipids as PL-PC 32:0, PC 30:0 and PC 32:1 or converted to other long-chain fatty acids (e.g.; oleic (C18:1) or linoleic (C18:2) acids) by elongases and desaturases (Kihara, A. 2012; He et al., 2015).

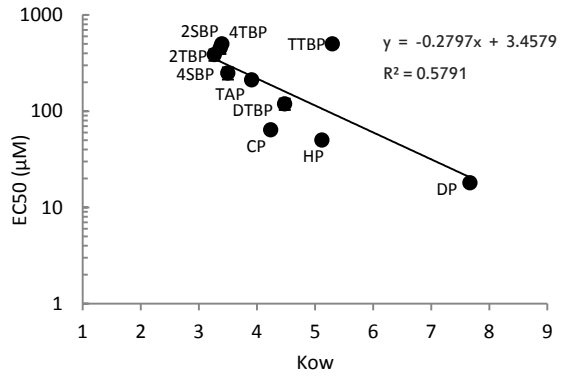


Figure 5. Plot of the relationship between APs hydrophobicity (log K_{ow}) and cytotoxicity (EC_{50}) in JEG-3 cells.

Interestingly, HP, DP and TTBP at concentrations of 10 and/or 20 µM, induced significant alterations in the relative abundance of cellular lipids. However, the global changes in lipid composition induced by these compounds are completely different. Comparing the individual lipid species, it was found that no toxic concentrations (20 µM) of HP increased saturated and mono- unsaturated species (PC 32:0, PC 32:1, PC 34:1, PC 36:1 and DAG 32:0) in placental cells, whereas the most polyunsaturated ones, including TAGs (e.g.: TAGs 56:4, 56:5 and 58:5), PL-PCs (e.g.: PL-PCs 36:4, 36:5, 38:5, 38:6 and 40:4), DAGs (e.g.: DAGs 32:2, 34:2 and 38:4) and PCs (e.g. PCs 34:3, 38:4 and 40:7) were decreased. High levels of ROS are likely to be the cause of lipid peroxidation because polyunsaturated lipids are very vulnerable (De la Haba et al., 2013). Accordingly, 20 µM HP generated high levels of ROS (>25-fold), and consequently led to a general decrease of polyunsaturated species, which are the more susceptible to peroxidation. This is of particular concern since the fetus compartment needs to be provided with preferably long chain polyunsaturated fatty acids from maternal TAGs, from which are released by lipoprotein lipase activity of placental cells (Coleman and Haynes, 1987; Gude et al., 2004). Thus, due to the unexpected and extraordinary responses of HP in ROS assays, together with the suspect of lipid peroxidation, the ability of HP to generate oxidative stress should be further investigated, particularly considering that oxidative stress has also often been related to inhibition of placenta aromatase as well as pregnancy complications, including spontaneous abortion, recurrent pregnancy loss and preeclampsia (Hempstock et al., 2003; Milczarek et al., 2008; Agarwal et al., 2012).

In contrast, DP at not toxic concentrations (10 µM), which did not induce ROS generation in placental cells, increased the amount of highly unsaturated TAGs (e.g.: TAGs 54:5, 56:6, 54:6, 58:5 and 58:6) and several PCs (e.g.: PCs 32:0, 32:1, 32:2, 34:1, 34:2, 34:3), whereas decreased their corresponding DAGs (DAGs 32:0, 32:1, 32:2, 34:1, 34:2, 34:3), suggesting that cells are producing PCs and TAGs at the expense of DAGs, in response to the presence of DP. The synthesis of PCs could response to an attempt to repair the damage caused in the cell membrane by the exposure to DP. Other explanation could be that DP induces proliferation and cells should produce lipids to cover cellular demande to perform mitosis. However, this hypothesis is not supported by the Alamar Blue assay, which did not detect any increase in cell proliferation.

It is worth to mention that the production of membrane and storage lipids occurs at the same time as the induction of P450 aromatase activity in cells exposed to 10 µM of DP, whereas the general depletion of lipids in presence of 20 µM of HP was accompanied by an inhibition of the enzyme. This fact evidence the need of further investigation of mechanisms of action of HP and DP to elucidate if there is an association between these responses.

Curiously, when JEG-3 cells were exposed to 20 µM of TTBP, which was no toxic for cells, a significant accumulation of TAGs was evidenced. Previous studies demonstrated the ability of several xenobiotics called obesogens, of inducing the storag energy as TAGs through diverse mechanisms. Among them, TBT acts by binding to the retinoid X receptor (RXR) and peroxisome proliferator activated receptor gamma (PPARy), which are key components in adipogenesis and their activation increases adipose mass in rodents and humans. It is known that prenatal exposure to some obesogens reprograms the exposed animals to be fat (Blumberg, 2011), and further rechearch is needed to know if TTBP acts by similar pathways.

The observed changes in the lipid profile of placental cells JEG-3 after exposure to HP, DP and TTBP may result in altered membrane fluidity, permeability and curvature, and useless storage of energy. It is well established that fluidity of the placental cell membranes plays a key role in modulating transport processes between fetus and mother, and that these species are modified during pregnancy, according to the needs of the growing fetus (Damiano, 2011).

Overall, this work evidenced DP, HP, CP and TTBP as the tested compounds that caused the most harmful effects (endocrine and/or lipid disruption, oxidative stress and cytotoxicity) in human placental cells. Because of the potential of HP to generate oxidative stress and to disrupt cellular lipids, but also of TTBP to induce TAG

accumulation, further studies are necessary to unravel the mode of action of these compounds and the consequences for the fetus health. In contrast, short chain APs (4TBP, 2TBP, 2SBP, 4SBP) were less toxic than long chain ones and did not act as endocrine disrupters in placental cells.

Acknowledgments

This work was supported by the project MIGRAPLAST, funded by the Spanish Ministry of Economy and Competitiveness (IPT-2011-0709-060000).

References

Agarwal A., Aponte-Mellado A., Premkumar, B.J., Shaman, A., Gupta, S. 2012. The effects of oxidative stress on female reproduction: a review. Reproductive Biology and Endocrinology 10: 49.

Azzouz, A., Rascón, A.J., Ballesteros, E. 2016. Simultaneous determination of parabens, alkylphenols, phenylphenols, bisphenol A and triclosan in human urine, blood and breast milk by continuous solid-phase extraction and gas chromatography–mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 119: 16–26.

Blumberg, B. 2011. Obesogens, stem cells and the developmental programming of obesity. J. Dev. Orig. Health Dis. 2, 3–8.

Bonefeld-Jørgensen, E.C., Long, M., Hofmeister, M.V., Vinggaard, A.M. 2007. Endocrine-Disrupting Potential of Bisphenol A, Bisphenol A Dimethacrylate, 4-N-Nonylphenol, and 4-N-Octylphenol in Vitro: New Data and a Brief Review. Environmental Health Perspectives 115: 69–76.

Brooke, D., Crookes, M., Johnson, I., Mitchell, R., Wattset, C. 2005. Prioritisation of Alkylphenols for Environmental Risk Assessment. Environment Agency. Retrieved from: http://a0768b4a8a31e106d8b0-50dc802554eb38a24458b98ff72d550b.r19.cf3.rackcdn.com/scho0205binf-e-e.pdf.

Chen, M.L., Chang, C.C., Shen, Y.J., Hung, J.H., Guo, B.R., Chuang, H.Y., Mao, I. F. 2008. Quantification of prenatal exposure and maternal-fetal transfer of nonylphenol. Chemosphere 73: 239–245.

Coleman, R. a, Haynes, E.B. 1987. Synthesis and release of fatty acids by human trophoblast cells in culture. J. Lipid Res. 28, 1335–41.Damiano, A.E. 2011. Review: Water channel proteins in the human placenta and fetal membranes. Placenta 32: 207–211.

De la Haba, C., Palacio, J.R., Martínez, P., Morros, A. 2013. Effect of oxidative stress on plasma membrane fluidity of THP-1 induced macrophages. Biochimica et Biophysica Acta (BBA) - Biomembranes 1828: 357–364.

Forte, M., Di Lorenzo, M., Carrizzo, A., Valiante, S., Vecchione, C., Laforgia, V., De Falco, M. 2016. Nonylphenol effects on human prostate non tumorigenic cells. Toxicology 357: 21–32.

Gude, N.M., Roberts, C.T., Kalionis, B., King, R.G. 2004. Growth and function of the normal human placenta. Thromb. Res. 114, 397–407.Hashimoto, Y., Moriguchi, Y., Oshima, H., Sawaguchi, M., Miyazaki, K., Nakamura, M. 2001. Measurement of estrogenic activity of chemicals for the development of new dental polymers. Toxicology in Vitro 15: 421–425.

Hempstock, J., Jauniaux, E., Greenwold, N., Burton, G.J. 2003. The contribution of placental oxidative stress to early pregnancy failure. Human Pathology 34: 1265–1275.

Herrera, E. 2002. Implications of dietary fatty acids during pregnancy on placental, fetal and postnatal development — A review. Placenta 23, Supplement A, Trophoblast Research 16, S9–S19

Huang, H., Leung, L.K. 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicology Letters 189: 248–252.

Huang, Y.F., Wang, P.W., Huang, L.W., Yang, W., Yu, C.J., Yang, S.H., Chiu, H.H., Chen, M.L. 2014. Nonylphenol in pregnant women and their matching fetuses: Placental transfer and potential risks of infants. Environmental Research 134: 143–148.

Jones, M.L., Mark, P.J., Waddell, B.J. 2014. Maternal dietary omega-3 fatty acids and placental function. Reproduction 147: 143-152.

Kihara, A. 2012. Very long-chain fatty acids: Elongation, physiology and related disorders. J. Biochem. 152, 387–395.

Kim, J., Kang, E.J., Park, M.N., Kim, J.E., Kim, S.C., Jeung, E.B., Lee, G. S., Hwang, D. Y., An, B.S. 2015. The adverse effect of 4-tert-octylphenol on fat metabolism in pregnant rats via regulation of lipogenic proteins. Environmental Toxicology and Pharmacology 40: 284–291.

Kontek, R., Jakubczak, M., Matlawska-Wasowska, K. 2014. The antioxidants, vitamin A and E but not vitamin C and melatonin enhance the proapoptotic effects of irinotecan in cancer cells in vitro. Toxicology in Vitro 28: 282–291.

LeBel, C., Ischiropoulos, H., Bondy, S. 1992. Evaluation of the probe of 2´,7´-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chemical Research in Toxicology 5: 227–231.

Lephart, E., Simpson, E. 1991. Assay of aromatase activity. Methods in Enzymology 206: 477–483.

Lepretti, M., Paolella, G., Giordano, D., Marabotti, A., Gay, F., Capaldo, A., Esposito, C., Caputo, I. 2015. 4-Nonylphenol reduces cell viability and induces apoptosis and ER-stress in a human epithelial intestinal cell line. Toxicology in Vitro 29: 1436–1444.

Masuno, H., Okamoto, S., Iwanami, J., Honda, K., Shiosaka, T., Kidani, T., Sakayama, K., Yamamoto, H. 2003. Effect of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicol. Sci. 75: 314–320.

Milczarek, R., Sokołowska, E., Hallmann, A., Kaletha, K., Klimek, J. 2008. NADPH- and iron-dependent lipid peroxidation inhibit aromatase activity in human placental microsomes. Journal of Steroid Biochemistry and Molecular Biology 110: 230–235.

Niki, E. 2014. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. Free Radical Biology & Medicine 66: 3–12.

Pérez-Albaladejo, E., Fernandes, D., Lacorte, S., Porte, C. 2017. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells. Toxicology in Vitro 38, 41–48.

Samson, M., Labrie, F., Luu-The, V. 2009. Specific estradiol biosynthetic pathway in choriocarcinoma (JEG-3) cell line. The Journal of Steroid Biochemistry and Molecular Biology 116, 154–159.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicology in Vitro 11: 107–119.

Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C. 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from 'modified' polystyrene. Environmental Health Perspectives 92: 167–173.

Szychowski, K.A., Rybczyńska-Tkaczyk, K., Leja, M. L., Wójtowicz, A. K., Gmiński, J. 2016. Tetrabromobisphenol A (TBBPA)-stimulated reactive oxygen species (ROS) production in cell-free model using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) assay-limitations of method. Environmental Science and Pollution Research, 23: 12246-12252.

Tan, B.L.L., Mohd, M.A. 2003. Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. Talanta 61: 385–391.

Tarpey, M.M., Fridovich, I. 2001. Methods of detection of vascular reactive species: Nitric Oxide, Superoxide, Hydrogen Peroxide, and Peroxynitrite. Circulation Research 89: 224–237.

Tetz, L.M., Kamau, P.W., Cheng, A.A., Meeker, J.D., Loch-Caruso, R. 2013. Troubleshooting the dichlorofluorescein assay to avoid artifacts in measurement of toxicant-stimulated cellular production of reactive oxidant species. Journal of Pharmacological and Toxicological Methods 67: 56–60.

Tollefsen, K.E., Blikstad, C., Eikvar, S., Finne, E.F., Gregersen, I.K. 2008. Cytotoxicity of alkylphenols and alkylated non-phenolics in a primary culture of rainbow trout (*Onchorhynchus mykiss*) hepatocytes. Ecotoxicology and Environmental Safety 69: 64–73.

Toosi, S., Orlow, S.J., Manga, P. 2012. Vitiligo-inducing phenols activate the unfolded protein response in melanocytes resulting in upregulation of IL6 and IL8. Journal of Investigative Dermatology 132: 2601–2609.

Vinggaard, A.M., Hnida, C., Breinholt, V., Larsen, J.C. 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicology in Vitro 14: 227–234.

Wolfgang, M.J., Lane, M.D. 2006. Control of energy homeostasis: role of enzymes and intermediates of fatty acid metabolism in the central nervous system. Annual Review of Nutrition 26: 23–44.

Xia, J., Wishart, D.S. 2016. Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. Current protocols in bioinformatics, 55, 14.10.1-14.10.91.

Xie, X., Lü, W., Chen, X. 2013. Binding of the endocrine disruptors 4-tert-octylphenol and 4-nonylphenol to human serum albumin. Journal of Hazardous Materials 248: 347–354.

Yang, F., Sarangarajan, R., Le Poole, I.C., Medrano, E.E., Boissy, R.E. 2000. The cytotoxicity and apoptosis induced by 4-tertiary butylphenol in human melanocytes are independent of tyrosinase activity. Journal of Investigative Dermatology 114: 157–164.

ARTÍCULO 7

Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells

E. Gorrochategui[1], E. Pérez-Albaladejo[1], J. Casas[2], S. Lacorte[1], C. Porte[2]

[1] Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain
[2] Department of Biomedicinal Chemistry, IQAC-CSIC, Barcelona, Spain

Toxicology and Applied Pharmacology 277 (2014) 124-130

RESUMEN

En este estudio se ha evaluado la citotoxicidad y el potencial de disrupción endocrina de diversos compuestos perfluorados (ácido perfluorobutanoico (PFBA), ácido perfluorohexanoico (PFHxA), ácido perfluorooctanoico (PFOA), ácido perfluorononanoico (PFNA), ácido perfluorododecanoico (PFDoA), perfluorobutanosulfonato (PFBS), perfluorohexanosulfonato (PFHxS) y perfluorooctanosulfonato (PFOS)) en la línea celular de placenta humana JEG-3. Únicamente los compuestos perfluorados de cadena larga (PFOS, PFDoA, PFNA y PFOA) mostraban citotoxicidad en las células JEG-3, con EC_{50} en el rango de 107 a 647 μM. Además, PFOS, PFOA y PFBS tenían un elevado potencial de para actuar como inhibidores de la actividad P450 aromatasa en las células JEG-3, con IC_{50} en el rango de 57 a 80 μM. Finalmente, la exposición de las células JEG-3 a una mezcla equitativa de los ocho compuestos (0,6 μM cada uno) condujo a un aumento relativo (hasta 3,4 veces) de diversas clases de lípidos, incluyendo fosfatidilcolinas (PCs), plasmalógeno PCs y liso-plasmalógeno PCs, lo que sugiere una interferencia de los compuestos perfluorados con los lípidos de membrana. En general, este trabajo destaca la capacidad de los compuestos perfluorados para

alterar la composición lipídica celular a concentraciones muy por debajo de las que generan citotoxicidad, y el potencial del compuesto de cadena corta PFBS, a menudo considerado un sustituto seguro de PFOS, para actuar como disruptor endocrino en las células de placenta JEG-3.

Toxicology and Applied Pharmacology 277 (2014) 124–130

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Perfluorinated chemicals: Differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells

Eva Gorrochategui [a], Elisabet Pérez-Albaladejo [a], Josefina Casas [b], Sílvia Lacorte [a,*], Cinta Porte [a,**]

[a] Department of Environmental Chemistry, IDAEA–CSIC, 08034 Barcelona, Catalonia, Spain
[b] Department of Biomedicinal Chemistry, IQAC–CSIC, 08034 Barcelona, Catalonia, Spain

ARTICLE INFO

Article history:
Received 17 January 2014
Revised 12 March 2014
Accepted 16 March 2014
Available online 26 March 2014

Keywords:
Perfluorinated chemicals
JEG-3
Cytotoxicity
CYP19
Phosphatidylcholines
PFBS

ABSTRACT

The cytotoxicity of eight perfluorinated chemicals (PFCs), namely, perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS) was assessed in the human placental choriocarcinoma cell line JEG-3. Only the long chain PFCs – PFOS, PFDoA, PFNA, PFOA – showed significant cytotoxicity in JEG-3 cells with EC50 values in the range of 107 to 647 μM. The observed cytotoxicity was to some extent related to a higher uptake of the longer chain PFCs by cells (PFDoA > PFOS ≫ PFNA > PFOA > PFHxA). Moreover, this work evidences a high potential of PFOS, PFOA and PFBS to act as aromatase inhibitors in placental cells with IC50s in the range of 57–80 μM, the inhibitory effect of PFBS being particularly important despite the rather low uptake of the compound by cells. Finally, exposure of JEG-3 cells to a mixture of the eight PFCs (0.6 μM each) led to a relative increase (up to 3.4-fold) of several lipid classes, including phosphatidylcholines (PCs), plasmalogen PC and lyso plasmalogen PC, which suggests an interference of PFCs with membrane lipids. Overall, this work highlights the ability of the PFC mixture to alter cellular lipid pattern at concentrations well below those that generate toxicity, and the potential of the short chain PFBS, often considered a safe substitute of PFOS, to significantly inhibit aromatase activity in placental cells.

Introduction

Perfluorinated chemicals (PFCs) represent a large group of compounds characterized by a hydrophobic fluorinated carbon tail attached to a polar hydrophilic head. PFCs are classified as perfluorinated sulfonic acids (PFSAs), perfluorinated carboxylic acids (PFCAs), fluorotelomer alcohols (FTOHs), high-molecular weight fluoropolymers and low-molecular weight perfluoroalkanamides. They are used in a variety of consumer products and have emerged as global environmental pollutants (Stahl et al., 2011). They are resistant to degradation and have a high accumulation potential; thus, once released in the environment, PFCs persist in aquatic organisms (Giesy and Kannan, 2002; Kannan et al., 2002). PFSAs and PFCAs are the most ubiquitous compounds and they have been detected in human blood (Ericson et al., 2007). Typical serum levels of PFOS and PFOA are in the range of 1 to 50 ng/mL, although concentrations up to 300 and 2000 ng/mL have been detected in occupationally exposed workers (Olsen et al., 1998).

Concerns about PFC toxicity have risen due not only to its widespread distribution and persistence in humans and the environment, but also to its toxicity and ability to act as endocrine disrupters and obesogens (Du et al., 2013; Hines et al., 2009). However, while the toxicity of PFOS and PFOA has been deeply investigated in the last decades, other PFC homologs have been rarely studied. PFCs of shorter chain length are expected to have similar functions to PFOS and be less bioaccumulative and less toxic. Thus, Buhrke et al. (2013) showed a positive correlation between carbon chain length of PFCAs and its toxicity in the human hepatocarcinoma cell line HepG2: the short chain length PFBA and PFHxA were less toxic than PFOA. Nonetheless, both PFOS and PFBS promoted expression of the estrogen and the androgen receptor at environmentally relevant concentrations and caused adverse hepato-histological effects in the amphibian *Xenopus laevis* at high concentrations (100–1000 μg/L), which opens the question of whether short chain PFCs are safe substitutes of PFOS (Lou et al., 2013).

