UNIVERSITAT DE BARCELONA

Evaluación del impacto ambiental asociado al uso de nuevos retardantes de llama

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“Hazlo, o no lo hagas, pero no lo intentes”

Maestro Yoda

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ADN	Ácido desoxirribonucleico.
BBB	Barrera hematoencefálica (del inglés blood-brain barrier).
BFR	Retardantes de llama bromados (del inglés brominated flame retardants).
BMF	Factor de biomagnificación (del inglés biomagnification factor).
BSAF	Factor de acumulación desde sedimento a biota (del inglés biota to sediment accumulation factor).
BTBPE	1,2-bis(2,4,6-tribromofenoxi)etano.
CA	Test del cometa (del inglés comet assay).
CAR	Receptor constitutivo de androstano (del inglés constitutive androstane receptor).
CFRs	Retardantes de llama clorados (del inglés chlorinated flame retardants).
CR	Clearance rate.
DBDPE	Decabromodifenil etano.
Dec 602	Decloran 602.
Dec 603	Decloran 603.
Dec 604	Decloran 604.
DCM	Diclorometano.
DP	Decloran plus.
DPMA	Monoaducto del DP (del inglés dechlorane plus mono adduct).
dw	Peso seco (del inglés dry weight).
EDARs	Estaciones de depuración de aguas residuales.
EI	Ionización electrónica (del inglés electron ionization).
ERA	Evaluación del riesgo ecológico (del inglés Ecological risk assesment)
EPA	Agencia de Protección Ambiental (del inglés Environmental Protection Agency).
ESI	Ionización por electrospray (del inglés electrospray ionization).
FR	Retardantes de llama (del inglés flame retardants).
GC	Cromatografía de gases (del inglés gas chromatography).
GPC	Cromatografía de permeabilidad en gel (del inglés gel permeation chromatography).
HBB	Hexabromobenceno.

HBCD	Hexabromociclododecano.
HCCPD	Hexaclorociclopentadieno.
HCDBCO	Hexaclorociclopentadienildibromo-ciclooctano.
HFRs	Retardantes de llama halogenados (del inglés halogenated flame retardants).
HNs	Norbornenos halogenados (del inglés halogenated norbornenes).
HNPs	Compuestos naturales halogenados (del inglés halogenated natural products).
HRMS	Espectrometría de masas de alta resolución (del inglés high resolution mass spectrometry).
iLODs:	Límite de detección instrumental (del inglés instrumental limits of detection).
IWC	Comisión ballenera internacional (del inglés international whaling comisión).
LC	Cromatografía de líquidos (del inglés liquid chromatography).
LDR	Relación longitud-cabeza (del inglés length diameter ratio).
LODs	Límites de detección (del inglés limits of detection).
lw	Peso lipídico (del inglés lipid weight).
MAE	Extracción asistida por microondas (del inglés microwave assisted extraction).
MeO-PBDEs	Polibromodifenil éteres metoxilados.
MHC-1	(1R,2S,4R,5R,1´E)-2-bromo-1-bromometil-1,4-dicloro-5-(2´-cloroetenil)-5-metilciclohexano.
mLODs	Límites de detección del método (del inglés method limits of detection).
MN	Test de micronúcleos (del inglés micronucleus assay).
MS	Espectrometría de masas (del inglés mass spectrometry).
MS-MS	Espectrometría de masas en tándem.
NCI	Ionización química negativa (del inglés negative chemical ionization).
OH-PBDEs	Polibromodifenil éteres hidroxilados (del inglés hydroxilated PBDEs).
PBDEs	Polibromodifenil éteres.
PBEB	Pentabromoetil benceno (del inglés pentabromoethyl benzene).
PCBs	Bifenilos policlorados (del inglés polychlorinated biphenyls).
PMBPs	Metil-bipirroles polihalogenados (del inglés polybromomethly bipyrroles).

PTV	Temperatura de vaporización programada (del inglés programable temperature vaporization).
PXR	Receptor X de pregnano (del inglés pregnane X receptor).
Q1	2,3,3´,4,4´,5,5´-heptacloro-1´-metil-1,2´-bipirrol.
QqQ	Triple cuadrupolo.
REACH	Registro, evaluación, autorización y restricción de sustancias químicas (del inglés Registration, Evaluation, Authorization and Restriction of Chemicals).
RoHS	Restricción de substancias peligrosas (del inglés Restrictions of Hazardous Substances).
SPLE	Extracción selectiva por líquidos presurizados (del inglés selective presurized liquid extraction).
SVHC	Substancias de alta prioridad (del inglés Substances of very High Concern).
TBB o EHTBB	2-etilhexil-2,3,4,5-tetrabromobenzoato.
TBBPA	Tetrabromobisfenol A.
TBBPA-DBPE	Tetrabromobisfenol A-bis(2,3-dibromopropiléter).
TBPH	Bis(2-ethylhexil)-3,4,5,6-tetrabromo-fthalato.
TetraBHD	2,5,7-dibromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno.
TL	Nivel trófico (del inglés trophic level).
TMF	Factor de biomagnificación trófico (del inglés trophic biomagnification factor).
TOC	Carbono orgánico total (del inglés total organic carbon).
TOF	Tiempo de vuelo (del inglés time of flight).
TriBHD	2,7-dibromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno.
UE	Unión Europea.
ZEC	Zonas especiales de conservación.

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SUMMARY

Halogenated flame retardants (HFRs) have been widely used for many years in order to prevent fires. They are present in a wide range of materials, such as furniture, textiles, electronic equipment, paints, and so on. Thus, they are continuously entering the environment since its demand is increasing every year, and they have become contaminants of concern to the scientific community. Besides, even if some of the most commonly used HFRs have been banned recently, other alternative FRs have appeared to replace them. On one hand, available information regarding "classical" HFRs, such as PBDEs and in less extent HBCD, is relative abundant since studies reporting its presence and behavior in the environment have critically increased in the last decade. On the other hand, information regarding the occurrence, behavior and effects of alternative HFRs such as dechlorane plus and related compounds is still scarce even though they have been used since the early 70s.

Consequently, the objectives of this thesis were mainly 5:

1. Development of analytical methodologies capable of determining these compounds with proper selectivity and sensitivity, especially for emerging HFRs.
2. Application of these methodologies in environmental samples, studying the presence of HFRs and the differences in contamination profiles.
3. Application of the developed methodologies in several biota samples corresponding to different trophic levels and diets (terrestrial or aquatic), determining the bioaccumulation and biomagnification capacities of HFRs
4. Comparison between concentrations of classical and alternative HFRs, evaluating possible trends indicating the shift from one family to another.
5. Evaluation of the toxicological effects of alternative FRs and comparison with the effects of classical FRs.

The development of a new methodology for the analysis of halogenated norbornenes, selective and sensitive enough for the detection of these compounds in areas far away from their production sources, was the first challenge of this thesis. When this thesis started most of studies focused on DP only, and this compound was included in already developed methodologies. Conversely, developed methodology was specifically adjusted to obtain the maximum selectivity and sensitivity for the analysis of HNs alone. This was achieved using gas chromatography coupled to tandem mass

spectrometry working in negative chemical ionization mode (GC-NCI-MS-MS) and represented the first methodology for the analysis of HNs by GC-MS-MS. The excellent sensitivity of NCI applied to halogenated compounds was combined with the great selectivity of the MS-MS. Similarly, the other methodology optimized during this thesis improved the existent methodology available in the laboratory for the analysis of PBDEs and other emerging BFRs. While the previous technique was GC-NCI-MS, the new methodology consisted in a GC-EI-MS-MS analysis, representing a great improvement in terms of selectivity while maintaining a good sensitivity. In short, two sensitive and selective methodologies were developed for the analysis of HFRs in several environmental (sediment and sludge) and biological (fish, blubber and bird eggs) matrices. This work can be found in detail in publications #1 and #2.

Sediments from Chile, Colombia and Spain were analyzed using the previously developed methodologies. PBDEs were found in the 3 study areas, showing an already proved worldwide presence. On the other hand, it was the first report of HNs in European sediments. Taking into account that HNs might have been out there for a while, more studies are needed to properly characterize the contamination of sediments by HNs in the future. Interestingly, DBDPE concentrations were similar to BDE-209 concentrations both in Colombia and Spain, maybe suggesting a shift between these two compounds. As expected due to the different industrial activity, the highest concentrations were found in Spain. Moreover, sludge from various wastewater treatment plants (WWTPs) from Spain was analyzed as well. It represented the first report of Dec 603 in sludge worldwide and showed that DP dominates the HN profile in sludge. Furthermore, DBDPE concentrations were in the same order that BDE-209 concentrations, as observed also in sediments. This work is compiled in publications #3 and #4.

Sediment and sludge can act as the first entrance point of HFRs in the trophic chain, or even re-introduce these contaminants after a long period of time accumulating them. Thus, after determining its presence in these matrices next logical step was the analysis of different species belonging to different trophic chains. Firstly, a total of 11 different species from an aquatic food web from Chile were sampled, divided into 3 categories (primary, secondary and tertiary consumers) and analyzed. Afterwards, blubber samples from aquatic top predators (dolphins) were obtained by biopsy sampling through Gulf of Cádiz and Strait of Gibraltar waters, and additional samples of blubber and brain were

collected from stranded animals along the Alboran sea coastline. In total, 5 different species resident in these waters were sampled. Finally, 14 bird species from Doñana Natural Space were monitored using their unborn eggs, collected after the ending of their breeding season. PBDEs, HBCD, emerging BFRs, HNs and HNPs were detected in almost all species, showing their worldwide presence and bioaccumulation capacity.

As shown in literature for other areas in the world, concentrations found during this thesis showed a geographical dependent behavior. While PBDEs dominated the contamination profile in Chile's biota, contribution of HNs in Spain was closer and even surpassed PBDEs in some cases. Moreover, all these results showed the presence of HNs in areas far away from production sources (USA and China) showing that they are used worldwide, and their bioaccumulation in species with a wide range of diets. In particular, DP was the main compound in species from Chile, while Dec 602 was the main compound both in aquatic and terrestrial species from Spain. All these differences might be due to a greater use of HNs in Europe. Results are showed in articles #5 to #8.

Bioaccumulation and biomagnification capacity of HNs was evaluated and compared to PBDEs. Dec 602 showed BSAF in the same order than BDE-47, while values for DP were similar to BDE-209. This is in agreement with the domination of HNs fingerprints by Dec 602, as BDE-47 usually dominates PBDEs profiles in species with aquatic diets. Likewise, Dec 602 showed a positive correlation with $\delta^{15}N$ both in birds and dolphins, suggesting biomagnification capacity either in aquatic or terrestrial food chains. This was also observed for PBDEs in general and BDE-47 in particular. On the contrary, DP did not present biomagnification capacity in any case. Overall, it seems that more attention should be paid to HNs due to their presence in a wide range of species, and to Dec 602 in particular considering its high concentrations in biota.

Analysis of blubber and brain samples from the same individuals allowed an evaluation of possible tissue specific accumulation of HFRs in 5 dolphin species. As expected, concentrations in blubber were higher than in brain, but there were some exceptions: BDE-153, HBB and TetraBHD levels were higher in brain than in blubber. Furthermore, ratio between PBDEs and HNs was considerably lower in brain than in blubber, even though PBDEs levels were still higher. All this shows that blubber might not be the proper tissue for the environmental monitoring of some compounds like HBB, barely detected in blubber but consistently detected in brain. Moreover, HNs seem to have a higher capacity to surpass the BBB than PBDEs.

Since eggs from 3 bird species (white stork, black kite and flamingo) collected in 1999, 2003, 2011, 2012 and 2013 were available, time trends occurring in these species could be evaluated. A decrease in the concentrations of PBDEs, specially the components included in the Penta-BDE mixture, was observed. This is in agreement with the restrictions over PBDEs in these years. On the contrary, HNs concentrations did not change during the decade studied. Since the use of HNs in Europe is not restricted yet, probably this timeframe was not enough to evaluate their time trends properly and a monitoring during the next years is recommended. This work is described in detail in publication #9.

Finally, in the last part of this thesis the toxicological effects of Deca-BDE and DP commercial mixtures were evaluated by *in vivo* exposures to Mediterranean mussels (*Mytilus galloprovincialis*). This work was done in Plymouth University during a predoctoral stay of 3 months under the supervision of Dr. Jha Awadhesh and Prof. James Readman. Deca-BDE mixture (which has a >90% of BDE-209) was chosen as it was the main PBDE mixture used in the recent years, and DP was chosen since it has been proposed as a BDE-209 substitute. Results showed that both compounds did not induce physiological alterations, but they did induce genetic alterations. Oxidative DNA damage and micronucleus induction were observed. Besides, DP caused similar or even higher damage than BDE-209 at lower concentrations.

CAPÍTULO 1

INTRODUCCIÓN GENERAL Y OBJETIVOS

1.1. Retardantes de llama

Los retardantes de llama (FRs, del inglés flame retardants) han sido usados para disminuir el riesgo de flamabilidad de los materiales desde mucho antes de la llegada de los compuestos químicos modernos, ya que los incendios siempre han representado una fuente importante de daños materiales y económicos, unidos a frecuentes pérdidas de vidas. Ya en tiempos de los egipcios se utilizaba una mezcla de alumbre para impedir que la madera ardiera tan fácilmente; posteriormente los romanos le añadirían vinagre (Alaee *et al.,* 2003).

Hoy en día se utilizan substancias químicas, pero el propósito sigue siendo el mismo: prevenir los incendios. Estas substancias se aplican a materiales muy diversos como textiles, plásticos, maderas, materiales eléctricos, etc. (Darnerud 2003). Los avances tecnológicos que se han producido en los últimos años han provocado la aparición de un gran número de polímeros con aplicaciones muy diversas y presentes en prácticamente todos los ámbitos. Además, la gran mayoría de los polímeros utilizados son derivados del petróleo y, por tanto, inflamables, lo que hace la presencia de los FRs más necesaria aún. El uso de polímeros aumentó desde las 145.000 hasta las 310.000 toneladas entre 1990 y 2000, suponiendo un aumento del 100% en una década. En consecuencia, la demanda de FRs también aumenta. En 2000 se estimaba en 1,2 millones de toneladas mientras que actualmente se prevé un aumento anual del 6%, llegando hasta una demanda de 2,6 millones de toneladas en 2016 (Vahabi *et al.*, 2015). Existen 4 familias principales de FRs: inorgánicos (representando el 50% de la producción anual), orgánicos halogenados (25%), organofosforados (20%) y basados en nitrógeno (5%) (Alaee *et al.,* 2003).

Para comprender mejor el mecanismo de actuación de los FRs conviene fijarse en el proceso de combustión. Éste consta de cuatro etapas: precalentamiento, volatilización o descomposición, combustión y propagación. La acción del FR consiste en inhibir una de estas cuatro etapas para impedir la combustión. Por ejemplo, el óxido de aluminio se deshidrata a temperaturas en torno a los 200 ºC en una reacción endotérmica que disminuye la temperatura del material, confiriendo a este óxido la capacidad de ser usado como FR ya que actúa en la fase de precalentamiento. Otros FRs actúan en fases más avanzadas de la reacción, siendo por tanto más efectivos (Troitzch 1900).

Existen dos clases principales de FRs, dependiendo de cómo se incorporan al polímero. Por un lado, los FRs de tipo reactivo se incorporan a la propia formulación del

polímero, mientras que por otro lado los FRs de tipo aditivo simplemente se incorporan al polímero. Los compuestos de tipo aditivo tienen más facilidad para pasar al medio ambiente ya que su unión con el polímero no es demasiado fuerte (Hutzinger y Thoma 1987).

El uso de HFRs se ha relacionado con la disminución del número de muertes, ya sea directas o a causa de la intoxicación por el humo, a causa de incendios en el Reino Unido. Las muertes totales pasaron de 950/año entre 1985 y 1990 a 750/año entre 1995 y 1998, mientras que las causadas por la inhalación de humo pasaron de 600 a 450 en los mismos periodos (Horrocks y Price 2001).

1.2. Retardantes de llama halogenados

Los retardantes de llama halogenados (HFRs, del inglés Halogenated Flame Retardants) son compuestos clorados o, principalmente, bromados. Actúan sobre la etapa de combustión capturando los radicales libres producidos durante la reacción, evitando la propagación de la misma e incluso la aparición de las llamas. Los halógenos son especialmente eficaces en ello, aunque no todos los compuestos halogenados pueden usarse como FRs (Troitzch 1900). Los compuestos fluorados, al ser muy estables, descomponen a temperaturas demasiado altas para que el flúor se libere a tiempo. Por el contrario, los compuestos yodados son poco estables y el yodo es liberado demasiado pronto. Por ello, los compuestos usados como HFRs son principalmente bromados y clorados. De hecho, la demanda total de retardantes de llama bromados (BFRs, del inglés brominated flame retardants) en el año 2000 representó un 36% de la demanda total de FRs (Alaee y Wenning 2002). Existen diferentes familias de BFRs; algunos como los Polibromodifenil éteres (PBDEs), el Hexabromociclodecano (HBCD) o el Tetrabromobisfenol A (TBBPA) han sido usados desde hace años, mientras que otros son más recientes y su uso aún no está tan extendido. Por otro lado, algunos ejemplos de FRs clorados (CFRs, del inglés chlorinated flame retardants) son el Decloran plus (DP), Decloran 602 (Dec 602), Decloran 603 (Dec 603) o el Decloran 604 (Dec 604).

La producción global de HFRs sufrió un aumento considerable entre 2001 y 2008. En el caso de los BFRs, su producción pasó a ser de unas 200.000 toneladas a 410.000 en todo el mundo. Así mismo, el volumen de producción de CFRs también se dobló, pasando de 82.000 a 190.000 toneladas (Shaw 2010).

Existen más de 175 compuestos diferentes listados como HFRs. A continuación se describen algunas características de los HFRs incluidos en esta tesis.

1.2.1. PBDEs

La estructura molecular de los PBDEs está formada por dos anillos de fenilo con uno o más átomos de bromo y unidos por un puente de oxígeno. Existen 209 congéneres diferentes dependiendo del grado de bromación y de la posición de los átomos de bromo en los anillos (Figura 1.1). La nomenclatura que se sigue es la misma que fue introducida para los diferentes congéneres de los bifenilos policlorados (PCBs, del inglés Polychlorinated bihpenyls) (Ballschmiter y Zell 1980).

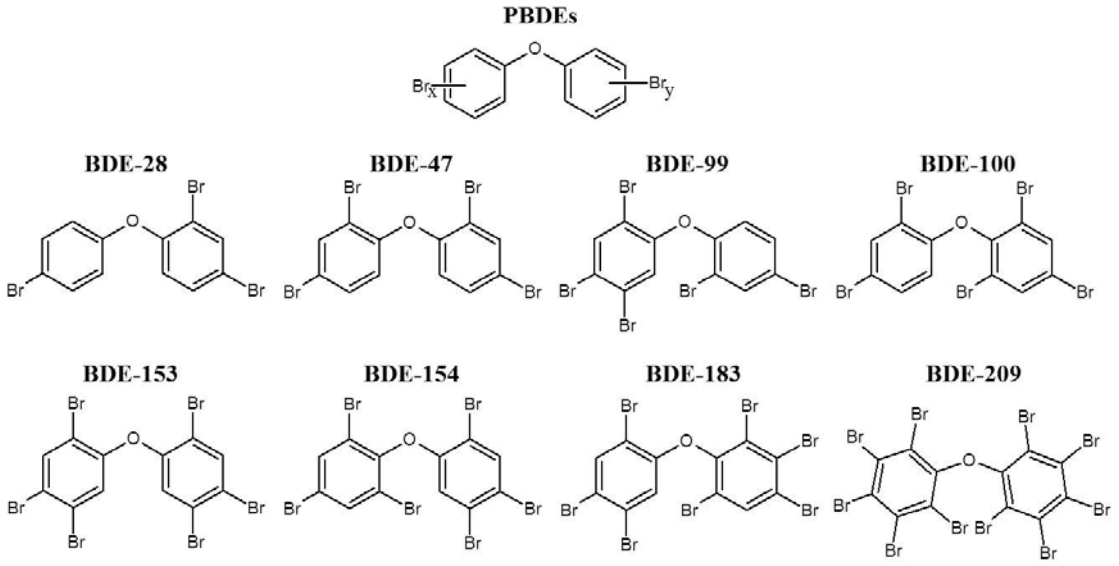

Figura 1.1. Estructura de los PBDEs incluidos en esta tesis.

Comercialmente existen en forma de 3 mezclas diferentes: Penta-BDE (24-37% de tetra-BDEs, 50-60% de penta-BDEs y 4-8% de hexa-BDEs), Octa-BDE (10-12% de hexa-BDEs, 44% de hepta-BDEs, 31-35% de octa-BDEs y 10-11% de nona-BDEs) y Deca-BDE (3% de nona-BDEs y 97% de deca-BDE o BDE-209). Las mezclas Penta-BDE y Octa-BDE han sido prohibidas en la Unión Europea (UE) y Norteamérica, mientras que actualmente la producción de Deca-BDE en las mismas zonas debería haber cesado, según el compromiso que asumieron los principales fabricantes para dejar de producir esta mezcla a finales de 2013 (Hess 2009; Schecter *et al.,* 2010). La legislación existente y sus consecuencias son discutidas en profundidad en el apartado 1.4.

La mezcla Penta-BDE era producida principalmente en Israel, Japón, EE.UU, UE y China, mientras que la Octa-BDE era producida también en Israel, EE.UU y Japón, además de en Holanda y Reino Unido. El volumen de producción anual de la mezcla Penta-BDE en EE.UU pasó de 22.000-111.000 toneladas en 2002 a 0 en 2006, mientras que el volumen de producción de la mezcla Octa-BDE pasó de 2.000-20.000 toneladas a 0, a causa de la prohibición de su uso e importación. En cambio, no existen datos sobre las zonas de producción de la mezcla Deca-BDE excepto en el caso de EE.UU, aunque es de suponer que se fabricaba en las mismas zonas (Jinhui *et al.,* 2015). El volumen de producción de la mezcla Deca-BDE se mantuvo constante entre 2002 y 2006 (111.000-222.000 toneladas) (Klosterhaus *et al.,* 2012). En 1999 se documentó que Norteamérica consumía el 50% de la producción global de PBDEs, concretamente el 97.5% de Penta-BDE, 36% de Octa-BDE y 44% de Deca-BDE, mientras que Asia absorbía el 53% de la producción global de Octa-BDE y 42% de Deca-BDE. El resto de la demanda era para Europa con el 3% de Penta-BDE, 12% de Octa-BDE y 14% de Deca-BDE (Hale *et al.*, 2003). En 2003 Asia acaparó el 40% de la demanda global, constituida en un 83% por Deca-BDE (Lee y Kim 2015).

Los materiales plásticos pueden contener hasta un 15% en peso de PBDEs, aunque normalmente el porcentaje oscila entre el 2 y 6%. En cambio, en otros polímeros como el poliuretano el contenido de PBDEs puede llegar hasta el 30% (Linares *et al.,* 2015). Más concretamente, la mezcla Penta-BDE es usada principalmente en materiales compuestos de poliuretano como colchones, muebles, etc., mientras que la mezcla Octa-BDE se usa en materiales de plástico más duros como cubiertas de ordenadores y monitores. La mezcla Deca-BDE es la que ha tenido un uso más extendido y prolongado, representando más del 80% de la producción total de PBDEs (Besis and Samara 2012). Se usa en plásticos como el poliestireno de alto impacto, equipamiento electrónico, recubrimientos de cables eléctricos, textiles y mobiliario. Además es aplicada también en vehículos, barcos y en el sector de la construcción (Costa y Giordano 2011; Guerra *et al.,* 2012a; Miller *et al.,* 2014)

1.2.2. HBCD

La reacción industrial mediante la cual se produce el HBCD es una adición de bromo al cis-trans-trans-1,5,9-ciclododecatrieno, que da lugar a una mezcla de 16 esteroisómeros, seis pares de enantiómeros y cuatro mesoformas (Heeb *et al.,* 2007). La mezcla comercial contiene los isómeros α-, β- y γ-HBCD en una proporción del 10-13%, 1-

10% y 75-89%, respectivamente (Figura 1.2), aunque se han indicado también los isómeros δ- y ε-HBCD en proporciones en torno al 0,5% (Heeb *et al.*, 2007). Los diferentes esteroisómeros presentan diferentes propiedades fisicoquímicas como polaridad o solubilidad en agua, atribuidas a las diferencias estructurales entre ellos. Por ejemplo, los isómeros α-, β- y γ-HBCD presentan solubilidades muy diferentes, siendo éstas de 49, 15 y 2 μg/L, respectivamente (Tomy *et al.*, 2004). Estas diferencias se trasladan también al medio ambiente, donde los diferentes isómeros tienen diferentes velocidades de absorción biológica y metabólica (Dirtu *et al.*, 2013).

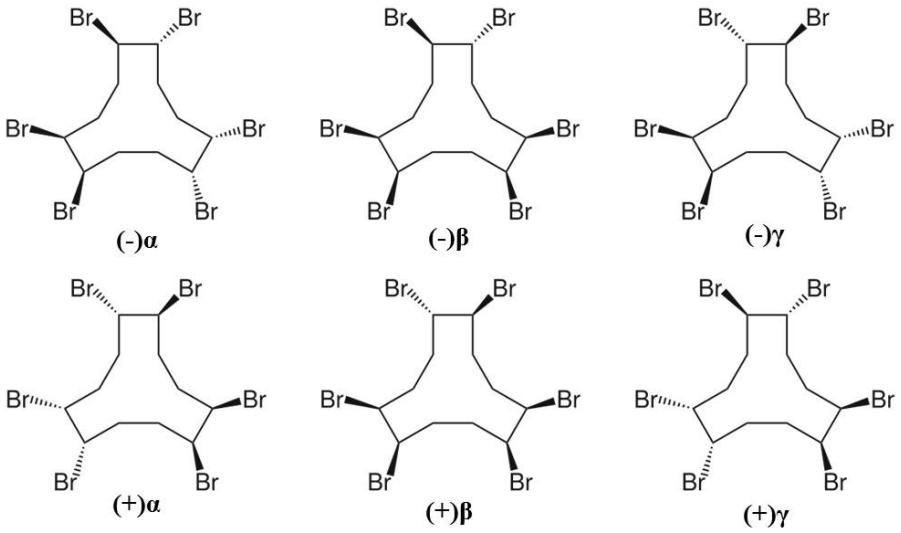

Figura 1.2. Estructuras de los principales isómeros y enantiómeros del HBCD.

El HBCD es utilizado principalmente en poliestireno, textiles y material electrónico doméstico (Covaci *et al.*, 2006). El HBCD fue considerado una alternativa potencial a los PBDEs cuando estos empezaron a llamar la atención de los organismos medioambientales (Al-Odaini *et al.*, 2015). Pese a llevar en el mercado desde la década de los 80, no fue hasta principios de siglo cuando su producción se incrementó considerablemente. En 2001 la demanda global era de unas 16.700 toneladas, en 2002 y 2003 la demanda fue de 21.500 y 22.000 toneladas respectivamente (Onogbosele y Scrimshaw 2014). En EE.UU su producción entre 2002 y 2006 fue de 111.000-222.000 toneladas por año (Klosterhaus *et al.*, 2012).

1.2.3. BFRs alternativos

La industria está en constante movimiento y, por necesidad, siempre un paso por delante de la legalidad vigente. Por ello, ya existen numerosos compuestos bromados

propuestos o ya usados como alternativas a los BFRs clásicos, además de los HNs. Algunos ejemplos son el hexabromobenceno (HBB, del inglés Hexabormobenzene), el pentabromoetil benceno (PBEB, del inglés pentabromoethyl benzene), el decabromodifenil etano (DBDPE, del inglés decabromodifenil ethane), los tres incluidos en esta tesis, y otros como el 1,2-*bis*(2,4,6-tribromofenoxi)etano (BTBPE), el 2-etilhexil-2,3,4,5-tetrabromobenzoato (TBB), el bis(2-etilhexil)-3,4,5,6-tetrabromo-ftalato (TBPH) o el hexaclorociclopentadienildibromo-ciclooctano (HCDBCO). No existe mucha información sobre su volumen de producción o usos aunque algunos ya han sido hallados en regiones remotas como la Antártida, probando que ya están siendo utilizados y empiezan a entrar en el medio ambiente. Al tener estructuras similares a otros BFRs (Figura 1.3) se cree que podrían presentar simulares propiedades tóxicas y comportamiento, aunque los estudios sobre ello son todavía escasos (Covaci *et al.*, 2011; de Wit *et al.*, 2010).

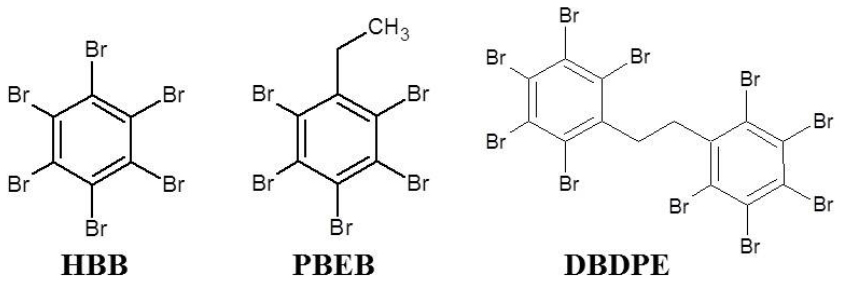

Figura 1.3. Estructuras de los 3 BFRs emergentes incluidos en esta tesis.

El DBDPE se está utilizando actualmente como substituto del BDE-209, aunque el elevado coste del producto limita su uso (Eljarrat *et al.*, 2005). De todos modos, la previsión es que se convierta en uno de los BFRs más utilizados en la industria termoplástica (Konstantinov *et al.*, 2006) por varios motivos. Primero, no produce furanos o dioxinas polibromadas tras exposición a la luz solar, al contrario que el BDE-209 (Kierkegaard *et al.*, 2004). Segundo, su biodisponibilidad parece ser menor que la del BDE-209, basándose en su elevado $LogK_{ow}$ que ha sido estimado en 11 (Chen *et al.*, 2013). De momento ya se usa comercialmente en distintos materiales como el poliestireno de alto impacto, algodón o poliéster (Kierkegaard *et al.*, 2004). No se produce en Europa, pero se importa en grandes cantidades (unas 2500 toneladas en 2001) y en 2006 ya era el segundo BFR aditivo más usado en China (Arias 2001; Xiao 2006). Además, en Japón la substitución de la mezcla Deca-BDE por el DBDPE ya

parece evidente pese a no estar impuesta en Asia (Watanabe 2010). Sus nombres comerciales son SAYTEX 8010 o Milebrome 8010.

El PBEB es un FR de tipo aditivo usado principalmente en textiles, adhesivos y recubrimientos eléctricos. Su pico más alto de producción fue en la década de los 70, especialmente en EE.UU donde se comercializaba bajo el nombre de FR-105 (Hoh *et al.*, 2005). Debido a que no se encuentra legislado se cree que su uso ha aumentado en los últimos años debido a las restricciones legales de otros FRs como los PBDEs, aunque está clasificado como un compuesto químico de bajo volumen de producción en la UE (Covaci *et al.*, 2011). Es un compuesto persistente en el medio ambiente y con capacidad de bioacumulación (Covaci *et al.*, 2011).

La producción del HBB tiene lugar principalmente en Japón y China a través de la Nippoh Chemical Corporation y la Shou Guang Longfa Chemical Corporation respectivamente y bajo el nombre de FR-B, mientras que no hay datos de su producción en la UE (Watanabe and Sakai 2003). El hecho de que se haya encontrado en diferentes muestras ambientales de Europa como sedimentos o peces demuestra que también tiene la capacidad de pasar desde los productos que lo contienen a diferentes compartimentos ambientales (Cruz *et al.*, 2015).

1.2.4. Decloran plus y compuestos relacionados

El DP ($C_{18}H_{12}Cl_{12}$), junto con sus análogos Dec 602 ($C_{14}H_4Cl_{12}O$), 603 ($C_{17}H_8Cl_{12}$) y 604 ($C_{13}H_4Br_4H_{16}$) son un grupo de norbornenos halogenados (HNs) que han sido fabricados durante más de 40 años por Hooper Chemicals and Plastics Corp., productora actualmente conocida como Oxychem ubicada en el río Niágara (Nueva York, EE.UU.) (Figura 1.4). Más recientemente se ha identificado otro punto de producción del DP en China (Anpon Electrochemical Co.), siendo estas fábricas las dos fuentes de origen que se conocen del DP y sus compuestos análogos (Sverko *et al.*, 2011; Xian *et al.*, 2011). Estos compuestos surgieron como alternativa al Mirex cuando su uso como FR fue prohibido en 1976 (EE.UU) al demostrarse su gran toxicidad y capacidad de bioacumulación. Más adelante, en 2001, sería incluido en la llamada “docena sucia” por la Convención de Estocolmo de Contaminantes Orgánicos Persistentes, prohibiéndose así su producción y uso también en Europa.

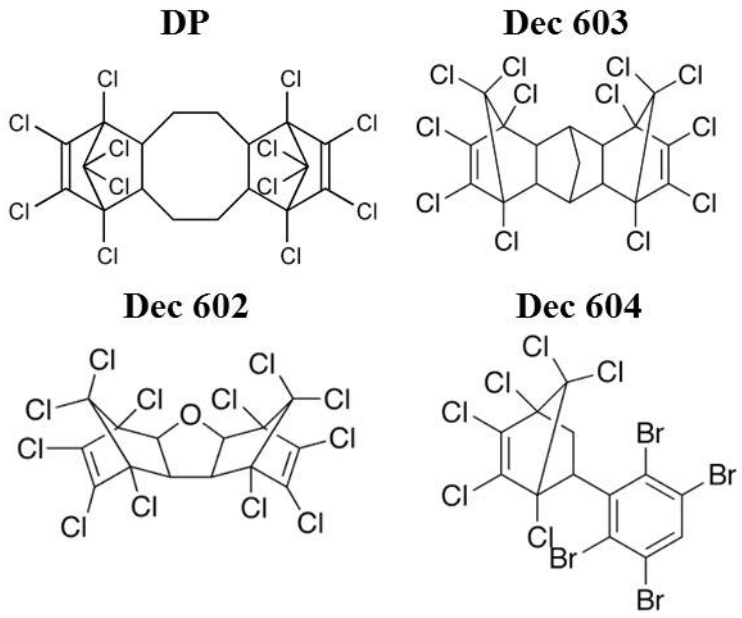

Figura 1.4. Estructuras de los diferentes norbornenos halogenados.

El DP presenta dos esteroisómeros y proviene de una reacción Diels-Alder donde 2 moles de hexaclorociclopentadieno (HCCPD) reaccionan con 1 mol de 1,5 ciclooctadieno. En las mezclas comerciales la proporción entre el *sin*-DP y *anti*-DP es de aproximadamente 1:3 (Xian *et al.*, 2011) y no puede estar presente en materiales plásticos en una cantidad superior al 35% en peso básico (Ren *et al.*, 2008). Existen 3 mezclas comerciales del DP (DP-25, DP-35 y DP-515) pero sólo difieren en el tamaño de partícula ya que la proporción de los dos isómeros no varía significativamente (Gauthier y Letcher 2009). En la Unión Europea está considerado un producto químico de bajo volumen de producción. Sin embargo, la Agencia de Protección Ambiental (EPA) de los EEUU lo cataloga como un compuesto químico de alto volumen de producción ya que se generan entre 500 y 5000 toneladas al año (HPV). Entre 2002 y 2006 su producción anual en EE.UU se mantuvo 2.000-20.000 (Klosterhaus *et al.*, 2012). Su ventaja en comparación con los BFRs es su mayor estabilidad térmica y su menor densidad y coste (Feo *et al.*, 2012). Se encuentra presente especialmente en recubrimientos de cables, plásticos y conexiones en ordenadores y televisiones, presencia que podría incrementarse en un futuro cercano ya que la Comisión Europea lo ha propuesto como una alternativa a la mezcla Deca-BDE (Sverko *et al.*, 2011).

Por otro lado, el Dec 602, Dec 603 y Dec 604 son compuestos generados por el mismo tipo de reacción de Diels-Alder que el DP, pero con un precursor distinto que reacciona con el HCCPD. En el caso del Dec 602 nuevamente 2 moles de HCCPD reaccionan con

1 mol de furano. En la formación del Dec 603 intervienen 2 moles de HCCPD que reaccionan con 1 mol de biciclo[2.2.1]hepta-2,5-dieno. Por último, y a diferencia de los anteriores, 1 mol de HCCPD reacciona con 1 mol de tetrabromoetenilbenceno para generar el Dec 604. Cabe señalar que el Dec 602 es el único que presenta un átomo de oxígeno en su estructura y que el Dec 604 presenta dos halógenos diferentes, cloro y bromo, aunque es el que menor grado de halogenación presenta de los 3. Estos compuestos también fueron patentados por Oxychem en los años 60 y 70 con la intención de mejorar las formulaciones de los polímeros para hacerlos menos inflamables. Básicamente son usados en los casos en los que el DP no puede ser usado en algunos materiales ya que no alcanza a cumplir los requisitos legales (Krackeler y Biddell 1976). Por ejemplo, está documentado el uso del Dec 602 en un 18% en peso en el Nylon-6 reforzado con fibra de vidrio, mientras que el Dec 604 se encuentra entre un 10-30% en peso en la silicona Molykote AS-810, usada en aplicaciones electromecánicas y como impureza en una forma comercial del Mirex (Shen *et al.*, 2011b). Además, el uso conjunto de estos dos compuestos está patentado para aislantes de cables. No obstante, existe mucha menos información sobre el uso del Dec 603, pese a estar patentado también su uso como FR por OxyChem. Sí está descrita su presencia como impureza en productos comerciales del Aldrin y Dieldrin, dos insecticidas usados en grandes cantidades en los años 70 pero que se encuentran actualmente prohibidos (Shen *et al.*, 2011a).

1.3. Propiedades fisicoquímicas

En la tabla 1.1 se muestran algunas de las propiedades fisicoquímicas de los HFRs incluidos en esta tesis. Se trata, como se puede ver, de moléculas muy hidrofóbicas ($\log K_{ow}$ entre 5,07 y 11,2) y de elevado peso molecular (pesos entre 407 y 971 g/mol). El HBCD representa un caso interesante ya que las propiedades estimadas utilizando la mezcla comercial no son para nada extrapolables a los isómeros individuales. Por ejemplo, la solubilidad de la mezcla comercial es de 65 μg/L, mientras que la de los isómeros por separado no llega ni mucho menos a ese valor. Sería interesante disponer de estos datos también para el DP o las mezclas comerciales de PBDEs, pero ningún estudio ha comparado ambos casos.

Tabla 1.1. Propiedades fisicoquímicas de los HFRs incluidos en esta tesis.

Compuesto	CAS	Pm	Vm (cm^3)	Solubilidad (µg/L)	$logK_{ow}$
BDE-28	41318-75-6	407	209	334	5,98
BDE-47	5436-43-1	486	224	94,7	6,02
BDE-99	32534-81-9	565	241	38,9	6,81
BDE-100	189084-64-8	565	241	54,1	6,86
BDE-153	68631-49-2	644	257	16,7	7,39
BDE-154	207122-15-4	644	257	0,87	7,39
BDE-183	207122-16-5	722	273	1,5	7,14
BDE-209	1163-19-5	959	322	0,14	9,97
HBB	87-82-1	546	187	3	6,07
PBEB	85-22-3	496	203	210	6,76
DBDPE	84852-53-9	971	344	0,095	11,2
α-HBCD	3194-55-6	642	299	48,8	5,07
β-HBCD	3194-55-6	642	299	14,7	5,12
γ-HBCD	3194-55-6	642	299	2,1	5,47
Dec 602	31107-44-5	614	300	0,0175	8,38
Dec 603	13560-92-4	638	324	0,0245*	8,24
Dec 604	34571-16-9	693	283	0,0375*	9,04
syn-DP	13560-89-9	654	358	249	9,51
anti-DP	13560-89-9	654	358	249	9,51

Pm: Peso molecular. Vm: Volumen molar. *: ng/L

1.4. Legislación

En este apartado se revisa la legislación existente sobre estos compuestos, que se encuentra además resumida en la Figura 1.5. Los PBDEs ya se encuentran prohibidos en su totalidad, las restricciones sobre el HBCD se han incrementado en los últimos años y, de momento, sobre los HNs y BFRs alternativos no pesa ninguna legislación firme.

PBDEs

Existen numerosas prohibiciones y restricciones sobre los PBDEs, especialmente centradas en las mezclas Penta-BDE y Octa-BDE. En Norteamérica fueron incluidas en el acta de protección medioambiental canadiense de 1999 y su producción fue cesada voluntariamente a principios de siglo; Canadá se anticipó así a las restricciones que posteriormente se aplicaron en 2006 y 2008 que establecieron la prohibición de fabricar o importar cualquier mezcla de PBDEs en Canadá (Miller *et al.*, 2014). De la misma manera, la EPA también estableció fuertes restricciones sobre estas mezclas en Estados Unidos en 2006, obligando a todas las compañías a avisar con 90 días de antelación a

cualquier importación o producción de estas mezclas (Sutton *et al.*, 2014). Además, todos los productores se comprometieron a detener la producción de la mezcla Deca-BDE a finales de 2013 (Ma *et al.*, 2013).

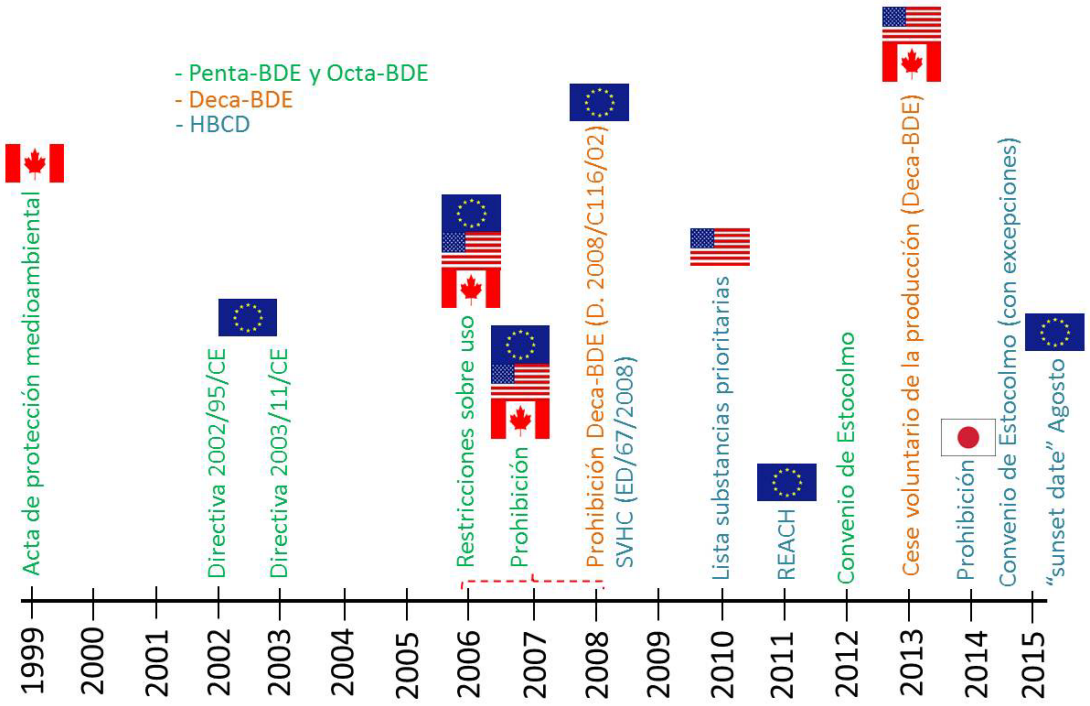

Figura 1.5. Cronología de la legislación existente sobre los HFRs incluidos en esta tesis.

En cuanto a Europa, los PBDEs se incluyeron en la restricción de substancias peligrosas (RoHS, del inglés Restrictions of Hazardous Substances) bajo la directiva 2002/95/CE. Posteriormente, la directiva 2003/11/EC prohibió la venta de las mezclas Penta-BDE y Octa-BDE en concentraciones superiores al 0,1% en masa. Además, desde julio de 2006 los equipamientos electrónicos fabricados en Europa no pueden contener PBDEs bajo ninguna circunstancia, según la directiva 2002/95/EC. Asimismo, la mezcla Deca-BDE fue prohibida en julio de 2008 por el tribunal europeo de justicia (2008/C 116/02), revocando así la excepción que se le había aplicado al Deca-BDE en la directiva 2005/717/EC. Los PBDEs cumplen los criterios establecidos en la Convención de Estocolmo para ser considerados POPs y por lo tanto fueron incluidos finalmente en 2012. Hasta la fecha, los PBDEs sólo están regulados en Norteamérica y Europa (de Wit *et al.*, 2010; Hoydal *et al.*, 2015; Muñoz-Arnanz *et al.*, 2011a), por lo que otras zonas como China o los países emergentes de América del Sur podrían seguir siendo potenciales focos de producción de estos compuestos (Zheng *et al.*, 2015). Además, el hecho de regularlos no implica una reducción total en las emisiones de estos

compuestos ya que los materiales fabricados previamente sí los contienen y por tanto es de esperar que la entrada de PBDEs en el medio ambiente prosiga, aunque cada vez en menor medida.

HBCD

Pese a que las restricciones sobre el HBCD no son comparables a las que pesan sobre los PBDEs, hay que comentar que en los últimos años se han tomado algunas medidas. Fue incluido en la lista de substancias prioritarias por la EPA en agosto de 2010 y en la lista de substancias de alta prioridad (SVHC, del inglés Substances of very High Concern) por la agencia europea de substancias y mezclas químicas en octubre de 2008 (decisión ED/67/2008). Posteriormente, en febrero de 2011, fue incluido en el anexo XIV del registro, evaluación, autorización y restricción de substancias químicas de la UE (REACH, del inglés Registration, Evaluation, Authorisation and Restriction of Chemicals). Por último, fue incluido en el Anexo A del convenio de Estocolmo en Mayo de 2013, quedando regulada su eliminación excepto en el poliestireno usado en edificios. A diferencia del caso de los PBDEs, fue un país asiático (Japón) el primero en prohibir específicamente la producción e importación de HBCD, siendo ésta efectiva en mayo de 2014. En cambio, en la UE su uso está permitido en las excepciones citadas anteriormente hasta la llamada "*sunset date*" (21 de Agosto de 2015) e incluso pasada esta fecha su uso podría ser autorizado por la UE siguiendo criterios excepcionales (Al-Odaini *et al.,* 2015; Andersen *et al.,* 2015; Jörundsdóttir *et al.,* 2013; Koch *et al.,* 2015).

HNs

Por el contrario, pese a que el DP ha sido propuesto como una alternativa a la mezcla Deca-BDE no existen muchas restricciones sobre las diversas aplicaciones de los decloranos. De momento el DP ha sido incluido en la lista de substancias domésticas de Canadá quedando regulado su uso en materiales plásticos, donde su contenido no puede exceder el 35% del peso básico del material (Ren *et al.,* 2008). Así mismo el Dec 602 y el Dec 604 se incluyen en la lista de substancias no domésticas de Canadá y en el sistema de información de substancias químicas europeo. Estas listas sólo establecen recomendaciones de uso y no restricciones propiamente dichas (Sverko *et al.,* 2011).

1.5. Compuestos halogenados naturales

Junto al gran número de contaminantes halogenados antropogénicos presentes en el medio ambiente se encuentran también los compuestos naturales halogenados (HNPs, del inglés halogenated natural products). Estos compuestos se originan casi exclusivamente en el medio marino debido a la gran abundancia de sales de cloro y bromo, así como de los propios átomos de Cl_2 y Br_2. En la actualidad se encuentran identificados más de 5000 HNPs, contando también los que contienen flúor o yodo (Gribble 1998; Gribble 2000; Gribble 2010; Vetter 2006). Microorganismos en simbiosis con esponjas o pequeños bivalvos, cianobacterias, algas o gusanos son considerados los principales productores de HNPs en el medio marino. Debido a que un estudio en profundidad de la presencia de HNPs en el medio ambiente no era el objetivo de esta tesis, se seleccionaron una serie de compuestos cuya presencia se estudió en las diferentes muestras marinas de las que se disponía. La presencia de estos compuestos y las concentraciones encontradas nos permiten poner en contexto la importancia de los niveles de HFRs encontrados en las mismas muestras, evaluando su contribución y posibles efectos en un medio que cuenta ya con una elevada presencia "de base" de compuestos orgánicos halogenados. Estos compuestos son los PBDEs metoxilados (MeO-PBDEs), el (1R,2S,4R,5R,1'E)-2-bromo-1-bromometil-1,4-dicloro-5-(2'-cloroetenil)-5-metilciclohexano (MHC-1), el 2,7-dibromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno (TriBHD) y 2,5,7-dibromo-4a-bromometil-1,1-dimetil-2,3,4,4a,9,9a-hexahidro-1H-xanteno (TetraBHD), y por último el 2,3,3',4,4',5,5'-heptacloro-1'-metil-1,2'-bipirrol (Q1) y otros metil-bipirroles polihalogenados (PMBPs, del inglés polybromomethyl bipyrroles) con la misma estructura que el Q1 pero con un patrón de halogenación diferente que consiste en la substitución de átomos de Cl por átomos de Br: BrCl-MBP (Q1 –Cl +Br), Br_2Cl_5-MBP, etc., hasta el Br_7-MBP (Figura 1. 6).

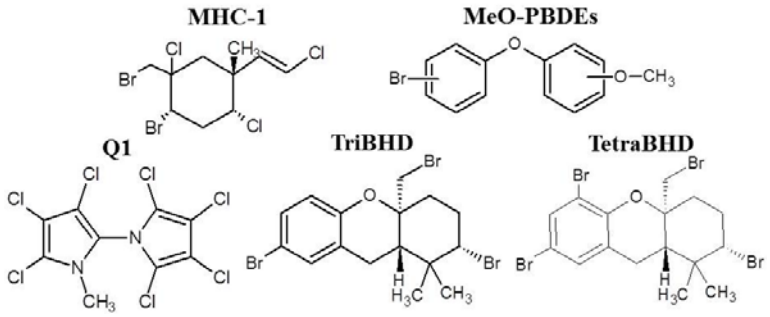

Figura 1.6. Estructuras de los diferentes HNPs incluidos en esta tesis.

La información sobre estos compuestos es bastante limitada comparada con la disponible sobre los contaminantes antropogénicos. Recientemente algunos HNPs como el Q1 han sido detectados en humanos, demostrándose así su capacidad de desplazarse a lo largo de la cadena trófica (Vetter *et al.,* 2000). Además del Q1, otros HNPs como los MeO-PBDEs o MHC-1 se han encontrado en mamíferos en la cima de la cadena trófica como osos polares o delfines (Hoh *et al.,* 2012; Shaul *et al.,* 2015; Vetter *et al.,* 2008). Es interesante ver como se encuentran en otros organismos además de sus productores y queda probada su capacidad de bioacumulación y relativa persistencia. En general se desconoce por qué los organismos marinos producen compuestos halogenados. Algunos compuestos específicos han demostrado tener propiedades beneficiosas para las esponjas, actuando como repelentes de depredadores o favoreciendo la regeneración de partes dañadas. En cambio, se cree que otras familias de HNPs como las incluidas en esta tesis podrían presentar las mismas propiedades que compuestos antropogénicos como los PBDEs o PCBs, presentando similares capacidades de persistencia y bioacumulación y potenciales efectos toxicológicos debido a sus estructuras moleculares, similares a otros compuestos halogenados (Gaul *et al.,* 2011; Gribble 2000; Gribble 2010; Vetter 2006; Vetter *et al.,* 2009). De hecho, el descubrimiento de los HNPs creó gran controversia en su momento ya que invalidaba una de las características de los POPs: que no se podían encontrar compuestos análogos en el medio ambiente. Hoy en día los HNPs están reconocidos como contaminantes del medio marino y productos de consumo humano procedentes del mismo (Vetter 2006).

Los MeO-PBDEs son un caso interesante ya que su estructura es muy similar a la de los PBDEs y durante un tiempo se dudó de su origen natural. No obstante, diversos estudios documentaron evidencia de su origen natural mediante el análisis de isótopos de carbono (Malmvärn *et al.,* 2005).

1.6. Presencia y comportamiento en el medio ambiente

En este apartado se pretende hacer una revisión de los niveles de HFRs y HNPs en la literatura mostrando su presencia en matrices ambientales y bióticas, así como una evaluación de su comportamiento en el medio ambiente. En algunos casos como el de los PBDEs la literatura existente es muy amplia, por lo que dichos niveles han sido revisados por distintos autores (Alaee *et al.,* 2003; Besis y Samara 2012; Betts 2010; Covaci *et al.,* 2003; Covaci *et al.,* 2007; Chen y Hale 2010; Chen *et al.,* 2012b; Hale *et*

al., 2006; Law *et al.*, 2006b; Law *et al.*, 2008; Ma *et al.*, 2012; Mikula y Svobodov 2006; Vonderheide 2009; Wang y Li 2010; Wiseman *et al.*, 2011; Yogui y Sericano 2009). En consecuencia, sólo se han incluido estudios recientes no incluidos en dichas revisiones, correspondientes a los años 2013, 2014 y 2015. Por otro lado se ha realizado una recopilación en más detalle para los HNs y BFRs alternativos dado que el número de estudios publicados es mucho menor y más reciente.

Al comparar niveles documentados por diferentes estudios hay que tener en cuenta que factores muy diversos pueden provocar diferencias substanciales en los resultados. Algunos, como la proximidad a zonas de producción o ambientes urbanos, se discuten más adelante. En líneas generales, por ejemplo en las depuradoras es importante tener en cuenta factores como el nivel de tratamiento de la depuradora, el caudal de entrada, técnicas usadas, población a la que se da servicio o carga industrial de la zona, que pueden afectar drásticamente a los niveles determinados (Zeng *et al.*, 2014a). Por otro lado, la comparación de concentraciones entre muestras de biota siempre debe llevarse a cabo teniendo en cuenta que incluso dos especies de peces o delfines podrán tener metabolismos o dietas muy diferentes que tendrán una influencia directa en los niveles de contaminación que presentarán (Weijs *et al.*, 2015).

1.6.1. Entrada al medio ambiente

Además de la migración de los HFRs desde los materiales que los contienen a diferentes compartimentos ambientales durante la vida útil de los mismos, los HFRs pueden entrar al medio ambiente siguiendo otras vías que, a primera vista, parecen más evitables. A continuación se detallan algunas posibles rutas de entrada (o reentrada) al medio ambiente.

Zonas de producción y zonas de elevada carga industrial

La producción de los PBDEs, y en menor medida del HBCD, se halla bastante extendida, ya que las compañías que los fabrican son varias y distribuidas por diferentes países. En cambio, las fuentes de producción de los decloranos están más localizadas ya que hasta la fecha sólo se conocen dos (figura 1.7). Pese a que, debido al aumento de los estudios sobre estos compuestos, actualmente se dispone de más información sobre su presencia global, al inicio de esta tesis (2011) la información sobre decloranos era escasa y la mayoría de estudios estaban centrados en las zonas de producción. Los

niveles encontrados eran relativamente altos y claramente atribuibles a la cercanía con las fuentes de emisión.

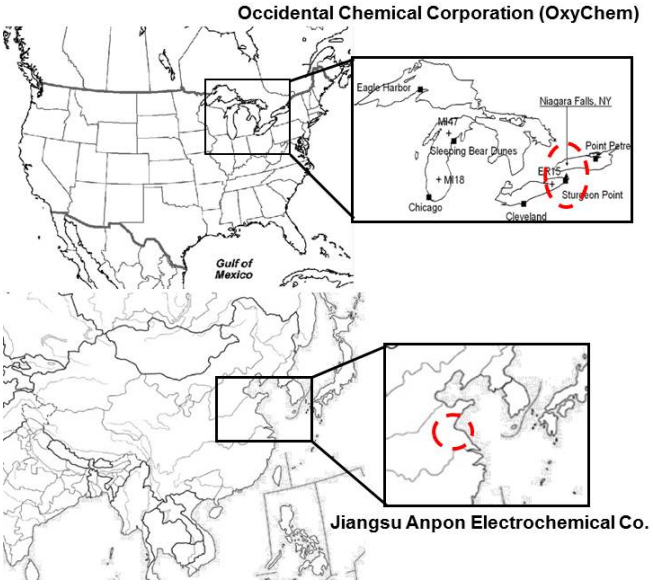

Figura 1.7. Zonas de producción conocidas de los decloranos.

e-Waste

El término e-waste, que es una abreviación de “electronic waste” (en castellano, “residuos electrónicos”), se usa para definir a todo el material electrónico (ordenadores y material asociado, televisiones, etc) que ha sido retirado de su uso. La cantidad de e-waste generada es enorme: para el periodo entre 1997 y 2004 se estimó que globalmente más de 500 millones de ordenadores quedaron obsoletos. Esto implica que se generaron unos 4 millones toneladas de e-waste en ese periodo. No existen datos más recientes, pero dada la expansión del sector informático en la última década es de esperar que esta cantidad se haya mantenido, como poco. Teniendo en cuenta que los HFRs se aplican en % en peso, y sabiendo que legalmente los materiales plásticos pueden contener hasta el 30% en peso de PBDEs o DP (ver apartados 1.2.1 y 1.2.4), entre 1997 y 2004 hasta 1,2 millones de toneladas de estos compuestos pudieron ser introducidos en el medio ambiente si consideramos el peor de los casos. Más concretamente, unos 145 millones de dispositivos electrónicos fueron desechados en la provincia de Guangdon, China. Esto supondría unas 261.000 toneladas de HFRs como PBDEs o DP solamente en esta región y año (Martin *et al.*, 2004).

Todo esto evidencia que, pese a que actualmente hay bastante atención puesta en las emisiones de los HFRs durante la producción y vida útil de los polímeros que los

contienen, se está subestimando en cierta manera el peligro del e-waste y otros deshechos que también contienen elevadas cantidades de HFRs como vía de reentrada de los HFRs al medio ambiente. De hecho, no parece casualidad que las concentraciones más altas en casi todas las matrices se hayan encontrado en China, ya que la mayoría del e-waste generado en Europa se envía a países asiáticos (China e India principalmente) debido al bajo coste de la mano de obra y las regulaciones menos estrictas sobre los tratamientos del residuo (Kumari *et al.*, 2014). Además, gran parte del e-waste que se envía a China se declara de manera fraudulenta como material reciclable y por tanto no pasa ni siquiera los pocos controles que debería (Schwarzer *et al.*, 2005).

En la figura 1.8 se muestra una comparativa entre concentraciones medias encontradas en diferentes especies de aves paseriformes de zonas donde se acumula e-waste en China, frente a otras encontradas en zonas del mismo país donde no se hace mención a ninguna planta de tratamiento de este tipo de residuos, viéndose claramente las diferencias entre zonas.

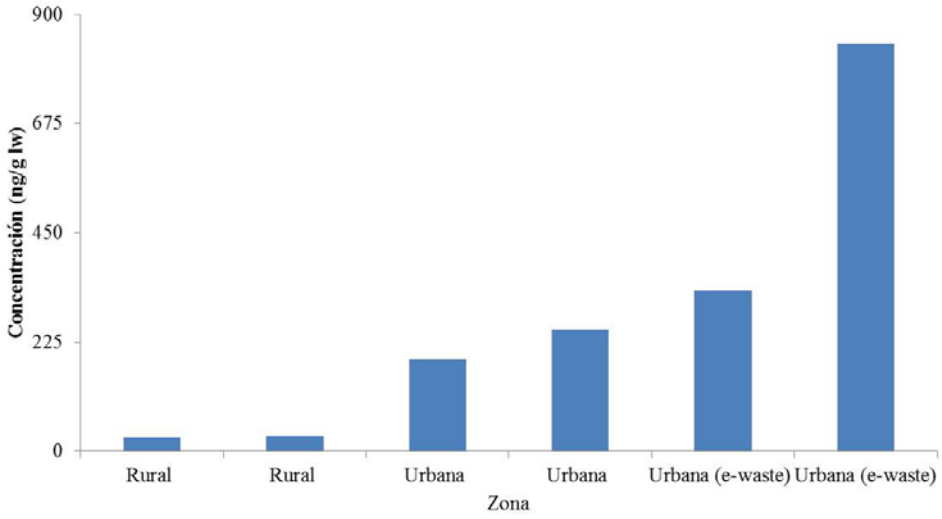

Figura 1.8. Concentraciones de PBDEs encontradas en diferentes zonas de China.

Vertederos

Todo lo explicado anteriormente no quiere decir que no haya que prestar atención a otros productos que contienen HFRs, como por ejemplo todo tipo de mobiliario, vehículos, textiles, etc. Estos materiales suelen acabar en vertederos, representando éstos otra de las principales fuentes de reentrada de los contaminantes en el medio ambiente (Stubbings y Harrad 2014). Se dispone de información fiable sobre el hecho

de que más del 80% de los deshechos que contenían BFRs (principalmente TBBPA, PBDEs y HBCD) acabaron en vertederos del Reino Unido y Norteamérica (Alcock *et al.*, 2003). Sólo en el Reino Unido unas 670.000 toneladas de mobiliario y 310.000 toneladas de textiles son desechadas anualmente, según se estimó mediante datos recolectados en 2010 y 2011 (Weil y Levchik 2008).

Al contrario que con el e-waste, la problemática que representan los vertederos a causa de los materiales y productos que van a parar a ellos sí que se ha reconocido y regulado. La directiva europea 1999/31/EC, que se implementó en 2001, clasificó los vertederos en 3 tipos, según los deshechos que albergaban (peligrosos, no peligrosos e inertes). Desde entonces se ha seguido avanzando en pos de un correcto control de los residuos. Por ejemplo, desde octubre de 2007 la Agencia Ambiental Europea obliga a tratar todos los residuos destinados a vertederos, para prevenir la emisión de contaminantes una vez allí o incluso durante el transporte de los mismos (Muenhor *et al.*, 2010). Actualmente es obligatorio que los vertederos tengan una membrana que actúe de barrera y separe los deshechos del suelo. Pese a que es una medida que a priori evita en gran parte la filtración de los contaminantes al subsuelo, esta protección no es total (Danon-Schaffer *et al.*, 2013a). Los posibles mecanismos de difusión desde los residuos del vertedero son principalmente 4 (Stubbings y Harrad 2014):

Emisión al aire: La volatilización de los HFRs directamente desde los productos es una de las vías más rápidas de reentrada al medio ambiente. Se ha visto que las emisiones de BDE-47 aumentaban considerablemente en función de la temperatura, observando un aumento de hasta 500 veces al cambiar de temperatura ambiente a unos 60 ºC. Además, la volatilización no está limitada a los compuestos más volátiles ya que el BDE-209 o HBCD, poco volátiles, también han demostrado esta capacidad (Wilford *et al.*, 2003).

Los HFRs también pueden llegar al aire a causa de la combustión de los residuos que, de hecho, es una práctica muy común y puede darse además accidentalmente. En el caso del HBCD se ha visto que es destruido a unos 805 ºC (Takigami *et al.*, 2014).

Filtración al subsuelo: Aunque a priori la solubilidad de los HFRs es bastante baja, la presencia de substancias húmicas puede interaccionar con ellos y aumentar su capacidad de lixiviación a causa de ciertas interacciones específicas (Choi *et al.*, 2009; Osako *et al.*, 2004). Una mayor acidez del medio ha demostrado tener también cierta influencia (Danon-Schaffer *et al.*, 2013b). A modo de confirmación, diversos estudios en

diferentes vertederos o simulaciones han confirmado este mecanismo como una posible vía de entrada (Daso *et al.,* 2013; Öman y Junestedt 2008).

Debromination y degradación: La debromination del BDE-209 ha sido más que contrastada por numerosos estudios, tanto en biota como en el medio ambiente (Covaci *et al.,* 2003; Covaci *et al.,* 2007; Ezechiáš *et al.,* 2014). La debromination en biota se discute más adelante. En medio ambiente puede darse a causa de procesos de reducción, de fotolisis o acción microbiana en medio anaeróbico (Danon-Schaffer y Mahecha-Botero 2010).

Abrasión y desgaste: El simple hecho de que los diferentes materiales choquen entre ellos puede ocasionar su erosión y la liberación de los contaminantes al medio ambiente. Del mismo modo, los efectos climatológicos también pueden descomponer el material, si no se aísla el vertedero con cuidado (Hale 2002).

Reutilización de los lodos de depuradora

Los HFRs son detectados en lodos de depuradora de manera frecuente, ya que tienen unas características altamente hidrofóbicas y son resistentes a los tratamientos primario, secundario y terciario aplicados en las depuradoras. A causa de nuevas regulaciones y protocolos establecidos en el tratamiento de aguas, la cantidad de lodos producida anualmente ha aumentado en los últimos años, creando una nueva problemática ambiental en relación a la gestión de dichos lodos. El problema radica en que estos lodos son también ricos en nutrientes y materia orgánica, lo que los hace ideales para ser utilizados como abono. De hecho, en algunos países como España esa es la aplicación predominante. En base a eso, la UE estableció una serie de regulaciones recogidas en la directiva 86/278/EEC con el objetivo de controlar esta actividad. No obstante, esta directiva no contempla la posibilidad de que esta aplicación pueda permitir la reentrada al medio ambiente de compuestos como los HFRs, que no son eliminados en el proceso de tratamiento de agua. No obstante, desde hace varios años se está preparando un borrador para una nueva directiva con el propósito de regular la presencia de algunos contaminantes orgánicos en lodos de depuradora. Una vez de vuelta al medio ambiente todos estos contaminantes pueden volver a ser bioacumulados por diferentes especies de animales y plantas, e incluso humanos. Por si fuera poco, los otros 2 mecanismos de eliminación del lodo no suponen una alternativa mucho mejor, ya que o son incinerados

o llevados a vertederos (De la Torre *et al.,* 2011; Konstantinov *et al.,* 2006; Wang *et al.,* 2007). Los lodos incinerados pueden generar la formación de dioxinas y furanos bromados (Magdziarz y Werle 2014), mientras que la problemática de los vertederos ya ha quedado reflejada en el apartado anterior.

1.6.2. PBDEs

Los PBDEs fueron detectados en el medio ambiente por primera vez en 1970 (DeCarlo 1979) pero no fue hasta que se detectaron en leche materna, y en niveles que aumentaban año a año, cuando la comunidad científica puso realmente su atención en ellos (Norén y Meironyté 2000). A lo largo de los años se han encontrado en una gran variedad de matrices y se ha demostrado su ubicuidad global (Tabla 1.2). En el caso de los PBDEs hay que tener en cuenta que pese a que los congéneres normalmente detectados son 8 (los mayoritarios en las mezclas comerciales), el número de compuestos estudiados varía de un estudio a otro. El compuesto más importante que suele no ser analizado es el BDE-209 debido a su mayor complejidad.

De todos los compuestos incluidos en esta tesis, los PBDEs son los más ubicuos ya que han sido detectados en prácticamente todo el globo, desde el círculo polar ártico hasta la Antártida (Möller *et al.,* 2012; Möller *et al.,* 2011). Incluso han sido hallados recientemente en sedimentos de África, concretamente en Uganda, aunque en concentraciones bastante bajas para tratarse de estos compuestos: 0,06-0,2 ng/g dw (peso seco, del inglés dry weight) (Ssebugere *et al.,* 2014). En Europa se han detectado recientemente en sedimentos de países como la República Checa o España en concentraciones comprendidas entre 0,12 y 812 ng/g dw (Cristale *et al.,* 2013; Hloušková *et al.,* 2014). En un estudio reciente en EE.UU se identificaron en concentraciones de hasta 21 ng/g dw, aunque el BDE-209 no se encontraba entre los compuestos analizados (Nilsen *et al.,* 2014). Del mismo modo, los PBDEs han sido detectados en muestras de aire de regiones muy dispares. Las concentraciones encontradas muestran una clara influencia de la región estudiada y los compuestos incluidos en el análisis. Los valores más elevados se determinaron en muestras tomadas en diferentes países de Europa, con concentraciones de hasta 1 ng/m^3 (Arellano *et al.,* 2014). Sorprendentemente los niveles en Uganda son similares a los determinados en EE.UU, e incluso superiores a los detectados en la costa del Mediterráneo, con máximos de 252, 220 y 19,9 pg/m^3 respectivamente (Arinaitwe *et al.,* 2014; Mulder *et al.,* 2015; Peverly *et al.,* 2015). Por último, los PBDEs se detectan con frecuencia y en

concentraciones considerables en lodos de depuradora. En China, concretamente en Shangai, se detectaron concentraciones de PBDEs entre 31 y 100 ng/g dw (Xiang *et al.*, 2014a); en un estudio sobre 94 estaciones de depuración de aguas residuales (EDARs) de EE.UU los niveles fueron mucho más altos y variados (0,06-5360 ng/g dw) evidenciando las grandes diferencias que puede haber entre depuradoras (Venkatesan y Halden 2014). En España también se han documentado niveles en EDARs repartidas por todo el país, con concentraciones de hasta 1200 ng/g dw (Cristale *et al.*, 2013; Gorga *et al.*, 2013). En todos los estudios donde es analizado, el BDE-209 es el compuesto más abundante en matrices ambientales.

La capacidad de bioacumulación de los PBDEs ha sido sobradamente probada, especialmente en los congéneres de menor grado de bromación, como el BDE-47, que suelen ser los que presentan mayores concentraciones. Se han encontrado en peces de numerosos rincones del globo, incluso en la Antártida, donde los niveles llegaron hasta los 74 ng/g lw (peso lipídico, del inglés lipid weight) (Lana *et al.*, 2014). Los niveles más altos documentados en los últimos años proceden de China, oscilando entre 140-1295 ng/g lw en carpas y pez cabeza de serpiente (Zeng *et al.*, 2014b). En España las concentraciones encontradas no superaron los 20 ng/g lw (Pardo *et al.*, 2014). Del mismo modo, han sido detectados en aves de diferentes regiones del planeta. Los niveles más elevados proceden de nuevo de China, donde se hallaron entre 12 y 3700 ng/g lw en diferentes especies de la familia de las paseriformes (Yu *et al.*, 2014). En Canadá, una de las zonas donde existe más literatura al respecto, recientemente los niveles variaron entre 8 y 486 ng/g lw en 3 especies de aves marinas (Miller *et al.*, 2014). En Europa, un reciente estudio en huevos de búho recogidos entre Bélgica y Francia reportó niveles de hasta 903 ng/g lw (Eulaers *et al.*, 2014). Asimismo, recientemente se han publicado las concentraciones de PBDEs más altas hasta la fecha (1710-28600 ng/g lw), que fueron encontradas en delfines varados en la costa Oeste de EE.UU (Adams *et al.*, 2014).

1.6.3. HBCD

El HBCD se detectó por primera vez en 1989 en peces del mar de Japón (Watanabe y Tatsukawa 1989) aunque en aquella época su uso era prácticamente residual y totalmente eclipsado por otros BFRs como los PBDEs. De hecho, el siguiente estudio enfocado al HBCD sería publicado casi 10 años después (Sellström *et al.*, 1998).

Tabla 1.2. Ejemplos de concentraciones de PBDEs encontradas en diferentes matrices ambientales y biológicas.

Matriz	Zona	n	(+)	Promedio (min-max)	Referencia
Sedimento (ng/g dw)	Australia	7	209	35 (0,3 – 55)	(Drage *et al.*, 2015)
	China	52	209	89 (0,23 – 250)	(Wang *et al.*, 2015b)
	Rep. Checa	16	209	117 (0,12 – 520)	(Hloušková *et al.*, 2014)
	Uganda	11*	99	0,15 (0,06 – 0,20)	(Ssebugere *et al.*, 2014)
	Méjico	39*	100	0,81 (0,4 – 1,7)	(Ruiz-Fernández *et al.*, 2014)
	EE.UU	11*	99	43,9 (nd – 20,9)	(Nilsen *et al.*, 2014)
	España	8	209	424 (88 – 812)	(Cristale *et al.*, 2013)
Aire (pg/m^3)	Mediterráneo	8	47	7,31 (nd – 19,9)	(Mulder *et al.*, 2015)
	Centroeuropa	14	209	532 (nd – 1015)	(Arellano *et al.*, 2014)
	Uganda	9*	47	29,2 (3,27 – 252)	(Arinaitwe *et al.*, 2014)
	EE.UU	8	47-209	51,5 (11 – 220)	(Peverly *et al.*, 2015)
Lodos de depuradora (ng/g dw)	China	18	209	47,6 (31 – 99,5)	(Xiang *et al.*, 2014a)
	EE.UU	40	209	292 (0,06 – 5360)	(Venkatesan y Halden 2014)
	España	8	209	850 (nd – 1220)	(Cristale *et al.*, 2013)
Peces (ng/g lw)	Antártida	7	47	4,88 (0,04 – 73,6)	(Lana *et al.*, 2014)
	Canadá	38	47	2570 (963 – 387)	(Houde *et al.*, 2014)
	China	39	47	324 (140 – 1295)	(Zeng *et al.*, 2014b)
	España	12*	47	3,87 (0,01 – 20,1)	(Pardo *et al.*, 2014)
Huevos de aves (ng/g lw)	Antártida	7*	47	1,25 (0,88 – 2,51)	(Colabuono *et al.*, 2014)
	Canadá	4*	47	111 (8,0 – 486)	(Miller *et al.*, 2014)
	China	16	209	320 (12 – 3700)	(Yu *et al.*, 2014)
	Bélgica	9*	47	(7,46 – 903)	(Eulaers *et al.*, 2014)
Delfines (ng/g lw)	EE.UU	13*	47	5970 (1710 – 28600)	(Adams *et al.*, 2014)
	EE.UU	6	nd	1375 (837 – 2380)	(Ellisor *et al.*, 2013)
	España	8*	47	510	(Méndez-Fernandez *et al.*, 2014)
	Brasil	39*	47	235 (7,9 – 764)	(Leonel *et al.*, 2014)

n: PBDEs analizados. *: BDE-209 no analizado. (+): PBDE más abundante. nd: No disponible

Desde entonces su presencia ha sido ubicua en todo tipo de matrices, tanto ambientales como biológicas (Law *et al.*, 2006c) a la vez que su uso iba en aumento (ver apartado 1.2.2). En la tabla 1.3 se muestran algunos ejemplos de trabajos publicados recientemente.

En sedimentos de China y EE.UU se determinó en niveles muy parecidos (0,07-0,52 y 0,1-1,6 ng/g dw, respectivamente) y con contribuciones del isómero α de un 20-40% (Letcher *et al.*, 2015; Zhang *et al.*, 2015). En muestras de aire recogidas en Canadá se encontraron concentraciones de hasta 4,7 pg/m^3 (Shoeib *et al.*, 2014). Por último, en lodos de depuradora de EE.UU se detectó el HBCD entre los 112 y los 140 ng/g dw (Letcher *et al.*, 2015), unas concentraciones muy superiores a las publicadas en China

(0,10-37,2 ng/g dw)(Xiang *et al.,* 2015). En todos estos casos la contribución del α-HBCD al valor total nunca superó el 50%.