Moreover, long chain PFCs can modulate the biosynthesis of gender-specific steroid hormones. Olsen et al. (1998) reported a 10% increase in estradiol levels among occupationally exposed workers who had the highest levels of serum PFOA (>30 ng/mL), although this association was confounded by body mass index. Decreased gene expression of key enzymes and transporters involved in steroidogenesis was observed in male rats exposed to PFDoA and male mice exposed to PFOS (Shi et al., 2007, 2009; Wan et al., 2011). Also, Zhao et al. (2010) showed a decrease in testosterone levels in isolated rat Leydig cells exposed to PFOA. All these studies seem to indicate that long chain PFCAs can act as endocrine disruptors, but their mechanisms of action are still unknown. A recent

* Corresponding author.
** Corresponding author. Fax: +34 932045904.
E-mail addresses: slbqam@cid.csic.es (S. Lacorte), cinta.porte@cid.csic.es (C. Porte).

http://dx.doi.org/10.1016/j.taap.2014.03.012
0041-008X/

work by Rosenmai et al. (2013) revealed that fluorochemicals present in food packaging materials can affect steroidogenesis through decreased Bzrp and increased CYP19 gene expression leading to lower androgen and higher estrogen levels. CYP19 aromatase plays a key role in catalyzing the irreversible conversion of androgens to estrogens and maintaining the androgen/estrogen hormonal balance. Thus, any interaction of chemicals with the expression of this enzyme or its catalytic activity is very likely to disrupt the internal hormonal balance between androgens and estrogens. In humans, aromatase activity has been reported in gonads, brain, ovaries, testis, placenta, adipose tissue, fetal liver, intestine, skin and brain. Numerous assays have been developed to evaluate the potential effects of chemicals on CYP19 aromatase, among them, the human recombinant microsomal aromatase assay that measures the direct effect of chemicals on aromatase catalytic activity in vitro (Vinggaard et al., 2000). Currently, the human placental choriocarcinoma JEG-3 cell line is also frequently used to assess CYP19 aromatase, since it allows the detection of changes in aromatase gene expression (Huang and Leung, 2009).

The structural resemblance of PFCs to fatty acids and the discovery that they bind to peroxisome proliferator-activated receptors (PPARs), nuclear receptors that play a key role in lipid metabolism and adipogenesis, have recently raised the concern that PFCs may disrupt lipid and weight regulation. Indeed, several studies suggest that exposure to PFOS and PFOA may be associated with increased cholesterol levels in humans (Nelson et al., 2010). Also, developmental exposure to low-doses of PFOA lead to increased weight in adult rats, with increased serum insulin and leptin, an effect not seen in animals exposed to high doses of PFOA (Hines et al., 2009). Interestingly, other environmental chemicals, termed obesogens, have been shown to induce obesity in adulthood after low-dose developmental exposure, while inducing weight loss at higher doses (Grün et al., 2006). In addition, PFOS has been shown to affect membrane properties (e.g. membrane fluidity, mitochondrial membrane potential) at concentrations below those associated with other adverse effects (Hu et al., 2003). Despite these evidences, more in-depth studies on the effects of PFCs on cellular lipidome and the physiological consequences for the cell, are still lacking.

Within this context, the aim of this study was to comparatively determine the cytotoxicity and ability to disrupt CYP19 aromatase activity of five perfluorinated carboxylic acids and three perfluorosulfonates of different chain lengths in the human placental choriocarcinoma cell line JEG-3. Special emphasis was placed on the analysis of cellular lipids by ultra-high performance liquid chromatography/mass spectrometry (UPLC/MS) in an attempt to roughly characterize major alterations of cellular lipids following exposure to a mixture of PFCs. This was designed as an exploratory analysis to be more fully investigated in future experiments with individual PFCs. The PFCs selected for the study were: perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS).

Materials and methods

Chemicals and reagents. Minimum Essential Medium, fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin G, streptomycin, PBS and trypsin–EDTA were from Gibco BRL Life Technologies (Paisley, Scotland, UK). PFBA, PFHxA, PFOA, PFNA, PFDoA and PFHxS were purchased from Sigma-Aldrich (Steinheim, Germany), except PFBS and PFOS which were obtained from Fluka (Austria). Stock standard solutions and serially diluted test solutions were prepared in ethanol, except for PFOS which was prepared in dimethyl sulfoxide (DMSO). These compounds were added to the complete growth medium so that the final solvent concentration never exceeded 0.4% (v/v), which was not cytotoxic. Perfluoro-n-(1,2,3,4-$^{13}C_4$)octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$)octanesulfonate (m-PFOS) from Wellington Laboratories (Ontario, Canada) were used as surrogate standards. HPLC grade water, ethanol (>99.8%) and acetonitrile (>99.8%) were purchased from Merck (Darmstadt, Germany).

Cell culture. JEG-3 cells derived from human placental carcinoma were obtained from American Type Culture Collection (ATCC HTB-36). They were grown in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate and 50 U/mL penicillin G/50 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C. Cells were routinely cultured in 75 cm^2 polystyrene flasks. When 90% confluence was reached, cells were dissociated with 0.25% (w/v) trypsin and 0.9 mM EDTA for subculturing and experiments. Experiments were carried out on confluent cell monolayers.

Cell viability. Two fluorescent dyes were used to monitor cell viability on JEG-3 cells (Schirmer et al., 1997). Metabolic activity was monitored with Alamar Blue (AB™, resazurin) and membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM). Cells were seeded at a rate of 25,000 cells per well (96-well plate) and allowed to attach overnight at 37 °C, 5% CO_2. After 24-hour exposure to PFCs, the medium was replaced by 100 μL of a solution of AB™/CFDA-AM, incubated for 1 h, and cell viability was measured using a fluorescence plate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths of 530/590 nm for AB™ and 485/530 nm for CFDA-AM. Results were recorded as relative fluorescence units.

Three independent sets of experiments were performed for each PFC. PFBA, PFBS, PFHxA and PFHxS were tested at 500 μM whereas PFOA, PFNA, PFDoA and PFOS were also tested at lower concentrations to obtain concentration–response curves. Within each experiment, addition of the test compound was done in septuplicate.

Uptake of PFCs. Cells were seeded at a rate of 100,000 cells per well (24-well plate) and allowed to attach overnight in an incubator at 37 °C, 5% CO_2. Cells were then dosed in triplicate with a mixture of eight PFCs at a concentration of 6.0 μM for each compound, and control cells were exposed to the carrier (0.4% ethanol). Given the intensive analytical work required to test eight PFCs individually at different concentrations, we decided to carry out an exploratory assay with a mixture of PFCs at a single non-toxic concentration, high enough to allow the detection of the fraction of PFCs absorbed by the cells. Right after dosing (time zero), and after 1, 3, 5, 8 and 24 h of incubation, the medium was aspirated, and the cells were rinsed with PBS, trypsinized and centrifuged at 3600 rpm for 10 min. The supernatant was aspirated and the cells were stored at −80 °C until analysis. Two independent sets of experiments were performed.

PFCs were extracted from JEG-3 cells following the method of Fernández-Sanjuan et al. (2010), with some modifications. A surrogate standard solution containing m-PFOA and m-PFOS was added to the cell pellets followed by 1 mL of acetonitrile. Samples were shaken and extracted in an ultrasonic bath for 10 min (×3); the supernatant was transferred to a new vial and purified by adding 25 mg of activated carbon and 50 μL of glacial acetic acid. The obtained supernatant was evaporated to dryness and reconstituted in 15% methanol/acetonitrile (60:40) and 85% water.

PFCs were analyzed using an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, USA) connected to a Triple Quadrupole Detector. An XBridge™ C_{18} column (3.5 μm particle size, 50 mm × 4.6 mm, Waters, Ireland) was used as mobile phase residue trap to remove any PFC traces from the mobile phases and tubing. The analysis was performed on an Acquity UPLC BEH C_{18} column (1.7 μm particle size, 50 mm × 2.1 mm, Waters, Ireland) connected to an Acquity UPLC BEH C_{18} (1.7 μm particle size, 5 mm × 2.1 mm, Waters, Ireland) VanGuard™ pre-column at a flow rate of 0.4 mL/min at a column temperature of 40 °C. The mobile phase was 20 mM NH_4OAc/

acetonitrile (90:10) (A)/methanol/acetonitrile (60:40) (B). Gradient elution started at 15% of B and continued to 95% of B in 8 min. Initial conditions were attained in 2 min and the system was stabilized for 3 min. PFCs were measured under negative electrospray ionization (ESI) and acquisition was performed in Multiple Reaction Monitoring (MRM) using one or two transitions from parent to product ion to identify each compound. The elution times, transitions used as well as the dwell times, cone voltages and collision energies are given in Table 1. m-PFOA was used as an internal standard to quantify PFBA, PFHxA, PFOA, PFNA and PFHxS, while m-PFOS was used to quantify PFDoA, PFBS and PFOS. Samples were extracted and analyzed in batches together with a procedural blank in order to control any external contamination during the analytical process. Instrument detection limits (LOD_{inst}) were calculated with a standard solution at a concentration of 0.001 ng/μL of each compound by using three times the signal-to-noise ratio value. Method detection limits (LOD_{method}) were calculated in the same way, using spiked cells at a concentration of 0.6 μM (Table 1). No traces of PFCs were detected in procedural blanks.

Cellular P450 aromatase (CYP19) activity. The assay was adapted from the method of Lephart and Simpson (1991) that measures the amount of ^3H_2O formed during the aromatization of [1β-^{3}H] androstenedione (^{3}H-AD) by JEG-3 cells. PFC test solutions ranged from 3 nM to 500 μM; the most cytotoxic PFCs (PFOS, PFDoA, PFNA and PFOA) were tested at an upper concentration of 100 μM, while PFBA, PFHxA, PFBS and PFHxS were tested at 500 μM and below.

Cells were seeded at a rate of 100,000 cells per well (24-well plate) and allowed to attach overnight in an incubator at 37 °C, 5% CO_2. After 24-hour exposure to the different PFCs, the cells were washed with PBS and incubated for 1 h in the presence of 39.5 nM of ^{3}H-AD in DPBS–Glucose. Under these conditions, titrated water production was linear over time. The reaction was stopped by placing the plate on ice and aspirating 1 mL of medium that was extracted with 3 mL of dichloromethane (×3). The amount of titrated water formed was counted in an aliquot of the aqueous phase (Tri-Carb 2100TR, Packard). Aromatase activity was expressed in pmol/min/mg protein.

Analysis of lipids in JEG-3 cells. Cells were seeded at a rate of 100,000 cells per well (24-well plate) and allowed to attach overnight. The medium was then replaced with a medium containing a mixture of the eight PFCs at a concentration of 0.6 and 6 μM each, or the solvent (0.4% ethanol). After 24 h incubation, the medium was aspirated; the cells were rinsed with PBS and trypsinized.

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 cell pellets, vortexed and extracted in an ultrasonic bath for 5 min (×2). The extracts were evaporated to dryness and stored at −20 °C in an argon atmosphere. Lipids were measured using an Acquity UPLC system (Waters, USA) connected to a Time of Flight (LCT Premier XE) Detector with an Acquity UPLC BEH C_8 column (1.7 μm particle size, 100 mm × 2.1 mm, Waters, Ireland) at a flow rate of 0.3 mL/min and column temperature of 30 °C. The mobile phase was methanol with 1 mM ammonium formiate and 0.2% formic acid (A)/water with 2 mM ammonium formiate and 0.2% formic acid (B). Gradient elution started at 80% of A, increased to 90% A in 3 min, held for 3 min, increased to 99% A in 9 min and held for 3 min. Initial conditions were attained in 2 min and the system was stabilized for 3 min. Phosphatidylcholine (PC), plasmalogen PC, lyso plasmalogen PC, diacylglycerol (DAG), triacylglycerol (TAG) and cholesterol ester (CE) were analyzed under positive ESI. Positive identification of the lipids was based on the accurate mass measurement with an error < 5 ppm and its LC relative retention time, compared to that of the standard (±2%) (Garanto et al., 2013). Glycerophospholipids, diacylglycerol, triacylglycerol and cholesteryl esters were annotated as <lipid subclass><total fatty acyl chain length>:<total number of unsaturated bonds>.

Curve fitting and statistical analysis. Statistical significance was assessed with non-parametric Mann–Whitney U and Kruskal–Wallis tests by using Stata 12. $P < 0.05$ was considered statistically significant. The concentrations which caused a 50% decline on cell viability (EC50) and on enzyme activity (IC50) were calculated using SigmaPlot 11.0.

Results

Cytotoxicity

No significant cytotoxicity was observed for the shortest chain length PFCs (PFBA, PFHxA, PFBS and PFHxS) after 24 h incubation with the human placental choriocarcinoma cell line JEG-3. In contrast, cell viability decreased to 55–59% following exposure to 500 μM PFOA, while PFOS, PFDoA and PFNA caused a decline in cell viability higher than 90% (Fig. 1). Both, CFDA-AM and Alamar Blue gave a similar response. PFOA, PFOS, PFDoA and PFNA were tested at lower concentrations to obtain the corresponding EC_{50}-values (Table 2). PFOS was the most cytotoxic compound for JEG-3 cells, with EC_{50} in the range of

Table 1
Elution times, MRM transitions monitored, optimized cone voltages, collision energies and quality parameters for the analyzed PFCs.

PFCs	Elution time (min)	Transition (m/z)	Cone voltage (V)	Collision energy (eV)	LOD_{instr} (pg)	LOD_{met} (pg)	Recoveries[a] (±SD)
PFBA	0.99	213 > 169	16	10	3.57	4.74	87 ± 8
PFBS	3.38	299 > 80	50	29	4.05	3.19	146 ± 15
		299 > 99		26			
PFHxA	3.77	313 > 119	15	8	3.33	3.84	98 ± 13
		313 > 269		22			
PFHxS	5.20	399 > 80	45	40	8.82	28.1	106 ± 7
		399 > 99		40			
PFOA	5.52	413 > 169	19	11	1.90	5.34	98 ± 1
		413 > 369		20			
m-PFOA	5.52	417 > 372	17	11	n.a.	n.a.	n.a.
PFNA	6.12	463 > 419	16	8	1.58	4.04	87 ± 7
PFOS	6.40	499 > 80	50	42	5.77	17.1	101 ± 11
		499 > 99		42			
m-PFOS	6.40	503 > 80	52	39	n.a.	n.a.	n.a.
		503 > 99		39			
PFDoA	7.53	613 > 169	20	10	5.73	9.21	70 ± 9
		613 > 569		28			

*In all cases the dwell time is 0.07 s.
n.d.: not determined.
[a] Values obtained in culture medium spiked with the mixture of PFCs at 6 μM.

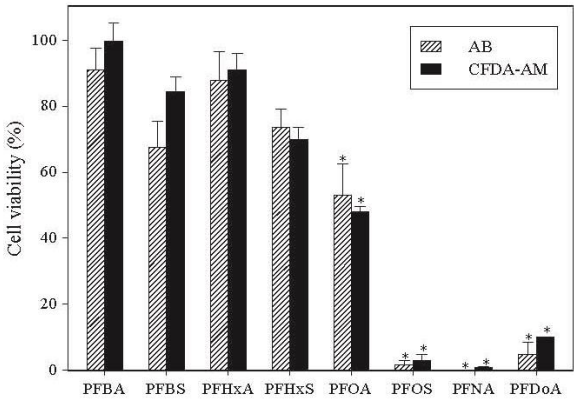


Fig. 1. Cytotoxicity of PFCs tested at a concentration of 500 μM in JEG-3 cells. Cell viability expressed as percentage of viable cells referred to control cells (exposed to the solvent). Values are mean ± SEM (n = 3). *Statistical significant differences with respect to the control ($P < 0.05$).

107–125 μM, followed by PFDoA and PFNA (181–220 μM) and PFOA (594–647 μM) (Table 2).

Uptake of PFCs

The differential bioavailability of PFCs in the in-vitro system was assessed by measuring the fraction retained in the cells right after exposure (time 0) to the eight PFC mixture (6 μM), and 1, 3, 5, 8 and 24 h later. Under the assay conditions, PFDoA and PFOS exhibited the highest concentration in JEG-3 cells. Right after dosing, a concentration of 149 pmol/mg cell protein was detected for PFDoA, which reached the maximum cellular concentration 5 h later (1000–1200 pmol/mg cell protein); no significant increase was detected thereafter (Fig. 2A). Similarly, a high concentration of PFOS was detected in the cells right after exposure (127 pmol/mg cell protein), and the maximum cellular concentration was reached after 5 h of exposure (340–470 pmol/mg cell protein). Comparatively, lower concentrations were detected for PFNA, PFOA and PFHxA at time 0 (3–10 pmol/mg cell protein) and 24 h later (10–39 pmol/mg cell protein) (Fig. 2B). Interestingly, maximal cellular concentration of PFHxA was reached after 1 h of exposure, while PFOA reached equilibrium after 3 h of exposure, and the long chain PFNA after 5 h. The concentration of PFBA, PFBS and PFHxS in the cells was below detection limit under our assay conditions.

P450 aromatase (CYP19) activity

CYP19 activity in JEG-3 cells following 24 h exposure to a wide range of PFC concentrations (3 nM–500 μM) is presented in Figs. 3 & 4. Fig. 3 summarizes the data obtained for the shorter chain PFCs (PFBA,

Table 2
Estimated EC_{50} of PFCs in the Alamar Blue and CFDA-AM cytotoxicity assays, and estimated IC_{50} for CYP19 activity. Values are mean ± SD (n = 3).

Compound	EC_{50} (μM)		IC_{50} (μM) CYP19
	Alamar Blue	CFDA-AM	
PFBA	–	–	n.d.
PFBS	–	–	68 ± 11
PFHxA	–	–	n.d.
PFHxS	–	–	298 ± 29
PFOA	594 ± 19	647 ± 22	80 ± 4
PFNA	213 ± 3	220 ± 3	132 ± 51
PFDoA	181 ± 10	219 ± 16	518 ± 562
PFOS	107 ± 9	125 ± 6	57 ± 4

–: No significant cytotoxicity at the highest concentration tested (500 μM).
n.d.: Not determined, low potential to inhibit CYP19 aromatase.

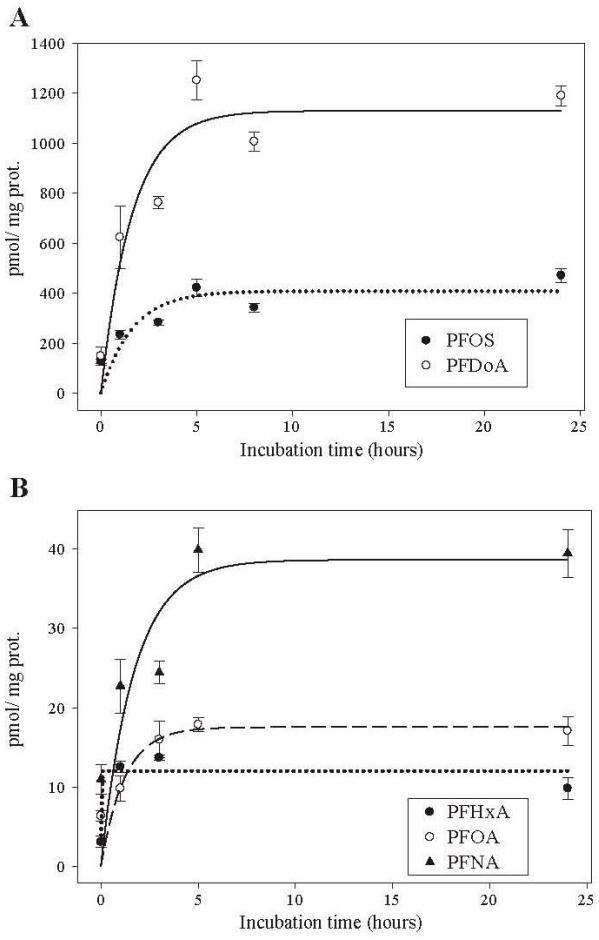


Fig. 2. Time-dependent concentration of (A) PFOS and PFDoA, and (B) PFHxS, PFOA and PFNA in JEG-3 cells exposed to a mixture of eight PFCs at a nominal concentration of 6 μM. Values are mean ± SEM (n = 3). Concentrations of PFBA, PFBS and PFHxS were below detection limit.

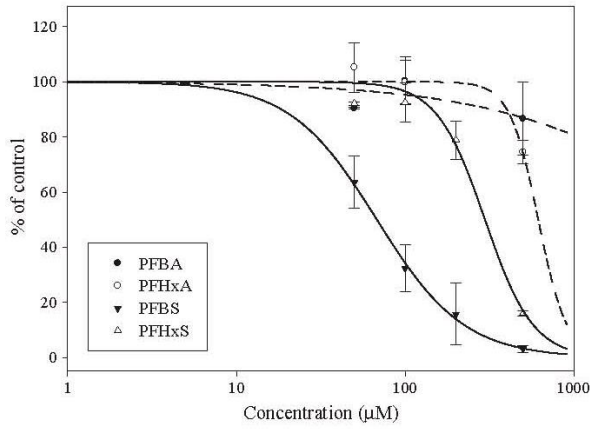


Fig. 3. Aromatase activity after exposure to PFBA, PFHxA, PFBS and PFHxS in the range of 1 μM to 500 μM. Values are relative to control cells (set to 100%) as mean ± SEM (n = 3). CYP19 aromatase activity in control cells was 0.252 ± 0.02 pmol/min/mg protein.

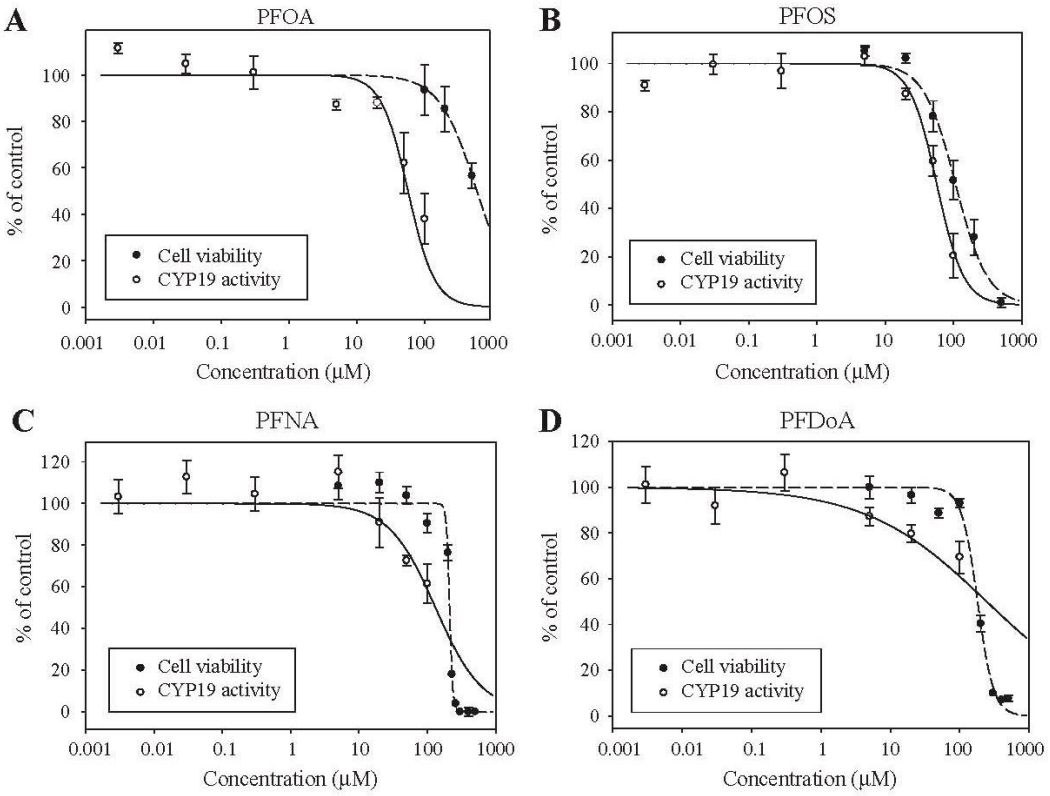


Fig. 4. Aromatase activity and percentage of cell viability in JEG-3 cells following 24 h exposure to different concentrations of PFOA (A), PFOS (B), PFNA (C) and PFDoA (D). Values are relative to control cells (set to 100%) as mean ± SEM (n = 3). CYP19 aromatase activity in control cells was 0.252 ± 0.02 pmol/min/mg protein.

PFHxA, PFBS and PFHxS) which were not cytotoxic for the cells. The sulfonates PFBS and PFHxS were stronger inhibitors of aromatase activity (97 and 84% inhibition when tested at 500 μM) than the corresponding acidic compounds (PFBA and PFHxA: 13 and 26% inhibition). Exposure of JEG-3 cells to the shorter chain PFCs resulted in a concentration dependent inhibition of aromatase activity, with an IC_{50} of 298 ± 29 μM for PFHxS and 68 ± 11 μM for PFBS (Table 2).

Conversely, the potential of longer chain PFCs (PFOA, PFOS, PFNA and PFDoA) to act as CYP19 inhibitors was to some extent affected by their toxicity, particularly for PFOS, as both toxicity and CYP19 inhibition curves were closely related (Fig. 4). Thus, 48% cell death and 80% inhibition of aromatase activity were observed for 100 μM PFOS, and IC_{50} (57 ± 4 μM) and EC_{50} (107–125 μM) differed only by a factor of 2. In contrast, PFOA was not cytotoxic but acted as a strong inhibitor of CYP19 activity (62% inhibition) when tested at 100 μM, with an IC_{50} of 80 ± 4 μM, comparable to the ones obtained for PFOS and PFBS. PFNA and PFDoA were weaker inhibitors of CYP19 aromatase: a 39% inhibition and a 31% inhibition were detected at 100 μM, when the toxicity of these compounds was very low (Fig. 4).

Changes in cell lipidome

A total of 45 lipids belonging to the classes of PC (16), plasmalogen PC (10), lyso-plasmalogen PC (1), DAG (6), TAG (10) and CE (2) were detected by UPLC-TOF ESI positive mode. Relative changes in lipid content of JEG-3 cells exposed for 24 h to the mixture of PFCs at 0.6 and 6 μM are indicated in Fig. 5. A statistically significant increase (2- to 3-fold) of PC, plasmalogen PC and lyso plasmalogen PC was detected together with a minor increase of TAG (30%), and no significant changes in the relative abundance of DAG and CE. The effects were more evident in cells exposed to 0.6 μM PFC mixture than in those exposed to 6 μM PFC.

The identified compounds were the following: lyso plasmalogen PC 18:1; plasmalogen PC (34:3, 34:2, 34:1, 34:0, 36:4, 36:3, 36:2, 36:1, 36:0, 38:4); PC (32:2, 32:0, 34:4, 34:3, 34:2, 34:1, 36:6, 36:3, 36:1,

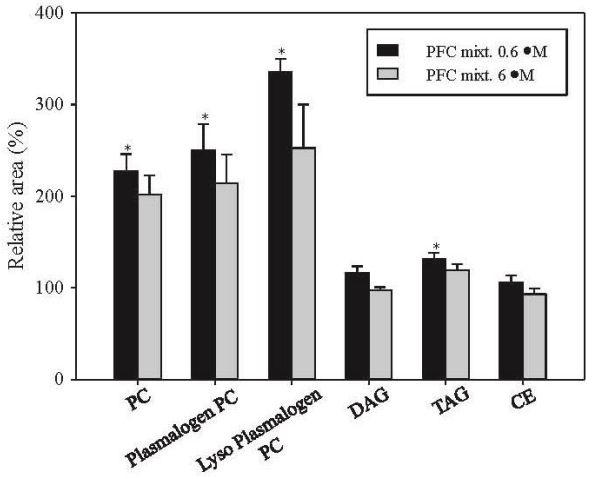


Fig. 5. Relative changes in lipid content of JEG-3 cells exposed to the mixture of PFCs at 0.6 and 6 μM. Lipids are grouped in families of PCs (16), PC-plasmalogens (10), LPC-plasmalogens (1), DGs (6), TGs (10) and ChEs (2). Values are relative to controls (set to 100%) and are mean ± SEM (n = 3). *Statistical significant differences with respect to the control ($P < 0.05$).

38:8, 38:7, 38:6, 38:4, 38:3, 38:2, 38:0); TAG (48:2, 48:1, 50:3, 50:1, 52:3, 52:2, 52:1, 54:3, 54:2, 54:1); CE (22:6, 18:1), and DAG (32:1, 32:0, 34:2, 34:0, 36:2, 36:1).

Discussion

PFCs with long fluorinated carbon chains, namely PFDoA, PFNA, PFOA and PFOS, were cytotoxic to JEG-3 cells, while the short chain ones (PFBA, PFBS, PFHxA and PFHxS) showed no cytotoxicity at the highest concentration tested (500 μM). Similarly, Kleszczyński et al. (2007) measured EC_{50}-values of different PFCAs in human colon carcinoma HCT116 cells showing that cytotoxicity increased with the elongation of the fluorocarbon chain; EC_{50}-values ranged from 124 for perfluorotetradecanoic acid (PFTeA) to 4154 μM for PFHxA. Also, Buhrke et al. (2013), by using the human hepatocarcinoma cell line HepG2 as an in vitro model for human hepatocytes, showed a positive correlation between the carbon chain length of the respective PFCA and its cytotoxicity. The EC_{50}-values obtained in JEG-3 cells are in agreement with those reported by Kleszczyński et al. (2007), but 9- to 25-fold higher than those reported in HepG2, which suggests a higher sensitivity of the latter to PFCs exposure in comparison to JEG-3 or HCT116 cells. Our work also evidenced a comparatively higher toxicity of the perfluorosulfonates than PFCAs of the same chain length, which is particularly evident for PFOS with an EC_{50} 5-fold lower than PFOA (Table 2).

Interestingly, length of the fluorocarbon chain was not the only factor affecting PFC absorption by JEG-3 cells. Among the eight analytes, PFDoA and PFOS showed the highest intracellular concentration (1190 and 470 pmol/mg protein after 24 h exposure), while the concentration of the long chain PFNA was 10- to 30-fold lower (Fig. 2). PFC residues detected in JEG-3 cells after 24 h exposure were as follows: PFDoA > PFOS ≫ PFNA > PFOA ~ PFHxA, being shorter chain PFCs (PFBA, PFBS, PFHxS) below detection limit. The cytotoxic effect of PFCs, although to some extent affected by the absorption of the compounds, did not follow the same pattern: PFOS > PFDoA ~ PFNA > PFOA > PFHxA. Interestingly, the non cytotoxic compounds (PFBA, PFHxA, PFBS and PFHxS) were the ones that were not detected in JEG-3 cells or that showed the lowest cell residue after 24 h exposure (Figs. 1 & 2).

Moreover, this work evidences a high potential of PFOS, PFOA and PFBS to act as aromatase inhibitors in placental cells. It is important to stress that not only PFBS, but also PFHxS significantly inhibited CYP19 aromatase activity despite the fact that the measured uptake of the compounds by cells was below detection limit. Therefore, both PFBS and PFHxS may exert the inhibitory effect on CYP19 aromatase activity at rather low endogenous cellular concentrations. These findings are of particular relevance since at present, producers of fluorochemicals are replacing long-chain for short-chain PFCs, which are expected to have a lower impact on environment and human health.

Reproductive toxicity following exposure to long chain PFCs has often been reported in animals and human cells. Thus, PFOA alters female pubertal timing in multiple strains of mice (Yang et al., 2009), and both PFOA and PFOS have been reported to delay pubertal timing in girls, but not in boys (Lopez-Espinosa et al., 2011). Exposure to PFOS (3×10^{-8}–3×10^{-7} M) increased estradiol concentration in H295R cell medium and increased CYP19 expression; however, exposure to 500 mg/L PFOS, decreased expression of CYP19a and CYP19b and changed the expression pattern of estrogen receptor in zebrafish embryos (Du et al., 2013). Similarly, Ankley et al. (2005) and Shi et al. (2008) found that PFOS exposure reduced aromatase activity in fathead minnow and gene expression zebrafish embryos, which is consistent with our findings, although in the present study, the inhibitory effect of PFOS is very closely related to its cytotoxic effect (Fig. 4B).

Concerning effects on JEG-3 cell lipidome, exposure to the mixture of PFCs produced a high increase in lyso plasmalogen PC, plasmalogen PC and PC, the major components of cell membranes, and a relatively low increase in TAG. The strongest alteration in cell lipids was registered at the lowest concentration tested (0.6 μM). These results suggest the ability of PFCs to interact with cellular membranes, possibly inducing the synthesis of PCs and plasmenyl-PCs as a defense mechanism of cells. Xie et al (2010a) reported that PFOS may cause adverse biological effects by altering the fluidity of lipid assemblies. Also PFOA has a high tendency to partition into phosphatidylcholine bilayers and to alter their phase behavior (Xie et al., 2010b). Furthermore, PFOA and PFOS exposures in PPARα knock-out mice have shown changes in gene expression indicative of lipotoxicity and altered fatty acid metabolism (Rosen et al., 2008, 2010).

Levels of PFOS and PFOA in human serum are in the range of 1 to 50 ng/mL, which corresponds to concentrations of 2 and 100 nM, respectively. However, the concentrations in occupationally exposed workers rise to 0.6 and 4.8 μM, respectively (Olsen et al., 1998). Thus, although we report significant alterations in JEG-3 cell lipidome at concentrations about two orders of magnitude above the PFC levels reported in human serum, the observed alterations are very likely to occur in occupationally exposed workers. Moreover, future studies are needed to investigate the ability of individual components of the PFC mixture to alter lipid profiles, as well as to determine the lowest concentration of individual and combined PFCs that leads to a significant alteration of cellular lipids.

Overall, this work contributes to the better knowledge of the effects of PFCs in human cells indicating an interference with cellular lipids at concentrations well below those associated with other adverse effects, such as cytotoxicity or endocrine disruption. The work also highlights: (a) the ability of PFOS and PFOA, particularly the shorter chain PFBS and PFHxS, to inhibit CYP19 aromatase activity in human placental cells, and (b) the importance of measuring the uptake of xenobiotics by cells in further in-vitro studies in order to establish a more realistic concentration–effect scenario.

Acknowledgments

This study was financed by the Ministry of Education, Science and Innovation in Spain, with the project INNPACTO [IPT-2011-0709-060000] and the Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya Grant 2009SGR1072. Dr. R. Chaler, D. Fanjul and M. Comesaña are acknowledged for MS support.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Ankley, G.T., Kuehl, D.W., Kahl, M.D., Jensen, K.M., Linnum, A., Leino, R.L., Villeneuve, D.A., 2005. Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 24, 2316–2324.

Buhrke, T., Kibellus, A., Lampen, A., 2013. In vitro toxicological characterization of perfluorinated carboxylic acids with different carbon chain lengths. Toxicol. Lett. 218, 97–104.

Christie, W.W., 1985. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. J. Lipid Res. 26, 507–512.

Du, G., Hu, J., Huang, H., Qin, Y., Han, X., Wu, D., Song, L., Xia, Y., Wang, X., 2013. Perfluorooctane sulfonate (PFOS) affects hormone receptor activity, steroidogenesis, and expression of endocrine-related genes in vitro and in vivo. Environ. Toxicol. Chem. 32, 353–360.

Ericson, I., Gómez, M., Nadal, M., van Bavel, B., Lindström, G., Domingo, J.L., 2007. Perfluorinated chemicals in blood of residents in Catalonia (Spain) in relation to age and gender: a pilot study. Environ. Int. 33, 616–623.

Fernández-Sanjuan, M., Meyer, J., Damásio, J., Faria, M., Barata, C., Lacorte, S., 2010. Screening of perfluorinated chemicals (PFCs) in various aquatic organisms. Anal. Bioanal. Chem. 398, 1447–1456.

Garanto, A., Mandal, N.A., Egido-Gabás, M., Marfany, G., Fabriàs, G., Anderson, R.E., Casas, J., Gonzàlez-Duarte, R., 2013. Specific sphingolipid content decrease in *Cerkl* knockdown mouse retinas. Exp. Eye Res. 110, 96–106.

Giesy, J.P., Kannan, K., 2002. Perfluorochemical surfactants in the environment. Environ. Sci. Technol. 36, 146A–152A.

Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B., 2006. Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Mol. Endocrinol. 20, 2141–2155.

Hines, E.P., White, S.S., Stanko, J.P., Gibbs-Flournoy, E.A., Lau, C., Fenton, S.E., 2009. Phenotypic dichotomy following developmental exposure to perfluorooctanoic acid (PFOA) in female CD-1 mice: low doses induce elevated serum leptin and insulin, and overweight in mid-life. Mol. Cell. Endocrinol. 304, 97–105.

Hu, W.Y., Jones, P.D., DeCoen, W., King, L., Fraker, P., Newsted, J., Giesy, J.P., 2003. Alterations in cell membrane properties caused by perfluorinated compounds. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135, 77–88.

Huang, H., Leung, L.K., 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol. Lett. 189, 248–252.

Kannan, K., Corsolini, S., Falandysz, J., Oehme, G., Focardi, S., Giesy, J.P., 2002. Perfluorooctanesulfonate and related fluorinated hydrocarbons in marine mammals, fishes, and birds from coasts of the Baltic and the Mediterranean Seas. Environ. Sci. Technol. 36, 3210–3216.

Kleszczyński, K., Gardzielewski, P., Mulkiewicz, E., Stepnowski, P., Składanowski, A.C., 2007. Analysis of structure–cytotoxicity in vitro relationship (SAR) for perfluorinated carboxylic acids. Toxicol. in Vitro 21, 1206–1211.

Lephart, E.D., Simpson, E.R., 1991. Assay of aromatase activity. Methods Enzymol. 206, 477–483.

Lopez-Espinosa, M.J., Fletcher, T., Armstrong, B., Genser, B., Dhatariya, K., Mondal, D., Ducatman, A., Leonardi, G., 2011. Association of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) with age of puberty among children living near a chemical plant. Environ. Sci. Technol. 45, 8160–8166.

Lou, Q.Q., Zhang, Y.F., Zhou, Z., Shi, Y.L., Ge, Y.N., Ren, D.K., Xu, H.M., Zhao, Y.X., Wei, W.J., Qin, Z.F., 2013. Effects of perfluorooctanesulfonate and perfluorobutanesulfonate on the growth and sexual development of *Xenopus laevis*. Ecotoxicology 22, 1133–1144.

Nelson, J.W., Hatch, E.E., Webster, T.F., 2010. Exposure to polyfluoroalkyl chemicals and cholesterol, body weight, and insulin resistance in the general U.S. population. Environ. Health Perspect. 118, 197–202.

Olsen, G.W., Gilliland, F.D., Burlew, M.M., Burris, J.M., Mandel, J.S., Mandel, J.H., 1998. An epidemiologic investigation of reproductive hormones in men with occupational exposure to perfluorooctanoic acid. J. Occup. Environ. Med. 40, 614–622.

Rosen, M.B., Abbott, B.D., Wolf, D.C., Corton, J.C., Wood, C.R., Schmid, J.E., Das, K.P., Zehr, R. D., Blair, E.T., Lau, C., 2008. Gene profiling in the livers of wild-type and PPARalpha-null mice exposed to perfluorooctanoic acid. Toxicol. Pathol. 36, 592–607.

Rosen, M.B., Schmid, J.R., Corton, J.C., Zehr, R.D., Das, K.P., Abbott, B.D., Lau, C., 2010. Gene expression profiling in wild-type and PPARalpha-null mice exposed to perfluorooctane sulfonate reveals PPARalpha-independent effects. PPAR Res. 2010. http://dx.doi.org/10.1155/2010/794739.

Rosenmai, A.K., Nielsen, F.K., Pedersen, M., Hadrup, N., Trier, X., Christensen, J.H., Vinggaard, A.M., 2013. Fluorochemicals used in food packaging inhibit male sex hormone synthesis. Toxicol. Appl. Pharmacol. 266, 132–142.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. in Vitro 11, 107–119.

Shi, Z., Zhang, H., Liu, Y., Xu, M., Dai, J., 2007. Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. Toxicol. Sci. 98, 206–215.

Shi, X., Du, Y., Lam, P.K., Wu, R.S., Zhou, B., 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicol. Appl. Pharmacol. 230, 23–32.

Shi, Z., Ding, L., Zhang, H., Feng, Y., Xu, M., Dai, J., 2009. Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. Toxicol. Lett. 188, 192–200.

Stahl, T., Mattern, D., Brunn, H., 2011. Toxicology of perfluorinated compounds. Environ. Sci. Eur. 23–38, 1–52.

Vinggaard, A.M., Hnida, C., Breinholt, V., Larsen, J.C., 2000. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol. in Vitro 14, 227–234.

Wan, H.T., Zhao, Y., Wong, M.H., Giesy, J.P., Wong, C.K.C., 2011. Testicular signaling is the potential target of perfluorooctanesulfonate-mediated subfertility in male mice. Biol. Reprod. 84, 1016–1023.

Xie, W., Ludewig, G., Wang, K., Lehmler, H.J., 2010a. Model and cell membrane partitioning of perfluorooctanesulfonate is independent of the lipid chain length. Colloids Surf. B: Biointerfaces 76, 128–136.

Xie, W., Bothun, G.D., Lehmler, H.J., 2010b. Partitioning of perfluorooctanoate into phosphatidylcholine bilayers is chain length-independent. Chem. Phys. Lipids 163, 300–308.

Yang, C., Tan, Y.S., Harkema, J.R., Haslam, S.Z., 2009. Differential effects of peripubertal exposure to perfluorooctanoic acid on mammary gland development in C57Bl/6 and Balb/c mouse strains. Reprod. Toxicol. 27, 299–306.

Zhao, B., Chu, Y., Hardy, D.O., Li,, X.k, Ge, R.S., 2010. Inhibition of 3β- and 17β-hydroxysteroid dehydrogenase activities in rat Leydig cells by perfluorooctane acid. J. Steroid Biochem. Mol. Biol. 118, 13–17.

V. Discusión de Resultados

En esta tesis se ha estudiado el uso de modelos biológicos *in vitro* para la evaluación de la toxicidad y los mecanismos de acción de contaminantes ambientales, tanto mezclas de xenobióticos como compuestos individuales, con especial énfasis en citotoxicidad, estrés oxidativo, disrupción endocrina, activación del metabolismo de xenobióticos y alteración de los niveles de lípidos en la célula.