Al igual que los PBDEs, la capacidad de bioacumulación del HBCD ha sido sobradamente demostrada. En individuos de roncador y pez gato marino muestreados en China se observaron concentraciones de hasta 161 ng/g lw (Zhang *et al.,* 2015) mientras que en España estaban entre 4,4 y 18 ng/g lw en las diferentes especies de peces estudiadas (Eljarrat *et al.,* 2014). Del mismo modo, en 3 especies de aves marinas estudiadas en Canadá se determinaron niveles comprendidos entre los 11 y los 213 ng/g lw, valores en el mismo rango que los hallados en búhos de Bélgica (0,38-239 ng/g lw). No obstante, el valor medio en Canadá fue el triple que el obtenido en este segundo estudio (Eulaers *et al.,* 2014; Miller *et al.,* 2014). Por último, su presencia también ha sido estudiada en delfines, siendo identificados en 16 especies residentes en el Pacífico (cerca de Hawái) en concentraciones de hasta 990 ng/g lw (Bachman *et al.,* 2014), mientras que en individuos de marsopa común muestreados en la costa del Reino Unido las concentraciones llegaron hasta los 19.208 ng/g lw (Law *et al.,* 2012). En todas estos estudios el isómero α fue el más abundante, siendo su contribución del 100% en algunos casos. Por lo visto en biota el α-HBCD es el isómero que presenta una mayor capacidad de bioacumulación

Tabla 1.3. Ejemplos de niveles del HBCD en diferentes matrices ambientales y biológicas.

Matriz	Zona	% α-HBCD	HBCD	Referencia
Sedimento (ng/g dw)	China	40 - 65	(0,07 – 0,52)	(Zhang *et al.,* 2015)
	EE.UU	42	0,48 (0,1 – 1,6)	(Letcher *et al.,* 2015)
Aire (pg/m^3)	Canadá	n.d	1,39 (nd – 4,69)	(Shoeib *et al.,* 2014)
Lodo de depuradora (ng/g dw)	EE.UU	13	126 (112 – 140)	(Letcher *et al.,* 2015)
	China	20 - 48	4,7 (0,10 – 37,2)	(Xiang *et al.,* 2015)
Peces (ng/g lw)	China	60 - 100	(10,1 – 161)	(Zhang *et al.,* 2015)
	España	54 - 81	10,6 (4,44 – 18,1)	(Eljarrat *et al.,* 2014)
Huevos de aves (ng/g lw)	Canadá	-	60,5 (11 – 213)	(Miller *et al.,* 2014)
	Bélgica	80 - 100	20,2 (0,38 – 239)	(Eulaers *et al.,* 2014)
Delfines (ng/g lw)	Hawái	-	33,9 (2,42 – 990)	(Bachman *et al.,* 2014)
	Reino Unido	63 – 100	2666 (10 – 19208)	(Law *et al.,* 2012)

nd: No disponible

1.6.4. Decloranos

El DP fue detectado por primera vez en 2006, cuando en un estudio en sedimentos procedentes de la región de los grandes lagos de Canadá se observaron 2 compuestos desconocidos que resultaron ser el *syn*- y *anti*-DP (Hoh *et al.*, 2006). Ya en 2010 se identificaron el Dec 602, Dec 603 y Dec 604 en sedimentos de la misma zona a partir de picos desconocidos de espectro similar al DP (Sverko *et al.*, 2010). Muchos estudios sobre estos compuestos se han llevado a cabo en áreas cercanas a las 2 únicas fuentes de producción conocidas (Norteamérica y China) donde los niveles son mucho más altos que en otras zonas más alejadas.

En la tabla 1.4 se incluyen algunos ejemplos de los niveles de DP en diferentes matrices ambientales. En aire los niveles son muy variados a nivel global. Primero de todo cabe destacar que el DP ha demostrado capacidad de transporte a larga distancia, encontrándose en zonas muy cercanas al Ártico aunque a niveles bajos que no superaban los 5 pg/g m^3 (Möller *et al.*, 2011). También se ha determinado en muestras de aire de los Grandes Lagos en niveles bastante altos (nd – 490 pg/m^3). En China, en una zona cercana a la compañía productora de DP, con concentraciones extremadamente altas que van desde los 7,7 a los 27 ng/m^3 siendo las concentraciones más altas de DP hasta la fecha (Ren *et al.*, 2008). En España las concentraciones varían entre 0,8 y 11 pg/m^3 (De la Torre *et al.*, 2010a).

En sedimentos los niveles existentes hasta la fecha son relativamente bajos comparados con otros HFRs. En los Grandes Lagos las concentraciones son cercanas a los 100 ng/g dw, mientras que en otras zonas de Norteamérica las concentraciones de DP no superaban 1 ng/g dw (Qiu *et al.*, 2007; Shen *et al.*, 2010b; Sverko *et al.*, 2008). Sorprendentemente, en China los niveles documentados son inferiores a los norteamericanos (0,6 – 9,27 ng/g dw) (Hong *et al.*, 2010; Ma *et al.*, 2011; Wang *et al.*, 2010). Ambos isómeros del DP también están presentes en diferentes EDARs de China y España. Los niveles en 31 EDARs distribuidas por todo el territorio español van desde 2,45 hasta 94 ng/g dw (De la Torre *et al.*, 2010b) mientras que en China se determinaron máximos de hasta 298 ng/g dw (Xiang *et al.*, 2014b). Esto demuestra que, al igual que otros HFRs, el DP no es eliminado en los procesos de depuración del agua, por lo menos en su totalidad, quedando retenido en los residuos del proceso.

Tabla 1.4. Ejemplos de niveles de DP en diferentes matrices ambientales.

Matriz	Zona	*syn*-DP	*anti*-DP	f_{anti}	∑DP
Aire (pg/m^3)	Canadá*[1]	nr	nr	0,65 – 0,71	nd - 490
	China*[2]	nr	nr	0,61 – 0,85	7737 – 26734
	China[3]	nr	nr	nr	nd – 3,9
	España[4]	nr	nr	nr	0,80 – 11
	Atlántico (N)[5]	nr	nr	nr	0,05 – 4,20
Suelos (ng/g dw)	China*[6]	nr	nr	0,61 – 0,87	5,1 – 13400
	China[7]	0,19 - 280	0,64 - 910	0,67 – 0,85	0,83 – 1200
Sedimento (ng/g lw)	Canadá*[8]	nr	nr	nr	0,01 – 105
	EE.UU[9]	0,03 – 0,3	0,06 – 0,6	nr	0,1 – 0,9
	China*[10]	nr	nr	nr	0,6 – 9,27
	China[11]	nr	nr	nr	nd – 0,16
	Noruega[a,12]	0,3 – 5,4	0,3 – 15,9	0,2 - 1	0,3 – 21,4
	Mar del Norte[a,12]	1 - 918	1 - 611	0,21 - 1	1 - 1159
	Paquistán[13]	0,06 – 8,47	0,04 – 4,02	0,6 – 0,68	0,10 – 12,5
Lodos de depuradora (ng/g dw)	España[14]	0,9 – 19,2	1,55 – 75,1	0,62 – 0,80	2,45 – 93,8
	China[15]	0,05 – 19,5	0,06 – 74,5	0,61 – 1,00	nd - 298
	China[16]	0,1 – 0,71	0,31 – 2,56	0,60 – 0,94	0,51 – 3,02
	Canadá[17]	n.r	n.r	n.r	119

* Muestras procedentes de zonas cerca de los focos de producción del DP. nr: No reportado. [1]Venier *et al.* 2008. [2]Ren *et al.* 2008. [3]Swerko *et al.* 2008. [4]De la Torre *et al.* 2010. [5]Möller *et al.* 2011. [6]Wang *et al.* 2010. [7]Ma *et al* 2011. [8]Qiu *et al.* 2007. [9]Swerko *et al.* 2008. [10]Wang *et al.* 2010. [11]Hong *et al.* 2010. [12]Na *et al.* 2015. [13]Mahmood *et al.* 20015. [14]De la Torre *et al.* 2010b. [15]Xiang *et al.* 2014. [16]Zeng *et al.* 2014. [17]Davis *et al.* 2010.

Asimismo, el DP también ha demostrado capacidad de bioacumulación, determinándose en muestras de biota pertenecientes a todos los niveles tróficos (Tabla 1.5). En plancton y zooplancton los niveles han ido de 2 y 0,5-4,4 ng/g lw, respectivamente (Tomy *et al.*, 2007). En diferentes especies de bivalvos de EE.UU y Europa la concentración media es de 0,43 ng/g lw y 0,02 ng/g lw, respectivamente (Schlabach 2011). Estos niveles son considerablemente inferiores a los de China, especialmente en especies residentes cerca de una zona con elevada carga industrial donde la concentración media encontrada fue de 190 ng/g lw (Wu *et al.*, 2010b).

Del mismo modo, el DP se determinó en diferentes especies de peces procedentes de zonas muy diversas. En Canadá las concentraciones oscilaban entre 0,04 y 2,6 ng/g lw (Hoh *et al.*, 2006; Tomy *et al.*, 2007), similares a las de Brasil (0,32-6,26) (De La Torre *et al.*, 2012). De nuevo China presenta las concentraciones más altas con valores que van desde los 254 a 1971 ng/g lw (Zhang *et al.*, 2011). En Corea del Sur se compararon los niveles determinados en muestras de 5 especies de peces procedentes de 15 zonas industriales y 7 rurales, siendo los niveles muy diferentes (1,6-126 ng/g lw y 0,44-2,7 ng/g lw, respectivamente) (Kang *et al.*, 2010). El DP se ha determinado en aves de

Norteamérica, China y España. En Norteamérica se observaron niveles bastante altos en huevos de halcones peregrinos recogidos en la región de los Grandes Lagos, con valores entre 7,5 y 209 ng/g lw, mientras que en España los niveles en huevos de la misma especie no superaban los 3 ng/g lw. Se observaron también diferencias entre individuos con una dieta acuática e individuos con una dieta terrestre, lo que ilustra la influencia de la dieta en la acumulación de estos contaminantes (Guerra *et al.*, 2011b). Por otro lado, en huevos de cigüeña recogidos en la zona de Madrid los niveles oscilaron entre los 0,8 y 19,6 ng/g lw (Muñoz-Arnanz *et al.*, 2011b). De nuevo, los niveles en Norteamérica son bastante superiores en este caso a los españoles.

Por último, el DP también ha sido detectado en diferentes especies de delfines, animales en la cima de sus cadenas tróficas. En un estudio donde se analizaba la grasa de dos especies de delfín residentes en el mar de China, el delfín rosado y la marsopa sin aleta, se hallaron concentraciones de DP de 0,45-5,1 ng/g lw y 1,74-63,7 ng/g lw, respectivamente. Además la frecuencia de detección del DP fue prácticamente del 100% (Zhu *et al.*, 2014). En Brasil se halló DP en el hígado de especímenes de franciscana, con concentraciones entre 0,32 y 6,3 ng/g lw y demostrando que el DP también tiene capacidad para acumularse en el hígado de mamíferos marinos. Ya en Norteamérica, se han detectado niveles de DP comprendidos entre 0,2 y 7,1 ng/g lw en grasa de individuos de delfín mular varados a lo largo de la costa de California (Shaul *et al.*, 2015). Por último, los únicos datos sobre la presencia de DP en Europa se documentaron en la costa del Reino Unido, concretamente en muestras de grasa de marsopa común, con concentraciones entre 0,12 y 0,42 ng/g lw (Law *et al.*, 2013).

Fanti

Las mezclas comerciales del DP contienen ambos isómeros, en una proporción de aproximadamente *syn*-DP (1): *anti*-DP (3). El cociente conocido como f_{anti} consiste en la división de la concentración de *anti*-DP por la total de DP. Esta relación oscila entre 0,65 y 0,75 en las mezclas comerciales pero se ha observado que en el medio ambiente no se mantiene en diversas matrices, especialmente en biota. Por ello, este valor (o la f_{syn}, donde se usa la concentración de *syn*-DP) se usa muchas veces como indicativo de la acumulación específica de uno de los dos isómeros, o la mayor degradación del otro, permitiendo entender un poco más el comportamiento del DP en el medio ambiente (Sverko *et al.*, 2011; Xian *et al.*, 2011).

Tabla 1.5. Ejemplos de niveles de DP en diferentes especies (ng/g lw).

Matriz	Zona	*syn*-DP	*anti*-DP	f_{anti}	∑DP
Plancton	Canadá[1]	0,72	1,33	0,65	2,05
Zooplancton	Norteamérica[1]	0,12 – 1,31	<0,002 – 3,11	0,70 – 0,77	0,50 – 4,42
Bivalvos	EE.UU[1]	0,43	0,0015		0,43
	Noruega[2]				0,021
	China[3] (industrial)	64	126	0,66	190
	China[4] (rural)	1,9	2,2	0,54	4,10
Peces	Canadá[1,5]	0,01 – 0,45	<0,002 – 0,76	0,44 – 0,96	0,04 – 2,6
	China[6]	134 - 1062	30 - 1212	0,12 – 0,71	254 – 1971
	Corea[7] (industrial)	0,25 - 13	0,56 – 30	0,67 – 0,70	1,6 – 126
	Corea[7] (rural)	0,4	1,2	0,75	0,44 – 2,7
	Brasil[8]			0,71	0,32 – 6,26
	Alemania[9]			0,40 – 0,97	0,14 - 112
Aves	Norteamérica[10]			0,50 – 0,70	7,5 – 209
	España[10,11]	0,04 – 0,27	0,07 – 0,13	0,66 – 0,80	0,30 – 19,6
	China (Sur)[12]				nd - 220
	China (Norte)[13]	6,4 - 280	37 - 1080		1,3 - 1360
Delfines	China[14]			0,35 – 0,81	0,45 – 63,7
	EE.UU[15]	0,08 – 3,8	0.06 – 3.3		0,2 – 7,1
	Brasil[8]	0,16 – 1,37	0,23 – 4,89	0,55 - 1	0,32 – 6,26
	Reino Unido[16]	0,06 – 0,17	0,06 – 0,36	0,5 – 0,86	0,12 – 0,42

[1]Tommy *et al.* 2007. [2]Schlabach *et al.* 2011. [3]Wu *et al.* 2010. [4]Jia *et al.* 2011. [5]Hoh *et al.* 2006. [6]Zhang *et al.* 2011. [7]Kang *et al.* 2010. [8]De la Torre *et al.* 2012. [9]Sühring *et al.* 2014. [10]Guerra *et al.* 2011b. [11]Muñoz-Aranz *et al.* 2011b. [12]Sun *et al.* 2013. [13]Chen *et al.* 2013. [14]Zhu *et al.* 2014. [15]Shaul *et al.* 2015. [16]Law *et al.* 2013.

La mayoría de estudios se centran en el DP, por lo que la información disponible sobre la presencia y comportamiento del Dec 602, Dec 603 y Dec 604 es más escasa. No obstante, estos compuestos han sido detectados en diferentes matrices ambientales y biológicas, especialmente el Dec 602 (Tabla 1.6).

En aire prácticamente no existen niveles documentados ya que estos compuestos suelen estar por debajo del límite de detección. En China se detectaron concentraciones de Dec 602 entre 4,1 y 5,1 pg/m^3 en una zona cercana a la fábrica de Anpon (particulado), mientras que en otro estudio las concentraciones fueron considerablemente inferiores no superando los 0,5 pg/m^3 (Wang *et al.*, 2010). Por el contrario, en España se identificaron niveles de Dec 602 de unos 0,30 pg/g m^3 (fase gas) en un estudio que comparaba muestras recogidas en entornos rurales y urbanos. Al contrario que en la mayoría de estudios existentes en bibliografía, no se observaron diferencias entre las diferentes zonas (De la Torre *et al.*, 2010a). En el único estudio hasta la fecha donde

algún declorano fue detectado en suelos, a excepción del DP, procede de China, donde se detectaron entre 0,11 y 52,5 ng/g dw de Dec 602 (Wang *et al.,* 2010). La matriz ambiental de la que se dispone más información sobre estos compuestos es, con diferencia, el sedimento. En China varios estudios han documentado concentraciones de Dec 602 entre 0,56 y 3,71 ng/g dw en diferentes puntos del país (Sun *et al.,* 2013; Wang *et al.,* 2010; Wang *et al.,* 2012). En la zona de los Grandes Lagos se han detectado tanto el Dec 602, Dec 603 y Dec 604 en intervalos de concentraciones de 0,006-11, 0,01-0,6 y 0,0008-8 ng/g dw, respectivamente. La gran variabilidad de los niveles viene dada por las diferencias existentes entre los diferentes lagos. Mientras que las concentraciones más altas se detectan en el lago Ontario, lugar muy cercano a la fábrica de OxyChem, los niveles en el resto de lagos son considerablemente más bajos (Shen *et al.,* 2012; Shen *et al.,* 2010a; Shen *et al.,* 2011a; Shen *et al.,* 2011b; Shen *et al.,* 2010b). Ya en Europa, se ha observado la presencia del Dec 602 en sedimentos marinos procedentes del Mar del Norte, en concentraciones entre 8 y 472 pg/g dw (Sühring *et al.,* 2015), así como en sedimentos del círculo polar ártico, donde se encontró este compuesto en concentraciones de hasta 1,4 pg/g dw. En el ártico también se observó la presencia del Dec 603 (1,2-3,4 pg/g dw) y Dec 604 (2,1-20 pg/g dw). A diferencia de en el resto de estudios consultados, el Dec 602 fue el compuesto con una menor contribución de los 3 (Möller *et al.,* 2010). Por último, el Dec 602 también se ha determinado en lodos de depuradora a bajas concentraciones tanto en China como en España (De la Torre *et al.,* 2010b; Qi *et al.,* 2010).

Por otro lado, los 3 compuestos han demostrado capacidad de bioacumulación, estando presentes en diferentes especies tanto acuáticas como terrestres. Anguilas procedentes de los ríos Elba y Rin, en Alemania, presentaron valores entre 0,06 y 48,8 ng/g lw de Dec 602 y entre 0,07 y 0,37 ng/g lw de Dec 603 (Sühring *et al.,* 2013). En Corea del Sur el Dec 602 estaba presente en diferentes especies de peces entre 0,24 y 2,3 ng/g lw mientras que en viruelas muestreadas en China las concentraciones de Dec 602 llegaron hasta los 20 ng/g lw. Además, en este último estudio se documentó también la presencia del Dec 603 en concentraciones de hasta 7,7 ng/g lw (Wang *et al.,* 2012). Por último, en los Grandes Lagos los niveles de Dec 602 oscilaron entre 0,47-34 ng/g lw, los del Dec 603 entre 0,01 y 0,55 ng/g lw, y los del Dec 604 entre 0,002 y 1,3 ng/g lw (Shen *et al.,* 2011a; Sverko *et al.,* 2010). Además, estos compuestos se han detectado también en aves. En huevos de gaviota recogidos en China se hallaron niveles de Dec 602 y Dec 603 de 0,04-3,2 y 0,01-1,1 ng/g lw, respectivamente (Peng *et al.,* 2014) mientras que en

huevos de halcón peregrino recogidos en Canadá y España las concentraciones fueron significativamente diferentes, al igual que en el caso del DP. En Canadá las concentraciones de Dec 602 oscilaban entre los 7,2 y los 211 ng/g lw, mientras que en España los valores estaban comprendidos entre nd y 25 ng/g lw. De igual modo, el Dec 603 se detectó en concentraciones entre los 5,3 y los 220 ng/g lw, mientras que en los individuos de España el rango de valores era de 1,5-7,5 ng/g lw. Por último las concentraciones del Dec 604 fueron de hasta 9,8 ng/g lw y 0,35 ng/g lw en Canadá y España, respectivamente.

Tabla 1.6. Ejemplos de niveles de los otros decloranos en matrices ambientales y biológicas.

Matriz	Zona	Dec 602	Dec 603	Dec 604
Aire (pg/m^3)	China[1]	4,1 – 5,14[a]		
	España[2]	0,30[b]		
Suelo (ng/g dw)	China[1]	0,11 – 52,5		
Lodo de depuradora (ng/g dw)	China[3]	1,0		
	España[4]	0,02		
Sedimento (ng/g dw)	China[1,5,6]	0,56 – 3,71		
	Canadá[7]	0,006 - 11	0,01 – 0,6	0,008 – 8,0
	Ártico*[,8]	nd – 1,4	1,2 – 3,4	2,1 – 20
	Mar del Norte*[,9]	8 - 472		
Peces (ng/g lw)	Alemania[10]	0,06 – 48,8	0,07 – 0,37	
	Corea[11]	0,24 – 2,3		
	China[6]	2,1 - 20	nd – 7,7	
	Canadá[7]	0,88 – 1,9	0,01 – 0,02	0,002 – 0,02
	Canadá[12]	0,47 - 34	0,014 – 0,55	0,06 – 1,3
Aves (ng/g lw)	China[13]	0,04 – 3,21	0,01 – 1,1	
	España[14]	nd - 25	1,5 – 7,5	nq – 0,35
	Canadá[14]	7,2 - 211	5,3 - 220	1,3 – 9,8
Delfines (ng/g lw)	Brasil[15]	0,12 – 0,94	0,25 – 1,99	

* pg/g lw. [a]Particulado. [b] Fase gas. [1]Wang *et al.* 2010. [2]De la Torre *et al.* 2010a. [3]Qi *et al.* 2010. [4]De la Torre *et al.* 2010b. [5]Sun *et al.* 2013. [6]Wang *et al.* 2012. [7]Shen *et al.* 2010a,b, 2011a,b, 2012. [8]Möller *et al.* 2010. [9]Sühring *et al.* 2015. [10]Sühring *et al.* 2013. [11]Kim *et al.* 2014. [12]Swerko *et al.* 2010. [13]Peng *et al.* 2014. [14]Guerra *et al.* 2011. [15]De la Torre *et al.* 2012.

Si el número de estudios que indican niveles del DP en mamíferos marinos ya es escaso, es entendible que los datos de Dec 602, Dec 603 y Dec 604 sean casi inexistentes. Hasta la fecha, solo un estudio en hígado de individuos de franciscanas de Brasil ha

documentado concentraciones de entre 0,12 y 0,94 ng/g lw y 0,25-1,99 ng/g lw de Dec 602 y Dec 603, respectivamente.

1.6.5. BFRs alternativos

Los estudios sobre los niveles de varios BFRs alternativos, incluyendo los estudiados en esta tesis, fueron revisados recientemente (Ezechiáš *et al.*, 2014). Al igual que para PBDEs, decloranos o HBCD, en la Tabla 1.7 se muestran algunos ejemplos de su presencia en diferentes matrices tanto ambientales como biológicas.

HBB

De manera similar al HBCD, algunos estudios ya indicaron su presencia a finales de los 90 (Watanabe *et al.*, 1986) pero no fue hasta mucho después cuando empezó a haber verdaderas evidencias sobre su presencia en el medio ambiente (Watanabe y Sakai 2003). Como la mayoría de los compuestos estudiados en esta tesis, el HBB ha demostrado capacidad de transporte a larga distancia siendo encontrado en el Ártico. Concretamente se encontraron entre 0,04 y 0,66 pg/m^3 en muestras de aire recogidas en 2011 (Möller *et al.*, 2011) así como hasta 2,6 ng/g lw en huevos de gaviota (Lee *et al.*, 2014).

En aire también se ha encontrado en niveles bastante altos en China, llegando a 6,5 pg/m^3 (Qiu *et al.*, 2010) y en EE.UU, con niveles entre 0,3 y 5,5 pg/m^3 (Ma *et al.*, 2013). Estos niveles son muy inferiores a los encontrados en Canadá, que oscilaban entre los 0,02 y 0,09 pg/m^3(Gouteux *et al.*, 2008).

En sedimentos destacan los niveles altos en China, con concentraciones de hasta 30.000 ng/g dw (8.672 ng/g dw de promedio) siendo las más altas de HFRs documentadas hasta la fecha (Wu *et al.*, 2010a). En otras zonas como España las concentraciones son mucho más bajas con valores que no superan los 2,4 ng/g dw (Guerra *et al.*, 2010).

En un estudio que abarcaba 17 depuradoras españolas los niveles de HBB oscilaron entre 1,78 y 5,71 ng/g dw (Gorga *et al.*, 2013). Estos valores son más altos que los determinados en una depuradora de Baltimore (EE.UU) donde las concentraciones iban 0,16 hasta 1,3 ng/g dw. Pese a que el tratamiento de esta última es similar al que utilizan el 80% de las depuradoras estadounidenses, muchos otros factores podrían estar detrás de estas diferencias, como se ha explicado anteriormente (Venkatesan y Halden 2014).

En cuanto a biota, el HBB ha demostrado su capacidad de bioacumulación en distintas especies y hábitats. En China se detectaron concentraciones muy altas (680-2.451 ng/g

lw) en diferentes especies de carpa obtenidas cerca de una zona de reciclado de e-waste (Verreault *et al.*, 2007). En aves, además de los niveles hallados en el Ártico mencionados previamente, también se ha detectado en gaviotas de los Grandes Lagos, aunque en niveles muy inferiores que van desde 0,24 a 0,53 ng/g lw (Gauthier *et al.*, 2007). Incluso se identificó en delfines en niveles relativamente altos (hasta 43 ng/g lw) (Alonso *et al.*, 2012). En general, pese a haber demostrado capacidad de bioacumulación y biomagnificación los estudios sobre su presencia en muestras ambientales, especialmente aire, son más abundantes (Venkatesan y Halden 2014).

PBEB

Los primeros niveles sobre el PBEB se describieron en muestras de aire de diferentes zonas de EE.UU, llegando estos hasta los 520 pg/m^3 (Hoh *et al.*, 2005). Al igual que el HBB, ha demostrado capacidad de transporte a larga distancia al detectarse su presencia en núcleos de hielo cerca del Ártico, aunque las concentraciones encontradas no superaban los 10 pg/L (Hermanson *et al.*, 2010). Asimismo, las concentraciones documentadas en el norte de Canadá no superaban los 0,1 pg/m^3.

En sedimentos procedentes de la cuenca del Ebro, en España, se determinaron máximos de 9,6 ng/g dw (Guerra *et al.*, 2010), mientras que en China se ha documentado una concentración media de 132 ng/g dw cerca de una planta de tratamiento de e-waste (Wu *et al.*, 2010a). De nuevo, los niveles en el país asiático son mucho más elevados que en el resto del mundo. En cuanto a lodos de depuradora, en diferentes EDARs del territorio español se detectaron concentraciones entre 2 y 2,3 ng/g dw (Gorga *et al.*, 2013). Estos niveles son superiores a los determinados en un estudio que comprendía 20 EDARs de Canadá, donde los niveles de PBEB iban desde 64 a 82 pg/g dw (Kim *et al.*, 2014).

EL PBEB también ha demostrado capacidad de bioacumulación, con niveles relativamente altos (3,98 – 25,6 ng/g lw) en peces de China (Wu *et al.*, 2010a) y a niveles más bajos (hasta 10,4 pg/g dw) en peces del Ártico (Wolschke *et al.*, 2015). También se detectó en huevos de gaviota recogidos en el Ártico, en concentraciones oscilando entre 0,03 y 0,23 ng/g lw (Lee *et al.*, 2014), así como en gaviotas de los Grandes Lagos donde las concentraciones eran algo más altas, llegando hasta los 1,4 ng/g lw (Gauthier *et al.*, 2007).

El caso del PBEB es un poco especial, ya que pese a estar considerado una alternativa a los PBDES no parece ser una opción a tener en cuenta por las compañías. Por ello,

existen dudas sobre si su detección en diferentes muestras ambientales responde a una gran persistencia o a su uso continuado en los últimos años (Wolschke *et al.*, 2015).

Tabla 1.7. Niveles de BFRs alternativos determinados recientemente en diferentes matrices ambientales y biológicas.

Matriz	Zona	HBB	PBEB	DBDPE
Aire (pg/m^3)	China	0,3 – 6,5[1]		23 - 3578[1]
	EE.UU	0,3 – 5,5[2]	0,1 - 520[3]	1,2 – 5,2[2]
	Canadá	0,02 – 0,09[4]	nd – 0,01[5]	
	Ártico	0,04 – 0,66[6]		
Sedimento (ng/g dw)	China	8672[7]	132[7]	38,8 - 1796[7]
	España	nd – 2,4[8]	nd – 9,6[8]	4,8 – 435[8,9]
	Suecia			24[10]
Lodo de depuradora (ng/g dw)	Suecia			32 – 160[10]
	Alemania			70 – 220[11]
	China			39 - 1995[12]
	EE.UU	0,16 – 1,3[13]		1,4 – 160[11]
	Canadá	0,19 – 0,28[14]	0,06 – 0,08[14]	0,019 – 0,032[11]
	España	1,78 – 5,71[15]	2,01 – 2,33[15]	38,2 – 257[15]
Peces (ng/g lw)	China	680 - 2451[16]	3,98 – 25,6[7]	nd – 338[7]
	Canadá			nd – 2,71[17]
Aves (ng/g lw)	China			9,6 - 800[18]
	EE.UU	0,24 – 0,53[19]	nd – 1,4[19]	
	Ártico	0,42 – 2,64[20]	0,03 – 0,23[20]	
Delfines (ng/g lw)	Brasil	<0,56 – 43,3[21]		<3,6 - 352[21]

[1]Qiu *et al.* 2010. [2]Ma *et al.* 2013. [3]Hoh *et al.* 2005. [4]Gouteux *et al.* 2008. [5]Hermanson *et al.* 2010. [6]Möller *et al.* 2011. [7]Wu *et al.* 2010a. [8]Guerra *et al.* 2010. [9]Cristale *et al.* 2013. [10]Kierkegaard *et al.* 2004. [11]Ricklund *et al.* 2008. [12]Shi *et al.* 2009. [13]Venkatesan 2014. [14]Kim *et al.* 2014. [15]Gorga *et al.* 2013. [16]Verreault *et al.* 2007. [17]Law *et al.* 2006a. [18]Luo *et al.* 2009. [19]Gauthier *et al.* 2007. [20]Lee *et al.* 2014. [21]Alonso *et al.* 2012.

DBDPE

Pese a estar documentado su uso desde principios de los 90 los primeros niveles en muestras ambientales son relativamente recientes (Kierkegaard *et al.*, 2004). Es, con bastante diferencia, el BFR emergente más estudiado y detectado de los 3 incluidos en esta tesis. Este hecho es de esperar ya que es una alternativa prácticamente específica de la mezcla Deca-BDE y el que tiene mayor volumen de fabricación, según los datos de producción disponibles.

Está presente en muestras de aire tanto de China como de EE.UU. En el país asiático se detectaron niveles muy variados que van desde los 23 a los 3578 pg/m^3 (Qiu *et al.*, 2010; Shi *et al.*, 2009), mientras que en EE.UU el rango de concentraciones es menor (1,2 – 5,2 pg/m^3) (Ma *et al.*, 2013). En sedimento los niveles más elevados son de nuevo

los encontrados en China, con concentraciones que van desde los 40 hasta los 1.796 ng/g dw (Shi *et al.,* 2009; Wu *et al.,* 2010a). En España se han llevado a cabo 2 estudios con concentraciones muy diferentes: por un lado, Guerra *et al.* con niveles entre 4,8 y 24 ng/g dw en sedimentos recogidos en la cuenca del Llobregat (Guerra *et al.,* 2010), mientras que Cristale *et al.* determinaron unas concentraciones considerablemente más elevadas (80 – 435 ng/g dw) en sedimentos procedentes de 3 diferentes ríos (Nalón, Arga y Besós) (Cristale *et al.,* 2013). En Suecia la concentración encontrada en una única muestra, 24 ng/g dw, entra en el rango alto de las concentraciones detectadas en el Llobregat (Kierkegaard *et al.,* 2004). Del mismo modo, existen numerosos estudios que documentan concentraciones de DBDPE en lodos de depuradora. En Europa los niveles más altos son los de España (38-257 ng/g dw)(Gorga *et al.,* 2013), Alemania (70-220 ng/g dw) (Ricklund *et al.,* 2008) y Suecia (32-160 ng/g dw)(Kierkegaard *et al.,* 2004). En cambio, en otros países como el Reino Unido los niveles existentes son inferiores (36 – 63 ng/g dw)(Ricklund *et al.,* 2008). De igual modo, las concentraciones reportadas en EE.UU son similares a las europeas con valores comprendidos entre 1,4 y 160 ng/g dw (Ricklund *et al.,* 2008). Por el contrario, los niveles hallados en China son de nuevo muy superiores a los del resto del globo. En un estudio en lodos de una depuradora cercana a una planta de reciclado de e-waste las concentraciones oscilaron entre 266 y 1.995 ng/g dw (Shi *et al.,* 2009) aunque en otras depuradoras más alejadas los niveles no superaban los 140 ng/g dw (Ricklund *et al.,* 2008).

La presencia del DBDPE en biota es prácticamente nula. Esto podría ser debido a su baja capacidad de bioacumulación; no obstante se creía lo mismo del BDE-209 y en los últimos años su presencia en biota ha sido más que demostrada. Se ha sugerido que, dadas las características del DBDPE, la no detección podría ser debida a problemas de sensibilidad de los métodos analíticos utilizados (Covaci *et al.,* 2011; Ricklund *et al.,* 2008). En peces procedentes de los Grandes Lagos los niveles han alcanzado valores de hasta 2,7 ng/g lw (Law *et al.,* 2006a) mientras que en China de nuevo las concentraciones son mucho más altas, llegando hasta unos 340 ng/g lw (Wu *et al.,* 2010a). En aves los únicos niveles existentes proceden de China. Es evidente que la diferencia viene influenciada en una gran parte por la zona de muestreo: cerca de una planta de tratamiento de e-waste se llegó a niveles máximos de 880 ng/g lw (Luo *et al.,* 2009) mientras que en otra zona las concentraciones no superaban los 2 ng/g lw (Gao *et al.,* 2009). No obstante, muchos otros factores pueden influir, como se verá más adelante. Por último, el DBDPE fue detectado también en delfines de la costa de Brasil,

llegando a concentraciones de hasta 352 ng/g lw (Alonso *et al.*, 2012). Estos datos evidencian que, al igual que ocurrió en su momento con el BDE-209, el DBDPE puede acumularse en diferentes especies pese a su elevado peso molecular y $\log K_{ow}$.

1.6.6. Compuestos halogenados naturales

Muchos de los HNPs se empezaron a descubrir como picos desconocidos en análisis de rutina de compuestos antropogénicos y posteriormente se ha ido probando su origen natural. De hecho, una queja habitual de los investigadores que estudian estos productos es que necesitan aportar evidencias muy concretas y detalladas para demostrar que se originaron naturalmente (Gribble 2010). Las variaciones en los niveles entre diferentes especies y zonas pueden ser incluso mayores que en el caso de los HFRs. Por ejemplo, existen zonas que albergan un gran número de especies productoras de estos compuestos y por tanto la exposición de los animales que se alimentan en la zona es más grande. Es el caso de la Gran Barrera de Coral de la costa australiana, donde se han encontrado los niveles más elevados.

A continuación se da una breve pincelada de la presencia de estos compuestos en el medio ambiente, que será ampliada en el capítulo 4.

MeO-PBDEs

Los compuestos análogos a los PBDEs, pero metoxilados, fueron detectados por primera vez en peces y focas del mar Báltico en 1997 (Haglund *et al.*, 1997). Más adelante se aportarían evidencias de su origen natural y no como metabolitos de los PBDEs (Gribble 1998; Haglund *et al.*, 1997). La investigación sobre las fuentes de origen de esta familia de compuestos tiene un largo recorrido y son varios los autores que proponen que los MeO-PBDEs pueden originarse a través de los PBDEs, aunque se considera una aportación menor a los MeO-PBDEs de origen natural (Fan *et al.*, 2014). Parece haber indicios de que los MeO-PBDEs con el grupo MeO- en la posición *meta o para* son producidos a partir de algunos PBDEs como el BDE-28 (Yu *et al.*, 2013) mientras que los MeO-PBDEs con el MeO- en la posición *orto* son productos naturales (Wan *et al.*, 2010). De hecho, esta teoría ha sido confirmada para el 6-MeO-BDE-47 y el 2´-MeO-BDE-68 (Teuten *et al.*, 2005). Por todo ello, los niveles de MeO-PBDEs en el medio ambiente son muy variados y pueden verse afectados por algunos factores difíciles de tener en cuenta.

PMBPs, PBHDs y MHC-1

La primera evidencia que se aportó sobre el origen natural del Q1 fue en 1999 cuando se identificó en muestras de focas de las costas africanas y antárticas (Vetter *et al.,* 1999). Más adelante, entre 2006 y 2007, se descubrirían el resto de PMBPs al analizar muestras de diferentes especies de delfines y detectar compuestos de estructura análoga al Q1 pero con diferente patrón de halogenación (Teuten *et al.,* 2006; Vetter *et al.,* 2007). El Q1 se ha identificado en zonas tan distintas como Chile, Nueva Zelanda o Australia, mostrando su distribución global y especialmente por el océano pacífico (Gaul *et al.,* 2011). El TriBHD y TetraBHD fueron identificados por primera vez en 2007 en peces del mediterráneo y en mejillones de Nueva Zelanda (Melcher *et al.,* 2007) y desde entonces se ha detectado en otras especies y zonas del mundo, como por ejemplo en cetáceos de EE.UU (Hoh *et al.,* 2012) o Australia (Losada *et al.,* 2009). En 2001 se identificó otro compuesto natural cuya estructura era una incógnita hasta entonces, el MHC-1 (Vetter *et al.,* 2001), que más adelante ha sido detectado en otras especies como delfines (Losada *et al.,* 2009) o mejillones (Hauler *et al.,* 2014).

1.6.7. Comportamiento en biota

En el apartado anterior hemos visto las principales rutas de entrada al medio ambiente. En este apartado se describen algunos de los principales procesos que componen el comportamiento de los HFRs en biota.

Bioacumulación

La bioacumulación se entiende como el proceso mediante el cual los contaminantes son incorporados por el organismo a través de la dieta, aunque en el caso de los peces u organismos filtradores también puede ser a través de las agallas (Macdonald y Bewers 1996). Ya se ha visto como todos los HFRs incluidos en esta tesis han sido identificados en diferentes especies de biota, tanto en organismos del nivel trófico primario (plancton) como en animales en las cimas de sus cadenas alimentarias (delfines). Algunos estudios han intentado identificar los mecanismos de incorporación por parte de las diferentes especies, y en ocasiones los datos obtenidos desmontan algunas teorías previas. Por ejemplo, el BDE-99 y BDE-153 demostraron capacidad de bioacumulación en mejillones pese a que su tamaño (9,6 Å) es superior al límite que permite atravesar las membranas celulares (9,4 Å). Además, tanto éstos como el BDE-47 mostraron coeficientes de incorporación hasta 10 veces superiores a las de algunos PCBs

(Gustafsson *et al.*, 1999). La capacidad de bioacumularse en organismos filtradores no es trivial ya que estos organismos filtran grandes volúmenes de agua (hasta 5-6 L/h) y por tanto su exposición a estos contaminantes es continua.

Otros organismos, como los peces, pueden incorporar los HFRs por 2 vías: a través de la dieta, y a través de las branquias, donde se concentra un gran número de glóbulos rojos que distribuirían rápidamente los contaminantes por el organismo (Borgå *et al.*, 2004). Varios estudios han demostrado la capacidad de los HFRs para acumularse en peces utilizando el factor de acumulación biota-sedimento (BSAF, del inglés biota to sediment accumulation factor) que consiste en un cociente entre la concentración en el pez (ng/g lw) y la concentración en sedimento (normalizada por el contenido de materia orgánica o TOC). Además, se ha probado que la dieta representa el principal mecanismo de bioacumulación para los diferentes congéneres de PBDEs, así como el HBCD. Se han encontrado BSAF > 1 para los PBDEs mayoritarios, incluido el BDE-209, así como para el HBCD (Arnot y Gobas 2006; Eljarrat *et al.*, 2004b). En cambio, la información disponible sobre los HNs al inicio de esta tesis era muy escasa.

En organismos más complejos como aves y mamíferos, la dieta juega aún un papel más importante ya que, salvo algunas excepciones, es el único mecanismo mediante el cual los individuos incorporan los HFRs. Se han observado claras diferencias entre aves de dieta acuática y dieta terrestre. Por ejemplo, al estudiar huevos de halcones peregrinos de España y Canadá se vio que los individuos con dieta terrestre habían acumulado menos Dec 603 que los individuos que seguían una dieta acuática (Guerra *et al.*, 2011b). En el caso de los PBDEs, aves con dieta terrestre como por ejemplo la cigüeña común acostumbran a acumular más BDE-209, teóricamente menos biodisponible que otros BDEs, mientras que en aves con dietas acuáticas como las gaviotas el compuesto mayoritario suele ser el BDE-47 (Chen *et al.*, 2012a; Muñoz-Arnanz *et al.*, 2011a). Se consideró que las diferencias entre las contribuciones de los diferentes PBDEs entre especies con diferente dieta (cernícalos, gaviotas y estorninos) se debían principalmente a esta misma (Chen *et al.*, 2012a) aunque el diferente metabolismo de las especies podría tener algo que ver también.

Biomagnificación

El estudio de la capacidad de bioacumulación de los contaminantes puede llevar a conclusiones equivocadas si se lleva a cabo en especies en posiciones altas de la cadena trófica, debido a que la biomagnificación también juega un papel relevante. La

biomagnificación se refiere al proceso mediante el cual la concentración de un determinado contaminante o familia de contaminantes en biota aumenta a medida que subimos en la cadena trófica (Macdonald y Bewers 1996). Si la concentración aumenta a medida que lo hace el nivel o la posición trófica, se considera que el contaminante en cuestión presenta capacidad de biomagnificación. Si, por el contrario, la concentración no aumenta, se considera que no se observa biomagnificación (Figura 1.9). La posición y nivel trófico se establecen mediante el análisis de isótopos estables de nitrógeno. Los organismos en niveles tróficos altos presentan un enriquecimiento del isótopo ^{15}N debido a una excreción preferencial del isótopo más ligero, el ^{14}N; por lo tanto cuanto más alta es la relación $^{15}N/^{14}N$, también expresada como $\delta^{15}N$, más arriba en la cadena trófica se encuentra el individuo.

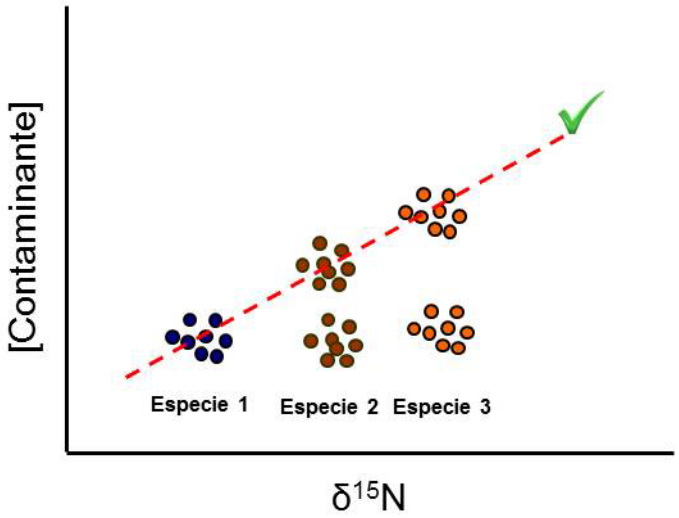

Figura 1.9. Caracterización de la biomagnificación de los contaminantes.

Una vez se ha visto que el contaminante puede biomagnificarse, es posible calcular el factor de biomagnificación trófico (TMF, del inglés biomagnification factor), que básicamente será la pendiente de la recta obtenida. Es decir, en una ecuación de recta típica de y=ax+b, el valor del término “a” es considerado el TMF de este compuesto. Este valor indicará el aumento de la concentración al subir en la cadena trófica (biomagnificación, si es positivo) o de biodilución, si se observa una disminución al subir en la cadena trófica (valor negativo). Tanto PBDEs como HBCD han demostrado capacidad de biomagnificación (Losada *et al.*, 2009; Poma *et al.*, 2014).

En ocasiones también se calcula el coeficiente de biomagnificación (BMF, del inglés biomagnification factor). Este coeficiente se calcula dividiendo la concentración

encontrada en el predador por la encontrada en la presa, ambas en peso lipídico. BMFs > 1 indican capacidad de biomagnificación, mientras que BMFs < 1 suelen interpretarse como biodilución (Borgå *et al.,* 2004). El BMF_{TL} es el mismo coeficiente pero incorporando el nivel trófico (TL, del inglés trophic level) a la fórmula (Weijs *et al.*, 2009). Tanto PBDEs como el HBCD han mostrado BMFs > 1 en diferentes cadenas tróficas acuáticas y terrestres (Jia *et al.,* 2011; Losada *et al.,* 2009; Voorspoels *et al.*, 2007; Wu *et al.,* 2010b).

Debromination y decloración

Tanto en peces, aves y mamíferos se ha demostrado que el BDE-209 puede sufrir procesos de debromination, dando lugar a congéneres de grado de bromación más bajo (Hakk y Letcher 2003). Además, se ha demostrado que el BDE-154 y BDE-153 también pueden dar lugar a PBDEs de menor grado de bromación, concretamente BDE-99 y BDE-47 en el caso del BDE-154, y BDE-100 y BDE-49 en el caso del BDE-153 (Ikonomou *et al.*, 2002). Esto tiene varias implicaciones. Por un lado, es una fuente de compuestos como el BDE-47 que debe ser tenida en cuenta a la hora de estudiar su comportamiento en el medio ambiente. Por otro, puede causar que la "entrada" de compuestos ya prohibidos como el BDE-47 se continúe produciendo, aunque en menor medida.

Aunque de momento sólo existen estudios en aves, se conoce que el DP puede dar lugar a dos compuestos fruto de pérdida de un átomo de cloro: $aCl_{11}DP$ y $aCl_{10}DP$. Pese a haber sido hallados en halcones peregrinos y cigüeñas, sus concentraciones fueron muy inferiores a las del DP. Por tanto, pese a que su análisis completa la caracterización de la contaminación por DP de momento no parece haber evidencias de que sean compuestos excesivamente relevantes (Guerra *et al.,* 2011b; Muñoz-Arnanz *et al.,* 2011b).

También se han identificado productos de debromination del HBCD producidos por la acción de microbacterias en medios acuáticos y en depuradoras (Davis *et al.,* 2006) aunque de momento se desconoce si ésta puede producirse en biota.

1.7. Toxicidad

En este apartado se pretende dar una visión global de los efectos tóxicos que pueden presentar los HFRs. Posteriormente, en el capítulo 5, se discutirán los casos concretos de PBDEs y HNs focalizando especialmente en el BDE-209 y DP.

A medida que iba resultando cada vez más evidente que los PBDEs presentaban una alta capacidad de bioacumulación y diseminación a lo largo del globo los estudios sobre sus posibles efectos nocivos aumentaron. De todos modos, aún queda mucho por explicar en cuanto al comportamiento de estos compuestos en comparación a otros más estudiados como pueden ser los PCBs (Buratovic *et al.*, 2014; Costa *et al.*, 2014; Dou *et al.*, 2014). Una de los efectos más peligrosos de los PCBs es la capacidad de afectar al funcionamiento del sistema endocrino, que influye en procesos tan importantes como el desarrollo las funciones cerebrales y sexuales, metabolismo y crecimiento, etc. Debido a las similitudes de las estructuras de algunos contaminantes, éstos actúan igual que las hormonas tiroideas modificando el funcionamiento normal del sistema endocrino. Se ha demostrado que los PBDEs tienen esta capacidad; son por tanto disruptores endocrinos que pueden provocar trastornos en el crecimiento y desarrollo del individuo así como daños en el sistema neurológico. Los mecanismos no están del todo claros pero podrían incluir actividad estrogénica y androgénica, variaciones en las uniones a ciertos receptores como el receptor constitutivo de androstano (CAR) y el receptor X de pregnano (PXR), y también disrupciones en las hormonas tiroideas. También se ha demostrado que pueden provocar daños en el hígado de ratones (Erratico *et al.*, 2011). Incluso existen estudios que sugieren que los PBDEs pueden formar aductos con la molécula de ADN (ácido desoxirribonucleico) aunque las implicaciones de este hecho requieren más estudios (Lyons *et al.*, 2004). Asimismo, los PBDEs pueden causar distintos tipos de daño oxidativo como daños en la cadena del ADN, disfunción mitocondrial o inducción a la apoptosis (Costa *et al.*, 2014).

Pese a que el HBCD no presenta una estructura similar a las hormonas tiroideas, y por tanto no debería interferir con sus mecanismos, se ha visto que en organismos expuestos a él se producían alteraciones en el sistema tiroideo (Germer 2006 y Palace 2008). En ratones ha sido capaz de afectar a procesos cerebrales (Saegusa 2009). Además de todo esto, las distintas propiedades fisicoquímicas de los 3 isómeros más abundantes les confieren propiedades ligeramente diferentes. Muchos estudios evalúan el efecto del HBCD como una mezcla de isómeros, mientras que otros utilizan sólo el α-HBCD. Es recomendable realizar estos estudios utilizando isómeros individuales con el objetivo de caracterizar bien sus efectos (Koch *et al.*, 2015). La información disponible aún es insuficiente para caracterizar completamente el peligro que representa el HBCD para el medio ambiente (Marvin *et al.*, 2011).

La toxicidad de los BFRs emergentes no ha sido estudiada en profundidad, aunque algunos ensayos han revelado que también suponen un riesgo para el medio ambiente. Por ejemplo, el HBB causó daños por estrés oxidativo en hígado de carpas doradas así como inhibición de los canales de Na^+ y K^+ (Feng *et al.,* 2014). Así mismo, también se han observado inhibiciones en procesos enzimáticos claves del hígado en ratones, aunque solo en las fases iniciales de la exposición in vivo (Frydrych *et al.,* 2005). Del mismo modo el DBDPE ha mostrado ser capaz de causar daños en hígado de peces (Feng *et al.,* 2013) y en ratones. En estos últimos además provocó alteraciones en diferentes procesos enzimáticos, causando por ejemplo una disminución en los niveles de glucosa (Sun *et al.,* 2014).

Del mismo modo, los estudios sobre los HNs son más escasos ya que las evidencias de su capacidad de bioacumulación en diferentes organismos son más recientes y todavía no tan abundantes como las existentes sobre los PBDEs. No obstante, cada vez más estudios demuestran que tienen una capacidad de bioacumulación nada despreciable, por lo que el interés en sus posibles efectos tóxicos ha crecido exponencialmente y ya se ha demostrado que tienen la capacidad de causar daños hepáticos en ratones y peces (Liang *et al.,* 2014; Wu *et al.,* 2013). De hecho los estudios sobre el DP y compuestos análogos en los últimos años han aumentado exponencialmente, aunque suelen estar centrados exclusivamente en el DP. El DP ha demostrado, al igual que los PBDEs, ser capaz de inducir la apoptosis e interferir en algunos procesos metabólicos. Se observó que el DP interfirió en algunas rutas de expresión de proteínas y en los canales de Ca^{2+} en peces expuestos a concentraciones elevadas (1, 10 y 100 μg/g ww) de DP. En este estudio se demostró que el DP puede comportarse igual que los PBDEs o el HBCD en cuanto a efectos toxicológicos se refiere (Liang *et al.,* 2014). El hecho de que los HFRs interfieran en los canales de Ca^{2+} debe ser tenido en cuenta ya es uno de los reguladores del transporte a través de membranas celulares y por tanto podría ser uno de los mecanismos de entrada de estos compuestos directamente a las células (Kodavanti y Ward 2005).

Por último, se cree que los MeO-PBDEs pueden presentar similares efectos que los PBDEs debido al gran parecido de sus estructuras. Además, pueden dar lugar a OH-PBDEs, compuestos de mayor toxicidad que los MeO-PBDEs e incluso los PBDEs (Wang *et al.,* 2015a). Los posibles efectos toxicológicos del resto de HNPs aún no ha sido estudiado.

1.8. Análisis

El desarrollo de metodologías para el análisis de FRs en el medio ambiente no es fácil debido a la complejidad de las diversas matrices, bajas concentraciones de los analitos, contaminación cruzada, etc. Algunos compuestos como el BDE-209 o el HBCD supusieron un reto durante muchos años debido a sus complicadas propiedades fisicoquímicas, aunque en los últimos años las metodologías de análisis han avanzado mucho permitiendo un análisis fiable y reproducible.

1.8.1. Preparación de muestra

En general, los HFR tienen propiedades fisicoquímicas similares. Son muy hidrofóbicos, estables, y de pesos moleculares en general elevados. Por ello, las mismas metodologías utilizadas en el análisis de otros compuestos como los PCBs, PBBs y otros compuestos orgánicos son aplicables para la extracción de estos compuestos. En la tabla 1.8 se muestran las técnicas de extracción y purificación más utilizadas.

Muestreo, preservación y pretratamiento

La toma de muestra y su preservación son pasos críticos que repercuten en todo el proceso posterior. Al fin y al cabo, los resultados que se obtienen en el laboratorio van referidos a la muestra analizada por lo que una mala estrategia de muestreo o mala conservación de la muestra darán lugar a un resultado impreciso y/o incorrecto. Por ejemplo, algunos compuestos como el BDE-209 son fotosensibles y por ello las muestras deben preservarse lejos de la luz del día y a ser posible a 4 ºC o menos (Covaci *et al.,* 2003; Eljarrat *et al.,* 2002; Kierkegaard *et al.,* 2009).

Debido a que estos compuestos se extraen usando solventes orgánicos, el agua de la muestra debe ser eliminada en la medida de lo posible. La liofilización suele ser la técnica más usada en todo tipo de muestras; algunos autores eliminan el agua de los sedimentos o polvo manteniéndolos a temperatura elevada durante 24h aunque la liofilización es un proceso más recomendable (Eljarrat *et al.,* 2004a). Se recomienda homogeneizar la muestra ya que la extracción posterior será más efectiva y el peso de muestra más representativo (Covaci *et al.,* 2007; Hyötyläinen y Hartonen 2002).

Extracción

La naturaleza de la matriz a analizar resulta determinante a la hora de elegir la técnica de extracción a utilizar. Así mismo, la cantidad de muestra requerida puede afectar en la decisión: algunas técnicas de extracción necesitan gran cantidad de muestra para ser realmente efectivas, y no siempre se dispone de ella. Del mismo modo, algunas técnicas no están recomendadas para grandes cantidades de muestra cosa que supone un riesgo en casos donde se espera que los niveles de contaminación sean bajos (Fulara y Czaplicka 2012).

Aunque los estudios sobre la presencia de FRs en agua son escasos ya que su elevado $LogK_{ow}$ hace que se encuentren principalmente en el material particulado (Fulara y Czaplicka 2012), su presencia en el medio acuático ha sido estudiada usando diversas técnicas:

Líquido-Líquido (LE, del inglés liquid extraction): Es la técnica más utilizada, usando hexano, ter-butil-éter o diclorometano (DCM). Necesita que el analito y la matriz tengan solubilidades diferentes ya que se basa en la mayor afinidad del analito por una de las fases. La eficiencia de esta técnica depende en gran medida de la naturaleza del solvente elegido, temperatura y otros factores que puedan modificar el equilibrio en favor del disolvente elegido (pH, salinidad…). Si se dan las condiciones puede ser una técnica eficaz, rápida y selectiva, pero tiene la desventaja de necesitar elevados volúmenes de muestra y disolvente (Vonderheide 2009).

Extracción en fase sólida (SPE, del inglés solid phase extraction): Otra técnica cuyo uso está bastante extendido y que en agua se aplica utilizando C_{18} como fase estacionaria (Rezaee *et al.,* 2010). Se basa en la retención de los analitos contenidos en fase gas o líquida en una fase estacionaria a la cual son afines. Posteriormente los analitos serán eluidos utilizando un disolvente adecuado. Esta técnica, además de como técnica de extracción propiamente dicha, puede utilizarse para concentrar muestras de agua a un determinado volumen. En agua, la principal desventaja que presenta es la posible saturación de los cartuchos y pérdida de efectividad de extracción si el volumen utilizado es demasiado alto (Fontana *et al.,* 2009). Una de sus principales ventajas es que puede acoplarse a técnicas de análisis y por tanto realizar la extracción, purificación y análisis instrumental conjuntamente, reduciendo el tiempo de análisis y la variabilidad provocada por el error humano.

Extracción por sorción a barra agitadora (SBSE, del inglés stir bar sorptive extraction): En esta técnica los contaminantes se concentran en el sorbente que recubre una barra agitadora y presenta la ventaja de ser una técnica rápida y que necesita poco volumen de solvente y muestra (Llorca-Porcel *et al.,* 2006).

Extracción por punto de nube (CPE, del inglés cloud point extraction): Se basa en la separación en 2 fases de una disolución al alcanzar una temperatura determinada (punto de nube) a causa de los tensoactivos que ésta contiene. Una vez producida esta separación, se descarta la fase que no contiene los analitos de interés. Se llega a factores de concentración considerables, pero presenta el inconveniente de que es necesario eliminar los tensoactivos antes de realizar el análisis instrumental del extracto (Wang *et al.,* 2007).

Microextracción líquido-líquido dispersiva (DLLME, del inglés dispersive liquid-liquid micro extraction): Se utiliza un agente extractante no miscible con el agua y un agente dispersante miscible tanto con el extractante como con el agua. Se forma una nube en donde el agente extractante se encuentra en forma de pequeñas gotas y por tanto la superficie de contacto con el agua se incrementa en gran medida, aumentando la eficiencia de la extracción L-L. El principal inconveniente es la recuperación del extractante y en ocasiones las interferencias causadas por el agente dispersante (Regueiro *et al.,* 2009).

En matrices sólidas las metodologías utilizadas son bastante diferentes aunque se suelen basar en metodologías sólido líquido:

Soxhlet o extracción mediante líquidos presurizados (PLE, del inglés presurized liquid extraction): Son técnicas muy similares y, de hecho, la PLE no deja de ser una evolución moderna del Soxhlet. Habitualmente se usa una mezcla de disolventes (1:1) como por ejemplo hexano:DCM o hexano:acetona (Alonso *et al.,* 2014; De La Cal *et al.,* 2003; Eljarrat *et al.,* 2004b; Siddique *et al.,* 2012). La PLE se considera más efectiva que el Soxhlet y mucho más óptima ya que requiere menor cantidad de disolvente, permite extraer un mayor número de muestras simultáneas y el tiempo de extracción se reduce considerablemente. Es la técnica más utilizada actualmente pese a que su optimización es más compleja: parámetros como presión, temperatura, tiempo de calentamiento y estático o número de ciclos de extracción deben ser optimizados. De hecho, actualmente el Soxhlet ha sido casi totalmente reemplazado por nuevas

metodologías y su uso ha quedado reducido a una metodología de comparación con los nuevos métodos optimizados (De La Cal *et al.*, 2003). A modo de ejemplo puede verse la comparación realizada por De la Cal *et al.* (2003) entre los porcentajes de recuperación mediante Soxhlet y PLE (Figura 1.10). Como puede verse las recuperaciones son similares y el PLE proporciona menor error.

Dispersión en fase sólida (MSPD, del inglés matrix solid phase dispersion): Evita pasos previos de tratamiento de muestra antes de la extracción. La muestra se dispersa a lo largo de un soporte, normalmente C_{18}, desde donde luego es extraida. Proporciona buenas recuperaciones y es rápida, aunque su uso está menos extendido que las anteriores metodologías descritas para el análisis de HFRs (Dopico-García *et al.*, 2007; Muñoz-Arnanz *et al.*, 2011a).

Extracción asistida por microondas (MAE, del inglés microwave assisted extraction): Consiste en la aplicación de microondas para elevar la temperatura de extracción. Al igual que la PLE, el hecho de trabajar a temperatura y presión altas reduce el volumen de disolvente de extracción sin pérdida, e incluso aumento, de la eficacia, además de reducir el tiempo de extracción respecto a la LLE. Sin embargo, el extracto debe ser filtrado posteriormente y tiene un coste moderadamente alto (Wang y Li 2010).

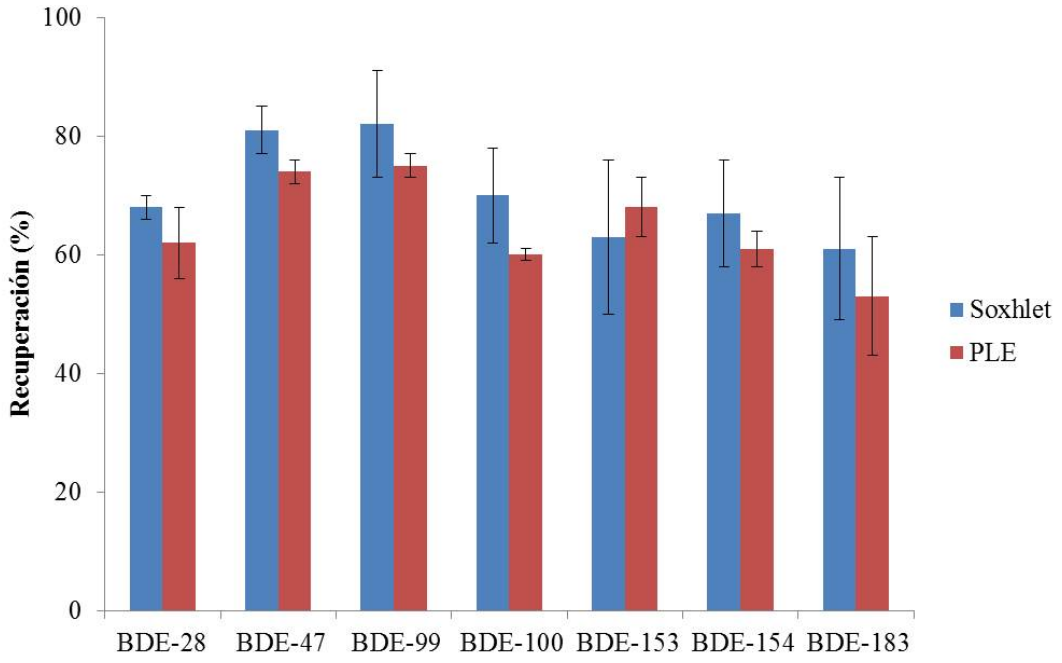

Figura 1.10. Comparación entre las metodologías de Soxhlet y PLE (datos de De la Cal *et al.* 2003).

Purificación

Muchos extractos, especialmente los obtenidos de matrices complejas como las biológicas, requieren un proceso de purificación antes de poder ser analizados si se pretende conseguir una buena identificación y cuantificación de los contaminantes deseados. Este proceso es también necesario en matrices a priori menos complejas como los sedimentos. Además, en muestras de biota la materia grasa que contiene los HFRs debe ser eliminada, y a ser posible determinada, antes del análisis instrumental (Covaci *et al.*, 2003).

Extracción selectiva por líquidos presurizados (SPLE, del inglés selective presurized liquid extraction): Se trata de una combinación entre la PLE y la SPE. Consiste en rellenar la celda de extracción utilizada en la PLE con algún absorbente (alúmina, sílica o florisil) antes de añadir la muestra. De este modo el extracto que se obtiene ya ha sido purificado, lo que comporta un evidente ahorro de tiempo y disolvente. Sin embargo, esta técnica presenta una serie de inconvenientes: no es posible determinar la grasa, necesaria para expresar los resultados en unidades lipídicas, y en muestras muy complejas puede ser necesaria una SPE posterior igualmente. Es una técnica utilizada especialmente en sedimentos.

Tratamiento ácido: Esta técnica utiliza un ácido concentrado, normalmente H_2SO_4, que elimina la materia orgánica por oxidación. Al añadirlo al extracto, que debe estar en un disolvente inmiscible con el ácido (hexano, por ejemplo), los HFRs son liberados de la grasa y pasan a la fase orgánica, mientras que toda la materia orgánica que había en el extracto es destruida y los residuos son descartados antes de proceder con la purificación del extracto. Pese a que esta técnica es muy efectiva como técnica de purificación, también puede ocasionar la pérdida de algunos compuestos no estables en ácido. Además al ser un procedimiento manual puede ocasionar malas recuperaciones si no es llevado a cabo con cuidado, o en matrices muy sucias donde sean necesarias muchas adiciones de ácido.

Cromatografía de permeabilidad en gel (GPC, del inglés gel permeation chromatography). La GPC es un tratamiento menos destructivo que permite el análisis de compuestos que son degradados por el ácido. Se basa en la exclusión por tamaño de partícula, ya que las moléculas orgánicas quedan retenidas en una columna mientras que las moléculas de interés, más pequeñas, pasan más fácilmente a través de los poros de la columna. En general, el tratamiento ácido permite obtener extractos más limpios y, pese

a que pueda parecer lo contrario, requiere menos tiempo que la GPC. Esto es debido a que el tratamiento ácido permite tratar varias muestras simultáneamente, mientras que la GPC requiere de inyecciones individuales y secuenciales. En general, la GPC sólo está recomendada cuando los analitos de interés no son resistentes al ácido y para muestras con bajo contenido lipídico (Guerra *et al.*, 2011b; Jia *et al.*, 2011; Kang *et al.*, 2010; Muñoz-Arnanz *et al.*, 2011b).

Tabla 1.8. Principales técnicas de extracción y purificación usadas en el análisis de los HFRs.

Matrices	Compuestos	Extracción	Purificación
Sedimento	PBDEs, HFRs emergentes , HNs	Soxhlet (Hex:DCM (3:1)) PLE Hex:DCM (1:1)	Sílica ácida Alúmina neutra
Aire	PBDEs, HFRs emergentes , HNs	Soxhlet (Hexano:Acetona (1:1))	Alúmina
Lodos	PBDEs, HFRs emergentes, HNs	PLE Hexano:DCM (1:1)	Sílica ácida, alúmina neutral y carbono activo
Biota	PBDEs, HFRs emergentes , HNs	PLE Hex:DCM (1:1)	1-Tratamiento ácido 2- Alúmina
	HNs	LE (Hexane)	Sílica y alúmina
	PBDEs, HNPs	PLE Hex:DCM (1:2)	GPC
	PBDEs, HFRs emergentes	Soxhlet (DCM)	1- GPC 2- Sílica
	HNs	MSPD	Sílica ácida y básica
Pelo humano	PBDEs	L-L Hexano	Fluorisil
Leche materna	PBDEs, HNs	L-L Hexano:DCM (1:1)	GPC

SPE: Cuando se utiliza para purificar extractos de muestras sólidas la SPE se aplica utilizando alúmina, sílica o florisil como sorbentes (Booij *et al.*, 2002; Guerra *et al.*, 2012a; Hoh *et al.*, 2006). En algunos casos ésta se substituye o complementa con columnas de sílica o alúmina, mientras que en algunas metodologías de extracción por PLE se añade un sorbente en la extracción. Además, se suele utilizar también para concentrar el extracto.

1.8.2. Análisis instrumental

Dado que los compuestos estudiados en esta tesis se determinan a nivel traza, es necesario el análisis mediante espectrometría de masas (MS, del inglés mass spectrometry) debido a la elevada sensibilidad y selectividad que proporciona respecto a otras técnicas. Además, debido a la existencia de numerosos compuestos que pueden interferir con la determinación, es necesario trabajar con técnicas de cromatografía previas al análisis mediante MS.

Cromatografía

Respecto a la separación cromatográfica, la cromatografía de gases (GC, del inglés gas chromatography) es la técnica más utilizada para el análisis de los HFR, debido a los valores de presión de vapor, volatilidad, etc. de los compuestos habitualmente de interés (Król *et al.*, 2012). La elección de la columna es un factor crítico ya que su longitud, conector o fase estacionaria pueden tener una gran influencia en el análisis de los HFRs (Korytár *et al.*, 2006). Fases estacionarias apolares como la DB-5 son las más usadas habitualmente, con una longitud de columna que normalmente está en torno a los 30 m. Por el contrario, fases estacionarias usadas comúnmente para el análisis de otros compuestos (como por ejemplo la DB-XLB) no son adecuadas para el análisis de compuestos que, como muchos HFRs, presentan una elevada masa molecular (Kierkegaard *et al.*, 2004). Por otro lado, el BDE-209 se degrada a causa del elevado tiempo de retención, por lo que si se quiere analizar este compuesto la columna debe ser de 15 m (Eljarrat *et al.*, 2004a).

De hecho, es necesario llegar a un compromiso dependiendo de los compuestos y matrices que se quieren analizar, ya que las columnas presentan ventajas y desventajas. Por ejemplo, aunque las columnas de 15 m permiten analizar compuestos que se degradarían en las columnas de 30 m, las co-eluciones aumentan. Este problema es especialmente importante cuando la identificación utilizada no es lo suficientemente selectiva. Por ello, muchos autores analizan muestras complejas de biota utilizando columnas de 30 m sacrificando así la identificación del BDE-209, considerando que la presencia de este compuesto en biota es normalmente residual (Alonso *et al.*, 2012).

Otro aspecto importante es el sistema de inyección. Los 3 sistemas más comúnmente utilizados son el split/splitless, on-column y temperatura de vaporización programada (PTV, del inglés programable temperature vaporization). La temperatura del inyector y la interfase también son importantes ya que los HFRs pueden degradarse a temperaturas

elevadas. Normalmente estas temperaturas oscilan entre 250 y 300 ºC (Kierkegaard *et al.*, 2009; Stapleton 2006).

Recientemente nuevas técnicas como la cromatografía de gases bidimensional (GCxGC) han cobrado importancia en el análisis de los HFRs debido al superior poder de resolución que proporciona y que ha demostrado su eficacia en el análisis de otros compuestos polihalogenados. Mediante esta técnica los problemas ocasionados por la co-elución de varias substancias se pueden resolver prácticamente en su totalidad cuando se acopla a un espectrómetro de masas de tiempo de vuelo (TOF, del inglés time of flight). No obstante, esta técnica presenta una serie de inconvenientes. Por un lado, el tratamiento posterior de los datos es muy complejo debido a la gran cantidad de información generada, por lo que se requiere experiencia y ordenadores muy potentes. Además, la cuantificación no es tan asequible como con las otras técnicas (Korytár *et al.*, 2006; Wang y Li 2010).

Por otra parte, el análisis mediante la cromatografía de líquidos (LC, del inglés liquid chromatography) es una alternativa para el análisis de compuestos menos estables térmicamente (Bacaloni *et al.*, 2009). En este caso fases estacionarias apolares como el C_{18} son las que mejores resultados proporcionan en el análisis de HFRs (Zhou *et al.*, 2010). Algunos compuestos cuyo análisis se lleva a cabo mediante LC son el HBCD o el TBBPA. En el caso del HBCD su análisis por GC no permite diferenciar entre sus diferentes diasteroisómeros ya que en las condiciones a las que se trabaja se produce una interconversión entre los diferentes diasteroisómeros o degradación de los mismos (Covaci *et al.*, 2003). Dado que los diferentes isómeros del HBCD presentan características diferentes, poder diferenciarlos es clave y hace su análisis por GC no recomendable. En cambio, la LC permite una correcta separación e identificación de los principales diasteroisómeros e incluso el análisis enantiomérico (Guerra *et al.*, 2011a). El análisis se lleva a cabo mediante LC-MS (o LC-MS-MS). La técnica de ionización más utilizada es la ionización por electrospray (ESI, del inglés electrospray ionization). Pese a que esta técnica presenta un problema grave con el efecto matriz, poniendo el peligro los resultados, el hecho de poder usar patrones isotópicamente marcados soluciona en gran medida el problema (Guerra *et al.*, 2012b; Guerra *et al.*, 2008). Otra potencial aplicación de la LC es el análisis de metabolitos de los HFRs. Algunos de estos metabolitos, como los PBDEs hidroxilados (OH-PBDEs, del inglés hydroxilated PBDEs) presentan características diferentes a los PBDEs como son una mayor polaridad y no volátil que no permiten su análisis mediante GC. En estos casos la LC se convierte

en una alternativa a la derivatización previa para poder analizarlos por GC (Feo *et al.*, 2013).

Espectrometría de masas

Las 3 técnicas más extendidas para el análisis de los HFRs son la MS, la espectrometría de masas en tándem (MS-MS) y la espectrometría de masas de alta resolución (HRMS, del inglés high resolution mass spectrometry). La HRMS es la técnica que mejor selectividad y sensibilidad proporciona y es siempre la técnica utilizada si existe la posibilidad. No obstante, su elevado coste hace que no todos los laboratorios puedan permitírselo y por ello la MS y MS-MS, cuyo coste es considerablemente menor, son las más utilizadas.

Espectrometría de masas (cuadrupolo simple)

El análisis de los HFRs mediante GC-MS está muy extendido, ya sea trabajando en ionización química negativa (NCI, del inglés negative chemical ionization) o en impacto electrónico (EI, del inglés electron ionization) (Eljarrat *et al.*, 2002; Lacorte y Guillamon 2008). EI es la fuente de ionización más común, pero no siempre supone la mejor opción. En concreto, la NCI proporciona una gran sensibilidad para los compuestos halogenados ya que la pérdida de un ion de cloro o bromo se produce con mucha facilidad siendo superior en este aspecto a la EI. Por ello, cuando la técnica utilizada es GC-MS el modo de ionización más común es la NCI. No obstante, la GC-MS (NCI) presenta un problema de selectividad que puede ser de gran importancia si se analizan matrices complejas y con un gran número de compuestos halogenados. En estos casos los iones monitorizados en NCI suelen ser los iones de cloro y bromo (*m/z* 79 y *m/z* 35) salvo algunas excepciones, y por ello las co-eluciones representan un problema difícil de resolver. Además, esto conlleva la imposibilidad de usar patrones marcados para cuantificar mediante la técnica de dilución isotópica. En cambio, los iones monitorizados en EI suelen ser más específicos del compuesto en cuestión; se monitorizan los fragmentos $[M\text{-}Br_2]^{-\cdot}$ e incluso muchas veces el ion utilizado es el molecular (Eljarrat *et al.*, 2002). Además, la fragmentación de las moléculas puede utilizarse para la identificación, y el uso de patrones marcados es posible ya que se monitorizan fragmentos más grandes de la molécula. Por otro lado, algunos autores proponen el uso de la NCI para la determinación de los HFRs y confirmar luego los resultados utilizando EI (Covaci *et al.*, 2003; Sánchez-Avila *et al.*, 2011).

Espectrometría de masas en tándem

El análisis por GC-NCI-MS es muy sensible en compuestos halogenados, pero poco selectivo. Por otro lado, el análisis por GC-EI-MS es selectivo, pero poco sensible, mientras que el análisis mediante GC-EI-MS-MS o GC-NCI-MS-MS permite mantener o incluso aumentar la sensibilidad y, lo que es más importante, la selectividad. Por tanto, es la metodología que proporciona los mejores límites de detección (LODs, del inglés limits of detection) a modo de alternativa a la HRMS (Tabla 1.9).

Tabla 1.9. Comparación de diferentes técnicas de detección. Modificada de Covaci *et al.* (2003).

	NCI-MS	**EI-MS**	**IT-MS-MS**	**QqQ-MS-MS**	**TOF-MS**	**HRMS**
Sensibilidad	**	*	**	**	**	***
Selectividad	*	**	**	**	**	***
Precisión	**	***	***	***	**	***
Coste	*	*	**	**	**	***

La principal ventaja que presenta esta técnica es la gran selectividad que proporciona el hecho de monitorizar la transición entre un ion padre y un fragmento del mismo, al contrario que en MS donde sólo se monitoriza un ion. Además, en muchos casos donde por MS se monitorizaban masas comunes entre el compuesto nativo y marcado permite el uso del método de cuantificación mediante dilución isotópica, obteniéndose un resultado mucho más fiable. Así mismo, el ruido de fondo también es reducido considerablemente, hecho de gran importancia en muestras que presentan bajos niveles de contaminación (Lacorte *et al.*, 2010; Losada *et al.*, 2010; Pirard *et al.*, 2006; Sánchez-Avila *et al.*, 2011).

Por último, otras técnicas como la trampa de iones (IT, del inglés ion trap) acoplada a la MS-MS han demostrado ser muy útiles a la hora de analizar HFRs debido a la posibilidad de identificar diferentes patrones de fragmentación según la posición de los halógenos en la molécula (Covaci *et al.*, 2007; Larrazábal *et al.*, 2004).