Por un lado, se investiga el impacto que la actividad humana (puertos, ríos, efluentes de WWTP) ejerce sobre la zona costera y, en particular, en el sustrato bentónico, mediante el uso de una línea celular de peces (PLHC-1), una línea celular de riñón de mono (COS-7) transfectada con zfPxr, y fracciones microsomales de ovario de lubina (*Dicentrarchus labrax*), como modelos biológicos en una batería de ensayos *in vitro* que investigan diferentes CYPs y receptores nucleares implicados en el metabolismo celular (Tema 1; artículos del 1 al 4 y resultados adicionales). Se discute la utilidad de los diferentes bioensayos para su uso en estudios de biovigilancia, sustituyendo al menos parcialmente a los estudios basados en el uso de biomarcadores en organismos centinela.

Por otro lado, se estudia la toxicidad potencial de diferentes grupos de contaminantes emergentes, mediante el uso de una línea celular de placenta humana (JEG-3) y su fracción subcelular S9, en experimentos *in vitro* que estudian su citotoxicidad, su potencial de disrupción endocrina y su capacidad de generar estrés oxidativo. A partir de ahí, se investiga la biodisponibilidad de los compuestos en el medio de cultivo, así como el impacto de los alquilfenoles en la composición lipídica celular (Tema 2; artículos del 5 al 7).

Los resultados obtenidos se discuten a continuación.

V.1. TEMA 1.

EVALUACIÓN DE LA CALIDAD AMBIENTAL DE SEDIMENTOS MARINOS

En este tema se ha investigado el impacto de la contaminación sobre el sustrato bentónico en zonas costeras, a través del análisis de los sedimentos procedentes de diferentes áreas del Mar Mediterráneo (Italia y Croacia; 13 muestras de sedimento), Mar Negro (Rumanía; 10), Estuario del Algarve (Portugal; 14), Bahía de Paranaguá (Brasil; 8), y Antártida (12). La caracterización del riesgo de muestras ambientales complejas como son los sedimentos marinos, requiere utilizar diferentes bioensayos que proporcionen información acerca de los diferentes modos de acción de la mezcla de contaminantes presentes en la muestra. Mediante la batería de bioensayos *in vitro* realizada, se han podido detectar diferentes niveles de citotoxicidad, presencia de agentes disruptores endocrinos, agonistas de los receptores AhR y zfPxr, y agentes pro-oxidantes, en los extractos de sedimentos. Los sedimentos de los puertos y deltas/estuarios eran los que mostraban mayor **citotoxicidad** en la línea celular de peces PLHC-1. Cabe mencionar que, dado que la citotoxicidad es una consecuencia asegurada tras la exposición celular a una determinada concentración de cualquier muestra ambiental y generalmente se manifiesta a concentraciones más altas que a las cuales ocurre la inducción de respuestas metabólicas, dicho ensayo se implementa más como una medida de control de calidad que verifica que la mortalidad celular no está implicada en la inducción de respuesta observada, que como un marcador en sí. Es decir, los efectos subletales son los que tienen un mayor valor desde un punto de vista de caracterización del riesgo toxicológico. En la Tabla 3 se muestra una perspectiva global de los resultados obtenidos, mediante un código de

colores que facilita la visualización de la magnitud del efecto. El color verde indica efecto a altas concentraciones (baja potencia), y las transiciones al rojo indican presencia de contaminantes muy activos (elevada potencia). El código de colores se basa en un valor medio (amarillo) correspondiente a: 50 % de viabilidad (AB), 30 mg eQsed/mL (R_{BNF} y REC_{20}), inducción ROS de 2.5 veces, y 75 % de actividad aromatasa (CYP19). Así, los tonos rojos indican un elevado impacto antropogénico en la zona y los verdes señalan zonas relativamente limpias, mientras que los matices amarillos indican zonas intermedias.

ACTIVACIÓN DEL METABOLISMO DE XENOBIÓTICOS

El sistema enzimático CYP1A, inducido por la unión de un ligando al receptor AhR, juega un importante papel en el metabolismo de xenobióticos, y se ha utilizado con éxito como biomarcador de exposición a contaminantes ambientales en las células de peces PLHC-1. Los sedimentos que inducían una mayor respuesta CYP1A, pertenecen principalmente a los puertos: Constanza, Mangalia (ambos en el Mar Negro), Ravenna (Mar Mediterráneo), Split (Bahía de Kaštela , Mar Mediterráneo) y Portimao (estuario del Algarve); a las desembocaduras de los ríos: Danubio (Mar Negro), Po (Mar Mediterráneo), Jadro (Bahía de Kaštela) y Arade (estuario del Algarve); y a las zonas influenciadas por tráfico de barcos: aguas abiertas a 20 millas del puerto de Constanza, otra zona similar a 35 millas frente a la desembocadura del Po, Bahía de Kaštela, y zonas al noroeste del Mar de Weddell en la Antártida (Tabla 3). Hasta la fecha, este es el primer trabajo que demuestra la presencia de inductores de actividad EROD en sedimentos marinos de la Antártida, y evidencia la necesidad de biovigilancia ambiental en este remoto y frágil ecosistema.

Tabla 3: Perspectiva global de la calidad ambiental de los sedimentos marinos estudiados, basada en el porcentaje de viabilidad celular (AB) a 60 mg eQsed/mL, inducción CYP1A (R_{BNF}), activación zfPxr (REC_{20}), inducción máxima de ROS, y disrupción endocrina (CYP19: porcentaje de actividad P450 aromatasa a 2 mg eQsed/mL). El color verde indica efecto a altas concentraciones (baja potencia), y las transiciones al rojo indican presencia de contaminantes muy activos (elevada potencia). El código de colores parte del amarillo (valor medio), correspondiendo a: 50 % de viabilidad (AB), 30 mg eQsed/mL (R_{BNF} y REC_{20}), 2.5 veces ROS, y 75 % de actividad aromatasa (CYP19). n.a.: ensayo no realizado.

Area	Criteria	Site	AB	CYP1A	zfPxr	ROS	CYP19
Black Sea	Danube mouth north	B1					
(Rumania)	Danube mouth	B2					
	Danube mouth south	B3					
	Touristic resort/upstream Constanta	B4					
	Urban WWTP	B5					
	Roadstead of the Constanta harbour	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	Po mouth	A7					
(Italy)	Po mouth	A4					
	Po influenced area (35 nautical miles from Pila mouth)	A8					
	City harbor (Ravenna), petrochemical industry	A2					
	Reference area	A1					
Kastela Bay	Industrial, Jadro River discharges	S1B					n.a.
(Croatia)	Former chloralkali plant, marine traffic (Port of Split)	S1C					n.a.
	Marine traffic	S3					n.a.
	Marine traffic	S4					n.a.
Brac Channel	Marine traffic (Split Ferry port)	S5					n.a.
(Croatia)	Zrnovnica River discharge	S9					n.a.
	Center of the channel, Split WWTP	S11					n.a.
	Cetina River discharge	S15					n.a.
Paranaguá	Antonina Bay, agriculture, harbour	P1			n.a.		
(Brazil)	Paranaguá harbor, industrial activities	P4			n.a.		
	Urban and industrial activities, Itibere River discharge	P5			n.a.		
	Cotinga Island (runoff from urban effluents)	P7			n.a.		
	Cobras Island-Galheta Channel, (boat traffic)	P10			n.a.		
	DNOS Channel (boat traffic)	P12			n.a.		
	agriculture, protected area	P16			n.a.		
	Low traffic, preserved area	P17			n.a.		
					n.a.		
Arade	Near the estuary mouth (boat traffic)	Ar1			n.a.		
(Portugal)	Harbour of Portimao	Ar2			n.a.		
	Shipyard and fishing harbour	Ar3			n.a.		
	Waste water effluents of the city	Ar4			n.a.		
	Marina resort and waste water effluents	Ar5			n.a.		
	Highway bridge (car traffic)	Ar6			n.a.		
	Animal husbandry, agriculture and city of Silves	Ar7			n.a.		
Guadiana	Coastal area outside the estuary, beach zone (ref)	G1			n.a.		
(Portugal)	Estuary mouth (boat traffic)	G2			n.a.		
	Harbour of Vila Real de St. Antonio	G3			n.a.		
	Harbour of Ayamonte	G4			n.a.		
	Waste water effluents of the city Vila Real St. Antonio	G5			n.a.		
	Highway bridge (car traffic)	G6			n.a.		
	Abandoned mines, cattle breeding and agriculture	G7			n.a.		
Wedell Sea	Southern Weddell Sea/ Filchner shelf (remote area)	108			n.a.	n.a.	n.a.
(Antarctica)		145			n.a.	n.a.	n.a.
		153			n.a.	n.a.	n.a.
		198			n.a.	n.a.	n.a.
	Northwestern Wedell Sea (boat traffic)	120			n.a.	n.a.	n.a.
		163			n.a.	n.a.	n.a.
	Drake Passage / Bransfield Strait (boat traffic)	202			n.a.	n.a.	n.a.
		238			n.a.	n.a.	n.a.
	Eastern Weddell Sea / Austasen shelf (scientific bases)	260			n.a.	n.a.	n.a.
		276			n.a.	n.a.	n.a.
		279			n.a.	n.a.	n.a.
		283			n.a.	n.a.	n.a.

Por el contrario, otros extractos de sedimentos generaban muy poca o ninguna respuesta CYP1A en las células de peces, incluyendo los extractos correspondientes al Canal de Brač y los estuarios del Guadiana y Paranaguá, indicando que se trata de zonas relativamente limpias de agonistas de AhR. No obstante, no se debe descartar la posibilidad de que exista una mayor dilución o movilización de contaminantes hacia mar abierto por tratarse de zonas más expuestas a las corrientes marinas. Esta teoría se refleja en el caso de la costa de Croacia, donde el Canal de Brač está expuesto a aguas abiertas y presenta menor concentración de agentes inductores de CYP1A, mientras que la Bahía de Kaštela muestra un mayor impacto estando en aguas más confinadas, con menor tasa de renovación, lo que favorece la acumulación de material particulado y contaminantes en esta última (Tabla 3). Dicha teoría también explica la respuesta gradual de activación zfPxr, desde el punto más interior de la Bahía de Kaštela (S1B) hacia las zonas más expuestas (S11 y S15). Así mismo, en el Delta del Danubio también se ha observado una respuesta gradual de nordeste a suroeste, en los ensayos de citotoxicidad, inducción CYP1A, activación zfPxr y generación de estrés oxidativo, poniendo de manifiesto la redistribución de la materia particulada y los contaminantes asociados, a favor de las corrientes marinas principales, por efecto de Coriolis, a lo largo de la costa del Mar Negro. Comparativamente, los sedimentos de la desembocadura del río Danubio, cuya superficie y caudal abarcan los 2900 km y 6500 m^3/s, está significativamente más impactada que la de otros ríos menos caudalosos, como el río Po (650 km; 1500 m^3/s). Este hecho también se explica por el transporte de los contaminantes a favor de corriente, desde el foco de descarga hasta el mar, de forma que en el delta se combinan las mezclas de

compuestos provenientes de distintas fuentes, aumentando su concentración disponible en los sedimentos.

Recientemente, Lechner et al. (2014) observó un gradiente de microplásticos en las aguas del Danubio: 4 g/s en Viena (Austria), 17 g/s en Pančevo (Serbia), y 48 g/s en la desembocadura del Danubio, lo cual probablemente refleja el comportamiento de otros contaminantes que acaban en la costa de Rumanía.

Curiosamente, las células expuestas a los extractos de sedimentos de la desembocadura del estuario de Arade (afectada por tráfico de barcos), Antonina Bay (agricultura y actividades portuarias) y puerto de Paranaguá (actividades portuarias e industriales) no mostraban actividad EROD, a pesar de generar citotoxicidad. No se puede descartar la presencia de inhibidores de la actividad CYP1A en los extractos de sedimentos (ej. benzo[a]antraceno-7,12-quinona, benzo[a]fluorenona, fluoranteno y/o metales, entre otros). En este caso no se deben tener en cuenta los metales porque el método de extracción utilizado no es adecuado para obtener este tipo de compuestos (Rosado et al., 2016).

Los resultados para las muestras de los puertos de Constanza, Mangalia y Ravenna, delta del Po y noroeste del Mar de Weddell generan una curva dosis-respuesta acampanada. En estos casos, la inducción de actividad EROD decrece a las concentraciones más altas (para facilitar la visualización, los gráficos de las publicaciones sólo muestran hasta la concentración de máxima actividad). Para los sedimentos del puerto de Constanza y Mar de Weddell, este hecho se explica por la citotoxicidad asociada, mientras que para el resto, se atribuye a efectos simultáneos de agentes inductores e inhibidores. En sus experimentos, Fent et al. (2000) observaron que la

interacción entre los PAHs presentes en mezclas de hasta ocho compuestos individuales mostraba una respuesta aditiva de actividad EROD. Mientras que en matrices ambientales complejas, se observaban efectos combinados de inducción e inhibición entre los compuestos presentes en la muestra (Bols et al., 1999; Bosveld et al., 2002; Brack et al. 2002 & 2003; Sadauskas-Henrique et al., 2017).

La interpretación, integración y comparación de los resultados obtenidos en los ensayos de actividad EROD y activación zfPxr es una tarea complicada cuando se tiene un número considerable de muestras. La respuesta máxima para cada muestra, no ofrece información sobre la concentración a la cual ocurre, mientras que la EC_{50} no proporciona el valor de inducción máxima. En este sentido, el uso de controles positivos permite calibrar el sistema en cada ensayo. En este trabajo se propone la aplicación de los índices REC_{20} (concentración de muestra requerida para inducir zfPxr en la misma medida que el 20 % de la respuesta máxima del control positivo clotrimazol) y R_{BNF} (concentración de muestra requerida para inducir CYP1A en la misma medida que el control positivo, 1 µM β-naftoflavona) en los ensayos de activación zfPxr y actividad EROD, respectivamente. Estos índices integran el nivel de respuesta y la concentración de muestra a la cual ocurre, que junto con el valor de inducción máxima permiten comparar resultados de diferentes laboratorios, así como comparar entre la calidad ambiental de diferentes áreas marinas del mundo, solventando los inconvenientes citados anteriormente.

Los índices R_{BNF} y REC_{20} han permitido clasificar los sedimentos del Mar Negro y Mar Adriático (artículos 1 y 2) en tres grupos, de acuerdo con la presencia de agentes inductores de CYP1A y/o de ligandos de zfPxr. Al primer grupo pertenecen el puerto de Constanza, los puntos más interiores

de la Bahía de Kaštela , y la desembocadura sur del río Danubio, con un alto contenido de agonistas AhR y zfPxr (R_{BNF} y REC_{20} < 14 mg eQsed/mL). El segundo grupo, con valores R_{BNF} y REC_{20} entre 18 y 32 mg eQsed/mL, abarca la zona de la desembocadura del Po, la costa de Ravenna, el Canal de Brač (excepto S11), el puerto de Mangalia y el punto situado en mar abierto frente al puerto de Constanza (B7) influenciado por líneas regulares de barcos. Y el tercer grupo incluye las áreas menos impactadas, correspondientes a la desembocadura norte del Danubio, alrededores de Constanza (que incluyen las áreas turísticas de Cazino Mamaia y Costinesti), el área de referencia A1 en Italia, y el punto central del Canal de Brač (S11) (R_{BNF} y REC_{20} > 32 mg eQsed/mL). El hecho de que la mayoría de los extractos de sedimentos con capacidad para inducir una respuesta CYP1A a través del receptor AhR mostraran una respuesta análoga en los ensayos de activación zfPxr, sugiere la existencia de ligandos comunes para ambos receptores en los extractos de sedimentos. Sin embargo, no todas las muestras que activaban zfPxr podían inducir actividad EROD (ej. Canal de Brač; Tabla 3), lo que sugiere que la gama de ligandos de zfPxr es, comparativamente, más amplia que la de AhR.

Actualmente, no cabe duda de la promiscuidad del receptor zfPxr, dado que sus ligandos abarcan una larga y variada lista de xenobióticos, incluyendo pesticidas, plastificantes y compuestos industriales, así como moléculas endógenas, como precursores del colesterol y hormonas esteroideas (Milnes et al., 2008; Ekins et al., 2008). Estudios previos demostraron la activación de PXR humano por muestras de aguas de río, afectadas por descargas agrícolas y urbanas, que contenían alquilfenoles, hormonas, productos farmacéuticos, pesticidas, PCBs, BPA y efluentes de plantas de tratamiento de aguas (Creusot et al., 2010; Kinani et al., 2010). Hasta lo que sabemos,

este es el primer estudio que investiga la capacidad de mezclas de contaminantes presentes en sedimentos marinos, para activar Pxr de pez cebra *in vitro*. El receptor zfPxr regula la expresión coordinada de genes implicados en el metabolismo de xenobióticos, incluyendo *pxr, cyp3a y mdr1* (Bresolin et al., 2005; Wassmur et al., 2010), y actúa como detector de xenobióticos con una importante función como protector celular, muy implicado en la regulación de la biotransformación. Aunque la activación de zfPxr representa un marcador de exposición a xenobióticos, la ausencia de activación no debe considerarse estrictamente una prueba de ausencia de xenobióticos en la muestra. Ciertamente, los extractos de sedimentos contienen una variedad de compuestos que podrían contribuir de forma aditiva, inhibitoria o sinérgica a la respuesta de activación zfPxr y AhR. Estos hechos evidencian la necesidad de investigar múltiples vías del metabolismo cuando se trata de mezclas complejas de contaminantes, en estudios de caracterización del riesgo ambiental.

Por otro lado, es importante mencionar que, aunque clásicamente se relaciona la toxicidad del compuesto únicamente con la concentración de la muestra en el pocillo de la placa de cultivo, se ha observado que el volumen de exposición también influye significativamente en los ensayos *in vitro*. Por ejemplo, en el ensayo de actividad EROD, se detectó un aumento en la respuesta celular cuando el volumen de exposición se incrementaba sin variar la concentración del extracto en el pocillo (Figura 14; no publicado). Esto se debe a que el número de moles en el pocillo es directamente proporcional al volumen, y por lo tanto, un mayor volumen implica más moles disponibles para las células, y un mayor efecto. Así mismo, a lo largo de este trabajo se optimizó la concentración de 7-etoxiresorufina (sustrato para determinar la actividad EROD), pasando de 2 µM a 4 µM, dado que en

el primer caso, en algunas muestras la concentración de sustrato era limitante. Estos datos son de una particular relevancia dado que el volumen no se acostumbra a indicar en las publicaciones científicas, pero tienen una gran repercusión en los resultados del estudio.

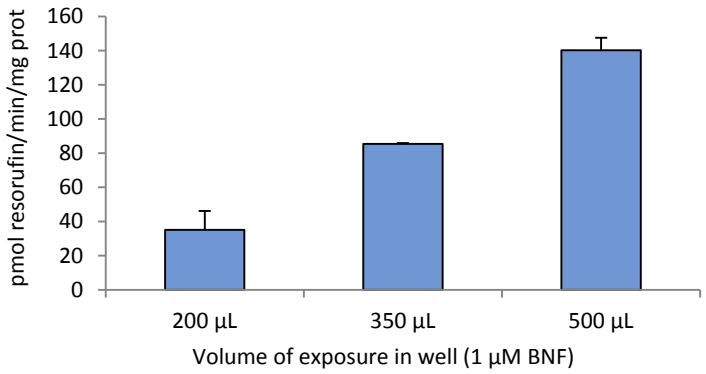


Figura 14. Aumento de actividad EROD en células PLHC-1 expuestas durante 24 h a 1 µM de BNF, según el volumen final de exposición. La concentración y volumen de sustrato (7-etoxiresorufina) en el ensayo eran 4 µM y 500 µL, respectivamente.

ESTRÉS OXIDATIVO

Paralelamente a los ensayos para detectar la activación de los sistemas de biotransformación de xenobióticos, se han realizado experimentos para determinar la generación de especies reactivas de oxígeno en las células de peces PLHC-1 expuestas a los extractos de sedimentos. Prácticamente la totalidad de los sedimentos del Mar Negro y Mar Adriático italiano, generaban niveles significativos de ROS que incrementaban a lo largo del tiempo de exposición (180 min), indicando que el tiempo de reparación, en su caso, supera estas tres horas. Aunque tres horas está dentro del tiempo que aún se considera estrés oxidativo agudo, unos niveles continuados de ROS apuntarían a un estrés oxidativo crónico, o incluso de permanencia indefinida (Lushchak, 2016). Curiosamente, los sedimentos procedentes de zonas urbanas costeras (Constanza, Ravenna y Costinesti), eran los que

generaban los niveles de ROS más elevados (hasta 4.5 veces más que las células control). Estos niveles de ROS triplican a aquellos generados por los sedimentos del Canal de Brač (Croacia) y del estuario del Algarve, y doblan a los inducidos por los extractos del complejo estuárico de la Bahía de Paranaguá, así como de otros estuarios y áreas costeras del Mar Cantábrico (Schnell et al., 2013).