Espectrometría de masas de alta resolución

La HRMS proporciona una elevada sensibilidad y selectividad ya que permite monitorizar la masa exacta de la molécula. Sin embargo, debido a que no todos los laboratorios pueden disponer de ella su uso es más reducido. Además, en el caso concreto de los norbornenos halogenados su uso no está recomendado ya que la ionización mediante EI (la técnica normalmente asociada a GC-HRMS) produce un

fragmento muy intenso de masa 270 ($C_5Cl_6^+$) que es un fragmento muy común en compuestos organoclorados. Por tanto, la selectividad se ve reducida considerablemente (De la Torre *et al.,* 2010b). En los últimos años, debido al gran número de compuestos tanto naturales como antropogénicos cuya presencia se va descubriendo, existe un creciente interés en el campo del llamado "non-target analysis". Este análisis persigue la identificación de nuevos compuestos mediante su patrón de fragmentación y masa exacta para incorporarlos a bibliotecas ya existentes. A diferencia de los análisis normales mediante HRMs de sector magnético, estos análisis se llevan a cabo en equipos Orbitrap. Es especialmente interesante el análisis de metabolitos y/o productos de degradación de los compuestos originales ya que permite una mejor caracterización del impacto total de un compuesto en el medio ambiente (Hoh *et al.,* 2012; Shaul *et al.,* 2015). En la tabla 1.10 se listan los fragmentos utilizados en cada técnica.

Tabla 1.10. Fragmentos estudiados según las diferencias técnicas utilizadas en GC (Cuantificación / confirmación). n.d: No disponible.

Compuesto	MS (NCI)	MS (EI)	MS-MS	HRMS
Tri-BDEs	79 / 81	406 / 408	409>249 / 407>247	405,8026 / 407,8006
Tetra-BDEs	79 / 81	484 / 486	486>326 / 488>328	483,7131 / 485,7121
Penta-BDEs	79 / 81	564 / 566	566>406 / 568>408	563,6215 / 565,6195
Hexa-BDEs	79 / 81	642 / 644	646>486 / 648>488	641,5320 / 643,5300
Hepta-BDEs	79 / 81	722 / 724	561>402 / 563>404	721,4405 / 723,4398
Deca-BDE	79 / 81	797 / 801	799>640 / 801>642	957,1699 / 959,1679
DBDPE	79 / 81	485 / 487	485>325 / 485>404	969,2063 / 971,2043
HBB	79 / 81	550 / 552	550>390 / 552>313	549,5058 / 551,5038
PBEB	79 / 81	485 / 487	485>325 / 485>406	499,6266 / 501,6246
Dec 602	612 / 35	273 / 275	612>35 / 612>37	271,8102 / 273,8072
Dec 603	638 / 35	263 / 265	638>35 / 638>37	262,8570 / 264,8540
Dec 604	79 / 81	419 / 421	460>79 / 504>79	417,7026 / 419,7006
DP	654 / 35	273 / 275	654>35 / 654>37	271,8102 / 273,8072
MeO-Tri-BDEs	79 / 81	434 / 436	n.d	435,8133 / 437,8113
MeO-Tetra-BDEs	79 / 81	512 / 514	516>356 / 516>358	513,7237 / 515,7217
MeO-Penta-BDEs	79 / 81	590 / 592	596>434 / 596>433	n.d
MHC-1	79 / 81	396 / 398	n.d	395,8430 / 397,8440
TriBHD	79 / 81	467	n.d	465,8966
TetraBHD	79 / 81	546	n.d	545,8050
Q1	386 / 388	387	n.d	385,8086
$BrCl_6$-MBP	432 / 434	n.d	n.d	n.d
Br_2Cl_5-MBP	476 / 478	n.d	n.d	n.d
Br_3Cl_4-MBP	520 / 522	n.d	n.d	n.d
Br_4Cl_3-MBP	563 / 565	n.d	n.d	n.d
Br_5Cl_2-MBP	607 / 609	n.d	n.d	n.d
Br_6Cl-MBP	653 / 655	575	n.d	647,5084
Br_7-MBP	699 / 701	619	n.d	n.d

1.9. Justificación de la tesis

El uso de los HFRs está extendido a nivel global debido a que las ventajas que aportan en cuanto a prevención de incendios, con todo lo que ello implica, les confieren una gran importancia. Esta gran demanda y uso extendido hace que sean introducidos continuamente en el medio ambiente ya que la gran demanda que existe genera elevadas cotas de producción. Además, pese a que muchos de los HFRs usados durante décadas se encuentran actualmente prohibidos, la elevada vida útil de los materiales que los contienen hace que puedan seguir siendo liberados mucho después de que su uso haya sido prohibido.

El grado de conocimiento respecto a la presencia y comportamiento de los HFRs varía dependiendo de la familia. En el caso de los PBDEs, su presencia en prácticamente todo el globo ha sido sobradamente probada, así como su capacidad de bioacumulación. Más recientemente se ha descubierto que el BDE-209, cuya capacidad de bioacumulación era prácticamente despreciada, puede acumularse en grandes cantidades en algunos organismos; incluso se ha visto que es el PBDE con mayor contribución en aves o moluscos. Además, se ha visto como los PBDEs presentan capacidad de biomagnificación a lo largo de la cadena trófica. En cambio, el conocimiento que se tiene sobre otras familias como los HNs es más limitado. De hecho, los estudios sobre su presencia en el medio ambiente han crecido exponencialmente en los últimos años demostrando así el creciente interés que recae en estos compuestos. Del mismo modo, la información que se tiene sobre su comportamiento en el medio ambiente y efectos en los organismos que lo habitan es escasa y un aspecto en el que se están centrando un gran número de investigadores de todo el mundo.

Gracias a las evidencias presentadas por la comunidad científica, las primeras restricciones sobre los PBDEs han sido aplicadas y en Norteamérica y la Unión Europea su uso ya ha sido prohibido. El HBCD sigue el mismo camino, y debido a que la industria sigue sintetizando nuevos HFRs que quedan fuera de estas medidas legales es necesario continuar aportando información sobre su presencia, comportamiento y efectos con el fin de evaluar si es aconsejable su uso o si, por el contrario, estos nuevos HFRs también deben ser sometidos a restricciones por parte de los organismos competentes.

1.10. Objetivos

Considerando todo lo expuesto anteriormente y con el objetivo global de evaluar el impacto ambiental asociado al uso de los nuevos HFRs, al inicio de la presente Tesis Doctoral se establecieron los siguientes objetivos específicos:

1. Desarrollo de metodologías analíticas sensibles y selectivas para el análisis de HFRs, especialmente los emergentes (HNs, PBEB, HBB y DBDPE) en matrices ambientales y biológicas.

2. Aplicación de las metodologías desarrolladas en muestras ambientales, estudiando la presencia de HFRs y sus diferentes patrones de acumulación.

3. Aplicación de las metodologías desarrolladas en diferentes muestras de biota correspondientes a diferentes niveles de las cadenas tróficas acuáticas y terrestres, estudiando la capacidad de bioacumulación y biomagnificación de los HFRs.

4. Comparación de los niveles de FRs clásicos con los de FRs alternativos, y evaluación de posibles tendencias que indiquen cambios en el uso de unos u otros.

5. Evaluación de los efectos toxicológicos de algunos HFRs.

1.11. Plan de trabajo y estructura de la tesis

Esta tesis ha sido escrita como compendio de publicaciones y está dividida en 6 capítulos principales. En el capítulo 1 se ha introducido la problemática asociada a los HFRs, sus propiedades fisicoquímicas, los mecanismos de entrada de los mismos al medio ambiente, su comportamiento en biota y concentraciones encontradas en el medio ambiente. Se introducen los compuestos estudiados en esta tesis y las metodologías más habituales de análisis. Por último, se expone el objetivo global y los objetivos específicos planteados.

Los capítulos 2, 3, 4 y 5 exponen el trabajo desarrollado y los resultados obtenidos. Cada capítulo empieza con una introducción donde se explica el trabajo realizado en el marco del mismo, añadiendo o ampliando algunos conceptos vistos en el capítulo 1. Posteriormente se adjuntan los trabajos publicados incluidos en el capítulo y se desarrolla una discusión global de los resultados, poniéndolos en contexto con la bibliografía existente. Finalmente se exponen las conclusiones extraídas en base al trabajo realizado.

En el capítulo 2 se discute sobre el desarrollo de nuevas metodologías analíticas llevado a cabo durante esta tesis, incluyéndose 2 publicaciones científicas, para el análisis medioambiental de HFRs. Las técnicas analíticas desarrolladas y utilizadas en los diferentes trabajos de la presente tesis se basan en el uso de GC-MS-MS.

- Publicación científica #1: *Analytical method for the determination of halogenated norbornene flame retardants in environmental and biota matrices by Gas Chromatography coupled to Tandem Mass Spectrometry*. E. Barón, E.Eljarrat, D.Barceló. J. of Chromatography, 1248, 154-160 (2012).

- Publicación científica #2: *Gas chromatography/tandem mass spectrometry method for the simultaneous analysis of 19 brominated compounds in environmental and biological samples.* Barón, E.; Eljarrat, E.; Barceló, D. Analytical and Bioanalytical Chemistry, 406 (29), 7667-7676 (2014).

En el capítulo 3 se discute la presencia de HFRs en diversas muestras ambientales de diferente origen geográfico, concretamente sedimentos procedentes de Chile, Colombia y España, y lodos de depuradora de diferentes EDARs españolas. Se demuestra que los HNs también están presentes en estas matrices, al igual que los PBDEs o el HBCD. Se incluyen otras 2 publicaciones científicas:

- Publicación científica #3: *Occurrence of hydrophobic organic pollutants (BFRs and UV-Filters) in sediments from South America.* E. Barón, P. Gago, M. Gorga, I. Rudolph, G. Mendoza, A.M. Zapata, S. Díaz-Cruz, R. Barra, W. Ocampo, M. Páez, R.M. Darbra, E. Eljarrat, D. Barceló. Chemosphere, 92 (3), 309-316 (2013).

- Publicación científica #4: *Occurrence of classic and emerging halogenated flame retardants in sediment and sludge from Ebro and Llobregat river basins (Spain).* Barón, E.; Santín, G.; Eljarrat, E.; Barceló, D. Journal of Hazardous Materials. 265, 288-295 (2014).

En el capítulo 4, que representa el apartado más extenso de esta tesis, se discute sobre la presencia y comportamiento de los HNs y otros HFRs en diferentes especies pertenecientes a cadenas tróficas acuáticas (Chile y Mediterráneo Sur) y terrestres (Doñana). Se aportan datos sobre la capacidad de bioacumulación y biomagnificación de los HNs, combinando los resultados obtenidos mediante el análisis químico con los resultados de isótopos estables de nitrógeno. También se evalúan posibles tendencias temporales en la contaminación por HFRs. Se incluyen 5 publicaciones científicas:

- Publicación científica #5: *Emerging and historical halogenated flame retardants in an aquatic food web from Chile*. E. Barón, I. Rudolph, R. Barra, E. Eljarrat, D. Barceló. Environment International, 461-462, 258-264 (2013).

- Publicación científica #6: *Bioaccumulation and biomagnification of classical flame retardants, related halogenated natural compounds and alternative flame retardants in three delphinids from Southern European waters*. Barón, E.; Giménez, J.; Verborgh, P.; Gauffier, P.; De Stephanis, R.; Eljarrat, E.; Barceló, D. Environmental Pollution, 203, 107-155 (2015).

- Publicación científica #7: *Halogenated natural products in dolphins: brain-blubber distribution and comparison with halogenated flame retardants*. Barón E., Hauler C., Gallistl C., Giménez J., Gauffier, P., Castillo, J. J., Fernández-Maldonado, C., de Stephanis R., Vetter W., Eljarrat E., Barceló D. Environmental Science & Technology, 49, 9073-9083 (2015).

- Publicación científica #8: *Bioaccumulation and biomagnification of emerging and classical flame retardants in bird eggs of 14 species from Doñana Natural Space and*

surrounding areas (South-western Spain). Barón, E.; Mañez, M.; Andreu, A.C.; Sergio, F.; Hiraldo, F.; Eljarrat, E.; Barceló, D. Environment International, 68, 118-126 (2014).

- Publicación científica #9: *Temporal trends in classical and alternative flame retardants in bird eggs from Doñana Natural Space and surrounding areas (South-western Spain) between 1999 and 2013.* Barón, E., Bosch, C, Máñez, M., Andreu, A, Sergio, F., Hiraldo, F., Eljarrat, E., Barceló, D. Chemosphere, 138, 316-323 (2015).

El capítulo 5 aporta información sobre los efectos toxicológicos del BDE-209 y DP, exponiendo además el trabajo realizado durante la estancia pre doctoral en la Universidad de Plymouth donde se realizaron exposiciones *in vivo* de BDE-209 y DP en mejillones. Se incluye el siguiente artículo:

- Publicación científica #10: *Evaluation of the genetic and physiological effects of decabromodiphenyl ether (BDE-209) and dechlorane plus (DP) in Mytilus galloprovincialis by in vivo exposure.* Barón, E., Dissanayake, A., Vila, J., Crowther, C., Readman, J., Jha, A., Eljarrat, E., Barceló, D. Enviado a la Environmental Science & Technology (2015).

Por último, en el capítulo 6 se exponen las conclusiones generales de esta tesis en base a la problemática expuesta en el capítulo 1, los objetivos específicos planteados y los resultados obtenidos y discutidos en los capítulos 2, 3, 4 y 5.

CAPÍTULO 2

DESARROLLO DE MÉTODOS ANALÍTICOS

2.1. Introducción

Los norbornenos halogenados presentan características fisicoquímicas similares a otros contaminantes como los PBDEs o PCBs. Por tanto, es lógico suponer que las mismas técnicas de extracción, purificación y análisis instrumental que han demostrado ser apropiadas para la determinación de estos contaminantes sean válidas también para los HNs. De hecho, a la fecha de inicio de esta tesis prácticamente todos los métodos aplicados en los estudios sobre los HNs incorporaban estos compuestos a las metodologías pero rara vez se aportaban los datos sobre recuperaciones o LODs del método (mLODs, del inglés method limits of detection). Exceptuando algunos estudios donde se utilizaba LC-MS, con mLODs demasiado elevados y no adecuados para el análisis a concentraciones bajas, las técnicas más comunes eran (y siguen siendo) la GC-NCI-MS, cuya aplicabilidad era satisfactoria debido a que los estudios publicados se habían realizado en zonas cercanas a las fábricas de producción y por tanto los niveles ambientales eran elevados, y la GC-HRMS (Tabla 2.1). Después de desarrollar una metodología para el análisis de los HNs mediante GC-NCI-MS se vio que los límites de detección que ésta proporcionaba no eran suficientes para el análisis de estos compuestos en zonas alejadas de las fábricas de producción, donde los niveles eran mucho más bajos, por lo que se decidió optimizar una metodología mediante GC-MS-MS con el objetivo de lograr LODs más bajos. Pese a que la identificación de los HNs por NCI-MS es relativamente selectiva, al monitorizar en la mayoría de los casos los iones moleculares, el riesgo de co-eluciones sigue suponiendo un problema. Por ejemplo, en sedimentos de Canadá se identificó una substancia que co-eluia con el *syn*-DP en columnas DB-5 (Sverko *et al.,* 2010). Al trabajar con MS-MS también se aumenta la selectividad, uno de los motivos por los que se eligió esta técnica, que dio lugar a la publicación #1: *Analytical method for the determination of halogenated norbornene flame retardants in environmental and biota matrices by gas chromatography coupled to tandem mass spectrometry*. En su momento fue la primera metodología desarrollada por GC-MS-MS para el análisis de los HNs. En estos años han aparecido otras, demostrándose que su uso se ha extendido, aunque aún es inferior al de las metodologías más clásicas (Guo *et al.,* 2014; Sühring *et al.,* 2013; Zhang *et al.,* 2015).

Tabla 2.1. Ejemplos de las columnas y técnicas más usadas en el análisis de PBDEs y HNs.

Matriz	Columna	Técnica
Sedimento	HT-8 50m×0.22mm×0.25µm	GC-NCI-MS
	DB-5MS 15m×0.1mm×0.1 µm	GC-NCI-MS
	HT-5MS 15m×0.25mm×0.1µm	GC-EI-MS-MS
Aire	DB-5MS 30m×0.25mm×0.25µm	GC-NCI-MS
Lodos	DB-5MS 60m×0.25mm×0.25µm	GC-HRMS
Biota	DB-5MS 15m×0.10mm×0.1 µm	GC-NCI-MS
	DB-5HT 5m×0.25mm×0.10 µm	GC-HRMS
	HT-5MS 15m×0.25mm×0.1µm	GC-EI-MS-MS
	DB-5 30m×0.25mm×0.25µm	GC-HRMS
	Rtx 15m×0.25mm×0.10 µm	GC-EI-MS-MS GC-NCI-MS
	DB-5HT 15m×0.25mm×0.10µm	GC-HRMS
	DB-5HT 15m×0.10mm×0.10µm	GC-HRMS
Pelo humano	ZB-5MS 15m×0.25mm×0.1 µm	GC-EI-MS

Por otro lado, otro de los objetivos que se plantearon fue desarrollar una metodología para el análisis de los PBDEs, MeO-PBDEs y BFRs emergentes mediante GC-MS-MS. Como ya se ha explicado en el apartado 1.8, la GC-NCI-MS es una técnica que proporciona una gran sensibilidad en el análisis de estos compuestos. Sin embargo, presenta una falta de selectividad importante al monitorizar normalmente iones tan poco específicos, al estar presentes en un sinfín de moléculas de diferentes familias y orígenes. Las co-eluciones representan un problema crítico ya que normalmente el ión monitorizado es el mismo y no permite diferenciar entre diferentes compuestos, como se ha visto en el capítulo 1. De igual modo, no es posible trabajar con la técnica de cuantificación por dilución isotópica ya que no se puede diferenciar entre los bromos o cloros procedentes del compuesto nativo y del marcado, obteniéndose una única señal. Por otro lado, la GC-EI-MS que es más selectiva y sí permite el uso de la dilución isotópica, carece de la sensibilidad necesaria en la mayoría de los casos. Así pues, se buscaba una técnica que aportara selectividad y sensibilidad, descartando la HRMS debido a su elevado coste. El nuevo método está detallado en la publicación #2: *Gas chromatography/tandem mass spectrometry method for the simultaneous analysis of 19 brominated compounds in environmental and biological samples*. Pese a que los PBDEs y otros BFRs se han analizado tradicionalmente por GC-NCI-MS (Eljarrat *et al.*, 2004; Eljarrat *et al.*, 2002; Hyötyläinen y Hartonen 2002; Lacorte y Guillamon 2008) la tendencia actual es analizarlos mediante GC-EI-MS-MS. Básicamente, se trata de

sacrificar algo de sensibilidad en pos de una mayor selectividad y correcta identificación de los contaminantes. Sumando todos los PBDEs y otros BFRs, HNPs, BFRs emergentes y otros compuestos que pueden contener bromo, se obtiene un número muy alto de compuestos bromados a separar en una columna de sólo 15 m. La MS-MS permite diferenciar entre diferentes compuestos bromados que puedan co-eluir y proporciona resultados más fiables, además de reducir posibles interferencias de matriz (Camino-Sánchez *et al.*, 2011; Cristale *et al.*, 2012; Sánchez-Avila *et al.*, 2011).

2.2. Metodologías utilizadas y optimizadas

Pese a que los compuestos estudiados en esta tesis son a priori parecidos, su análisis requiere el uso de diferentes metodologías. Algunas habían sido optimizadas previamente en el laboratorio, mientras que otras tuvieron que ser desarrolladas. En el laboratorio se disponía de una metodología para el análisis de PBDEs y BFRs emergentes mediante GC-NCI-MS. Se buscó ir un paso más allá y desarrollar la metodología mediante GC-MS-MS con la idea de conseguir una identificación y cuantificación más fiable de estos BFRs. Por el contrario, no se disponía de metodología para el análisis de los HNs, por lo que primero se optimizó un método mediante GC-MS para luego desarrollar una metodología mediante GC-MS-MS, al ver que se necesitaba conseguir unos límites de detección inferiores. Estos 2 procesos se explican en más detalle en el apartado 2.3.

En el caso de los HNPs, dado que su análisis se realizó durante una estancia de 2 semanas en la Universidad de Hohenheim (Alemania), con excepción de los MeO-PBDEs, el método analítico fue el disponible en su laboratorio mediante GC-NCI-MS (Hauler *et al.*, 2014). El análisis del HBCD se llevó a cabo mediante LC-ESI-MS-MS siguiendo el protocolo optimizado previamente en el laboratorio, con sólo una ligera modificación en la rampa cromatográfica para reducir el tiempo de análisis (Guerra *et al.*, 2008; Guerra *et al.*, 2011a). Como se ha explicado en el capítulo 1, el análisis del HBCD por GC no es recomendable ya que no es posible diferenciar entre los distintos isómeros, hecho que al tener éstos diferente comportamiento en el medio ambiente ocasiona una pérdida de información crucial.

Publicación científica #1

Analytical method for the determination of halogenated norbornene flame retardants in environmental and biota matrices by gas chromatography coupled to tandem mass spectrometry

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Analytical method for the determination of halogenated norbornene flame retardants in environmental and biota matrices by gas chromatography coupled to tandem mass spectrometry

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ABSTRACT

A new methodology for the analysis of Dechlorane Plus and related compounds (DPMA, Dec 602, Dec 603 and Dec 604) by gas chromatography coupled to negative chemical ionization tandem mass spectrometry (GC–NCI–MS–MS) was developed for three different matrices, including environmental (sediment and sludge) and biota (fish) samples. Analytical parameters such as linearity, repeatability and reproducibility, recoveries, limits of detection and limits of quantification were evaluated, showing satisfactory values for the developed methodology. Moreover, a comparison with the analysis by GC–NCI–MS was carried out. Method limits of detection (MDLs), ranging between 0.12 and 1.26 pg/g dw, 1.16–2.90 pg/g dw and 2.30–21.1 pg/g lw for sediment, sludge and fish respectively, were much better than those obtained by GC–MS, with improvement factor up to 320. The applicability of the developed methodology was demonstrated by the analysis of real samples collected in a non-producing area, the Ebro river basin (Spain). DP values were up to 1.61 ng/g dw, 18.8 ng/g dw and 2.24 ng/g lw for sediment, sludge and fish samples, respectively.

1. Introduction

Halogenated flame retardants (HFRs) are a group of chemicals which are added to different materials such as textiles, furniture or polymers in order to prevent fires. They usually are structurally different chlorinated or brominated compounds [1]. Dechlorane 602 ($C_{14}H_4Cl_{12}O$, Dec 602), 603 ($C_{17}H_8Cl_{12}$, Dec 603), 604 ($C_{13}H_4Br_4H_{16}$, Dec 604), and Dechlorane Plus ($C_{18}H_{12}Cl_{12}$, DP) were introduced as replacements for Mirex ($C_{10}Cl_{12}$), an organochlorine pesticide which was also used as flame retardant in the 1960s but was banned due to its toxicological properties in the 1970s [2]. DP is classified as a high production volume chemical in USA but a low production volume chemical in EU [3,4], and exists in commercial products as two isomers: *syn*- and *anti*-DP, with a ratio of 1:3 respectively. These two stereoisomers come from the Diels–Alder reaction of 2 mol of hexachlorocyclopentadiene with 1 mol of 1,5-cyclooctadiene [5]. DP has been produced for many years in China (Anpon Electrochemical Co.) and North America (Oxychem) but it was not detected in the environment until 2006 [6]. DP has been found in environmental matrices such as sediment [2,7–10], air [3,11–13] or water [10,14], and also in biological matrices such as fish [15–17], eggs [18–21], blood [22] and hair [23]. Despite it was not supposed to have the same bioavailability as Mirex, reported levels show that DP has bioaccumulation capacity [24]. Due to its high degree of chlorination, Dec 602, Dec 603 and Dec 604 are supposed to have similar environmental behavior and toxicological properties than DP and Mirex. However, it has not been reported yet [25,26]. DP monoadduct ($C_{13}H_{12}Cl_6$, DPMA) can be generated from DP in a retro-Diels–Alder process, so it was also included in some published studies [21,26].

Currently, the number of scientific publications regarding to dechloranes is increasing. However, the knowledge about the environmental occurrence, fate and behavior of these halogenated norbornene flame retardants is still limited. Moreover, most of the studies are done near to manufacturing areas and, consequently, concentration levels are higher than those expected in other places of the world. The methodologies usually applied for the analysis of dechloranes are gas chromatography coupled to mass spectrometry (GC–MS) and GC coupled to high resolution mass spectrometry (HRMS) [27]. GC-HRMS provides excellent selectivity and sensitivity but, on the other hand, it is expensive and not all the laboratories can afford it. GC–MS has a lower price but selectivity

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and sensitivity are worse than in HRMS. This fact is not important for the analysis of samples from the manufacturing zones, where both techniques can be applied due to the existent high levels. Nevertheless, in non-producing areas with expected much lower levels the use of GC–MS may not give an adequate sensitivity. Regarding the sample preparation techniques, and as dechloranes have physical and chemical properties of some polybromodiphenyl ethers (PBDEs), the methods are typically based on quantitative organic analysis with GC–MS detection. Most laboratories use the PBDE or PCB processed extracts as this is where the dechlorane compounds are found. However, to our knowledge, analytical parameters for dechlorane determinations applying the same methodologies used for the determination of PBDEs or PCBs have never been reported.

The aim of this study was to develop a methodology for the analysis of halogenated norbornene flame retardants in sediment, fish and sewage sludge by GC coupled to tandem mass spectrometry (GC–MS–MS), an instrumental technique which provides good sensitivity and selectivity. This is the first work that applied GC–MS–MS for the analysis of dechloranes as an alternative to the expensive GC–HRMS. In addition, analytical parameters such as recoveries, reproducibility, limits of detection (LODs) and limits of quantification (LOQs) of the methods are also evaluated. Finally, and in order to check the applicability of the developed method for samples coming from non-producing areas, sediment, sewage sludge and fish samples from the Ebro river basin (Spain) were analyzed. In this sense, this work represents also the first report of halogenated norbornene flame retardants in a Spanish aquatic ecosystem.

2. Materials and methods

2.1. Standards and reagents

Syn- and *anti*-isomers of DP (CAS#13560-89-9) were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Dec 602 (95%, CAS#31107-44-5), Dec 603 (98%, CAS#13560-92-4) and Dec 604 (98%, CAS#34571-16-9) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). Mirex (98%, CAS#2385-85-5) and DPMA (98%, CAS#135821-04-4) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). ^{13}C-syn-DP (98%, CAS#135821-03-3), used as internal standard, was also obtained from Cambridge Isotope Laboratories Inc (Andover, MA).

Alumina (0.063–0.2 mm) and copper (<63 μm) were obtained from Merck (Darmstadt, Germany). Al–N and silica cartridges were obtained from Biotage. Dichloromethane and hexane, solvents for organic trace analysis, were purchased from Merck.

2.2. Sample collection

Samples analyzed in this study were sediments and fish (*Barbus barbus*, *Silurus glanis* and *Cyprinus carpio*) collected along five different sampling points from the Ebro river basin (Spain), and sewage sludge from six wastewater treatment plants (WWTPs), Logroño, Pamplona, Tudela, Zaragoza, Lleida and Tortosa, along also the Ebro river basin (Spain). All the samples were transferred to the laboratory at a temperature of 4 °C, frozen at −20 °C before being freeze dried. The lyophilized samples were grounded and homogenized by sieving through a stainless steel 2-mm sieve, and stored in sealed containers at −20 °C until analysis.

2.3. Sample preparation

The sample preparation methodologies applied were similar to those previously optimized for extraction and purification of different PBDE congeners with some modifications [28,29]. In order to estimate the analytical parameters of the different methodologies for each studied matrix (recoveries, reproducibility, limits of detection and quantification), four different replicates were carried out with samples spiked with the selected analytes (20 ng of each compound). Moreover, for each matrix, a blank was prepared to subtract the natural content of the different compounds in each material.

It is important to note that fish and sludge are dirtier matrices than sediment, and that because different sample preparation techniques were applied. For sediment, a selective pressurized liquid extraction (SPLE) method, in which extraction and purification of the sample were carried out simultaneously, was applied. However, this method cannot provide clean extracts for fish and sludge samples. For fish, a treatment with sulfuric acid is mandatory. In the case of sludge, even with the acid attack, a full cleaning of the extract was not achieved. So it was necessary to carry out an additional step of purification by SPE silica cartridges.

2.3.1. Sediment

Before extraction, 2 g dry weight (dw) were spiked with 10 ng of internal standard (^{13}C-syn-DP). Samples were kept overnight to equilibrate prior to SPLE. The extraction was done using an ASE 350 system (Dionex, Sunnywale, CA, USA). Spiked samples were grown with alumina and copper (1:2:2) and loaded into a 22 mL extraction cell previously loaded with 8 g of alumina. Death volume was filled with hydromatrix. The cell was filled with a hexane:dichloromethane mixture (1:1) until a pressure of 1500 psi was reached and was heated to 100 °C. Five minutes of oven heat-up and 2 static cycles of 10 min were made. Final extraction volume was about 35 mL. Extracts were concentrated to incipient dryness and re-dissolved with toluene for a final volume of 40 μL.

2.3.2. Fish

Before extraction, 3 g dw were spiked with 10 ng of internal standard (^{13}C-syn-DP). Samples were kept overnight to equilibrate prior to the pressurized liquid extraction (PLE). The sample was loaded in a 11 mL extraction cell and hydromatrix was used to fill the dead volume. Extraction conditions were the same as the described for sediments. Resulting extracts were solvent removed for gravimetric lipid determination and subsequently re-dissolved in hexane. The solution was then treated with concentrated sulfuric acid and the organic phase was cleaned by solid phase extraction (SPE) using alumina (5 g) cartridges. Cartridges were conditioned with 20 mL of hexane before sample was loaded, and 20 mL of hexane:dichloromethane (1:2) were used for the elution. Finally, extracts were concentrated following the same procedure as that for sediments.

2.3.3. Sewage sludge

Before extraction, 1.5 g dw were spiked with 10 ng of internal standard (^{13}C-syn-DP). Samples were kept overnight to equilibrate. Spiked samples were ground with copper (1:2) and loaded into an 11 mL extraction cell. Death volume was filled with hydromatrix and the same PLE conditions used for fish samples were also applied to sludge. Resulting extracts were treated with concentrated sulfuric acid, and two steps of SPE were done in order to obtain a clean extract. Silica (2 g) cartridges were first used, with 20 mL of hexane for the conditioning step and 20 mL of hexane for elution step. Alumina (5 g) cartridges were then used in the same way as for fish samples. Resulting extracts were concentrated and re-dissolved as described before.

Table 1
Retention time, ions, transitions and collision energies (CE) selected for the dechlorane study by GC–NCI–MS and GC–NCI–MS–MS.

Compound	Rt (min)	MS		MS–MS			
		Ion 1 (*m/z*)	Ion 2 (*m/z*)	Transition 1 (*m/z*)	CE 1 (eV)	Transition 2 (*m/z*)	CE 2 (eV)
DPMA	13.4	35	237	237 > 35	45	237 > 37	45
Dec 602	17.8	612	35	612 > 35	35	237 > 35	30
Dec 603	20.6	638	35	638 > 35	15	638 > 37	15
Dec 604	21.0	79	81	460 > 79	20	504 > 79	20
syn-DP	22.4	654	35	654 > 35	35	654 > 37	35
anti-DP	22.8	654	35	654 > 35	35	654 > 37	35
^{13}C-syn-DP	22.4	664	35	664 > 35	30	664 > 37	30

2.4. Instrumental determination

2.4.1. Gas chromatography–mass spectrometry

GC–negative chemical ionization (NCI)–MS and GC–electron impact (EI)–MS analyses were performed on an Agilent 7890C gas chromatograph connected to an Agilent 5975A mass spectrometer. A DB-5ms capillary column (15 m × 0.1 mm i.d., 0.1 μm film thickness) was used with helium as the carrier gas. The temperature program started at 80 °C (held for 2 min) to 300 °C (held for 10 min) at 10 °C/min, using the splitless injection mode during 1 min. With that chromatographic programme, all the analytes were completely separated in a total run time of 34 min (Table 1).

The sensitivity obtained for EI was lower than that obtained for NCI. Thus, NCI was selected as the ionization mode for further optimization experiments. Different NCI parameters were studied in order to optimize the signal, including the selection of the chemical ionization moderating gas (ammonia versus methane), ion source temperature (ST) (modifying its value from 150 to 300 °C), gas flow (GF) (between 1.25 and 2.5 mL/min), electron energy (EI) (between 50 and 200 eV) and emission current (EC) (modifying its value from 80 to 200 eV).

Selected ion monitoring (SIM) mode was applied in order to enhance the sensitivity. The experiments were carried out monitoring the two most intense peaks from the NCI spectra. Table 1 lists the ions monitored for each selected analyte. The most intense peaks were used for quantification purposes, and the second ones for confirmation.

2.4.2. Gas chromatography/tandem mass spectrometry

GC–NCI–MS–MS analyses were performed on an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quad. The same chromatographic conditions used for GC–MS experiments were used for tandem MS studies. Also, the same NCI source conditions used for GC–MS were applied to the MS–MS experiments. Additional parameters, such as collision energies (modifying its value from 1 to 45 eV) were optimized (Table 1).

Selective reaction monitoring (SRM) mode was applied in order to enhance the sensitivity. The experiments were carried out monitoring the two most intense transitions (Table 1). The most intense transitions were used for quantification purposes, and the seconds for confirmation criteria.

3. Results and discussion

3.1. Optimization for GC–MS

Different parameters were studied in order to optimize the signal by GC–NCI–MS. The selection of the optimal chemical ionization moderating gas was carried out by comparing the signals obtained working with ammonia and methane. Whereas the use of ammonia or methane does not influence the signal of PBDEs [30], different behavior was observed for dechloranes. Methane showed better instrumental LODs (iLODs) (from 2.6 to 11 times lower) than ammonium. This could be due to the different proton affinity (PA) of the analytes, which could cause that methane (PA 551 kJ mol^{-1}) gives better results than ammonium (PA 854 kJ mol^{-1}). Furthermore, NH_4^+ could form a complex with Cl^- that could change different equilibriums in the source. According to these results, the selection of the chemical ionization moderating gas is a critical parameter for obtaining a method with a good sensitivity for dechloranes, since iLODs considerably improve in all cases with the use of methane.

Other MS parameters were also evaluated in order to maximize the signal. The GF was tested modifying its value between 1.25, 1.5, 1.75, 2, 2.25 and 2.5 mL/min. The optimum value was established working at 2.25 mL/min. The ST was tested modifying its value between 150, 175, 200, 250 and 300 °C, and the optimal value was set at 175 °C. Higher values of ST showed more fragmentation of the molecular ion of DP, resulting in a decrease of the intensity of the peak, while at lower temperatures the molecular ion showed less fragmentation. In this case also, different behavior was observed comparing with PBDE analysis, for which high ST, i.e. 250 °C, are usually recommended. The EC was checked modifying its value between 80, 100, 150 and 200 eV. The best sensitivity was obtained for the highest value tested, 200 eV. However, working at higher values of EC was not recommended. Finally, the EI was tested modifying its value between 50, 100, 150 and 200 eV, and selecting the 150 eV as the optimal value.

3.2. Optimization for GC–MS–MS

The same GC–NCI–MS conditions were applied to the MS–MS experiments. Once the optimal transitions were established,

Table 2
Quality parameters obtained for the optimized GC–NCI–MS–MS: comparison with the GC–NCI–MS method.

Compound	Precision (% RSD, $n = 5$)		Linearity		iLOD (injected fg)	
	Between run	Within run	Range (pg/μL)	R^2	MS	MS–MS
DPMA	6.5	5.6	0.5–900	0.997	13	43
Dec 602	2.9	4.1	0.5–900	0.999	96	40
Dec 603	8.3	4.3	0.5–900	0.997	5.0	75
Dec 604	8.6	5.9	0.5–900	0.997	17	20
syn-DP	7.1	2.3	0.5–900	0.999	12	21
anti-DP	5.5	4.5	0.5–900	0.998	9.0	17

collision energies for each analyte were optimized modifying its value from 1 to 45 eV. Selected values are presented in Table 1.

Analytical parameters, such as linearity, precision of the method and instrumental limits of detection were calculated for the developed GC–NCI–MS–MS method. Table 2 shows the results obtained. In order to evaluate the precision of the method, five consecutive injections were performed under the optimum conditions during the same day (within run) and in five different days (between run). The relative standard deviations (RSD) between the five values were calculated for all the selected analytes. RSD values ranged from 2.3 to 5.9% in intra-day tests and from 2.9 to 8.6% in inter-day tests, being always <10%, indicating good intra- and inter-assay variation. For the linearity study, calibration curves were determined for all the selected analytes. Good correlations were obtained within the interval studied, with correlation coefficients ranging between 0.997 and 0.999. The instrumental limits of detection (iLODs), defined as the minimum amount of analyte which produces a peak with a signal-to-noise (S/N) ratio equal to 3, were determined for dechloranes applying both techniques, NCI–MS and NCI–MS–MS. The iLODs varied from 5 to 96, and from 17 to 75 injected fg for NCI–MS and NCI–MS–MS, respectively. The use of MS–MS does not seem to offer significant improvement in terms of iLODs, being even slightly higher than those obtained by MS. This fact was expected analysing standard solutions. The main advantage of the use of a more selective technique, such as MS–MS, must be appreciated with the analysis of real samples with complex matrices.

3.3. Analytical parameters of the developed methods

Table 3 shows the analytical parameters obtained for the developed methods for dechlorane analysis by GC–NCI–MS–MS in the three studied matrices. Recovery tests were carried out by spiking 20 ng of each compound; four replicates and a procedural blank were made for each matrix. Stored samples of fish, sediment and sludge from the Ebro River were chosen to carry out the tests. Recoveries ranged from 65 to 114%, 57–76% and 82–99% for sediment, sludge and fish samples, respectively. As expected, and due to the differences among the matrices, best recoveries were obtained for sediment, followed by fish, while the worse values were obtained for sludge samples. However, in all the cases, obtained recoveries are acceptable for all the analytes, with some exceptions for DPMA. DPMA was well recovered in sediment tests (93%) whereas it was lost in fish and sludge samples. The acid treatment applied in these matrices could be an explanation, since this treatment can fragment the DPMA molecule, due to the attack at the free double bond of the mono adduct. As regards the reproducibility of the methods, RSD values were also acceptable in all cases, with values between 4.3–11, 6.4–12 and 2.8–22% for sediment, sludge and fish samples, respectively.

Method limit of detections (MDL) and method limit of quantifications (MQL) were also calculated for each selected analyte in the 3 studied matrices using the recovery tests for each matrix. MQL is defined as the minimum amount of analyte which produces a peak with a signal-to-noise (S/N) ratio equal to 10. As expected, MDLs obtained for sediment were the lowest, with values ranging from 0.12 to 1.26 pg/g dw. Higher MDLs (between 1.4 and 15 times) were obtained for sludge samples, with values between 1.16 and 2.90 pg/g dw, but always in the low pg/g level. In fish samples, MDL values ranged from 2.30 to 21.1 pg/g lw. Between the different selected analytes, the worst results were obtained for the Dec 602, whereas the better results were found for the Dec 603.

Results obtained applying MS–MS were compared with those obtained by single MS (Table 3). In all the cases, the use of MS–MS provides better MDL than those obtained by single MS. In sediment samples, improvement factors move between 9 and 24, with the

Table 3
Recoveries, relative standard deviations (RSDs) and method limits of detection (MDLs) of GC–NCI–MS–MS analysis of selected analytes in sediment, sludge and fish samples: comparison with the GC–NCI–MS method.

Analyte	Sediment (n = 4)						Sludge (n = 4)						Fish (n = 4)					
	Blank (pg/g dw)	R (%)	RSD (%)	MS–MS (pg/g dw)		MS (pg/g dw)	Blank (pg/g dw)	R (%)	RSD (%)	MS–MS (pg/g dw)		MS (pg/g dw)	Blank (pg/g lw)	R (%)	RSD (%)	MS–MS (pg/g lw)		MSc (pg/g lw)
				MDL	MQL	MDL				MDL	MQL	MDL				MDL	MQL	MDL
DPMA	nd	93	5.0	0.4	1.2	43	–	–	–	–	–	–	–	–	–	–	–	–
Dec 602	nd	114	4.3	1.3	4.2	16	nq	60	12	1.8	6.0	575	565	97	12	21	70	278
Dec 603	128	86	5.7	0.1	0.3	1.6	82	66	10	1.2	3.9	36	179	88	22	7.3	24	24
Dec 604	nd	65	5.7	0.2	0.6	4.6	nd	57	11	2.9	9.7	645	nd	99	14	7	24	252
syn-DP	107	86	7.5	0.3	0.9	2.3	642	76	7.0	1.7	5.8	32	13	86	2.8	5.5	18	21
anti-DP	282	104	11	0.2	0.5	2.1	709	72	6.4	1.4	4.7	42	14	82	4.8	2.3	7.7	20

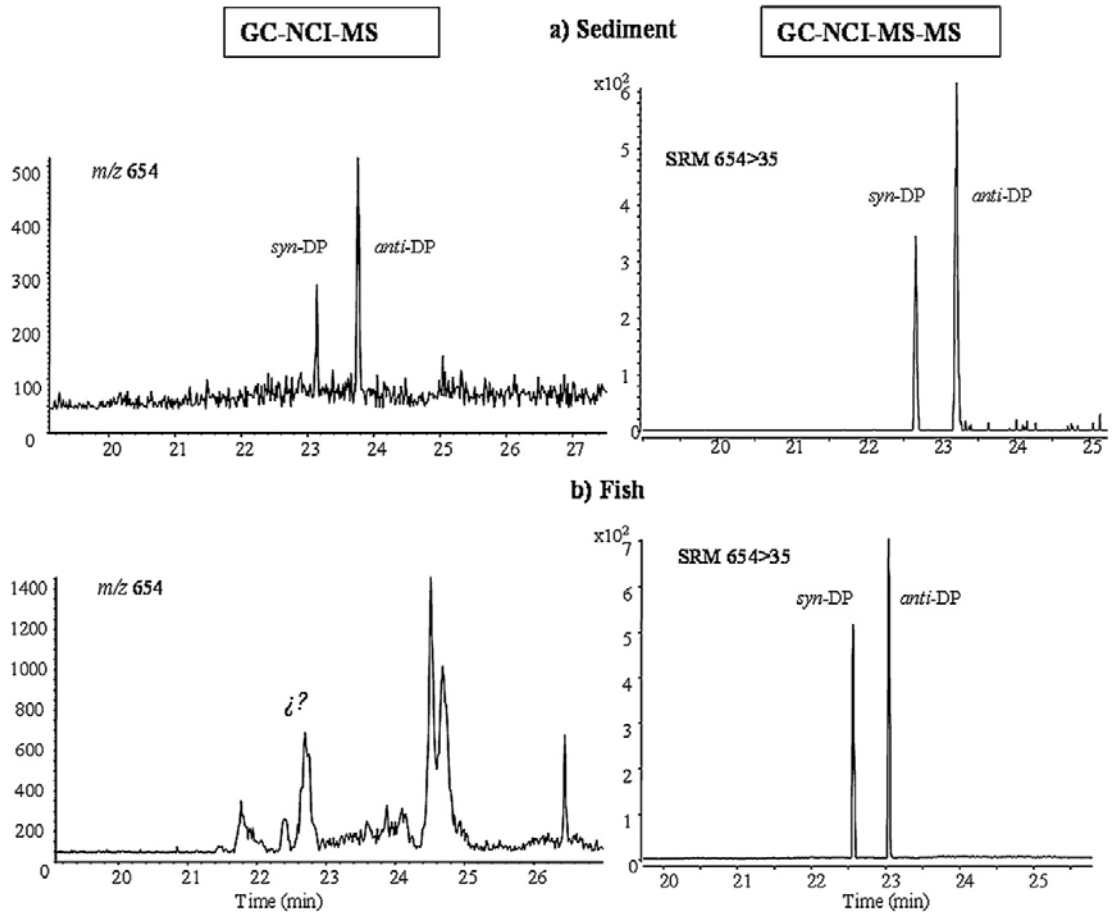

Fig. 1. Chromatograms obtained for the MS and MS–MS analysis of DP in (a) sediment sample and (b) fish sample.

exception of DPMA for which a great improvement was observed (more than 100 times). Similarly, improvement factors were calculated for sludge samples, with values ranging between 19 and 320. Finally, MDLs in fish samples decreased between 3 and 36 times in MS–MS method. Thus, it can be seen that with the most complex matrices (sewage sludge) the improvement is more evident.

The advantage of using SRM with a triple quadrupole MS–MS is the high sensitivity obtained for multi-analyte quantitative assays. SRM assays are particularly useful for the specific analysis of target compounds in complex mixtures and matrices. All the analytes can be accurately detected and quantified because their SRM ion transitions are mutually exclusive, allowing total exclusion of background and interferences. The product ion signal often decreases, but the percentage decrease in noise is much larger for real samples. Thus, the signal to noise ratios and LODs improve due to the noise reduction. This noise reduction is evident when comparing our chromatograms obtained by MS and MS–MS (Fig. 1). For DP, either in sediment or fish, the use of MS–MS increased the sensitivity in comparison with the use of MS: a considerable reduction of noise was observed, especially in the case of fish matrices. Selectivity increase was also noticed when working with the SRM 654>35, obtaining only signals corresponding to the two DP isomers. Other unknown peaks appeared when using the SIM (*m/z* 654), which could cause miss-identification when DP environmental levels were low.

Our MDLs were compared with other reported values in the literature. Published values of MDL for DP, Dec 602, Dec 603 and Dec 604 are very limited. Some works were carried out applying GC–MS, such as one published work analysing fish samples [17], with MDL values of 0.6 and 1.3 pg/g dw (or 12 and 26 pg/g lw) for *syn*- and *anti*-DP respectively. These values are five times higher than those obtained in our study by GC–MS–MS. Another work [32], in this case with sludge samples, reported MDLs of 15 and 25 pg/g dw for *syn*- and *anti*-DP respectively. These values were about 10–15 times higher than our MDLs obtained by GC–MS–MS. Other published work [25] reported MDL values obtained by GC–HRMS: MDLs for DP, Dec 602, 603 and 604 ranged from 0.5 to 1 pg/g dw and from 0.5 to 15 pg/g lw, in sediment and fish samples respectively. These values were quite similar to those obtained in our study by GC–MS–MS. Thus, the MS–MS method improved the sensitivity obtained by MS, and it is virtually as sensitive as the HRMS.

3.4. Application to real samples

The developed GC–MS–MS method was finally applied to the determination of halogenated norbornene flame retardants in sediment, sludge and fish samples from the Ebro river basin (Table 4). These results represented the first report of concentration levels of these emerging contaminants in a Spanish aquatic ecosystem. Dec 602, Dec 603, *syn*-DP and *anti*-DP were detected in the three selected matrices, with frequency of detection ranging from 17 to 100%. Dec 604 was not detected in any sample, probably because is not used in this area. Also DPMA was not detected. This compound could be present in fish samples, as it is expected that its

Table 4
Concentration levels (range and median) of dechloranes in sediment (ng/g dw), sludge (ng/g dw) and fish (ng/g lw) samples.

	Dec 602	Dec 603	*syn*-DP	*anti*-DP	F_{anti}	Total DP
Sediment						
fd (%)	25	50	100	100	–	100
Range	nd-0.14	nd-0.26	0.02–0.28	0.05–1.33	0.69–0.83	0.07–1.61
Median	0.14	0.14	0.05	0.13	0.74	0.18
Sludge						
fd (%)	17	100	100	83	–	100
Range	nd-0.24	0.08–0.6	0.85–11.2	nd-11.9	0.63–0.74	2.58–18.8
Median	0.24	0.13	2.33	5.92	0.67	8.69
Fish						
fd (%)[a]	57	88	75	88	–	88
Range	nd-86.1	nd-4.52	nd-0.96	nd-1.28	0.43–0.71	nd-2.24
Median	15.9	1.09	0.52	0.54	0.57	0.88

nd = not detected.
[a] Frequency of detection.

bioavailability is higher than that of DP due to its smaller molecular size. However, as already mentioned above, the acid treatment step applied to fish samples leads to total DPMA loss. We would require the development of a different sample preparation methodology, i.e., substituting the fat removal with an acid attack by a gel permeation chromatography (GPC), to be able to determine the DPMA presence in these fish samples.

In sediment samples, concentration levels ranged from nd to 0.14 ng/g dw for Dec 602, 0.02–0.26 ng/g dw for Dec 603 and 0.07–1.61 ng/g dw for DP. Our Dec 602 and Dec 603 levels are lower than those reported in the Great Lakes (0.001–11 and 0.15–0.28 ng/g dw for Dec 602 and Dec 603 respectively) [26] and in coastal sediments from China (up to 2 ng/g dw for Dec 602) [27]. On the other hand, our DP levels (mean values of 0.05 and 0.13 ng/g dw for *syn*- and *anti*-DP respectively) are considerably lower than those reported from Canada (up to 300 ng/g dw for total DP) [8,9,25] and China (up to 7000 ng/g dw) [7,10]. These differences were expected, since the two DP production facilities are located in North America and China.

Concentration levels in fish ranged from nd to 86.1 ng/g lw for Dec 602, nd to 4.52 ng/g lw for Dec 603 and nd to 2.24 ng/g lw for DP. Unlike to sediment samples, in fish samples Dec 602 presented higher levels than those of DP. This finding is consistent with the previous reported levels in the Great Lakes region, where the authors also found higher values for Dec 602 than for DP (0.47–34 ng/g lw for Dec 602 and 0.03–1.3 ng/g lw for DP) [25]. The differences in $\log K_{OW}$ values for Dec 602 and DP (7.1 and 9.0, respectively) could be an explanation of this trend. Concentration levels for Dec 602 (median value of 16 ng/g lw) are similar to those obtained in fishes from Lake Ontario (18 ng/g lw) but higher than those obtained in other lakes of the Great Lakes region (0.9 ng/g lw) [25]. Regarding to DP, total DP levels ranged from 0.10 to 2.24 ng/g lw, being similar to those reported from the Lake Ontario (0.2–1.9 ng/g lw) [16], but lower than those in China (from 20 to 2000 ng/g lw) [15] or Korea (from 0.6 to 130 ng/g lw) [31].

Finally, concentration levels found in sludge samples ranged from nd to 0.24 ng/g dw for Dec 602, 0.08–0.60 ng/g dw for Dec 603 and 2.58–18.8 ng/g dw for DP. Our DP levels are slightly lower than those found in another study from Spain, with values up to 33 ng/g dw [32] and from North Carolina, with values up to 45 ng/g dw [33]. On the other hand, Dec 602 and Dec 603 levels were much higher than those reported in China (0.01 ng/g dw for Dec 602) [14] and in Spain (up to 0.02 ng/g dw for both Dec 602 and Dec 603) [34].

In order to evaluate the different behavior of both DP isomers, the fraction of *anti*-DP (F_{anti}) is estimated as the concentration of the *anti*-DP divided by the total DP (sum of concentrations of *syn*- and *anti*-DP). In environmental samples (sediments and sludge), the F_{anti} values ranged from 0.63 to 0.83, being similar to those obtained from the technical mixtures (0.75) [21]. However, when we move to fish samples, F_{anti} values decreased (from 0.43 to 0.71). As argued by other authors [26], one possible explanation could be a higher bioaccumulation capacity of the *syn*-DP or because the *anti*-DP could be easily degraded, as has been suggested in other studies.

4. Conclusions

For the first time, an analytical methodology for the analysis of Dechlorane Plus and related compounds by GC–NCI–MS–MS has been optimized. Instrumental parameters such as linearity, reproducibility, repeatability and sensitivity have been calculated. Other parameters such as recovery, reproducibility, MDLs and MQLs were also calculated for two environmental matrices (sediment and sludge) and one biological matrix (fish). Recoveries ranged from 57 to 114%, always with acceptable relative standard deviations. MDLs and MQLs obtained by GC–MS–MS are always lower than those obtained by GC–MS methods, up to 300 times lower. Therefore, this new methodology is adequate for the analysis of these compounds at low environmental levels, giving good sensitivity and selectivity and being a good alternative to the use of the most costly high resolution (HR)MS.

Optimized methodologies were applied to real samples of sediment, sludge and fish from the Ebro river basin (Spain). Dec 602, Dec 603, *syn*-DP and *anti*-DP were detected in the three selected matrices. The detection of these emerging pollutants shows their widespread presence in environment, and therefore the need to carry out further studies in order to better understand their fate and occurrence. Hence the importance to have a methodology with the appropriate sensitivity and selectivity, especially on non-producing areas where lower levels are expected. The analytical methodology developed in this study meets all these requirements, besides being simpler and less expensive than HRMS.

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Publicación científica #2

Gas chromatography/tandem mass spectrometry method for the simultaneous analysis of 19 brominated compounds in environmental and biological samples

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RESEARCH PAPER

Gas chromatography/tandem mass spectrometry method for the simultaneous analysis of 19 brominated compounds in environmental and biological samples

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Abstract A methodology for the simultaneous analysis of eight polybrominated diphenyl ethers (PBDEs); eight methoxylated PBDEs (MeO-PBDEs); and three emerging flame retardants, hexabromobenzene (HBB), pentabromoethyl benzene (PBEB), and decabromodiphenyl ethane (DBDPE) by gas chromatography coupled to tandem mass spectrometry (GC-MS-MS) was developed for two environmental matrices (sediment and sludge) and three biological matrices (fish, dolphin blubber, and bird eggs). The use of selective reaction monitoring (SRM) allows a high selectivity, which is critical in the analysis of complex samples like blubber. Analytical parameters such as linearity, reproducibility, or accuracy were evaluated. Method limits of detection and quantification were evaluated and compared with GC-EI-MS and GC-NCI-MS. Method detection limits were valid for the environmental analysis in all cases, with values between 0.01 and 1.65 ng/g dw for sediment, 0.05 and 2.78 ng/g dw for sludge, 0.04 and 10.6 ng/g lw for fish, 0.01 and 1.11 ng/g lw for dolphin blubber, and 0.03 and 3.20 ng/g lw for bird eggs. The developed method was applied to five samples of each matrix. PBDEs were detected in all samples, while MeO-PBDEs were only detected in dolphin blubber. DBDPE was detected in sediment and sludge.

Keywords Emerging BFRs · GC-MS-MS · MeO-PBDEs · PBDEs

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Introduction

Brominated flame retardants (BFRs) are one of the most used families of flame retardants (FRs), and its demand increases every year. They are used in a wide range of materials such as textiles, furniture, or wire coat materials, where they represent a considerable amount of the total product weight [1]. One of the main BFR families is polybrominated diphenyl ethers (PBDEs) which have been widely used in great amounts for many years. However, this situation has changed and penta- and octa-BDE mixtures are already banned in USA and EU, while the production and usage of deca-BDE formulation is decreasing [2]. Thus, other alternative FRs such as hexabromobenzene (HBB), pentabromoethyl benzene (PBEB), and decabromo diphenyl ethane (DBDPE) have been proposed as an alternative [3]. In contrast to the few studies reporting environmental levels for these new compounds, PBDEs have been found since 1970 [4] in several environmental and biological matrices such as sediment [5], sludge [6], air [7], fish [8], bird eggs [9], or cetaceans [10]. Besides, they have been found in remote places such the Artic, proving their wide-range transport [11].

On the other hand, other brominated compounds naturally produced by sponges or red algae have proved to be present in the environment in similar levels to PBDEs, sometimes even at higher concentrations [12]. Methoxylated PBDEs (MeO-PBDEs) represent an example of these halogenated natural products (HNPs). They have been found in several cetaceans [13] and also in fish [14] around the world.

Brominated compounds were normally analyzed by GC-NCI-MS, which provides a great sensitivity. But, on the other hand, it is a low selectivity technique. Thus, problems related to the coelution of some relevant compounds might occur. The objective of this work was to develop a selective technique for the analysis of these brominated compounds in environmental (sediment and sludge) and biological (fish, dolphin blubber,

and bird egg) matrices by gas chromatography coupled to tandem mass spectrometry (GC-MS-MS). Other works have pointed out the importance of using GC-MS-MS for the analysis of some of these compounds [15–17], but this work is the first which includes such a wide range of matrices and the simultaneous determination of all these compounds. In addition to the selectivity obtained by using MS-MS instead of MS, this technique allows the usage of mass-labeled standards for isotope dilution quantification. Some analytical parameters that are not usually given, such as accuracy, together with recovery values and limits of detection were evaluated.

Materials and methods

Standards and reagents

Method 1614 Surrogate Stock Solution (PAR Solution) containing tri-BDE-28, tetra-BDE-47, penta-BDE-99, penta-BDE-100, hexa-BDE-153, hexa-BDE-154, hepta-BDE-183, and deca-BDE-209; a mixture of MeO-PBDEs containing 5-MeO-BDE-47, 6-MeO-BDE-47, 4-MeO-BDE-49, 2-MeO-BDE-68, 5′-MeO-BDE-99, 5-MeO-BDE-100, 4′-MeO-BDE-101, and 4-MeO-BDE-103; and also HBB, DBDPE, and PBEB were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Method 1614 Labeled Surrogate Stock Solution containing the mass labeled PBDEs (^{13}C-BDE-28, ^{13}C-BDE-47, ^{13}C-BDE-99, ^{13}C-BDE-100, ^{13}C-BDE-154, ^{13}C-BDE-153, ^{13}C-BDE-183, and ^{13}C-BDE-209) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA)

Alumina (0.063–0.2 mm) and copper (<63 μm) were obtained from Merck (Darmstadt, Germany). Al-N and silica cartridges were obtained from Biotage. Dichloromethane and hexane, solvents for organic trace analysis, were purchased from Merck.

Sample collection

Several samples of sediment, sludge, fish, dolphin blubber, and bird egg were analyzed in order to evaluate the performance of the developed methodology in real samples. Among the Llobregat River Basin (Spain), five sediment samples from five different sampling points were collected with a Van Veen drag while five different sludge samples were collected from five different waste water treatment plants (WWTPs). Moreover, five fish samples from different sampling points of the same river were collected by DC electric pulse. Furthermore, dolphin blubber samples from *Tursiops truncatus* were obtained by biopsy sampling in the Gulf of Cadiz, and white stork egg samples that had failed to hatch were collected from Doñana National Park, in southern Spain. All the samples were homogenized and freeze-dried. Sediment and sludge samples were sieved (120 μm). All samples were kept at −20 °C until analysis.

Sample preparation

The sample methodologies used were similar to those previously optimized for the extraction and purification of PBDEs [18–20]. In the case of sediment, selective pressurized liquid extraction (SPLE) was applied. However, this methodology could not be applied for the other matrices since all (sludge, fish, dolphin blubber, and bird egg) require a more complex cleanup and lipid content determination in the case of biota. Normal PLE was used in these cases. Both SPLE and PLE were carried out in an ASE 350 system (Dionex, Sunnywale, CA, USA).

Sediment

Before extraction, 1 g dry weight (dw) was spiked with 5 ng of the mass labeled PBDEs (50 ng for ^{13}C-BDE-209). Samples were kept overnight to equilibrate and were grown with alumina and copper (1:2:2) and loaded into a 22-mL extraction cell previously filled with alumina (6 g). Hexane:dichloromethane (1:1) were used as extraction solvents, with a temperature of 100 °C and a pressure of 1500 psi. Two static cycles of 10 min were made. Extracts were concentrated to incipient dryness and reconstituted in 40 μL of toluene prior to instrument analysis.

Sludge

Before extraction, 1.5 g dw were spiked with 10 ng of ^{13}C-PBDEs and 100 ng of ^{13}C-BDE-209. Samples were grown with copper (1:2) and loaded into the extraction cell. PLE conditions were the same as the ones described before. Resulting extracts were treated with sulphuric acid and then purified with two consecutive solid phase extraction (SPE) since sludge needs a more complex cleanup than the other matrices. The first one was done using silica cartridges (2 g) and the second one using alumina cartridges (5 g). Resulting extracts were concentrated and reconstituted as described before.

Biota samples

Before extraction, 1 g of sample was spiked with 5 ng of ^{13}C-PBDEs and 50 ng of ^{13}C-BDE-209. Samples were kept overnight to equilibrate and then loaded into an 11-mL extraction cell. PLE extractions were done using the same parameters than for sediments. Extracts were evaporated and, after gravimetric determination of the lipid content, redissolved in hexane. Fat was removed with $H_2SO_{4(conc.)}$, and the extracts were

purified with SPE using alumina cartridges (AL-N, 5 g). Resulting extracts were concentrated to 40 μL in toluene.

Instrumental determination

GC-MS-MS analyses were performed on an Agilent Technologies 7890A GC system coupled to a 7000A GC/MS Triple Quadrupole. A DB-5ms capillary column (15 m×0.1 mm i.d., 0.1 μm film thickness) was used for the chromatographic separation with helium as carrier gas. Different temperature ramps were tested. The final gradient started at 140 °C, held for 1 min and then ramped to 310 at 10 °C/min and held for 10 min, for a total run time of 36.5 min.

Two different ionization modes, negative chemical ionization and electron ionization (NCI and EI, respectively), were tested. It is well known that NCI provides a greater sensitivity for halogenated compounds than EI, while on the other hand, EI is a more selective technique. Obtained transitions for PBDEs using NCI-MS-MS did not provide acceptable results in terms of sensitivity. Moreover, the use of EI allows the quantification by isotope dilution, which is far more reliable than using other non-labeled standards. Besides, we compared also EI-MS with EI-MS-MS. The selectivity obtained when using MS-MS is especially important when analyzing complex matrices since there are lot of possible interferences. After comparing the different methodologies, EI-MS-MS was chosen. Selective reaction monitoring (SRM) mode was used with two transitions monitored for each compound. The most intense transitions were used for quantification purposes, and the second ones for confirmation criteria comparing the SRM1/SRM2 ratio calculated for the standards with the ratio found in the samples. Table 1 shows the retention time (Rt) and collision energies (CE) for all the compounds studied. Other MS parameters such as ion source temperature (T), gas flow (GF), injector temperature (IT), and transfer line temperature (LT) were also optimized in order to increase the signal. The optimum values were 300 °C, 2.25 ml/min, 280 °C, and 280 °C, respectively. Ionization energy was set at 70 eV since it is the standard energy used in this technique.

Analytical parameters

Linearity was determined by a six-point calibration curve including all the analytes, with concentrations ranging from 10 to 1000 pg/μL, and the internal standards in a concentration of 100 pg/μL. Standards were prepared in toluene and stored at −20 °C. Repeatability was measured by the relative standard deviation (RSD) of five consecutive injections (intra-assay) and three injections on four different days (inter-assay). Accuracy was measured by the percent deviation (%Dev) of the nominal concentration both for intra- and inter-assays. Both repeatability and accuracy were assessed in three different concentration levels: low level (0.025 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 0.25 ng/ μL for BDE-209; and 0.06 ng/ μL for DBDPE), medium level (0.2 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 2 ng/μL for BDE-209; and 0.5 ng/μL for DBDPE);,and high level (1 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 10 ng/μL for BDE-209; and 2.5 ng/μL for DBDPE).

Instrumental detection limits (IDLs) were determined for each compound as the minimum amount of analyte that gave a signal to noise ratio (S/N) of 3, and the instrumental quantification limits (IQLs) were determined as the minimum amount of analyte that gave a S/N of 10.

Recovery, repeatability, and accuracy were also measured (intra- and inter-assay) for the five matrices studied at three concentration levels by spiking 1 g of sample with 5 ng of PBDEs, MeO-PBDEs, HBB, and PBEB, 50 ng of BDE-209 and 25 ng of DBDPE (low level); 20 ng of PBDEs, MeO-PBDEs, HBB, and PBEB, 200 ng of BDE-209 and 100 ng of DBDPE (medium level); and 50 ng of PBDEs, MeO-PBDEs, HBB, and PBEB, 500 ng of BDE-209 and 250 ng of DBDPE (high level). Five replicates were done for each matrix and level except for dolphin blubber, where due to sample availability only three replicates were made for medium and high levels. Three blank samples were made for each matrix in order to evaluate the presence of these compounds in the matrices used, correcting the value obtained when the contribution was higher than 5 %. However, the contribution never exceeded a 10 % of the total value except for dolphin blubber, which was one of the reasons to only choose medium and high level for this matrix.

Method detection and quantification limits (MDL and MQL, respectively) were calculated using low level points for sediment, sludge, fish, and bird egg, and medium level for dolphin blubber, by the same method used to calculate IDLs and IQLs.

Results and discussion

GC-MS-MS conditions

Two different columns were tested using different temperature programs: a DB-5ms capillary column (15 m×0.1 mm i.d., 0.1 μm film thickness) and a DB-5ms capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness). A 30-m column is used in some studies to achieve a total separation of all the analytes but, on the other hand, BDE-209 is completely lost due to thermal degradation also due to the long retention time in these columns [21]. Since the separation obtained for all the analytes was satisfactory for all the compounds with the temperature program used, the 15-m column was chosen.

Several MS parameters were optimized in order to maximize the signal. The GF was tested modifying its value

Table 1 Retention times, two transitions (SRM_1 and SRM_2), and the collision energies (eV) of each one

	Compound	Rt	SRM_1	CE_1	SRM_2	CE_2
PBDEs	BDE-28	10.8	408>246	25	408>248	25
	BDE-47	12.8	486>326	30	488>328	30
	BDE-100	14.1	406>297	30	564>404	30
	BDE-99	14.6	406>297	30	564>404	30
	BDE-154	15.6	486>377	30	644>484	30
	BDE-153	16.2	486>377	30	644>484	30
	BDE-183	17.7	721>562	30	721>564	30
	BDE-209	23.2	298>220	25	361>280	25
MeO-PBDEs	2-MBDE-68	13.5	516>356	25	516>358	25
	6-MBDE-47	13.8	516>356	25	516>358	25
	5-MBDE-47	14.2	516>356	25	516>358	25
	4-MBDE-99	14.4	516>356	25	516>358	25
	5-MBDE-100	15.1	596>434	30	594>436	30
	4-MBDE-100	15.2	596>434	30	594>436	30
	5-MBDE-99	15.8	596>434	30	594>436	30
	4-MBDE-101	15.9	596>434	30	594>436	30
Emerging BFRs	HBB	17.1	468>308	25	468>310	25
	PBEB	11.1	500>485	30	485>406	30
	DBDPE	24.4	485>406	25	325>165	25

Rt retention times, *CE* collision energies

between 1.25 and 2.5 mL/min (1.25, 1.5, 1.75, 2, 2.25, and 2.5). The optimal value was 2.25 mL/min. ST was tested modifying its value from 150 to 300 °C (150, 200, 250, and 300 °C). The optimal value was set at 250 °C. Higher temperatures caused the degradation of the high brominated compounds such as BDE-209.

The fragmentations of the precursor ion for PBDEs proved to be dependent on the bromination degree. Sánchez-Ávila et al. [15] reported different fragmentation patterns for tri-, tetra-, penta-, hexa-, hepta-, and deca-BDEs. For tri- and tetra-BDEs, parent ions $[M]^+$ and $[M+2]^+$ gave di-brominated product ions ($[M-2Br]^+$ and $[M-2Br+2]^+$, respectively). Penta- and hexa-BDEs had the same parent ions, $[M-2Br]^+$ and $[M+2]^+$, but different product ions. Penta-BDEs gave $[M-COBr]^+$ and $[M-2Br+2]^+$, while hexa-BDEs gave $[M-4Br]^+$ and $[M-2Br+2]^+$. The fragmentation described by Sánchez-Ávila et al. for hepta-BDEs and deca-BDE was the same as for hexa-BDEs. However, these transitions were not optimal for hepta-BDE (BDE-183) and deca-BDE (BDE-209). We found other transitions that gave a much better response: for BDE-183, $[M]^+$ as parent ion gave $[M-2Br]^+$ and $[M-2Br+2]^+$ as product ions and for BDE-209, parent ions $[M-O-8Br]^+$ and $[M-CO-7Br]^+$ gave $[M-O-9Br]^+$ and $[M-CO-8Br]^+$ as product ions. The same transitions were used by Law et al. [22].

Regarding MeO-PBDEs, MeO-tetra-, and MeO-penta-BDEs presented the same pattern. For MeO-tetra-BDEs parent ion $[M+4]^+$ gave $[M-2Br+2]^+$ and $[M-2Br+4]^+$ as product ions, while for MeO-penta-BDEs, parent ion $[M+6]^+$ gave $[M-2Br+2]^+$ and $[M-2Br+4]^+$ as products ions. Despite the fact that other fragmentation patterns have been described for the *ortho*- substituted MeO-PBDEs [23], with $[M-Br-CH3]^+$ as product ion, the most abundant transitions were the ones described before and were considered good enough in terms of selectivity and sensitivity.

Emerging FRs, HBB, PBEB, and DBDPE presented different fragmentation patterns. For HBB, parent ion $[M-Br]^+$ gave $[M-3Br]^+$ and $[M-3Br+2]^+$ as product ions and for PBEB, parent ion $[M+4]^+$ gave $[M-CH3+4]^+$ as product ion and $[M-CH3+4]^+$ was also used as parent ion with $[M-Br-CH3+4]^+$ as product ion. Finally, two product ions were used for DBDPE, $[M-6Br]^+$ and $[M-8Br-5H]^+$, which gave $[M-7Br]^+$ and $[M-10Br-5H]^+$ as product ions.

Analytical parameters

Table 2 shows the instrumental parameters evaluated: linearity, sensitivity, and reproducibility. Regarding linearity, the calibration curve was linear at a range from 10 to 1000 pg/μL, with correlation coefficients (r) between 0.9986 and 0.9999. IDLs ranged from 0.11 to 16.3 injected pg, while IQLs ranged from 0.35 to 54.3 injected pg. For reproducibility, RSD values were lower than 20 % for all the analytes both for inter- and intra-assay experiments and at the three concentration levels. In intra-assay experiments, RSD values ranged from 1.3 to 18 %. Similarly, values in inter-assay experiments ranged from 2.6 to 19 %.

Table 2 Instrumental detection and quantification limits, in injected pg, and reproducibility (relative standard deviation, %) for intra- and interday experiments at the three concentration levels

		R^2	IDL	IQL	Intraday			Interday		
					1	2	3	1	2	3
PBDEs	BDE-28	0.998	0.11	0.35	1.73	3.63	1.75	2.58	3.27	9.55
	BDE-47	0.999	0.18	0.61	4.38	2.32	1.27	7.44	6.25	8.72
	BDE-100	0.996	0.43	1.44	6.34	2.14	4.77	8.71	5.22	8.75
	BDE-99	0.998	0.54	1.81	11.9	4.54	3.75	12.9	7.81	7.29
	BDE-154	0.997	2.27	7.58	5.99	7.40	5.25	5.98	6.62	10.3
	BDE-153	0.997	3.57	11.9	7.32	5.39	8.02	12.4	5.02	7.22
	BDE-183	0.998	12.5	41.7	14.1	9.38	11.4	16.4	14.8	13.8
	BDE-209	0.997	16.3	54.4	7.65	1.45	2.77	7.71	9.37	3.00
MeO-PBDEs	2-MBDE-68	0.999	1.06	3.52	14.3	4.12	8.23	13.1	3.74	7.44
	6-MBDE-47	0.999	0.83	2.78	11.8	3.09	6.79	10.7	2.80	6.28
	5-MBDE-47	0.996	0.47	1.57	11.3	3.52	8.74	11.6	4.18	7.84
	4-MBDE-99	0.996	1.14	3.79	9.09	3.98	7.31	8.85	3.84	6.57
	5-MBDE-100	0.997	3.41	11.4	11.4	8.22	12.4	10.3	8.06	11.7
	4-MBDE-100	0.998	9.38	31.3	17.1	7.22	9.44	15.4	10.6	8.56
	5-MBDE-99	0.998	5.77	19.2	5.60	11.3	11.6	7.93	10.1	10.9
	4-MBDE-101	0.998	4.69	15.6	13.8	10.7	10.3	12.7	11.4	9.49
Emerging BFRs	HBB	0.999	1.32	4.39	6.73	9.25	9.68	6.09	8.29	8.67
	PBEB	0.999	0.90	3.01	8.52	5.07	6.04	8.48	5.34	18.9
	DBDPE	0.996	6.25	20.8	7.14	3.54	18.2	13.2	18.8	15.5

Level 1: 0.025 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 0.25 ng/ μL for BDE-209; and 0.06 ng/μL for DBDPE.
Level 2: 0.2 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 2 ng/μL for BDE-209; and 0.5 ng/μL for DBDPE.
Level 3: 1 ng/μL for PBDEs, MeO-PBDEs, HBB, and PBEB; 10 ng/μL for BDE-209; and 2.5 ng/ μL for DBDPE

IDLs instrumental detection limits, *IQLs* instrumental quantification limits, *RSD* relative standard deviation

Recoveries, reproducibility, accuracy, and MDLs and MQLs were calculated for the five different matrices at three different concentration levels (Table 3). As said before, blanks were used to subtract the natural content that samples might had but the values found never surpassed a contribution of 10 % to the total value.

Even though the extraction methodologies used had already proved to provide good recoveries in these matrices, recovery values for these experiments were calculated. Recoveries for PBDEs ranged from 75 to 96 %, from 52 to 67 %, from 57 to 77 %, from 53 to 82 %, and from 57 to 87 % in sediment, sludge, fish, bird egg, and dolphin blubber, respectively. MeO-PBDEs were well recovered as well, with values ranging from 78 to 91 %, from 53 to 68 %, from 51 to 77 %, from 58 to 83 %, and from 70 to 77 % in sediment, sludge, fish, bird egg, and blubber, respectively. Finally, HBB, PBEB, and DBDPE recoveries ranged from 103 to 105 % in sediment, from 52 to 66 % in sludge, from 68 to 80 % in fish, from 70 to 78 % in bird eggs, and from 71 to 76 % in dolphin blubber. As expected, sediment was the matrix which presented the best recovery values and sludge was the matrix which gave the lowest ones.

Regarding intraday assays, RSD values ranged from 0.9 to 7.5 % in sediment, from 1.1 to 18 % in sludge, from 1.1 to 12 % in fish, from 1.81 to 18.0 % in dolphin blubber, and from 2.5 to 11 % in bird egg. For interday assays, RSD values ranged from 1.7 to 17 % in sediment, from 2.7 to 19 % in sludge, from 3.2 to 19 % in fish, from 2.33 to 13.3 % in dolphin blubber, and from 2.0 to 20 % in bird egg.

Regarding the accuracy, expressed as percent deviation (%Dev), values obtained for intra- and inter-assays were satisfactory for all the matrices at the three levels since values of the |%Dev| were always lower than 15 %. In sediment, values ranged from −8.2 to 10 %; in sludge, values ranged from −13 to 13 %; in fish values ranged from −11 to 15 %, while in dolphin blubber, values ranged from −9.12 to 10.7 %. Finally, values in bird egg ranged from −13 to 15 %. As happened for the precision, accuracy values were not statistically different between intra- and inter- assays in most of the cases. This is attributed to the fact that the methodology provides consistent values through time, even though some variations, despite being in acceptable ranges, are quite high. Moreover, not positive or negative values prevailed over the other for the same matrix.

Table 3 Recovery values, method detection and quantification limits for the five matrices studies

		Sediment (ng/g dw)			Sludge (ng/g dw)			Fish (ng/g lw)			Dolphin blubber (ng/g lw)			Bird egg (ng/g lw)		
		R (%)	MDL	MQL	R (%)	MDL	MQL	R (%)	MDL	MQL	R (%)	MDL	MQL	R (%)	MDL	MQL
PBDEs	BDE-28	96	0.01	0.03	65	0.05	0.17	72	0.04	0.12	87	0.01	0.04	82	0.03	0.09
	BDE-47	96	0.01	0.04	67	0.05	0.17	77	0.05	0.18	81	0.01	0.05	72	0.03	0.10
	BDE-100	91	0.02	0.07	65	0.11	0.38	67	0.20	0.67	77	0.06	0.19	67	0.12	0.41
	BDE-99	75	0.03	0.09	61	0.10	0.33	63	0.29	0.97	81	0.03	0.09	73	0.13	0.45
	BDE-154	95	0.08	0.25	65	0.63	2.08	59	0.43	1.42	75	0.21	0.69	57	0.35	1.17
	BDE-153	87	0.12	0.39	59	0.79	2.63	73	0.64	2.13	71	0.13	0.42	53	0.31	1.02
	BDE-183	95	0.39	1.32	59	1.36	4.55	61	3.19	10.6	56	1.39	4.63	61	1.51	5.02
	BDE-209	83	1.65	5.49	51	2.78	9.26	57	10.6	35.4	62	1.11	3.69	57	3.20	10.7
MeO-PBDEs	2-MBDE-68	87	0.07	0.22	62	0.22	0.74	68	1.06	3.54	77	0.09	0.31	58	0.59	1.95
	6-MBDE-47	87	0.05	0.16	60	1.36	4.55	77	0.43	1.42	75	0.24	0.79	67	0.25	0.85
	5-MBDE-47	91	0.03	0.09	53	1.00	3.33	65	0.43	1.42	71	0.06	0.19	65	0.28	0.92
	4-MBDE-99	89	0.07	0.24	53	0.75	2.50	51	2.13	7.09	74	0.16	0.53	61	0.46	1.55
	5-MBDE-100	78	0.13	0.43	56	1.36	4.55	66	1.99	6.64	76	0.31	1.04	76	0.59	1.98
	4-MBDE-100	89	0.38	1.25	68	1.67	5.56	73	2.20	7.33	77	1.59	5.29	83	2.01	6.70
	5-MBDE-99	91	0.16	0.53	55	1.00	3.33	63	3.75	12.5	70	0.47	1.57	63	0.54	1.80
	4-MBDE-101	80	0.22	0.75	64	1.50	5.00	74	3.19	10.6	76	0.80	2.68	73	0.83	2.76
Emerging BFRs	HBB	105	0.03	0.11	66	0.35	1.16	80	0.20	0.67	76	0.06	0.21	75	0.12	0.39
	PBEB	104	0.04	0.14	64	0.56	1.85	70	0.18	0.61	71	0.06	0.19	70	0.14	0.47
	DBDPE	103	0.11	0.37	52	0.94	3.12	58	9.66	32.2	72	1.06	3.52	78	3.54	11.8

R recovery, *MDLs* method detection limits, *MQLs* method quantification limits

Furthermore, MDLs and MQLs are shown in Table 3. The low brominated PBDEs presented better MDLs and MQLs in all cases, while high brominated PBDEs presented the highest values in all the matrices. The same was observed for MeO-PBDEs since the tetra-brominated congeners gave lower values than the penta-brominated congeners. Values obtained for sediment were considerably lower than in sludge, as expected, since sediment is a cleaner matrix. With the exception of BDE-28 and BDE-47, where MDLs in sediment were only up to five times lower, in some cases, the values in sediments were up to 12 times lower than in sludge (i.e., PBEB and HBB). Regarding biological matrices, dolphin blubber and bird egg showed similar MDLs (0.01–1.59 ng/g lw and 0.03–3.54 ng/g lw, respectively), while fish showed slightly higher values (0.04–10.6 ng/g lw). The sensitivity of our developed method was compared with previous published works analyzing PBDEs and MeO-PBDEs by EI-MS-MS. Our IDLs are lower than those reported by Sanchez-Ávila [15] which ranged from 1.5 to 10 injected pg. In this work, BDE-183 and BDE-209, the less sensitive BDE congeners, were not included. So, our IDLs for PBDEs ranged from 0.11 to 3.57 injected pg, which represent an improvement of IDL values up to 13 times less. Similarly, the MDLs they reported for sediment (11 to 44 ng/g dw) were also higher than those obtained in our study (0.008 to 1.68 ng/g dw). On the other hand, our IDLs are similar to those reported by Losada et al. (0.9 to 2.5 injected pg for PBDEs and 0.4 to 1.5 injected pg for MeO-PBDEs). Losada et al. also reported MDLs in whale blubber which were lower than those reported in our study, with values ranging from 0.03 to 0.29 ng/g lw and from 0.05 to 0.18 ng/g lw for MeO-PBDEs and PBDEs, respectively [14].

Due to its instability at the high temperatures, the analysis of BDE-209 by GC-MS-MS represents a challenge since it is difficult to obtain a proper parent ion. Most of the published works do not include this compound in the method. Our IDLs were comparable to those obtained by Law et al. [22], who also included BDE-209 with values ranging from 0.5 to 75 injected pg. Law et al. also reported IDLs for HBB and PBEB, which were similar to those we obtained: 0.9 toward 0.4 and 1.3 toward 1.0 for HBB and PBEB for Law et al. and our methodology, respectively.

EI-MS versus EI-MS-MS

EI-MS and EI-MS-MS were compared in terms of sensitivity. Table 4 shows the MDLs obtained by both methodologies for the several matrices studied spiked at low level, with the exception of dolphin blubber where the medium level was used for the calculations. Since MS-MS provides much better

Table 4 Method detection limits by EI-MS and EI-MS-MS for all the compounds studied

		Sediment (ng/g dw)		Sludge (ng/g dw)		Fish (ng/g lw)		Dolphin blubber (ng/g lw)		Bird egg (ng/g lw)	
		MS	MS-MS	MS	MS-MS	MS	MS-MS	MS	MS-MS	MS	MS-MS
PBDEs	BDE-28	0.06	0.01	0.18	0.05	0.28	0.04	0.11	0.01	0.42	0.03
	BDE-47	0.03	0.01	0.10	0.05	0.32	0.05	0.15	0.01	0.52	0.03
	BDE-100	0.06	0.02	0.44	0.11	0.88	0.20	0.34	0.06	1.37	0.12
	BDE-99	0.06	0.03	0.27	0.10	1.46	0.29	0.43	0.03	1.69	0.13
	BDE-154	0.33	0.08	1.50	0.63	11.2	0.43	0.42	0.21	1.35	0.35
	BDE-153	0.30	0.12	1.76	0.79	11.3	0.64	0.59	0.13	1.33	0.31
	BDE-183	2.54	0.39	–	1.36	4.11	3.19	2.17	1.39	–	1.51
	BDE-209	–	1.65	–	2.78	–	10.6	53.1	1.11	185	3.20
MeO-PBDEs	2-MBDE-68	0.23	0.07	4.11	0.22	15.4	1.06	0.61	0.09	1.53	0.59
	6-MBDE-47	0.16	0.05	1.52	1.36	2.87	0.43	0.53	0.24	0.50	0.25
	5-MBDE-47	0.24	0.03	2.05	1.00	5.25	0.43	0.56	0.06	1.78	0.28
	4-MBDE-99	0.17	0.07	1.89	0.75	5.08	2.13	0.42	0.16	0.92	0.46
	5-MBDE-100	0.54	0.13	5.17	1.36	10.5	1.99	0.44	0.31	1.70	0.59
	4-MBDE-100	0.73	0.38	6.98	1.67	15.0	2.20	0.49	1.59	1.59	2.01
	5-MBDE-99	0.56	0.16	8.88	1.00	18.1	3.75	0.81	0.47	2.77	0.54
	4-MBDE-101	0.76	0.22	8.20	1.50	22.9	3.19	0.53	0.80	2.06	0.83
Emerging BFRs	HBB	–	0.03	–	0.35	3.99	0.20	0.98	0.06	5.64	0.12
	PBEB	0.16	0.04	2.12	0.56	3.54	0.18	0.22	0.06	0.18	0.14
	DBDPE	3.44	0.11	18.5	0.94	72.4	9.66	2.88	1.06	7.35	3.54

MDLs method detection limits

selectivity than MS, the signal to noise (S/N) ratio decreases considerably, providing better MDLs or even allowing the determination of the compound while it was not possible by EI-MS. For instance, BDE-209 could not be determined by EI-MS in sediment, sludge, and fish, while it could be determined by EI-MS-MS. When using EI-MS-MS, MDLs improved considerably: from 2 to 8 times in sediment, from 2 to 18 times in sludge, from 2 to 26 times in fish, from 1.5 to 48 times in dolphin blubber, and from 1.3 to 47 times in bird egg.

Moreover, Fig. 1 shows several chromatograms from real samples of fish and sludge where the difference between EI-MS and EI-MS-MS can be clearly seen. The use of MS-MS allows the correct identification of BDE-100 and BDE-99 due to its higher selectivity, while several unknown peaks appear when using MS. In addition, BDE-209 could not be determined in sludge by MS, whereas the S/N is reduced considerably with the use of MS-MS and thus BDE-209 can be correctly identified and determined.

Application to real samples

The optimized methodology was applied to five different samples for each matrix, as described in section "Sample collection." Different PBDEs were detected in all matrices, while MeO-PBDEs were only detected in dolphin blubber. This fact was expectable since, as explained before, MeO-PBDEs are only found in marine environment. Unfortunately, we could not obtain marine samples of the other matrices. On the other hand, HBB and PBEB were not detected in any sample, whereas DBDPE was only detected in environmental samples (sediment and sludge). Results are summarized in Table 5. Several PBDEs (from tetra-brominated to deca-brominated) were detected in sediments from the Llobregat River Basin, with BDE-209 as the most abundant compound. Total PBDE levels ranged from 2.50 to 48.1 ng/g dw and were slightly higher than the ones that Labandeira et al. reported for the same river in 2007 [19]. On the other hand, Guerra et al. reported higher levels (from 22 to 136 ng/g dw) in sediments from the same river [5], so there is a great variation on the levels depending on the sampling points and year. DBDPE was also detected with levels ranging from not detected (nd) to 30.7 ng/g dw. Kierkegaard et al. detected similar levels (24 ng/g dw) in sediment samples from the Netherlands [24].

Regarding sludge, several PBDEs and DBDPE were detected. Total PBDE levels ranged from nd to 250 ng/g dw and, in the same way than sediments, BDE-209 was the most abundant compound; DBDPE levels ranged from nd to 100 ng/g dw. Our values are clearly lower than those reported by De la Torre et al. (from 58 to 2606 ng/g dw) [25] and Gorga

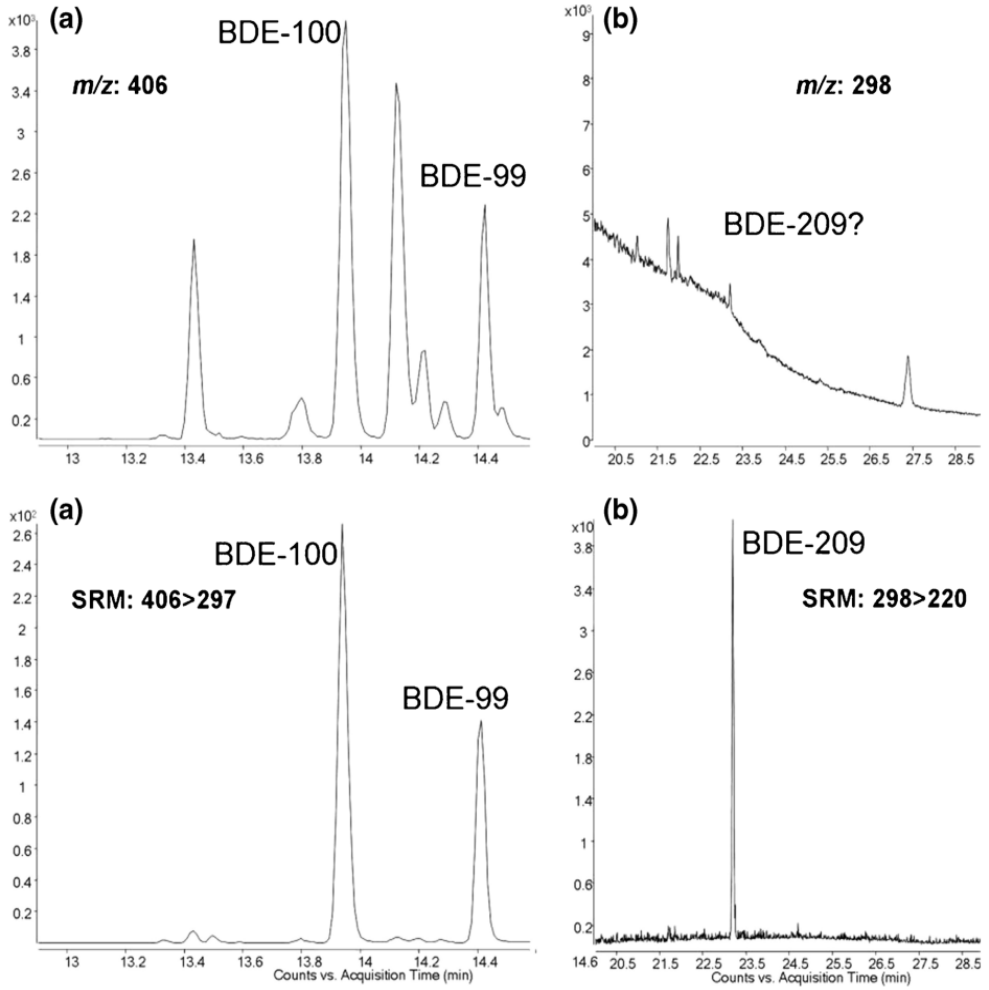

Fig. 1 Comparison between SIM-EI (*up chromatograms*) and SRM-EI (*down chromatograms*) of the same **a** fish and **b** sludge samples

et al. (up to 2303 ng/g dw) [6] in different WWTPs from Spain. On the other hand, our values were similar to ones reported by De la Torre et al., which ranged from 3.24 to 125 ng/g dw [26].

PBDEs were also detected in fish samples. In this case, BDE-47 was the most abundant compound, which is in agreement with other published works. Total PBDE levels ranged from nd to 248 ng/g lw and were in the same range than other study carried out in the Llobregat river by Labandeira et al., who reported PBDE concentrations ranging from 28.8 and 744 ng/g lw [19]. However, since the fish species are different, these results have to be compared with caution. PBDEs have been analyzed in fish worldwide, with a great variability on the levels reported [27].

Table 5 Levels found in sediment and sludge (ng/g dw) and in fish, dolphin blubber, and bird egg (ng/g lw). $N=5$ in all matrices

	Sediment	Sludge	Fish	Blubber	Bird egg
PBDEs	2.51–48.1	nd–250	nd–248	15.5–1350	5.51–40.4
MeO-PBDEs	nd	nd	nd	50.1–1244	nd
HBB	nd	nd	nd	nd	nd
PBEB	nd	nd	nd	nd	nd
DBDPE	nd–30.7	nd–100	nd	nd	nd

nd not detected

Moreover, both PBDEs and MeO-PBDEs were detected in dolphin blubber. BDE-47 was the most abundant PBDE, and 6-MeO-BDE-47 was the most abundant MeO-PBDE. Total PBDE levels ranged from 15.5 to 1350 ng/g lw while total MeO-PBDE levels ranged from 50.1 to 1244 ng/g lw. Dolphins are known to be at the top of the food chain and usually present high PBDE and MeO-PBDE burdens. Recently, Alonso et al. reviewed all the studies reporting PBDEs and MeO-PBDEs around the world [13]. Our results are in the middle of the total range

since there are studies which report concentrations up to 13,000 ng/g lw of PBDEs or MeO-PBDEs.

Finally, PBDEs were also detected in white stork eggs, with levels ranging from 5.51 to 40.4 ng/g lw. BDE-209 was the most abundant compound, which is surprising but in agreement with Muñoz-Aranz et al., who reported the predominance of BDE-209 also in white stork eggs from Doñana National Park [28]. Our values are lower than the ones reported for the same species in the same location, which ranged from 2.92 to 129 ng/g lw [28]. However, these samples were taken in 1999–2000, which could explain this variation. To our knowledge, the higher PBDE values reported for bird eggs were reported for peregrine falcons from the Great Lakes, with values ranging from 530 to 38,000 ng/g lw [9]

Conclusions

An analytical methodology for the simultaneous analysis of eight PBDE congeners (from tri- to deca-BDEs), eight MeO-PBDEs, and three emerging BFRs in two environmental matrices (sediment and sludge) and three biological matrices (fish, dolphin blubber, and bird egg) by GC-EI-MS-MS was developed. The methodology provided MDLs and MQLs adequate for the analysis of these compounds in the environment, and other analytical parameters such as accuracy or precision were also evaluated for all the matrices. Furthermore, differences between NCI and EI, and between EI-MS and EI-MS-MS were studied. Even though NCI is more sensible than EI, the improvement on the selectivity of the EI was considered the main factor to take into account considering the problems that occur in environmental analysis. In addition, EI allows the use of mass labeled standards providing a more reliable quantification. Besides, EI-MS-MS proved to be more sensitive than EI-MS.

The methodology was applied to several samples for each matrix studied. PBDEs were detected in the five matrices, with different levels and congener distributions in environmental and biological samples. DBDPE was only detected in sediment and sludge, while MeO-PBDEs were only detected in the only marine matrix, dolphin blubber.

This methodology allows the reliable determination of these compounds in a wide number of matrices. Important analytical parameters, which are rarely given in other works, were satisfactory and met the requirements for this kind of analysis.

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2.3. Discusión

Las dos metodologías desarrolladas han permitido el análisis de los diferentes HFRs en diferentes muestras ambientales y biológicas de manera sensible y selectiva, aportando nuevos datos sobre su presencia y comportamiento en el medio ambiente. Esta información, especialmente para los HNs, aún es relativamente escasa. A continuación se explica el proceso seguido para el desarrollo de ambos métodos.

2.3.1. Decloranos

Se ha desarrollado una metodología para el análisis del Dec 602, Dec 603, Dec 604, *syn*- y *anti*-DP mediante GC-NCI-MS-MS, previa optimización de una metodología mediante GC-NCI-MS, utilizando CH_4 como gas reactivo. También se incluyeron el mono aducto del DP (DPMA, del inglés dechlorane plus mono adduct) y el mirex. El primero es producto de una reacción Diels-Alder inversa que origina el mono aducto del DP (Figura 2.1). Existen evidencias de que este compuesto está presente tanto en sedimentos como en biota (Guerra *et al.,* 2011b; Sverko *et al.,* 2011). No obstante, el doble enlace expuesto que presenta hace que no sea estable en H_2SO_4 y por tanto no es posible detectarlo en biota o lodos con nuestra metodología de purificación actual. Pese a que en un principio se planteó la posibilidad de optimizar una metodología de purificación mediante GPC, se acabó descartando la idea por falta de tiempo y por tanto la determinación del DPMA quedó limitada a las muestras de sedimentos.

Como se ha comentado, en la fecha de comienzo de esta tesis la mayoría de metodologías para el análisis de estos compuestos (normalmente sólo el DP) eran GC-NCI-MS o GC-HRMS (Feo *et al.,* 2012; Kolic *et al.,* 2009; Sverko *et al.,* 2011; Xian *et al.,* 2011). Tras la evidencia de su presencia en el medio ambiente, se habían incluido en los métodos de rutina ya que por sus características fisicoquímicas encajaban bien con los métodos de extracción habituales. Sin embargo, en general había un vacío en cuanto a la información sobre sus recuperaciones y límites de detección, por lo que no quedaba claro si realmente analizarlos utilizando las mismas condiciones que para otros HFRs era la mejor opción. Primero se desarrolló un programa de temperatura que permitiera separar bien todos los compuestos incluyendo los 2 isómeros del DP, y se optimizaron los iones monitorizados mediante SIM (Tabla 1 del Artículo #1). No obstante, se vio que los mLODs no eran suficientes para la detección de concentraciones bajas así que se decidió optimizar una metodología mediante GC-MS-MS.

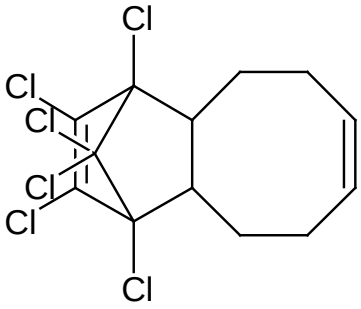

Figura 2.1. Estructura del DPMA.

Se realizó un primer estudio para valorar el modo de ionización óptimo para estos analitos. La sensibilidad obtenida mediante GC-EI-MS-MS fue unas 100 veces inferior a la que proporcionaba la GC-NCI-MS-MS. Esto fue seguramente debido a que mediante NCI se podía monitorizar la pérdida de los iones de cloro, una pérdida muy favorable en estas condiciones, mientras que por EI no se consiguió una transición suficientemente abundante. Curiosamente, los LODs instrumentales (iLODs, del inglés instrumental limits of detection) no variaban tanto entre GC-NCI-MS y GC-NCI-MS-MS (Tabla 2 del Artículo #1) mientras que mejoraron considerablemente al aplicar el método a muestras reales (Tabla 3 y Figura 1 del Artículo #1). Esto demuestra que la mejora en sensibilidad vino causada principalmente por la drástica reducción del ruido de fondo, mejorando los mLODs entre 2 y 36 veces en sedimento, 9 y 116 veces en lodos de depuradora, y entre 19 y 300 veces en peces. El hecho de que se mejorara más en matrices más complejas como lodos y peces permite ver la importancia de la selectividad de la MS-MS a la hora de conseguir mejores LODs. En la figura 2.2 se muestran varios cromatogramas donde la mejora obtenida con la nueva metodología se hace evidente.

El resto de autores que actualmente analizan los HNs mediante MS-MS lo hacen usando EI (Guo *et al.*, 2014; Sühring *et al.*, 2015a; Sühring *et al.*, 2015b; Zhang *et al.*, 2015). En la tabla 2.2 se resumen los diferentes mLODs de estas metodologías. Puede observarse claramente como la NCI-MS-MS desarrollada en esta tesis permite detectar concentraciones bastante más bajas que la EI-MS-MS.

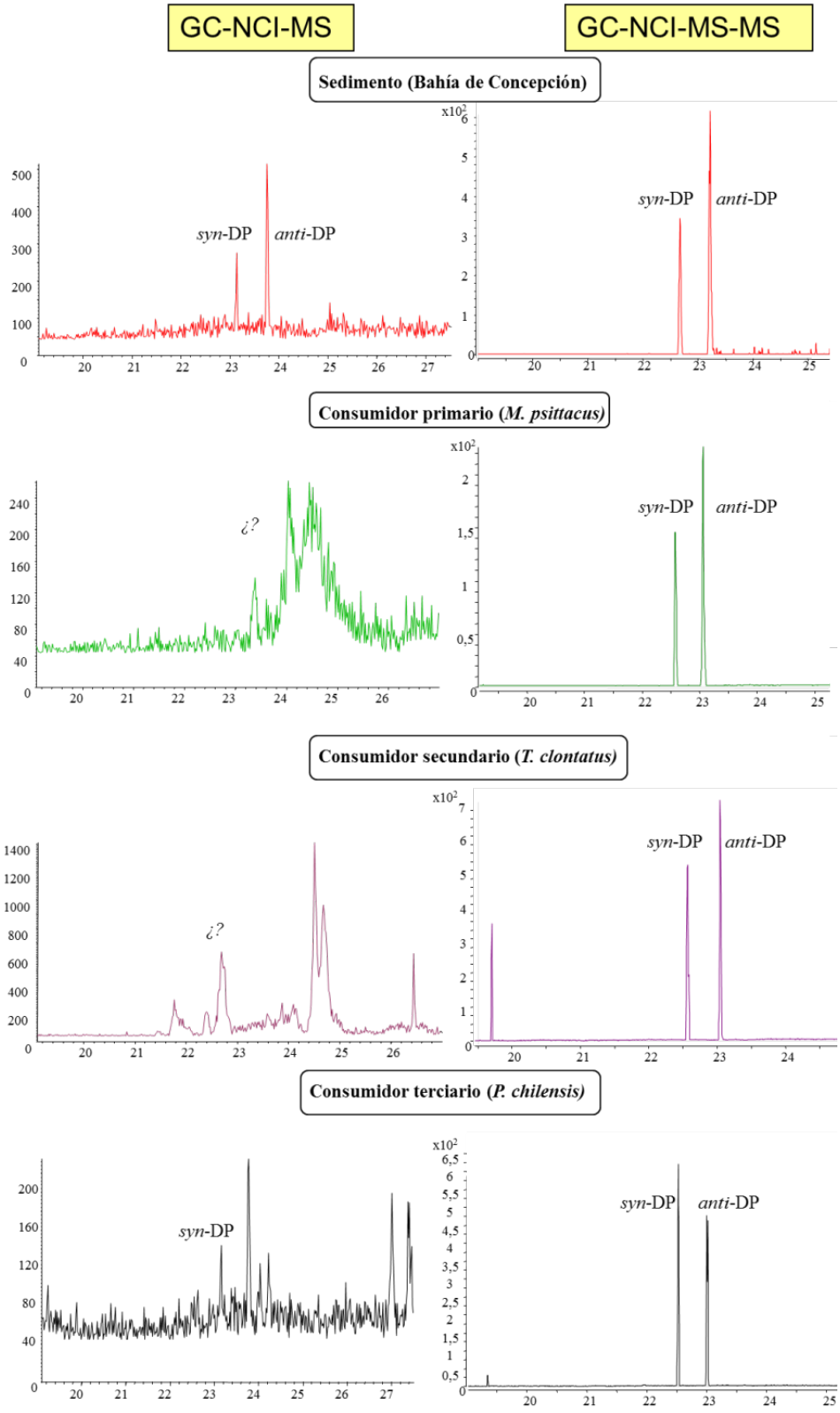

Figura 2.2. Cromatogramas de los diferentes decloranos en diferentes tipos de muestra obtenidos mediante su análisis por GC-NCI-MS y GC-NCI-MS-MS.

Tabla 2.2. Límites de detección de las diferentes metodologías GC-MS-MS disponibles en la literatura.

	Barón *et al. 2012*	Sühring *et al. 2015*	Zhang *et al. 2014*	Guo *et al .2014*	Barón *et al.2012*	Zhang *et al.2015*
	Sedimento (pg/g dw)				Biota (pg/g lw)	
Dec 602	1,3	13	n.r	n.r	21	n.r
Dec 603	0,1	52	n.r	n.r	7,3	n.r
Dec 604	0,2	355	n.r	n.r	7	n.r
syn-DP	0,3	42	1,2	644	5,5	200
anti-DP	0,2	59	1,2	644	21	200

n.r: No reportado

2.3.2. PBDEs y BFRs emergentes

Al inicio de esta tesis la metodología utilizada en el laboratorio para el análisis de PBDEs era la GC-NCI-MS, con NH_3 como gas reactivo (De La Cal *et al.,* 2003; Eljarrat *et al.,* 2004). Pese a la elevada sensibilidad que proporciona esta técnica, la baja selectividad de los iones del bromo representa un serio problema especialmente en muestras de biota, que justamente constituyen una gran parte de esta tesis. Por ello, y en base a la tendencia en los últimos años al uso de la GC-EI-MS-MS para este tipo de análisis, se decidió desarrollar esta metodología para el análisis de PBDEs, BFRs emergentes y MeO-PBDEs (los únicos HNPs incluidos en el método) y validarla en diferentes matrices ambientales y biológicas. Se optimizaron las diferentes transiciones y se adaptó el programa de temperatura de manera que permitiera la correcta separación cromatográfica de los compuestos de interés (Tabla 1 del Artículo #2). El método mostró buena reproducibilidad y repetitividad, nunca superando el 20% instrumental o en matriz (Tablas 2 y 3 del artículo #2). El análisis del BDE-209 es especialmente problemático y su detección y cuantificación, ya complejas y difíciles en MS, son aún más complicadas por MS-MS. Con esta metodología se consiguieron mLODs bastante aceptables (Tabla 3 del Artículo #2). En la figura 2.3 se muestra una comparativa entre los mLODs obtenidos mediante MS y MS-MS con EI como fuente de ionización para los PBDEs y BFRs emergentes. Pese a que la diferencia no es tan pronunciada como en el caso de los HNs, en muchos casos el uso de MS-MS permitió la detección del BDE-209, proporcionando además valores más bajos para el resto de compuestos que la MS. Los mLODs obtenidos eran comparables a los reportados por los otros estudios de

PBDEs mediante MS-MS (Cristale *et al.*, 2012; Law *et al.*, 2013; Losada *et al.*, 2010; Sánchez-Avila *et al.*, 2011); esta discusión puede encontrarse en el apartado de resultados y discusión del artículo #2.

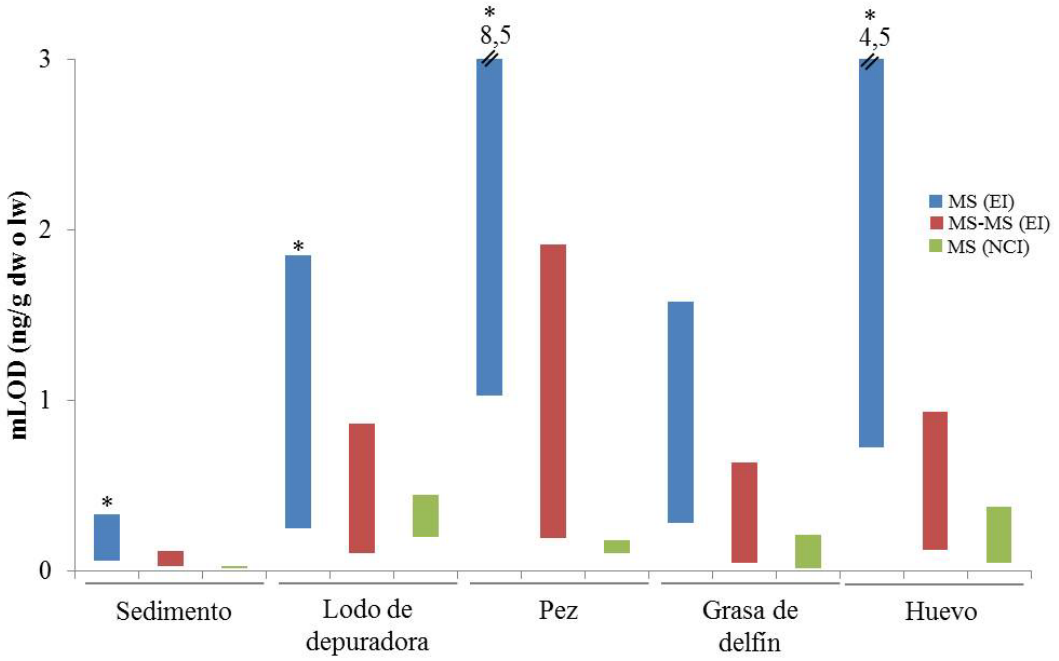

Figura 2.3. Comparativa entre los mLODs obtenidos mediante MS y MS-MS en diferentes matrices; las barras representan los valores entre el 25 y 75 del percentil. * BDE-209 no detectado.

2.3.3. ¿Por qué no un análisis multiresiduo?

Evidentemente, la idea inicial era desarrollar una metodología que permitiera el análisis conjunto de todos los compuestos de interés. Sin embargo, al optimizar la metodología para los HNs se identificaron ciertas características que, por lo menos con la instrumentación de la que se disponía, lo harían imposible. Primero de todo, no fue posible obtener una transición lo suficientemente abundante para los HNs mediante EI, que es la fuente de ionización utilizada en el análisis de los BFRs por MS-MS. Al ver que la NCI-MS-MS daba muy buenos resultados para los HNs se intentó obtener transiciones con una intensidad suficiente para los PBDEs, pero la pérdida de bromo es un proceso tan favorable y que produce una señal tan intensa que no se consiguió un ion padre suficientemente abundante. Otro elemento que ilustra las diferentes características de estas dos familias es la comparación entre los LODs obtenidos utilizando CH_4 y NH_3 como gases de reacción. Mientras que para los PBDEs no parece haber mucha diferencia, en el caso de los HNs se puede ver claramente como es un factor a tener en cuenta (Tabla 2.3).

Tabla 2.3. Comparación entre los LODs (fg inyectados) obtenidos mediante 2 gases de reacción diferentes.

	DPMA	**Dec 602**	**Dec 603**	**Dec 604**	***syn*-DP**	***anti*-DP**	**PBDEs**
Amonio	47	926	30	187	61	23	30-210
Metano	13	96	5	17	12	9	56-177

Un método conjunto mediante GC-NCI-MS hubiera sido posible, asumiendo que los LODs de los HNs podrían no ser suficientes para el análisis en ciertas zonas donde se esperaban niveles bajos, pero como se ha explicado la prioridad era trabajar con MS-MS y conseguir una identificación sensible y selectiva. De este modo, pese a necesitar 2 inyecciones (más otra mediante LC-MS-MS si se quiere analizar el HBCD) se consiguen unos resultados más seguros y con una sensibilidad adecuada para el análisis ambiental (Tabla 2.4).

Tabla 2.4. Transiciones, LODs y LOQs instrumentales (pg inyectados) de las metodologías definitivas utilizadas para el análisis de HFRs en el transcurso de la tesis.

	Compuesto	SRM_1	SRM_2	LOD	LOQ
PBDEs	BDE-28	408>246	408>248	0,11	0,35
	BDE-47	486>326	486>328	0,18	0,61
	BDE-100	406>297	564>404	0,43	1,44
	BDE-99	406>297	564>404	0,54	1,81
	BDE-154	486>377	644>484	2,27	7,58
	BDE-153	486>377	644>484	3,57	11,9
	BDE-183	721>562	721>564	12,5	41,7
	BDE-209	298>220	361>280	16,3	54,4
BFRs emergentes	HBB	468>308	468>310	1,32	4,39
	PBEB	500>485	485>406	0,9	3,01
	DBDPE	485>406	325>165	6,25	20,8
HBCD*	α-HBCD	639>79	639>81	0,5	1,7
	β-HBCD	639>79	639>81	0,5	1,8
	γ-HBCD	639>79	639>81	1,2	4
HNs**	Dec 602	612>35	612>37	96	320
	Dec 603	638>35	638>37	5,0	17
	Dec 604	460>79	504>79	17	57
	syn-DP	654>35	654>37	12	40
	anti-DP	654>35	654>37	9,0	30

*Guerra *et al.* (2008). ** fg inyectados.

En general, los mejores LODs se obtuvieron para los decloranos, seguidos por el HBCD, los PBDEs y BFRs emergentes. En el caso del HBCD los LODs son buenos pese a trabajar en LC debido al uso de la MS-MS. Por lo que respecta a las diferentes matrices incluidas, el sedimento presentó los mejores LODs para todos los compuestos, como era de esperar al ser una matriz relativamente sencilla. Mientras que en PBDEs y BFRs emergentes los LODs para lodos y huevos eran ligeramente inferiores a los obtenidos en peces, en el caso de los HNs se obtuvieron mejores LODs para peces que para las otras 2 matrices. Para todos los compuestos los LODs en grasa de delfín fueron inferiores a las del resto (excepto sedimentos). Seguramente, el hecho de que las otras matrices presenten materia orgánica más compleja podría ser una explicación.

En cualquier caso, como se mostrará en los capítulos posteriores los métodos desarrollados han resultado ser útiles para los estudios de niveles ambientales.

CAPÍTULO 3

PRESENCIA EN MUESTRAS AMBIENTALES

3.1. Introducción

Como se ha comentado en el capítulo 1, la emisión de la mayoría de HFRs desde los productos que lo contienen puede producirse en 2 periodos diferentes: durante la vida útil del producto o material en cuestión, y una vez éste ha sido retirado. Asimismo se puede producir la re-entrada de los contaminantes al medio ambiente desde otras fuentes secundarias como los lodos de depuradora o sedimentos, por lo que compuestos que ya estaban "fijos" en una zona pueden volver a entrar en juego. Una vez liberados de nuevo pueden tanto acumularse en los organismos cercanos, volverse a depositar en las capas sedimentarias superficiales, o volatilizarse a la atmósfera. Este último proceso es el que permite su transporte a otras zonas, ya que la mayoría de los HFRs son altamente estables y difícilmente degradados en la atmósfera. En el caso de los lodos la problemática viene dada mayoritariamente por el tratamiento que se les da: si no son destruidos en condiciones óptimas se emitirán los HFRs, y si son usados en la agricultura se contaminará tanto el terreno cultivado como posiblemente plantas y animales. El análisis de este tipo de muestras permite obtener datos sobre la presencia de los HFRs en este compartimento ambiental concreto, poder evaluar el impacto de la actividad humana en el medio ambiente, o estudiar posibles diferencias de perfiles entre zonas. Además, las concentraciones obtenidas pueden ser usadas para llevar a cabo evaluaciones del riesgo ecológico (ERA, del inglés ecological risk assesment) de estos compuestos, lo que a medio plazo puede contribuir en la mejora de las estrategias de gestión ambiental (Barakat *et al.,* 2012). A continuación se resume brevemente los mecanismos mediante los cuales los HFRs se acumulan en las 2 matrices ambientales estudiadas en esta tesis: sedimento y lodos de depuradora.

Sedimentos

El medio acuático se puede dividir en 3 compartimentos principales: agua, sedimentos y material particulado o materia orgánica en suspensión. Al ser los HFRs altamente hidrofóbicos será más probable encontrarlos ya sea en el material particulado o en el sedimento; contaminantes adsorbidos en el particulado acabarán sedimentando por deposición en zonas con poco flujo como lagos o remansos, por lo que se ha de prestar atención a las características geológicas a la hora de planificar un estudio (Bigus *et al.*, 2014). No obstante, a la hora de considerar los factores que influyen en el proceso de sedimentación se deben tener en cuenta también otros factores. Estos son la presión,

temperatura, tamaño de partícula, composición del sedimento, porosidad o cantidad de materia orgánica, que influirán en las concentraciones de los diferentes contaminantes (Fei *et al.,* 2011; Lors *et al.,* 2012; Mechlińska *et al.,* 2009). Por todo ello, las diferencias encontradas entre sedimentos de distintas zonas pueden ser debidas, por lo menos parcialmente, a otros factores además de las diferentes emisiones o usos de los HFRs. Normalmente las concentraciones se normalizan con el TOC para reducir esta variabilidad en la medida de lo posible y hacer las comparaciones más fiables.

Lodos

En el capítulo 1 se ha introducido la problemática de la reutilización de los lodos de depuradora en la agricultura. Esta alternativa es más económica que la incineración y de hecho es la recomendada por la UE siempre que el lodo cumpla con unos requisitos mínimos (Düring y Gäth 2002), pero al mismo tiempo puede provocar la re-inserción de contaminantes que ya habían salido del sistema ambiental. Los lodos de depuradora acumulan prácticamente la totalidad de los HFRs que entran en la depuradora (Katsoyiannis y Samara 2007), por lo que técnicamente ésta los eliminaría del medio ambiente… si no fuera por el tratamiento posterior que se le da a los lodos (Dimitriou-Christidis *et al.,* 2015). Por todo ello, el análisis de esta matriz es importante tanto para hacerse una idea de las concentraciones presentes e identificar posibles focos de contaminación, como para aportar evidencias de la necesidad de utilizar pre-tratamientos antes de su uso posterior (Joo *et al.,* 2015; Zuloaga *et al.,* 2012).

3.2. Estudios en muestras de sedimento y lodos de depuradora

En este capítulo se incluyen 2 trabajos diferentes realizados en el marco de 2 proyectos de investigación. En el primero se estudió la presencia de los PBDEs, BFRs emergentes, HBCD y otros HFRs en sedimentos procedentes de Colombia (3 zonas, 13 muestras en total) y Chile (5 zonas, 19 muestras en total) en el marco del proyecto titulado "**Evaluación del impacto ambiental de los retardantes de llama bromados en ecosistemas acuáticos de América Latina** (BROMACUA)". Este trabajo se recoge en la publicación #3: *Occurrence of hydrophobic organic pollutants (BFRs and UV-filters) in sediments from South America.* En este trabajo se integraron también los compuestos de la familia de filtros solares, que no serán discutidos aquí al tratarse de otra línea de investigación no relacionada con esta tesis, y por el contrario no se pudieron analizar los

HNs al no disponer todavía de la metodología necesaria. Por otro lado, se analizaron muestras de sedimentos y lodos de depuradora procedentes de las cuencas hidrográficas del Ebro y Llobregat en busca de PBDEs, HNS y BFRs emergentes, en el marco del proyecto "**Evaluación y predicción de los efectos del cambio global en la cantidad y la calidad del agua en ríos ibéricos** (SCARCE)". El muestreo se realizó en diversos puntos a lo largo de las 2 cuencas: en el Ebro se analizaron 19 muestras de sedimento y 6 lodos procedentes de diferentes EDARs, mientras que en el Llobregat se analizaron 14 sedimentos y 1 sola depuradora. Este trabajo dio lugar a la publicación #4: *Occurrence of classic and emerging halogenated flame retardants in sediment and sludge from Ebro and Llobregat river basins (Spain).*

Publicación científica #3

Occurrence of hydrophobic organic pollutants (BFRs and UV-filters) in sediments from South America

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Occurrence of hydrophobic organic pollutants (BFRs and UV-filters) in sediments from South America

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HIGHLIGHTS

- BFRs and UV-F levels were comparable to those reported in other regions of the world.
- The contamination was greater in Colombian sites.
- Contribution of BFRs was higher than that of UV-F for almost all sediments.
- Deca-BDE was the formulation used in Colombia, whereas Penta-BDE was also applied in Chile.
- DBDPE was detected only in Chilean samples.

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ABSTRACT

In the present study the occurrence of emerging hydrophobic organic pollutants in sediment samples from South America (Chile and Colombia) was investigated for the first time. Nineteen Chilean and thirteen Colombian sediment samples were analyzed in order to determine their content of brominated flame retardants (BFRs) (including PBDEs and emerging BFRs) as well as UV filters (UV-F). Samples were collected from neighboring aquatic ecosystems highly urbanized and industrialized in Colombia (Magdalena River area) and Chile (Biobio region). Different analytical procedures were applied depending on the selected analytes, based on chromatographic and mass spectrometric methodologies (GC–MS and LC–MS–MS). In general, concentration levels of both BFRs (up to 2.43 and 143 ng g^{-1} dw of PBDEs in Chile and Colombia, respectively) and UV-F (nd–2.96 and nd–54.4 ng g^{-1} dw in Chile and Colombia, respectively) were in the low range of published data, and the contribution of BFRs was higher than that of UV-F for almost all the sampled sediments.

1. Introduction

Many studies have arrived to the conclusion that sediments constitute a sink for chemical compounds that are hydrophobic. The contamination of sediments may pose an unacceptable risk to aquatic organisms, which tend to bioaccumulate persistent organic pollutants (POPs), and to wildlife and humans through the ingestion of contaminated fish and shellfish.

Brominated flame retardants (BFRs) are a structurally diverse group of chemicals that are added to polymers which are used in plastics, textiles, electronic circuitry and other materials to reduce the risk of fire. Some of them are ubiquitous, and many have been detected in biota, sediments, air, water, marine mammals and even in human milk (Law et al., 2006). Most of the research conducted has been focused on the study of polybrominated diphenyl ethers (PBDEs) due to their persistence and hydrophobicity, two characteristics that make them amenable to bioaccumulation and biomagnifications (Eljarrat et al., 2004a). Due to their toxicological effect, the production and use of PBDE formulations have been banned in Europe, and Penta- and Octa-BDE formulations are now banned in North America.

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In response to the increasing international regulations on BFR formulations, alternative additive flame retardants for achieving commercial product fire safety standards are being developed and used. Some of these non-BDE BFRs are tetrabromobisphenol a (TBBPA), hexabromocyclododecane (HBCD), pentabromoethylbenzene (PBEB), hexabromobenzene (HBB) and decabromodiphenylethane (DBDPE). These compounds have been detected in environmental samples from Europe and North America, including sediment, sludge and dust (Eljarrat et al., 2005; Hoh et al., 2005; Stapleton et al., 2008), biota (Fernie and Letcher, 2010) and humans (Eljarrat et al., 2009).

More recently, the research on new hydrophobic organic pollutants, the UV filters (UV-F), has raised the scientific interest. UV-F are considered as environmental contaminants of increasing concern since the most commonly used are known to cause endocrine disrupting effects, to interfere with the thyroid axis, and with the development of reproductive organs and brain in both aquatic and terrestrial organisms (Fent et al., 2008; Brausch and Rand, 2011). As a consequence of the raised concern about the harmful effects of solar radiation, these compounds have been increasingly used from last decades. UV-F are extensively used in personal care products to protect the skin and hair from the deleterious effect of sunlight and, as well as BFRs, are present in a wide variety of industrial goods such as paints, plastics or textiles in this case to prevent degradation of polymers and pigments (Zenker et al., 2008). Residues of more polar organic UV-F have been found in all kind of water matrices (Balmer et al., 2005; Rodil et al., 2008; Tarazona et al., 2010). Like BFRs, more lipophilic compounds tend to accumulate in sediments (Gago-Ferrero et al., 2011a) and sewage sludge (Gago-Ferrero et al., 2011b), and bioaccumulate in aquatic organisms (Fent et al., 2010; Gago-Ferrero et al., 2012) and humans, being detected in human breast milk (Schlumpf et al., 2010).

Information on levels, trends and effects of persistent and emerging organic contaminants is quite scarce in South America. In this sense, the BROMACUA project aims to gather baseline information on levels of BFRs and UV-F in aquatic ecosystems as a way to contribute to the knowledge of the levels of these pollutants in developing countries. The present study investigated the occurrence of emerging BFRs including TBBPA, HBCD, PBEB, HBB and DBDPE together with PBDE congeners, as well as eight different UV-F compounds (Benzophenone 3 (BP3), 4-methylbenzylidene camphor (4-MBC), octocrylene (OCT), ethylhexyl methoxycinnamate (EHMC), octyl dimethyl PABA (OD-PABA), 4-hydroxybenzophenone (4HB), 2,4-dihydroxybenzophenone (BP1), and 4,4′-dihydroxybenzophenone (4DHB)) in sediments. The samples were taken from neighboring aquatic ecosystems of highly urbanized and industrialized areas in Colombia and Chile. As far as the authors know, this is the first time that BFRs as well as UV-F have been analyzed in sediment samples from South America.

2. Experimental

2.1. Area of study

The selected areas of study were neighboring aquatic ecosystems of Colombia and Chile that were highly urbanized and industrialized. Nineteen sampling stations were selected in Chile (Fig. 1a), including river areas, estuary and coastal bays in the Biobio region (South Central Chile). Sampling sites were selected according to different criteria such as being located close to some important discharges of chemicals, food processing and urban discharges. Along the Biobio river basin a total of 326 populated localities are located, of which 17 are cities (INE-Chile, 2012). The economic sectors that predominate in this river basin are related to forestry, agriculture and industry (mainly represented by metallurgical, chemical, oil refining, textiles, pulp industries, among others). As it can be seen in Fig. 1a, the study was divided in four different areas. Coronel Bay area is highly intervened and affected by human activities, ranging from the impact of wastewater from the town of Coronel to the presence of a large concentration of fishing industries (fish meal production) and a power generation station (thermal coal) that discharge their wastes through pipelines into the Bay. San Vicente Bay concentrates an important industrial complex, whose main activities are related to fish production, steel and petrochemicals. The river sediments were collected at sampling sites in the Biobio river located at the mouth of the river, downstream suspected sources of pollution such as urban sewage, oil refinery and a paper mill.

In the case of Colombia, 13 sampling stations were selected in the area of influence of the Magdalena River in its intersection with the Caribbean Sea (Fig. 1b). Sampling sites included natural and urban areas from the river-city boundary. Barranquilla city, located on the west bank of the river, has an industrial district and a major sea-river port. Chemical, petrochemical, pharmaceutical, metal mechanical, agrochemical, and fishing are common activities in this area. Moreover, the Magdalena river basin is the largest river system in Colombia being an important fluvial stream for economic purposes. It has the highest sediment yield of any other large river in South America (Restrepo et al., 2006). The main cities of the country, including Bogotá, Medellín, Cali and Barranquilla, are located into the basin. Inadequate municipal wastewater treatment infrastructure is a pervasive problem in Colombia (Blackman, 2009). Consequently, water and sediment pollution problems that probably affect ecosystems and biodiversity are present. A site called "Caño Clarín" was monitored to detect the probability of migration of pollutants from the river to the "Ciénaga Grande de Santa Marta", a special Ramsar site. Other selected sites were located in the river mouth itself (Bocas de Ceniza), in a coastal wetland (Mallorquin swamp) and across the west coastal line where wind and water flows facilitate the movement of pollutants.

The sample campaign was done in December 2009 and April 2010 in Chile and Colombia, respectively (Table 1). Samples were taken with a petit-ponar type dredge, stored in aluminum foil, sealed in plastic bags and conserved on ice until the arrival into the laboratory. Sediment samples were freeze dried, and lyophilized material was ground, homogenized, and stored in sealed containers at −20 °C until chemical analysis. Moreover, a sediment characterization was performed according to the methodology proposed by Byers et al. (1978), and textural classification according to Wentworth (1922).

2.2. Chemicals

The PBDE native compounds stock solution BFR-PAR, BDE-77, BDE-181 and $^{13}C_{12}$-BDE-209 were purchased from Wellington Laboratories (Guelph, Ontario, Canada). The components of BFR-PAR solution include different PBDE congeners (from di- to deca-brominated) as well as PBEB, HBB and DBDPE. TBBPA, ^{13}C-TBBPA, d_{18}-α-, d_{18}-β-, and d_{18}-γ-HBCD were also obtained from Wellington Laboratories Inc. BPA and d_{16}-BPA were from Aldrich Chemical Co. (WI, USA). α-, β-, and γ-HBCD were obtained from Cambridge Isotope Laboratories Inc. (WI, USA). MonoBBPA, DiBBPA, and TriBBPA were a kind of gift from Dr. Göran Marsh (Dep. of Environmental Chemistry, Stockholm University, Sweden). Analytical standards of BP3, OC, OD-PABA, BP1, 4HB, 4DHB and the isotopically labeled compound BP-d_{10}, used as internal standard (IS), were obtained from Sigma–Aldrich (Steinheim, Germany). 4MBC was supplied by Dr. Ehrenstorfer (Augsburg, Germany) and EHMC by Merck (Darmstadt, Germany).

Individual stock standard solutions were prepared on a weight basis and stored at −20 °C. A mixture of all selected standards was prepared by appropriate dilution of individual stock solutions. Stock solutions of internal standards were also prepared and stored

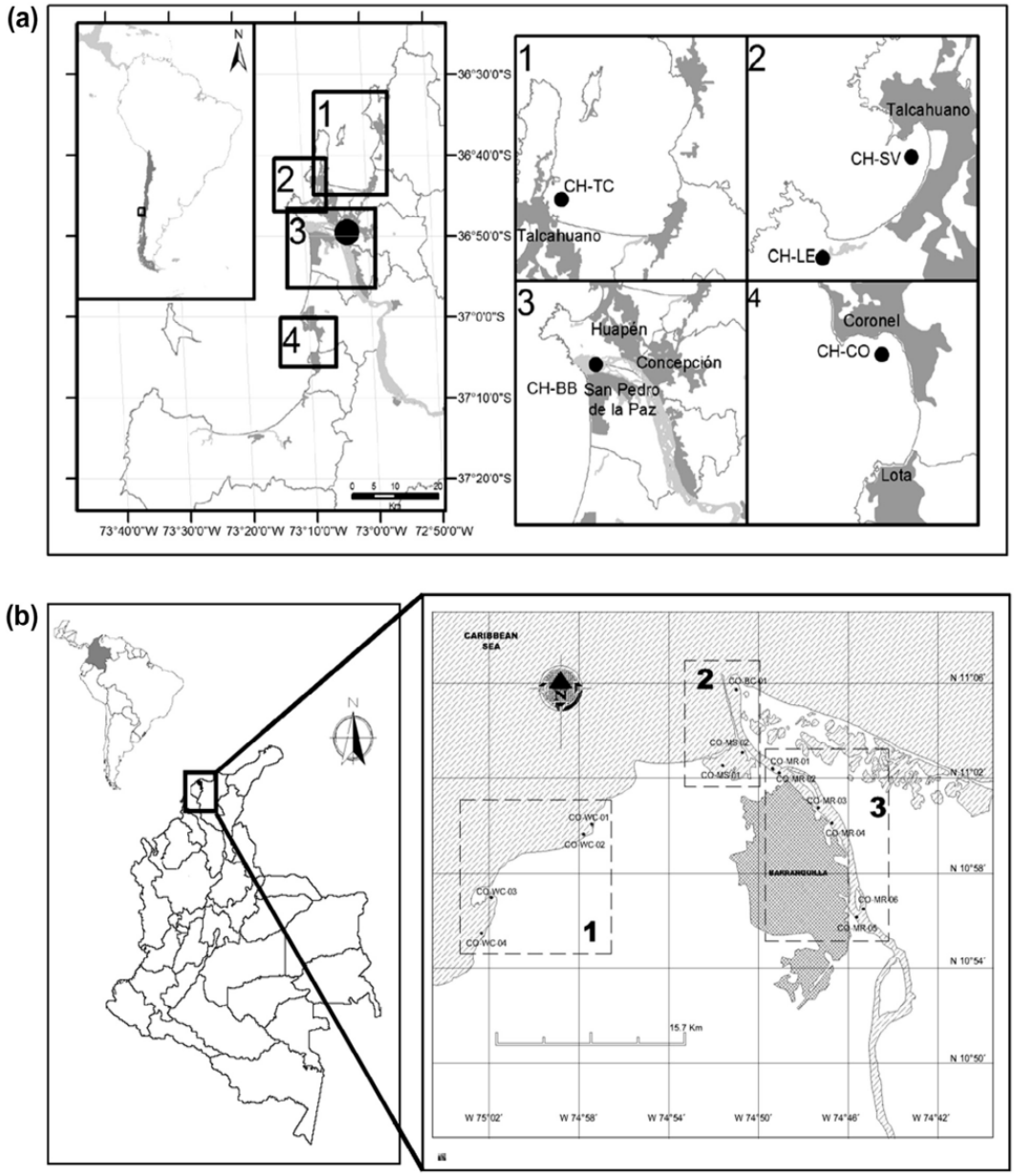

Fig. 1. Sediment sampling locations in (a) Chile: 1, Concepción Bay; 2, San Vicente Bay and Lenga Estuary; 3, Biobio river; 4, Coronel Bay; and (b) Colombia: 1, West coastal line; 2, River mouth; 3, Magdalena river-city boundary.

at −20 °C. A mixture of these standards, used for internal standard calibration, was also prepared by diluting the individual stock solutions.

All solvents and other reagents were provided by Merk (Darmstadt, Germany). N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Syringe filters (0.45 μm) were obtained from Whatman (London, UK). Pressurized liquid extraction cellulose filters were purchased from Dionex Corporation (Sunnyvale, CA, USA).

2.3. Analytical methodology for BFRs

BFR determinations were carried out using two different analytical approaches. First of all, PBDEs together with PBEB, HBB and DBDPE were analyzed by gas chromatography (GC) coupled to mass spectrometry (MS) working with negative ion chemical ionization (NICI). On the other hand, HBCD isomers, TBBPA and related compounds (BPA, MonoBBPA, DiBBPA and TriBBPA) were determined by means of liquid chromatography (LC) coupled to tandem mass spectrometry (MS–MS).

2.3.1. Analysis by GC–MS

One gram dry weight (dw) of sediment sample was spiked with internal standards (10 ng BDE-77, 10 ng BDE-181 and 500 ng $^{13}C_{12}$-BDE-209). Spiked samples were kept overnight to equilibrate. In order to reduce time of analysis, a selective pressurized liquid extraction (SPLE) method that automatically and rapidly achieves quantitative and selective extraction of BFRs was used (De la Cal

Table 1
Information related with the location and physico-chemical parameters of sediment samples collected in this study.

Matrix	Location	Sample code	Latitude–longitude	Sediment parameters				
				% Clay	% Sand	% Gravel	Texture classification (Wentworth, 1922)	% TOC
Samples collected in Chile								
Coastal sediment	Concepción Bay	CH-TC-01	S36°43′13″ W73°06′41″	29	71	0	Sandy clay loam	5.7
		CH-TC-02	S36°43′14″ W73°05′18″	31	69	0	Sandy clay loam	4.4
		CH-TC-03	S36°43′16″ W73°05′16″	11	89	0	Loamy sand	2.9
Coastal sediment	San Vicente Bay	CH-SV-01	S36°43′32″ W73°07′50″	75	25	0	Clay	10.3
		CH-SV-02	S36°43′32″ W73°07′49″	83	17	0	Clay	12.4
		CH-SV-03	S36°43′38″ W73°07′43″	73	27	0	Clay	12.1
		CH-SV-04	S36°44′18″ W73°07′53″	21	79	0	Sandy clay loam	3.7
Estuarine sediment	Lenga Estuary	CH-LE-01	S36°46′09.0″ W73°09′46.3″	42	58	0	Sandy clay	8.7
		CH-LE-02	S36°46′06.5″ W73°10′17.0″	45	55	0	Sandy clay	6.1
River sediment	Biobio River	CH-BB-01	S36°49′39.4″ W73°05′47.5″	1	99	0	Sand	1.3
		CH-BB-02	S36°46′10.6″ W73°06′24.2″	0	100	0	Sand	<1.0
		CH-BB-03	S36°46′10.4″ W73°06′28.0″	0	100	0	Sand	<1.0
		CH-BB-04	S36°46′06.2″ W73°07′14.2″	0	100	0	Sand	<1.0
		CH-BB-05	S36°46′06.5″ W73°03′20.5″	0	100	0	Sand	<1.0
		CH-BB-06	S36°46′17.7″ W73°10′09.7″	0	100	0	Sand	<1.0
Coastal sediment	Coronel Bay	CH-CO-01	S37°01′36″ W73°09′20″	100	0.0	0	Clay	15.4
		CH-CO-02	S37°01′42″ W73°09′36″	5	95	0	Sand	11.6
		CH-CO-03	S37°01′42″ W73°09′52″	88	12	0	Clay	17.4
		CH-CO-04	S37°02′10,5″ W73°09′12″	81	19	0	Clay	14.2
Samples collected in Colombia								
Coastal sediment	West Coastal Line	CO-WC-01	N11°03′23.2″ W74°50′34.0″	0	100	0	Sand	<1.0
		CO-WC-02	N11°00′28.8″ W74°57′16.3″	0	100	0	Sand	<1.0
		CO-WC-03	N10°59′29.5″ W74°57′37.3″	0	100	0	Sand	1.1
		CO-WC-04	N10°56′51.6″ W75°01′41.2″	0	100	0	Sand	<1.0
Estuarine sediment	Bocas de Ceniza and Mallorquin Swamp	CO-BC-01	N11°05′38.0″ W74°50′45.1″	0	100	0	Sand	1.2
		CO-MS-01	N11°02′47.5″ W74°51′11.9″	0	100	0	Sand	1.0
		CO-MS-02	N11°03′23.4″ W74°51′12.1″	0	100	0	Sand	<1.0
Estuarine sediment	Magdalena River	CO-MR-01	N11°02′34.9″ W74°49′23.5″	0	100	0	Sand	1.0
		CO-MR-02	N11°02′08.1″ W74°48′43.3″	0	100	0	Sand	1.0
		CO-MR-03	N11°00′35.5″ W74°46′44.7″	0	100	0	Sand	<1.0
		CO-MR-04	N10°59′41.5″ W74°46′00.1″	0	100	0	Sand	1.1
		CO-MR-05	N10°56′46.6″ W74°45′30.9″	0	100	0	Sand	2.1
		CO-MR-06	N10°57′3.12″ W74°44′53.3″	0	100	0	Sand	1.3

et al., 2003). Two grams copper were added to the cell to remove sulfur interferences. SPLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA) using aluminum oxide neutral and Hydromatrix (Varian Inc., Palo Alto, USA). The extraction cell was heated to 100 °C and extraction was carried out using a mixture of hexane:CH_2Cl_2 (1:1). The volume of the resulting extract was about 35 mL. Extracts were finally concentrated to incipient dryness and re-dissolved with 50 μL of toluene prior to analysis by GC-NICI–MS. GC-NICI–MS analyses were performed on a trace GC ultra gas chromatograph connected to a dual stage quadrupole mass spectrometer (Thermo Electron, Texas, USA). A DB–5MS capillary column (15 m × 0.25 mm i.d., 0.1 μm film thickness) was used with ammonia as the carrier gas at an ion source pressure of 1.9×10^{-4} Torr. The temperature program was from 140 °C (held for 2 min) to 325 °C (held for 10 min) at 10 °C min^{-1}. Injection was carried out by splitless mode for 1 min with an injector temperature of 250 °C (Eljarrat et al., 2004b). Experiments were carried out monitoring the two most abundant isotope peaks from the mass spectra corresponding to $m/z = 79$ and 81 $[Br]^-$ for di- to nona-BDEs, PBEB, HBB and DBDPE, $m/z = 487$ and 489 for BDE-209 and $m/z = 497$ and 498 for $^{13}C_{12}$-BDE-209. The identification of selected analytes was based on the following restrictive criteria: (i) retention time for all monitored ions for a given analyte should maximize simultaneously ± 1s, with signal to noise ratio >3 for each; and (ii) the ratio between the two monitored ions should be within 15% of the theoretical value (calculated upon standards). The quantification of di- to penta-BDEs, PBEB and HBB was carried out by internal standard procedure using BDE-77, whereas for hexa- to hepta-BDEs, BDE-181 standard was used. In the case of deca-BDE and DBDPE, the quantification was carried out using $^{13}C_{12}$-BDE-209 as internal standard.

2.3.2. Analysis by LC–MS–MS

Sediment sample of 0.5 g dw was spiked with 50 μL of a mixture of d_{18}-α-HBCD and d_{18}-γ-HBCD, and 50 μL of ^{13}C-TBBPA, both solutions at concentrations of 5 ng μL^{-1}. Spiked samples were kept overnight to equilibrate and extracted by sonication with 10 mL of dichloromethane:methanol (1:9, v/v). The obtained extracts were cleaned with SPE C18 cartridges. Finally, extracts were concentrated to incipient dryness and re-dissolved with 50 μL of d_{18}-β-HBCD and 50 μL of d_{16}-BPA at 5 ng μL^{-1}, and 150 μL of methanol prior to analysis by LC–MS–MS. The LC system used was an Symbiosis Pico (Spark Holland, Emmen, The Netherlands) with a Symmetry C18 column (2.1 mm × 150 mm, 5 μm) preceded by a C18 guard column (2.1 × 10 mm) supplied by Waters (Massachusetts, USA). Experiments were carried out in negative ionization mode using H_2O:methanol (3:1 v/v) as eluent A and methanol as eluent B, at a flow rate of 0.25 mL min^{-1}. The injection volume was set at 10 μL. The elution program started at an initial composition of 100% A and was ramped to 0% eluent A in 8 min, then eluent A increased to 10% in 17 min and initial conditions were reached again in 3 min and returned to the starting conditions in 15 min. Mass spectrometric analysis was performed with a hybrid triple quadrupole/linear ion trap Applied Biosystem MSD Sciex 4000QTRAP™

(Applied Biosystems, Foster City, CA, USA) instrument equipped with an electrospray (ESI) Turbo spray interface. All data were acquired and processed using Analyst 1.4.2 Software. For target quantitative analyses, data acquisition was performed in selected reaction monitoring (SRM). The MS–MS detection conditions were optimized previously to afford the highest relative intensity: curtain gas (CUR) at 50 psi, collision gas (CAD) at 4.5×10^{-5} Torr, temperature of the turbo gas in the TurboIonSpray™ source (TEM) at 350 °C, ion source gas 1 (GS1) and ion source gas 2 (GS2) at 50 psi (Guerra et al., 2008).

2.4. Analytical methodology for UV-F

Background contamination in the laboratory is a common problem observed in the determination of UV-F at environmental trace levels. As precautionary measures all glassware used was previously washed and heated overnight at 380 °C and further sequentially rinsed with HPLC grade water, ethanol and acetone, and immediately used. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. Since many of the compounds analyzed undergo photodegradation and the samples may suffer the exposure to light during the procedure, all samples and stock standard solutions were always covered with aluminum foil and stored in the dark.

Similarly to BFR analysis, sediment samples were extracted and purified by SPLE. Aliquots of 1 g of alumina (previously heated at 130 °C overnight, and then allowed to cool down in a desiccator before use) were placed at the outflow side of each cell onto two cellulose filters. Under optimized conditions, aliquots of 1 g dw sediment were mixed in the PLE extraction cells with alumina in order to perform the in-cell purification. Finally, the PLE extract was brought to 25 mL with methanol. A 2 mL aliquot of this solution was passed through 0.45 μm syringe filter to a LC-vial, and evaporated to dryness under a gentle stream of nitrogen in a TurboVap LV evaporator from Zymark (Zymark, Hopkin, MA). The dried extracts were reconstituted in 250 μL of acetonitrile containing the IS.

Chromatographic separations were performed on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 2 μm) LC column supplied by Merck with an Acquity UPLC chromatograph (Waters). The column temperature was kept at 50 °C. Separation was performed with a binary mobile phase at a flow rate of 0.4 mL min^{-1}. The optimized separation conditions were as follows: solvent A consisted of H_2O and solvent B acetonitrile, both with 0.3% formic acid. The gradient elution started with 5% eluent B, increasing to 80% in 2 min. and raising to 100% in the following 9 min kept constant for 2 min, then return to initial conditions in 2 min, and finally three additional min to allow the equilibration of the column. The sample volume injected was 10 μL.

The ultra performance LC (UPLC) instrument was coupled to a triple quadrupole mass spectrometer (Waters). Acquisition was achieved in electrospray positive mode (ESI(+)) using SRM mode recording two transitions for each compound for enhanced sensitivity and selectivity. For each compound, two characteristic fragments of the protonated molecule $[M+H]^+$ were monitored. The most abundant transition was used for quantification, whereas the second most abundant one was used for confirmation. This procedure was in compliance with the European Council Directive 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. More information about the analytical method can be found in a previous work from Gago-Ferrero et al. (2011a).

Table 2
Recoveries, relative standard deviations (RSDs) and method limits of detection (LOD) and quantification (LOQ) of analysis of BFRs and UV-F in sediment samples.

Compound	Recovery (%)	RSD (%)	LOD[a] (ng g^{-1} dw)	LOQ[a] (ng g^{-1} dw)
Brominated flame retardants				
Di-BDEs	87–105	0.7–4	*20–41*	*67–136*
Tri-BDEs	57–101	3–5	*15–19*	*50–63*
Tetra-BDEs	64–88	0.8–8	*4.7–11*	*15–36*
Penta-BDEs	96–110	0.8–7	*7.7–24*	*25–81*
Hexa-BDEs	74–105	2–8	*8.4–22*	*28–74*
Hepta-BDEs	82–102	0.6–8	*1.0–13*	*29–43*
Octa-BDEs	66–102	2–6	*21–36*	*70–120*
Nona-BDEs	56–71	2–8	*13–18*	*43–61*
Deca-BDE	83	6	*25*	*37*
PBEB	58	3	*18*	*60*
HBB	62	5	*9.1*	*30*
DBDPE	102	6	*86*	*288*
BPA	106	7	3.7	9.5
Mono-BBPA	88	8	0.6	1.9
Di-BBPA	105	6	2.0	6.7
Tri-BBPA	126	7	28	92
TBBPA	110	9	2.7	8.9
α-HBCD	53	11	1.6	5.3
β-HBCD	57	13	1.4	4.6
γ-HBCD	89	3	2.2	8.2
UV-filters				
4MBC	89	6	1.1	3.6
OCT	85	7	9.9	33
EHMC	90	6	4.1	14
OD PABA	120	4	0.7	2.5
BP3	125	10	0.4	1.3
BP1	58	16	4.6	15
4HB	80	8	0.7	2.3
4DHB	120	9	0.8	2.7

[a] Italic values corresponded to LODs and LOQs expressed in pg g^{-1} dw.

2.5. Quality assurance/quality control

Method blank samples were performed to check for interferences or contamination from solvents and glassware. No presence of analytes of interest was observed. Quality assurances of the described methods were evaluated by measuring parameters as linearity, sensitivity, recoveries, and reproducibility. The limits of detection (LOD) defined as three times the noise level, and the limit of quantification (LOQ), defined as 10 times the noise level, were calculated and presented in Table 2.

3. Results and discussion

3.1. BFR contamination levels

The levels of BFRs in sediment samples collected for this study are presented in Table 3. PBDEs were detected in all the sediment samples from Chile at concentrations ranging from 0.03 to 2.43 ng g^{-1} dw. The Lenga estuary and Coronel bay were the most contaminated zones, with mean values of 1.84 ng g^{-1} dw and 1.57 ng g^{-1} dw, respectively. After them San Vicente and Concepcion bays presenting a similar level of contamination (mean values of 1.01 and 0.95 ng g^{-1} dw, respectively), and finally, the less contaminated area corresponded to Biobio river (mean value of 0.31 ng g^{-1} dw) (Fig. 2). This pattern of contamination was also previously observed with other pollutants in these areas, such as PAHs, with the highest values found in the Lenga estuary (Pozo et al., 2011).

With regard to the sediments from Colombia, PBDEs were detected in five out of 13 analyzed samples. However, in these samples, PBDE contamination was greater than that observed for

Table 3
Concentration levels of BFRs and UV-F in sediment samples collected in Chile and Colombia. Results expressed as range of concentrations in ng g^{-1} dw.

	Chile					Colombia		
	Concepción Bay ($n = 3$)	San Vicente Bay ($n = 4$)	Lenga Estuary ($n = 2$)	Biobio River ($n = 6$)	Coronel Bay ($n = 4$)	West Coastal Line ($n = 4$)	Bocas de Ceniza and Mallorquin Swamp ($n = 3$)	Magdalena River ($n = 6$)
Brominated flame retardants								
BDE-47	nd	nd–0.33	nd	nd	0.15–0.63	nd	nd	nd
BDE-100	nd–0.09	nd	0.19–0.30	nd–0.03	nd–0.21	nd	nd	nd
BDE-99	nd–0.17	nd–0.53	0.75–0.83	nd	0.19–0.73	nd	nd	nd
BDE-154	nd	nd	nd–0.59	nd	nd	nd	nd	nd
BDE-183	nd	nd–0.32	nd	nd	nd	nd	nd	nd
BDE-209	nd–1.72	0.73–0.93	0.50–0.51	nd–0.39	nd–0.85	nq	nq–143	nq–55.8
PBEB	nd–0.08	nd	nd	nd–0.15	nd–0.11	nd	nd	nd
DBDPE	1.62–2.26	nd–2.16	1.12–1.96	nd–2.23	nd–1.91	nd	nd	nd
Di-BBPA	nq–210	1230–1328	7.83–51.3	nd–164	21.7–685	nd	nd	nd
Tri-BBPA	4.50–8.40	nd–6.63	nq	3.33–7.03	nd–4.25	nd	nd	nd–0.27
TBBPA	nd	nd	nd	nd	nd	nd	nd–0.58	nd
γ-HBCD	nd	nd	nd	nd	nd	nd	nd	nd–0.33
UV filters								
BP3	nd	nd–1.42	nd–2.96	nd–1.05	nd	nd–2.52	nd–4.85	nd–5.38
4MBC	nd	nd	nd	nd	nd	nd–7.90	nd–17.2	nd
EHMC	nd	nd	nd	nd	nd	nd–17.8	nd–39.0	nd–47.1

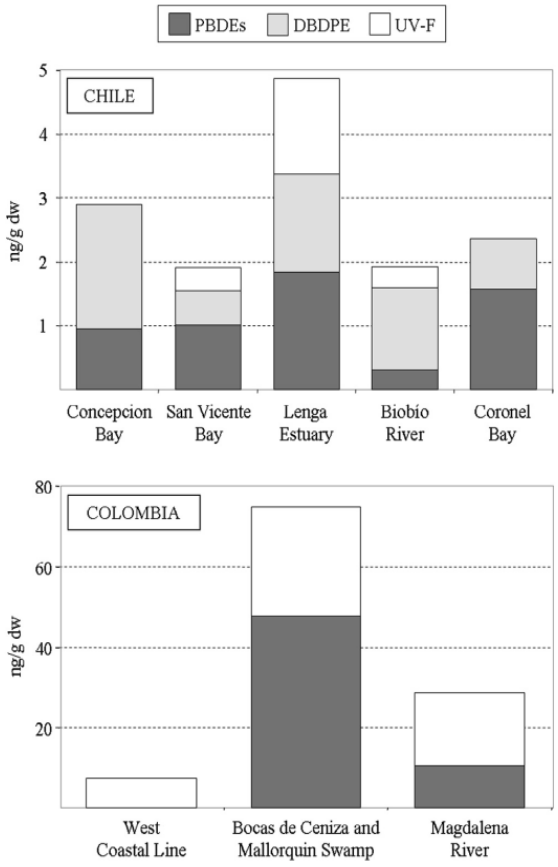

Fig. 2. Median values of BFRs and UV-F in each selected area of study (Chile and Colombia).

Chilean sediments, with levels ranging up to 143 ng g^{-1} dw. The higher contamination was observed for estuarine sediments from Bocas de Ceniza and Mallorquin swamp (mean value of 47.8 ng g^{-1} dw), followed by the sediments from Magdalena river (mean value of 10.6 ng g^{-1} dw). Finally, the sediment collected at the west coastal line does not presented PBDE contamination, probably due to the high plume dispersion and sediment transport (Fig. 2). There are important differences in terms of circulation of water, fluxes, and amount of particles between the Magdalena river mouth and the estuary. Strong littoral currents predominantly flow toward the west and are the result of open ocean swells generated by NE trade winds, and the muddy plume of the river spans more than 90 km in length which could explain the high dispersion of BFRs and the consequent lower detection in the respective samples. Likewise, high rates of total solids are present in the water at the river mouth meaning high sediment yields which also help disperse organic pollutants. The highest value (143 ng g^{-1} dw) appeared in the sediment sample collected at the center of the Mallorquin swamp. This area has historically received discharges with non treated domestic wastes from Barranquilla. Very quiet waters and the high sedimentation processes suffered in this wetland may contribute to explain such levels. Another high level of contamination (55.8 ng g^{-1} dw) was found in one of the Magdalena river sediments, downstream an industrial free zone. In general, the presence of BFRs in Colombian river systems could be probably due to poor e-waste management since many toxic electronic wastes are commonly disposed in landfills.

Of 38 PBDEs congeners included in our analytical work, only six different PBDEs were detected: tetra-BDE-47, penta-BDE-99, penta-BDE-100, hexa-BDE-154, hepta-BDE-183 and deca-BDE-209. The dominance of BDE-209 in the total PBDEs is unquestionable, similar to findings in reported studies with sediments from different geographical areas (Eljarrat et al., 2004b). In this study, BDE-209 constituted between 24% and 100% of the total PBDE contamination. It is important to note that in all sediment samples from Colombia with detectable PBDE levels, the pollution is due to exclusively the BDE-209. The presence of BDE-209 indicates the use of commercial formulations of deca-BDE in the studied area. The presence of BDE-47, BDE-99 and BDE-100 in Chilean sediments was attributed to the use of penta-formulations.

Unfortunately, there are no data on PBDE levels in sediments of South America. However, there are a lot of studies reporting levels in other geographical areas. In order to compare the levels found in this study with those of other areas, the comparison will be made with data published during this year (2012). Levels of PBDEs were determined in sediments from the Scheldt estuary (the Netherlands-Belgium), with an average concentration of 115 ng g^{-1} dw. The total amount of PBDEs was mainly coming from BDE-209, in a 99% of the cases (van Ael et al., 2012). These findings are in

agreement with the results of the present study in Colombia. PBDEs were also measured in sediments from Southern California Bight, with a geometric mean and maximum levels of 4.7 and 560 ng g^{-1} dw (Dodder et al., 2012). Although these maximum levels are higher than those found in this study, the average concentration is of the same order as in the Chilean samples. Several recent studies were focused on China. PBDEs of sediment samples from the coastal East China Sea were measured, with levels of BDE-209 and ΣPBDEs (sum of BDE-28, -47, -99, -100, -153, -154 and -183) between 0.3–44.6 and nd–8.0 ng g^{-1} dw, respectively (Li et al., 2012). In another study, sediment samples from rivers of Shanghai were analyzed (Wu et al., 2013). PBDEs were detected at concentrations from 11.0 to 64.1 ng g^{-1} dw, with an average value of 29.7 ng g^{-1} dw. BDE-209 was the predominant congener accounting for more than 97% of total PBDEs. Once again, these results are in accordance with Colombian data.