El estrés oxidativo resulta en daños a proteínas, ácidos nucleicos, lípidos y carbohidratos, e incluso muerte celular. Así, los niveles altos de ROS están implicados en el envejecimiento y en varios procesos patológicos. Realmente es muy complicado definir cuál es el grado en que la presencia de agentes pro-oxidantes en el medio acuático contribuye a la reducción del bienestar físico de los animales, enfermedades, susceptibilidad a infecciones y parásitos, crecimiento, desarrollo, reproducción y supervivencia (Livingstone, 2001). Las sustancias que inducen ROS son compuestos implicados en ciclos redox, incluyendo quinonas, compuestos nitroaromáticos, nitroaminas y herbicidas, pero también algunos PAHs, PCBs y otros hidrocarburos halogenados, dioxinas y metales. El ensayo ROS utilizado en este estudio (H_2DCF-DA), ofrece información cualitativa sobre la presencia de agentes capaces de incrementar los niveles de ROS por encima del nivel homeostático celular, aunque no da información sobre los procesos por los cuales ocurre.

La presencia de agentes generadores de ROS en los sedimentos, no estaba relacionada con la inducción CYP1A ni con la activación de zfPxr en muchos casos. Previamente se había observado que el fungicida hexaclorobenceno estimulaba la producción de ROS en el cerebro de carpa (*C. carpio*), pero no inducía actividad CYP1A en sus hepatocitos (Smeets et al., 1999; Song et al., 2006). Lushchak (2016) lo explicaba por dos mecanismos posibles: unión del

compuesto a los citocromos y, si no se metaboliza fácilmente, desacoplamiento de la cadena de electrones de la monooxigenasa, favoreciendo la producción de ROS; y/o entrada del principal metabolito, pentaclorofenol, en reacciones de autooxidación con producción de ROS.

En la mayoría de estudios, se investigan los niveles de ROS inducidos por contaminantes individuales. Sin embargo, el uso de mezclas de contaminantes presentes en matrices ambientales sería más realista (Lushchak, 2016). En este sentido, la puerta sigue abierta a futuras investigaciones que esclarezcan los mecanismos por los cuales se incrementan los niveles de ROS en presencia de estas matrices ambientales, y cuáles son los compuestos responsables.

DISRUPCIÓN ENDOCRINA

Debido a su implicación clave en el desarrollo de los peces y demás vertebrados, la inhibición de la actividad del enzima P450 aromatasa, codificada por *cyp19*, por la mezcla de xenobióticos presentes en los sedimentos marinos, merece una especial consideración. La fracción microsomal de ovario de lubina (*Dicentrarchus labrax*) se ha utilizado como herramienta para detectar la presencia de agentes disruptores endocrinos en los extractos de sedimentos del Mar Negro, Mar Adriático, Bahía de Paranaguá, y estuario del Algarve. A concentraciones elevadas (60 mg eQsed/mL), prácticamente la totalidad de los sedimentos eran capaces de inhibir la actividad P450 aromatasa en los microsomas ováricos de lubina. Sin embargo, aquellos obtenidos en la desembocadura del Danubio, áreas alrededor de la ciudad de Constanza (puerto, área turística de Costinesti, mar abierto a 20 millas náuticas), el resort Marina del estuario y el área bajo

el puente de la autopista que cruza el río Arade, así como la zona G7 afectada por minas abandonadas, ganadería y agricultura del estuario del río Guadiana, generan mayor preocupación por su capacidad para inhibir la actividad CYP19 de 34 % hasta 55 % a concentraciones de 2 mg eQsed/mL (Tabla 3; artículos 1, 3 y 4), evidenciando la presencia de agentes disruptores endocrinos potencialmente efectivos en estas áreas. Ciertamente, no es fácil extrapolar los resultados obtenidos *in vitro* a sistemas *in vivo* y determinar si estas concentraciones son ambientalmente relevantes. Sin embargo, el hecho de que algunas holoturias puedan llegar a ingerir más de 50 g de sedimentos marinos diariamente (Martín et al., 2017), sugiere que estos y otros animales que habitan el bentos marino (equinoideos, asteroideos, ofiuras, poliquetos, bivalvos, rajiformes, pleuronectiformes y múlidos, entre otros), podrían estar expuestos de manera crónica a los disruptores endocrinos presentes en los sedimentos de las citadas áreas. A través de estos organismos, los disruptores endocrinos se integrarían en la red trófica, alcanzando a las especies de interés comercial y a los seres humanos.

En estudios químicos previos, se detectaron diferentes compuestos disruptores endocrinos en agua y sedimentos del estuario del Algarve, en el orden de ng/g (compuestos organoestánnicos, herbicidas, pesticidas, PAHs, nonilfenol y bisfenol A; Almeida et al., 2007) y en bilis de peces (nonilfenol; Fernandes et al., 2009). Aunque a menudo estos y otros contaminantes no analizados se encuentran a bajas concentraciones, ciertamente contribuyen a los efectos aditivos, sinérgicos o antagónicos de la mezcla de compuestos. Se ha detectado una inhibición de la actividad P450 aromatasa en peces de los ríos Ebro y Sena expuestos a descargas urbanas y pesticidas (Lavado et al., 2004; Gerbron et al., 2014). Kroon et al. (2015) encontraron

correlaciones entre la disrupción del sistema endocrino de peces (*Lates calcarifer* y *Plectropomus* sp.) de la Gran Barrera de Coral australiana, y la presencia de pesticidas provenientes de escorrentías agrícolas. Curiosamente, no encontraron asociación entre dicho efecto y los efluentes de las plantas de tratamiento de aguas, descargas urbanas e industriales propias de la zona, atribuyéndolo a la estricta normativa reguladora de estos aspectos del parque marino. De acuerdo con ellos, no se han detectado inhibidores de la actividad aromatasa en los sedimentos cercanos a los efluentes urbanos y WWTP de Vila Real de San Antonio (Guadiana) y Constanza (Mar Negro), ni en la zona P5 de la Bahía de Paranaguá, influenciada por actividades urbanas e industriales.

Los efectos de los disruptores endocrinos en la biología de los peces son numerosos e incluyen el desarrollo de oocitos femeninos en testículos de macho (intersex), alteración de la temporalidad del ciclo biológico, disminución de la fecundidad, calidad de los oocitos y reclutamiento, e incluso colapso de la población (Kidd et al., 2007; Ortiz-Zarragoitia et al., 2014).

Desde una perspectiva global, se ha evidenciado el impacto de puertos, ciudades, estuarios y deltas fluviales en términos de acumulación de contaminantes o sustancias bioactivas en los sedimentos costeros, y que los ríos de mayor caudal aportan más cantidad de sustancias bioactivas a las áreas marinas costeras, que los ríos menos caudalosos. Además, las características hidrográficas contribuyen al transporte, distribución y acumulación de xenobióticos en el medio acuático, de manera que, a gran escala, los sedimentos de los mares más cerrados están más impactados que las zonas con aguas abiertas al océano.

Finalmente, mencionar que la detección de una respuesta en los bioensayos de actividad EROD y activación de zfPxr, ofrecen información sobre la activación del metabolismo y de los mecanismos de defensa y reparación que probablemente protegen de efectos letales causados por la exposición a xenobióticos. Sin embargo, las respuestas detectadas en los ensayos de generación de ROS y de actividad P450 aromatasa ofrecen una medida del potencial para provocar estrés oxidativo y alterar la funcionalidad del sistema endocrino, lo que probablemente causará daños o enfermedades en el individuo y/o en la descendencia, incluso años después de la exposición. De este modo, y desde una perspectiva de riesgo ambiental, los agentes disruptores endocrinos y los compuestos que generan estrés oxidativo, presentes en el medio marino tienen una relevancia crucial porque ponen en riesgo a las poblaciones salvajes y al ecosistema marino en general. Como mínimo, los ensayos indicadores de contaminación ambiental deben cubrir aspectos relevantes que representen la inducción del metabolismo de xenobióticos, disrupción endocrina y respuestas de estrés oxidativo (Escher et al., 2014). Aquí proponemos los ensayos de actividad EROD, activación de Pxr, actividad P450 aromatasa y generación de ROS, además de la comprobación previa de la citotoxicidad, como aquellos que deben incluirse en cualquier batería de ensayos rutinaria para un primer cribado. La batería de ensayos propuesta cumple el objetivo de caracterizar la calidad ambiental del ecosistema marino a escala local, de una manera rápida, sensible, reproducible y relativamente económica, mediante ensayos *in vitro* basados en el uso de cultivos celulares y fracciones subcelulares, contribuyendo así a la reducción y/o reemplazo de los animales de experimentación.

V.2. TEMA 2.

EVALUACIÓN DE LA TOXICIDAD DE CONTAMINANTES EMERGENTES

En esta parte se investiga el potencial tóxico de diferentes compuestos plastificantes, ftalatos, bisfenol A y alquilfenoles, y compuestos perfluorados en la línea celular de placenta humana JEG-3. Para ello se han utilizado diferentes técnicas *in vitro* que estiman la citotoxicidad celular aguda, generación de estrés oxidativo, modulación de la esteroidogénesis y alteración de la composición lipídica celular. Debido a su carácter hidrofóbico, se ha determinado la concentración experimental de los compuestos plastificantes en el medio de exposición. Aunque en un principio se investigaron únicamente OP y NP como alquilfenoles modelo dado que son los más ampliamente distribuidos en el ambiente, los resultados obtenidos en cuanto a disrupción endocrina se refiere, nos animaron a continuar la investigación de otros alquilfenoles, algunos considerados como potenciales sustitutos de los primeros, y que por el contrario, están escasamente estudiados.

Entre los alquilfenoles estudiados, los de menor peso molecular generaban poca o ninguna citotoxicidad en las células de placenta. En general, los alquilfenoles eran más citotóxicos cuantos más larga era la cadena alquilada, de manera que inducían una respuesta gradual y dependiente del número de carbonos de la cola, siendo los APs de doce a siete carbonos (DP, NP, OP y HP), los más citotóxicos (EC_{50} de 18 a 50 μM), seguidos por aquellos con cinco y cuatro carbonos (TAP, 4SBP, 2TBP, 2SBP y 4TBP) y DTBP, con dos cadenas alquílicas (EC_{50} > 119 μM). Además, se ha observado una asociación (R^2 = 0.58) entre citotoxicidad (EC_{50} AB) e hidrofobicidad (log K_{ow}) de los alquilfenoles, excepto para TTBP, que aunque hidrofóbico

(log K_{ow} > 5) no reducía la viabilidad celular, y HP, que mostraba mayor citotoxicidad de la esperada.

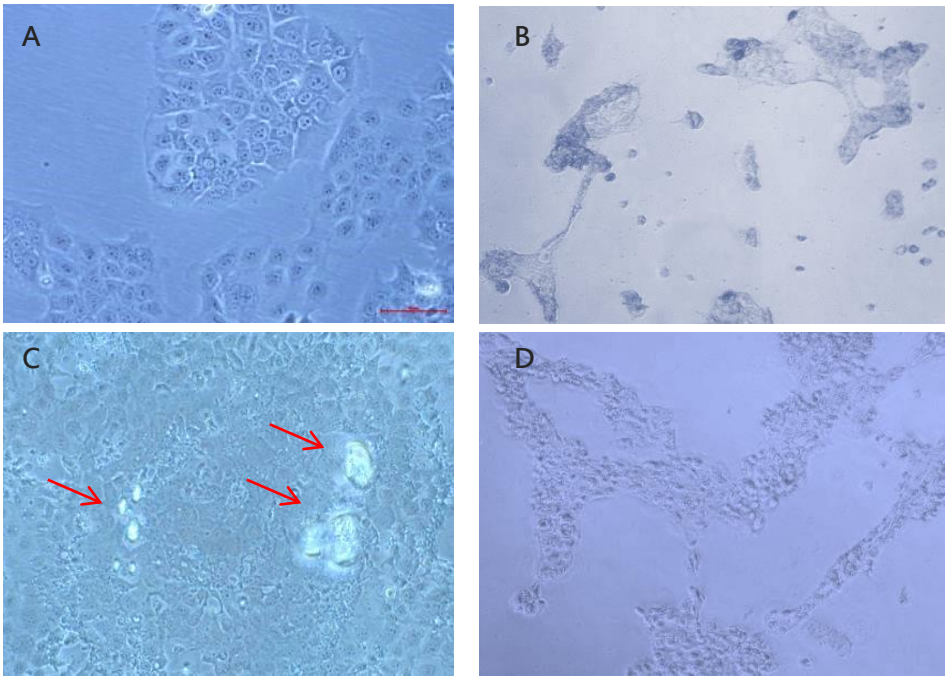


Figura 15. Microfotografías (x10) de células JEG-3 sanas (A), y tras 24 horas de exposición a 500 µM de NP (B), BBP (C), y BPA (D). En las células sanas se observan las estructuras celulares intactas, con las membranas citoplasmáticas y nucleares bien delimitadas. Las células expuestas a NP se han despegado de la placa de cultivo, probablemente asociado a las propiedades detergentes del compuesto, y únicamente quedan los restos celulares degradados. En presencia de BBP, se aprecian cambios morfológicos en las células, aunque estas siguen pegadas a la placa y no se detecta reducción en su número. Además, se puede ver la precipitación de BBP (flechas rojas) debida a su baja solubilidad. La exposición a BPA resulta en una importante reducción del número de células y un notable cambio morfológico donde sólo se observan vestigios celulares y ya no se aprecian núcleos, membranas, ni otras estructuras, indicando mortalidad celular.

Hasta lo que sabemos, no existen demasiados estudios previos sobre la toxicidad de estos alquilfenoles en células de placenta humana. Sin embargo, Tollefsen et al., (2008) publicaron resultados similares de citotoxicidad de HP, DP y TTBP en hepatocitos de trucha arcoíris (*Oncorhynchus mykiss*). Por el contrario, los alquilfenoles de cadena corta (2TBP, 4TBP, 4SBP y DTBP) mostraban mayor toxicidad en las células de peces (EC_{50} entre 28 y 217 µM) comparado con las células JEG-3 (EC_{50} entre

120 y >500 μM), lo cual sugiere que las células humanas presentan un metabolismo y mecanismos de detoxificación más desarrollados.

Los "pseudo-alquilfenoles" BPA y CP, aunque sólo se diferencian en un grupo hidroxilo en el anillo bencénico, diferían significativamente en su citotoxicidad. Así, CP era más citotóxico (EC_{50} ~ 65 μM) que BPA, cuya EC_{50} como mínimo doblaba la de CP (EC_{50}138-218 μM). Para los ftalatos apenas se detectó citotoxicidad en las células expuestas a la concentración más alta estudiada (500 μM). La Figura 15 muestra los efectos nocivos en la morfología celular de las células JEG-3 tras 24 horas de exposición a algunos de los compuestos estudiados.

Respecto a los compuestos perfluorados, aquellos que presentaban cadenas fluorocarbonadas más largas eran citotóxicos para las células de placenta, mientras que aquellos con cadenas cortas mostraban poca o ninguna toxicidad. Para una misma longitud de cadena, los perfluorosulfonatos tienden a ser más citotóxicos comparado con sus correspondientes ácidos carboxílicos, particularmente PFOS (EC_{50} cinco veces menor que PFOA) lo cual se ha atribuido a la elevada absorción de este compuesto por las células JEG-3 (concentración intracelular de PFOS tras 24 h de exposición ~ 470 pmol/mg proteína) comparado con PFOA (< 20 pmol/mg proteína).

GENERACIÓN DE ROS POR CONTAMINANTES EMERGENTES

Los ensayos de estrés oxidativo, determinaron que BPA, CP y todos los alquilfenoles excepto 2TBP, DTBP y TTBP, generaban niveles significativos de ROS. Entre ellos, destacaban 4SBP y TAP, que generaban hasta seis veces más ROS que el control, y sobre todo HP, que sorprendentemente inducía

43 veces más, a 40 µM. El método de H_2DCF-DA para la detección de ROS intracelular, aunque muy utilizado, ha sido también muy discutido por algunos autores por sus limitaciones. Algunos compuestos, como tetrabromobisphenol A (TBBPA), pueden estimular la conversión de H_2DCF a DCF incluso en ausencia de células (Tetz et al., 2013; Szychowski et al., 2016). Siguiendo este ejemplo, se han realizado ensayos con H_2DCF-DA activado con NaOH para obtener H_2DCF (ya que de otro modo no podría oxidarse y emitir fluorescencia), en presencia de HP y en ausencia de células, para determinar si HP seguía el mismo comportamiento que TBBPA. Los resultados mostraron que HP no tenía la habilidad de oxidar H_2DCF a DCF en ausencia de células. También se comprobó que HP no emitía fluorescencia en la misma longitud de onda que DCF, se repitieron los ensayos con nuevos stocks de HP diluido en varios disolventes (etanol 0.1%, y DMSO 0.5% y 0.1%), y con diferentes medios de cultivo (L-15-ex y DPBS-glucosa). En todos los casos, los resultados eran similares, HP generaba niveles extraordinarios de ROS. También se realizaron ensayos TBARS (thiobarbituric acid reactive susbstances). El ensayo TBARS permite detectar el malondialdehído (MDA) generado por los hidroperóxidos de lípidos (moléculas que se forman como subproductos de la peroxidación lipídica), usando ácido tiobarbitúrico como reactivo. No se observó respuesta de HP en estos ensayos. Sin embargo, se debe tener en cuenta que el MDA es sólo uno de los numerosos productos formados por la descomposición de los productos de la peroxidación lipídica, pero no se genera en todos los casos. Por este motivo los resultados negativos del ensayo TBARS para HP, no deben tomarse como concluyentes de que no existe peroxidación lipídica. Hasta el momento no hemos averiguado si los resultados de generación de ROS en JEG-3 por HP son efectivamente reales, de modo que se necesita profundizar esta parte de la investigación, particularmente considerando

que el estrés oxidativo está relacionado con algunas complicaciones en el embarazo, incluyendo aborto espontáneo, parto prematuro y preeclampsia (Ferguson et al., 2016). Los ftalatos por su parte, no producían niveles significativos de ROS a las concentraciones estudiadas (de 5 a 500 µM).

Por otro lado, se añadió vitamina E (un antioxidante) a las células expuestas a los plastificantes, para ver si disminuía la citotoxicidad supuestamente asociada al estrés oxidativo de los compuestos. Curiosamente, se observó un incremento significativo de la citotoxicidad de la mayoría de ellos (BPA, CP, 2SBP, 4TBP, 2TBP, TAP, HP, OP, NP y DP). Actualmente, existe controversia sobre si los efectos de los antioxidantes resultan beneficiosos o perjudiciales para la célula ante la exposición a los xenobióticos, incluyendo medicamentos. De acuerdo con nuestros resultados, Aydoğan et al. (2008) detectaron un incremento del daño oxidativo cerebral en ratas expuestas a BPA, OP y NP coadministrados con otro antioxidante, vitamina C. Igualmente, en su reciente estudio, Kontek et al. (2014) observaron un incremento en la muerte por apoptosis en células A549 y HT29 expuestas a irinotecan (medicamento contra el cáncer de colon) conjuntamente con 25 µM vitamina E. Por el contrario, Thibaut et al. (2008) demostraron que la viabilidad de las células de peces PLHC-1 tras 24 h de exposición a fluoxetina y paroxetina (medicamentos antidepresivos) aumentaba un 32 % y 60 %, respectivamente, en presencia de 40 µM de vitamina E, aunque en este caso las células fueron pretratadas con vitamina E durante una hora antes de la exposición. Así mismo, Gallo et al. (2010) determinaron que las vitaminas C y E a concentraciones entre 0.001 y 50 nM, protegían las células JEG-3 contra la citotoxicidad de la nicotina (0-100 pg/mL), y apoyaban la vinculación entre dicha citotoxicidad y el estrés oxidativo causado por exceso de ROS. Estos datos sugieren que el supuesto beneficio de los

antioxidantes para la célula debe ser estudiado individualmente en cada caso, ya que pueden actuar de manera opuesta dependiendo del xenobiótico, la dosis de ambos (xenobiótico y antioxidante) y el método de exposición.

DISRUPCIÓN ENDOCRINA: ALTERACIÓN DE LA SÍNTESIS DE ESTRÓGENOS EN LAS CÉLULAS DE PLACENTA

Los disruptores endocrinos pueden afectar a la actividad P450 aromatasa directamente reaccionando con el complejo enzimático o a través de otros mecanismos que resultan en una regulación anómala de la actividad del enzima. Los ensayos de actividad aromatasa realizados con la fracción subcelular S9 obtenida de las células de placenta e incubada con los compuestos plastificantes (DEHP, BBP, DBP, DMP, NP, OP y BPA), estimaban la interacción de los compuestos con el enzima aromatasa directamente sin la necesidad de atravesar la membrana plasmática. Estos ensayos permitieron detectar el potencial de BBP y NP (IC_{50} ~ 40 µM) para inhibir la actividad aromatasa, seguidos por DBP, OP y BPA (IC_{50} ~ 64-94 µM). Por el contrario, DEHP y DMP no alteraban la actividad del enzima.