As expected due to the lower industrial use, the presence of the non-BDE BFRs (PBEB, HBB, DBDPE, HBCD and TBBPA) is lower than that of PBDEs. HBB was not detected in any sample, HBCD was detected only in two samples from Colombia at concentration levels lower than 0.33 ng g^{-1} dw, and PBEB was detected only in four samples from Chile and at concentration levels lower than 0.15 ng g^{-1} dw. DBDPE was detected in 12 of 19 analyzed sediments from Chile (from nd to 2.26 ng g^{-1} dw), but it was not detected in Colombian sediments indicating their lack of use in that country. DBDPE applications are similar as for BDE-209 with the advantage of no production of dibenzo-*p*-dioxins and no formation of furans under pyrolysis conditions (Jakab et al., 2003; Kierkegaard et al., 2004). Generally, the reported levels of DBDPE were lower than those of BDE-209 (Kierkegaard et al., 2009). In the present study it is observed that in 58% of Chilean samples, DBDPE concentrations are higher than BDE-209, with ratios between 2.2 and 6.2. This could indicate a higher input in the use of DBDPE in this area.

With reference to TBBPA, this BFR was detected only in two Colombian samples. However, high levels of their related compounds were detected in Chilean sediments, with values between nd to 1328 ng g^{-1} dw and nd to 8.40 ng g^{-1} dw for Di-BBPA and Tri-BBPA respectively. The presence of these related compounds may be due to the TBBPA degradation. Debromination of TBBPA could occur during the wastewater treatment, or other abiotic debromination processes (Chu et al., 2005).

3.2. UV-F contamination levels

UV-F concentrations in sediment samples analyzed are summarized in Table 3. Just one out to eight compounds, BP3, was detected in the sediment samples from Chile in a range of concentration from 1.05 to 2.96 ng g^{-1} dw. This compound is used in personal care products or as an additive in materials that have to be protected from sunlight-initiated disrupting (FDA, 1999; Council Directive, 1976) and have been detected previously in river sediment samples of Spain (Gago-Ferrero et al., 2011a) and China (Zhang et al., 2011) in the same range of concentration.

Higher levels of UV-F were detected in several sites of Colombia. In this case three out to eight compounds, BP3, 4MBC and EHMC, were present in the samples. EHMC was detected at a frequency of 38% and with the highest concentrations in the analyzed samples, above 47 ng g^{-1} dw in some cases. 4MBC was present in the 23% of the samples in the range from 7.9 to 17.2 ng g^{-1} dw. BP3 was the most ubiquitous compound and its levels were higher than in Chilean samples, 77% frequency of detection at concentrations from <LOQ to 5.38 ng g^{-1} dw. It is noteworthy that the highly lipophilic UV-F OC, which usually is the most ubiquitous UV-F compound in solid environmental matrices, was not present in any studied sediment. This fact suggests that the production and usage profiles of UV-F are different among countries.

All these compounds have been found to have estrogenic hormonal activity and multiple endocrine-disrupting activities (Fent et al., 2008; Christen et al., 2011). Adverse effects on fecundity and reproduction have also been observed for BP3 (Coronado et al., 2008). 4MBC has also shown high estrogenic potency and its use in cosmetic products is not allowed in the USA or Japan legislation. However, these adverse effects take place at higher concentrations than those detected in the published environmental studies.

The presence of such substances is influenced by the extended use of solar protection creams since sun rays are very strong in this area. Aquatic tourist activities, which are frequent in this area, are also a factor to take into account and may play an important role in the determined levels. However, a possibly even more important factor could be wastewater treatment plants (WWTPs) discharges, from urban and industrial areas. Monitoring data of WWTPs indicates that current techniques are not effective at all removing UV-F. Several of them, including BP3, EHMC and 4MBC, were found in untreated and treated wastewater in different countries (Li et al., 2007; Negreira et al., 2009a), as well as in sewage sludge (Gago-Ferrero et al., 2011b). Measurable values of these compounds have been determined at relatively high values in raw and treated water. This effect could be more important if, as commented in the area of study section, historically urban and industrial effluents have been released to the river without cleaning treatment, which multiplies the presence of organic anthropogenic pollutants in the water and sediments.

High differences between the low levels found in Chile versus the levels in Colombia can be explained because the second is a tropical country with high solar radiation levels, and the use of personal care products containing UV-F is probably much higher than in Chile. Moreover, the tourism activity in Caribbean Colombian beaches could be responsible by the higher UV-F concentrations in water compared to the Chilean case.

4. Conclusions

The occurrence of emerging hydrophobic organic pollutants in sediment samples from Chile (Biobio region) and Colombia (Magdalena River area) was investigated for the first time. Different BFRs were analyzed, showing the presence of PBDEs in both selected areas at concentration levels up to 2.43 and 143 ng g^{-1} dw in Chile and Colombia, respectively. These results are comparable to those reported recently in other regions of the world. Different PBDE patterns were observed, with Deca-BDE formulation being the unique used in Colombia, whereas Chilean samples showed also the use of Penta-BDE formulations. As regards the non-PBDE BFRs, their presence was less frequent and at low concentration levels, with the exception of DBDPE. This replacement of Deca-BDE was detected in Chilean samples, but not in Colombian sediments indicating their lack of use in that country.

As regards UV-F presence, only three out of the total of compounds included in the analytical work where detected at concentration levels ranging between nd to 2.96 and nd–54.4 ng g^{-1} dw in Chile and Colombia, respectively. Similarly to BFRs, the contamination was greater in Colombian sites. It is also important to notice that in almost all of the samples, UV-F levels were lower than those found for BFRs for the same site (Fig. 2).

The present findings provide evidence that currently, emerging contaminants such as non-PBDE BFRs and UV-F are present in the South American environment. Further measurements are required to better understand their fate and occurrence of these compounds. Moreover, although the levels found in this study were

not extremely high, it is expected that in the future the concentration levels may increase. Due to the toxicological effect of PBDEs, their production and use has been banned in Europe and North America. One would expect that this will be the same situation in the near future in South American countries, in particular considering that Penta-BDE and Octa-BDE mixtures have been already included in the Stockholm Convention and that parties should enforce their gradual elimination. And, in order to meet the commercial product fire safety standards, non-PBDE formulations, such as DBDPE, HBB and PBEB, are likely to be used more frequently. Therefore, it will be necessary to continue these monitoring programmes in order to detect a possible raise in pollution levels due to increased use and application.

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Occurrence of classic and emerging halogenated flame retardants in sediment and sludge from Ebro and Llobregat river basins (Spain)

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Occurrence of classic and emerging halogenated flame retardants in sediment and sludge from Ebro and Llobregat river basins (Spain)

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HIGHLIGHTS

- Classical and emerging FRs were analyzed in sediments and sludge from two Iberian river basins.
- Halogenated norbornenes were detected in sediments from Spain for the first time.
- Although BDE-209 was still the most abundant congener, levels of DBDPE were close.
- Although concentration levels of halogenated norbornenes were low, its use is expected to increase during the next years.

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ABSTRACT

Classic (polybromodiphenyl ethers, PBDEs) and emerging halogenated flame retardants such as hexabromobenzene (HBB), pentabromoetilbenzene (PBEB), decabromodiphenyl ethane (DBDPE), Dechlorane 602 (Dec 602), Dechlorane 603 (Dec 603), Dechlorane 604 (Dec 604) and Dechlorane plus (DP) were analyzed in 33 sediments and 7 sludges from two Iberian river basins, Ebro and Llobregat. In sediment samples, PBDE levels ranged between nd and 44.3 ng/g dw with BDE-209 being the most abundant congener. Levels of DBDPE and halogenated norbornenes ranged between nd and 31.5 ng/g dw and between nq and 3.74 ng/g dw, respectively. This is the first study to report halogenated norbornene levels in sediment samples from Spain. PBDE, DBDPE and halogenated norbornene levels in sludge ranged from 13 to 340, nq to 124 and 2.7 to 19 ng/g dw, respectively. HBB and PBEB were not detected in any sample. Levels of classic and emerging HFRs were compared. Our results suggest that DBDPE is the most frequently used compound to replace BDE-209, whereas the use of halogenated norbornenes is still low.

1. Introduction

Flame retardants (FRs) are a group of compounds which have been widely used for many years in order to prevent fires. One of the most widely used FRs are the polybromodiphenyl ethers (PBDEs), which are present in a wide range of materials such as plastics, furniture, vehicles and electronic devices [1]. They have been found in the environment in different environmental matrices such as air [2], sediment [3], water [4] and sludge [5], and also in different biological matrices such as fish [6], bird eggs [7] and humans [8]. Moreover, several toxic properties have been reported for PBDEs. For example, they can act as endocrine disruptors and affect neurological, thyroid and liver activity [9–11]. As a result, PentaBDE and OctaBDE mixtures were banned by the EU in 2001 and since 2006 their presence in polymeric formulations and other compounds is being reduced both in Europe and in North America [12]. Furthermore, DecaBDE is also banned in Europe, although there are exceptions for certain applications, and its production in North America is slowly decreasing and the plan is to stop its production by the end of 2013 [13]. In consequence, some new FRs have been proposed as an alternative for PBDEs. They are called emerging FRs and some examples are hexabromobenzene (HBB), pentabromoethylbenzene (PBEB), decabromodiphenyl ethane (DBDPE) [14] and halogenated norbornenes (Dechlorane 602 (Dec 602), Dechlorane 603 (Dec 603), Dechlorane 604 (Dec 604) and Dechlorane plus (DP)) [15]. There is still a lack of information regarding their behavior and occurrence in the environment, but in recent years the interest in these compounds has greatly increased. They have been found in several environmental and biological matrices such as sediment [16], air [17], sludge [18], water [19], fish [20], eggs [21] and humans [22]. Besides, they seem to have similar toxic properties to PBDEs [23].

The aim of this study was to evaluate the presence of both classical and emerging FRs in sediment and sludge samples from two

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Iberian river basins, and also to establish whether the emerging FRs are really gaining more relevance in comparison to the banned PBDEs. Moreover, this study provides information about the presence of emerging HFRs in Spain which might be useful when establishing new legislation restrictions over these compounds.

2. Sampling

Within the framework of the SCARCE project sediment and sludge samples from two Iberian river basins, Ebro and Llobregat, were collected during 2010. Nineteen different sampling points for sediment collection and six wastewater treatment plants (WWTPs) for sludge were selected along the Ebro river basin, while 14 sediments and one sludge sites were analyzed in the Llobregat river basin (Fig. 1). After collection, samples were stored at −20 °C prior to lyophilization and instrumental analysis.

These two rivers have high levels of industrial activity. The Ebro river is the most important river in Spain. It also flows into the Ebro Delta, which was designated as Natural Park and it is in intensive agricultural use for rice, fruit (in particular citrus), and vegetables. The Ebro is largely regulated by dams and channels, which have altered its hydrological and sedimentary regime. On the other hand, the Llobregat river is the second longest river in Catalonia and receives extensive urban and industrial waste water discharges as well as surface runoff from agricultural areas that cannot be diluted by its natural flow. The river is heavily managed in its lower course and water that was previously lost to the sea is now pumped upstream to increase the natural flow, recharge the delta wetlands and control seawater incursion. This river is one of Barcelona's major drinking water resources.

3. Materials and methods

3.1. Standards

The standard mixture of PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209), HBB, DBDPE and PBEB were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). BDE-77, BDE-181 and ^{13}C-BDE-209, used as internal standards, were also purchased from Wellington Laboratories Inc.

Syn- and *anti*-isomers of DP were purchased from Wellington Laboratories Inc. Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). ^{13}C-*syn*-DP (98%), used as internal standard, was also obtained from Cambridge Isotope Laboratories Inc. (Andover, MA).

Alumina (0.063–0.2 mm) and copper (<63 μm) were obtained from Merck (Darmstadt, Germany). Al–N and Silica cartridges were obtained from Biotage. Dichloromethane and hexane, solvents for organic trace analysis, were purchased from Merck.

3.2. Sample treatment

The methodologies applied for the sample treatment of sediment and sludge were based on methods described previously [24,25]. The methodology applied for sludge was more complex, since it is a dirtier matrix than sediment.

For sediment a selective pressurized liquid extraction (SPLE), where extraction and purification are done simultaneously, was used. Before extraction, 2 g dry weight (dw) of sediment were spiked with the surrogate standards (5 ng of BDE-77, BDE-181 and ^{13}C-*syn*-DP, and 50 ng of ^{13}C-BDE-209) and were kept overnight to equilibrate. SPLE was carried out using an ASE 350 system (Dionex, Sunnywale, CA, USA). Spiked samples were ground with alumina and copper (1:2:2) and loaded into a 22 mL extraction cell previously loaded with 8 g of alumina. Dead volume was filled with hidromatrix. A hexane:dichloromethane (DCM) mixture (1:1) was used as extraction solvent, while pressure and temperature were settled at 1500 psi and 100 °C respectively. 5 min of oven heat-up and two static cycles of 10 min were made. Final extraction volume was about 35 mL. Extracts were concentrated to incipient dryness and re-dissolved with toluene for a final volume of 40 μL.

Regarding the sludge samples, 1.5 g dw were spiked with the internal standards (5 ng of BDE-77, BDE-181 and ^{13}C-*syn*-DP, and 50 ng of ^{13}C-BDE-209) and were kept overnight to equilibrate. Spiked samples were ground with copper (1:2) and loaded into an 11 mL extraction cell. Dead volume was filled with hidromatrix and PLE was carried out using the same pressure, temperature and conditions as for sediment samples. Resulting extracts were treated with concentrated sulfuric acid (H_2SO_4), and two steps of solid phase extraction (SPE) were done in order to obtain a clean extract. Silica (2 g) cartridges were first used, with 20 mL of hexane for the conditioning step and 20 mL of hexane for elution step. Alumina (5 g) cartridges were then used with 20 mL of hexane for conditioning and 20 mL of hexane:DCM (1:2) for the elution step. Resulting extracts were concentrated and re-dissolved as described before.

3.3. Instrumental analysis

PBDEs and emerging BFRs (HBB, PBEB and DBDPE) were analyzed by an Agilent 7890C gas chromatograph connected to an Agilent 5975A Network mass spectrometer, working in negative chemical ionization mode (NCI) using NH_4^+ as reagent gas. The instrumental conditions and elution program had been previously developed [3,26]. Briefly, temperature started at 140 °C, was held for 2 min and then ramped to 325 °C at 10 °C/min. Final temperature was held 10 min. Source temperature was set at 250 °C. Selected ion monitoring (SIM) mode was applied in order to enhance the sensitivity. Experiments were carried out monitoring the two most intense peaks from the NCI spectra. Ions monitored were *m/z* 79 and 81 for all PBDEs and emerging BFRs with the exception of BDE-209 and ^{13}C-BDE-209, where the two ions monitored were *m/z* 487 and 489, and *m/z* 497 and 499, respectively. The most intense peaks were used for quantification purposes, and the second ones for confirmation.

On the other hand, halogenated norbornenes were analyzed using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole, working in NCI using CH_4^+ as reagent gas. Our previous work showed the advantages of MS–MS against the single quadrupole MS [27]. Temperature started at 80 °C, was held for 2 min and then ramped to 300 °C in 10 °C/min. Final temperature was maintained for 10 min. Source temperature was set at 175 °C and electron energy and emission current were set at 200 and 150 eV, respectively. Selective reaction monitoring (SRM) mode was applied in order to enhance the sensitivity. The two most intense transitions were used for quantification and confirmation purposes. The most intense transition was used for the quantification and the second transition was used for the confirmation.

3.4. Quality control

Recoveries, method limits of detection (mLODs), defined as the three times the noise level, and method limits of quantification (mLOQ), defined as 10 times the noise level, are showed in Table 1. Regarding individual PBDE congeners, recoveries were between 53% and 83% (RSD < 10%) in sediments, and between 31% and 81% (RSD < 14%) in sludge. Values for emerging BFRs were between 58% and 102% (RSD < 6%) in sediments and between 30% and 50% (RSD < 12%) in sludge. Finally, recoveries of halogenated norbornenes were between 65% and 114% in sediments (RSD < 11%) and between 57% and 72% (RSD < 12%) in sludge. Since sludge is a

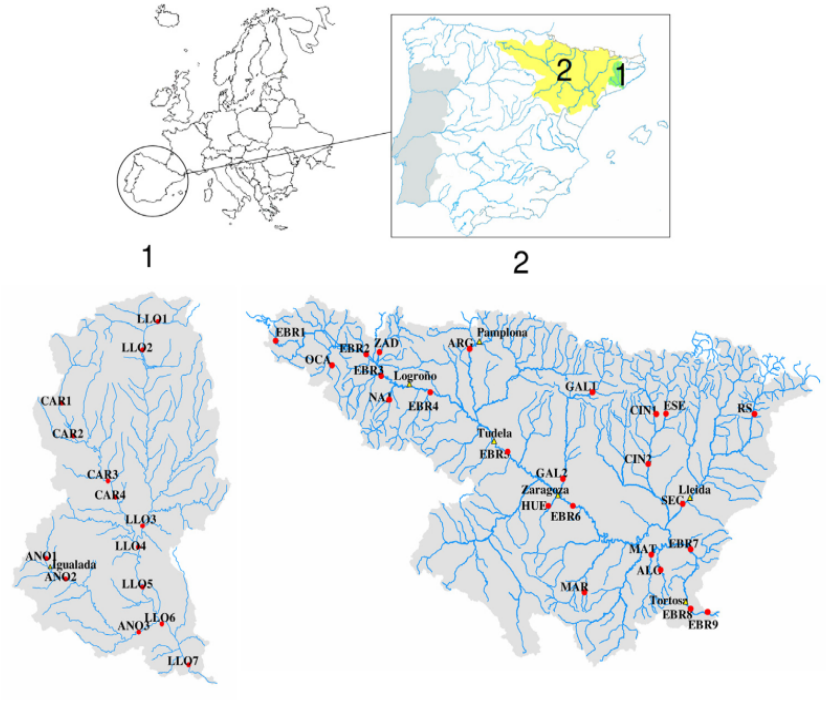

Fig. 1. Sampling points in the Llobregat (1) and Ebro (2) river basins for sediments (circle) and sludge (triangle).

dirtier matrix than sediment and more complex clean-up process is needed, the fact that the recovery values were lower than in sediment was considered normal. Moreover, the mLODs obtained for sediments were better than the ones obtained for sludge.

In sediments, mLODs for PBDEs and emerging BFRs were between 8.1 and 41 pg/g dw and between 9.1 and 86 pg/g dw, respectively. Values for halogenated norbornenes were lower, ranging from 0.1 to 1.3 pg/g dw. Regarding to sludge, mLODs for PBDEs and emerging BFRs ranged from 0.19 to 0.67 ng/g dw and from 0.17 to 3.63 ng/g dw, while values for halogenated norbornenes were between 0.012 and 0.029 ng/g dw.

The linearity of the method was checked using calibration curves made from standard solutions at five concentration levels (ranging from 10 pg injected to 5000 pg injected). Matrix effect was evaluated for all the compounds. Values of the ion suppression or enhancement were lower than 15% for sediments and lower than 35% for sludge, which was considered acceptable.

4. Results and discussion

4.1. Sediment samples

Table 2 shows the concentration levels found for PBDEs and emerging BFRs in Ebro and Llobregat river basins. Several PBDEs (BDE-47, BDE-100, BDE-99, BDE-154, BDE-153, BDE-183 and BDE-209) were detected in all the samples from the Llobregat river basin and in 18 out of 19 samples from the Ebro river basin. BDE-209 was the most abundant compound, with a contribution between

Table 1
Recoveries (%), RSD (%), mLODs and mLOQs for all the compounds in the two matrices studied.

	Sediment				Sludge			
	Recovery (%)	RSD (%)	mLOD (pg/g dw)	mLOQ (pg/g dw)	Recovery (%)	RSD (%)	mLOD (ng/g dw)	mLOQ (ng/g dw)
Dec 602	114	4	1.3	4.3	60	12	0.018	0.06
Dec 603	86	6	0.1	0.33	66	10	0.012	0.04
Dec 604	65	6	0.2	0.67	57	11	0.029	0.10
syn-DP	86	8	0.3	1.00	76	7	0.017	0.06
anti-DP	104	11	0.2	0.67	72	6	0.014	0.05
BDE-28	62	6	19	63	31	13	0.33	1.10
BDE-47	74	2	9	30	63	14	0.21	0.70
BDE-100	60	1	8.1	27	46	7.4	0.19	0.63
BDE-99	75	2	18	61	75	12	0.2	0.67
BDE-154	61	3	21	70	30	5.8	0.21	0.70
BDE-153	68	5	29	95	31	11	0.67	2.23
BDE-183	53	10	41	137	38	5.9	0.21	0.70
BDE-209	83	6	25	83	81	9.3	0.2	0.67
HBB	62	5	9.1	30	30	7.4	0.56	1.86
PBEB	58	3	18	60	50	12	0.17	0.57
DBDPE	102	6	86	286	44	6	3.63	12.09

PBDEs: Polybromodiphenyl ethers. **DBDPE**: Decabromodiphenyl ethane. **HBB**: Hexabromobenzene. **PBEB**: Pentabromoetilbenzene. **Dec 602, 603, 604**: Dechlorane 602, 603, 604. **DP**: Dechlorane plus. **RSD**: Relative standard deviation. **mLOD**: Method limit of detection. **mLOQ**: Method limit of quantification.

Table 2
Concentrations of PBDEs and DBDPE in sediments from Llobregat and Ebro river basins (ng/g dw).

	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	BDE-183	BDE-209	ΣPBDEs	DBDPE
Llobregat river basin									
LLO1	nd	nd	0.15	nd	nd	nd	2.38	2.53	7.14
LLO2	0.30	nd	0.53	nd	nd	nd	1.47	2.30	7.40
LLO3	0.11	nd	0.18	nd	nd	0.08	5.91	6.30	5.22
CAR1	nd	nd	nd	nd	nd	nd	1.50	1.50	3.19
CAR2	nd	nd	0.15	nd	nd	0.11	2.13	2.40	16.6
CAR3	nd	nd	nd	nd	nd	0.03	5.01	5.03	2.12
CAR4	0.70	nd	nd	nd	nd	0.01	43.6	44.3	25.6
LLO4	nd	0.09	0.35	0.04	nd	0.06	12.6	13.2	10.5
LLO5	nd	0.04	0.11	0.02	nd	0.03	14.9	15.1	13.3
ANO1	nd	nd	0.29	nd	nd	0.18	3.84	4.31	15.4
ANO2	nd	nd	nd	nd	nd	nd	29.1	29.1	nd
ANO3	nd	nd	nd	nd	nd	nd	2.11	2.11	7.91
LLO6	nd	0.02	0.07	nd	0.01	nd	2.08	2.18	20.8
LLO7	Nd	nd	0.49	0.03	nd	0.18	32.9	33.6	21.9
Ebro river basin									
EBR1	0.06	nd	3.48	nd	0.47	nd	28.9	33.0	18.4
OCA	nd	nd	nd	nd	nd	nd	nd	nd	nd
EBR2	nq	nd	0.02	nd	nd	nd	6.01	6.03	3.52
ZAD	nd	nd	nd	4.29	nd	nd	15.2	19.5	12.5
EBR3	nd	0.03	0.05	0.02	0.03	0.05	14.8	15.0	24.2
NAJ	0.10	nd	0.21	nd	nd	nd	9.42	9.73	13.2
ARG	0.28	nd	0.29	nd	nq	nd	19.5	20.1	18.6
EBR4	nd	nq	nd	nd	0.10	0.04	3.12	3.25	6.69
GAL1	nd	nd	nd	nd	nd	nd	2.09	2.09	6.36
GAL2	0.02	nd	0.10	nd	nd	nd	1.58	1.71	7.25
HUE	nd	nd	1.31	nd	nd	nd	1.13	2.43	1.97
EBR6	0.13	nd	0.30	nd	nd	nd	22.4	22.9	31.5
MAR	0.16	nd	0.48	nd	nd	nd	nd	0.64	nd
ESE	nd	nd	nd	nd	nd	nd	2.50	2.50	9.31
CIN1	nd	nd	nd	nd	nd	nd	2.62	2.62	1.63
CIN2	1.39	nd	3.23	0.62	2.18	9.14	20.7	37.3	22.7
RS	nd	nd	nd	nd	nd	nd	3.76	3.76	6.0
ALG	nd	nd	nd	nd	nd	nd	3.01	3.01	nd
EBR9	0.14	nd	nd	nd	nd	nd	0.22	0.36	nd

nd: Below detection limit. **PBDEs**: Polybromodiphenyl ethers. **DBDPE**: Decabromodiphenyl ethane.

64% and 100% of the total PBDE burden in Llobregat samples and between 47% and 100% in Ebro samples. Total PBDE levels ranged from 1.50 to 44.3 and from nd to 37.3 ng/g dw in Llobregat and Ebro river basin, respectively. Differences in concentration levels between the two river basins were not significant.

Our results are consistent with other published levels in previous works. Regarding the Llobregat river basin, in 2007 Labandeira et al. [25] reported total PBDEs levels in sediments from this river ranging from 2.5 to 9.8 ng/g dw. Although our values are higher than values reported in 2007 by Labandeira et al. [25], they are lower than those reported by Guerra et al. [3] (22–136 ng/g dw) in 2010. This difference could be attributed to the fact that the use of PBDEs has been restricted in recent years, but also to the fact that the different studies do not share the same sampling points. Regarding the Ebro river basin, Eljarrat et al. [28] reported total PBDE levels ranging from 2.4 to 42 ng/g dw in sediments from four sampling sites, which are similar to our values, but the fact that in that case 40 BDE congeners were analyzed should be taken into account. Higher levels than those we report were found (from 11.1 to 14,400 ng/g dw) in sediments from a sampling area near an industrial park in the Ebro river basin [29].

Some other data on PBDEs in river sediment are available. Ma et al. reviewed the published data of PBDEs in sediment from China until 2012 [30]. PBDEs were detected in sediments from different rivers in the mainland of China. Total PBDE levels ranged from 0.16 to 7340 ng/g dw, with a high variability among the different areas. Recently, Chen et al. [31] reported high levels of PBDEs (ΣPBDE ranging from 17 to 588 ng/g dw) in China, although these values were lower than reported in 2005 in the same area [32]. On the other hand, lower levels than we observed were found in San Francisco Bay (USA), with a ΣPBDE concentration ranging from 2 to 8 ng/g dw [33]. Hale et al. reported low ΣPBDE levels (up to 0.5 ng/g dw, BDE-209 not included) in river sediments from Virginia. However, a high concentration of penta-BDEs was found near to a polyurethane foam manufacturing facility in North Carolina (135 ng/g dw) and BDE-209 were really high (up to 3190 ng/g dw) [34]. In Europe, total PBDE levels ranging from 0.06 to 84 ng/g dw were reported by Sawal et al. [35] for 32 sediments from the Danube river and its tributaries.

There is a great variation of PBDEs levels worldwide, with higher levels associated to the highly industrialized areas. However, several studies report that total PBDEs levels are decreasing year by year [36,37], probably due to the recent restrictions over the production and use of PBDEs.

Regarding emerging BFRs, HBB and PBEB were not detected in any sample. However, DBDPE was detected both in Llobregat and Ebro river basins (Table 2). DBDPE was detected in 13 out of 14 samples from the Llobregat river, with values ranging from nd to 25.6 ng/g dw, and in 15 out of 19 samples from the Ebro river with values ranging from nd to 31.5 ng/g dw. These values are slightly lower than those found by Wang et al. [38] in river sediments from Northern China (from 16 to 68 ng/g) and similar to those detected by Kierkegaard et al. [39] in Western Scheldt, Netherlands (mean value of 24 ng/g dw).

It is interesting to study the ratio between concentration levels of BDE-209 and their substitute DBDPE ($R_{BDE\text{-}209/DBDPE}$). Fig. 2 shows these $R_{BDE\text{-}209/DBDPE}$ values in sediment samples. For Llobregat river basin, $R_{BDE\text{-}209/DBDPE}$ ranged from 0.1 to 2.4 with a mean value of 0.8; and, for Ebro river basin, $R_{BDE\text{-}209/DBDPE}$ ranged from 0.2 to 1.7 with the same mean value of 0.8. Thus, BDE-209 and DBDPE levels were quite similar. Wei et al. [40] reported a $R_{BDE\text{-}209/DBDPE}$ value of 2.1 in sediments from Arkansas. Furthermore, He et al. [41] found two

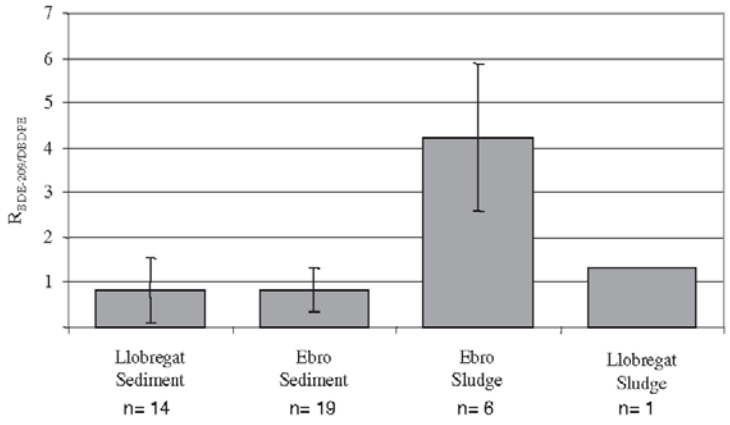

Fig. 2. Mean values (and associated standard deviations) of the ratio between brominated diphenyl ether 209 (BDE-209) and decabromodiphenyl ethane (DBDPE) concentrations ($R_{BDE-209/DBDPE}$) in sediment and sludge samples from Ebro and Llobregat river basins.

interesting tendencies in sediments from Southern China: firstly, BDE-209 levels were much lower in sediments collected in 2010 (from 1.3 to 2400 ng/g dw) than in sediments collected in 2002 (21 to 7340 ng/g dw). Secondly, the mean value for DBDPE slightly increased in those 10 years, being 153 ng/g dw in 2002 and 200 ng/g dw in 2010, for the same sediment samples. Both facts may indicate that the restrictions over the use of BDE-209 are being followed and DBDPE is being used as its substitute. Unfortunately, there are no other reported levels of DBDPE in river sediment from Spain, so this tendency can not be confirmed with our results. Nevertheless, BDE-209 levels seem to decrease over the years, as suggested by Yang et al. [37].

As regards halogenated norbornenes, Dec 602, Dec 603 and both isomers of DP were detected both in Llobregat and Ebro river basins (Table 3), whereas Dec 604 was not found in any sediment sample. Halogenated norbornenes were detected in 13 out of 14 Llobregat river samples with total levels ranging from 0.02 to 3.68 ng/g dw, and in 17 out of 19 of the Ebro river samples with total levels ranging from nq to 3.74 ng/g dw. Like PBDEs, no difference was found between the two river basins. However, Dec 602 and Dec 603 were most often detected in Llobregat river basin (frequency of detection of 35% and 57%, respectively) than in Ebro river basin (frequencies of 10% and 21%, respectively). Since Llobregat is a short river with a high density of industries and WWTPs, this behavior could be expected. Regarding DP, total levels ranged from nd to 1.39 ng/g dw in Llobregat river and from nq to 1.64 in Ebro river. This is the first study to report concentrations of halogenated norbornenes in sediments from Spain. Regarding river sediments from other places of the world, in China Sun et al. [42] found concentrations of Dec 602, Dec 603 and DP ranging from nd to 0.05, nd to 0.026 and nd to 1.1, respectively, which is similar to what we found. Similarly, Wang et al. found total DP levels up to 1.3 ng/g dw in river sediments from South China [43]. On the other hand, DP was found in sediments from Lake Ontario and Lake Erie at concentrations up to 586 and 8.62 ng/g dw, respectively [44], which are considerably higher than our values. This may be due to the fact that the OxyChem factory, which produces the DP, is located in the Great Lakes [15].

F_{anti} values (the isomeric ratio of the two DP isomers, expressed as the ratio of the amount of *anti*-DP relative to the total amount of both isomers) were calculated and mean values were 0.73 and 0.75 for the Ebro and the Llobregat rivers, respectively. Differences between F_{anti} values could indicate a different environmental behavior of both isomers. However, in our case, F_{anti} values in sediment samples were similar to those found in commercial mixtures (F_{anti} values ranging from 0.64 to 0.80) [45]. This is in agreement with Swerko et al. [15] who concluded that F_{anti} tends to remain similar to the commercial mixture in sediment samples.

4.2. Sludge samples

Several classical PBDEs (BDE-47, BDE-100, BDE-99, BDE-154, BDE-153 and BDE-209) and emerging HFRs (DBDPE, Dec 602, Dec 603, *syn*-DP and *anti*-DP) were detected in sludge samples collected at different WWTPs (Table 4). BDE-209 was the most abundant PBDE congener, with levels ranging from nq to 319 ng/g dw. Other compounds with lower bromination levels were found at lower concentrations (from nd to 17.2 ng/g dw), with tetra-BDE-47 and penta-BDE-99 being the most abundant. The sample collected at WWTP of Lleida showed the highest total PBDE level (343 ng/g dw) while the lowest level was found at Pamplona (20.7 ng/g dw). It should be note that BDE-209, which clearly dominates the BDE profile in sludge, could not be quantified in this WWTP. No correlation was found with the total flow (m^3/day) or equivalent population (Supporting information 1)

Our total PBDE values in sludge were lower than those previously reported in Spanish sewage sludge. De la Torre et al. [46] found values from 58 to 2606 ng/g dw in sludge collected at 31 WWTPs from Spain. Other authors found similar values, ranging from 197 to 1185 ng/g dw [47], from 21 to 737 ng/g dw [48] or from nd to 2326 ng/g dw [5]. It is interesting to note that when we compare our results with those reported for Spanish sludge samples collected some years ago, BDE-209 values have clearly decreased. For example, Gorga et al. reported levels of BDE-209 up to 2303 ng/g dw [5] while our maximum is 319 ng/g dw. This tendency could be explained by the ban of BDE-209 usage. Comparing with levels in other countries, higher values, with total PBDEs levels ranging from 5.1 to 1115 ng/g dw, were found in sludge samples from different WWTPs from China [49]. On the other hand, reported levels in sludge from different USA WWTPs were also considerably higher (1085 to 8080 ng/g dw), probably due to the fact that BDE-209 was used in the USA at higher amounts than in Europe [34].

As regards emerging BFRs, HBB and PBEB were not detected in any sample. However DBDPE was detected in all the analyzed samples with values ranging from nq to 124 ng/g dw. These values are quite similar to those reported by [36] in Spanish samples from 31 different WWTPs (3.24 to 125 ng/g dw). These samples were collected in 2006, four years before the collection of the present study. Whereas the range of concentration was similar for both studies, the mean value was different: 45 ng/g dw for the 2006

Table 3
Concentrations levels of halogenated norbornenes in sediments from Llobregat and Ebro river basins (ng/g dw).

	Dec-602	Dec-603	*syn*-DP	*anti*-DP	F_{anti}	ΣDechloranes
Llobregat river basin						
LLO1	nd	nd	0.02	0.08	0.77	0.11
LLO2	nd	nd	nd	0.02	1.00	0.02
LLO3	nd	0.02	0.05	0.09	0.64	0.16
CAR1	0.06	0.01	0.05	0.12	0.72	0.23
CAR2	nd	nd	nd	nd	–	nd
CAR3	nd	1.04	0.06	0.25	0.80	1.36
CAR4	0.08	0.08	0.19	0.24	0.56	0.59
LLO4	0.13	0.76	0.34	1.05	0.75	2.28
LLO5	0.09	0.12	0.12	0.40	0.77	0.73
ANO1	nd	0.06	0.06	0.15	0.73	0.27
ANO2	0.79	0.66	0.73	1.50	0.67	3.68
ANO3	nd	nd	0.04	0.16	0.79	0.20
LLO6	nd	nd	0.08	0.17	0.69	0.24
LLO7	nd	0.17	0.34	0.95	0.74	1.46
Ebro river basin						
EBR1	nd	nd	0.06	0.17	0.75	0.23
OCA	nd	nd	0.03	0.09	0.72	0.12
EBR2	nd	0.02	0.02	0.05	0.72	0.10
ZAD	nd	nd	0.46	1.19	0.72	1.64
EBR3	nd	0.26	0.28	1.33	0.83	1.88
NAJ	nd	nd	0.06	0.14	0.70	0.20
ARG	nd	0.03	0.11	0.22	0.67	0.36
EBR4	0.14	nd	0.04	0.09	0.69	0.26
GAL1	nd	nd	0.03	0.06	0.67	0.08
GAL2	nd	nd	0.04	0.11	0.76	0.15
HUE	1.91	nd	0.50	1.33	0.73	3.74
EBR6	nd	nq	nq	nq	–	nq
MAR	nd	nd	0.06	0.08	0.56	0.14
ESE	nd	nd	0.01	0.03	0.86	0.03
CIN1	nd	nq	nq	nq	–	nq
CIN2	nd	nq	0.001	0.01	0.89	0.01
RS	nd	nd	0.08	0.16	0.68	0.24
ALG	nd	nq	0.03	0.07	0.73	0.09
EBR9	nd	0.30	0.07	0.16	0.72	0.53

nd: Below detection limit. **nq**: Below quantification limit. **Dec 602, 603**: Dechlorane 602, 603. **DP**: Dechlorane plus.

sampling, and 74 ng/g dw for the 2010 sampling, which may indicate an increase of DBDPE use in Spain. In addition, Gorga et al. [5] found mean DBDPE concentrations of 57 ng/g dw for sludge samples collected in 2009. However, studies reporting the presence of DBDPE in sludge are still limited. Ricklund et al. [50] found DBDPE in sludge from Stockholm with values ranging from 60 to 95 ng/g dw, matching reasonably well with our data.

Fig. 2 shows the $R_{BDE\text{-}209/DBDPE}$ values in sludge samples. $R_{BDE\text{-}209/DBDPE}$ ranged from 2.6 to 6.4 with a mean value of 4.2 for the Ebro river basin, indicating that BDE-209 levels were higher than those of DBDPE. For the unique sludge sample collected at the Llobregat river, $R_{BDE\text{-}209/DBDPE}$ was 1.3; in this case, BDE-209 and DBDPE levels were similar.

Regarding the halogenated norbornenes, Dec 602 was only detected in one sludge sample, Tortosa, at concentration level of 0.24 ng/g dw. In contrast, Dec 603 and both isomers of DP were detected in all the samples, with values ranging from 0.08 to 0.60, 0.85 to 11.2 and nq to 11.9 ng/g dw, respectively. Unlike the case

Table 4
Concentrations levels of PBDEs, DBDPE and halogenated norbornenes in sewage sludge from different Ebro and Llobregat WWTPs (ng/g dw).

	Ebro						Llobregat
	Lleida	Logroño	Pamplona	Tortosa	Tudela	Zaragoza	Igualada
PBDEs and emerging BFRs							
BDE-47	17.2	6.53	7.24	7.28	7.94	4.31	4.50
BDE-100	3.88	1.40	1.60	1.59	2.04	nd	2.30
BDE-99	nd	8.09	10.3	10.1	1.17	5.84	6.41
BDE-154	nd	0.49	0.61	0.69	0.99	0.43	nd
BDE-153	2.99	nd	0.97	1.00	1.56	0.65	nd
BDE-209	319	214	nq	233	225	153	150
DBDPE	124	nq	112	52.5	35.1	43.7	112
$R_{BDE\text{-}209/DBDPE}$	2.56	–	–	4.43	6.42	3.51	1.34
Halogenated norbornenes							
Dec-602	nd	nd	nd	0.24	nd	nd	nd
Dec-603	0.60	0.08	0.09	0.42	0.16	0.08	nd
syn-DP	11.2	0.85	2.42	2.16	6.94	2.24	1.50
anti-DP	nq	1.73	6.80	3.93	11.9	5.92	3.81
F_{anti}	–	0.67	0.74	0.65	0.63	0.73	0.72
ΣDechloranes	11.6	2.66	9.31	6.75	19.0	8.24	5.31

nd: Below detection limit. **nq:** Below quantification limit. **PBDEs:** Polybromodiphenyl ether. **DBDPE:** Decabromodiphenyl ethane. **Dec 602, 603:** Dechlorane 602, 603. **DP:** Dechlorane plus.

of PBDEs, the sludge sample which showed the highest levels of halogenated norbornenes was located in Tudela, while Lleida, which had the highest PBDE levels, had the second highest level of norbornenes. Similar to sediment samples, F_{anti} values in sludge (ranging from 0.63 to 0.74) were similar to those found in commercial mixtures. Studies reporting the presence of halogenated norbornenes in sludge are very scarce. De la Torre et al. [51] found slightly higher values of total DP (2.5 to 94 ng/g dw) in 31 different sludge from Spain. Kolic et al. [52] found about 119 ng/g dw of DP in sludge from one WWTP from Toronto, Canada.

5. Conclusions

PBDEs, emerging BFRs and halogenated norbornenes were analyzed in sediment and sludge samples from two Iberian river basins. Different PBDE congeners, DBDPE, Dec 602, Dec 603 and both isomers of DP were detected. This is the first time that halogenated norbornene levels were reported in sediment samples from Spain. Gathering information regarding HFRs in sludge is important since 65% of the sludge is used for agricultural purposes. Since these contaminants have been found in sludge the agricultural soils could be affected. The new information is also useful in order to establish new legislation over the application of sewage sludge

Our results seem to indicate that emerging FRs are slowly replacing the "classical" PBDEs. Whereas halogenated norbornene levels are still lower than those of PBDEs, DBDPE concentrations seem to have been increasing in recent years. It is expected that this behavior will continue in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2013.10.069.

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Supporting information

		Flow m^3/day	Eq. habitants	Treatment	Sludge Treatment
Llobregat	Igualada	20000	285660	Secondary	Anaerobic digestion + centrifugation
Ebro	Pamplona	129600	550000	Secondary	Anaerobic digestion + centrifugation
	Logrono	103680	466560	Secondary	Anaerobic digestion +centrifuge
	Tudela	24000	80000	Secondary	Aerobic digestion + centrifugation
	Zaragoza	34560	1200000	Secondary	Thickening + incineration
	Lleida	87500	186666	Secondary	Anaerobic digestion + centrifugation
	Tortosa	10296	46827	Secondary	-

3.3. Discusión

En las publicaciones #3 y #4 se describen los resultados obtenidos en el análisis de las muestras de Chile, Colombia y España. A continuación se discuten las concentraciones presentes en sedimento y lodos de depuradora, así como la influencia de las diferentes zonas de estudio. Como se ha discutido en el capítulo 1, es muy importante caracterizar el área de estudio ya que ésta tiene una gran influencia en la presencia o no de los HFRs en la zona, así como la abundancia de los mismos. Por ello, es interesante comparar 2 regiones tan diferentes como España, un país miembro de la UE y por tanto suscrito a todas las regulaciones detalladas en el apartado 1.4, con otras donde las únicas regulaciones vigentes son las de carácter internacional, como el convenio de Estocolmo. Pese a que los tres países firmaron el convenio en la misma fecha (23/05/2001), en Chile esta firma fue ratificada en 2005 y en Colombia en 2008 (en España lo fue en 2004). Además, las diferencias en cuanto al desarrollo industrial y nivel de vida de los diferentes países también juegan un papel importante y comprobar si éstas influyen en las concentraciones de HFRs no carece de interés.

3.3.1. Sedimentos

Tanto en los sedimentos recogidos en Chile y Colombia como a lo largo de las cuencas hidrográficas del Ebro y Llobregat, en España, se detectaron diferentes congéneres de PBDEs (BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 y BDE-209), cuya presencia a lo largo del globo es de sobra conocida, aunque fue la primera vez que se estudiaron en Chile y Colombia. Mientras que en Chile se determinaron prácticamente todos los congéneres incluidos en nuestra metodología analítica, en Colombia sólo se detectó el BDE-209. Además, se detectaron algunos BFRs emergentes como el PBEB en Chile y el DBDPE en los 3 países. En el caso de España se detectaron varios compuestos de la familia de los HNs, el Dec 602, Dec 603 y DP, pero no el Dec 604.

Observando los resultados obtenidos en Sudamérica, es de suponer que en Colombia la única mezcla utilizada es la Deca-BDE mientras que en Chile también se aplicaba la Penta-BDE, pese a que el BDE-209 presentaba la contribución más alta a las concentraciones totales de PBDEs. El hecho de encontrar DBDPE y PBEB en Chile y no en Colombia podría ser debido a que en Chile se empezaron a buscar alternativas a los PBDEs, o por lo menos a la mezcla Penta-BDE, cosa que no ocurrió en Colombia.

Estas muestras se recogieron entre 2009 y 2010, por lo que estos perfiles podrían haber cambiado desde entonces. De hecho, recientemente se llevó a cabo otro estudio en la misma bahía de Concepción (Chile) y los perfiles obtenidos fueron bastante diferentes (Pozo *et al.*, 2015). En muestras de sedimento recogidas en 2013 se vio como los niveles de BDE-209, así como los de otros PBDEs de menor grado de bromación, presentaban un perfil diferente al encontrado en la misma zona durante el estudio realizado en esta tesis (Tabla 3.1).

Tabla 3.1. Comparativa entre concentraciones en sedimentos procedentes de la Bahía de Concepción (ng/g dw).

	Pozo *et al.* (2013)			**Artículo #3**
Sedimentos (n)	1	1	1	3
Penta-BDE	0,09	0,05	0,02	0,3
BDE-209	21	5	2	1,7

n: Número de muestras en cada punto de muestreo. Penta-BDE: ∑BDE-47, BDE-99, BDE-100.

En 2010 hubo un fuerte tsunami que afectó a toda la zona de muestreo y de hecho podría haber tenido influencia en este aumento de las concentraciones del BDE-209, ya que en la bahía de Concepción se acumularon numerosos deshechos arrastrados por el tsunami. Si éstos contenían BDE-209 se podría esperar un aumento de la presencia del mismo en la zona. No obstante, la disminución observada en las concentraciones de los componentes mayoritarios de la mezcla Penta-BDE podría sugerir que su uso se detuvo. El HBCD sólo se detectó en muestras aisladas de Colombia, por lo que se podría asumir que su uso en Sudamérica no está muy extendido, o por lo menos no lo estaba en 2010. Lamentablemente en el momento en el que se analizaron estas muestras no se disponía aún de la metodología para el análisis de los HNs y ningún otro estudio los ha analizado en América del Sur. De cara al futuro sería interesante hacerlo, para evaluar si realmente están también presentes en ese continente o no.

En los sedimentos de los 2 ríos españoles, las concentraciones de HNs fueron unas 10 veces inferiores a las de los clásicos PBDEs, que al igual que en Chile estuvieron dominadas por la presencia del BDE-209 con contribuciones siempre superiores al 64%. Pese a no observarse diferencias significativas entre ambas cuencas en cuanto a valores de concentración, sí pudieron observarse diferencias en cuanto a los perfiles a lo largo de las cuencas (Figura 3.1).

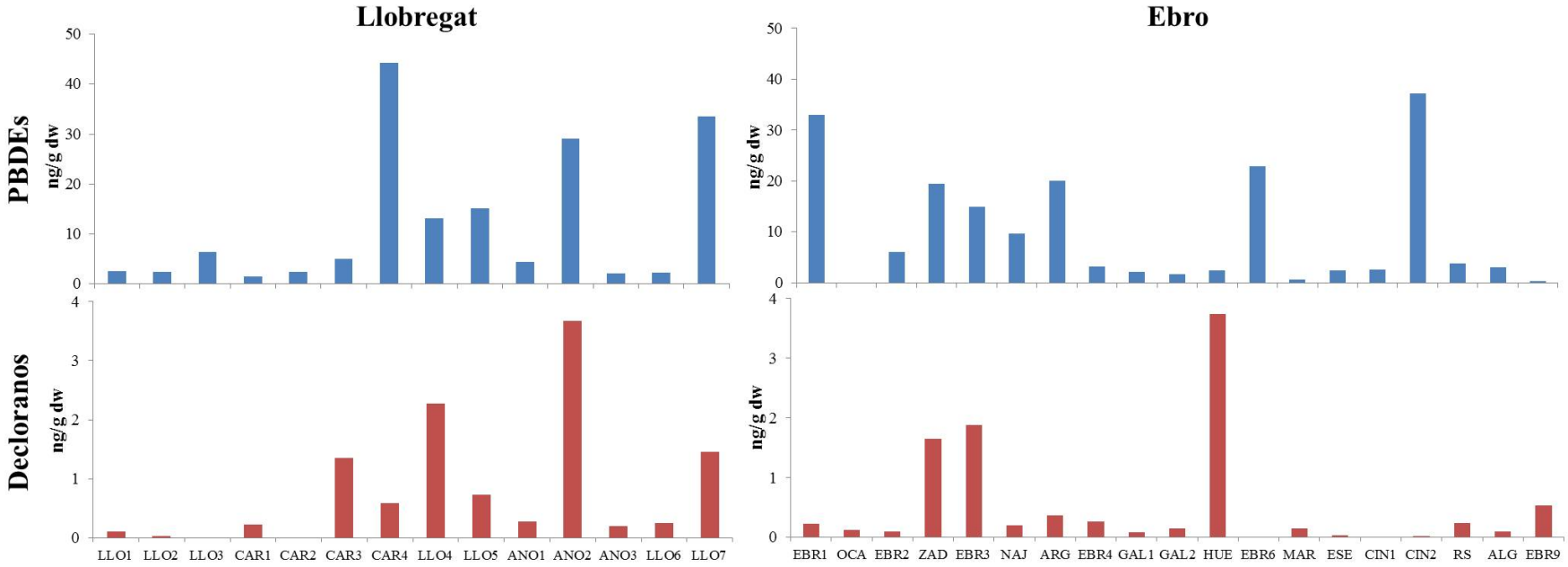

Figura 3.1. Concentraciones encontradas a lo largo de las cuencas hidrográficas del Llobregat y Ebro

Pese a que las evidencias sugerían que la contaminación por HFRs representaba un problema global, los estudios en ciertas regiones eran escasos o inexistentes. Por ejemplo, el artículo #3 fue el primer trabajo que reportaba concentraciones de HFRs en sedimentos de Sudamérica. Además, el artículo #4 aporta nuevos datos en las cuencas del Ebro y Llobregat que, pese haber sido monitorizadas previamente, permiten llevar un seguimiento de la contaminación por HFRs. En el futuro será interesante ver si la contaminación de los ya prohibidos PBDEs se reduce. Mientras que la presencia de PBDEs ya había sido estudiada con anterioridad, esta es la primera evidencia de la presencia de los HNs en ríos españoles. El compuesto encontrado en mayores concentraciones fue el DP (nd-1,82 ng/g dw) y además estaba presente en el 93% de las muestras. Por otro lado el Dec 602 y Dec 603 mostraron una presencia menor, aunque no despreciable (36 y 39%). No obstante, en la Figura 3.1 se ve claramente como las concentraciones de HNs aún no se acercan a las de los PBDEs.

En la figura 3.2 se comparan las concentraciones encontradas para los PBDEs en diferentes zonas estudiadas en esta tesis, utilizando los datos aquí obtenidos así como otros de la literatura para tener una perspectiva más completa (Guerra *et al.*, 2010; Labandeira *et al.*, 2007; Ruiz-Fernández *et al.*, 2014).

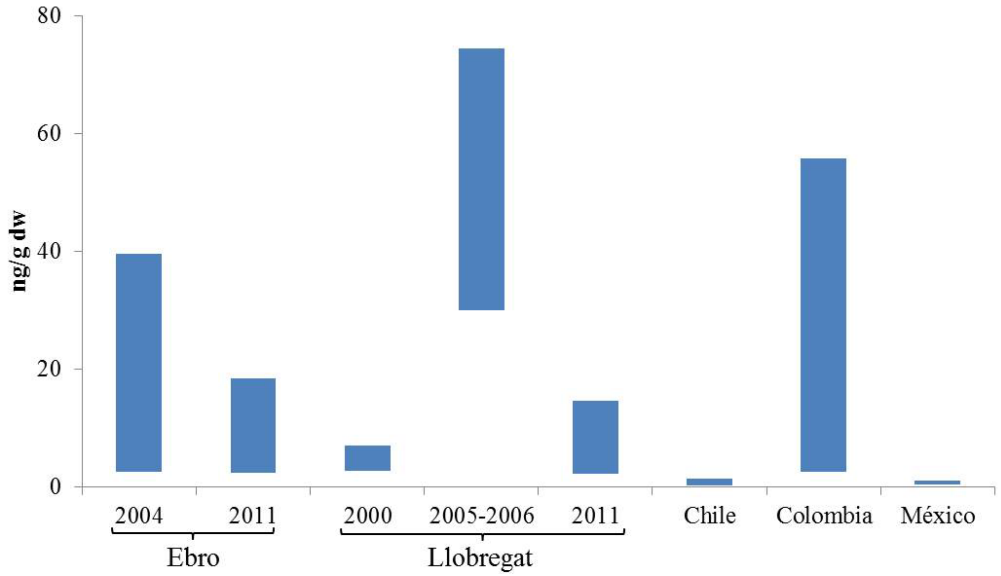

Figura 3.1. Concentraciones de PBDEs entre el primer y el tercer cuartil en las diferentes zonas estudiadas en esta tesis. Ebro 2004: Guerra *et al.* 2010. Llobregat 2000, 2005-2006: Labandeira *et al.* 2007. México: Ruiz-Fernández *et al.* 2014.

Al comparar distintas zonas, la influencia del punto de muestreo puede ser de gran importancia. Por ejemplo, las elevadas concentraciones encontradas en un punto de

muestreo de Colombia se atribuyeron a la proximidad de este punto con Barranquilla y al poco movimiento de agua de la zona, que favorecería la concentración de contaminación en ese punto. Al comparar la misma cuenca hidrográfica y observar como existe también gran variabilidad en los niveles encontrados se puede llegar a la misma conclusión, ya que a lo largo de la cuenca la actividad industrial no es uniforme. Por ejemplo, cerca de una zona de elevada carga industrial en el Ebro se hallaron valores de hasta 14.400 ng/g dw (Eljarrat *et al.*, 2007). Este estudio ha sido excluido de la figura 3.2. Por otro lado, se observa un aumento de las concentraciones entre el 2000 y 2006 en el Llobregat, y una disminución en 2011, coherente también con lo que parece ocurrir en el Ebro. Obviando las diferencias entre puntos de muestreo y considerando la cuenca como un global, esto podría indicar un aumento del uso de la mezcla Deca-BDE a principios de la década, y una disminución posterior a causa de su inclusión en el convenio de Estocolmo. En los artículos #3 y #4 puede encontrarse una discusión más amplia sobre niveles en otras zonas como China o EE.UU disponibles en la bibliografía. Las concentraciones en estas zonas son considerablemente más altas que las estudiadas en esta tesis (Chen *et al.*, 2013; Hale *et al.*, 2003; Klosterhaus *et al.*, 2012; Ma *et al.*, 2013; Mai *et al.*, 2005). En el futuro puede ser interesante realizar un estudio similar para ver si los perfiles presentados en la figura 3.1 han cambiado.

En el caso concreto de los HNs, el artículo #4 presenta los primeros datos en sedimentos en España, demostrándose su presencia a lo largo de las 2 cuencas. De cara al futuro será interesante ver si las concentraciones encontradas, en el orden de los pg/g dw, van en aumento en respuesta a la prohibición del uso de la mezcla Deca-BDE, ya que el DP ha sido propuesto como un posible substituto. Estos niveles son muy inferiores a los determinados cerca de las zonas de producción de China y EE.UU y similares a algunos otros estudios llevados a cabo en otras zonas (Tabla 3.2). Destacan los niveles relativamente altos hallados en sedimentos de río en Paquistán, que muestran de nuevo la influencia de zonas industriales en la presencia de estos contaminantes incluso en áreas donde no habían sido estudiados hasta la fecha (Mahmood *et al.*, 2015), hecho que en sedimentos ha sido caracterizado previamente (Qi *et al.*, 2010; Tomy *et al.*, 2007; Wang *et al.*, 2010). Además en un embalse cercano a una planta de reciclado de e-waste las concentraciones de DP superaron a las de otros contaminantes clásicos como el PCB-153 o el BDE-47 (Wu *et al.*, 2010). Respecto al Dec 602 o Dec 603 las concentraciones encontradas en los ríos españoles son considerablemente inferiores a

las encontradas cerca de las zonas de producción, pero similares a las de otras zonas (Shen *et al.*, 2011; Sun *et al.*, 2013; Wang *et al.*, 2012).

El hecho de que los HNs también hayan sido identificados en diferentes zonas no relacionadas entre si demuestra que también suponen una problemática global, al igual que los PBDEs, por lo que más estudios son necesarios con el fin de caracterizar correctamente su problemática en sedimentos. Cabe recordar que, una vez acumulados en el sedimento, pueden acumularse en diferentes organismos y entrar así en la cadena trófica (Li *et al.*, 2014). Sería interesante conseguir, en un futuro, que la presumible globalidad de los HNs esté tan bien caracterizada como la de los PBDEs.

Tabla 3.2. Comparativa entre las concentraciones de HNs en sedimentos de distintas zonas (ng/g dw).

País	Zona	Dec 602	Dec 603	Dec 604	DP
España	Llobregat	nd-0,79 (0,23)	nd-1,04 (0,22)	n.d	nd-2,23 (0,51)
	Ebro	nd-1,19 (0,19)	nd-0,29 (0,15)	n.d	nd-1,82 (0,38)
Paquistán	Chenab	n.a	n.a	n.a	0,1-12,5 (1,85)
Ártico	King's bay	nd-1,4 (0,7)[a]	0,2-3,4 (1,5)[a]	1,9-20 (7,9)[a]	0,12-0,86 (0,35)
China	Songhua	n.a	n.a	n.a	nd-0,16 (0,04)
	Hong Kong	n.a	n.a	n.a	0,15-2,5 (0,86)
	Embalse (Sur, Industrial)	n.a	n.a	n.a	(7590)
	Huai'an	0,56-3,7 (2,1)	n.a	n.a	1,9-8,0 (4,9)
	Dailing	nd-0,058 (0,042)	n.a	n.a	0,89-2,1 (1,2)
Canada	Lago Winnipeg	n.d	n.d	n.d	(0,03)
	Lago Eire	n.d	n.d	n.d	(0,2)
	Lago Ontario	0,008-21 (6,3)	0,001-0,82 (0,33)	0,005-10 (3,9)	0,06-160 (72)
	Niagara	0,04-1,4 (0,36)	0,008-280 (0,04)	0,01-1,6 (0,22)	2,5-62 (17)

[a]: pg/g dw. **n.d**: No detectado. **n.a**: No analizado

3.3.2. Lodos de depuradora

Tanto los mismos congéneres de PBDEs detectados en sedimento como el DBDPE, Dec 602, Dec 603 y ambos isómeros del DP fueron detectados en los lodos de las 7 EDARs estudiadas. No obstante, cabe destacar que el Dec 602 sólo fue detectado en la depuradora de Tortosa. En el caso del Dec 603 el artículo #4 supuso el primer trabajo que muestra su presencia en EDARs. De nuevo el BDE-209 fue el compuesto más abundante, al igual que en sedimentos y en otros estudios llevados a cabo en lodos de depuradora de España (De la Torre *et al.*, 2011; Gorga *et al.*, 2013). Como ya se ha visto, la zona de estudio tiene una gran importancia. Los lodos de depuradora suelen presentar niveles muy elevados de los contaminantes más hidrofóbicos, debido a un efecto de concentración, así que no es sorprendente observar una gran variabilidad al comparar los resultados obtenidos en el artículo #4 con los obtenidos en otras depuradoras distribuidas por todo el territorio español (Figura 3.3).

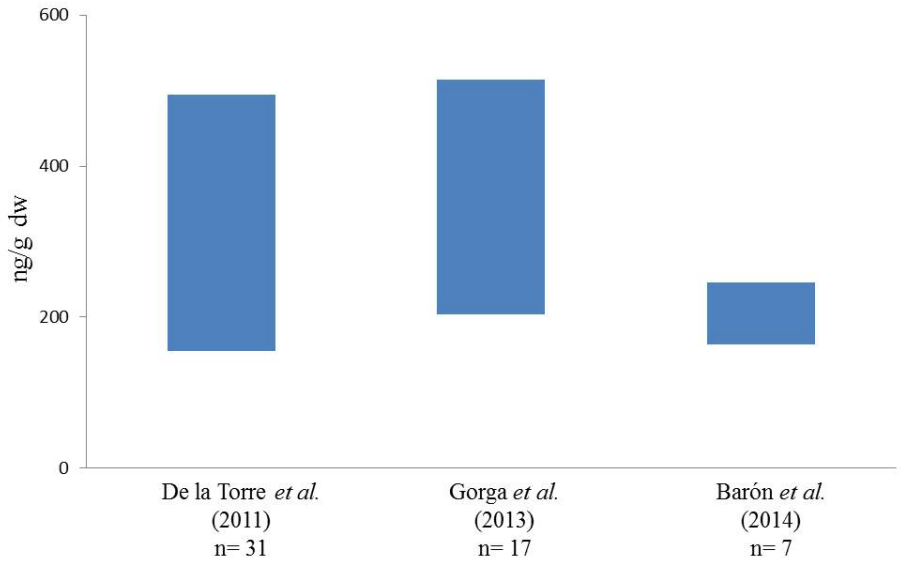

Figura 3.2. Concentraciones de PBDEs entre el 1er y 3er cuartil encontradas en EDARS de todo el territorio Español.

Del mismo modo, al comparar las concentraciones de DP en el Ebro y Llobregat con las publicadas por De la Torre *et al.* (2011) en 31 depuradoras distribuidas a lo largo de toda España, se observa también la gran variabilidad existente. Las estudiadas en esta tesis se encuentran en el rango bajo de concentraciones, al igual que en el caso de los PBDEs (Figuras 3.3 y 3.4). En el artículo #4 se detectaron HNs en concentraciones comprendidas entre 2,7 y 19 ng/g dw (las concentraciones de PBDEs oscilaron entre 13 y 340 ng/g dw). Como se puede ver la diferencia no es tan abismal como en el caso de

los sedimentos, pero aun así los PBDEs siguen siendo más abundantes seguramente debido a un mayor uso. El Dec 602 se detectó en el 14% de los lodos, el Dec 603 en el 85%, el syn-DP en el 100% y el anti-DP en el 85%, siendo de nuevo el compuesto más abundante de la familia. De nuevo, esto demuestra su ubicuidad pese a sus relativamente bajas concentraciones, existiendo asimismo el peligro de que un aumento en su uso unido a la citada ubicuidad de lugar a una problemática mucho mayor.

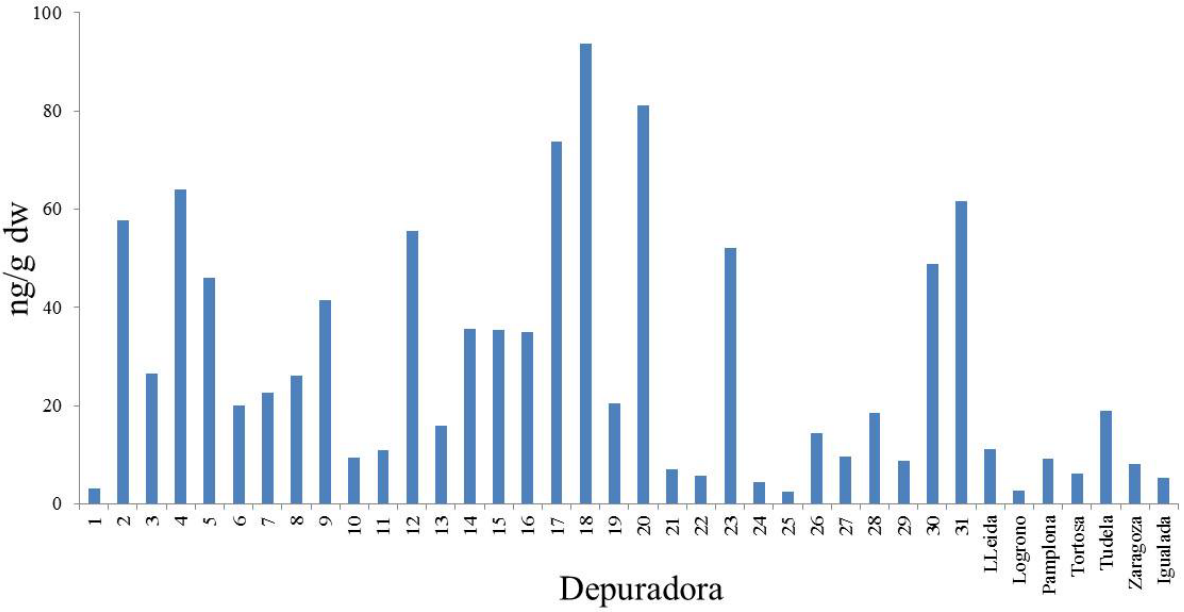

Figura 3.3. Concentraciones de DP encontradas en 38 depuradoras españolas (1-31 datos de de la Torre *et al.* 2011).

Las concentraciones de DP encontradas en España (2,45-18,0 ng/g dw) son inferiores a las encontradas en una región de China con elevada carga industrial (nd-298 ng/g dw) y a la encontrada en una sola depuradora de Canadá (119 ng/g dw). Por el momento la investigación sobre la presencia de estos contaminantes en lodos de depuradora es limitada. Dado que en España en concreto casi el 50% se usa en la agricultura (De la Torre *et al.*, 2011), no es un asunto trivial y sería necesario continuar aportando nuevos datos.

3.3.3. BDE-209 y sus substitutos

Pese a que los HFRs pueden entrar al medio ambiente incluso varios años después de que se hayan retirado del uso los productos que los contienen, las restricciones sobre algunos de ellos han de afectar por fuerza a sus niveles en el medio ambiente. Por ello,

es interesante comparar los niveles de los retardantes de llama clásicos (PBDEs) con los de los BFRs emergentes o HNs. Es de suponer que transcurridos varios años estas diferencias serán mucho menores o incluso podrían revertirse, por lo que es interesante aportar datos actuales que más adelante sirvan como referencia. Además, en algunas zonas ya se empieza a ver como las concentraciones de unos y otros van siendo cada vez más similares, especialmente las del DBDPE y BDE-209.

En las figuras 2 de los artículos #3 y #4 puede verse una comparación entre los niveles de BDE-209 y DBDPE, uno de los compuestos propuestos específicamente como substituto de la mezcla Deca-BDE. En sedimentos se puede ver como las concentraciones de DBDPE superan a las del BDE-209 en 3 de las 5 zonas estudiadas en Chile, mientras que no se detectó en Colombia. Esto parece sugerir que Chile ha empezado a reemplazar el Deca-BDE por el DBDPE, mientras que en Colombia el BFR alternativo aún no se aplica. De igual modo, las concentraciones en el Ebro y Llobregat fueron bastante similares. Por el contrario, en fangos las concentraciones de BDE-209 fueron muy superiores, cosa que es consistente con otros trabajos publicados en España (De la Torre *et al.*, 2012; Gorga *et al.*, 2013) y también en otras zonas como Corea (Lee *et al.*, 2014). Además, las concentraciones de BDE-209 suelen estar correlacionadas con las del DBDPE, lo que sugiere que provienen de fuentes similares. En la figura 3.5 se muestra un ejemplo de la correlación encontrada en sedimentos del Ebro, correlación que también se dio en los lodos de depuradoras ($p < 0.05$, R^2=0,704) (De la Torre *et al.*, 2012).

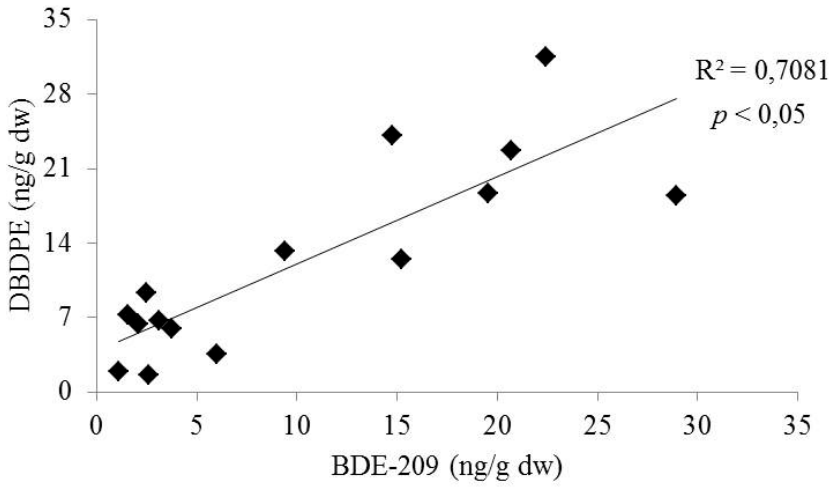

Figura 3.4. Correlación entre las concentraciones de BDE-209 y DBDPE (Sedimentos Ebro).

3.4. Conclusiones

Finalmente, las conclusiones a raíz del trabajo realizado en este capítulo son las siguientes:

- Se ha detectado por primera vez la presencia de HFRs en sedimentos de Chile y Colombia, demostrando que estos compuestos también representan un problema en esa región. Además, se ha confirmado la presencia del DP en lodos de diferentes depuradoras españolas y detectado por primera vez el Dec 603 en esta matriz. Se vio que el DP es el HN más abundante tanto en sedimentos como en lodos.
- Existen marcadas diferencias entre distintos países como Chile y Colombia, que sugieren un uso diferente de las mezclas comerciales Penta-BDE y Deca-BDE, así como la implantación del uso del DBDPE en el caso de Chile.
- Pese a no observarse diferencias en las concentraciones globales entre las cuencas del Ebro y Llobregat, sí se vieron diferencias en cuando a los perfiles de contaminación a lo largo de las mismas. Además, la posible aplicación de los lodos en agricultura podría agravar el problema siendo un foco importante de reintroducción de los contaminantes al medio ambiente.
- Se observó correlación entre las concentraciones de BDE-209 y DBDPE lo que podría indicar que se han estado utilizando en productos similares. Además, los niveles del DBDPE eran similares, e incluso superiores en algunos casos, a los del BDE-209, lo que podría indicar la aplicación de este BFR emergente en substitución del ya prohibido BDE-209.

CAPÍTULO 4

BIOACUMULACIÓN Y BIOMAGNIFICACIÓN EN BIOTA

4.1. Introducción

En el capítulo anterior se ha discutido la presencia de diferentes HFRs en muestras ambientales como sedimentos y fangos. Su acumulación en estas matrices representa su vía de entrada en las diferentes cadenas tróficas. Desde allí se acumulan en diversas especies que van desde pequeños invertebrados hasta grandes mamíferos o aves rapaces, tanto en medio acuático como terrestre (Weijs *et al.*, 2015). Pese a que se ha visto que algunos microorganismos acumulan los HFRs tras descomponer los materiales poliméricos que los contienen, la mayoría los acumulan una vez estos han sido liberados en el medio ambiente.

En este capítulo se pretendía demostrar la capacidad de los diferentes HFRs para acumularse en diferentes niveles de la cadena trófica, ver si los perfiles de contaminación estaban influenciados por la dieta, y estudiar los procesos de biomagnificación. Pese a que esta información ya estaba disponible para los PBDEs y en menor medida para el HBCD, los datos respecto a los HNs eran muy limitados y daban lugar a interpretaciones ambiguas. La comparación de su comportamiento con el de los 2 primeros, más estudiado, dará una idea de la problemática real de estos compuestos emergentes en el medioambiente.

La presencia de HFRs en 3 niveles tróficos de la Bahía de Concepción (Chile) fue estudiada como uno de los objetivos del proyecto **"Evaluación del impacto ambiental de los retardantes de llama bromados en ecosistemas acuáticos de América Latina"** (BROMACUA) y los resultados están recogidos en la publicación #5: *Occurrence and behavior of natural and anthropogenic (emerging and historical) halogenated compounds in marine biota from the Coast of Concepción (Chile).* Dado que no se tuvo la oportunidad de muestrear especies en los niveles más altos de la cadena trófica, donde la información sobre posibles procesos de biomagnificación es más relevante, se consideró necesario ampliar el estudio a otras especies que ocuparan niveles tróficos superiores. Por ello, y en el marco del proyecto FLAME **("Estudio de los contaminantes retardantes de llama (flame retardants) en el Golfo de Cádiz: implicaciones para la conservación"**) se estudiaron 5 especies de delfines con diferentes posiciones tróficas procedentes del Estrecho de Gibraltar, Golfo de Cádiz y Mar de Alborán. Se pudieron estudiar procesos de biomagnificación a lo largo de una misma cadena trófica, constatar que incluso entre zonas muy cercanas entre sí existen diferencias significativas en las concentraciones encontradas, y también la acumulación

específica de algunos compuestos en cerebro. Este trabajo se recoge en las publicaciones #6 (*Bioaccumulation and biomagnification of classical flame retardants, related halogenated natural compounds and alternative flame retardants in three delphinids from southern European waters*) y #7 (*Halogenated natural products in dolphins: Brain-blubber distribution and comparison with halogenated flame retardants*). Con el objetivo de ampliar el estudio a organismos con una dieta principalmente terrestre y evaluar posibles diferencias, se analizaron huevos no eclosionados de 15 especies diferentes de aves del Parque Nacional de Doñana, dentro del marco del proyecto de título "**Evaluación del impacto sobre la fauna del Parque Nacional de Doñana asociado al uso de nuevos contaminantes retardantes de llama**" (IMPAR). Este trabajo permitió evaluar las diferencias de acumulación entre diferentes especies, las capacidades de biomagnificación de diferentes contaminantes, así como las tendencias temporales entre 2003 y 2012, dando lugar a las publicaciones #8 (*Bioaccumulation and biomagnification of emerging and classical flame retardants in bird eggs of 14 species from Doñana Natural Space and surrounding areas (South-western Spain*) y #9 (*Temporal trends in classical and alternative flame retardants in bird eggs from Doñana Natural Space and surrounding areas (south-western Spain) between 1999 and 2013*).

A continuación se amplían o introducen algunos conceptos de importancia específica de este capítulo.

4.2. Bioindicadores

Los bioindicadores son especies usadas para monitorizar el estado del medio ambiente. Son especies concretas, aunque a veces se usan conjuntos de especies, abundantes en una misma zona, presentes en distintas regiones lo que permite realizar comparaciones, y que presentan unas respuestas concretas a la contaminación. Ya sea porque acumulan los contaminantes, porque la contaminación afecta a su morfología o porque su comportamiento o población se altera, estas especies permiten evaluar el estado del medio ambiente (Carignan y Villard 2002; Niemi y McDonald 2004). También se suele usar el concepto de "especies centinela" para referirse a los bioindicadores que se encuentran en posiciones tróficas altas, como las aves, mamíferos marinos o terrestres e incluso animales de compañía (Basu *et al.,* 2007). El aumento del desarrollo, tanto en países emergentes como en el primer mundo, implica un aumento de la actividad

industrial y la demanda de recursos. Por ello, la actividad humana cada vez tiene un impacto mayor en el medio ambiente. Así pues, identificar correctamente las especies que nos permiten evaluar el efecto de dicha actividad humana es de vital importancia (Gentes *et al.,* 2015).