Los ensayos de actividad aromatasa realizados con las células JEG-3 intactas, permitieron integrar entrada de los compuestos en la célula y su metabolismo, regulación de la actividad del enzima a través de unión de dichos compuestos a receptores celulares (ej. receptor de estrógenos) y/o modulación de las vías de transducción de señal, entre otros. De esta manera, se pudo detectar el efecto estrogénico de los alquilfenoles DP, NP, OP, CP (a concentraciones entre 5 y 30 µM), 2TBP y el ftalato DMP (100-200 µM), induciendo la actividad aromatasa entre 30 % y 50 %. Es importante

recalcar que la actividad aromatasa aumentaba en presencia de NP y OP en un rango de concentraciones notablemente estrecho (20-30 μM), para luego caer drásticamente a concentraciones más altas, debido a su citotoxicidad, pero también por el efecto inhibidor que tienen estos compuestos sobre el enzima, tal y como se observa en el ensayo S9. Este hecho es de particular relevancia, dado que los estudios de toxicología tienden a trabajar con factores de dilución elevados (ej. 1:10) para cubrir grandes rangos de concentraciones, con lo cual pueden no detectarse los efectos que ocurren a un rango más pequeño de concentraciones. Hasta donde sabemos, este es el primer trabajo que muestra el incremento de actividad aromatasa en células de placenta humana expuestas a concentraciones de NP y OP cercanas a su EC_{50} de citotoxicidad. Los mecanismos a través de los cuales ocurre este incremento pueden ser variados, incluyendo daños en los mecanismos de detoxificación, regulación al alza de la expresión del gen *cyp19*, regulación a nivel post-transcripcional y/o cambios en vías de señalización.

En el caso de BPA, se ha observado una inhibición de la actividad aromatasa tanto en la fracción S9 como en las células intactas, con una IC_{50} algo más baja en las células, indicando que quizás existe una inhibición en la expresión del enzima. Sin embargo, no se puede descartar que ocurran varios mecanismos conjuntamente tal y como se deduce al integrar la información de este y otros trabajos publicados. Por ejemplo, Nativelle-Serpentini et al. (2003) observaron una inhibición de la actividad aromatasa en células JEG-3 tras 18 horas de exposición a BPA (25-100 μM), mientras que exposiciones cortas de 2 a 6 horas inducían dicha actividad, atribuyéndolo a una interacción directa con el enzima, ya que no encontraron alteraciones en la tasa de expresión de *cyp19*. Sin embargo,

años más tarde, Huang and Leung (2009) y Quignot et al. (2012), detectaron una disminución de los niveles de mRNA de CYP19 en células JEG-3 expuestas a BPA (5-100 µM) durante 24 h. La reducción de la tasa de transcripción del gen *cyp19* también fue observada por Watanabe et al. (2012) en células ováricas KGN y osteoblásticas SV-HFO humanas expuestas a BPA.

Mientras que BPA inhibía la síntesis de estrógenos en las células de placenta a dosis inferiores a su EC_{50}, la aparente inhibición de la actividad aromatasa generada por CP (de estructura muy similar a BPA) se ha asociado a su citotoxicidad dada la similitud entre sus EC_{50} e IC_{50}, y la superposición de las curvas dosis-respuesta para ambos ensayos, citotoxicidad y aromatasa. De igual manera ocurría para los alquilfenoles 4SBP, 2TBP, TAP y DTBP. Sorprendentemente, TTBP, con tres sustituyentes en el anillo fenólico, no reducía la viabilidad de las células JEG-3, pero era uno de los APs con mayor potencial de disrupción endocrina, junto con DP, NP, OP, HP y DTBP (IC_{50} entre 14 y 100 µM). Este comportamiento también ha sido observado en los ftalatos DBP y BBP, que no eran citotóxicos, pero eran capaces de inhibir un 50 % la actividad aromatasa a concentraciones de 104 y 167 µM, respectivamente. Es importante enfatizar que estas IC_{50} son hasta cuatro veces mayores que las calculadas para el ensayo S9 (64 y 39 µM). Por otro lado, los ftalatos DMP y DEHP no mostraron un efecto inhibitorio significativo de la actividad aromatasa en las células JEG-3.

Además, se ha evidenciado el potencial de los perfluorosulfonatos PFBS y PFOS, el ácido carboxílico PFOA (IC_{50} entre 57 y 80 µM), y en menor medida PFNA y PFHxS (IC_{50} 132 y 300 µM, respectivamente) para inhibir la actividad aromatasa en las células JEG-3. Contrariamente, Kjeldsen and Bonefeld-Jørgensen (2013a) no observaron alteraciones en la actividad aromatasa de

células JEG-3 expuestas a estos y otros compuestos perfluorados, aunque sus exposiciones eran de 18 h y las nuestras de 24 h, lo que puede variar la sensibilidad del ensayo. Sin embargo, sí que se han observado respuestas de inhibición de la actividad aromatasa en peces expuestos a PFOS (Ankley et al., 2005). El hecho de que los perfluorados de cadena corta PFBS y PFHxS actúen como disruptores endocrinos es de particular relevancia, dado que la industria tiende a introducirlos en la fabricación de artículos cotidianos, reemplazando a los perfluorados de cadena larga, porque se suponía que tenían menor toxicidad y un menor impacto en la salud humana.

ANÁLISIS DE LA CONCENTRACIÓN EXPERIMENTAL FRENTE A LA CONCENTRACIÓN NOMINAL DE LOS COMPUESTOS EN EL MEDIO DE CULTIVO

La escasa respuesta observada para los ftalatos en los ensayos *in vitro* con células intactas, generaba dudas sobre su solubilidad en el medio, por lo que se realizaron análisis químicos con el objetivo de averiguar la concentración de estos y otros plastificantes (BPA, NP, OP, DMP, DEHP, DBP y BBP) en el medio de cultivo.

Los análisis de biodisponibilidad determinaron diferencias significativas entre la concentración nominal (teórica) y la concentración experimental (observada) de los compuestos. Así mismo, se detectaron pequeñas cantidades de DEHP y DBP (0.15-0.60 µM) en el medio de cultivo utilizado como blanco, probablemente debido a la migración desde la botella de plástico al contenido de la misma. En un estudio reciente, Keresztes et al. (2013) también observaron la migración de estos compuestos al agua contenida en botellas de tereftalato de polietileno (PET).

Las diferencias observadas entre las concentraciones nominales y las concentraciones experimentales de los alquilfenoles NP y OP son aceptables cuando se trata de concentraciones menores de 30 µM. Es decir, la EC_{50} calculada a partir de las concentraciones nominales, aunque tiende a ser más alta, no difiere significativamente de la EC_{50} calculada con las concentraciones experimentales de estos compuestos en el medio de cultivo. A concentraciones más altas, NP y OP, por su condición de surfactantes, probablemente tienden a acumularse en las interfaces del sistema (medio-aire y medio-plástico). Además, la temperatura relativamente elevada (37 ºC) propia de los cultivos de células de mamíferos, facilita la evaporación de los compuestos. De hecho, tras una exposición a OP durante 24 horas a concentraciones de 100 a 500 µM, se detectaron concentraciones en el rango de 4 a 18 µg/mL (18 a 83 µM) en el medio de cultivo de los pocillos adyacentes (datos no publicados). Además, Heringa et al. (2004) detectaron pérdidas de OP atribuidas a la unión a proteínas del medio, poniendo de manifiesto la complejidad de los medios de cultivos celulares.

Por el contrario, las concentraciones experimentales obtenidas para los ftalatos DBP, BBP y DEHP, distaban mucho de las nominales, incluso a bajas concentraciones. Para determinar si estas pérdidas se debían a que los compuestos se adherían al plástico de la placa de cultivo, o si quizás se unían a los componentes del suero fetal bobino (FBS), se realizaron análisis en pocillos de vidrio, y en medio con y sin FBS. Curiosamente, cuando se analizó el medio de cultivo en ausencia de FBS, las concentraciones observadas eran incluso menores que las detectadas en el medio suplementado con FBS, de lo que se deduce que la unión de estos compuestos a los componentes del suero es insignificante. De hecho, las

observaciones microscópicas evidenciaban la formación de gotas en el fondo de los pocillos de vidrio/plástico, así como en la superficie del medio de cultivo sin FBS, cuando se incubaba con DEHP, DBP o BBP a concentraciones mayores de 20 µM. Además, también se observó precipitación de BBP y DBP en el medio con FBS, pero a concentraciones a partir de 100 µM (Figura 16).

Así, la aparente insensibilidad del sistema *in vitro* a los ftalatos, está asociada a la elevada hidrofobicidad y baja solubilidad de los compuestos en el medio de cultivo. Otra contribución podrían ser las pérdidas asociadas al plástico de las puntas típicas de las pipetas de laboratorio. Consecuentemente, las IC_{50} de BBP y DBP, calculadas a partir de las concentraciones experimentales (~ 14 µM), resultaron ser mucho más bajas que las que se calcularon con las concentraciones nominales (104 y 168 µM), poniendo de manifiesto el potencial disruptor endocrino de estos compuestos, antes infravalorado.

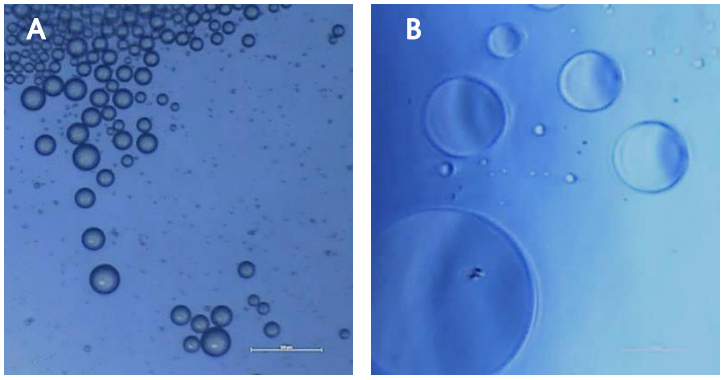


Figura 16. Microfotografías (x10) que muestran las gotas de BBP (200 µM) en (A) el fondo del pocillo de la placa, y en (B) la superficie del medio de cultivo sin FBS.

En la figura 17 se muestra una visión global de los resultados obtenidos para citotoxicidad y disrupción endocrina. En general, el alquilfenol DP es el contaminante de mayor relevancia desde un punto de vista toxicológico,

coincidiendo con que es el más hidrofóbico de su grupo y el que tiene un mayor número de carbonos en la cadena alquilada. Dentro del grupo de los alquilfenoles, la toxicidad está asociada a la hidrofobicidad, excepto para TTBP, que es un potente disruptor endocrino, aunque no genera citotoxicidad. Los compuestos perfluorados, PFOS, PFBS y PFOA, y BPA se sitúan en el mismo nivel que TTBP, con una capacidad similar de actuar como disruptores endocrinos, inhibiendo la actividad P450 aromatasa en células de placenta.

Las mujeres embarazadas son un colectivo particularmente vulnerable porque el complejo enzimático P450 aromatasa es crucial para la producción de estrógenos y el balance de esteroides sexuales necesario para el desarrollo fetal y embrionario, y se ha demostrado que los plastificantes pueden cruzar la barrera placentaria. Los estudios disponibles en bibliografía sobre los efectos de la exposición a ftalatos durante el embarazo muestran resultados a menudo contradictorios. Sin embargo, muchos estudios coinciden en que la exposición a niveles moderados (< 11 µg/L) de ftalatos y sus metabolitos (principalmente DBP y DEHP) está relacionada con alteraciones en la duración del embarazo y con una reducción de la distancia anogenital (Marie et al., 2015; Philips et al., 2017). Respecto a BPA, Leclerc et al. (2014) detectaron niveles de BPA significativamente más elevados (hasta 0.44 µM) en tejido placentario de mujeres embarazadas con preeclampsia, comparados con aquellos observados en mujeres con presión arterial normal (hasta 0.14 µM). Además, en estas últimas, observaron una distribución similar entre los niveles de BPA detectados en los compartimentos maternal, placentario y fetal; mientras que en mujeres con preeclampsia existía una mayor concentración en la placenta.

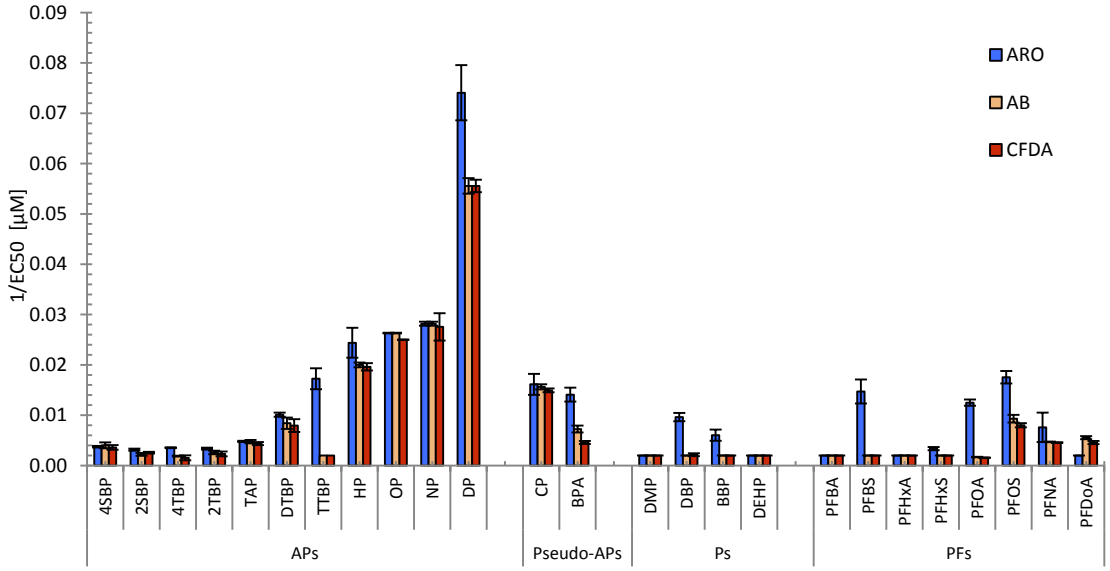


Figura 17. Inversa de la concentración a la cual hay un 50 % de respuesta, en los ensayos de inhibición de la actividad P450 aromatasa (ARO) y citotoxicidad (AB y CFDA). Los valores más altos indican efectos a menor concentración. Los valores de ARO que superan a los valores de AB y CFDA indican que la inhibición de la actividad aromatasa ocurre a dosis no letales. El error se ha calculado dividiendo la desviación estándar entre el cuadrado de la EC_{50}.

La detección de PFOS y PFOA en sangre humana se ha reducido durante los últimos años, mientras que aumenta la de los perfluorados de cadena corta PFBS y PFHxS (Glynn et al., 2012; Toms et al., 2014; Yang et al., 2016). Esto se debe a que estos últimos se están implementando como sustitutos de PFOS y PFOA, porque se espera que sean menos dañinos para la salud humana y ambiental. Aunque las concentraciones detectadas en humanos distan de aquellas con un efecto bioactivo, su potencial de persistencia y bioacumulación convierte a los compuestos perfluorados en contaminantes preocupantes a largo plazo.

En general, la modulación de la actividad P450 aromatasa por DBP, OP y NP (EC_{50} experimentales en el rango de 5 a 40 µM) es preocupante dado que ocurre a concentraciones cercanas a aquellas detectadas en cordón

umbilical humano (DBP ~ 21 µM; OP ~ 6 µM; NP ~ 5 µM) de bebés nacidos con poco peso en China (Lin et al., 2008).

DISRUPCIÓN DE LA COMPOSICIÓN LIPÍDICA CELULAR

La condición de surfactante que caracteriza a los alquilfenoles generó la hipótesis de que podrían alterar la funcionalidad de las membranas celulares y otros componentes lipídicos. Este trabajo investigó la identificación de posibles alteraciones en la composición lipídica de las células de placenta humana JEG-3 expuestas durante 24 h a 10 y 20 µM HP, 10 µM DP y 20 µM TTBP, prestando especial atención a lípidos tales como fosfatidilcolinas (PCs), plasmalógenos PCs (PL-PCs), diacilgliceroles (DAGs) y triacilgliceroles (TAGs). La síntesis de estos lípidos está estrictamente regulada en la célula, de manera que cualquier alteración en el balance de estas moléculas podría resultar en cambios en la fluidez y dinamismo de las membranas celulares, compactación defectuosa de los lípidos de la bicapa, fallos en la gestión de la energía, en el transporte o en la transducción de señales, y daños en la integridad celular (Wolfgang and Lane, 2006).

Bajo las condiciones del ensayo, se han detectado 69 especies lipídicas, que incluyen 20 PCs, 18 PL-PCs, 11 DAGs y 20 TAGs. Las PCs eran el grupo más abundante, abarcando un 45 % de la concentración total de los lípidos detectados. Las especies lipídicas más comunes contenían cadenas acilo di- o mono-insaturadas (ej. PC 32:1, PC 34:1, PC 34:2, PC 36:2, PL-PC 32:1, PL-PC 34:1, DAG 34:1, DAG 36:2, TAG 50:1 y TAG 52:2). Esto coincide con el hecho de que la célula obtiene grandes cantidades de ácido palmítico (16:0) y ácido esteárico (18:0), que junto con ácido oleico (18:1) y ácido linoleico (18:2), se utilizan rápidamente para sintetizar lípidos de membrana y TAGs.

Los DAGs eran significativamente menos abundantes (4 %) que el resto de lípidos. Este grupo de lípidos se utilizan como como moléculas intermedias en las vías de síntesis, o como segundos mensajeros, entre otras funciones (Hermansson et al., 2011; Kihara, 2012).

El análisis PLS-DA (partial least square - discriminant analysis) reveló claras diferencias en los perfiles lipídicos de las células expuestas a los alquilfenoles HP, DP y TTBP. Sin embargo, los cambios observados en la composición lipídica inducida por dichos compuestos eran completamente distintos. Cuando se compararon las especies lipídicas individualmente, se observó que 20 μM HP (concentración no citotóxica) inducía la acumulación de especies lipídicas saturadas y monoinsaturadas (PC 32:0, PC 32:1, PC 34:1, PC 36:1 y DAG 32:0) en las células de placenta, mientras que reducía el 40% de las especies detectadas (28 especies de 69) pertenecientes a todos los grupos lipídicos investigados (PCs, PL-PCs, DAGs y TAGs), particularmente aquellas poliinsaturadas. Los niveles elevados de ROS pueden generar fácilmente peróxidos de lípidos, atacando principalmente a las especies poliinsaturadas, debido a que los dobles enlaces son más vulnerables a la oxidación (De la Haba et al., 2013). De acuerdo con esto, 20 μM HP generaba elevados niveles de ROS (>25 veces el control), que pueden producir peroxidación lipídica en las células JEG-3, resultando en una reducción generalizada de las especies poliinsaturadas, las más vulnerables a la peroxidación. Así mismo, no sería descabellado pensar que los elevados niveles de ROS podrían estar indirectamente asociados a la inhibición de la actividad P450 aromatasa observada en presencia de 20 μM de HP. Milczarek et al., (2008) demostraron que los peróxidos de lípidos, consecuencia de elevados niveles de ROS, provocaban una degradación del enzima P450 aromatasa en microsomas de células primarias de placenta

humana, resultando en una reducción de la actividad del enzima. Así, la extraordinaria respuesta observada en los ensayos ROS y la sospecha de que resulte en peroxidación lipídica, evidencian la necesidad de investigar en profundidad la capacidad de HP para generar estrés oxidativo.

Por el contrario, 10 µM DP incrementaba principalmente los niveles de los TAGs poliinsaturados (ej. TAG 54:5, TAG 54:6, TAG 56:6, TAG 58:5 y TAG 58:6), así como numerosas PCs (ej. PC 32:0, PC 32:1, PC 32:2, PC 34:1, PC 34:2, PC 34:3 y PC 36:4), a la vez que reducía la abundancia de los correspondientes DAGs (ej. DAG 32:0, DAG 32:1, DAG 32:2, DAG 34:1, DAG 34:2 y DAG 34:3), lo cual sugiere que las células sintetizan lípidos de membrana y TAGs a partir de los DAGs disponibles, como respuesta a la presencia de DP, posiblemente para reparar los posibles daños en la membrana plasmática causados por DP. También cabría pensar que DP induce proliferación celular y se sintetizan lípidos para cubrir la demanda para realizar la mitosis. Sin embargo, esta hipótesis no se soporta dado que los ensayos Alamar Blue no detectaron ningún aumento en la proliferación celular en presencia de HP.

Cabe mencionar que las especies PC 30:0, PC 32:0 y PC 32:1, cuya abundancia aumentaba en presencia de dosis no tóxicas de HP y DP, forman parte de la composición del surfactante alveolar en los pulmones, y su concentración está asociada a las necesidades fisiológicas respiratorias del individuo. En el caso del feto, los niveles de surfactante en los pulmones pueden influir en la fecha del parto (Bernhard et al., 2001; White, 2006; Ghayee and Auchus, 2007), por lo que se deduce que la exposición a HP y DP podrían influir en este proceso.

Sorprendentemente, la exposición a 20 μM TTBP, compuesto no tóxico, evidenció una acumulación de TAGs en las células de placenta. La placenta juega un papel clave en la transferencia de ácidos grasos preferentemente poliinsaturados al feto, que se liberan a partir de los TAGs maternos gracias a la actividad lipoproteína lipasa de las células de dicho órgano (Coleman and Hynes, 1987; Gude et al., 2004). Así, un aumento de TAGs en las células de placenta, probablemente regulará la actividad del enzima resultando alteraciones en la tasa de transferencia de ácidos grasos al compartimento fetal. Numerosos estudios previos han demostrado la capacidad de algunos xenobióticos para inducir obesogénesis a través del almacenaje de energía en forma de TAGs por diversos mecanismos. Por ejemplo, el biocida tributilestaño (TBT) es capaz de activar los receptores RXR y PPARγ, altamente implicados en el incremento de la grasa adiposa en humanos. Se ha demostrado que la exposición prenatal a este tipo de sustancias puede reprogramar a los organismos para ser obesos favoreciendo el almacenaje de un exceso de calorías en forma de grasa corporal (Blumberg, 2011). En este sentido, sería necesario determinar si los mecanismos de acción de TTBP son similares a otros obesógenos (ej. TBT), así como las consecuencias para la salud del feto.

VI. CONCLUSIONES

Este trabajo ha dado lugar a las siguientes conclusiones:

1. El uso integrado de ensayos de citotoxicidad, disrupción endocrina, generación de ROS, inducción CYP1A y activación zfPxr, en distintos modelos celulares, es una herramienta útil para evaluar la calidad de sedimentos costeros afectados por diferentes fuentes de contaminación, y ha permitido clasificar las áreas marinas según su nivel de presión antropogénica.

2. Entre las áreas marinas estudiadas, destacan los puertos, las desembocaduras de grandes ríos (Danubio y Po), y la Bahía de Kaštela, como las más impactadas. Destacar así mismo la presencia de agonistas de AhR en los sedimentos de zonas remotas situadas en la Antártida.

3. Entre los contaminantes investigados, los alquilfenoles muestran mayor citotoxicidad y potencial de disrupción endocrina en células de placenta humana, particularmente DP, seguido de NP, OP, HP y TTBP. La toxicidad de los alquilfenoles está significantemente asociada a su hidrofobicidad, de manera que los compuestos con un coeficiente log K_{ow} mayor, son los más tóxicos.

4. La capacidad de los alquilfenoles de cadena larga DP, OP y NP, de inducir la actividad P450 aromatasa en un rango de concentraciones tan reducido (10-30 µM) y tan cercano a su citotoxicidad, evidencia la necesidad de diseñar los ensayos de exposición con factores de dilución de la muestra lo suficientemente bajos, para facilitar la detección de estas respuestas.

5. Los compuestos perfluorados PFOS, PFOA y PFBS, inhiben la actividad P450 aromatasa en células de placenta, con IC_{50} en el rango de 57 a 80 µM. Esto es de particular relevancia dado que, debido a su menor toxicidad, PFBS se está utilizando como sustituto de PFOS y PFOA.

6. Los contaminantes NP, OP y DBP modulan la actividad P450 aromatasa en células de placenta a concentraciones ambientalmente relevantes (5-40 μM). Dichas concentraciones han sido detectadas en cordón umbilical de bebés nacidos con problemas de poco peso.

7. La aparente insensibilidad de las células JEG-3 a la exposición a ftalatos está asociada a su elevada hidrofobicidad y baja solubilidad en el medio de cultivo. Es necesario determinar las concentraciones experimentales frente a las dosis teóricas en el medio de cultivo, para así no subestimar la toxicidad de estos compuestos.

8. La cromatografía líquida de alta resolución acoplada a espectrometría de masas ha permitido describir el perfil lipídico de las células de placenta JEG-3, así como la detección de alteraciones en dicho perfil tras exposición a alquilfenoles. La exposición a DP y TTBP a concentraciones de 10 y 20 μM, respectivamente, inducen la acumulación de triacilglicéridos en dichas células.

9. El perfil de lípidos en las células de placenta expuestas a 20 μM de HP muestra una reducción significativa de las especies lipídicas más poliinsaturadas, las más susceptibles a oxidación. Dicho efecto, junto con la elevada respuesta observada en el ensayo de estrés oxidativo, sugieren que HP promueve la peroxidación lipídica.

VII. BIBLIOGRAFÍA

Adir, M., Combelles, C.M.H., Mansur, A., Ophir, L., Hourvitz, A., Orvieto, R., Dor, J., Machtinger, R. 2017. Dibutyl phthalate impairs steroidogenesis and a subset of LH-dependent genes in cultured human mural granulosa cell *in vitro*. Reprod. Toxicol. 69, 13-18.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D. 1996. Molecular biology of the cell. Ed. Omega, ISBN 84-282-1011-X. pp 172-174.

Almeida, C., Serôdio, P., Florêncio, M.H., Nogueira, J.M.F. 2007. New strategies to screen for endocrine-disrupting chemicals in the Portuguese marine environment utilizing large volume injection-capillary gas chromatography-mass spectrometry combined with retention time locking libraries (LVI-GC-MS-RTL). Anal. Bioanal. Chem. 387, 2569-2583.

Andersen, H., Vinggaard, A.M., Rasmussen, T., Gjermandsen, I.M., Bonefeld-Jørgensen, E., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. Toxicol. Appl. Pharmacol. 179, 1–12.

Ankley, G.T., Kuehl, D.W., Kalhl, M.D., Jensen, K.M., Linnum, A., Leino, R.L., Villeneuve, D.A. 2005. Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 24, 2316-2324.

Aydoğan, M., Korkmaz, A., Barlas, N., Kolankaya, D. 2008. The effect of vitamin C on bisphenol A, nonylphenol and octylphenol induced brain damages of male rats. Toxicology 249, 35–39.

Bahn, R.S., Worsham, A., Speeg, K.V., Ascoli, M., Rabin, D. 1981. Characterization of steroid production in cultured human choriocarcinoma cells. J. Clin. Endocrinol. Metab. 52, 447-450.

Baumard, P., Budzinski, H., Michon, Q., Garrigues, P., Burgeot, T., Bellocq, J. 1998. Origin and bioavailability of PAHs in the Mediterranean Sea from mussel and sediment records. Estuar. Coast. Shelf Sci. 47, 77-90.

Bechi, N., Ietta, F., Romagnoli, R., Focardi, S., Corsi, I., Buffi, C., Paulesu, L. 2006. Estrogen-like response to p-nonylphenol in human first trimester placenta and BeWo choriocarcinoma cells. Toxicol. Sci. 93, 75-81.

Benachour, N., Aris, A. 2009. Toxic effects of low doses of Bisphenol A on human placental cells. Toxicol. Appl. Pharmacol. 241, 322-328.

Bernhard, W., Hoffmann, S., Dombrowsky, H., Rau, G.A., Kamlage, A., Kappler, M., Haitsma, J.J., Freihorst, J., Von der Hardt, H., Poets, C.F. 2001. Phosphatidylcholine molecular species in lung surfactant composition in relation to respiratory rate and lung development. Am. J. Respir. Cell Mol. Biol. 25, 725–731.

Birnie-Gauvin, K., Costantini, D., Cooke, S.J., Willmore, W.G. 2017. A comparative and evolutionary approach to oxidative stress in fish: A review. Fish Fish. 1-15.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M. 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol. Sci. 54, 138-153.

Blumberg, B. 2011. Obesogens, stem cells and the developmental programming of obesity. J. Dev. Orig. Health Dis. 2, 3–8.

Blumberg, B., Sabbagh, W., Juguilon, H., Bolado, J., Meter, C.M. Van, Ong, E.S., Evans, R.M. 1998. SXR, a novel steroid and xenobiotic- sensing nuclear receptor. Genes Dev. 3195–3205.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K. 2005. Chapter 2. Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. Environ. Toxicol. 6, 43–84.

Bols, N.C., Schirmer, K., Joyce, E.M., Dixon, D.G., Greenberg, B.M., Whyte, J.J. 1999. Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-O-deethylase activity in a trout liver cell line. Ecotoxicol. Environ. Saf. 44, 118–28.

Bonefeld-Jørgensen, E.C., Autrup, H., Hansen, J.C. 1997. Effect of toxaphene on estrogen receptor functions in human breast cancer cells. Carcinogenesis 18, 1651–1654.

Bonefeld-Jørgensen, E.C., Grünfeld, H.T., Gjermandsen, I.M. 2005. Effect of pesticides on estrogen receptor transactivation *in vitro*: A comparison of stable transfected MVLN and transient transfected MCF-7 cells. Mol. Cell. Endocrinol. 244, 20–30.

Bosveld, A.T.C., de Bie, P.A.F., Van Den Brink, N.W., Jongepier, H., Klomp, A. V. 2002. *In vitro* EROD induction equivalency factors for the 10 PAHs generally monitored in risk assessment studies in The Netherlands. Chemosphere 49, 75–83.

Brack, W., Schirmer, K. 2003. Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P4501A-inducers in a contaminated sediment. Environ. Sci. Technol. 37, 3062–3070.

Brack, W., Schirmer, K., Kind, T., Schrader, S., Schüürmann, G., 2002. Effect-directed fractionation and identification of cytochrome P4501A-inducing halogenated aromatic hydrocarbons in a contaminated sediment. Environ. Toxicol. Chem. 21, 2654–62.

Bresolin, T., de Freitas Rebelo, M., Celso Dias Bainy, A. 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 140, 403-407.

Bretaud, S., Lee, S., Guo, S. 2004. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. Neurotoxicol. Teratol. 26, 857–864.

Brooke, D., Crookes, M., Johnson, I., Mitchell, R., Watts, C. 2005. Prioritisation of Alkylphenols for Environmental Risk Assessment 80. Published by: Environment Agency, Bristol, ISBN 1-84432-369-2.

Brossa, L., Marcé, R.M., Borrull, F., Pocurull, E. 2005. Occurrence of twenty-six endocrine-disrupting compounds in environmental water samples from Catalonia, Spain. Environ. Toxicol. Chem. 24, 261-267.

Brueggemeier, R.W., Hackett, J.C., Diaz-Cruz, E.S. 2005. Aromatase inhibitors in the treatment of breast cancer. Endocr. Rev. 26, 331-345.

Brügger, B. 2014. Lipidomics: analysis of the lipid composition of cells and subcellular organelles by electrospray ionization mass spectrometry. Annu. Rev. Biochem. 83, 79–98.

Casals-Casas, C., Desvergne, B. 2011. Endocrine disruptors: from endocrine to metabolic disruption. Annu. Rev. Physiol. 73, 135-162.

Chapman, P.M., Wang, F., Janssen, C., Persoone, G., Allen, H.E. 1998. Ecotoxicology of metals in aquatic sediments: binding and release, bioavailability, risk assessment, and remediation. Can. J. Fish. Aquat. Sci. 55, 2221-2243.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31-62.

Clayton, E.M., Todd, M., Dowd, J.B., Aiello, A.E. 2011. The impact of bisphenol A and triclosan on immune parameters in the U.S. population, NHANES 2003-2006. Environ. Health Perspect. 119, 390-396.

Coatu, V., Tiganus, D., Oros, A., Lazar, L., 2013. Analysis of hazardous substance contamination of the marine ecosystem in the Romanian Black Sea coast, part of the Marine Strategy Framework Directive (2008/56/EEC) implementation, in: Recherches Marines. 144-186.

Coleman, R., Haynes, E.B. 1987. Synthesis and release of fatty acids by human trophoblast cells in culture. J. Lipid Res. 28, 1335–41.

Cook, H.W., McMaster, C.R. 2002. Fatty acid desaturation and chain elongation in eukaryotes. Chapter 7. Biochem. Lipids, Lipoproteins Membr. 36, 181–204.

Covaci, A., Geens, T., Roosens, L., Ali, N., Van Den Eede, N., Ionas, A., Malarvannan, G., Dirtu, A., 2012. Human exposure and health risks to emerging organic contaminants, in: Barceló, D. (Ed.), Emerging Organic Contaminants and Human Health. Hdb Env Chem, Springer-Verlag, Berlin Heidelberg, 243–306.

Creusot, N., Kinani, S., Balaguer, P., Tapie, N., Lemenach, K., Maillot-Maréchal, E., Porcher, J.M., Budzinski, H., Aït-Aïssa, S. 2010. Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: Screening of chemicals and application to water samples. Anal. Bioanal. Chem. 396, 569-583.

Das, S., Majumder, S., Gupta, S., Dutta, S., Mukherjee, D. 2016. Effects of phenol on ovarian P450arom gene expression and aromatase activity *in vivo* and antioxidant metabolism in common carp *Cyprinus carpio*. Fish. Physiol. Biochem. 42, 275-286.

Dayeh, V.R., Schrmer, K., Lee, L.E.J., Bols, N.C., Schirmer, K., Lee, L.E.J., Bols, N.C. 2005. Rainbow trout gill cell line microplate cytotoxicitiy test. Small-scale Freshw. Toxic. Investig. 1, 473–503.

De la Haba, C., Palacio, J.R., Martínez, P., Morros, A. 2013. Effect of oxidative stress on plasma membrane fluidity of THP-1 induced macrophages. Biochim. Biophys. Acta - Biomembr. 1828, 357–364.

Desdoits-Lethimonier, C., Albert, O., Le Bizec, B., Perdu, E., Zalko, D., Courant, F., Lesné, L., Guillé, F., Dejucq-Rainsford, N., Jégou, B. 2012. Human testis steroidogenesis is inhibited by phthalates. Hum. Reprod. 27, 1451-1459.

Díaz-Cruz, M.S., López de Alda, M.J., Barceló, D. 2003. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. TrAC Trends Anal. Chem. 22, 340–351.

Dimastrogiovanni, G., Córdoba, M., Navarro, I., Porte, C. 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquat. Toxicol. 165, 277-285.

Ekambaram, P., Narayanan, M., Jayachandran, T. 2014. Changes in oxidative stress and antioxidant status in stressed fish brain. Int. J. Sci. Res. 3, 164-170.

Ekins, S., Reschly, E.J., Hagey, L.R., Krasowski, M.D. 2008. Evolution of pharmacologic specificity in the pregnane X receptor. BMC Evol. Biol. 8, 103.

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe, B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraishi, F., Snyder, S., Su, G., Tang, J.Y.M., Burg, B. Van Der, Linden, S.C. Van Der, Werner, I., Westerheide, S.D., Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X., Leusch, F.D.L. 2014. Benchmarking organic

micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environ. Sci. Technol. 48, 1940-1956.

Fahy, E., Subramaniam, S., Brown, H. A., Glass, C. K., Merrill, A. H., Murphy, R. C., Raetz, C.R.H., Russell, D.W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., Van Meer, G. VanNieuwenhze, M.S., White, S.H., Witztum, J.L., Dennis, E.A. 2005. A comprehensive classification system for lipids. J. Lipid Res. 46, 839-861.

Fasano, E., Bono-Blay, F., Cirillo, T., Montuori, P., Lacorte, S. 2012. Migration of phthalates, alkylphenols, bisphenol a and di(2-ethylhexyl)adipate from food packaging. Food Control. 27, 132-138.

Fent, K. 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. In Vitro. 15, 477-488.

Fent, K., Bätscher, R. 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: Demonstration of additive interactions. Environ. Toxicol. Chem. 19, 2047–2058.

Ferguson, K.K., Chen, Y.-H., VanderWeele, T.J., McElrath, T.F., Meeker, J.D., Mukherjee, B. 2016. Mediation of the relationship between maternal phthalate exposure and preterm birth by oxidative stress with repeated measurements across pregnancy. Environ. Health Perspect. 125, 488-494.

Fernandes, D., Bebianno, M.J., Porte, C. 2009. Assessing pollutant exposure in cultured and wild sea bass (*Dicentrarchus labrax*) from the Iberian Peninsula. Ecotoxicology 18, 1043–1050.

Fernandez, M.F., Arrebola, J.P., Taoufiki, J., Navalón, A., Ballesteros, O., Pulgar, R., Vilchez, J.L., Olea, N. 2007. Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reprod. Toxicol. 24, 259–64.

Gallo, C., Renzi, P., Loizzo, S., Loizzo, A., Piacente, S., Festa, M., Caputo, M., Tecce, M.F., Capasso, A. 2010. Potential therapeutic effects of vitamin E and C on placental oxidative stress induced by nicotine: an *in vitro* evidence. Open Biochem J 4, 77–82.

Geens, T., Neels, H., Covaci, A. 2012. Distribution of bisphenol-A, triclosan and N-nonylphenol in human adipose tissue, liver and brain. Chemosphere, 87, 796-802.

Gerbron, M., Geraudie, P., Fernandes, D., Rotchell, J.M., Porte, C., Minier, C. 2014. Evidence of altered fertility in female roach (*Rutilus rutilus*) from the River Seine (France). Environ. Pollut. 191, 58-62.

Ghayee, H.K., Auchus, R.J. 2007. Basic concepts and recent developments in human steroid hormone biosynthesis. Endocr. Metab. Disord. 8, 289-300.

Gibson, G. G., Skett, P. 2001. Introduction to drug metabolism. Nelson Thornes, ISBN 0748760113.

Glynn, A., Berger, U., Bignert, A., Ullah, S., Aune, M., Lignell, S., Darnerud, P.O. 2012. Perfluorinated alkyl acids in blood serum from primiparous

women in Sweden: Serial sampling during pregnancy and nursing, and temporal trends 1996-2010. Environ. Sci. Technol. 46, 9071–9079.

Göhner, C., Svensson-Arvelund, J., Pfarrer, C., Häger, J.-D., Faas, M., Ernerudh, J., Cline, J.M., Dixon, D., Buse, E., Markert, U.R. 2014. The placenta in toxicology. Part IV: battery of toxicological test systems based on human placenta. Toxicol. Pathol. 42, 345–351.

Goksøyr, A. 2006. Endocrine disruptors in the marine environment. Journal of Toxicol. Environ. Health. 69, 175-184.

Grün, F., Blumberg, B. 2009. Minireview: The case for obesogens. Mol. Endocrinol. 23, 1127-1134.

Grün, F., Venkatesan, R.N., Tabb, M.M., Zhou, C., Cao, J., Hemmati, D., Blumberg, B. 2002. Benzoate X receptors α and β are pharmacologically distinct and do not function as xenobiotic receptors. J. Biol. Chem. 277, 43691–43697.

Grünfeld, H., Bonefeld-Jørgensen, E. 2004. Effect of *in vitro* estrogenic pesticides on human oestrogen receptor α and β mRNA levels. Toxicol. Lett. 151, 467–480.

Gude, N.M., Roberts, C.T., Kalionis, B., King, R.G. 2004. Growth and function of the normal human placenta. Thromb. Res. 114, 397-407.

Haggarty, P. 2004. Effect of placental function on fatty acid requirements during pregnancy. Eur. J. Clin. Nutr. 58, 1559–1570.

Halliwell, B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 141, 312-322.

Halliwell, B., Gutteridge, J.M.C. 2015. Free radicals in biology and medicine, 5th ed. Oxford: Clarendon Press, ISBN 978-0-19-871747-8.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298-300.

Harris, c., Scott, A.P., Johnson, A.C., Panter, G.H., Sheahan, M.R., Sumpter, J.P. 2014. Principles of sound ecotoxicology. Environ. Sci. Technol. 48, 3100-3111.

Hassan, Z.K., Elobeid, M.A., Virk, P., Omer, S.A., Elamin, M., Daghestani, M.H., Alolayan, E.M. 2012. Bisphenol A induces hepatotoxicity through oxidative stress in rat model. Oxid. Med. Cell. Longev. 1-6.

Hasselberg, L., Meier, S., Svardal, A. 2004. Effects of alkylphenols on redox status in first spawning Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 69, 95–105.

Heringa, M.B., Schreurs, R.H.M.M., Busser, F., Van Der Saag, P.T., Van Der Burg, B., Hermens, J.L.M. 2004. Toward more useful *in vitro* toxicity data with measured free concentrations. Environ. Sci. Technol. 38, 6263–6270.

Hermansson, M., Hokynar, K., Somerharju, P. 2011. Mechanisms of glycerophospholipid homeostasis in mammalian cells. Prog. Lipid Res. 50, 240–257.

Herrera, E. 2002. Implications of dietary fatty acids during pregnancy on placental, fetal and postnatal development - a review. Placenta 23 Suppl A, S9–S19.

Heudorf, U., Mersch-Sundermann, V., Angerer, J. 2007. Phthalates: Toxicology and exposure. Int. J. Hyg. Environ. Health. 210, 623-634.

Hoffman, D. J. 2002. Handbook of Ecotoxicology. 2nd ed. Boca Raton, FL: CRC Press, ISBN 978-1-4200-3250-5.

Huang, H., Leung, L.K. 2009. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol. Lett. 189, 248–252.

Innis, S.M. 2005. Essential fatty acid transfer and fetal development. Placenta 26, S70–S75.

Iso, T., Watanabe, T., Iwamoto, T., Shimamoto, A., Furuichi, Y. 2006. DNA damage caused by bisphenol A and estradiol through estrogenic activity. Biol. Pharm. Bull. 29, 206-210.

Jaeger R.J., Rubin R.J. 1970. Plasticizers from plastic devices: extraction, metabolism and accumulation by biological systems. Science. 170, 460-462.