4.3. Medio marino

En un primer estudio se muestrearon diferentes especies a lo largo de la Bahía de Concepción (Chile) durante febrero de 2010. Se trata de un área con una elevada carga industrial ya que la industria petrolera tiene una gran presencia en la zona. Además, una serie de desastres medioambientales recientes (vertido de petróleo en 2007 y terremoto en 2010) hacen que sea interesante evaluar la presencia de HFRs en las diferentes especies que la pueblan. Algunos de los puertos más importantes de Chile se encuentran allí, como los de Penco o Talcahuano. El muestreo se llevó a cabo concretamente en la Bahía San Vicente (sistema de agua marina) y el Estuario Lenga (sistema de agua dulce y marina). Es una zona muy rica en biodiversidad que alberga más de 100 especies. Entre ellas se seleccionaron diferentes organismos para cubrir diferentes niveles tróficos: consumidores primarios, secundarios y terciarios.

Por otro lado, el estudio de la presencia de los HFRs y los HNPs en 5 especies de delfines en aguas del sur-oeste del mediterráneo representa una parte importante de este bloque. Los delfines son considerados buenos bioindicadores ya que su salud y población no solo refleja el estrés al que están sometidos ellos mismos, sino también el de especies en niveles inferiores de su mismo ecosistema (Wells *et al.,* 2004). Además, su esperanza de vida es bastante alta y presentan una gran acumulación de contaminantes acumulados a lo largo de la misma. El estrés sobre los cetáceos ha aumentado en las últimas décadas, afectándolos a ellos y a sus presas. En consecuencia, la comisión ballenera internacional (IWC, del inglés international whaling commision) ha promovido el monitoreo de estas especies para definir su estado de salud y cómo están siendo afectados por la contaminación (Fossi *et al.*, 2013).

Toda el área abarcada en el estudio sobre la presencia de HFRs en cetáceos presenta unas características muy singulares debido a la gran actividad humana que tiene lugar y a su situación geográfica. Es un punto donde se intercambian las aguas del Atlántico y el Mediterráneo, representado el único punto que los conecta. El tráfico marítimo es muy importante, sin contar la cercanía a África (el puerto de Algeciras es uno de los

más importantes de Europa). Esto no es impedimento para que muchas especies de cetáceos circulen por él cada día, tanto especies en proceso de migración entre el Atlántico y el Mediterráneo como poblaciones residentes en la zona. Esto ha añadido las actividades recreativas de avistamiento de cetáceos al ya de por si saturado tránsito marítimo, exponiendo aún más a la fauna a la contaminación generada por ello. Actualmente la costa española está protegida por el Parque Natural del Estrecho (Cañadas *et al.,* 2005; De Stephanis *et al.,* 2008a; De Stephanis *et al.,* 2008b; Senigaglia *et al.,* 2012).

4.3.1. Métodos de muestreo en delfines

Todas las especies de cetáceos se encuentran protegidas, por lo que no es ni legal ni ético capturar individuos sea cual sea la finalidad. Por ello, existen 3 formas principales de obtener muestras con el propósito de estudiar estos animales: biopsias, capturas accidentales o varamientos (Figura 4.1). El muestreo mediante biopsia causa daños temporales que curan con el tiempo, ya que sólo se perfora la capa superficial de piel del animal para obtener una pequeña cantidad de muestra de grasa (Giménez *et al.,* 2011; Macfarlane 2015). Dependiendo de la especie y las dificultades para aproximarse a ella se usa un arpón con punta hueca o una ballesta con flechas también de punta hueca (Figura 4.1).

Figura 4.1. De izquierda a derecha: muestreo por biopsia, captura accidental y varamiento.

Por otro lado, en ocasiones los delfines mueren atrapados en redes de pescadores o aparecen varados. En ese caso, si en la zona existe algún protocolo al respecto, personal especializado evalúa el estado del individuo y tras practicar una autopsia se archivan las muestras para su posterior uso. Las ventajas de la biopsia son obviamente que es una técnica no invasiva, ya que no causa daños permanentes al animal, y que permite el diseño de un estudio a medida, pero a cambio la cantidad de muestra es pequeña, no se

puede disponer de otros tejidos, y si se quiere más información sobre el individuo en concreto (por ejemplo el sexo) se necesitan llevar a cabo análisis genéticos. En cambio, el principal problema de trabajar con individuos muertos es que tanto las especies disponibles, como la cantidad de muestras, son totalmente aleatorias.

4.3.2. Especies estudiadas

En la Bahía de Concepción se analizaron 11 especies en total (Figura 4.2), clasificadas en 3 niveles tróficos en base a su dieta (ver apartado 4.5). Estas especies, divididas en tres grupos, fueron, por parte de los consumidores primarios: picoroco (*Austromegabalanus pstittacus*), lapa (*Fisurella sp.*), almeja (*Venus antiqua*), navaja (*Tagelus dombeii*) y piure (*Pyura chilensis*). Secundarios: jaiba mora (*Homalaspis plana*), panchote (*Taliepus dentatus*), bilagai (*Cheilodactylus variegatus*), castañuela común (*Chromis crusma*). Por último, los consumidores terciarios fueron el rollizo (*Pinguipes chilensis*) y el loco (*Concholepas concholepas*). Los peces se obtuvieron mediante pesca submarina, mientras que los bivalvos y crustáceos se recolectaron manualmente o por medio de trampas. Este muestreo fue llevado a cabo por investigadores de la Universidad de Concepción (Chile).

Figura 4.2. Especies estudiadas en la bahía de Concepción. De izquierda a derecha, Consumidores primarios: picoroco, lapa, almeja, navaja y piure. Consumidores secundarios: bilagai, jaiba mora, panchote y castañuela. Consumidores terciarios: rollizo y loco.

Las 5 especies de cetáceos estudiadas fueron el delfín común (*Delphinus delphis*), delfín listado (*Stenella coureloalba*), delfín nariz de botella (*Tursiops truncatus*), calderón

común (*Globicephala melas*) y el calderón o delfín gris (*Grampus griseus*), que se pueden ver en la figura 4.3. Diferentes muestras de común, nariz de botella y calderón común se obtuvieron mediante biopsia en 2 campañas de muestreo en 2011 y 2012 a lo largo del Golfo de Cádiz y el Estrecho de Gibraltar, mientras que muestras de grasa y cerebro de las 5 especies se obtuvieron de individuos varados a lo largo de la costa del mar de Alborán entre 2004 y 2011. Todas las muestras de cetáceos se obtuvieron gracias a la colaboración con investigadores de la Estación Biológica de Doñana (CSIC).

Figura 4.3. Especies de cetáceos estudiadas. 1: Delfín común. 2: Delfín listado. 3: Calderón gris. 4: Calderón común. 5: Delfín nariz de botella.

4.4. Medio terrestre

El uso de aves como bioindicadores está aceptado desde hace bastante tiempo, ya que cumplen muchos de los criterios necesarios para ello. Son fáciles de identificar, las diferentes especies y su dieta están bien caracterizadas, así como su biología y ecología, y la gran variedad de especies existentes abarca varios niveles de la cadena trófica lo que permite llevar a cabo estudios de biomagnificación. Por otro lado, la toma de muestra se puede hacer sin necesidad de matar al individuo ya sea capturándolo o utilizando huevos o plumas. Además, debido a que en una misma zona suelen anidar un gran número de individuos, se puede obtener un número relativamente elevado de muestras de una misma especie en un mismo lugar y se puede llevar a cabo un control fiable del estado de contaminación del mismo. Las poblaciones son relativamente estables durante el tiempo por lo que un cambio significativo puede ser atribuido a un episodio concreto como una contaminación elevada o vertido. Por todo ello, los datos sobre contaminación en aves son abundantes y, al tener las especies presencia en

bastantes zonas del globo existe la posibilidad de comparar entre diferentes zonas (Fox 2001; Gentes *et al.*, 2015; Wan *et al.*, 2008).

4.4.1. Muestreo

Las muestras de huevos no eclosionados se obtuvieron gracias a la colaboración con el Equipo de Seguimiento de la Estación Biológica de Doñana (CSIC), que recolectaron los huevos que no habían eclosionado una vez acabado el periodo de cría. Este es un método no invasivo con el principal inconveniente de que no se dispone de información adicional, como por ejemplo si el huevo fue el primero o el último (en las especies que ponen más de un huevo por tanda) pero con la ventaja de que no pone en peligro la población de la especie. Eso sí, presenta algunos inconvenientes como el hecho de limitar la información a hembras en edad de reproducción y sólo al periodo de cría. En otros estudios se cogen huevos a priori fértiles, se captura al animal, se toma una muestra de sangre, y se le libera de nuevo (Bertolero *et al.*, 2015), mientras que en otros se utilizan individuos muertos naturalmente o en centros de recuperación, con lo que se tiene acceso a diferentes tejidos (Chen *et al.*, 2013). Por otro lado, mientras que en este estudio se decidió analizar cada huevo individualmente, hay otros autores que prefieren hacer una muestra combinada entre varios huevos de una misma especie para reducir la variabilidad intra-especie (Wang *et al.*, 2015). Todos estos factores deben ser tenidos en cuenta a la hora de comparar diferentes estudios.

4.4.2. Especies estudiadas

Se estudiaron huevos de 14 especies recogidos entre 2010 y 2012 a lo largo del Parque Nacional de Doñana. Las especies correspondían a diferentes familias, detalladas a continuación: falconiformes: milano negro (*Milvus migrans*), milano real (*Milvus milvus*), aguilucho lagunero (*Circus aeruginosus*), águila calzada (*Aquila pennata*), cernícalo (*Falco tinnunculus*) y elanio común (*Elanus caeruleus*); ciconiformes: morito común (*Plegadis falcinellus*), garza imperial (*Ardea purpurea*) y cigüeña blanca (*Ciconia ciconia*); charadriformes: gaviota picofina (*Chroicocephalus genei*), gaviota reidora (*Chroicocephalus ridibundus*) y pagaza piconegra (*Gelochelidon nilotica*); strigiformes: lechuza común (*Tyto alba*); y por último anseniformes: ánade fiso (*Anas strepera*). Posteriormente se decidió evaluar posibles tendencias temporales y usando el archivo de muestras de la Estación Biológica de Doñana se analizaron muestras

recogidas entre 2003 y 2012 de cigüeña común, milano negro y flamenco común (*Phoenicopterus roseus*). (Figura 4.4).

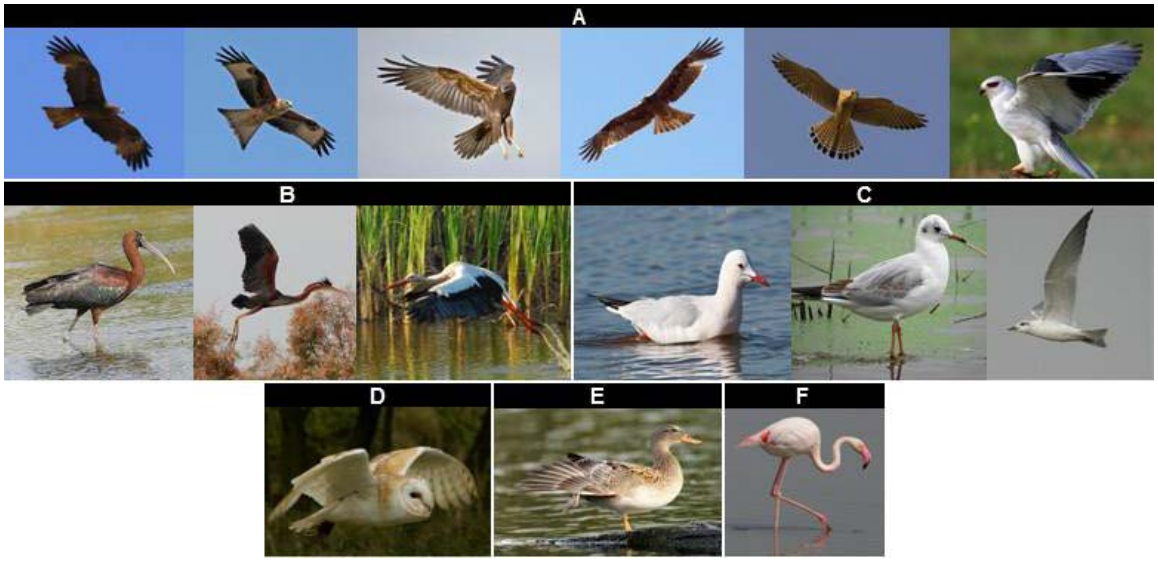

Figura 4.4. Especies de aves estudiadas. De izquierda a derecha: A (Falconiformes): milano negro, milano real, aguilucho lagunero, águila calzada, cernícalo, elanio común. B (Ciconiformes): morito común, garza imperial, cigüeña blanca. C (Charadriformes): gaviota picofina, gaviota reidora, pagaza piconegra. D (Strigiformes): lechuza común. E (Anseniformes): ánade fiso. F (Phoenicopteriforme): flamenco común.

4.5. Análisis de isótopos estables

El análisis de isótopos estables es una metodología que permite investigar los procesos que tienen lugar a lo largo de una cadena trófica y establecer unos niveles tróficos diferenciando desde organismos productores a depredadores en la cima de la cadena. Normalmente se utiliza una combinación del análisis de isótopos de carbono y nitrógeno. Los resultados se expresan utilizando relaciones ($\delta^{15}N$ o $\delta^{13}C$) entre el isótopo más pesado y el más ligero ($^{15}N/^{14}N$ o $^{13}C/^{12}C$, utilizando los mismos ejemplos) corregidos por el ratio de unos estándares comerciales. Esta técnica ha demostrado ser más efectiva que las que se usaban tradicionalmente, como el análisis de contenidos estomacales, ya que sus resultados integran periodos de tiempo más grandes y no dependen sólo de la dieta más reciente del animal (Jardine *et al.*, 2006). El $\delta^{15}N$ proporciona información sobre la posición trófica del animal, mientras que el $\delta^{13}C$ permite hacerse una idea del tipo de dieta que seguía (Gentes *et al.*, 2012). Evaluar la capacidad de biomagnificación de un contaminante o de una familia entera permite hacerse una idea de la verdadera problemática que representa, ya que si existe capacidad de biomagnificación significa que los organismos en niveles tróficos altos lo acumulan

en mayor medida y por tanto están más expuestos a sus posibles efectos tóxicos tanto agudos como crónicos. Además, da una idea del peligro que representa para poblaciones humanas debido a una posible exposición a través de la dieta, con lo cual esta información puede ayudar a establecer una legislación adecuada sobre contaminantes como los HFRs (Gentes *et al.,* 2012; Jardine *et al.,* 2006). La combinación del análisis de isótopos estables con los niveles de contaminación es una aproximación novedosa y que cada vez tiene más relevancia en el estudio de los procesos de biomagnificación.

No obstante, el análisis isotópico debe interpretarse con cuidado ya que existen algunas fuentes de variabilidad. Por ejemplo, si el animal se encuentra en un periodo de hambruna o en una fase de alto consumo energético verá incrementado su valor de $\delta^{15}N$. Es por ello que no es recomendable utilizar valores previos de $\delta^{15}N$ o $\delta^{13}C$ y éstos deben ser siempre medidos de nuevo ya que, pese a que no se producirá una variación crítica, ésta sí puede ser suficiente para afectar a los resultados (Jardine *et al.,* 2006). Además, las comparaciones entre los valores de $\delta^{15}N$ deben realizarse siempre en especies que habiten un mismo ecosistema. Si mezclamos especies de diferentes ecosistemas podemos cometer errores. Por ejemplo, las zonas agrícolas pueden verse enriquecidas de nutrientes, provocando así unos valores anómalos de $\delta^{15}N$ y conllevando a una sobreestimación del nivel trófico de las especies de dicho ecosistema.

Publicación científica #5

Occurrence and behavior of natural and anthropogenic (emerging and historical) halogenated compounds in marine biota from the Coast of Concepción (Chile)

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Occurrence and behavior of natural and anthropogenic (emerging and historical) halogenated compounds in marine biota from the Coast of Concepcion (Chile)

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HIGHLIGHTS

- Anthropogenic and natural halogenated compounds were detected in Chilean biota from different trophic levels.
- Concentration levels of classical FRs (PBDEs) were higher than those of emerging FRs.
- PBDEs and MeO-PBDEs showed biomagnification capacity (BMF>1), whereas halogenated norbornenes presented BMF<1.
- Biomagnification capacity of naturally occurring MeO-PBDEs was higher than that of PBDEs.

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ABSTRACT

Fifty-five biota samples from the Coast of Concepcion (Chile) were analyzed for PBDEs, emerging brominated FRs, halogenated norbornenes and naturally-occurring MeO-PBDEs. PBDEs, MeO-PBDEs and halogenated norbornenes were detected at concentration levels ranging from 11 to 170, nd to 118 and nd to 5.8 ng/g lw, respectively. However, emerging brominated FRs such as decabromodiphenylethane (DBDPE), hexabromobenzene (HBB) and pentabromoethylbenzene (PBEB) were not detected in any sample.

Bioaccumulation and bioconcentration processes were evaluated for the different families of compounds. Biomagnification factors (BMFs) were calculated, and some PBDE congeners (BDE-28, BDE-183 and BDE-209) as well as MeO-PBDEs presented BMF > 1, being values of the naturally occurring MeO-PBDEs higher than those obtained for PBDEs. As regards halogenated norbornenes, BMF < 1 were found.

1. Introduction

Flame retardants (FRs) are a group of compounds added or applied to a material to increase the fire resistance of that product. The most widely used families of compounds for this purpose are the halogenated flame retardants (HFRs). Among them, polybrominated diphenyl ethers (PBDEs) have been used for many years and in great amounts, and exist in 3 main commercial mixtures: Penta-BDE, Octa-BDE and Deca-BDE. They have been found in environmental matrices, such as sediments (Hale et al., 2006), air (Harner et al., 2006) or sludge (Guerra et al., 2010), and also in biological matrices such as fish (Christensen et al., 2002), bird eggs (Guerra et al., 2012) or breast milk (Lacorte and Ikonomou, 2009). Different toxicological properties of PBDEs have been reported: effects on the thyroid hormone homeostasis (Freitas et al., 2011), interaction with the estrogenic receptor (Montaño et al., 2012) or the fact that they can act as endocrine disruptors (Simon et al., 2010). As a result, Penta-BDE and Octa-BDE mixtures are currently listed and consequently banned due to the Stockholm Convention (Renner, 2004). The use of Deca-BDE mixture in the EU was also banned in 2008 (Justice, 2008) and it has been said that its production in the USA will stop by the end of 2013 (Covaci et al., 2011).

Due to these restrictions, different compounds have been proposed as an alternative to PBDEs, including emerging chemicals such as decabromodiphenylethane (DBDPE), hexabromobenzene (HBB) and pentabromoethylbenzene (PBEB). Moreover, halogenated norbornenes were first used as substitutes for Mirex when it was banned as FR in 1976 and now dechlorane plus (DP) has been proposed by the UE as

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an alternative to Deca-BDE. Although they have been used for many years, they were not found in the environment until 2006 (Hoh et al., 2006). Since then, different studies have reported levels of these contaminants in sediment (Shen et al., 2011b; Sverko et al., 2008), air (Venier and Hites, 2008) or sludge (De la Torre et al., 2010), and also in biological matrices such as fish (Shen et al., 2011a), eggs (Guerra et al., 2011) or human serum (Zheng et al., 2010). These levels are still lower than other classical FRs such as PBDEs. However, they will probably increase due to the recent restrictions over PBDEs.

On the other hand, more than 4000 naturally-produced halogenated molecules have been found in the marine environment, such as methoxylated PBDEs (MeO-PBDEs). Their main sources seem to be blue mussels, sponges and red algaes (Malmvärn et al., 2005), and they have been measured at high concentrations in marine animals, especially in top predators such as dolphins (Alonso et al., 2012) or killer whales (Nomiyama et al., 2011a). Besides, some studies show that they may have similar toxic properties to PBDEs (Wiseman et al., 2011).

The aim of this study was to evaluate the concentration levels of "classical" and emerging HFRs, as well as naturally occurring brominated compounds, in an aquatic food web from an area in the South Central Chilean coast. Moreover, the results will be of interest in order to study the bioaccumulation and bioconcentration processes of emerging FRs. To the best of our knowledge, this is the first study which reports levels of HFRs in an industrial area from Chile. In addition, there is a lack of studies about MeO-PBDEs and halogenated norbornenes in the Southern hemisphere.

2. Materials and methods

2.1. Study area and sample collection

The samples analyzed were collected during February of 2010 in the frame of the BROMACUA project. In a previous sampling campaign, sediments from 4 different areas were sampled in order to evaluate the area with the highest contamination levels of FRs (Barón et al., 2013). Thus, in the sampling campaign during the earlier 2010 the Lenga estuary was chosen to carry out the study in biota samples (Fig. 1). However, it was not possible to collect all the biota samples in this place so samples were collected in the nearby area called Chome and Perone. This area has a high industrial charge, being part of a large petrochemical complex. In fact, an industrial growth of 2.1% was estimated between November of 2010 and November of 2011 (INE, 2011). In recent years this area has suffered heavy environmental damages: a fuel spill of 350 m^3 in 2007 and an earthquake with its epicenter at 150 km of the estuary in 2010.

A total of 55 biota samples, divided into 3 different trophic levels (primary, secondary and tertiary consumers), were collected. This division was made based in the alimentation source of the species. Species listed as primary consumers were 5 different species of filtering (they get their nutrients by directly filtrating water): giant barnacle (*Austromegabalanus pstittacus*, MP), keyhole limpet (*Fisurella* sp., FS), sea squirt (*Pyura chilensis*, PC), clam (*Venus antiqua*, VA) and razor shell clam (*Tagelus dombeii*, NV). The species listed as secondary consumers were 2 crustaceans, crab (*Homalaspis plana*, HP) and crab with Spanish common name "panchote" (*Taliepus dentatus*, TD), and 2 fish, peruvian morwong (*Cheilodactylus variegatus*, CV) and damselfish (*Chromis crusma*, CA). These 4 species are herbivorous or they eat small organisms. Finally, tertiary consumers were the two available predators: sandperches (*Pinguipes chilensis*, PR) and Chilean abalone (*Concholepas concholepas*, CC). In Supporting information 1 the number of samples collected for each species as well as the number of individuals used for each pool sample is listed.

2.2. Standards and reagents

The standard mixture of PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209), the standard mixture of MeO-PBDEs (5-MeO-BDE-47, 6-MeO-BDE-47, 4′-MeO-BDE-49, 2′-MeO-BDE-68, 5′-MeO-BDE-99, 5′-MeO-BDE-100, 4′-MeO-BDe-101 and 4′-MeO-BDE-103), PBEB, HBB, DBDPE, BDE-181 and ^{13}C-BDE-209, used as internal standards, and *syn*- and *anti*-isomers of DP were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). Mirex (98%) and ^{13}C-*syn*-DP (98%), used as internal standard, were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA).

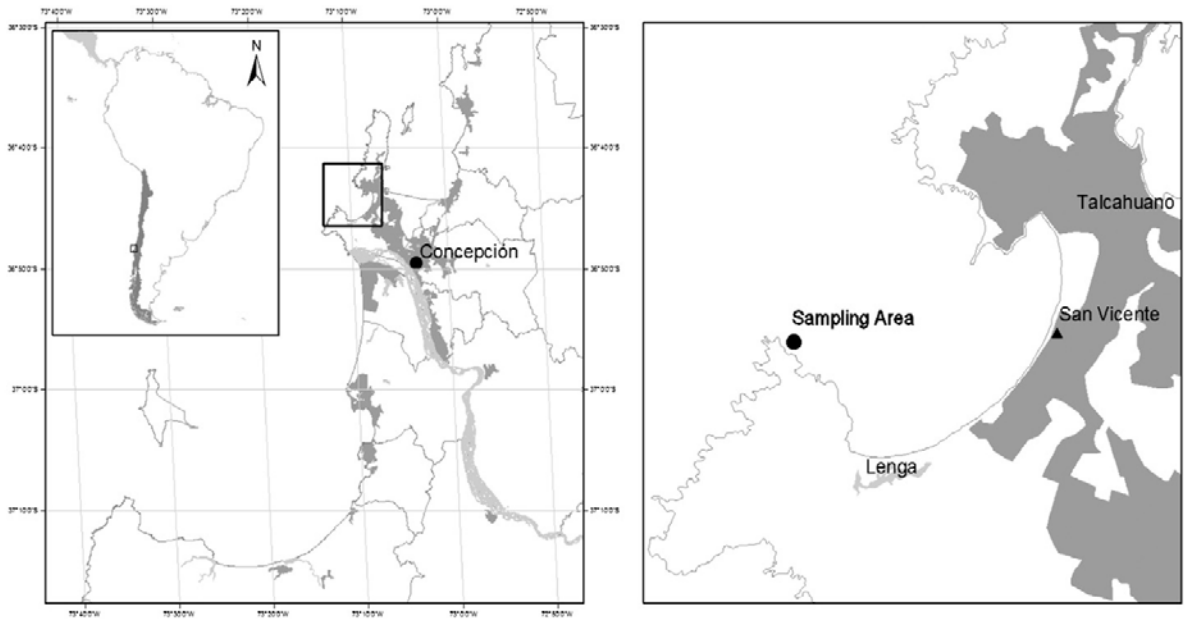

Fig. 1. Map of Chilean coast showing the study area for biota collection.

Al-N cartridges were obtained from Biotage. Dichloromethane and hexane, solvents for organic trace analysis, were purchased from Merck (Darmstadt, Germany).

2.3. Sample preparation

The sample preparation method applied had been previously optimized (De La Cal et al., 2003; Labandeira et al., 2007). Biota samples were freeze dried. Lyophilized samples were ground and homogenized and stored in sealed containers at −20 °C until analysis. Before extraction, one gram dry weight (dw) of each sample was spiked with the surrogate standards (5 ng of BDE-181, 50 ng of ^{13}C-BDE-209 and 5 ng of ^{13}C-*syn*-DP). Samples were kept overnight to equilibrate prior to the pressurized liquid extraction (PLE). Spiked samples were loaded into an 11 mL extraction cell and the death volume was filled with diatomeus earth. PLE was carried out using hexane: dichloromethane (1:1) as extraction solvent mixture. Two static cycles of 10 min. were made at 100 °C and 1500 psi. Flush volume was set at 8 mL and purge time was set at 90s. After the extraction, lipid content was determined gravimetrically and the resulting extracts were re-dissolved in hexane. Then, fat was removed by H_2SO_4 treatment. The clean up of the organic phase was carried out using solid phase extraction (SPE) with alumina cartridges (AL-N). Cartridges were conditioned with 20 mL of hexane and eluted with 20 mL of a mixture of hexane:DCM (1:2). Extracts were then evaporated with N_2 to incipient dryness and reconstituted in 40 μL prior to the instrumental analysis.

2.4. Instrumental analysis

PBDEs, MeO-PBDEs and emerging BFRs (HBB, PBEB and DBDPE) were analyzed by an Agilent 7890C gas chromatograph connected to an Agilent 5975A Network mass spectrometer, working in negative chemical ionization mode (NCI) using NH_4^+ as reagent gas. The instrumental conditions and elution program had been previously developed (Eljarrat et al., 2004; Guerra et al., 2010). Briefly, temperature started at 140 °C, was hold for 2 min. and then ramped to 325 °C at 10 °C/min. Final temperature was hold 10 min. Source temperature was set at 250 °C. Selected ion monitoring (SIM) mode was applied in order to enhance the sensitivity. Experiments were carried out monitoring the two most intense peaks from the NCI spectra. Ions monitored were *m/z* 79 and 81 for all PBDEs, MeO-PBDEs and emerging BFRs with the exception of BDE-209 and ^{13}C-BDE-209, where the two ions monitored were *m/z* 487 and 489, and m/z 497 and 499, respectively. The most intense peaks were used for quantification purposes, and the second ones for confirmation.

On the other hand, halogenated norbornenes were analyzed using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole, working in NCI using CH_4^+ as reagent gas. The advantages of this methodology are showed in our previous work (Barón et al., 2012). Temperature started at 80 °C, was hold for 2 min and then ramped to 300 °C in 10 °C/min. Final temperature was maintained for 10 min. Source temperature was set at 175 °C and electron energy and emission current were set at 200 and 150 eV, respectively. Selective reaction monitoring (SRM) mode was applied in order to enhance the sensitivity. The experiments were carried out monitoring the two most intense transitions (Supporting information 2). The most intense transitions were used for quantification purposes, and the seconds for confirmation criteria.

2.5. Quality assurance/quality control

Quality parameters of the method were previously evaluated. The method showed good recoveries for PBDEs (55 to 69%), MeO-PBDEs (55 to 89%), emerging BFRs (50 to 98%) and halogenated norbornenes (82 to 97%) (Table 1). The identification and confirmation of PBDEs, MeO-PBDEs, emerging BFRs and halogenated norbornenes was based on the following criteria: (i) Simultaneous responses to the two selected ions or transitions (*m/z*1 and *m/z*2, or SRM1 and SRM2) were needed, (ii) The area of signal must be at least 3 times higher than the signal noise, and (iii) the difference in the relative intensity of a peak respect to theoretical values obtained with standard solutions can not exceed ± 15%. 5 procedural blanks were made to prevent interferences and contamination.

Method detection limits (mLODs) were determined for each congener as the minimum amount of analyte which produces a peak with a signal-to-noise ratio of 3, and the method limits of quantification (mLOQs) were determined as the minimum amount of analyte which produces a peak with a signal-to-noise ratio of 10. The recoveries, mLODs and LOQs of the different families studied are showed in Table 1.

3. Results and discussion

3.1. Brominated flame retardant levels

Table 2 shows the range of concentrations for the different BFRs detected in the biota samples corresponding to different aquatic trophic levels (primary, secondary and tertiary consumers). PBDEs were detected in all the samples analyzed, whereas emerging BFRs (HBB, PBEB and DBDPE) were not detected in any sample. Seven different congeners were detected, including BDE-28, BDE-47, BDE-99, BDE-100, BDE-154, BDE-183 and BDE-209. Total PBDE values ranged between 24 and 86 ng/g lw.

As regards primary consumers, total PBDE levels were similar for the five different species, with values between 30 and 47 ng/g lw. BDE-100 was not detected in any sample, whereas BDE-209 was the most predominant congener in all the samples accounting from 65 to 95% of the total PBDE contribution. (Booij et al., 2002) suggested that BDE-209 levels measured in mussels were dominated by the concentrations found in ingested particles in the gut. This fact could explain our high BDE-209 levels, since our samples were not depurated (Moon et al., 2007).

Significant differences were found for the species included as secondary consumers. A clear differentiation between crustaceans (crab and "panchote") and fishes (peruvian morwong and damselfish) was observed. Total PBDE levels were higher in crustaceans (72–86 ng/g lw) than in fish (24–42 ng/g lw). Moreover, different congener pattern was also observed. Crustacean samples were dominated by BDE-209 (with a contribution between 70 and 83% of the total), but also other PBDE congeners presented a significant contribution, i.e., BDE-100 and BDE-183. In contrast, fish samples presented predominant levels of BDE-47, with lower contributions to the total PBDE level for the higher brominated congeners BDE-183 and BDE-209 than the contribution they had in crustaceans. Moreover, other PBDE congeners, such as BDE-99, BDE-100 and BDE-154 were not detected. This differential behavior may be explained by the high metabolic capacity of the fish, which could degrade the higher brominated congeners to lower ones. For instance, BDE-99 and BDE-100 could be converted to BDE-47, as has been suggested in other studies (Athanasiadou et al., 2008; Erratico et al., 2011; Qiu et al., 2009).

Finally, total PBDE levels in the tertiary consumers are similar in the two species studied, with values of 32 and 44 ng/g lw. Again, it was observed a predominance of BDE-209, accounting 81 – 83% of the total PBDE contribution. The BDE-209 predominance is surprising due to the

Table 1
Range of recoveries (%), mLODs and mLOQs (ng/g lw) for the different families studied.

	Recoveries	mLODs	mLOQs
PBDEs	55–69	0.1–0.9	0.2–2.8
MeO-PBDEs	55–89	0.1–0.2	0.3–0.6
Emerging BFRs	50–98	0.1–2.1	0.6–6.8
Hal. norbornenes	82–97	5.5–21[a]	7.7–70[a]

[a] pg/g lw.

Table 2
Range of concentrations (expressed in ng/g lipid weight (lw)) for the PBDE and MeO-PBDEs congeners.

	Primary consumers					Secondary consumers				Tertiary consumers	
	MP (n = 7)	FS (n = 3)	VA (n = 6)	NV (n = 7)	PC (n = 6)	TD (n = 4)	HP (n = 1)	CV (n = 2)	CA (n = 4)	CC (n = 4)	PR (n = 13)
BDE-28	nd–25	nd	nd	nd	nd–8.6	nd	nd	nd	nd	nd–13	nd
BDE-47	1.2–7.9	nd–2.0	nd	1.1–2.7	nd	nd–3.7	1.7	5.0–5.8	1.4–42	nd–4.5	nd–1.4
BDE-100	nd	nd	nd	nd	nd	nd–3.8	nd	nd	nd	nd	nd
BDE-99	nd–6.6	nd–0.4	nd	nd	nd	3.5–11	2.6	nd	nd	nd–3.7	nd–2.8
BDE-154	nd	nd–0.4	nd–5.6	nd	nd	nd	1.4	nd	nd	nd	nd
BDE-183	nd–1.0	nd–9.8	nd	nd–2.1	nd–25	nd–6.5	115	nd–1.1	nd–2.7	nd–2.2	nd–30
BDE-209	5.5–63	23–38	25–35	30–46	18–30	25–131	50	7.8–14	15–24	26–45	11–47
ΣPBDEs	11–111	31–40	25–35	32–47	18–29	29–152	170	14–20	19–67	24–58	13–69
	30–47[a]					72–86[a]		24–42[a]		32–44[a]	
6-MBDE-47	2.7–21	nd	3.8–8.9	1.8–47	nd–13	nd–30	2.9	3.0–4.3	2.9–11	29–71	nd–47
2-MBDE-68	nd–2.7	nd	nd–1.6	1.7–4.7	nd–6.8	nd	nd	5.0–7.0	2.8–9.8	10–46	nd–0.8
4-MBDE-49	nd	nd	nd	nd	nd	nd–6.3	nd	nd	nd	nd	nd
5-MBDE-47	nd	nd	nd–1.5	nd	nd	nd	nd	0.3–1.0	0.5–7.5	nd	nd–1.8
5-MBDE-99	nd	nd	nd	nd	nd	nd	nd	2.4–3.6	nd	nd	nd
5-MBDE-100	nd	nd	nd	nd–1.3	nd	nd	nd	nd	nd	nd	nd
4-MBDE-101	nd	nd	nd	nd	nd–1.6	nd	nd	nd	nd	nd	nd–1.6
4-MBDE-103	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd–4.0	nd
ΣMeO-PBDEs	2.7–21	nd	3.8–8.9	3.5–49	nd–20	nd–37	2.9	13–14	6.3–29	39–118	0.5–48
	nd–12[a]					2.9–17[a]				6.8–64[a]	

nd: Below limit of detection. MP: *Austromegabalanus pstittacus*. FS: *Fisurella* sp. VA: *Venus antiqua*. NV: *Tagelus dombeii*. PC: *Pyura chilensis*. TD: *Taliepus dentatus*. HP: *Homalapsis plana*. CV: *Cheilodactylus variegatus*. CA: *Chromis crusma*. CC: *Concholepas concholepas*. PR: *Pinguipes chilensis*.
[a] Total range of the mean values found for each specie in the three trophic levels.

fact that it is the congener with lower bioaccumulation capacity due to its large molecular size. Even though this is not in agreement with most of the published studies with biota samples, (Muñoz-Arnanz et al., 2011) also found a similar behavior in white storks from Spain, while (Moon et al., 2007) also found that BDE-209 was the predominant PBDE congener in different bivalves from Korean coastal waters.

Total PBDE levels were similar for all the samples analyzed, regardless of whether samples corresponded to primary, secondary o tertiary consumers. Only values found for crustaceans appear to be slightly higher. In any case, we must keep in mind that while we are differentiating between three levels of consumers, the differences in their trophic level are minimal. Hence, it is difficult to conclude a bioaccumulation along the food chain. To do this, it would be necessary to expand the range of trophic levels studied, including samples of organisms with higher trophic level than that studied here.

Total PBDE levels found in this study were compared with those obtained in a previous work carried out in Chile (Montory et al., 2010). In this study, samples of chinook salmon (*Oncorhynchus tshawytscha*) from Chilean Patagonia, a virgin region in Chile, were analyzed and PBDE levels were around 25 ng/g lw, being slightly lower than those found in our study. If we compare with studies in fish carried out in other parts of the world, our values were higher than those found in the Artic for polar cod (*Boreogadus saida*) (3.5 ng/g lw, mean value) (Wolkers et al., 2004), but lower than those found in carp (*Cyprinus carpio*) from Virgina watersheds, USA (about 7000 ng/g lw, medium) (Hale et al., 2001). Regarding PBDE levels in mussels, our total PBDE levels were slightly lower than the reported in bivalves from San Francisco (35 to 104 ng/g lw) (Oros et al., 2005) but higher than the levels found in blue mussels from Greenland (about 5 ng/g lw) (Christensen et al., 2002). (Ramu et al., 2007) found total PBDE levels ranging from 0.46 to 440 ng/g lw in mussels from Cambodia, China, India, Indonesia, Japan and Korea, while (Bianco et al., 2010) reported total PBDE levels ranging from 3.1 to 106 ng/g lw in mussels from the Mediterranean sea. Finally, (Webster et al., 2009) reported total PBDE levels ranging from 3 to 186 ng/g lw in mussels from Scotland. As can be seen, the contamination of mussels with PBDEs occurs worldwide.

3.2. MeO-PBDE levels

Table 2 shows the range of concentrations for the 8 different MeO-PBDEs analyzed in the biota samples. The naturally-occurring MeO-PBDEs were detected in all the samples with the exception of those corresponding to keyhole limpet. For primary consumers, total MeO-PBDE values ranged from nd to 11.6 ng/g lw. 6-MeO-BDE-47 and 2-MeO-BDE-68 were the most abundant congeners, with values ranging from nd to 10 and from nd to 2.7 ng/g lw, respectively. Other methoxylated congeners were also detected but in lower levels as well as lower frequency: 5-MeO-BDE-47 (nd to 1.5 ng/g lw), 5-MeO-BDE-100 (nd to 1.3 ng/g lw) and 4-MeO-BDE-101 (nd to 1.6 ng/g lw).

Regarding secondary consumers, total MeO-PBDE values ranged from 2.9 to 17 ng/g lw, and differences between the two families (fish and crustaceans) were also observed. While 6-MeO-BDE-47 was the predominant congener in crustacean, with values ranging from nd to 30 ng/g lw, and 2-MeO-BDE-68 was not detected in any sample, fish samples presented a similar contribution of both 6-MeO-BDE-47 and 2-MeO-BDE-68 congeners (from 2.9 to 11 and from 2.8 to 9.8 ng/g lw, respectively). Moreover, 5-MeO-BDE-47 was also detected in fish samples, whereas it was not detected in crustacean samples.

For tertiary consumers, total MeO-PBDE values ranged from 6.8 to 64 ng/g lw. Again, differences were observed between the two fish species studied. Higher levels were found for Chilean abalone, with 6-MeO-BDE-47 levels ranging from 29 to 71 ng/g lw and 2-MeO-BDE-68 levels from 10 to 46 ng/g lw. Concentrations found for sandperches were in the range of nd to 47 and nd to 0.8 ng/g lw for 6-MeO-BDE-47 and 2-MeO-BDE-68, respectively.

As it was observed for PBDEs, values showed significant differences between species. This may be associated to the different metabolic capacity of the species to incorporate or transform the different MeO-PBDE congeners. Furthermore, the differences observed for MeO-PBDEs among species may be explained also by the variability in the production of these two congeners from natural sources.

If we compare the concentration levels obtained for the different consumers, and in contrast with the behavior observed for PBDEs, it seems that MeO-PBDEs were bioaccumulated along the food chain: primary and secondary consumers presented similar values, with slightly higher values for secondary ones. However, levels for samples corresponding to tertiary consumers were the highest, with an increase of 5 times approximately with respect those obtained for primary and secondary consumers.

Naturally-occurring MeO-PBDE levels found in this study were compared with those published in the literature. However, the number of

studies which refer to these compounds is limited. Values obtained are lower than the values reported on blue mussels (*Mytitus edulis*), with a mean value of total MeO-PBDEs of 250 ng/g lw (Löfstrand et al., 2011) but higher than the values found in fish by (Losada et al., 2010): 7.5 and 13 ng/g lw for salmon (*Salmo salar*) and gilthead seabream (*Sparus aurata*), respectively. Generally, 6-MeO-BDE-47 and 2-MeO-BDE-68 are the most abundant MeO-PBDEs reported in bibliography. However, other congeners have been occasionally reported (Alonso et al., 2012; Nomiyama et al., 2011b). It seems that the MeO-tetra-PBDE levels are normally higher than the levels for MeO-tri- or MeO-penta-PBDEs (Covaci et al., 2008). The source of the MeO-PBDEs could also affect to the congeners found in the different organisms. Hence, if the MeO-PBDEs came from sponges or related organisms or from algaes or related organisms different congeners could be found (Vetter, 2006). This fact could explain the different congener profiles for MeO-PBDEs in the different species studied.

3.3. Halogenated norbornenes

Table 3 showed the range of concentrations for the halogenated norbornenes in the biota samples. Dec 603 and the two DP isomers were detected, whereas Dec 602 and Dec 604 were not found in any sample. In general, DP was detected with a higher frequency than Dec 603, and their concentration levels were also higher.

Regarding the primary consumers, DP was detected in the 5 species studied, with levels ranging between nd and 9.8 ng/g lw. The highest levels were found in keyhole limpet and in razor shell clam. On the other hand, Dec 603 was only detected in the sea squirt at lower level than DP. DP was also detected in 3 out of the 4 secondary consumers studied, with values ranging from 0.04 to 1.1 ng/g lw. In contrast to the differences found for PBDEs and MeO-PBDEs between crustaceans and fish, for the halogenated norbornenes this difference was not observed. Dec 603 was detected at similar DP levels in dalmselfish and at lower DP concentrations in "panchote". Finally, any of the halogenated norbornenes were found in one of the tertiary consumer species, the Chilean abalone. However, both isomers of DP and Dec 603 were detected in sandperches. Range values of 0.04 to 0.7, nd to 0.9 and nd to 1.2 ng/g lw were found for Dec 603, *syn*-DP and *anti*-DP, respectively. Dec 603 levels in this tertiary consumer were the highest found in all the analyzed species, and its detection frequency was similar to that of DP. It seems that Dec 603 increased with the trophic level, from maximum values up to 0.2 and 0.2 for the primary and secondary consumers, until concentrations up to 0.7 ng/g lw for the tertiary consumers. This finding could indicate a biomagnification capacity for Dec 603, altought Dec 603 is not detected in many species and more studies should be carried out in order to confirm this behavior. This behavior was not observed for DP. As for PBDEs, total DP levels were lower for secondary (nd – 1.1 ng/g lw) and tertiary (nd – 2.0 ng/g lw) consumers than for primary (nd – 9.8 ng/g lw) ones. This could be due to the capacity of these species to metabolize or transform this compound. There is a lack of information about degradation and/or metabolic products of DP, but it has been observed that the DP molecule could break to form DP monoadduct (DPMA) or other dechlorinated products (Sverko et al., 2011). Future works must include the determination of this degradation product in order to confirm this hypothesis.

There is a lack of studies reporting levels of halogenated norbornenes in marine fish. Moreover, the studies in the literature for river or lake fish were scarce. Our DP levels are considerably lower than those found in Korean river fish (between 0.6 and 130 ng/g lw) (Kang et al., 2010) or in the Great Lakes (between 0.2 and 7.2 ng/g lw) (Sverko et al., 2011). Furthermore, the mean value found in Chinese oysters (Jia et al., 2011) was about 35 ng/g lw, higher than our values in filtering species. These higher values are expected because all these studies were done near to the production sources of DP. Finally, (Sühring et al., 2013) reported DP values in eels from two German river basins, with values up to 34 ng/g lw. These results were lower than those found in China but higher than those in USA or in our study in Chile.

Although DP exists as 3 different commercial mixtures (DP-25, DP-35 and DP-515) the only difference among them is the particle size. The isomeric ratio (F_{anti}, expressed as the amount of *anti*-DP divided by the total amount of both isomers) in the 3 mixtures is about 0.7 (Hoh et al., 2006). However, several studies suggested a different behavior of the two isomers: this ratio is maintained in sediments, while in biota samples it tends to decrease, indicating a higher bioaccumulation potential for the *syn*-isomer or an easier degradation or metabolization of the *anti*-isomer (Sverko et al., 2011; Xian et al., 2011).

Regarding to our F_{anti} values (Table 2), they are similar for primary and secondary consumers (0.60 and 0.61 respectively), being not significantly different from the value of commercial mixture (about 0.7). However, the value of F_{anti} for the tertiary consumers was 0.47, which was significantly different from the value of the commercial mixture (t test, $p < 0.01$). This lowering in the F_{anti} value may indicate that *syn*-DP is more bioaccumulative than *anti*-DP, or that tertiary consumers can degrade the *anti*-DP isomer. This fact is in agreement with other studies in biota (Gauthier and Letcher, 2009; Tomy et al., 2008; Wu et al., 2010).

Table 3
Range of concentrations (expressed in ng/g lipid weight (lw)), Fanti and frequency of detection of DP (%) for halogenated norbornenes (HNs).

	Dec603	*Syn*-DP	*Anti*-DP	Σ*DP*	F_{anti}	Fd (%)	Total HNs
Primary consumers							
MP (n = 7)	nd	nd–0.3	nd–0.3	nd–0.6	0.5–0.9	71	nd–9.8
FS (n = 3)	nd	0.8–2.9	1.5–7.0	2.3–9.8	0.5–0.7	100	
VA (n = 6)	nd	nd–0.2	nd–0.3	nd–0.4	0.5–0.7	75	
NV (n = 7)	nd	nd–0.5	nd–5.4	nd–5.8	0.5–0.9	85	
PC (n = 6)	0.04–0.2	nd–0.1	nd–0.2	nd–0.3	0.5–0.6	83	
Secondary consumers							
TD (n = 4)	0.1	0.1–0.4	0.1–0.8	0.3–1.1	0.5–0.8	100	nd–1.1
HP (n = 1)	nd	nd	nd	–	–	0	
CV (n = 2)	nd	0.04	0.04–0.1	0.08–0.1	0.5–0.6	100	
CA (n = 4)	0.1–0.2	0.01–0.1	0.03–0.2	0.04–0.3	0.6–0.7	100	
Tertiary consumers							
C (n = 4)	nd	nd	nd	–	–	0	nd-2.0
PR (n = 13)	0.04–0.7	nd–0.9	nd–1.2	nd–2.0	0.4–0.5	69	

nd: Below limit of detection. MP: *Austromegabalanus pstittacus*. FS: *Fisurella* sp. VA: *Venus antiqua*. NV: *Tagelus dombeii*. PC: *Pyura chilensis*. TD: *Taliepus dentatus*. HP: *Homalapsis plana*. CV: *Cheilodactylus variegatus*. CA: *Chromis crusma*. CC: *Concholepas concholepas*. PR: *Pinguipes chilensis*.

3.4. Anthropogenic vs natural levels

Due to the fact that DP and its analogues have been proposed as substitutes of PBDEs, especially of BDE-209, it is interesting to compare the levels found for the "classical" and the emerging families. There are no specific restrictions for the use of PBDEs in Chile so, naturally, the PBDE levels where higher than the dechlorane levels in all cases (Fig. 2). It is expected that in the future, when PBDEs are banned in South America, the levels of emerging FRs will increase and the differences between "classical" and emerging FRs will decrease.

It is also interesting to compare levels of anthropogenic PBDEs with those of naturally-occurring MeO-PBDEs (Fig. 2). PBDE levels were higher than MeO-PBDEs for primary consumers, being the PBDE contribution up to the 81.6% of the total. Similar contribution was observed for secondary consumers, with 83% of the total corresponding to PBDEs. However, for tertiary consumers the contribution of PBDEs and MeO-PBDEs was similar (51.6% and 48%, respectively). This may indicate that tertiary consumers are able to metabolize PBDEs but not MeO-PBDEs, or that MeO-PBDEs have higher biomagnification capacity than PBDEs.

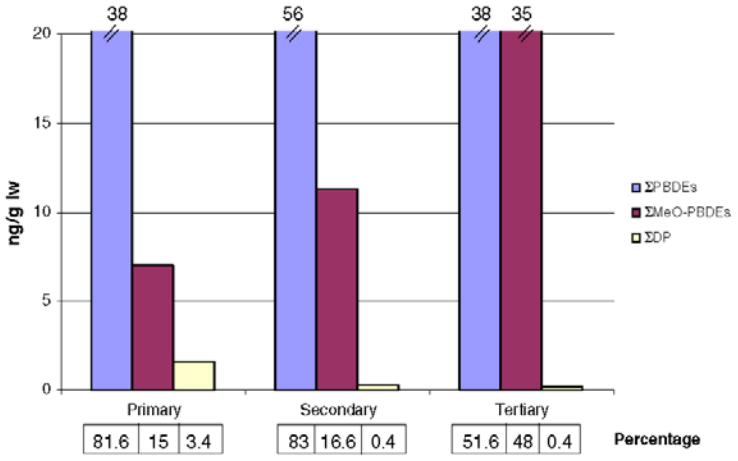

Fig. 2. Total contribution of PBDEs, MeO-PBDEs and DP in primary, secondary and tertiary consumers.

3.5. Biomagnification factor

The biomagnification factor (BMF), defined as the ratio of the concentrations of the predator and prey, both normalized to lipid weight (Borgá et al., 2004), was evaluated. A BMF > 1 means that predators have a lower capacity to metabolize the compound than their prey, or also that they can accumulate higher amounts. In contrast, a BMF < 1 means that the predator is able to metabolize the compound, or also that it can not accumulate as much as the prey (Weijs et al., 2009).

Three different predator-pray relationships were identified between the species selected in this study. Chilean abalone is known to have giant barnacle and sandperches among his preys. Moreover, peruvian morwong can feed from keyhole limpet. BMFs were calculated for these cases. For Chilean abalone and sandperches we found a BMF > 1 for BDE-183, BDE-209, 6-MeO-BDE-47 and 2-MeO-BDE-68 (1.5, 1.3, 2.5 and 1.8 respectively). Regarding Chilean abalone and giant barnacle, again 2 PBDEs and 2 MeO-PBDEs had BMF > 1: BDE-28 (3.1), BDE-209 (1.5), 6-MeO-BDE-47 (6.8) and 2-MeO-BDE-68 (9.4). In contrast, for peruvian morwong and keyhole limpet, only BDE-47 had a BMF > 1 (13.8). In general, MeO-PBDEs had higher BMF than PBDEs. This behavior would explain the increase in the percentage contribution of MeO-PBDEs against PBDEs when we move from primary to the tertiary consumers, as discussed in the previous section.

This behavior is in contrast with results obtained for seals and porpoises from the North Sea (Weijs et al., 2009) or for bivalves, Arctic cod, sculpin and salmon (Kelly et al., 2008). Nevertheless, BMF > 1 have been found for MeO-PBDEs in several studies (Kelly et al., 2008; Losada et al., 2009; Weijs et al., 2009).

4. Conclusions

PBDEs, MeO-PBDEs and halogenated norbornenes were detected in an aquatic food web from Chile, with total values ranging from 11 to 170, nd to 118 and nd to 5.8 g/g lw, respectively. However, emerging brominated FRs such as HBB, PBEB and DBDPE were not detected in any sample. As expected, "classical" PBDE levels were higher than those of the emerging halogenated norbornenes. Further studies must be carried out in order to assess possible changes in this trend due to the restricted use of PBDEs and increasing use of halogenated norbornenes applied as an alternative to the formers.

Some PBDE congeners (BDE-28, BDE-183 and BDE-209) showed biomagnification capacity, as well as MeO-PBDEs. However, BMFs of the naturally occurring MeO-PBDEs were higher than those obtained for PBDEs. These results could explain the increase in the percentage contribution of MeO-PBDEs against PBDEs when we move from primary to the tertiary consumers. On the other hand, BMF > 1 was found for halogenated norbornenes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.05.006.

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Supporting information 1: Complete sample information.

Site	Sampling date	Common name	Specie	(n) Pool	Code name
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP1
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP2
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP3
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP4
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP5
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP6
Lenga	15/07/2010	Giant barnacle	*Megabalanus psittacus*	3 to 5	MP7
Lenga	03/09/2010	Clam	*Venus antiqua*	3	VA1
Lenga	03/09/2010	Clam	*Venus antiqua*	3	VA2
Lenga	03/09/2010	Clam	*Venus antiqua*	3	VA3
Lenga	03/09/2010	Clam	*Venus antiqua*	3	VA4
Lenga	03/09/2010	Keyhole limpet	*Fisurella sp1*	1	FS1
Lenga	03/09/2010	Keyhole limpet	*Fisurella sp1*	1	FS2
Lenga	03/09/2010	Keyhole limpet	*Fisurella sp2*	1	FS3
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV1
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV2
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV3
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV4
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV5
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV6
Lenga	03/09/2010	Razor shell clam	*Tagelus dombeii*	5	NV7
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC1
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC2
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC3
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC4
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC5
Lenga	15/07/2010	Sea squirt	*Pyura chilensis*	15 to 20	PC6
Lenga	03/09/2010	Panchote	*Taliepus clontatus*	3	TD1
Lenga	03/09/2010	Panchote	*Taliepus clontatus*	4	TD2
Lenga	03/09/2010	Panchote	*Taliepus clontatus*	3	TD3
Lenga	03/09/2010	Panchote	*Taliepus clontatus*	3	TD4
Lenga	03/09/2010	Crab	*Homilapsis plana*	1	HP1
Lenga	15/07/2010	Damselfish	*Cromis Crusma*	1	CA1
Lenga	15/07/2010	Damselfish	*Cromis Crusma*	1	CA2
Lenga	15/07/2010	Damselfish	*Cromis Crusma*	1	CA3
Lenga	15/07/2010	Damselfish	*Cromis Crusma*	1	CA4
Lenga	15/07/2010	Peruvian morwong	*Chelodactilus variegatus*	1	CV1
Lenga	15/07/2010	Peruvian morwong	*Chelodactilus variegatus*	1	CV2

Site	Date	Name	Specie	(n) Pool	Code name
Lenga	03/09/2010	Chilean abalone	*Concholepas concholepas*	1	CC1
Lenga	03/09/2010	Chilean abalone	*Concholepas concholepas*	1	CC2
Lenga	03/09/2010	Chilean abalone	*Concholepas concholepas*	1	CC3
Lenga	03/09/2010	Chilean abalone	*Concholepas concholepas*	1	CC4
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR1
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR2
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR3
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR4
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR5
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR6
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR7
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR8
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR9
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR10
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR11
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR12
Lenga	15/07/2010	Sandperches	*Pinguipes chilensis*	1	PR13

Supporting information 2: Transitions used for quantification and confirmation, collision energy (eV) and ratio of the halogenated norbornenes

	SRM_1	CE_1 (eV)	SRM_2	CE_2 (eV)	Ratio
Dec 602	612>35	35	237>35	30	2,4
Dec 603	638>35	15	638>37	15	3,1
Dec 604	460>79	20	504>79	20	1,5
syn-DP	654>35	35	654>37	35	3,2
anti-DP	654>35	35	654>37	35	3,3
^{13}C-*syn*-DP	664>35	30	664>37	30	3,1

Supporting information 3: Levels of PBDEs, MeO-PBDEs and Halogenated norbornenes in each sample (ng/g lw)

	PBDEs						
Specie	**28**	**47**	**100**	**99**	**154**	**183**	**209**
Primary consumers							
FS1	nd	0.60	nd	0.37	0.43	9.36	22.9
FS2	nd		nd	nd	nd	nd	31.3
FS3	nd	1.96	nd	nd	nd	nd	38.2
MP1	1.55	1.30	nd	nd	nd	0.96	11.9
MP2	nd	3.10	nd	2.22	nd	nd	63.2
MP3	nd	7.04	nd	6.58	nd	nd	46.7
MP4	nd	1.87	nd	1.62	nd	nd	19.3
MP5	nd	2.22	nd	1.64	nd	nd	9.70
MP6	24.7	7.90	nd	6.50	nd	nd	72.7
MP7	2.08	1.21	nd	0.97	nd	0.77	5.45
NV1	nd	1.86	nd	nd	nd	nd	29.6
NV2	nd	1.12	nd	nd	nd	nd	45.5
NV3	nd	2.66	nd	nd	nd	2.06	35.2
NV5	nd	1.84	nd	nd	nd	nd	32.9
NV6	nd	1.46	nd	nd	nd	nd	33.6
NV7	nd	1.31	nd	nd	nd	nd	39.4
VA1	nd	nd	nd	nd	nd	nd	27.8
VA2	nd	nd	nd	nd	5.61	nd	24.7
VA3	nd	nd	nd	nd	nd	nd	28.3
VA4	nd	nd	nd	nd	nd	nd	35.2
PC1	nd	nd	nd	nd	nd	54.5	28.6
PC3	nd	nd	nd	nd	nd	nd	24.2
PC4	8.61	nd	nd	nd	nd	nd	18.6
PC5	nd	nd	nd	nd	nd	nd	30.4
PC6	nd	nd	nd	nd	nd	nd	17.6
Secondary consumers							
TD1	nd	nd	nd	3.54	nd		25.2
TD2	nd	2.09	nd	7.63	nd	4.66	74.3
TD3	nd	nd	3.78	10.5	nd	6.49	132
TD4	nd	3.68	3.53	9.19	nd	5.11	52.8
HP	nd	1.66	nd	2.62	1.39	115	49.8
CA1	nd	26.1	nd	nd	nd	nd	23.6
CA2	nd	42.3	nd	nd	nd	nd	79.4
CA3	nd	29.3	nd	nd	nd	2.74	14.7
CA4	nd	1.36	nd	nd	nd	1.01	16.4
CV1	nd	4.95	nd	nd	nd	1.09	14.2
CV2	nd	5.77	nd	nd	nd	nd	7.75
Tertiary consumers							
CC1	nd	1.93	nd	3.65	nd	2.24	26.4
CC2	12.62	nd	nd	nd	nd	nd	45.3
CC3	nd	4.50	nd	nd	nd	nd	31.1
CC4	8.63	nd	nd	nd	nd	nd	39.9
PR1	nd	0.81	nd	nd	nd	0.26	14.2
PR2	nd	0.93	nd	nd	nd	30.4	37.3

PR3	nd	0.36	nd	nd	nd	1.28	19.6
PR4	nd	0.65	nd	1.66	nd	nd	31.2
PR5	nd	1.41	nd	2.78	nd	3.29	39.7
PR6	nd	nd	nd	nd	nd	2.68	20.1
PR7	nd	1.37	nd	1.05	nd	3.94	25.0
PR9	nd	0.28	nd	0.88	nd	1.41	17.3
PR0	nd	0.69	nd	1.40	nd	2.19	47.4
PR11	nd	0.76	nd	nd	nd	1.56	30.9
PR12	nd	0.23	nd	0.40	nd	0.65	11.4
PR13	nd	nd	nd	1.31	nd	2.93	25.4

	MeO-PBDEs							
Specie	6MBDE47	2MBDE68	4MBDE49	5MBDE47	5MBDE99	5MBDE100	4MBDE101	4MBDE103
	Primary consumers							
FS1	nd	nd	nd	nd	nd	nd	nd	nd
FS2	nd	nd	nd	nd	nd	nd	nd	nd
FS3	nd	nd	nd	nd	nd	nd	nd	nd
MP1	21.1	nd	nd	nd	nd	nd	nd	nd
MP2	8.60	2.68	nd	nd	nd	nd	nd	nd
MP3	9.86	2.33	nd	nd	nd	nd	nd	nd
MP4	4.82	1.82	nd	nd	nd	nd	nd	nd
MP5	4.07	2.45	nd	nd	nd	nd	nd	nd
MP6	21.1	nd	nd	nd	nd	nd	nd	nd
MP7	2.65	nd	nd	nd	nd	nd	nd	nd
NV1	2.24	3.05	nd	nd	nd	1.25		
NV2	1.83	1.72	nd	nd	nd	nd	nd	nd
NV3	7.30	4.65	nd	nd	nd	nd	nd	nd
NV5	46.5	2.29	nd	nd	nd	nd	nd	nd
NV6	3.07	1.97	nd	nd	nd	nd	nd	nd
NV7	3.09	2.46	nd	nd	nd	nd	nd	nd
VA1	8.90	nd	nd	nd	nd	nd	nd	nd
VA2	6.97	1.64	nd	nd	nd	nd	nd	nd
VA3	3.82	nd	nd	nd	nd	nd	nd	nd
VA4	5.80	nd	nd	1.37	nd	nd	nd	nd
PC1	13.4	6.78	nd	nd	nd	nd	nd	nd
PC3	7.37	4.66	nd	nd	nd	nd	6.21	nd
PC4	9.24	nd	nd	nd	nd	nd	nd	nd
PC5	nd	nd	nd	nd	nd	nd	nd	nd
PC6	nd	nd	nd	nd	nd	nd	nd	nd
	Secondary consumers							
TD1	nd	nd	nd	nd	nd	nd	nd	nd
TD2	10.6	nd	nd	nd	nd	nd	3.42	nd
TD3	30.4	nd	6.34	nd	nd	nd	nd	nd
TD4	nd	nd	nd	nd	nd	nd	nd	nd
HP	2.94	nd	nd	nd	nd	nd	nd	nd
CA1	6.78	4.63	nd	4.91	nd	nd	nd	nd
CA2	11.2	9.80	nd	7.47	nd	nd	nd	nd

CA3	6.27	5.15	nd	4.92	nd	nd	nd	nd
CA4	2.91	2.82	nd	0.54	nd	nd	nd	nd
CV1	3.02	5.00	nd	1.04	3.56	nd	nd	nd
CV2	4.33	6.95	nd	0.29	2.42	nd	nd	nd
				Tertiary consumers				
CC1	29.1	9.98	nd	nd	nd	nd	nd	nd
CC2	29.5	14.4	nd	nd	nd	nd	nd	4.04
CC3	71.3	46.3	nd	nd	nd	nd	nd	nd
CC4	34.0	15.4	nd	nd	nd	nd	nd	nd
PR1	1.23	0.27	nd	0.47	nd	nd	nd	nd
PR2	2.01	nd	nd	nd	nd	nd	nd	nd
PR3	0.57	nd	nd	nd	nd	nd	nd	nd
PR4	1.34	nd	nd	nd	nd	nd	0.95	nd
PR5	2.56	0.43	nd	1.80	nd	nd	1.58	nd
PR6	10.13	nd	nd	nd	nd	nd	nd	nd
PR7	3.33	0.75	nd	0.74	nd	nd	nd	nd
PR9	0.49	nd	nd	nd	nd	nd	0.37	nd
PR0	47.2	nd	nd	0.65	nd	nd	0.55	nd
PR11	3.95	nd	nd	nd	nd	nd	0.00	nd
PR12	0.21	nd	nd	nd	nd	nd	0.24	nd
PR13	nd	nd	nd	0.70	nd	nd	0.82	nd

Halogenated norborenenes

Specie	**Dec 602**	**Dec 603**	**Dec 604**	***syn*-DP**	***anti*-DP**
FS1	nd	nd	nd	2.87	6.97
FS2	nd	nd	nd	0.84	1.47
FS3	nd	nd	nd	2.79	2.52
MP1	nd	nd	nd	0.03	0.04
MP2	nd	nd	nd	nd	0.02
MP3	nd	nd	nd	0.05	0.05
MP4	nd	nd	nd	nd	nd
MP5	nd	nd	nd	nd	nd
MP6	nd	nd	nd	0.27	0.34
MP7	nd	nd	nd	0.01	nd
NV1	nd	nd	nd	0.44	0.47
NV2	nd	nd	nd	0.24	1.40
NV3	nd	nd	nd	0.32	0.63
NV4	nd	nd	nd	0.52	0.67
NV5	nd	nd	nd	nd	nd
NV6	nd	nd	nd	0.41	5.40
NV7	nd	nd	nd	0.49	0.68
VA1	nd	nd	nd	0.20	0.18
VA2	nd	nd	nd	0.12	0.30
VA3	nd	nd	nd	0.08	0.11
VA4	nd	nd	nd	nd	nd

PC1	nd	0.08	nd	0.14	0.17
PC2	nd	nd	nd	nd	nd
PC3	nd	0.13	nd	0.07	0.16
PC4	nd	0.15	nd	0.11	0.10
PC5	nd	nd	nd	0.13	0.19
PC6	nd	0.04	nd	0.06	0.07
			Secondary consumers		
TD1	nd	nd	nd	0.13	0.14
TD2	nd	nd	nd	0.17	0.79
TD3	nd	nd	nd	0.39	0.69
TD4	nd	0.11	nd	0.44	0.53
HP	nd	nd	nd	nd	nd
CA1	nd	0.14	nd	0.03	0.06
CA2	nd	nd	nd	0.13	0.17
CA3	nd	0.15	nd	0.06	0.14
CA4	nd	nd	nd	0.01	0.03
CV1	nd	nd	nd	0.04	0.05
CV2	nd	nd	nd	0.04	0.04
			Tertiary consumers		
CC1	nd	nd	nd	nd	nd
CC2	nd	nd	nd	nd	nd
CC3	nd	nd	nd	nd	nd
CC4	nd	nd	nd	nd	nd
PR1	nd	0.19	nd	0.21	0.19
PR2	nd	0.07	nd	0.10	0.11
PR3	nd	nd	nd	0.87	0.76
PR4	nd	0.04	nd	0.05	0.06
PR5	nd	nd	nd	0.05	0.05
PR6	nd	0.68	nd	0.86	1.15
PR7	nd	nd	nd	nd	nd
PR8	nd	nd	nd	nd	nd
PR9	nd	nd	nd	nd	nd
PR10	nd	nd	nd	0.39	0.23
PR11	nd	0.35	nd	0.11	0.06
PR12	nd	0.24	nd	0.22	0.15
PR13	nd	nd	nd	nd	nd

Publicación científica #6

Bioaccumulation and biomagnification of classical flame retardants, related halogenated natural compounds and alternative flame retardants in three delphinids from southern European waters

E. Barón, J. Giménez, P. Verborgh, P. Gauffier, R. de Stephanis, E. Eljarrat, D. Barceló

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PBDEs

ABSTRACT

Occurrence and behaviour of classical (PBDEs) and alternative (HNs, HBB, PBEB, DBDPE and HBCD) flame retardants, together with naturally produced MeO-PBDEs, were studied in short-beaked common dolphin (*Delphinus delphis*), bottlenose dolphin (*Tursiops truncatus*) and long-finned pilot whale (*Globicephala melas*) in two sampling locations from Southern European waters. PBDEs, Dec 602, Dec 603, DP, α-HBCD and two MeO-PBDEs were detected in all three species. ∑PBDEs were between 17 and 2680 ng/g lw; ∑HNs were between 1.1 and 59 ng/g lw; α-HBCD levels ranged between 3.2 and 641 ng/g lw; ∑MeO-PBDEs were between 34 and 1966 ng/g lw. Bottlenose dolphins were the most contaminated species and some individuals could present health risk for endocrine disruption since levels found were above the reported threshold (1500 ng/g lw). Stable isotope analysis was used to evaluate the biomagnification capacity of these compounds. PBDEs, MeO-PBDEs and Dec 602 showed a significant positive correlation with trophic position.

1. Introduction

Odontocetes accumulate high concentrations of anthropogenic contaminants, many considered persistent, bioaccumulative and toxic (PBT) (Fossi et al., 2013a). Nowadays, there is no evidence that PBT chemicals are provoking direct mortalities of marine mammals, however these lipophilic contaminants cause immune and reproductive dysfunction that can have consequences at the population level (Hammond et al., 2005; Ross et al., 1995).

Flame retardants (FRs) have been used for many years in order to prevent fires, being applied to a wide range of materials such as textiles, furniture, electronic materials and so on (Alaee et al., 2003). Halogenated flame retardants (HFRs) proved to be really effective and became one of the most used families of FRs, with an annual production constantly increasing (Birnbaum and Staskal, 2004). Some examples are polybromodiphenyl ethers (PBDEs) or Mirex. These compounds have been found in the environment, both in environmental matrices such as sediment (Hu et al., 2010), sludge (Gorga et al., 2013) or air (Harner et al., 2006), and in biological samples such as fish (Guerra et al., 2010), birds (Barón et al., 2014b), cetaceans (Alonso et al., 2012) or even humans (Covaci et al., 2003). PBDEs are lipophilic, persistent and toxic to wildlife and humans (Alaee et al., 2003; de Wit et al., 2010). In mammals, they present potential health risks such as thyroid disruption, neurobiological development and foetal toxicity/teratogenicity (Alonso et al., 2014). Due to its presence in the environment and its

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proved toxicity, Penta- and Octa-BDE mixtures were banned in 2006, while the production of Deca-BDE mixture was planned to be stopped by the end of 2013 (Schecter et al., 2010). As a result, other alternative FRs were developed, and were given the name of emerging FRs (EFRs). Some examples of brominated EFRs are hexabromociclododecane (HBCD), hexabromobenzene (HBB), pentabromoethyl benzene (PBEB) and decabromodihpenyl ethane (DBDPE). The family of halogenated norbornenes (HNs) includes some chlorinated FRs such as dechlorane 602 (Dec 602), 603 (Dec 603), 604 (Dec 604) and dechlorane plus (DP), with its *syn*- and *anti*-isomers. DP was also the replacement of Mirex when this compound was banned as FR in 1976 (Sverko et al., 2011). With the exception of HBCD, included in the list of global elimination compounds under the Stockholm Convention in May 2013 (Al-Odaini et al., 2015), these compounds are still not legislated, even though they have been found in several environmental and biological matrices (Alonso et al., 2014; Covaci et al., 2011; Guerra et al., 2011; Sverko et al., 2011; Xian et al., 2011). Besides, despite HBCD was included in the Stockholm convention, its use is still permitted in expanded polystyrene and extruded polystyrene in buildings, as long as materials are clearly labelled, indicating that they contain HBCD (Al-Odaini et al., 2015). DP can interfere with the metabolism of some species and has been associated to proteins regulating the apoptosis and cell differentiation in liver cells (Liang et al., 2014). DBDPE and DP have been proposed as a specific alternative for BDE-209 (De la Torre et al., 2011; De la Torre et al., 2010) and consequently their concentrations in the environment might increase in the next years. Despite the increase of studies focussing on these contaminants in recent years, more information regarding their bioaccumulation and biomagnification capacities is needed. To date, there is only one study reporting the presence of DP in cetaceans from Brazil (De La Torre et al., 2012a), while PBDEs have been found in cetaceans at high concentrations worldwide, as reviewed by Alonso et al. (2014). Furthermore, some studies have revealed that cetaceans can be sensible to some families of organic contaminants (Fossi et al., 2013b) and, consequently, the study of the bioaccumulation of these contaminants, including FRs, is of special interest.

On the other hand, methoxylated PBDEs (MeO-PBDEs) are naturally produced halogenated compounds, called halogenated natural products (HNPs). There are more than 4000 HNPs mainly produced by algae, sponges or bacteria (Vetter, 2006) and, consequently, they are considered only a marine problem. These compounds have been found in different species like fish (Barón et al., 2013), polar bears (Letcher et al., 2009) or cetaceans (Alonso et al., 2012). Several studies report that its toxic potential could be similar to dioxins (Su et al., 2012). Furthermore, MeO-PBDEs are structurally similar to compounds shown to exert toxic effects such as endocrine disruption (Fu et al., 1995).

One of the main concerns about Persistent Organic Pollutants (POPs) is their bioaccumulation capacity (Alaee et al., 2003). Besides, it gets worse if the contaminant can also biomagnify in wild animal populations. Previous studies suggest that the uptake of organohalogen compounds occurs through the diet rather than directly from the environment (Vetter et al., 2002). Stable isotope analysis has become a powerful tool to study dietary exposure and biomagnification of contaminants in wild animal populations (Cullon et al., 2012; Chouvelon et al., 2012). δ^{15}N is commonly taken as indicator of trophic level (Cabana and Rasmussen, 1996), due to the increase of δ^{15}N from prey to predator throughout the food web (Jardine et al., 2006) and thus the correlation between the concentration of the contaminant in the sample (lipid-normalized) with the δ^{15}N is used to evaluate the biomagnification capacity of the contaminant.

Despite the fact that the Southern Iberian Peninsula is of special interest for cetaceans because of its diversity of species (Cañadas et al., 2005; De Stephanis et al., 2008; Esteban et al., 2014; Verborgh et al., 2009) and its location as the unique natural connexion between the Mediterranean Sea and the Atlantic Ocean, there is only one recent study reporting FRs in the area, in striped dolphin (*Stenella coeruloalba*) (Fossi et al., 2013a). Publications about these organic contaminants in cetaceans are not rare, except for halogenated norbornenes, however most studies were undertaken in both west and east coasts of USA, the Baltic Sea, Japan's Sea and the south east coast of Brazil (Alonso et al., 2014).

The aim of this work was to evaluate the presence of several classical and emerging FRs in the blubber from three cetacean species from southern Iberian Peninsula waters, to compare the anthropogenic burden with the levels of naturally-produced MeO-PBDEs and to evaluate the biomagnification capacity of these different FR families.

2. Materials and methods

2.1. Sampling

A total of 67 blubber samples from 3 cetacean species were obtained by remote biopsy sampling in the Strait of Gibraltar and the Gulf of Cadiz during 2012. Bottlenose dolphins and long-finned pilot whales were sampled via a crossbow and a modified dart with sterilised stainless-steel biopsy tips designed by Finn Larssen, following the protocols described in Giménez et al. (2011) to ensure a low impact sampling method. A pole and smaller biopsy tips were used to sample bow-riding individuals of common dolphins. All samples were collected under a special permit from the Spanish Ministry of Environment. Adults and sub adults were the main target and no calf under 2 years-old was sampled. In the Gulf of Cádiz, 15 samples of short-beaked common dolphin (*Delphinus delphis*) and 20 samples of bottlenose dolphin (*Tursiops truncatus*) were obtained. Furthermore, 2 samples of short-beaked common dolphin, 20 samples of bottlenose dolphin and 10 samples of long-finned pilot whale (*Globicephala melas*) were obtained in the Strait of Gibraltar.

2.2. Nitrogen stable isotope determination

Prior to the isotope determination, lipid content was extracted from the sample with several rinses of chloroform:methanol (2:1) solution in order to reduce the isotopic variability due to the differential content of lipids (Logan et al., 2008). Each sample was covered with the solvent mixture for 24 h, solvent was then removed and fresh solvent was added. This process was repeated for at least 3 times until the solvent appeared clean. Samples were dried at 50 °C for 24 h. Subsamples of powdered materials were weighed to the nearest μg and placed into tin capsules for δ^{15}N determinations. Isotopic analyses were carried out at the "Laboratorio de Isótopos Estables of the Estación Biológica de Doñana" (LIE-EBD, Spain; www.ebd.csic.es/lie/index.html). All samples were combusted at 1020 °C using a continuous flow isotope-ratio mass spectrometry system by means of Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The isotopic compositions are reported in the conventional delta (δ) per mil notation (‰), relative to atmospheric N_2 (δ^{15}N). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ±0.2‰ for δ^{15}N. The standards used were: EBD-23 (cow horn, internal standard), LIE-BB (whale baleen, internal standard) and LIE-PA (feathers of Razorbill, internal standard). These laboratory standards were previously calibrated with international standards supplied by the International Atomic Energy Agency (IAEA,

Vienna).