Jamieson, A.J., Malkocs, T., Piertney, S.B., Fujii, T., Zhang, Z. 2017. Bioaccumulation of persistent organic pollutants in the deepest ocean fauna. Nat. Ecol. Evol. 1, 0051.

Jones, M.L., Mark, P.J., Waddell, B.J. 2014. Maternal dietary omega-3 fatty acids and placental function. Reproduction, 147.

Keresztes, S., Tatár, E., Czégény, Z., Záray, G., Mihucz, V.G. 2013. Study on the leaching of phthalates from polyethylene terephthalate bottles into mineral water. Sci. Total Environ. 458, 451–458.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897-8901.

Kihara, A. 2012. Very long-chain fatty acids: Elongation, physiology and related disorders. J. Biochem. 152, 387–395.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.M., Aït-Aïssa, S. 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut. 158, 74-83.

Kjeldsen, L.S., Bonefeld-Jørgensen, E.C. 2013a. Perfluorinated compounds affect the function of sex hormone receptors. Environ. Sci. Pollut. Res. 20, 8031-8044.

Kjeldsen, L.S., Ghisari, M., Bonefeld-Jørgensen, E.C. 2013b. Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. Toxicol. Appl. Pharmacol. 272, 453-464.

Kliewer, S.A. 2003. The nuclear pregnane X receptor regulates xenobiotic detoxification. J. Nutr. 133, 2444S-2447S.

Kliewer, S.A., Goodwin, B., Willson, T. M. 2002. The Nuclear Pregnane X Receptor: A Key Regulator of Xenobiotic Metabolism. Endocr. Rev. 23, 687-702.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., McKee, D.D., Oliver, B.B., Willson, T.M., Zetterström, R.H.,

Perlmann, T., Lehmann, J.M. 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell, 92, 73–82.

Kontek, R., Jakubczak, M., Matlawska-Wasowska, K. 2014. The antioxidants, vitamin A and E but not vitamin C and melatonin enhance the proapoptotic effects of irinotecan in cancer cells in vitro. Toxicol. Vitr. 28, 282–291.

Krahmer, N., Farese, R.V., Walther, T.C. 2013. Balancing the fat: lipid droplets and human disease. EMBO Mol. Med. 5, 973–983.

Kraugerud, M., Zimmer, K.E., Ropstad, E., Verhaegen, S. 2011. Perfluorinated compounds differentially affect steroidogenesis and viability in the human adrenocortical carcinoma (H295R) *in vitro* cell assay. Toxicol. Lett. 205, 62-68.

Kretschmer, X.C., Baldwin, W.S. 2005. CAR and PXR: xenosensors of endocrine disrupters? Chem. Biol. Interact. 55, 111-28.

Kroon, F.J., Hook, S.E., Jones, D., Metcalfe, S., Henderson, B., Smith, R., Warne, M.S.J., Turner, R.D., McKeown, A., Westcott, D.A. 2015. Altered transcription levels of endocrine associated genes in two fisheries species collected from the Great Barrier Reef catchment and lagoon. Mar. Environ. Res. 104, 51-61.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol. Appl. Pharmacol. 196, 247-257.

Lechner, A., Keckeis, H., Lumesberger-Loisl, F., Zens, B., Krusch, R., Tritthart, M., Glas, M., Schludermann, E. 2014. The Danube so colourful: a potpourri of plastic litter outnumbers fish larvae in Europe's second largest river. Environ. Pollut. 188, 177–81.

Leclerc, F., Dubois, M.F., Aris, A. 2014. Maternal, placental and fetal exposure to bisphenol A in women with and without preeclampsia. Hipertension in Pregnancy, 33, 341-348.

Li, Z., Gibson, M., Liu, C., Hu, H. 2013. Seasonal variation of nonylphenol concentrations and fluxes with influence of flooding in the Daliao River Estuary, China. Environmental Monitoring and Assesssment, 185, 5221-5230.

Lille-Langøy, R., Goldstone, J.V., Rusten, M., Milnes, M.R., Male, R., Stegeman, J.J., Blumberg, B., Goksøyr, A. 2015. Environmental contaminants activate human and polar bear (*Ursus Maritimus*) Pregnane X Receptors (PXR, NR1I2) differently. Toxicology and Applied Pharmacology, 284, 54-64.

Lin, L., Zheng, L., Gu, Y., Wang, J., Zhang, Y., Song, W. 2008. Levels of environmental endocrine disruptors in umbilical cord blood and maternal blood of low-birth-weight infants. Chinese J. Prev. Med. 42, 177–180.

Livingstone, D.R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Lushchak, V.I. 2016. Contaminant-induced oxidative stress in fish: a mechanistic approach. Fish Physiol. Biochem. 42, 711–747.

Marie, C., Vendittelli, F., Sauvant-Rochat, M.P. 2015. Obstetrical outcomes and biomarkers to assess exposure to phthalates: A review. Environ. Int. 83, 116–136.

Martín, J., Zafra-gómez, A., Hidalgo, F., Ibáñez-yuste, A.J., Alonso, E., Vilchez, J.L. 2017. Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (*Holothuria tubulosa*) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography - tandem mass spect. Talanta 166, 336-348.

Martínez-Bueno, M. J., Gomez, M.J., Herrera, S., Hernando, M.D., Agüera, A., Fernández-Alba, A.R. 2012. Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: two years pilot survey monitoring. Environmental Pollution, 164, 267-73.

Matic, M., Mahns, A., Tsoli, M., Corradin, A., Polly, P., Robertson, G.R. 2007. Pregnane X receptor: promiscuous regulator of detoxification pathways. Cell Biology, 39: 478-483.

Mattison, D. 2010. Environmental exposures and development. Curr. Opin. Pediatr. 22, 208–218.

Meeker, J., Calafat, A., Hauser, R. 2010. Urinary bisphenol A concentration in relation to serum thyroid and reproductive hormones in men from an infertility clinic. Environ. Sci. Technol. 44, 1458-1463.

Méndez-Fernandez, P., Webster, L., Chouvelon, T., Bustamante, P., Ferreira, M., González, A.F., López, A., Moffat, C.F., Pierce, G.J., Read, F.L., Russell, M., Santos, M.B., Spitz, J., Vingada, J. V., Caurant, F. 2014. An assessment of contaminant concentrations in toothed whale species of the NW Iberian Peninsula: Part I. Persistent organic pollutants. Sci. Total Environ. 484, 196–205.

Michałowicz, J. 2014. Bisphenol A - Sources, toxicity and biotransformation. Environ. Toxicol. Pharmacol. 37, 738-758.

Milczarek, R., Sokołowska, E., Hallmann, A., Kaletha, K., Klimek, J. 2008. NADPH- and iron-dependent lipid peroxidation inhibit aromatase activity in human placental microsomes. J. Steroid Biochem. Mol. Biol. 110, 230–235.

Mills, L.J., Gutjahr-Gobell, R.E., Zaroogian, G.E., Horowitz, D. B., Laws, S.C. 2014. Modulation of aromatase activity as a mode of action for endocrine disrupting chemicals in a marine fish. Aquat. Toxicol. 147, 140-150.

Milnes, M. R., Garcia, A., Grossman, E., Grün, F., Shiotsugu, J., Tabb, M. M., Kawashima, Y., Katsu, Y., Watanabe, H., Iguchi, T., Blumberg, B. 2008. Activation of steroid and xenobiotic receptor (SXR, NR1/2) and its orthologs in laboratory, toxicologic, and genome model species. Environ. Health Persp. 116, 880-885.

Moore, L.B. 2002. Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR), and Benzoate X Receptor (BXR) define three

pharmacologically distinct classes of nuclear receptors. Mol. Endocrinol. 16, 977–986.

Muth-Köhne, E., Westphal-Settele, K., Brückner, J., Konradi, S., Schiller, V., Schäfers, C., Teigeler, M., Fenske, M. 2016. Linking the response of endocrine regulated genes to adverse effects on sex differentiation improves comprehension of aromatase inhibition in a fish sexual development test. Aquat. Toxicol. 176, 116–127.

Nativelle-Serpentini, C., Richard, S., Séralini, G.-E., Sourdaine, P. 2003. Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells. Toxicol. Vitr. 17, 413–422.

Navarro-Martín, L., Blázquez, M., Piferrer, F. 2009. Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. General and Comparative Endocrinology, 160, 3-11

Newman, M.C. 2001. Fundamentals of ecotoxicology: the Science of Pollution. CRC Press. Taylor & Francis Group, Boca Raton. ISBN 978-14665-8229-3. pp 680.

Olsen, G.W., Butenhoff, J.L., Zobel, L.R. 2009. Perfluoroalkyl chemicals and human fetal development: An epidemiologic review with clinical and toxicological perspectives. Reprod. Toxicol. 27, 212-230.

Ortiz-Zarragoitia, M., Bizarro, C., Rojo-Bartolomé, I., De Cerio, O.D., Cajaraville, M.P., Cancio, I. 2014. Mugilid fish are sentinels of exposure

to endocrine disrupting compounds in coastal and estuarine environments. Mar. Drugs 12, 4756-4782.

Philips, E.M., Jaddoe, V.W.V., Trasande, L. 2017. Effects of early exposure to phthalates and bisphenols on cardiometabolic outcomes in pregnancy and childhood. Reprod. Toxicol. 68, 105–118.

Piferrer, F., Blázquez, M., Navarro, L., González, A. 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen. Comp. Endocrinol. 142, 102–110.

Quignot, N., Desmots, S., Barouki, R., Lemazurier, E. 2012. A comparison of two human cell lines and two rat gonadal cell primary cultures as *in vitro* screening tools for aromatase modulation. Toxicol. Vitr. 26, 107–118.

Rahman, M., Brazel, C.S. 2004. The plasticizer market: an assessment of traditional plasticizers and research trends to meet new challenges. Progress in Polymer Science, 29, 1223-1248.

Repetto, M., Repetto, G. 2009. Toxicología fundamental, Ed. Díaz de Santos. ISBN: 978-84-7978-898-8.

Rosado, D., Usero, J., Morillo, J. 2016. Ability of 3 extraction methods (BCR, Tessier and protease K) to estimate bioavailable metals in sediments from Huelva estuary (Southwestern Spain). Mar. Pollut. Bull. 102, 65-71.

Russo, M.V., Avino, P., Perugini, L., Notardonato, I. 2015. Extraction and GC-MS analysis of phthalate esters in food matrices: a review. Royal Society of Chemistry, 5, 37023-37043.

Sadauskas-Henrique, H., Duarte, R.M., Gagnon, M.M., Almeida-Val, V.M.F. 2017. Validation of a suite of biomarkers of fish health in the tropical bioindicator species, tambaqui (*Colossoma macropomum*). Ecol. Indic. 73, 443-451.

Sánchez-Valle, V., Méndez-Sánchez, N. 2013. Estrés oxidativo, antioxidantes y enfermedad. Rev. Invest. Med. Sur. Mex. 20, 161-168.

Sanderson, J.T. 2009. Placental and fetal steroidogenesis. Methods Mol. Biol. 550,127-136.

Sanderson, J.T., Letcher, R.J., Heneweer, M. 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male. Environ. Health Persp. 109, 1027–1031.

Sarasquete, C., Segner, H. 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. Sci. Total Environ. 247, 313-332.

Schirmer, K., 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology, 224, 163-183.

Schirmer, K., Chan, G., Bols, N.C. 2000. Transitory metabolic disruption and cytotoxicity elicited by benzo[a]pyrene in two cell lines from rainbow trout liver. J. Biochem. Mol. Toxicol. 14, 262-276.

Schirmer, K., Dayeh, V.R., Bopp, S., Russold, S., Bols, N.C. 2004. Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. Toxicology 205, 211-221.

Schnell, S., Olivares, A., Piña, B., Echavarri-Erasun, B., Lacorte, S., Porte, C. 2013. The combined use of the PLHC-1 cell line and the recombinant yeast assay to assess the environmental quality of estuarine and coastal sediments. Mar. Pollut. Bull. 77, 282–9.

Sen, N., Liu, X., Craig, Z.R. 2016. Short term exposure to di-n-butyl phthalate (DBP) disrupts ovarian function in young CD-1 mice. Reprod. Toxicol. 53, 15-22.

Shea, K.M. 2003. Pediatric exposure and potential toxicity of phthalate plasticizers. Pediatrics, 111, 1467-1474.

Smeets, J.M., Van Holsteijn, I., Giesy, J.P., VandenBerg, M. 1999. The anti-estrogenicity of Ah receptor agonists in carp (*Cyprinus carpio*) hepatocytes. Toxicol. Sci. 52, 178–188.

Soclo, H., Garrigues, P., Ewald, M. 2000. Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Cotonou (Benin) and Aquitaine (France) areas. Mar. Pollut. Bull. 40, 387-396.

Song, S.B., Xu, Y., Zhou, B.S. 2006. Effects of hexachlorobenzene on antioxidant status of liver and brain of common carp (*Cyprinus carpio*). Chemosphere 65, 699–706.

Sonnenschein, C., Soto, A.M. 1998. An updated review of environmental estrogen and androgen mimics and antagonists. J. Steroid Biochem. Mol. Biol. 65, 143-150.

Soto, A.M., Chung, K.L., Sonnenschein, C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ. Health Persp. 102, 380–383.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernández, M.F., Olea, N., Olea Serrano, F. 1995. The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Environ. Health Persp. 103, 113-122.

Stephensen, E., Sturve, J., Förlin, L. 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 133, 435–442.

Stuart, M., Lapworth, D., Crane, E., Hart, A. 2012. Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1-21.

Szychowski, K.A., Rybczyńska-Tkaczyk, K., Leja, M.L., Wójtowicz, A.K., Gmiński, J. 2016. Tetrabromobisphenol A (TBBPA)-stimulated reactive oxygen species (ROS) production in cell-free model using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) assay-limitations of method. Environ. Sci. Pollut. Res. 23, 12246-12252.

Tavakoly Sany, S.B., Hashim, R., Rezayi, M., Salleh, A., Safari, O. 2014. A review of strategies to monitor water and sediment quality for a

sustainability assessment of marine environment. Environ. Sci. Pollut. Res. 21, 813-833.

Testai, E., Epstein, M., Emri, I., Hartemann, P., Hoet, P., Leitgeb, N., Martínez Martinez, L., Proykova, A., Rizzo, L., Rodriguez-Farré, E., Rushton, L., Rydzynski, K., Samaras, T., Testai, E., Vermeire, T., Hartemann, P., Rastogi, S.C., Bernauer, U., Piersma, A., De Jong, W., Gulliksson, H., Sharpe, R., Schubert, D., Rodríguez-Farre, E. 2016. The safety of medical devices containing DEHP plasticized PVC or other plasticizers on neonates and other groups possibly at risk (2015 update). Regul. Toxicol. Pharmacol. 76, 209-210.

Tetz, L.M., Cheng, A.A., Korte, C.S., Giese, R.W., Wang, P., Harris, C., Meeker, J.D., Loch-Caruso, R. 2013. Mono-2-ethylhexyl phthalate induces oxidative stress responses in human placental cells *in vitro*. Toxicol. Appl. Pharmacol. 268, 47–54.

Thibaut, R., Porte, C. 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: Cytotoxicity and interactions with cytochrome P450 1A. Toxicol. Vitr. 22, 1128–1135.

Tobiszewski, M., Namieśnik, J. 2012. PAH diagnostic ratios for the identification of pollution emission sources. Environ. Pollut. 162, 110-119.

Tollefsen, K.E., Blikstad, C., Eikvar, S., Farmen Finne, E., Katharina Gregersen, I. 2008. Cytotoxicity of alkylphenols and alkylated non-phenolics in a primary culture of rainbow trout (*Onchorhynchus mykiss*) hepatocytes. Ecotoxicol. Environ. Saf. 69, 64–73.

Toms, L.-M.L., Thompson, J., Rotander, A., Hobson, P., Calafat, A.M., Kato, K., Ye, X., Broomhall, S., Harden, F., Mueller, J.F. 2014. Decline in perfluorooctane sulfonate and perfluorooctanoate serum concentrations in an Australian population from 2002 to 2011. Environ. Int. 71, 74–80.

Tran, B.C., Teil, M.J., Blanchard, M., Alliot, F., Chevreuil, M. 2015. BPA and phthalate fate in a sewage network and an elementary river of France: influence of hydroclimatic conditions. Chemosphere, 119, 43-51.

Uno, T., Ishizuka, M., Itakura, T. 2012. Cytochrome P450 (CYP) in fish. Environmental Toxicology and Pharmacology, 34, 1-13.

Van Cauwenberghe, L., Janssen, C. 2014. Microplastics in bivalves cultured for human consumption. Environmental Pollution, 193, 65-70.

Van Drooge, B.L., Garriga, G., Koinig, K.A., Psenner, R., Pechan, P., Grimalt, J.O. 2014. Sensitivity of a remote alpine system to the Stockholm and LRTAP regulations in POP emissions. Atmosphere, 5, 198-210.

Van Meer, G., Voelker, D.R., Feigenson, G.W. 2008. Membrane lipids: where they are and how they behave. Nature reviews, 9, 112-124.

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V. 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24, 139-177.

Villa, S., Migliorati, S., Monti, G.S., Holoubek, I., Vighi, M. 2016. Risk of POP mixtures on the Arctic food chain. Environ. Toxicol. Chem. 1-12.

Villeneuve, D.L., Kannan, K., Khim, J.S., Falandysz, J., Nikiforov, V.A., Blankenship, A.L., Giesy, J.P. 2000. Relative potencies of individual

polychlorinated naphthalenes to induce dioxin-like responses in fish and mammalian *in vitro* bioassays. Arch. Environ. Contam. Toxicol. 39, 273-281.

Villeneuve, D.L., Knoebl, I., Kahl, M.D., Jensen, K.M., Hammermeister, D.E., Greene, K.J., Blake, L.S., Ankley, G.T. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 76, 353–368.

Wassmur, B., Gräns, J., Kling, P., Celander, M.C. 2010. Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 100, 91-100.

Watanabe, M., Ohno, S., Nakajin, S. 2012. Effects of bisphenol A on the expression of cytochrome P450 aromatase (CYP19) in human fetal osteoblastic and granulosa cell-like cell lines. Toxicol. Lett. 210, 95–9.

Wenning, R.J., Ingersoll, C.G. 2002. Use of sediment quality guidelines and related tools for the assessment of contaminated sediments: Executive summary booklet of a Society of Environ. Toxicol. Chem. (SETAC) Pellston Workshop.

White, P.C. 2006. Ontogeny of adrenal steroid biosynthesis: why girls will be girls. J. Clin. Invest. 116, 872-874.

Wolfgang, M.J., Lane, M.D. 2006. Control of energy homeostasis: role of enzymes and intermediates of fatty acid metabolism in the central nervous system. Annu. Rev. Nutr. 26, 23–44.

Wright, J., George, S., Martinez-Lara, E., Carpene, E., Kindt, M. 2000. Levels of cellular glutathione and metallothionein affect the toxicity of oxidative stressors in an established carp cell line. Mar. Environ. Res. 50, 503–508.

Wu, F., Tian, F.J., Lin, Y., Xu, W.M. 2016. Oxidative Stress: Placenta Function and dysfunction. Am. J. Reprod. Immunol. 76, 258-271.

Xu, J., Osuga, Y., Yano, T., Morita, Y., Tang, X., Fujiwara, T., Takai, Y., Matsumi, H., Koga, K., Taketani, Y., Tsutsumi, O. 2002. Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells. Biochem. Biophys. Res. Commun. 292, 456-462.

Yamada, H., Furuta, I., Kato, E.H., Kataoka, S., Usuki, Y., Kobashi, G., Sata, F., Kishi, R., Fujimoto, S. 2002. Maternal serum and amniotic fluid bisphenol A concentrations in the early second trimester. Reprod. Toxicol. 16, 735–739.

Yang, L., Li, J., Lai, J., Luan, H., Cai, Z., Wang, Y., Zhao, Y., Wu, Y. 2016. Placental transfer of perfluoroalkyl substances and associations with thyroid hormones: Beijing Prenatal Exposure Study. Nat. Publ. Gr. 2–10.

Zhang, W.L., Luo, Y., Zhang, L., Cai, Q., Pan, X.J. 2013. Known and emerging factors modulating estrogenic effects of endocrine-disrupting chemicals. Environ. Rev. 22, 87-98.

Zhao, B., Chu, Y., Hardy, D.O., Li, X., Ge, R.S. 2010. Inhibition of 3β- and 17β-hydroxysteroid dehydrogenase activities in rat Leydig cells by perfluorooctane acid. J. Steroid Biochem. Mol. Biol. 118, 13-17.

Ziv-Gal, A., Zelieann, C., Wang, W., Flaws, J. 2013. Bisphenol A inhibits cultured mouse ovarian follicle growth partially via the aryl hydrocarbon receptor signaling pathway. Reprod. Toxicol. 42, 58-67.

Zuo, Y., Zhu, Z., Alshanqiti, M., Michael, J., Deng, Y. 2015. Bisphenol A and the related alkylphenol contaminants crustaceans and their potential bioeffects. Adv. Environ. Res. 4, 39-48.