2.3. Contaminant analysis

2.3.1. Sample treatment

Sample extraction methodology was based on previous works (Eljarrat et al., 2002; Guerra et al., 2010) and it is explained in detail in the Supplementary Information.

2.3.2. Instrumental analysis

Instrumental analysis of PBDEs, MeO-PBDEs and emerging FRs (HBB, PBEB and DBDPE) was carried out by gas chromatography coupled to tandem mass spectrometry (GC-MS-MS) using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole. PBDEs, MeO-PBDEs, HBB, PBEB and DBDPE were analysed following the protocol optimized in (Barón et al., 2014a) while halogenated norbornenes were analysed as described in (Barón et al., 2012). Moreover, HBCD was analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) as described in (Guerra et al., 2008). Methodologies are described in detail in the supplementary information.

Recoveries, method detection limits (MDLs) and method quantification limits (MQLs) are showed in Table 1.

2.4. Data analysis

Stable isotopes and contaminant levels were compared for common and bottlenose dolphins in the Strait of Gibraltar and Gulf of Cadiz to evaluate the difference between the populations inhabiting these two areas. Comparisons between species of the same area were also performed. Data were tested for normality and homogeneity of variances using the Shapiro–Wilks test of normality and an F test. Differences between groups were tested using a t-test or ANOVA using p-value ≤ 0.05 to determine statistical difference. Statistical analyses were conducted using the open-source statistical programming language R v.3.1.1 (http://cran.r-project.org).

In order to evaluate the biomagnification capacity of these compounds we studied the relationships between nitrogen stable isotope and contaminant loads of different species sampled in the same area, *i.e.* common dolphins and bottlenose dolphins in the Gulf of Cadiz, and bottlenose dolphins and pilot whales in the Strait of Gibraltar. The relationship was analysed through standard regression. The two samples of common dolphin from the Strait of Gibraltar were not included in this analysis due to the low sample size.

Table 1
Recoveries (%), MDLs and MQLs (ng/g lw) of all the analysed compounds.

		R (%)	RSD (%)	MDLs	MQLs
PBDEs	BDE-28	87	1.8	0.01	0.03
	BDE-47	81	11	0.01	0.03
	BDE-100	77	8.1	0.06	0.20
	BDE-99	81	6.0	0.03	0.10
	BDE-154	75	8.1	0.21	0.70
	BDE-153	71	7.4	0.13	0.43
	BDE-183	56	5.2	1.39	4.63
	BDE-209	62	6.1	1.11	3.70
MeO-PBDEs	2-MeO-BDE-68	77	7.7	0.09	0.30
	6-MeO-BDE-47	75	10	0.24	0.80
	5-MeO-BDE-47	71	3.1	0.06	0.20
	4-MeO-BDE-99	74	4.7	0.16	0.53
	5-MeO-BDE-100	76	5.2	0.31	1.03
	4-MeO-BDE-100	77	4.4	1.59	5.30
	5-MeO-BDE-99	70	4.1	0.47	1.57
	4-MeO-BDE-101	76	2.4	0.8	2.67
Emerging BFRs	HBB	76	7.8	0.06	0.20
	PBEB	71	4.1	0.06	0.20
	DBDPE	72	11	1.06	3.53
HNs	Dec 602	75	8	0.05	0.16
	Dec 603	80	12	0.04	0.14
	Dec 604	68	14	0.15	0.50
	syn-DP	87	2.7	0.02	0.07
	anti-DP	84	4.7	0.01	0.03
HBCD	α-HBCD	95	3.1	0.37	1.23
	β-HBCD	85	4.7	0.41	1.37
	γ-HBCD	105	8.3	0.99	3.33

R: Recovery. MDLs: Method detection limits. MQLs: Method quantification limits. RSD: relative standard deviation. PBDEs: Polybromodiphenyl ethers. MeO-PBDEs: Methoxylated Polybromodiphenyl ethers. BFRs: Brominated flame retardants. HNs: Halogenated norbornenes. HBCD: Hexabromociclododecane.

3. Results

3.1. Nitrogen stable isotope analysis

Bottlenose dolphins had the highest $\delta^{15}N$ values of all 3 cetacean species in both areas (mean ± sd, 14.23 ± 0.70‰ and 13.39 ± 0.36‰ for the Gulf of Cádiz and Strait of Gibraltar respectively), followed by pilot whales (12.89 ± 0.34‰) and common dolphins (12.82 ± 0.33‰ in the Gulf of Cádiz and 11.89 ± 0.55‰ in the Strait of Gibraltar).

Since $\delta^{15}N$ values were statistically different between species from the same area ($t = -9.20$, $df = 29.50$, p-value <0.01 for the Gulf of Cádiz and $F = 19$, $df = 31$, p-value<0.01 for the Strait of Gibraltar), their trophic position was considered useful to carry out biomagnification studies: bottlenose dolphins presented the highest trophic position followed by pilot whales, and finally common dolphins (Fig. 1).

3.2. PBDE levels

Several PBDEs were detected in the three species and in both locations. Congeners detected were BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-209, while the emerging brominated FRs analysed, HBB, PBEB and DBDPE were not detected in any sample (Table 2). Variation in PBDE levels among species was

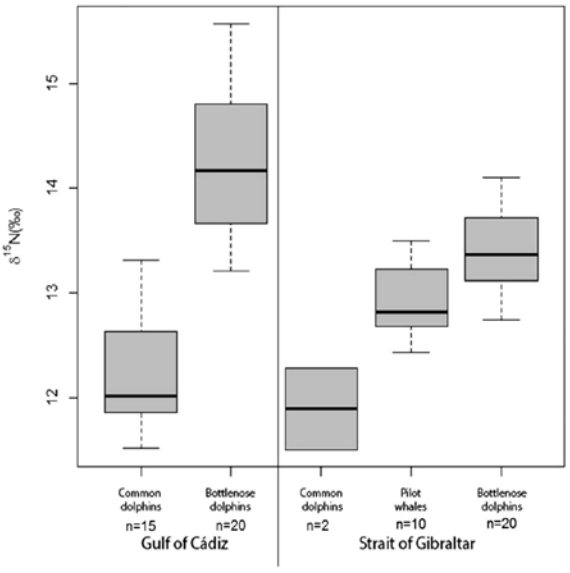

Fig. 1. Box plot of $\delta^{15}N$ values by species and sampling area.

Table 2
Levels of PBDEs, MeO-PBDEs, HNs and HBCD (ng/g lw) for the 3 cetacean species by area (mean [range]), and detection frequencies (DF, %).

	Gulf of Cádiz		Strait of Gibraltar		
	Common dolphin (n = 15)	Bottlenose dolphin (n = 20)	Common dolphin (n = 2)	Bottlenose dolphin (n = 20)	Pilot whale (n = 10)
BDE-28	6.77 [nd-14.8]	3.93 [0.44–8.65]	8.12 [7.54–8.70]	2.47 [nd-9.72]	2.91 [nd-6.73]
BDE-47	129 [nd-268]	556 [nd-1250]	116 [56.3–175]	594 [nd-1441]	178 [nd-287]
BDE-100	25.0 [nd-47.2]	156 [nd-403]	52.8 [nd-52.8]	248 [nd-517]	41.9 [nd-71.3]
BDE-99	23.0 [nd-43.8]	24.2 [nd-57.0]	26.5 [nd-26.5]	35.3 [59.7]	39.1 [nd-117]
BDE-154	24.9 [nd-47.9]	59.2 [nd-132]	37.0 [nd-37.0]	174 [nd-295]	nd [-]
BDE-153	9.46 [nd-20.7]	95.9 [nd-177]	nd [–]	203 [nd-457]	11.3 [15.5]
BDE-209	20.9 [nd-50.6]	18.8 [nq-73.5]	16.7 [10.5–22.9]	10.8 [nd-30.4]	12.6 [nd-35.6]
∑PBDEs	203 [nd-422]	813 [17.3–1947]	199 [74.3–323]	1184 [nd-2328]	240 [nd-423]
DF PBDEs	93	100	100	95	70
2-MeO-BDE-68	34.6 [nd-88.3]	72.0 [nd-387]	81.0 [nd-81.0]	16.9 [nd-71.3]	6.30 [nd-10.8]
6-MeO-BDE-47	190 [nd-377]	707 [34.2–1855]	359 [nd-359]	459 [nd-1124]	308 [nd-542]
∑MeO-PBDEs	225 [nd-447]	775 [34.2–1966]	440 [nd-440]	475 [nd-1124]	312 [nd-553]
DF MeO-PBDEs	93	100	50	95	70
Dec 602	1.76 [nd-2.83]	9.54 [1.22–21.5]	1.92 [nq-1.92]	10.9 [nd-29.6]	2.33 [nd-2.98]
Dec 603	1.16 [nd-5.67]	7.70 [0.84–22.5]	0.09 [nq-0.09]	2.22 [nd-8.67]	2.11 [nd-4.58]
syn-DP	2.74 [nd-14.2]	2.79 [nd-12.6]	7.60 [0.31–14.9]	2.48 [nd-8.1]	1.50 [nd-4.23]
anti-DP	1.83 [nd-12.9]	2.15 [nd-10.1]	5.94 [0.37–11.6]	2.10 [nd-10.6]	1.18 [nd-2.93]
f_{anti}	0.36 [0.11–0.54]	0.49 [0.16–0.72]	0.47 [0.44–0.49]	0.48 [0.02–0.87]	0.49 [0.33–0.71]
∑DP	4.57 [nd-27.1]	4.95 [nd-22.7]	13.5 [0.61–26.5]	4.58 [nd-18.6]	2.67 [nd-7.17]
∑HNs	6.87 [nd-29.4]	21.7 [4.99–58.7]	14.5 [2.61–26.5]	14.9 [nd-34.6]	6.21 [nd-12.5]
DF HNs	93	100	100	95	70
α-HBCD	30.2 [nd-70.2]	50.9 [nd-109]	107 [nd-107]	300 [nd-641]	144 [nd-180]
DF HBCD	64	45	50	70	20

PBDEs: Polybromodiphenyl ethers. MeO-PBDEs: Methoxylated Polybromodiphenyl ethers. HNs: Halogenated norbornenes. HBCD: Hexabromociclododecane. f_{anti}: Ratio of the concentration of *anti*-DP and ∑DP. DF: Frequency of detection. nd: below limit of detection. nq: below limit of quantification.

observed, but variation was also substantial within the same species. ∑PBDE mean values were similar for common dolphins and long-finned pilot whales (mean [range], 203 [nd (below limit of detection)-422] and 240 [nd-423] ng/g lw, respectively); conversely bottlenose dolphins presented higher values (994 [nd-2328] ng/g lw). No information was available on the age, sex or body condition of the individuals, so intra-species variation can be considered normal since individuals with different characteristics might be pooled together. This fact implies that comparisons between species have to be made with caution since age and sex are two important factors that can affect the concentrations.

In order to investigate possible differences between areas, results from the same species were compared from both locations. ∑PBDE values of bottlenose dolphins from the Gulf of Cádiz (813 [17.3–1947] ng/g lw) were similar to those of individuals from the Strait of Gibraltar (1184 [nd-2328] ng/g lw; $t = 1.952$, d.f = 38, $p > 0.05$). Regarding common dolphins, ∑PBDE values were similar in the Gulf of Cadiz (203 [nd-422] ng/g lw) and in the Strait of Gibraltar (199 [74.3–323] ng/g lw; $t = 0.049$, d.f = 14, $p > 0.1$). On the other hand, bottlenose dolphin presented higher levels than common dolphin in the Gulf of Cádiz ($t = 4.405$, d.f = 32, $p < 0.001$) and also in the Strait of Gibraltar, but in this case differences were not significant ($t = 1.984$, d.f = 20, $p = 0.06$). However, the number of common dolphin samples from the Strait of Gibraltar (n = 2) limits the interpretation of these results.

Recently, Alonso et al. (2014) reviewed PBDE levels in cetacean blubber worldwide and reported mean levels of PBDEs in common dolphins from the NE Atlantic ranging from 422 to 758 ng/g lw, levels higher than the obtained in the present study (203 ng/g lw). Furthermore, PBDE levels in bottlenose dolphins in the current study (994 ng/g lw) are in the low range of the levels found in the North West and East Atlantic, with mean values ranging from 30 to 7055 ng/g lw and from 7040 to 8800 ng/g lw, respectively, but they are higher than mean values found for individuals of the South West Pacific, with values ranging from 80 to 209 ng/g lw. Similarly, mean values in pilot whales (240 ng/g lw) were higher than those reported in the SW Pacific (6–32 ng/g lw) but in the low range of the levels reported in NE Atlantic (from 51 to 3038 ng/g lw). In addition, PBDE levels of striped dolphin from the Strait of Gibraltar ranged from 100 to 172 ng/g lw (Fossi et al., 2013b) being lower than the values of the rest of species analysed in this area. Most values were below the upper limit of threshold level (1500 ng/g lw) associated with thyroid endocrine disruption in juvenile grey seals (Hall et al., 2003). However, for the most contaminated species, bottlenose dolphins, 6 individuals from the Strait of Gibraltar and 1 from the Gulf of Cadiz presented values higher than the threshold, potentially putting them at risk for hyperthyroidism and associated thyrotoxicosis. Different species can show different sensitivities to the same chemical, but unfortunately no data is available for dolphins.

3.3. MeO-PBDE levels

MeO-PBDEs were also detected in all the species and in both locations studied. Only two MeO-BDEs were detected, 2'-MeO-BDE-68 and 6-MeO-BDE-47, both tetra-brominated congeners (Table 2). These two compounds are the two main MeO-PBDEs normally found in marine mammals and in fact many studies only focus in these two compounds, as discussed by Alonso et al. (2014). 6-MeO-BDE-47 contributed between the 60% and 100% to the ∑MeO-PBDEs value. Similarly to PBDEs data, variation of ∑MeO-PBDE levels within the same species was also substantial; moreover, ∑MeO-PBDEs were also similar for common dolphins and pilot whales (240 [nd-447] and 312 [nd-553] ng/g lw, respectively), and bottlenose dolphin was the most contaminated species (628 [nd-1966] ng/g lw).

Regarding the variation between sampling sites, MeO-PBDEs burden for bottlenose dolphins was significantly higher in the Gulf of Cádiz (775 [34.2–1966] ng/g lw) than in the Strait of Gibraltar (475 [nd-1124] ng/g lw; $t = 2.257$, d.f = 38, $p = 0.03$). A different behaviour was observed in common dolphin, where mean ∑MeO-PBDEs values were similar in both areas; 225 [nd-447] ng/g

lw in the Gulf of Cádiz and 440 [nd-440] ng/g lw in the Strait of Gibraltar ($t = 1.861$, d.f = 13, $p = 0.08$). Again, it is difficult to come to conclusions for common dolphins, but the case of bottlenose dolphins, where more samples were available, suggests that individuals from the Gulf of Cádiz are more exposed to the naturally-produced MeO-PBDEs.

∑PBDEs and ∑MeO-PBDEs levels were similar in common dolphin and in pilot whale samples ($t = 0.848$, d.f = 28, $p > 0.1$ and $t = 0.835$, d.f = 13, $p > 0.1$ for common dolphin and pilot whale, respectively). Regarding bottlenose dolphins, individuals from the Gulf of Cádiz showed no differences between anthropogenic and natural compounds ($t = 0.242$, d.f = 39, $p > 0.1$). However we did find differences for bottlenose dolphins of the Strait of Gibraltar, where ∑PBDE levels were significantly higher than ∑MeO-PBDEs ($t = 4.178$, d.f = 37, $p < 0.001$) (Fig. 2).

Contrary to the numerous PBDEs studies, only a few studies from the South West Pacific reported levels of MeO-PBDEs for the species included in the present study (Melcher et al., 2005; Vetter et al., 2002). Levels in the Pacific Ocean were much higher than our findings in southern European waters, with mean values ranging from 1960 to 5435 ng/g lw in common dolphins (240 ng/g lw in our study), from 2095 to 13,145 ng/g lw in bottlenose dolphins (628 ng/g lw in our study) and from 144 to 856 ng/g lw in pilot whales (312 ng/g lw in our study). These differences could be attributed to the presence of the Great Barrier Reef, which supports many species of algae and sponges which might produce these compounds (Vetter et al., 2002). To our knowledge, our study represents the first report on MeO-PBDE levels in cetaceans from southern European waters.

3.4. Halogenated norbornene levels

Dec 602, Dec 603 and both isomers of DP were detected in all the species, while Dec 604 was not detected in any sample (Table 2). ∑HNs in common dolphin were 7.89 (nd-29.4) ng/g lw; for bottlenose dolphins values were 18.4 (nd-58.7) ng/g lw; and for pilot whale values were 6.25 (nd-12.5) ng/g lw. DP and Dec 602 were the most abundant compounds in common and bottlenose dolphins from the Gulf of Cadiz contributing, when detected, between 17% and 92% and between 23 and 94% of the total ∑HN value, while Dec 603 was detected in lower amounts. Similar to PBDEs and MeO-PBDEs, bottlenose dolphins presented the highest levels of the three species. No significant difference was found between bottlenose individuals from the Gulf of Cadiz and Strait of Gibraltar ($t = 1.648$, d.f = 38, $p > 0.1$). In addition, ∑HNs in common dolphins (6.87 [nd-29.4] ng/g lw) was much lower than in bottlenose dolphins (21.7 [4.99–58.7] ng/g lw) in the Gulf of Cádiz ($t = 3.291$, d.f = 32, $p = 0.002$) while this difference was not significant in the Strait ($t = 0.047$, d.f = 20, $p > 0.1$) where ∑HNs was 14.5 (2.61–26.5) ng/g lw and 14.9 (nd-34.6) ng/g lw in common and bottlenose dolphins, respectively.

The different behaviour of the *syn*- and *anti*-DP isomers was evaluated by comparing the f_{anti} values (concentration of *anti*-DP divided by the total DP concentration, lipid normalized) for the three species. While f_{anti} is around 0.65–0.75 in commercial mixture, several studies have proved that this value can change in the environment either due to a higher bioaccumulation capacity of the *syn*-isomer, or because the *anti*-isomer can be degraded easier (Sverko et al., 2011; Xian et al., 2011). Values of f_{anti} in common dolphin were lower than in bottlenose dolphin ($t = 2.340$, d.f = 48, $p < 0.05$) but similar to values in pilot whale ($t = 1.979$, d.f = 20, $p > 0.05$). Furthermore, no difference was observed between bottlenose dolphins and pilot whales ($t = 0.079$, d.f = 41, $p > 0.1$). Moreover, values seemed to be lower than in the commercial mixture. Values were 0.37 [0.11–0.54] for common dolphins, 0.49 [0.02–0.87] for bottlenose dolphins, and 0.49 [0.33–0.71] for pilot whale. This fact has been previously reported in biota samples such as fish (Sverko et al., 2011), but to our knowledge this is the first time it is observed in dolphins. As further work, it would be interesting to study the different toxic properties of both isomers of DP in cetaceans.

Studies reporting levels of halogenated norbornenes in cetaceans are still very scarce. In fact, only two other studies have been published to date (De La Torre et al., 2012a; Zhu et al., 2014). Both of them include DP but only one includes the other norbornenes as well. Zhu et al. (2014) reported concentrations of ∑DP ranging from 1.74 to 63.7 ng/g lw in blubber of Indo-pacific humpback dolphin (*Sousa chinensis*), a range slightly higher than our values for three delphinids. Moreover, De la Torre et al. (2012a) reported levels up to 0.38 [nd-0.98] ng/g lw of Dec 602, 0.75 [0.25–1.99] ng/g lw of Dec 603 and 1.53 [nd-6.26)] ng/g lw of DP in liver of Franciscana dolphin (*Pontoporia blainvillei*) from the south eastern coast

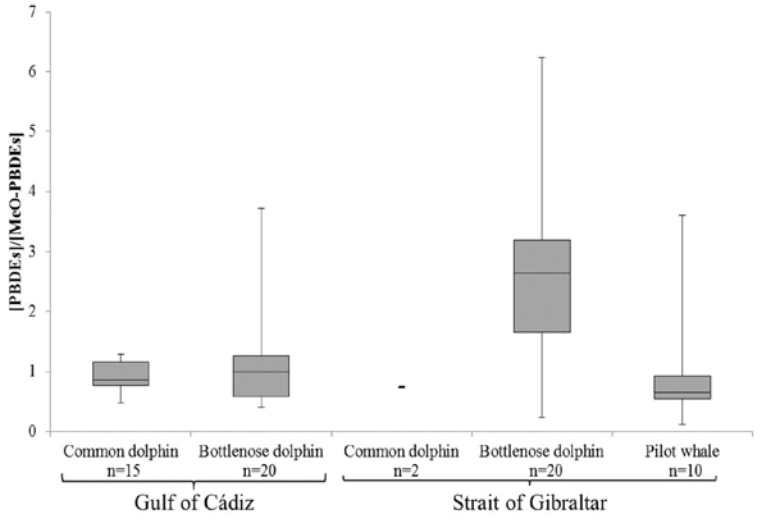

Fig. 2. Box plot of the ratio between ∑PBDE and ∑MeO-PBDE levels in the Gulf of Cádiz (2 species) and the Strait of Gibraltar (3 species).

of Brazil. ∑DP value is in the low-range of what we found in southern European waters, while levels for Dec 602 and Dec 603 are lower. However, since different species and matrices are studied, it is difficult to make any conclusion in this case.

3.5. HBCD levels

HBCD was detected in all the species, with detection frequencies ranging from 20% to 70%. Only α-HBCD was detected, which is in agreement with other studies that show the specific accumulation of this isomer in biota while its presence in the commercial mixture is only 10% (Covaci et al., 2006). HBCD levels in common dolphins were 37.8 [nd-107] ng/g lw; 202 [nd-641] ng/g lw for bottlenose dolphins; and 144 [nd-180] ng/g lw in pilot whales. Similarly to the other anthropogenic contaminants, bottlenose dolphin was the most contaminated species. Levels in the Strait of Gibraltar were significantly higher than in the Gulf of Cádiz: values for common dolphin were 30.2 [nd-70.2] ng/g lw in the Gulf of Cádiz and 107 [nd to 107] ng/g lw in the Strait of Gibraltar ($t = 3.348$, d.f = 9, $p = 0.01$); regarding bottlenose dolphins, mean values were 50.9 [nd-109] ng/g lw in the Gulf of Cádiz and 300 [nd-641] ng/g lw in the Strait of Gibraltar ($t = 4.202$, d.f = 22, $p < 0.001$). HCBD is the only anthropogenic contaminant to be higher in the Strait of Gibraltar than in the Gulf of Cádiz, and for both dolphin species.

HBCD in cetaceans have been studied mostly in the North Sea, China Sea and North West Atlantic (Covaci et al., 2006). To our knowledge, this is the first report of HBCD in cetaceans from southern European waters. Levels of HBCD in common dolphins from the north coast of Spain were up to 180 ng/g lw (mean), while in England and Ireland mean levels were up to 420 ng/g lw and 1200 ng/g lw, respectively (Law et al., 2012). These levels were all higher than the ones reported here.

3.6. Classical vs alternative FRs

Despite the fact that Penta- and Octa-BDE mixtures have been banned since 2006 and the production of Deca-BDE mixture should have ended at the end of 2013 (De la Torre et al., 2012b), many studies prove that they are still present in the environment. Due to its great stability and persistence, it is reasonable to assume that their levels in the environment, especially in top predators (*i.e.* dolphins) that have accumulated these contaminants for many years, will not decrease immediately (Law et al., 2014). On the other hand, levels of alternative FRs are expected to increase in the near future, since some of them have been proposed as alternatives to PBDEs and its use in different materials will probably increase. Interestingly, ∑Classic FRs (PBDE and HBCD) and ∑HNs ratio was similar in the Gulf of Cádiz than in the Strait of Gibraltar. In particular, mean ratio for common dolphin was 27 in the Gulf of Cádiz and 23 in the Strait, and was not significantly different ($t = 1.311$, d.f = 14, $p > 0.05$). Similarly, in bottlenose dolphins mean values were 40 and 70 in the Gulf of Cádiz and the Strait of Gibraltar, respectively ($t = 1.929$, d.f = 38, $p = 0.06$). On the other hand, classical FRs presented higher concentrations than alternative FRs, and ∑PBDE concentrations were higher than HBCD concentrations (Fig. 3).

3.7. Biomagnification studies

PBDEs and MeO-PBDEs proved to have biomagnification capacity (Fig. 4). PBDEs showed a significant positive correlation with trophic level in common and bottlenose dolphins from the Gulf of Cádiz ($R^2 = 0.5437$, $p < 0.001$), in the same way as MeO-PBDEs ($R^2 = 0.4602$, $p < 0.001$). This is in agreement with Losada et al. (2009) reporting the same behaviour for PBDEs and MeO-PBDEs

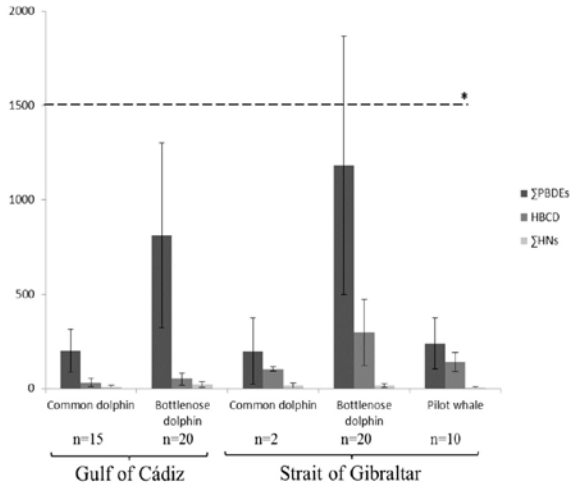

Fig. 3. Comparison between ∑PBDEs, HBCD and ∑Alternative FRs for the different species in the Gulf of Cádiz and the Strait of Gibraltar (means and confidence interval). *: Upper level of threshold that presented health effects attributed to PBDEs in grey seals (Hall et al., 2003).

in a marine food web, and shows the additional danger of these compounds for animals like dolphins which are at the top of their food web. Biomagnification in the Strait of Gibraltar was also evaluated in bottlenose dolphins and pilot whales but, surprisingly, in this case no biomagnification was observed for any group or individual contaminant.

Otherwise, a different behaviour was observed for HNs (Fig. 4). HNs did not show a significant correlation when considered together ($R^2 = 0.1112$, $p > 0.1$). However, while both DP and Dec 603 showed positive but low and not statistically significant correlation ($R^2 = 0.0974$, $p > 0.1$ and $R^2 = 0.0230$, $p > 0.1$ respectively), Dec 602 was significantly correlated positively with trophic level ($R^2 = 0.4778$, $p < 0.001$). Biomagnification data available in the literature for HNs, especially for DP, are ambiguous. In particular, Dec 602 and Dec 603 showed more biomagnification capacity than DP in fish from the Great Lakes and in bird eggs from Spain (Barón et al., 2014b; Sverko et al., 2011). As was suggested in these previous studies and consistent with our data in dolphins, more attention should be paid to Dec 602 and Dec 603 since their bioaccumulation and biomagnification capacities seem to be higher than DP. This also implies that future toxicological studies should focus on Dec 602 and Dec 603 as well, since, to the best of our knowledge, only toxicological properties of DP have been studied.

4. Conclusions

Seven PBDEs, two MeO-PBDEs, four HNs and α-HBCD were detected in three dolphin species from the Gulf of Cádiz and the Strait of Gibraltar. This is the second study reporting PBDEs in cetaceans from southern European waters and the first one reporting MeO-PBDE and halogenated norbornene levels. In terms of examined contaminants, bottlenose dolphins had the highest levels of all three species for all types of anthropogenic and naturally-produced contaminants, while common dolphins and long-finned pilot whales had similar lower values. No difference was found between the two areas overall. Our results show that the levels of the classical FRs (PBDEs and HBCD) are higher than levels of alternative FRs (HNs). Although most individuals were below the upper limit of threshold level (1500 ng/g lw) associated with endocrine disruption in grey seals (Hall et al., 2003), a total of 6 bottlenose dolphins

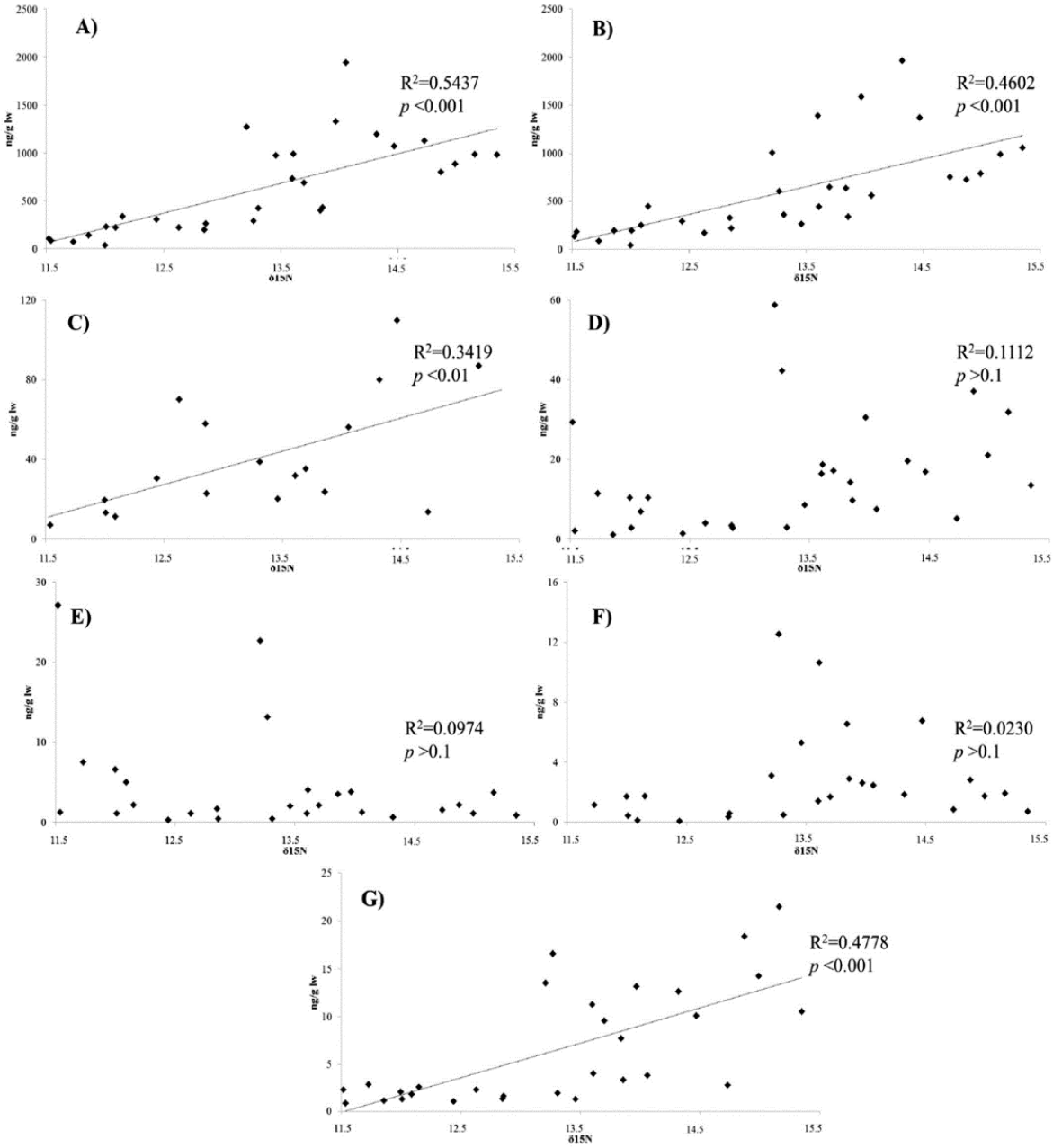

Fig. 4. Correlations between concentrations of several contaminant compounds and $\delta^{15}N$ in common and bottlenose dolphins from the Gulf of Cadiz. A): PBDEs. B): MeO-PBDEs. C): HBCD. D): HNs. E): DP. F): Dec 603. G) Dec 602.

of the Strait of Gibraltar and 1 of the Gulf of Cádiz could present possible health effects but unfortunately no data are available in dolphins, which could show different sensitivities to the same chemicals. Moreover, the contribution of halogenated natural compounds (MeO-PBDEs) was generally similar to the contribution of PBDEs, which confirms the importance of these compounds in the marine environment. With these results, baseline concentrations and patterns are now available to carry out future risk assessment studies and to monitor changes in anthropogenic impacts over time in the area.

Furthermore, we report correlational evidence of the biomagnification capacity of several compounds (PBDEs, MeO-BDEs and some HNs). Although Dec 602 is rarely included in the studies of halogenated norbornenes, here it showed high bioaccumulation and biomagnification capacity and should therefore be included in future contaminant studies.

Considering that dolphins are highly exposed to organic contaminants since they accumulate them during many years, and the relative high levels found here, toxicological studies of the effects of these compounds in cetaceans should be conducted in order to

assess the real danger these animals are subjected to. As sentinels of the marine environment, a monitoring of flame retardants and halogenated natural product concentrations in cetacean should be implemented and considered under the European Marine Strategy Framework Directive (Directive 2008/56/EC) to achieve a Good Environmental Status and a Favourable Conservation Status under EC Habitat Directive (Council Directive 92/43/EEC). The high levels found in three delphinids species strongly support that actions aiming at reducing the discharge of these compounds into the marine environment should be undertaken urgently to limit their bioavailability in marine food webs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.03.041.

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Supporting information

Chemicals

α-, β- and γ-HBCD and its deuterated congeners, together with *syn*- and *anti*- isomers of DP and ^{13}C-syn-DP were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Both native and mass-labelled PBDE mixtures, containing 8 PBDE congeners (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209, and the ^{13}C-labelled, respectively), MeO-PBDEs mixture (containing 5-MeO-BDE-47, 6-MeO-BDE-47, 4'-MeO-BDE-49, 2'-MeO-BDE-68, 5'-MeO-BDE-99, 5'-MeO-BDE-100, 4'-MeO-BDe-101 and 4'-MeO-BDE-103) as well as HBB, PBEB and DBDPE were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). ^{13}C-PBDEs, d_{18}-HBCD (α, β and γ) and ^{13}C-syn-DP were used as internal standards. Al-N cartridges were provided by Biotage.

Sample treatment

All the sample available (about 0.1-0.2 g) was spiked with 5 ng of ^{13}C-PBDEs and ^{13}C-*syn*-DP, 50 ng of ^{13}C-BDE-209 and 5 ng of d_{18}-HBCD (α-, β-, γ-). Samples were kept overnight to equilibrate prior to the pressurized liquid extraction (PLE). Samples were mixed with diatomeus earth and loaded into an 11 mL extraction cell. A mixture of hexane:dichloromethane (1:1) was used for the extraction, which consisted in 2 static cycles of 10 min at 100ºC and working at 1500 psi. Lipid content was determined gravimetrically after the extraction. Lipid content values ranged from 48 to 76% and no significant differences were found among species. Afterwards, organic content was re-dissolved in hexane and treated with H2SO4 (conc.) followed by a solid phase extraction (SPE) using alumina cartridges (Al-N, 5 g). Extracts were evaporated to incipient dryness and reconstituted to a final volume of 40 μL prior to the instrumental analysis.

Instrumental analysis

- Instrumental analysis of PBDEs, MeO-PBDEs and emerging FRs was carried out by gas chromatography coupled to tandem mass spectrometry (GC-MS-MS) using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole. PBDEs,

MeO-PBDEs, HBB, PBEB and DBDPE were analysed using electron ionization mode (EI) following the protocol optimized in (Barón *et al.,* 2014a). Temperature program started at 140ºC, held for 1 min, then ramped to 310ºC at 10 ºC/min and held for 10 min, for a total run time of 36.5 min. Source temperature was set at 250ºC.

- Halogenated norbornenes were analysed by negative chemical ionization mode (NCI) using CH_4^+ as reagent gas (Barón *et al.,* 2012). Temperature program started at 80ºC, held for 2 min and then ramped to 300ºC in 10ºC/min. Final temperature was maintained for 10 min for a total runtime of 34 min. Source temperature was set at 175ºC and electron energy and emission current were set at 200 and 150 eV, respectively.

- HBCD was analysed by liquid chromatograph coupled to tandem mass spectrometry (LC-MS-MS) using an Agilent HP 1100 binary pump LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap 4000QTRAP (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray (ESI) Turbospray interface, as described in (Guerra *et al.,* 2008). The elution program started at an initial composition of 100% A (H_2O:MeOH 3:1 v/v) and was ramped to 10% A in 17 min. Solvent B was MeOH. The initial conditions were reached again in 3 min and maintained for an additional 10 min for a total run time of 30 min. Source temperature was set at 350ºC.

- Selective reaction monitoring (SRM) mode was used to enhance sensitivity and selectivity in the aforementioned methodologies, monitoring two different transitions for each compound. The most intense was used for quantification and the second one for confirmation.

Publicación científica #7

Halogenated natural products in dolphins: Brain-blubber distribution and comparison with halogenated flame retardants

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Halogenated Natural Products in Dolphins: Brain−Blubber Distribution and Comparison with Halogenated Flame Retardants

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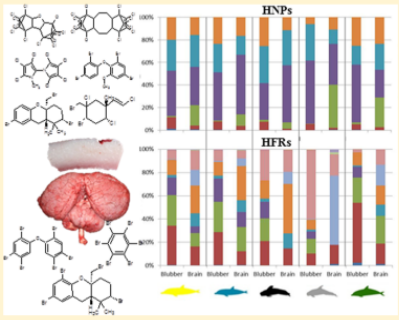
Supporting Information

ABSTRACT: Halogenated natural products (MHC-1, TriBHD, TetraBHD, MeO-PBDEs, Q1, and related PMBPs) and halogenated flame retardants (PBDEs, HBB, Dec 602, Dec 603, and DP) in blubber and brain are reported from five Alboran Sea delphinids (Spain). Both HNPs and HFRs were detected in brain, implying that they are able to surpass the blood−brain barrier and reach the brain, which represents a new finding for some compounds, such as Q1 and PMBPs, MHC-1, TriBHD, TetraBHD, or Dec 603. Moreover, some compounds (TetraBHD, BDE-153, or HBB) presented higher levels in brain than in blubber. This study evidence the high concentrations of HNPs in the marine environment, especially in top predators. It shows the importance of further monitoring these natural compounds and evaluating their potential toxicity, when most studies focus on anthropogenic compounds only. While no bioaccumulation was found for ∑HNPs, ∑HFRs increased significantly with body size for both common and striped dolphins. Studies evaluating BBB permeation mechanisms of these compounds together with their potential neurotoxic effects in dolphins are recommended.



INTRODUCTION

Halogenated flame retardants (HFRs) have been used for decades in order to reduce the flammability of a wide range of materials such as textiles, plastics, wood, or electronic furniture.[1] One of the most widely used HFRs were polybrominated diphenyl ethers (PBDEs) but, due to their proven bioaccumulation, persistence, wide-range transport, and toxicity, technical Penta- and Octa-BDE mixtures were banned in 2006, while the production of Deca-BDE was supposed to cease at the end of 2013 in North America and Europe.[2−6] PBDEs have been found in several environmental and biological samples from all over the world, such as sediment,[7] sludge,[8] air,[9] fish,[10] birds,[11] cetaceans,[12] and even humans.[13] Presently, in response to the legal restrictions on them, other brominated FRs have appeared as an alternative,[4] e.g., decabromodiphenyl ethane (DBDPE), pentabromoethylbenzene (PBEB), hexabromobenzene (HBB), or halogenated norbornene derivatives (HNs), which are a family of chlorinated FRs used for many years but not detected in the environment until 2006.[14] These are dechlorane 602 (Dec 602), dechlorane 603 (Dec 603), dechlorane 604 (Dec 604), and dechlorane plus (DP).[15] Since their first determination in 2006, HNs have been found in environmental matrices such as sediment,[16] water,[17] or air,[18] and also in biological matrices such as fish,[10] bird eggs,[19] dolphins,[20] or human blood.[21] Reported levels show that HNs have bioaccumulation capacity and might biomagnify among the trophic webs.[22,23]

Moreover, over 5000 halogenated natural products (HNPs) with different structures and thus belonging to different families have been described through the years.[24,25] It is believed that some microorganisms associated with sponges and algae are the main producers of these compounds and responsible for their presence in the environment.[26] To date, these compounds are

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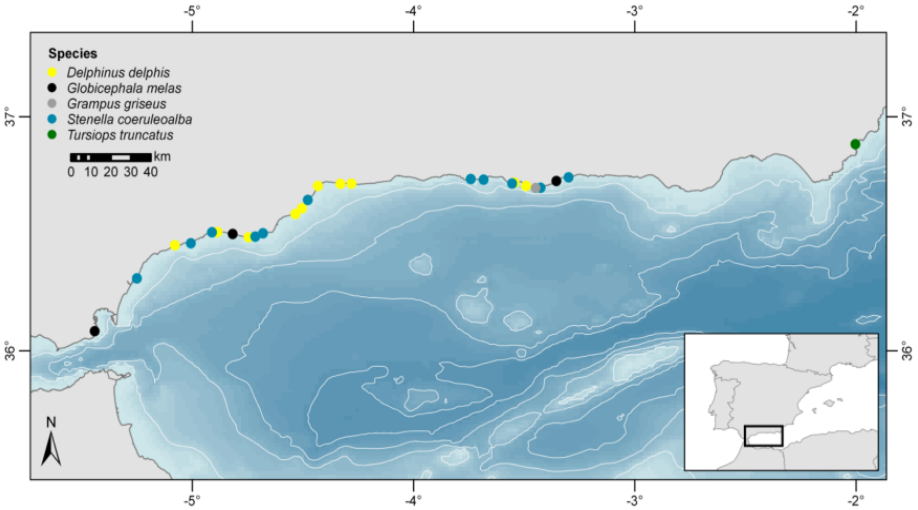

Figure 1. Location of the stranded individuals along the coastline. *D. delphis* (n = 10), *G. melas* (n = 3), *G. griseus* (n = 1), *S. coureloalba* (n = 11), and *T. truncatus* (n = 1).

mostly considered a marine problem, since only few compounds have been found scarcely in terrestrial species.[27] By contrast, several HNPs have been detected in marine species such as cetaceans,[28−30] fish,[31] birds,[32] and mollusks.[33] This proves that these compounds have bioaccumulation capacity, and should be included in the studies of bioaccumulation behaviors of other halogenated compounds that occur in the environment. Some examples of these HNPs are (1R,2S,4R,5R,1′E)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2′-chloroethenyl)-5-methylcyclohexane (MHC-1), 2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthene (TriBHD), 2,5,7-tribromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthene (TetraBHD), methoxylated PBDEs (MeO-BDEs), 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (Q1), and other polyhalogenated 1′-methyl-1,2′-bipyrroles related with Q1 but with different halogenation pattern which include substitution of chlorine by bromine (PMBPs). These compounds are $BrCl_6$-MBP, Br_2Cl_5-MBP, and so on, until Br_7-MBP. It is believed that the presence of HNPs in the marine environments is not due to a wide-range transport, but that they are produced worldwide by different marine organisms.[34] In addition, uptake by complex organisms, such as cetaceans, is believed to occur by diet rather than a direct uptake from water. However, the number of studies is still limited, and they often include a small number of samples. More information regarding the occurrence and behavior of these compounds is needed, especially considering that some congeners such as Q1 have also been found in human milk.[35]

Tissue distribution has been described as an important parameter to understand the migration and transformation processes of organic pollutants in biota, and also to identify which organs are the most exposed to the possible toxic effects of these compounds.[36,37] Despite the recent increase of the studies focusing on the toxicological properties of these compounds, information in marine mammals is still scarce, especially for the HNPs. Some examples of toxic effects of POPs in marine mammals are the depression of the immune system, which increases the risk of infections, reproductive failure, anemia, and hypothyroidism.[38−41] In particular, PBDEs induced cancer, reproductive and developmental disorders, endocrine disruption and alteration of the nervous system,[42] and caused thyroid hormone disruption in juvenile seals.[43] Furthermore, to date, only toxicological data of MeO-PBDEs are available. They presented multiple endocrine-disrupting effects in three in vitro studies with terrestrial mammals.[44] Further studies are needed regarding toxicity of HNPs in mammals, since it is interesting to assess whether some HNPs might cause similar toxicological effects as HFRs due to their similar structures and halogenation degree, or if they have adapted to them.[45]

The aim of this study was to evaluate the presence of several HNPs together with classical and alternative HFRs in blubber and brain from five dolphin species from Southern European waters. Moreover, for the first time, HNPs will be studied in brain samples of different dolphin species in order to study the blubber−brain distribution of these compounds.

MATERIALS AND METHODS

Sample collection. Blubber and brain samples of a total of 26 stranded dolphins were collected on the Alboran Sea coastline between 2004 and 2011 (Figure 1). The Alboran Sea connects the Mediterranean Sea with the Atlantic Ocean and provides an important corridor for migratory species.[46] In fact, it presents one of the highest densities of cetacean populations in the Mediterranean Sea.[47−49]

Five different species were sampled: 10 individuals (3 males, 7 females) of common dolphin (*Delphinus delphis*) with body lengths between 945 and 2000 mm, 11 individuals (8 males, 3 females) of striped dolphin (*Stenella coureloalba*) with body lengths between 980 and 2160 mm, 3 individuals (1 male, 2 females) of long-finned pilot whale (*Globicephala melas*) with body lengths between 3950 and 4700 mm, 1 individual of Risso's dolphin (*Grampus griseus*) of 2070 mm and 1 male of bottlenose dolphin (*Tursiops truncatus*) of 2070 mm. Information about the distribution of these species in the area can be found elsewhere.[47,48]

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Table 1. Mean [range] Values, Expressed in ng/g lw, of HNPs and HFRs in Blubber and Brain Tissues of the 5 Dolphin Species[a]

contaminants	common dolphin		striped dolphin		pilot whale		risso's dolphin[b]		bottlenose dolphin[b]	
	blubber	brain	blubber	brain	blubber	brain	blubber	brain	blubber	brain
MHC-1	45.0 [6.45−130]	3.30 [0.60−14.5]	15.0 [5.80−34.0]	1.60 [0.30−7.35]	4.70 [1.10−11.0]	0.65 [nd-0.65]	4.10	0.50	10.0	1.00
∑PBHDs	390 [nd-1620]	85.0 [nd-130]	270 [nd-1010]	120 [nd-430]	120 [32.5−240]	28.0 [nd-28.0]	370	50	180	110
∑PMBPs	2350 [nd-6120]	250 [nd-990]	2480 [nd-6980]	590 [nd-1740]	920 [580−1550]	330 [140−530]	5640	62.0	1160	190
∑MeO-PBDEs	690 [nd-2510]	63.0 [nd-220]	870 [nd-1660]	140 [nd-420]	360 [100−535]	47.0 [nd-76.0]	400	14.0	940	93.0
∑HNPs	3200 [245−6910]	360 [21.0−1100]	3410 [nd-9660]	725 [nd-2100]	1410 [720−2330]	280 [65.0−635]	6400	130	2290	390
∑PBDEs	1,000 [93.0−2040]	205 [6.90−790]	940 [100−2250]	510 [nd-1,670]	390 [190−490]	100 [35.0−230]	370	4.90	850	330
∑HNs	90.0 [4.50−320]	68.0 [18.5−315]	105 [12.0−380]	53.5 [nd-140]	140 [3.90−360]	38.0 [16.0−69.0]	560	6.30	24.0	64.0
HBB	7.20 [nd-8.10]	53.0 [nd-79.0]	8.20 [nd-8.20]	50.5 [nd-100]	nd	20.0 [11.0−38.0]	4.60	17.0	nd	84.0
∑HFRs	1,090 [150−2160]	320 [87.0−1150]	1,040 [150−2540]	610 [33.0−1860]	540 [195−850]	160 [60−340]	930	28.0	880	475

[a]Nd: not detected. [b]: Only one sample available. Blubber: DP and HBB detected in <25% of the samples. Brain: DP, Br_3Cl_4-MBP, Br_4Cl_3−BMP and Dec 603 detected in <25% of the samples. **PBHDs**: Polybrominated hexahydroxanthene derivatives. **PMBPs**: Polyhalogenated methyl bipyrroles. **MeO-PBDEs**: Methoxylated Polybromodiphenyl ethers. **HNPs**: Halogenated natural products. **PBDEs**: Polybromodiphenyl ethers. **HNs**: Halogenated norbornenes. **HBB**: Hexabromobenzene. **HFRs**: Halogenated flame retardants.

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Sample Treatment. Sample extraction methodology was based on previous works,[8,50] and it is described in detail in the Supporting Information (SI). Briefly, after adding the surrogate standards to 1 g of sample, extraction was performed by pressurized liquid extraction (PLE) followed by acid treatment with $H_2SO_{4(c)}$ and solid phase extraction (SPE) using Al−N cartridges. Lipid content was determined gravimetrically before acid treatment. Resulting extracts were concentrated to a final volume of 40 μL prior to instrumental analysis.

Instrumental Analysis. Instrumental analysis of PBDEs, emerging HFRs (HBB, PBEB, DBDPE, and HNs), and one family of HNPs (MeO-PBDEs) was carried out by gas chromatography coupled to tandem mass spectrometry (GC−MS−MS) using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole, following previously optimized protocols described in detail in the SI.[51,52] PBDEs, emerging HFRs, and MeO-PBDEs were analyzed by GC−MS−MS using electron ionization (EI), whereas HNs were analyzed by GC−MS−MS using negative chemical ionization (NCI).

The other HNPs (MHC-1, TriBHD, TetraBHD, and PBMPs) were analyzed by GC/NCI−MS using an Agilent 7890/5975C system in combination with an HP 7673 automatic injector (Agilent Technologies, Waldbronn, Germany).[53] Detailed information is given in the SI.

QA/QC. Identification and confirmation of all the compounds was based on three criteria: (a) simultaneous responses for the two monitored ions or transitions must be obtained at the same retention time than those of available standards; (b) signal-to-noise ratios (S/N) must be >3; and (c) relative peak intensity ratio must be within ±20% of the theoretical values obtained with standard solutions. In order to identify and correct possible interferences and contaminations, several procedural blanks were made. The contribution of the blank to the signal never exceeded 5% for any compound.

Recoveries were evaluated by spiking 10 ng of each compound in blubber samples. Four spiked replicates and three unspiked replicates were made; sample contamination did not exceed 10% of the spiked amount in any case. The method showed good recoveries for PBDEs (56 to 87%), emerging BFRs (71 to 76%), halogenated norbornenes (68 to 87%), MeO-PBDEs (70 to 77%), and the other HNPs (>70%), (SI Table S1). Reproducibility of the method was also satisfactory with relative standard deviations always below 15%. Method detection limits (MDLs) were determined for each congener, as the minimum amount of analyte which produced a peak with a S/N of 3, and the method quantification limits (MQLs) were determined as the minimum amount of analyte which produced a peak with a S/N of 10. MDLs and MQLs are shown in SI Table S1.

Data Analysis. Lipid normalized concentrations in blubber and brain were tested for normality and homogeneity of variances using the Shapiro−Wilks test of normality and the F test. Data were log-converted when needed and differences between species and/or tissues were tested using a t test considering a p-value ≤0.05 to determine statistical difference. Only common and striped dolphins were considered for the statistical analysis since sample size of pilot whale, Risso's and bottlenose dolphins were too low (3, 1, and 1 individuals, respectively). Statistical analyses were conducted using the open-source statistical programming language R v.3.1.1 (http://cran.r-project.org).

RESULTS AND DISCUSSION

Lipid Content. Lipid content in blubber samples ranged between 39% and 76% (mean 59% ± 22%). This is in agreement with other data in blubber of marine mammals, where lipid content usually ranges from 30% to 90%.[54] However, lipid content in brain samples was lower and more consistent, with values ranging from 10% to 30% (mean 16% ± 8%). No significant differences were found among species. Since all these compounds are lipophilic, all data were lipid normalized for a proper comparison between tissues.

HNPs. Results are summarized in Table 1 and in the SI (full data). Highest concentrations were obtained for PMBPs, being Q1 the most abundant compound, with values (mean [range]) of 1390 [nd-4110] ng/g lw in common; 1560 [nd-3680] ng/g lw in striped; 460 [440−505] ng/g lw in pilot; 3580 ng/g lw in Risso's; and 700 ng/g lw in bottlenose. Common and striped presented similar levels (t = 0.283, d.f = 18, p > 0.05), Risso's was in their high value range, and concentrations found in pilot and bottlenose were in their lower range. Moreover, the contribution of Q1 to the total PMBP burden was similar in all the species: 62% [40−71%] in common; 68% [53−89%] in striped; 59% [33−76%] in pilot; 64% in Risso's; and 61% in bottlenose. A total of 23 HNPs were detected in the blubber of the five delphinids, including MHC-1, both PBHDs, two MeO-PBDEs (6-MeO-BDE-47 and 2′-MeO-BDE-68), Q1 and 17 other PMBPs (5 $BrCl_6$-MBP, 3 Br_2Cl_5-MBP, 3 Br_3Cl_4-MBP, 2 Br_4Cl_3-MBP, 2 Br_5Cl_2-MBP, and 2 Br_6Cl-MBP). These values were slightly lower than the value previously reported in dolphins, up to 75−80%.[53,55] Vetter et al.[53] reported higher values of %Q1 in common and bottlenose (89% and 75%, respectively) in a study where 24 PMBPs were detected in Australian waters. Here, we found lower %Q1 values but also detected less PMBP congeners; comparing contaminant levels between species is challenging since a wide range of factors such as age, sex, or geographic location can affect contamination levels and profiles. However, relative abundance of the PMBP congeners was clearly related to its bromination degree ($BrCl_6$-MBP > Br_2Cl_5-MBP > Br_3Cl_4-MBP > Br_4Cl_3-MBP > Br_5Cl_2-MBP > Br_6Cl-MBP). $BrCl_6$-MBP represented 26−39% of the ∑PMBP burden, while the other PMBPs presented a contribution lower than 5%. This is in agreement with the contributions found by Vetter et al.[53] MHC-1 was the less abundant HNP but, interestingly, concentrations were significantly higher in common (430 [nd-1,750] ng/g lw) than in striped (315 [nd-1,030] ng/g lw) (t = 2.184, d.f = 17, p = 0.05). Regarding PBHDs, TriBHD concentrations were higher than TetraBHD for each species (t = 2.408, d.f = 17, p < 0.05 and t = 2.802, d.f = 17, p < 0.05 for common and striped, respectively).

Some HNPs such as TriBHD and TetraBHD were detected for the first time in blubber and brain samples of marine mammals from the Mediterranean Sea. Comparing with other areas, Q1 was found in bottlenose from Australia at higher concentrations (450−9,100 ng/g lw) than those reported in our study (700 ng/g lw).[56] The Great Barrier Reef hosts a vast amount of species which can produce HNPs, which could explain this high levels of Q1.[34] Q1 was also the main PMBP in bottlenose dolphins from Southern California Bight, proving its ubiquity worldwide. In addition, TriBHD and TetraBHDs were also detected in the same bottlenose dolphins.[29,30] Concerning MeO-PBDEs, mean [range] values were 690 [nd-2510] ng/g lw in common, 870 [nd-1660] ng/g lw in striped, 360 [100−

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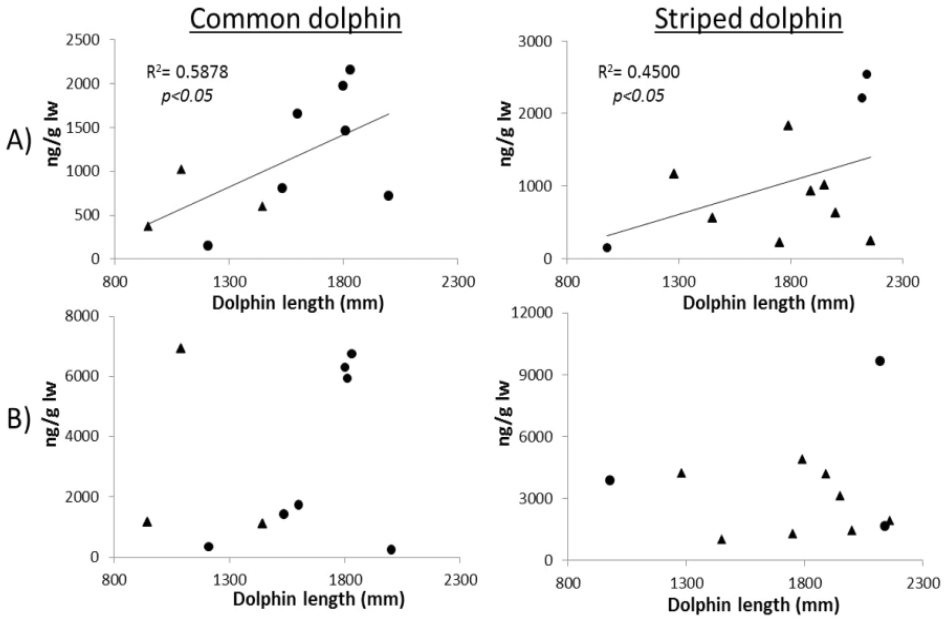

Figure 2. Length of dolphins versus concentrations of HFRs (A) and HNPs (B) in blubber. The lines are linear regression on the data. Circle: Females. Triangle: Males.

535] ng/g lw in pilot, 400 ng/g lw in Risso's, and 940 ng/g lw in bottlenose. Data regarding the five species included in this work are scarce and scattered around the world. Higher levels than the ones reported here were found in bottlenose dolphins from the Pacific Ocean[12] and its contributions were also higher than PBDEs in bottlenose dolphins from Southern California Bight.[29,30] In addition, similar values of MeO-PBDEs were found in blubber biopsies of common (240 ng/g lw), pilot (312 ng/g lw) and bottlenose (628 ng/g lw) from southern European waters.[57] Differences between the levels reported worldwide is affected by the different HNPs producers existing in each area, number of monitored congeners, and also by the different preys available, since dietary uptake is the main bioaccumulation route of these compounds. For example, MHC-1, TriBHD, and TetraBHD have been found in several fish species[26] as well as MeO-PBDEs.[58,59]

Additionally, 18 HNPs were detected in brain samples of the five species: MHC-1, both PBHDs, two MeO-PBDEs (6-MeO-BDE-47, and 2-MeO-BDE-68), Q1 and 13 other PMBPs (5 $BrCl_6$-MBPs, 3 Br_2Cl_5-MBPs, 2 Br_3Cl_4-MBPs, 2 Br_4Cl_3-MBPs, and 1 Br_5Cl_2-MBP). Similarly to blubber, Q1 was the most abundant compound in all species, with values of 135 [nd-410] ng/g lw in common; 460 [nd-1,300] ng/g lw in striped; 210 [71.5−340] ng/g lw in pilot; 46.0 ng/g lw in Risso's; and 96.0 ng/g lw in bottlenose. In this case, striped presented higher values than common (t = 2.176, d.f = 17, p = 0.05). The percentage contribution of Q1 to the total PMBP burden was slightly higher than in blubber: 63% [42−70%] in common; 77% [68−100%] in striped; 58% [52−65%] in pilot; 74% in Risso's and 52% in bottlenose. Again, $BrCl_6$-MBPs were the second most abundant PMBPs after Q1 and the abundance of the different congeners of PMBPs was also related to its halogenation pattern, similarly to blubber. MHC-1 presented low concentrations, but in this case no interspecies difference was observed.

Correlation with the length of the individual has been described as a good tool to evaluate the bioaccumulation potential of organic contaminants when other trophic levels cannot be sampled, since length can be related to age.[60] Figure 2 shows the measured concentrations of HNPs in blubber as a function of dolphin length. HNPs did not show a significant correlation neither in common or striped (R^2 = 0.04, p > 0.05; R^2 = −0.01, p > 0.05, respectively). This could suggest that the bioaccumulation capacity of HNPs over time is not high, probably due to metabolism, but they are continuously incorporated in high amounts by the dolphins, so their high burdens are maintained.[61] The offloading of contaminants from adult females to their offspring is a well-known phenomenon.[12] However, it was not observed in this study, which can be attributed to the low sample size or to the fact that most females were juvenile animals.[62−64] The relationship between length and age in odontocetes is normally explained by the Gompertz curves.[65] In this study, we have used length as a proxy of age, although it is true that when dolphins reach adulthood, the length stabilizes.[66] However, on the basis of the length ranges of common (945−2000 mm) and striped dolphins (980−2160 mm), and considering that in striped from the Mediterranean Sea, the asymptote was approached at a body length of 2000 mm in males and 1940 mm in females[64] (no data for common in this area are available), length was considered an adequate proxy to age.

Halogenated Flame Retardants. Several classical and alternative HFRs were detected in blubber of the five species including six PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154), Dec 602, Dec 603, and both isomers of DP and HBB. However, HBB was only detected in four samples (Table 1 and SI). As expected, BDE-47 was the most abundant PBDE and also the major contributor to the total HFR burden. BDE-47 concentrations were up to 370 [20−890] ng/g lw for common; 300 [30−725] ng/g lw for striped; 110

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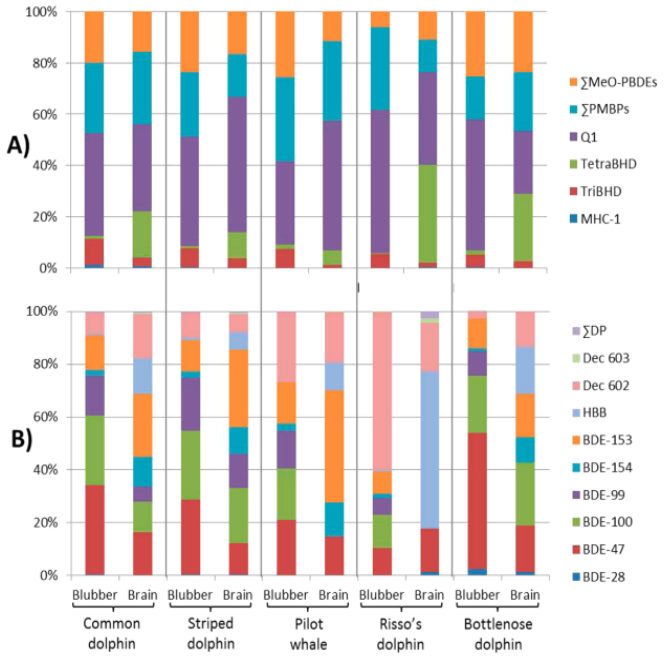

Figure 3. Contributions of the different contaminants to the total HNPs (A) or HFRs (B) burdens.

[75−160] ng/g lw for pilot; 90 ng/g lw for Risso's; and 450 ng/g lw for bottlenose. No significant differences were observed between common and striped neither for BDE-47 or ∑PBDEs (t = 0.6123, d.f = 20, p > 0.05; t = 0.2130, d.f = 20, p > 0.05, respectively). Dec 602 was the most abundant alternative HFR, with levels of 88.0 [4.50−320] ng/g lw in common, 100 [11.0−370] ng/g lw in striped; 140 [3.85−355] ng/g lw for pilot; 560 ng/g lw in Risso's; and 24.0 ng/g lw in bottlenose. Similar to PBDEs, no differences were found between common and striped (t = 0.335, d.f = 20, p > 0.05 for HNs and t = 0.308, d.f = 20, p > 0.05 for Dec 602). In this case, Risso's presented the highest HNs burden, but the fact that it is only one individual might mislead the interpretations. It has been documented that some species have difficulties to metabolize organochlorinated contaminants, but more individuals of Risso's would be needed to properly confirm this fact.[62]

Contribution of the *anti*-DP to the total DP was evaluated by the f_{anti} ratio ([*anti*-DP]/ [∑DP]). In the different commercial mixtures of DP, f_{anti} is around 0.7.[67] While this value is normally maintained in sediments or air, it normally decreases in biota, indicating that *syn*-DP might have more bioaccumulation capacity or that *anti*-DP is more likely to be degraded or metabolized.[15] The f_{anti} was calculated when both isomers were detected, and no interspecies difference was observed. Values ranged between 0.50 and 0.65, lower than in the commercial mixture. Unfortunately, these results have to be interpreted with caution since the detection frequency of DP was considerably low. Even if nearly all values were below the upper limit of threshold level associated with thyroid endocrine disruption, set up at 1500 ng/g lw in juvenile gray seals,[43] three individuals of common and three individuals of striped presented higher values in blubber. Thus, these individuals presented a higher risk of suffering hyperthyroidism and associated thyrotoxicosis, although histopathological analysis was not available to confirm it.

HFRs in blubber showed a significant positive correlation with the length of the individual both in common and striped (Figure 2, R^2 = 0.5878, p < 0.05 and R^2 = 0.4500, p < 0.05, respectively). This could be due to the fact that HFRs are accumulated by the dolphins over the years rather than in an acute exposure.

To date, there is only one published study reporting PBDE levels in this area.[68] Concentrations in striped dolphin blubber from the Strait of Gibraltar ranged from 100 to 172 ng/g lw[68] being lower than the range found in this study (100−2250 ng/g lw). Since the samples analyzed by Fossi et al. (2013) were obtained by biopsy sampling, results can be biased since no information regarding the size or sex of the individuals was available. Concerning the other four species, no other published levels from this area are available. Mean values in this study were 1000, 390, and 850 ng/g lw for common, pilot, and bottlenose, respectively, whereas mean PBDE concentrations in blubber biopsies of the same species from the Strait of Gibraltar and Gulf of Cádiz were 199, 240, and 1180 ng/g lw, respectively.[57] Worldwide, mean levels reported in common blubber (422−758 ng/g lw in individuals from NE Atlantic) are lower[12] but in the same order than mean levels found in this study. On the contrary, our mean concentration in blubber of pilot was in the low range of the mean levels reported in individuals from the NE Atlantic (51−3040 ng/g lw).[12] Our value in Risso's (370 ng/g lw) was lower than the mean value reported in individuals of NE Atlantic, up to 850 ng/g lw; while our value in bottlenose was in the same range of the mean values reported also in NE Atlantic (30−7060 ng/g lw).

Concerning brain samples, the six PBDEs detected in blubber, together with Dec 602, Dec 603, *syn*- and *anti*-DP and HBB were detected (Table 1 and SI), but in this case DP

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was only detected in five samples. The most abundant compounds were again BDE-47 of the classical HFRs and Dec 602 of the alternative HFRs. Values of BDE-47 and Dec 602 were respectively 65.0 [6.70−175] ng/g lw and 67.0 [17.0−310] ng/g lw for common; 90.0 [10.0−220] ng/g lw and 52.0 [14.0−140] ng/g lw for striped; 29.0 [9.85−39.0] ng/g lw and 38.0 [16.0−69.0] ng/g lw for pilot; 4.60 ng/g lw and 5.20 ng/g lw for Risso's; and finally 83.0 ng/g lw and 64.0 ng/g lw for bottlenose. Mean f_{anti} ranged from 0.20 to 0.65 and similarly to blubber no interspecies difference was observed.

Blubber and Brain Distribution. Contributions of the different contaminants to the total HNPs or ∑HFRs burdens are shown in Figure 3. Q1 and PMBPs stood for the 60−80% of the total HNP value both in blubber and brain, with no significant differences among species. Likewise, contributions of MeO-PBDEs and MHC-1 did not show any significant difference between both tissues. However, in the case of the two PBHDs studied, a different behavior was found. While TriBHD contributed with an average of 10%, 8%, 7%, 5%, and 6% to the ∑HNPs blubber burden of common, striped, pilot, Risso's, and bottlenose respectively, its contribution to the ∑HNPs brain concentration was 5%, 3%, 0.8%, 2%, and 3% for the same species. Thus, contribution of TriBHD was lower in brain than in blubber for each species (t = 2.693, d.f = 17, p < 0.05 for common and t = 2.419, d.f = 18, p < 0.05 for striped). On the contrary, both levels and contribution of TetraBHD were higher in brain than in blubber: while it contributed in a really low percentage in blubber (2%, 1%, 1%, 0.4%, and 2% of HNPs in common, striped, pilot, Risso's, and bottlenose, respectively), its presence in brain samples was considerably higher (26%, 13%, 4%, 38%, and 26% of HNPs for the same species). These differences were significant for common and striped (t = 2.313, d.f = 17, p < 0.05 and t = 2.201, d.f = 18, p < 0.05, respectively) and might suggest that TetraBHD has more affinity to the brain than the other HNPs, being able to penetrate the blood-brain barrier (BBB) more easily (Figure 4). Blubber/brain ratios of TriBHD were always higher than ratios for TetraBHD (d.f = 17, p < 0.05, t = 5.02 in common and d.f = 17, p < 0.05, t = 3.400 in striped).

Furthermore, some differences were also observed concerning anthropogenic compounds (Figure 3). BDE-153 and HBB presented higher contributions in brain than in blubber, showing more affinity for this tissue. Regarding BDE-153, mean contributions were 12%, 12%, 13%, 8%, and 11% of HFRs in blubber of common, striped, pilot, Risso's, and bottlenose, respectively, whereas contributions in brain were 20%, 29%, 36%, 0%, and 17% of HFRs in the same species (BDE-153 was not detected in the only brain sample of Risso's). Contributions were significantly higher in brain of common and striped (t = 4.493, d.f = 17, p < 0.05 and t = 11.795, d.f = 17, p < 0.05, respectively). Moreover, blubber/brain ratio of BDE-153 was always lower than ratio for BDE-47 (d.f = 17, p < 0.05, t = 2.815 in common and d.f = 17, p < 0.05, t = 3.171 in striped dolphin). In addition, HBB was only detected in two common and one striped blubber samples, while it contributed with the 26% and 14% to the ∑HFR contamination in brain (Figure 4). HBB was also detected in blubber and brain from the single individual of Risso's, contributing with the 0.5% and 60% to the total HFR burden, respectively.

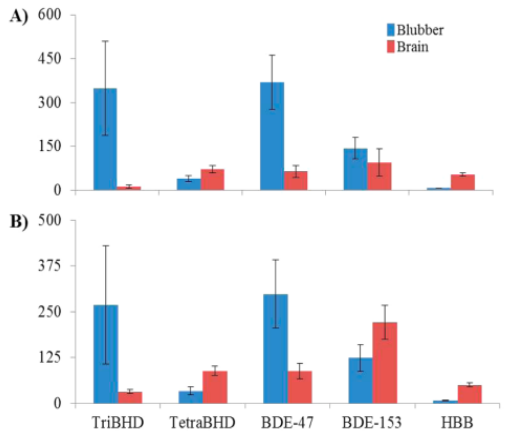

Figure 4. Concentrations (ng/g lw) found in blubber and brain of (A) common dolphin and (B) striped dolphin.

With the exception of TetraBHD, BDE-153, and HBB, levels of halogenated contaminants were higher in blubber than in brain samples. This is in agreement with other published studies.[69−71] In fact, more than 90% of the total POP burden in cetaceans is concentrated in blubber due to its high lipid content.[72] These compounds are generally highly lipophilic and with high molecular weights, thus they are accumulated mostly in blubber.[70] However, lipid is constantly mobilized from blubber since dolphins use it as an energy source and thus contaminants accumulated in blubber redistribute to blood, which is in direct contact with other tissues, and becomes the main source of organic contaminants in tissues such as liver or brain.[72] Furthermore, levels of POPs in blubber and blood have shown a high correlation both in captive and wild dolphins. More precisely, it was shown that PBDEs are mobilized from blubber to blood during blubber lipid mobilization. No relationship was observed between PBDE concentrations found in blubber and lipid content, either in total PBDE concentrations or individual BDEs. This is in agreement with the lack of relationship between PCB concentrations and lipid content in bottlenose dolphins. In contrast, Yordy et al. found a significant negative relationship between PCB plasma concentration and blubber lipid content.[72] Unfortunately, blood samples were not available in this study, and no information about blubber−blood distribution of HNPs or halogenated norbornenes is available.

Concerning accumulation in brain, the existence of the BBB should prevent the organic contaminants to enter the brain thanks to an active transport mechanism mediated by the P-glycoprotein.[73] However, the fact that both HNPs and HFRs were detected in brain samples implies that they were able to surpass the BBB and reach the brain, which represents a new finding for some compounds such as Q1 and related PMBPs, MHC-1, TriBHD, TetraBHD, or Dec 603. Several factors, such as molecular weight, lipid solubility, geometry, halogenation degree, or polarity, are key factors to determine the BBB permeation capacity of a compound.[37,74] Besides, it has been described that these pollutants could enter the BBB via two possible mechanisms: a lipid-mediated free diffusion or carrier-/receptor-mediated transport.[75] Due to the high lipophilicity of all molecules (SI Table S4), the first mechanism would explain why all the compounds can be found in brain even if their high molecular weight should be an impediment to penetrate through the BBB.[75] Moreover, the high contribution of TetraBHD, BDE-153, and HBB to the total burden in brain

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(Figures 3 and 4) is more likely to be related to the second mechanism since no significant correlations were found between brain concentrations and some physicochemical properties that might affect BBB permeability, such as molecular weight, polarizability, or molecular volume (SI Table S4). It has been reported that some molecules have an unexpected affinity to some BBB carrier-mediated transporters.[75,76] Furthermore, some transporters of the BBB are regulated by P450 enzymes,[75] and it has been documented that some of these enzymes such as CYP1A1 or CYP1B1 are upregulated by environmental toxins.[77] The higher contributions of some compounds in brain compared to blubber could be due to a higher affinity to some of these transporters, allowing specific compounds such as TetraBHD, BDE-153, or HBB to pass through the BBB more easily than the other congeners. This affinity might be related to the geometry or position of the halogens in the molecule. For example, the only difference between molecular structures of TriBHD and TetraBHD is an extra bromine atom, which could make TetraBHD a better target for a BBB transporter. In fact, enantioselective transport across the BBB has been documented for (−) and (+) HCH enantiomers, which shows the importance of molecular structure in the transport across BBB.[78,79]

Debromination processes of hexa-BDEs (BDE-154 > BDE-99 > BDE-47; BDE-153 > BDE-100 > BDE-49) have been previously reported.[80] Our results suggest that this debromination might occur in blubber, where contributions of BDE-154 and BDE-153 are lower, whereas it is not likely to occur in brain. Besides, contributions of BDE-47 were higher in blubber than in brain which could be a result of the debromination of BDE-154 (Figure 3). However, uptake of low brominated BDEs through diet is also another possibility and in fact compounds such as BDE-47 and BDE-99 have been reported as major PBDE contributors in fish.[81] However, limited information is available on the distribution of DP and its analogues in potential target tissues such as brain, whereas to our knowledge this is the first study reporting levels of Dec 603 in brain. Zhang et al. (2011)[82] studied the tissue distribution of DP in muscle, liver and brain of two fish species, mud carp (*Cirrhinus molitorella*) and northern snakehead (*Channa argus*), and found higher values of f_{anti} in brain compared to the other tissues. This was attributed to a high affinity of the *anti*-DP to the brain, maybe due to a higher capacity to penetrate through the BBB than the *syn*-DP. Our values of f_{anti} in brain were not different from blubber f_{anti}, and, in addition, DP had a low detection frequency, thus it was impossible to come to any conclusion. Similarly, Li et al. (2014)[71] did not find a significant syn- or anti- enrichment in brain from wild frogs (*Ranna limnocharis*).

Ratio of Natural and Anthropogenic Halogenated Compounds. Ratio between total natural and anthropogenic burdens is shown in Figure 5. In blubber, ∑HNPs/∑HFRs value was 2.75 [0.35−6.75] for common; 4.10 [1.90−8.30] in striped; 2.80 [2.10−3.70] in pilot; 6.90 in Risso's and 2.60 in bottlenose. Moreover, values in brain were 1.30 [0.10−2.20], 2.00 [1.00−5.20], 1.60 [0.80−2.20], 4.60, and 0.80 in the same species. No interspecific difference was observed for common and striped neither in blubber nor brain (t = 1.551, d.f = 18, p > 0.05 and t = 1.438, d.f = 18, p > 0.05, respectively). However, within the same species, the ratio in blubber was generally higher than in brain and this difference was statistically significant both for common and striped (t = 2.396, d.f = 19, p < 0.05 and t = 2.553, d.f = 17, p < 0.05, respectively). Considering that ∑HNPs concentrations were higher than ∑HFRs concentrations, these results suggest that anthropogenic contaminants might have a higher capacity to penetrate the BBB and accumulate in the brain, whereas natural products might have more difficulties going through the barrier, with the exception of TetraBHD. As discussed before the presence of most of these compounds in the brain might be due to a lipid-mediated free diffusion. Thus, since HFRs have higher LogP (6.22−11.65, SI Table S4) than HNPs (5.01−7.31), they are more likely to go through the BBB in higher amounts than HNPs.

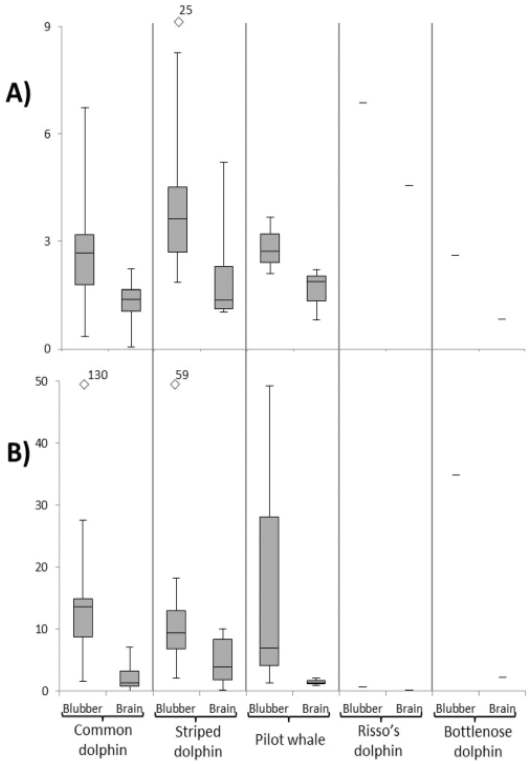

Figure 5. (A) Ratio between ∑HNPs and ∑HFRs concentrations (B) Ratio between ∑classic and ∑alternative concentrations.

On the other hand, the ratio between classical and alternative HFRs was also calculated (Figure 5). Again, no interspecies differences was found between common and striped (t = 0.794, d.f = 20, p > 0.05 in blubber and t = 1.743, d.f = 19, p > 0.05 in brain) whereas ratios were higher in blubber than in brain for each five species. Ratios in blubber and brain were respectively 12.0 [1.60−28.0] and 2.40 [0.10−7.15] (t = 3.837, d.f = 19, p < 0.05) in common; 9.85 [2.00−18.0] and 4.90 [0.20−10.0] in striped (t = 2.127, d.f = 20, p < 0.05); 19.0 [1.40−49.0] and 1.50 [1.00−2.15] in pilot; 0.65 and 0.20 in Risso's and 35.0 and 2.20 in bottlenose. These results might suggest that the alternative HFRs included in this study have more affinity to the brain tissue than the classical PBDEs, and thus more information regarding its neurotoxicity is needed. Furthermore, classical and emerging HFRs were also correlated (R^2 = 0.4909 and R^2 = 0.6818 (p < 0.05) for common and striped,

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respectively) which might imply that dolphins accumulated them through the same pathway.[83]

Overall, this study presented new data regarding the environmental behavior of HNPs and HFRs, evaluating its distribution between blubber and brain of five cetacean species and detecting some of these compounds in brain for the first time. Some compounds like HBB, BDE-153, or TetraBHD, presented higher concentrations (or contributions to total concentrations) in brain than in blubber. These data express the need for further study of the neurotoxic properties of these products, and the mechanisms that allow these compounds to surpass the BBB. Furthermore, classic and alternative HFRs presented some differences concerning their tissue distribution. Although HNPs presented higher concentrations than HFRs, there is still a huge information gap regarding the toxic properties of natural compounds. Hence, more attention should be paid to HNPs in the future.

■ ASSOCIATED CONTENT

Supporting Information

Detailed materials and methods, detailed HNPs and HFRs concentrations and structures, and correlation between classic and alternative FRs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02736.

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Notes

The authors declare no competing financial interest.

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HALOGENATED NATURAL PRODUCTS IN DOLPHINS: BRAIN-BLUBBER DISTRIBUTION AND COMPARISON WITH HALOGENATED FLAME RETARDANTS

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Table S1: Selected SRM or *m/z*, recoveries (%), MDLs and MQLs (ng/g lw) of the analysed compounds.

	Compound	R (%)	RSD (%)	SRM1 m/z1	SRM2 m/z2	MDLs (ng/g lw)	MQLs (ng/g lw)
PBDEs	BDE-28	87	1.8	408>246	408>248	0.01	0.03
	BDE-47	81	11	486>326	486>328	0.01	0.03
	BDE-100	77	8.1	406>297	564>404	0.06	0.20
	BDE-99	81	6.0	406>297	564>404	0.03	0.10
	BDE-154	75	8.1	486>377	644>484	0.21	0.70
	BDE-153	71	7.4	486>377	644>484	0.13	0.43
	BDE-183	56	5.2	721>562	721>564	1.39	4.63
	BDE-209	62	6.1	298>220	361>280	1.11	3.70
HNs	Dec 602	75	8	612>35	612>37	0.05	0.16
	Dec 603	80	12	638>35	638>37	0.04	0.14
	Dec 604	68	14	460>79	504>79	0.15	0.50
	syn-DP	87	2.7	654>35	654>37	0.02	0.07
	anti-DP	84	4.7	654>35	654>37	0.01	0.03
Emerging BFRs	HBB	76	7.8	468>308	468>310	0.06	0.20
	PBEB	71	4.1	500>485	485>406	0.06	0.20
	DBDPE	72	11	485>406	325>165	1.06	3.53
HNPs	2-MeO-BDE-68	77	7.7	516>356	516>358	0.09	0.30
	6-MeO-BDE-47	75	10	516>356	516>358	0.24	0.80
	5-MeO-BDE-47	71	3.1	516>356	516>358	0.06	0.20
	4-MeO-BDE-49	74	4.7	516>356	516>358	0.16	0.53
	5-MeO-BDE-100	76	5.2	596>434	594>436	0.31	1.03
	4-MeO-BDE-100	77	4.4	596>434	594>436	1.59	5.30
	5-MeO-BDE-99	70	4.1	596>434	594>436	0.47	1.57
	4-MeO-BDE-101	76	2.4	596>434	594>436	0.80	2.67
	MHC-1	>70*	<5%*	79	81	0.03	0.10
	TriBHD	>70*	<5%*	79	81	0.18	0.61
	TetraBHD	>70*	<5%*	79	81	0.12	0.41
	Q1	>70*	<5%*	386	388	0.02	0.05
	BrCl6-MBPs	>70*	<5%*	432	434	-	-
	Br2Cl5-MBPs	>70*	<5%*	476	478	-	-
	Br3Cl4-MBPs	>70*	<5%*	520	522	-	-
	Br4Cl3-MBPs	>70*	<5%*	563	565	-	-
	Br5Cl2-MBPs	>70*	<5%*	607	609	-	-
	Br6Cl-MBPs	>70*	<5%*	653	655	-	-

PBDEs: Polybromodiphenyl ethers. HNs: Halogenated norbornenes. MeO-PBDEs: Methoxilated Polybromodiphenyl ethers. R: Recovery. MDLs: Method detection limits. MQLs: Method quantification limits. RSD: relative standard deviation. * Recovery rates were estimated from results obtained in the German laboratory using a similar methodology

Table S2: Levels of HNPs in blubber and brain of the 5 species.

n=10		MHC-1	TriBHD	TetraBHD	∑ MHC-1 &BHDs	Q1	$BrCl_6$	Br_2Cl_5	Br_3Cl_4	Br_4Cl_3	Br_5Cl_2	Br_6Cl	∑BMPs	2-MBDE-68	6-MBDE-47	∑MeOBDEs	∑HNPs
Common dolphin (Blubber)	M	17.0	320	26.6	364	4108	1685	272	50.0	10.0	nd	nd	6125	49.5	373	423	6911
	M	30.3	88.6	14.1	133	408	148	17.2	2.71	0.59	nd	nd	576	45.1	387	432	1141
	M	88.5	146	26.2	260	370	181	21.3	4.87	1.06	nd	0.52	578	41.7	216	258	1096
	F	132	1575	42.4	1750	1554	2318	3.28	17.1	6.23	0.86	19.1	3918	114	972	1086	6755
	F	8.70	33.8	19.4	61.9	123	61.8	6.02	1.58	0.35	nd	nd	193	15.4	77.9	93.3	348
	F	30.4	503	67.3	601	1758	1181	204	49.3	9.91	3.73	5.43	3211	162	2344	2506	6317
	F	6.44	26.2	16.5	49.2	120	56.2	4.61	0.94	nd	nd	nd	182	91.5	1420	1512	1743
	F	59.4	250	32.5	342	520	241	36.8	8.24	2.03	0.37	0.86	810	47.0	236	283	1435
	F	29.3	200	115	345	3574	1823	123	20.9	6.30	nd	nd	5548	nd	59.1	59.1	5952
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	30.5	224	255	255
	Mean	**44.7**	**349**	**40.1**	**434**	**1393**	**855**	**76.4**	**17.3**	**4.06**	**1.65**	**6.48**	**2349**	**66.3**	**631**	**691**	**3195**
	Range	**nd-132**	**nd-1575**	**nd-115**	**nd-1750**	**nd-4108**	**nd-2318**	**nd-272**	**nd-50.0**	**nd-10.0**	**nd-3.73**	**nd-19.1**	**nd-6124**	**nd-162**	**59.1-2343**	**59.1-2506**	**254-6911**
Common dolphin (Brain)	M	1.53	5.68	119	126	159	94.7	6.98	nd	nd	nd	nd	261	nd	9.41	9.41	396
	M	3.23	2.69	89.6	95.5	104	50.9	4.36	0.81	nd	nd	nd	160	nd	nd	nd	255
	M	14.5	5.15	74.2	93.9	74.9	29.5	3.15	nd	nd	nd	nd	108	nd	30.2	30.2	232
	F	1.34	5.09	7.18	13.6	40.8	22.0	1.86	nd	nd	nd	nd	64.7	nd	146	146	224
	F	0.96	59.9	74.1	135	32.1	16.9	1.48	nd	nd	nd	nd	50.5	nd	9.36	9.36	195
	F	3.68	11.1	123	138	68.9	29.3	4.32	0.96	nd	nd	nd	104	nd	66.1	66.1	307
	F	0.60	8.29	22.5	31.4	288	116	16.7	4.08	nd	nd	nd	424	nd	218	218	674
	F	1.39	5.17	81.2	87.7	37.6	17.2	1.90	nd	nd	nd	nd	56.7	nd	21.3	21.3	166
	F	2.03	14.3	53.8	70.1	413	524	49.2	6.32	0.95	nd	nd	993	nd	43.1	43.1	1106
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	20.8	20.8	20.8
	Mean	**3.25**	**13.03**	**71.5**	**87.8**	**135**	**100**	**9.99**	**3.04**	**0.95**	**nd**	**nd**	**247**	**nd**	**62.7**	**62.7**	**358**
	Range	**nd-14.5**	**nd-60.0**	**nd-123**	**nd-138**	**nd-413**	**nd-524**	**nd-49.2**	**nd-6.32**	**nd-0.95**	-	-	**nd-993**	-	**nd-219**	**nd-218**	**21.0-1106**

n=11		MHC-1	TriBHD	TetraBHD	∑MHC-1 &BHDs	Q1	$BrCl_6$	Br_2Cl_5	Br_3Cl_4	Br_4Cl_3	Br_5Cl_2	Br_6Cl	∑BMPs	2-MBDE-68	6-MBDE-47	∑MeO-PBDEs	∑HNPs
Striped dolphin (Blubber)	M	13.3	295	45.2	354	1885	971	140	34.2	7.74	1.63	1.76	3042	127	1405	1532	4928
	M	33.6	56.5	12.0	102	732	155	24.3	5.28	0.48	nd	nd	917	40.8	253	293	1312
	M	7.95	490	53.8	552	1400	774	130	26.8	6.36	1.12	1.38	2340	95.7	1231	1326	4218
	M	5.81	86.2	17.5	110	837	351	42.5	7.26	1.56	0.21	nd	1240	nd	121	121	1471
	M	16.0	920	90.0	1026	3679	2509	613	133	31.8	5.28	7.44	6979	93.3	1567	1660	9665
	M	8.69	263	33.4	306	1156	598	118	27.9	6.04	1.34	1.38	1909	73.0	864	937	3151
	M	22.7	194	15.8	233	235	23.7	4.29	0.91	0.15	0.31	nd	265	49.3	484	532	1031
	M	11.1	91.9	22.2	125	2485	757.9	115	16.2	3.95	nd	nd	3378	7.26	742	749	4253
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	81.6	1559	1640	1640
	F	nd	187	27.1	215	2250	1030	134	23.9	4.72	0.79	nd	3443	24.3	200	224	3882
	F	16.9	96.2	18.8	132	927	302	54.4	11.2	1.32	nd	nd	1296	57.2	463	520	1948
	Mean	**15.1**	**268**	**33.6**	**315**	**1559**	**747**	**138**	**28.7**	**6.41**	**1.52**	**2.99**	**2481**	**64.9**	**808**	**867**	**3409**
	Range	**nd-33.6**	**nd-920**	**nd-90.0**	**nd-1026**	**nd-3679**	**ns-2509**	**nd-613**	**nd-133**	**nd-32.0**	**nd-5.28**	**nd-7.44**	**nd-6979**	**nd-127**	**121-1567**	**121-1660**	**1031-9665**
Striped dolphin (Brain)	M	0.44	6.67	26.7	33.8	442	199	17.4	3.85	nd	nd	nd	662	nd	192	192	888
	M	7.35	47.3	383	437	1168	28.8	7.26	3.17	nd	nd	nd	1207	nd	416	416	2061
	M	nd	7.75	38.3	46.1	242	94.6	11.1	2.66	nd	nd	nd	350	nd	199	199	595
	M	1.11	11.8	85.4	98.5	491	158	22.2	4.25	1.60	nd	nd	677	nd	262	262	1038
	M	0.96	21.0	46.9	68.9	1301	390	48.1	nd	nd	nd	nd	1738	nd	298	297	2105
	M	0.45	4.46	26.4	31.3	236	74.9	11.7	2.39	0.49	nd	nd	325	nd	73.2	73.2	430
	M	0.81	1.68	19.1	21.6	22.7	8.96	1.14	nd	nd	nd	nd	32.81	nd	49.6	49.6	104
	M	nd	187	89.2	276	6.96	nd	nd	nd	nd	nd	nd	6.96	nd	10.9	10.9	294
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	44.7	44.7	44.7
	F	0.33	3.86	85.7	89.9	211	80.0	6.73	nd	nd	nd	nd	298	nd	34.9	35.0	422
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Mean	**1.63**	**32.4**	**89.0**	**123**	**458**	**129.28**	**15.7**	**3.26**	**1.04**	**nd**	**nd**	**588**	**nd**	**158**	**144**	**726**
	Range	**nd-7.35**	**nd-187**	**nd-383**	**nd-437**	**nd-1301**	**nd-390**	**nd-48.1**	**nd-4.25**	**nd-1.60**	-	-	**nd-1738**	-	**nd-416**	**nd-416**	**nd-2105**

n=3		MHC-1	TriBHD	TetraBHD	∑ MHC-1 &BHDs	Q1	$BrCl_6$	Br_2Cl_5	Br_3Cl_4	Br_4Cl_3	Br_5Cl_2	Br_6Cl	∑BMPs	2-MBDE-68	6-MBDE-47	∑MeO-PBDEs	∑HNPs
Pilot whale (Blubber)	M	1.08	87.6	10.8	99.5	440	204	nd	nd	nd	nd	nd	643	22.7	423	446	1189
	F	11.0	26.3	6.22	43.5	437	119	18.3	2.56	nd	nd	nd	577	15.1	84.6	99.6	720
	F	2.16	196	44.9	244	506	1026	15.6	2.16	0.73	nd	nd	1550	49.5	486	535	2329
	Mean	**4.74**	**103**	**20.6**	**129**	**461**	**450**	**16.9**	**2.36**	**0.73**	**nd**	**nd**	**924**	**29.1**	**331**	**360**	**1413**
	Range	**1.1-11.0**	**26.3-196**	**6.2-44.9**	**43.5-244**	**437-506**	**119-1026**	**nd-18.3**	**nd-2.56**	**nd-0.73**	-	-	**577-1550**	**15.1-49.5**	**84.6-486**	**99.6-535**	**720-2329**
Pilot whale (Brain)	M	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	65.3	65.3	65.3
	F	0.65	5.02	22.93	28.6	341	174	14.1	nd	nd	nd	nd	530	nd	76.3	76.3	635
	F	nd	nd	nd	nd	71.5	60.6	3.38	0.61	nd	0.53	nd	137	nd	nd	nd	137
	Mean	**0.65**	**5.02**	**22.93**	**28.6**	**207**	**117**	**8.72**	**0.61**	**nd**	**0.53**	**nd**	**333**	**nd**	**47.2**	**47.2**	**279**
	Range	**nd-0.65**	**nd-5.02**	**nd-22.9**	**nd-28.6**	**nd-342**	**nd-174**	**nd-14.1**	**nd-0.61**	-	**nd-0.53**	-	**nd-530**	-	**nd-76.3**	**nd-76.3**	**nd-635**

n=1		MHC-1	TriBHD	TetraBHD	∑ MHC-1 &BHDs	Q1	$BrCl_6$	Br_2Cl_5	Br_3Cl_4	Br_4Cl_3	Br_5Cl_2	Br_6Cl	∑BMPs	2-MBDE-68	6-MBDE-47	∑MeO-PBDEs	∑HNPs
Risso's dolphin	?	4.09	344	22.3	370	3584	1904	146	nd	3.00	nd	nd	5637	33.7	362	396	6402
	?	0.54	2.15	48.5	51.2	45.7	16.2	nd	nd	nd	nd	nd	61.9	nd	14.1	14.1	127

n=1		MHC-1	TriBHD	TetraBHD	∑ MHC-1 &BHDs	Q1	$BrCl_6$	Br_2Cl_5	Br_3Cl_4	Br_4Cl_3	Br_5Cl_2	Br_6Cl	∑BMPs	2-MBDE-68	6-MBDE-47	∑MeO-PBDEs	∑HNPs
Bottlenose dolphin	M	10.1	136	44.2	190	699	434	17.8	2.11	nd	3.21	nd	1157	151	791	942	2289
	M	0.97	10.3	103	114	96.1	86.7	3.31	nd	nd	nd	nd	186	nd	92.6	92.6	393

M=Male. F= Female. nd= Not detected

Table S3: Levels of anthropogenic FRs in the 5 species.

n=10		BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	∑PBDEs	HBB	Dec 602	Dec 603	*syn*-DP	*anti*-DP	∑DP	∑HNs	∑FRs
Common dolphin (Blubber)	M	5.04	354	277	103	27.5	153	919	nd	105	0.21	nd	nd	nd	105	1024
	M	6.18	136	107	74.0	0.90	37.4	362	nd	13.0	0.06	nd	nd	nd	13.1	375
	M	6.50	174	160	172	6.83	40.9	560	6.33	34.7	0.23	nd	nd	nd	35.0	602
	F	13.3	892	753	130	18.5	238	2045	nd	113	0.55	nd	nd	nd	114	2159
	F	1.52	22.6	25.9	19.4	3.96	19.7	93.3	nd	50.0	0.68	3.68	3.82	7.49	58.1	151
	F	11.7	745	285	427	53.0	328	1849	nd	123	0.22	nd	nd	nd	124	1973
	F	4.73	551	393	334	63.9	290	1637	8.05	4.46	nd	nd	nd	nd	4.46	1650
	F	5.08	247	229	200	9.32	59.2	750	nd	51.9	0.28	nd	nd	nd	52.2	802
	F	0.68	365	447	147	24.3	159	1143	nd	319	3.23	nd	nd	nd	322	1465
	F	3.67	210	231	73.8	23.8	107	650	nd	66.5	0.78	0.75	0.78	1.53	68.8	719
	Mean	**5.84**	**370**	**291**	**168**	**23.2**	**143**	**1001**	**7.19**	**88.1**	**0.69**	**2.21**	**2.30**	**4.51**	**90.0**	**1092**
	Range	**0.68-13.3**	**22.6-892**	**26.0-753**	**19.4-427**	**0.90-63.9**	**19.9-328**	**93.3-2045**	**nd-8.05**	**4.46-319**	**nd-3.23**	**nd-3.68**	**nd-3.82**	**nd-7.49**	**4.46-322**	**151-2159**
Common dolphin (Brain)	M	0.21	20.2	24.5	19.3	22.6	38.3	125	57.4	52.8	1.04	nd	nd	nd	53.9	236
	M	0.16	20.3	nd	nd	18.8	39.1	78.3	61.8	56.0	nd	nd	nd	nd	56.0	196
	M	1.84	37.5	nd	nd	6.62	14.9	60.9	22.4	64.7	nd	nd	nd	nd	64.7	148
	F	1.36	131	nd	nd	nd	nd	132	nd	21.2	nd	nd	nd	nd	21.2	153
	F	0.19	6.67	nd	nd	nd	nd	6.87	54.0	26.0	nd	nd	nd	nd	26.0	86.9
	F	1.31	22.8	22.0	17.2	8.02	46.5	118	79.0	37.7	nd	nd	nd	nd	37.7	234
	F	2.07	155	nd	nd	51.2	105	313	nd	43.8	nd	nd	nd	nd	43.8	357
	F	1.08	33.32	27.2	28.4	20.8	32.5	143	48.7	41.8	nd	nd	nd	nd	41.8	234
	F	0.80	175	nd	nd	180	436	791	42.9	310	2.05	1.55	1.44	2.99	315	1150
	F	0.39	44.8	108	29.3	53.0	50.2	285	60.2	17.0	nd	1.24	0.26	1.50	18.5	364
	Mean	**0.94**	**64.6**	**45.3**	**23.5**	**45.1**	**95.2**	**205**	**53.3**	**67.1**	**1.54**	**1.39**	**0.85**	**2.24**	**68.0**	**316**
	Range	**0.16-2.07**	**6.67-175**	**nd-108**	**nd-29.3**	**nd-180**	**nd-436**	**6.87-791**	**nd-79.0**	**16.9-310**	**nd-2.05**	**nd-1.55**	**nd-1.44**	**nd-2.99**	**18.5-315**	**86.9-1149**

n=11		BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	∑PBDEs	HBB	Dec 602	Dec 603	*syn*-DP	*anti*-DP	∑DP	∑HNs	∑FRs
Stripped dolphin (Blubber)	M	8.53	582	576	267	63.2	217	1714	nd	117	0.33	nd	nd	nd	118	1831
	M	9.52	70.5	48.23	38.1	4.27	21.5	192	nd	21.3	0.07	nd	nd	nd	21.4	214
	M	5.95	380	162	157	26.9	133	865	nd	67.5	0.12	0.38	0.41	0.80	68.4	934
	M	0.75	194	178	109	19.1	76.5	578	nd	42.6	0.13	0.62	0.61	1.23	44.0	622
	M	5.02	725	522	349	37.8	195	1834	nd	369	1.30	3.50	3.63	7.14	377	2212
	M	4.67	273	308	163	29.3	136	913	nd	92.8	0.27	0.52	0.48	1.00	94.1	1007
	M	3.50	141	144	77.9	17.4	95.9	479	nd	74.2	0.12	nd	nd	nd	74.3	554
	M	8.57	119	138	759	17.0	107.9	1149	nd	19.3	0.11	nd	nd	nd	19.4	1169
	F	5.14	696	823	321	69.4	335	2250	nd	267	0.60	10.0	10.4	20.4	288	2538
	F	2.66	31.3	27.5	21.0	3.28	14.9	101	8.17	41.2	0.10	nd	nd	nd	41.3	150
	F	6.86	67.5	71.4	42.3	4.79	30.6	223	nd	11.1	0.06	0.53	0.49	1.02	12.2	236
	Mean	**5.56**	**298**	**273**	**209**	**26.6**	**124**	**936**	**8.17**	**102**	**0.29**	**2.59**	**2.67**	**5.27**	**105**	**1042**
	Range	**0.75-9.52**	**31.3-725**	**27.5-823**	**21.0-759**	**3.28-69.4**	**14.9-335**	**101-2250**	**nd-8.17**	**11.1-369**	**0.06-1.30**	**nd-10.0**	**nd-10.4**	**nd-20.4**	**12.2-377**	**150-2538**
Stripped dolphin (Brain)	M	1.09	117	90.6	41.0	75.7	262	587	23.0	37.5	nd	nd	nd	nd	37.5	647
	M	2.92	157	175	79.7	117	253	784	57.5	53.1	nd	nd	nd	nd	53.1	895
	M	1.31	77.6	85.0	49.9	79.3	160	453	49.2	56.1	nd	0.88	1.27	2.14	58.3	560
	M	22.4	106	117	69.8	101	201	617	100	74.8	nd	nd	nd	nd	74.8	792
	M	1.67	221	491	315	98.4	548	1674	52.8	136	nd	nd	nd	nd	136	1863
	M	0.70	55.7	63.1	58.2	35.8	113	327	30.7	59.6	nd	nd	nd	nd	59.6	417
	M	nd	18.5	nd	nd	nd	nd	18.5	nd	14.1	nd	nd	nd	nd	14.1	32.6
	M	0.07	9.99	nd	nd	28.3	34.1	72.4	96.5	23.0	nd	nd	nd	nd	23.0	192
	F	0.41	115	86.3	67.9	65.7	205	541	20.9	24.1	3.56	2.62	2.33	4.95	32.6	594
	F	nd	11.6	nd	nd	nd	nd	11.6	23.4	46.0	nd	nd	nd	nd	46.0	81.0
	F	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Mean	**3.82**	**88.9**	**158**	**97.29**	**75.0**	**222**	**509**	**50.5**	**52.4**	**3.56**	**1.75**	**1.80**	**3.55**	**53.5**	**607**
	Range	**nd-22.4**	**nd-221**	**nd-491**	**nd-315**	**nd-117**	**nd-548**	**nd-1674**	**nd-100**	**nd-136**	**nd-3.56**	**nd-2.62**	**nd-2.33**	**nd-4.95**	**nd-136**	**nd-1863**

n=3		BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	∑PBDEs	HBB	Dec 602	Dec 603	*syn*-DP	*anti*-DP	∑DP	∑HNs	∑FRs
Pilot whale (Blubber)	M	1.19	165	128	105	10.8	83.5	493	nd	69.7	0.25	0.31	0.33	0.64	70.6	564
	F	1.92	75.4	58.4	42.7	1.55	11.5	192	nd	3.85	0.03	nd	nd	nd	3.89	195
	F	2.45	93.4	127	84.0	31.2	157	495	nd	355	2.47	0.18	0.33	0.50	359	853
	Mean	**1.85**	**111**	**104**	**77.3**	**14.5**	**84.0**	**393**	**nd**	**143**	**0.92**	**0.24**	**0.33**	**0.57**	**144**	**537**
	Range	**1.19-2.45**	**75.4-165**	**58.4-128**	**42.7-105**	**1.55-31.2**	**11.5-157**	**192-494**	-	**3.85-355**	**0.03-2.47**	**nd-0.31**	**nd-0.33**	**nd-0.64**	**3.89-358**	**195-853**
Pilot whale (Brain)	M	0.37	39.4	nd	nd	nd	nd	39.8	12.2	28.9	0.60	nd	nd	nd	29.5	81.5
	F	0.54	36.6	nd	nd	42.8	151	230	37.6	69.1	0.30	nd	nd	nd	69.4	337
	F	nd	9.85	nd	nd	8.48	17.0	35.2	11.0	15.8	nd	nd	nd	nd	15.8	62.0
	Mean	**0.30**	**28.6**	**nd**	**nd**	**25.7**	**83.7**	**102**	**20.3**	**37.9**	**0.45**	**nd**	**nd**	**nd**	**38.2**	**160**
	Range	**nd-0.54**	**9.85-39.4**	-	-	**nd-42.8**	**nd-151**	**35.2-230**	**11.0-37.6**	**15.8-69.1**	**nd-0.60**	-	-	-	**15.8-69.4**	**62.0-337**

		BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	∑PBDEs	HBB	Dec 602	Dec 603	*syn*-DP	*anti*-DP	∑DP	∑HNs	∑FRs
Risso's dolphin	?	2.50	94.1	118	58.5	16.5	77.5	367	4.58	558	1.78	nd	nd	nd	560	931
	?	0.36	4.57	nd	nd	nd	nd	4.93	16.6	5.16	0.45	0.36	0.37	0.74	6.34	27.9

		BDE-28	BDE-47	BDE-100	BDE-99	BDE-154	BDE-153	∑PBDEs	HBB	Dec 602	Dec 603	*syn*-DP	*anti*-DP	∑DP	∑HNs	∑FRs
Bottlenose dolphin	M	20.3	454	189	82.4	9.81	97.8	853	nd	24.3	0.11	nd	nd	nd	24.4	877
	M	6.11	82.8	114	nd	45.6	77.6	326	84.0	64.1	nd	nd	nd	nd	64.1	474

M=Male. F= Female. nd= Not detected

Table S4: Compounds analysed and properties estimated by ACDLabs and ChemAxon

Compound	Source	Molecular weight	LogP*	Polarizability* (10^{-24} cm^3)	Molar volume[a] (cm^3)	Volume[b] ($Å^3$)
BDE-28	Anthropogenic	406.895	6.70 / 5.78	30.0 / 29.4	208.6	216.6
BDE-47	Anthropogenic	485.791	7.39 / 6.55	33.1 / 32.5	224.8	234.8
BDE-99	Anthropogenic	564.688	8.19 / 7.32	36.1 / 35.7	240.9	253.1
BDE-100	Anthropogenic	564.688	8.03 / 7.32	36.1 / 35.7	240.9	253.2
BDE-153	Anthropogenic	643.584	8.98 / 8.09	38.9 / 39.2	257.1	271.4
BDE-154	Anthropogenic	643.584	8.83 / 8.09	39.2 / 38.9	257.1	271.4
BDE-183	Anthropogenic	722.480	9.49 / 8.85	42.2 / 42.2	273.3	289.7
HBB	Anthropogenic	551.488	5.85 / 6.59	28.7 / 29.2	186.5	192.0
PBEB	Anthropogenic	500.645	6.40 / 6.77	29.4 / 29.3	203.2	207.7
DBDPE	Anthropogenic	971.222	11.1 / 12.2	54.4 / 54.5	344.8	370.2
Dec 602	Anthropogenic	613.617	8.38 / 7.01	45.7 / 46.1	299.5	356.1
Dec 603	Anthropogenic	637.681	8.24 / 7.85	49.7 / 50.0	324.4	388.8
Dec 604	Anthropogenic	692.505	9.01 / 8.84	45.4 / 45.1	282.5	328.9
syn-DP	Anthropogenic	653.724	9.51 / 9.07	52.4 / 52.5	358.1	415.4
anti-DP	Anthropogenic	653.724	9.51 / 9.07	52.4 / 52.5	358.1	415.5
5-MeO-BDE-47	Natural	515.817	6.66 / 6.39	35.7 / 35.1	248.8	260.9
6-MeO-BDE-47	Natural	515.817	6.34 / 6.39	35.7 / 35.1	248.8	260.8
4-MeO-BDE-49	Natural	515.817	6.80 / 6.39	35.7 / 35.1	248.8	260.8
2-MeO-BDE-68	Natural	515.817	6.33 / 6.39	35.7 / 35.1	248.8	261.0
5-MeO-BDE-99	Natural	594.714	7.46 / 7.16	38.8 / 38.3	264.9	279.2
5-MeO-BDE-100	Natural	594.714	7.46 / 7.16	38.8 / 38.3	264.9	279.2
4-MeO-BDE-101	Natural	594.714	7.46 / 7.16	38.8 / 38.3	264.9	279.2
4-MeO-BDE-103	Natural	594.714	7.46 / 7.16	38.8 / 38.3	264.9	279.2
MHC-1	Natural	399.377	5.05 / 4.97	30.2 / 29.5	232.1	242.8
TriBHD	Natural	467.034	7.10 / 6.02	37.1 / 36.3	277.2	294.7
TetraBHD	Natural	545.930	7.63 / 6.79	40.2 / 39.4	293.4	313.0
Q1	Natural	387.305	6.56 / 5.70	31.3 / 30.9	206.4	235.4

* (ACDLabs / ChemAxon) [a]: ACDLabs [b]: ChemAxon

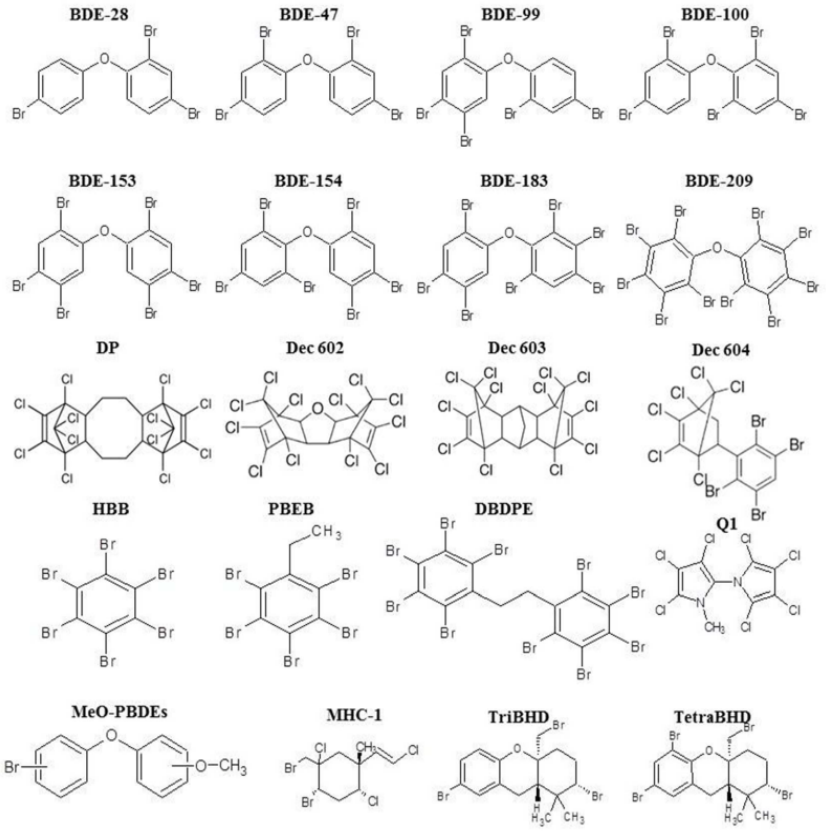

Figure S1: structures of all the compounds studied with the exception of MBPs related to Q1

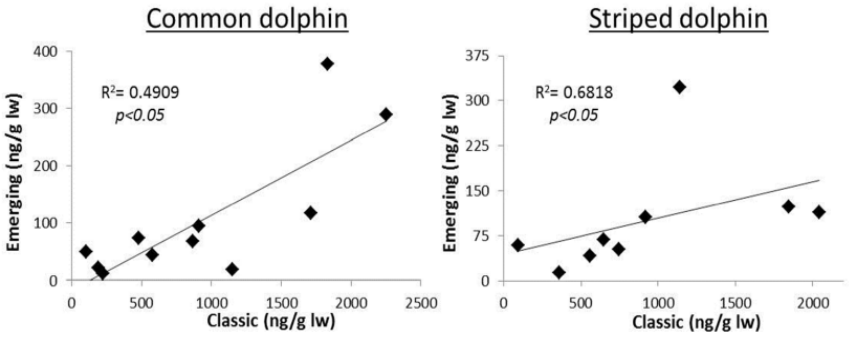

Figure S2: Classic HFRs concentrations versus emerging HFRs concentrations in blubber. The lines are linear regression on the data.

Nitrogen stable isotope determination

Procedure: Isotopic analyses were carried out at the Laboratorio de Isótopos Estables of the Estación Biológica de Doñana (LIE-EBD, Spain; www.ebd.csic.es/lie/index.html). The isotopic compositions are reported in the conventional delta (δ) per mil notation (‰), relative to atmospheric N_2 ($\delta^{15}N$). Prior to the isotope determination, lipid content was extracted from the sample with several rinses of chloroform: methanol (2:1, v/v) solution in order to reduce the isotopic variability due to the differential content of lipids.[1] Subsamples of powdered materials were weighed to the nearest µg and placed into tin capsules for $\delta^{15}N$ determinations. All samples were combusted at 1020 ºC using a continuous flow isotope-ratio mass spectrometry system by means of Flash HT Plus elemental analyzer coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The isotopic compositions are reported in the conventional delta (δ) per mil notation (‰), relative to atmospheric N_2 ($\delta^{15}N$). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ±0.2 ‰ for $\delta^{15}N$. The internal standards used were: EBD-23 (cow horn), LIE-BB (whale baleen), and LIE-PA (feathers of razorbill). These laboratory standards were previously calibrated with international standards supplied by the International Atomic Energy Agency (IAEA, Vienna).

Results: *Stable isotope analysis*

Risso's dolphin (n=1) had the highest $\delta^{15}N$, 13.5‰, followed by pilot whale (n=3) with a value of (mean [range]) 13.4‰ [12.2-15.3‰], bottlenose dolphin (n=1) with 13.2‰, striped dolphin (n=11) with 12.1‰ [10.9-15.0‰], and finally common dolphin (n=10) with 11.8‰ [10.6-13.6‰]. Biomagnification studies could not be carried out since no difference was found between common and striped dolphins (t=0.621, d.f=20, p>0.05), and sample size of

pilot whale, as well as Risso's and bottlenose dolphin was insufficient. These results are given with the only purpose of characterizing the species studied.

Chemicals and reagents

Both native and mass-labelled PBDE mixtures, containing 8 PBDE congeners (BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209, and the ^{13}C-labelled, respectively), MeO-PBDEs mixture (containing 5-MeO-BDE-47, 6-MeO-BDE-47, 4'-MeO-BDE-49, 2'-MeO-BDE-68, 5'-MeO-BDE-99, 5'-MeO-BDE-100, 4'-MeO-BDe-101 and 4'-MeO-BDE-103) as well as HBB, PBEB and DBDPE were purchased from Wellington Laboratories (Guelph, ON, Canada). *Syn*- and *anti*- isomers of DP and ^{13}C-*syn*-DP were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical (Toronto, ON, Canada). ^{13}C-PBDEs and ^{13}C-*syn*-DP were used as internal standards. Al-N cartridges were provided by Biotage (Uppsala, Sweeden). HNPs were synthesized or isolated from algae or sponges as reported elsewhere.[2]

Sample treatment

1 g of lyophilised sample was spiked with the surrogate standards (5 ng of ^{13}C-PBDEs and ^{13}C-*syn*-DP, 50 ng of ^{13}C-BDE-209) and was kept overnight to equilibrate prior to the pressurized liquid extraction (PLE) using an ASE 350 system (Dionex, Sunnywale, CA, USA). A mixture of hexane: dichloromethane (1:1) was used for the extraction, which consisted of 2 static cycles of 10 min at 100 ºC and working at 1500 psi. Lipid content was determined gravimetrically after the extraction. Afterwards, organic content was re-dissolved in *n*-hexane and treated with H_2SO_4 (conc.) followed by a solid phase extraction (SPE) using alumina cartridges (Al-N, 5 g). Extracts were evaporated to incipient dryness, then

perdeuterated α-l,2,3,4,5,6-hexachlorocyclohexane (α-PDHCH) was added as internal standard for HNP compounds, and reconstituted to a final volume of 40 µL prior to the instrumental analysis.

Instrumental analysis

PBDEs, MeO-PBDEs, HBB, PBEB, DBDPE: These compounds were analysed using electron ionization mode (EI). Temperature program started at 140 ºC, held for 1 min, then ramped to 310 ºC at 10 ºC/min and held for 10 min, for a total run time of 36.5 min. Source temperature was set at 250 ºC. Column used was a DB-5ms capillary column (15m x 0.1 mm i.d, 0.1 µm film thickness).

HNs: These compounds were analysed by negative chemical ionization mode (NCI) using CH_4 as reagent gas. Column used was a DB-5ms capillary column (15m x 0.1 mm i.d, 0.1 µm film thickness). Temperature program started at 80 ºC, held for 2 min and then ramped to 300 ºC in 10 ºC/min and held for 10 min for a total run time of 34 min. Source temperature was set at 175 ºC. Selective reaction monitoring (SRM) mode was used to enhance sensitivity and selectivity in the aforementioned methodologies, monitoring two different transitions for each compound. The most intense was used for quantification and the second one for confirmation.

Other HNPs: GC oven was programmed as follows: 60 ºC held for 2 min, ramped at 10 ºC/min to 300 ºC and held for 24 min for a total run time of 50 min. Source temperature was set at 150 ºC. Selective ion monitoring (SIM) was used for the detection and quantification of the different compounds using the two most abundant ions of the molecular ion for each compound. In this case, quantification was done using a response factor to the internal standard α-PDHCH. MBPs related to Q1 were quantified using the response factor obtained for Q1 since there were no available standards of these compounds.

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Publicación científica #8

Bioaccumulation and biomagnification of emerging and classical flame retardants in bird eggs of 14 species from Doñana Natural Space and surrounding areas (South-western Spain)

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ABSTRACT

The occurrence of classical (polybrominated diphenyl ethers, PBDEs) and emerging FRs (dechloranes, hexabromobenzene (HBB), pentabromoethyl benzene (PBEB) and decabromodiphenyl ethane (DBDPE)) in unborn eggs of 14 different species from Doñana Natural Space and surrounding areas was studied.
PBDEs, Dec-602, Dec-603 and DP were detected in all the species, whereas HBB, PBEB, DBDPE and Dec-604 were not detected in any sample. ΣPBDE and ΣDechlorane levels ranged from 1.40 to 90.7, and from 0.77 to 260 ng/g lw, respectively. BDE-209 was the most abundant BDE congener in almost all the species, whereas Dec-602 was the predominant among dechloranes. In general, levels of PBDEs and dechloranes were similar and even higher for dechloranes, probably indicating the increasing use of dechloranes as a result of legal restrictions on PBDEs. In both cases, the most contaminated specie was the white stork. Using stable isotope characterization, differences among species and possible biomagnification processes were also evaluated. PBDE levels increased as the trophic position increased, showing biomagnification capacity. The same behavior was observed for Dec-602 and Dec-603; however, DP levels were not linearly correlated with trophic level. These results show that more attention should be given to emerging FRs such as dechloranes since they show similar environmental behavior as PBDEs.

1. Introduction

Flame retardants (FRs) have been used for many years in order to prevent fires. Among them, halogenated compounds have shown great efficiency (Alaee et al., 2003). Polybrominated diphenyl ethers (PBDEs) have been used in great amounts for many years and their presence has been reported in different environmental and biological matrices such as sediment, sludge, dust, fish, bird eggs or cetaceans (Chen and Hale, 2010; Covaci et al., 2003; Tang et al., 2008). Due to their bioaccumulation capacity and toxic properties, the Penta- and Octa-BDE formulations were banned in Europe and North America in 2001 and since 2006 their presence in polymeric formulations is being reduced (Schecter et al., 2010). Furthermore, the Deca–BDE mixture is already banned in Europe and its production in North America is going to be stopped at the end of 2013 (Hess, 2009). On the other hand Mirex, a chlorinated compound used also as FR, was also banned in 1976 (Sverko et al., 2011).

Since the fire safety regulations needed to be reached, new compounds were developed and, consequently, they are considered emerging FRs. Some examples of emerging brominated FRs (BFRs) are hexabromobenzene (HBB), pentabromoethyl benzene (PBEB) or decabromodiphenyl ethane (DBDPE). These compounds have been found in different environmental and biological matrices (Covaci et al., 2011; Gorga et al., 2013; Guerra et al., 2012; Papachlimitzou et al., 2012). On the other hand, dechlorane 602 (Dec-602), dechlorane 603 (Dec-603), dechlorane 604 (Dec-604) and dechlorane plus (DP) were developed as an alternative for Mirex when it was banned (Shen et al., 2011). Despite the fact that they have been used for many years, they were found in the environment for the first time in 2006 (Hoh et al., 2006). Since then, they have proved their ubiquity as they have been found in different environmental and biological samples around the world. For instance, dechloranes have been found in sediment (Sverko et al., 2008), air (De la Torre et al., 2010a), dust (Zhu et al., 2007) or sludge (De la Torre et al., 2011). Regarding biota, dechloranes have been found both in aquatic and terrestrial organisms such as fish (Sverko et al., 2010), eels (Sühring et al., 2013), dolphins (De La Torre et al., 2012) or bird eggs (Guerra et al., 2011; Muñoz-Arnanz et al., 2010, 2011a,b). Moreover, most of the studies about DP are done close

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to the production sources located in the Great Lakes and China, where the concentrations are much higher than in other areas (Feo et al., 2012; Sverko et al., 2011; Xian et al., 2011). The number of studies in areas far away from the production sources is still scarce and more information is needed. In particular, in Spain DP and its analogs have been found both in environmental matrices, air and sludge (De la Torre et al., 2010a, 2010b), and in eggs from 4 different bird species (Guerra et al., 2011; Muñoz-Arnanz et al., 2010, 2011a, 2012).

In terms of biotic impacts, an additional problem is that birds can assimilate persistent organic pollutants (POPs) through the diet and, afterwards, transfer them to the eggs, with potential consequences for offspring (Bustnes et al., 2008). Thus, eggs are considered reliable bioindicators of POPs in birds (Weseloh et al., 1990). These compounds can affect bird behavior, the correct development of the chicks, causing malformations and reducing the shell thickness (Morales et al., 2012), or reproductive viability (Helander et al., 2002). The aim of this study was to evaluate the occurrence of the classical (PBDEs) and emerging FRs (dechloranes, HBB, PBEB and DBDPE) in unborn eggs of 14 different species from Doñana Natural Space and surrounding areas. Using stable isotope characterization, differences among species and possible biomagnification processes were also evaluated.

2. Sampling

Doñana Natural Space and surrounding areas, located in southwestern Spain, is considered a sanctuary for more than 300 bird species (Muñoz-Arnanz et al., 2010). Due to its important location, between 2 continents and close to the Atlantic Ocean and Mediterranean Sea, this area represents a strategic point where numerous birds breed, winter or stage during their migration (UNESCO, 2013) (Supporting information 1).

Several bird eggs that had failed to hatch were collected during three sampling campaigns in 2010, 2011 and 2012. Table 1 shows the information related to the different species studied, as well as main feeding habits and migratory behavior. Moreover, information regarding sampling locations, date of sampling, nest substrate, egg size, $\%H_2O$ and % lipid weight (lw) is presented in Supporting information 2. In total, 115 egg samples were collected corresponding to 14 different species. These species are grouped into five different orders. Within the order of falconiformes, samples were collected from 7 different species: black kite (*Milvus migrans*), red kite (*Milvus milvus*), western marsh harrier (*Circus aeruginosus*), booted eagle (*Aquila pennata*), common kestrel (*Falco tinnunculus*) and black-winged kite (*Elanus caeruleus*). Three different species of Ciconiiformes were also sampled: glossy ibis (*Plegadis falcinellus*), purple heron (*Ardea purpurea*) and white stork (*Ciconia ciconia*). Three other species belonging to the Charadriiformes were sampled: slender-billed gull (*Chroicocephalus genei*), black-headed gull (*Chroicocephalus ridibundus*) and gull-billed tern (*Gelochelidon nilotica*). Finally, the Strigiform barn owl (*Tyto alba*) and the Anseriform gadwall (*Anas strepera*) were also included in the study. Egg samples were collected opportunistically during nest checking and chick ringing operations, so that the number of samples per species depended on local abundance in the three study years. All the eggs were frozen and sent to the laboratory in individual and protected containers.

3. Materials and methods

3.1. Standards

The standard PBDE mixture, containing BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209, as well as HBB, DBDPE and PBEB were purchased from Wellington Laboratories Inc.

Table 1
Sample inventory, feeding and migratory behavior of the 14 bird species under study.

Order	Species	Scientific name	N	Feeding habits	Migratory behavior of breeding population
Falconiformes	Black kite	*Milvus migrans*	22	Predator and scavenger. It feeds on rabbits, birds (especially young), reptiles, amphibians, fish, insects and carrion (including meat from rubbish dumps, and road killed animals).	Migratory, wintering in sub-Saharan Africa
	Red kite	*Milvus milvus*	2	Predator and scavenger. It feeds on small or medium-sized mammals, birds, reptiles, amphibians, large insects and carrion (including meat from rubbish dumps, and road killed animals).	Resident and dispersive
	Western marsh harrier	*Circus aeruginosus*	1	It feeds mainly on rabbits, rodents, medium-sized birds, their offsprings and eggs.	Resident and dispersive
	Booted eagle	*Aquila pennata*	6	It feeds mainly on medium-sized birds; rabbits, reptiles and occasionally large insects.	Migratory, wintering in sub-Saharan Africa.
	Common kestrel	*Falco tinnunculus*	13	Their preys are usually small size mammals, small birds, reptiles and insects.	Resident and dispersive, and possibly partially migratory
	Black-winged kite	*Elanus caeruleus*	1	It feeds on small-sized mammals, birds and lizards.	Potentially resident, but some individuals make large-scale nomadic movements
Ciconiiformes	Glossy ibis	*Plegadis falcinellus*	4	It feeds mainly on aquatic beetles and dragonfly larvae; also Sharp-ribbed salamanders (*Pleurodeles waltl*) and small Carp (*Cyprinus carpio*).	Migratory and dispersive. Part of the population winters in Doñana
	Purple heron	*Ardea purpurea*	3	It feeds mainly on fish, amphibians, Odonata nymphs and aquatic beetles. Occasionally birds.	Migratory, wintering in sub-Saharan Africa
	White stork	*Ciconia ciconia*	34	It feeds on red-swamp crayfish (*Procambarus clarkii*), large insects, rodents, lizards, snakes, frogs, fish, bird eggs and nestlings, remains of human food.	Mainly migratory, but a part of the population is resident
Strigiformes	Barn owl	*Tyto alba*	1	It feeds mainly on small mammals, *Pelobates cultripes* and small birds.	Resident, but young birds make dispersive movements
Charadriiformes	Slender-billed gull	*Chroicocephalus genei*	3	Mainly fish and invertebrates (brine-shrimps *Artemia* spp., Diptera larvae, beetle larvae and adults).	Mainly migratory, but a small part of the population is resident
	Black-headed gull	*Chroicocephalus ridibundus*	7	Mainly insects and earthworms, but commonly supplemented by plant material and household or industrial waste.	Dispersive or partially migratory
	Gull-billed tern	*Gelochelidon nilotica*	8	It feeds mainly on insects and crustaceans; also amphibians and fish.	Migratory, wintering in sub-Saharan Africa
Anseriformes	Gadwall	*Anas strepera*	10	It mainly feeds on the vegetative part of plants (roots, leaves, tubers, bud and seeds of aquatic plants) and algae, with infrequent animal material, probably accidental.	Resident

(Guelph, ON, Canada). BDE-77, BDE-181 and ^{13}C-BDE-209, used as internal standards, where also purchased from Wellington Laboratories Inc. Dec-602 (95%), Dec-603 (98%) and Dec-604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). *Syn*- and *anti*-isomers of DP, together with ^{13}C-*syn*-DP, were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Al–N cartridges were obtained from Biotage and dichloromethane (DCM) and hexane were purchased from Merck.

3.2. Sample treatment

Egg samples were measured (larger diameter) and broken. The egg content was weighted, homogenized and freeze dried. Lyophilized samples were weighted and homogenized again and stored at −20 °C until analysis.

The sample extraction method applied had been previously optimized (De La Cal et al., 2003; Labandeira et al., 2007). 1.5 g dry weight (dw) of sample was spiked with 5 ng of BDE-77 and BDE-181, 50 ng of ^{13}C-BDE-209 and 5 ng of ^{13}C-*syn*-DP. Spiked samples were kept overnight to equilibrate. Pressurized liquid extraction (PLE) was used as extraction method. Samples were loaded into an 11 mL extraction cell and the dead volume was filled with diatomeus earth. PLE was carried out using a mixture of hexane: DCM (1:1) with 2 static cycles of 10 min at 100 °C and 1500 psi. Flush volume and purge time were 8 mL and 90 s respectively. After the extraction the lipid content was determined gravimetrically and the resulting extracts were re-dissolved in hexane and treated with H_2SO_4 (conc.) to remove fat. After the acid treatment the organic phase was cleaned by solid phase extraction (SPE) using Al–N (5 g) cartridges conditioned with 20 mL of hexane and eluted with 20 mL of hexane:DCM (1:2). Extracts were evaporated to incipient dryness and reconstituted to a final volume of 40 μL prior to the instrumental analysis.

3.3. Instrumental analysis

PBDEs and emerging BFRs (HBB, PBEB and DBDPE) were analyzed by an Agilent 7890C gas chromatograph connected to an Agilent 5975A Network mass spectrometer, working in negative chemical ionization mode (NCI) using NH_4^+ as reagent gas. The instrumental conditions and elution program were based in our previous works (Eljarrat et al., 2002, 2007). The elution program started at a temperature of 140 °C, was held for 2 min and then ramped to 325 °C at 10 °C/min. Final temperature was held for 10 min. The injector and source temperatures were set at 280 and 250 °C respectively. In order to enhance the sensitivity, selected ion monitoring (SIM) mode was applied. The two most intense peaks from the NCI spectra were monitored. Ions monitored were *m/z* 79 and 81 for all PBDEs and emerging BFRs with the exception of BDE-209 and ^{13}C-BDE-209, where the two ions monitored were *m/z* 487 and 489, and *m/z* 497 and 499, respectively. The most intense peaks were used for quantification purposes, and the second ones for confirmation.

On the other hand, our previous work (Barón et al., 2012) showed the advantages of MS–MS against the single quadrupole MS regarding the analysis of halogenated norbornenes. Thus, halogenated norbornenes were analyzed using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole, working in NCI using CH_4^+ as reagent gas. Temperature program started at 80 °C, was held for 2 min and then ramped to 300 °C in 10 °C/min. Final temperature was maintained for 10 min. Source temperature was set at 175 °C and electron energy and emission current were set at 200 and 150 eV, respectively. In order to enhance the sensitivity and selectivity, selective reaction monitoring (SRM) mode was applied. The most intense transition was used for the quantification and the second transition was used for confirmation. Transitions monitored were 654 > 35 and 654 > 37 for *syn*- and *anti*-DP, 460 > 79 and 504 > 79 for Dec-604, 638 > 35 and 638 > 37 for Dec-603, 612 > 35 and 612 > 37 for Dec-602, and 664 > 35 and 664 > 37 for ^{13}C-*syn*-DP.

3.4. QA/QC

Recoveries were evaluated by spiking 5 ng of each compound in purchased eggs. 4 replicates were made and the compounds found in the blank samples did not exceed a 5% of the amount spiked in any case. The method showed good recoveries for PBDEs (55 to 81%), emerging BFRs (57 to 79%) and halogenated norbornenes (73 to 89%). The identification and confirmation of PBDEs, emerging BFRs and halogenated norbornenes were based on the following criteria: (i) simultaneous responses to the two selected ions or transitions were needed, (ii) the area of signal must be at least 3 times higher than the signal noise, and (iii) the difference in the relative intensity of a peak respect to theoretical values obtained with standard solutions cannot exceed ± 15%. In order to prevent interferences and contamination several procedural blanks were made. The contribution of the blank to the signal never exceeded 5%.

Method detection limits (MDLs) were determined for each congener as the minimum amount of analyte which produces a peak with a signal-to-noise ratio of 3, and the method quantification limits (MQLs) were determined as the minimum amount of analyte which produces a peak with a signal-to-noise ratio of 10. For PBDEs, MDLs ranged from 0.05 to 0.45 ng/g lw and the MQLs ranged from 0.17 to 1.45 ng/g lw. For emerging BFRs MDLs ranged from 0.10 to 5 ng/g lw and MQLs ranged between 0.33 and 16 ng/g lw. Finally, MDLs for dechloranes ranged from 0.01 to 0.9 ng/g lw and MQLs ranged from 0.03 to 3.0 ng/g lw.

3.5. $\delta^{13}C$ and $\delta^{15}N$ determinations

Since the lipid content might interfere in the isotope determination, the fat was extracted from the sample using chloroform:MeOH (2:1). Approximately 0.5 g of sample was covered with the solvent mixture for 24 h, then the solvent was removed and fresh solvent was added. This process was repeated for at least 3 times until the solvent appeared clean. Samples were dried at 50 °C for 24 h. Subsamples were weighed to the nearest μg and placed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ determinations. Isotopic analyses were carried out at the Laboratorio de Isótopos Estables of the Estación Biológica de Doñana (LIE-EBD, Spain; www.ebd.csic.es/lie/index.html). All samples were combusted at 1020 °C using a continuous flow isotope-ratio mass spectrometry system by means of a Flash HT Plus elemental analyzer coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). Stable isotope ratios are expressed in the standard δ-notation (‰) relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Replicate assays of laboratory standards routinely inserted within the sampling sequence, and previously calibrated with international standards, indicated analytical measurement errors of ± 0.1‰ and ± 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

4. Results and discussion

4.1. PBDEs

PBDEs were detected in all the species, with total levels ranging from 1.40 (black-headed gull) to 90.7 ng/g lw (white stork) (Table 2). The most contaminated specie was the white stork with a mean value of 34.5 ng/g lw, followed by purple heron (mean value of 23.6 ng/g lw) and western marsh harrier (23.4 ng/g lw). Note that for the latter only one egg was collected. The next most-contaminated species were the booted eagle (mean value of 18.3 ng/g lw), red kite (14.2 ng/g lw), black kite (mean value of 13.6 ng/g lw), common kestrel (mean value of 12.7 ng/g lw) and glossy ibis (mean value of 11.1 ng/g lw). Finally, the least contaminated species were the charadriiformes, anseriformes and strigiformes, with mean values between 5.03 and 6.62 ng/g lw. However, the lowest ΣPBDE value corresponded to the unique available sample of black-winged kite (1.72 ng/g lw).

Table 2
Mean value (expressed in ng/g lw), associated standard deviation and range of PBDE concentrations in bird egg samples.

	Species	BDE-47	BDE-99	BDE-100	BDE-153	BDE-209	ΣPBDEs	% BDE-209
Falconiformes	Black kite	3.69 (3.88)	nd	7.26 (8.95)	3.42 (2.89)	9.90 (7.57)	13.6 (10.7)	78 (18)
		nd–14.3	nd	nd–13.6	nd–7.38	nd–22.1	nd–39.7	38–100
	Red kite	1.55	nd	nd	nd	12.7	14.2	89 (–)
		nq–1.55	nd	nd	nd	(nd–12.7)	(nd–14.2)	
	Western marsh harrier	4.15	4.98	2.10	nd	12.2	23.4	52
	Booted eagle	3.96 (2.56)	3.98 (–)	2.00 (2.48)	4.93 (6.73)	10.4 (7.41)	18.3 (8.33)	54 (27)
		nq–7.78	nd–3.98	nd–4.80	nd–9.69	nd–18.7	nd–30.7	17–83
	Common kestrel	3.32 (2.30)	nd	4.25 (0.57)	2.62 (1.57)	9.99 (8.57)	12.7 (9.37)	77 (29)
		nd–7.59	nd	nd–4.65	nd–4.70	nq–32.6	nq–39.6	15–100
	Black-winged kite	nq	nd	nd	nd	1.72	1.72	100
Ciconiiformes	Glossy ibis	2.42 (1.01)	nd	nq	1.20 (0.72)	6.54 (2.29)	11.1 (2.39)	57 (9.7)
		1.21–3.61	–	–	nq–2.00	3.78–4.48	8.49–14.2	44–65
	Purple heron	2.34 (1.07)	nd	14.9 (5.13)	3.44 (0.34)	4.02 (–)	23.6 (9.45)	17 (–)
		nq–3.09	–	9.03–18.4	nq–3.7	nd–4.02	13.1–31.3	0–17
	White stork	5.08 (4.28)	1.69 (0.94)	8.31 (7.52)	1.70 (1.42)	29.2 (15.2)	34.5 (21.0)	80 (16)
		nd–21.3	nd–2.75	nd–22.9	nd–4.82	nq–49.8	nq–90.7	50–100
Strigiformes	Barn owl	nd	nd	nd	nd	5.20	5.20	100
Charadriiformes	Slender-billed gull	3.06 (1.56)	nd	nd	nd	1.97 (0.61)	5.03 (1.00)	42 (21)
		1.45–3.14	–	–	–	1.30–2.47	3.92–5.29	22–63
	Black-headed gull	3.22 (1.49)	1.24 (1.05)	1.00 (0.04)	nd	3.82 (1.98)	5.98 (1.99)	67 (32)
		nd–4.75	nd–1.98	nd–1.05	–	1.40–6.86	1.40–11.9	32–100
	Gull-billed tern	2.14 (1.63)	nd	nd	nd	4.75 (1.64)	6.62 (3.19)	79 (17)
		nq–4.97	–	–	–	2.92–8.25	3.69–13.2	60–100
Anseriformes	Gadwall	2.17 (0.64)	1.85 (0.54)	nd	nd	1.65 (0.48)	5.66 (1.20)	36 (23)
		1.26–2.97	1.08–2.53	–	–	0.96–2.26	3.70–7.23	19–100

nd: below limit of detection; nq: below limit of quantification.

The variation in PBDE levels among different species was considerable, but variation was also substantial within species. Such variability is shown in Fig. 1. This fact has been reported in other studies about lyphofilic contaminants and is attributed to differences in diet composition (Voorspoels et al., 2007). In birds, factors such as age, body condition and habitat may affect the contaminants accumulated by the female, which are transferred to the egg (Herzke et al., 2002). High intraspecific variability was observed between levels obtained for species such as white stork, black kite, booted eagle and common kestrel. This variation could be explained by the migratory behavior of some of these species (see Table 1). Moreover, in the case of white storks, additional variation may be further promoted by the fact that part of the population migrates while another part winters in the regional surroundings of Doñana. On the contrary, resident species such as gadwall seem to show less inter-individual variation.

Five PBDE congeners were detected, including BDE-47, BDE-99, BDE-100, BDE-153 and BDE-209, whereas BDE-28, BDE-154 and BDE-183 were not detected in any sample. Moreover, BDE-209 was the most abundant BDE congener in almost all the species, with a percentage contribution to the total ΣPBDE values ranging from 30% in gadwall to 100% in black-winged kite and barn owl. The different feeding habits for gadwall, which correspond to an exclusively aquatic food web, could explain the low contribution of BDE-209 in this specie. Normally, the PBDE profile in biota samples presented a high contribution of the low-brominated BDEs, such as BDE-47, which present a higher bioaccumulation capacity than the high brominated congeners. This has been specially found in aquatic food webs (Muñoz-Arnanz et al., 2011b), but it is not so evident in terrestrial food webs. Nevertheless, birds might be exposed to BDE-209 by other routes such as the ingestion of the dust on the feathers (Voorspoels et al., 2006). Moreover, the predominance of BDE-209 in birds from Doñana National Park was also found by Muñoz-Arnanz et al. (2011b), who found a BDE-209 contribution of 44% to the total PBDE values in white storks. This high contribution has been attributed to different uptake routes rather than a different metabolic capacity. Furthermore, Morales et al. (2012) reported a contribution of BDE-209 to the total PBDE value of 89%, in eggs from two gull species, Yellow-legged gull (*Laurus michahellis*) and Audouin's gull (*Laurus audouinii*) from the Ebro river (Spain).

Several studies have reported PBDE levels in bird eggs around the world. Here only some examples are given regarding other studies with similar species analyzed. Some studies have been carried out in Spain. Muñoz-Arnanz et al. (2011b) reported ΣPBDE values ranging from 2.92 to 129 ng/g lw in white stork eggs collected during 1999–2000 from Doñana National Park, which are slightly higher than those reported in the present work (from nq to 90.7 ng/g lw). Morales et al. (2012) reported total PBDE levels ranging from 31.9 to 42.8 ng/g ww in eggs from two gull species (Yellow-legged gull and Audouin's gull) from the Ebro river (Spain).

Gauthier et al. (2007) reported total PBDE levels ranging from 1860 to 4980 ng/g olw in eggs of European herring gull (*Laurus argentatus*) from the Great Lakes. These values are much higher than the ones reported in our study. Moreover, in this case the BDE profile was dominated by low-brominated compounds such as BDE-47 or BDE-99. The higher values could be explained by the fact that sample collection was carried out between 1982 and 2006, time of very high use of PBDE formulations, especially in North America. The different profile could be due to a great use of the Penta-BDE formulation in North America (Morales et al., 2012). In addition, Guerra et al. (2012) reported really high PBDE levels in peregrine falcons from the Great Lakes, with levels ranging from 530 to 38,000 ng/g lw. Recently Sun et al. (2014) reported total PBDE values ranging from 53 to 423 ng/g lw in eggs from 4 different species collected in China between 2010 and 2012: light-vented bulbul (*Pycnonotus sinensis*), yellow-bellied prinia (*Prinia flaviventris*), plain prinia (*Prinia inornata*), and dark green white-eye (*Zosterops japonicus*). These values are higher than our values but lower than those reported in the Great Lakes. In addition, the contribution of BDE-209 was about 60% of the total PBDE burden, which agrees with what we found in our study.

4.2. Dechloranes and emerging BFRs

The three emerging BFRs included in our work, HBB, PBEB and DBDPE, were not detected in any sample. On the other hand, Dec-602, Dec-603 and DP were detected, whereas Dec-604 was not detected in any sample. Dechloranes were detected in all the species with total values ranging from 0.77 to 260 ng/g lw (Table 3). The most contaminated specie was the western marsh harrier (161 ng/g lw) but such

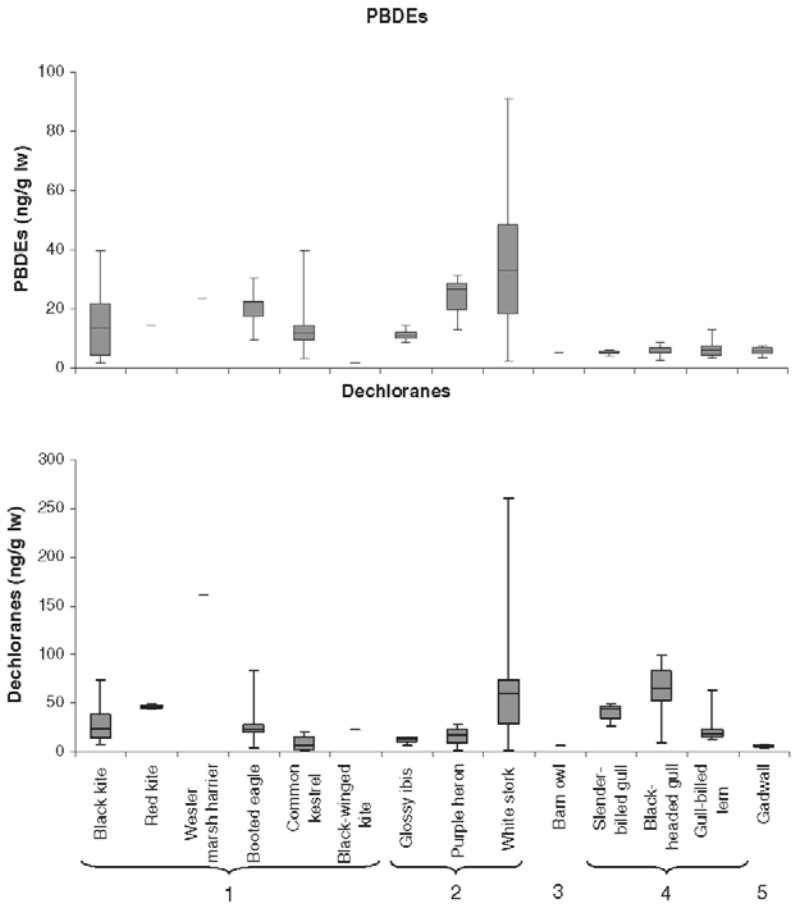

Fig. 1. Box plot for PBDE and dechlorane levels for each specie. 1 = Falconiformes; 2 = Ciconiiformes; 3 = Strigiformes; 4 = Charadriiformes; 5 = Anseriformes.

Table 3
Mean value (expressed in ng/g lw), associated standard deviation and range of Dechlorane concentrations in bird egg samples.

	Species	Dec-602	Dec-603	*syn*-DP	*anti*-DP	DP Total	F_{anti}	ΣDechloranes
Falconiformes	Black kite	17.4 (7.98)	11.5 (7.80)	5.19 (3.42)	10.9 (7.24)	16.1 (10.4)	0.68 (0.06)	30.9 (19.1)
		nd–32.7	0.71–29.3	nq–10.6	nq–24.3	nq–34.4	0.59–0.77	7.51–74.4
	Red kite	27.4 (1.06)	nq	4.50 (1.48)	14.0 (3.49)	18.5 (4.97)	0.76 (0.01)	45.9 (3.91)
		26.7–28.2	nq	3.46–5.55	11.5–16.5	15.0–22.0	0.75–0.77	43.2–48.7
	Western marsh harrier	88.9	69.2	0.85	1.91	2.76	0.69	161
	Booted eagle	49.9 (–)	12.4 (4.90)	4.43 (2.99)	8.89 (5.12)	13.3 (7.73)	0.68 (0.08)	29.9 (27.7)
		nd–49.9	nq–16.5	0.83–8.05	2.82–14.5	3.65–22.0	0.55–0.75	3.65–83.8
	Common kestrel	nd	5.88 (5.57)	2.45 (4.02)	2.15 (1.78)	4.33 (5.21)	0.60 (0.17)	8.88 (7.18)
		nd	1.02–18.9	nd–12.3	nd–4.72	nd–17.0	0.28–0.80	1.02–19.6
	Black-winged kite	nd (–)	20.9 (–)	0.63 (–)	1.08 (–)	1.72 (–)	0.63 (–)	22.6 (–)
Ciconiiformes	Glossy ibis	nq (–)	5.35 (3.45)	2.51 (1.30)	3.96 (1.80)	6.48 (3.10)	0.62 (0.03)	11.8 (3.59)
		–	1.20–9.05	1.00–3.99	1.99–4.81	2.99–10.0	0.60–0.67	7.02–15.0
	Purple heron	nq (–)	12.2 (5.30)	2.84 (2.40)	3.86 (2.98)	6.71 (5.38)	0.62 (0.07)	14.9 (13.3)
		–	Nq–16.0	0.23–4.95	0.54–6.31	0.77–11.3	0.56–0.70	0.77–27.3
	White stork	41.6 (32.7)	12.6 (13.6)	8.76 (9.14)	15.8 (14.6)	24.1 (22.0)	0.65 (0.10)	66.1 (57.6)
		nd–139	nd–54.2	nd–45.3	nd–73.4	nd–102	0.44–0.81	nd–260
Strigiformes	Barn owl	nd (–)	1.81 (–)	3.07 (–)	2.38 (–)	5.44 (–)	0.44 (–)	7.25 (–)
Charadriiformes	Slender-billed gull	33.1 (11.0)	5.39 (4.19)	0.41 (0.37)	0.47 (0.27)	0.87 (0.63)	0.57 (0.10)	39.4 (11.6)
		22.6–44.5	2.53–10.2	0.12–0.83	0.17–0.71	0.28–1.53	0.46–0.65	26.4–43.3
	Black-headed gull	35.4 (14.7)	23.6 (14.5)	1.30 (1.26)	3.10 (3.74)	4.40 (4.99)	0.68 (0.05)	63.4 (30.5)
		6.85–55.3	1.69–39.8	0.24–3.93	0.55–11.3	0.79–15.2	0.60–0.74	9.33–98.9
	Gull-billed tern	nd (–)	20.8 (16.3)	1.17 (1.07)	1.64 (0.61)	2.81 (1.51)	0.64 (0.17)	23.6 (16.6)
		–	10.2–60.0	0.16–3.38	0.96–2.56	1.25–5.70	0.41–0.88	13.3–63.5
Anseriformes	Gadwall	1.87 (0.40)	1.13 (0.52)	0.94 (0.20)	1.99 (0.92)	2.93 (0.94)	0.66 (0.10)	5.93 (1.33)
		1.22–2.38	0.68–2.12	0.61–1.20	1.20–3.75	2.00–4.93	0.53–0.81	4.29–8.27

value should be treated with caution because only one egg of this specie was collected. Excluding this unique sample, and similarly to PBDEs, the highest levels corresponded to the white stork, with a mean value of 66.1 ng/g lw, followed by black-headed gull (mean value of 63.4 ng/g lw), red kite (mean value of 45.9 ng/g lw) and slender-billed gull (mean value of 39.4 ng/g lw). Then, similar values were obtained for black kite, black-winged kite and booted eagle (mean values of 30.9, 30.7 and 29.9 ng/g lw, respectively). Slightly lower levels were found for gull-billed tern and barn owl (mean values of 23.6 and 22.6 ng/g lw). Finally, the lowest levels were for purple heron (mean value of 14.9 ng/g lw), glossy ibis (mean value of 11.8 ng/g lw), common kestrel (mean value of 8.88 ng/g lw) and gadwall (mean value of 5.93 ng/g lw).

As discussed for PBDEs, inter- and intra-specific variations are assumed as a result of different feeding habits and migratory behavior of each species (Table 1 and Fig. 1). Also, the variation in the compounds contribution was quite remarkable. In some species such as black kite, common kestrel or barn owl the contribution of DP in the total dechlorane burden represented 59%, 64% and 75% respectively. On the other hand, in other species, specifically the three species of charadriiformes (slender-billed gull, black-headed gull and gull-billed tern), DP represented only the 2%, 7% and 12% of the total dechloranes. DP has been the most studied compound of the dechlorane family, but the fact that it might not be the most predominant compound has been pointed out in some other studies (Barón et al., 2013; Guerra et al., 2011; Sverko et al., 2010). In fact, in our study levels of Dec-602 and 603 were often higher than DP levels, with maximum values for Dec-602 of 138 ng/g lw in white stork and 69.2 ng/g lw for Dec-603 in western marsh harrier. Although it has not been studied in birds, studies on fish samples indicated that Dec-602 might have more bioaccumulation and biomagnification potential than DP (Sverko et al., 2011). Moreover, the potential metabolic capacities of the different species must be also taken into account. Some dechlorination products have been described for DP (Guerra et al., 2011). Muñoz-Arnanz et al. (2011a) studied this possible dechlorination products in white storks from Doñana National Park, but only one compound was detected (anti-DP-1Cl) in about 10% of the samples. Thus, different metabolism mechanisms could also affect the compound distribution in each species.

The ratio between the *syn*- and *anti*-DP isomers was evaluated by comparing the f_{anti} values (amount of *anti*-DP divided by the total amount of both isomers) obtained for each bird egg sample. Table 3 showed that mean f_{anti} values ranged from 0.57 to 0.76, being similar to those found in commercial mixtures (between 0.65 and 0.75) (Xian et al., 2011). f_{anti} has been studied in different species showing a wide range of results. While some studies reported values lower than the values in commercial mixture (Barón et al., 2013; Sverko et al., 2010), indicating that the *syn*-DP has more bioaccumulation potential or that the *anti*-DP is easily degraded, other studies reported a predominance of the *anti*- isomer (De La Torre et al., 2012). This fact is especially clear in birds, for which all published studies reported f_{anti} values similar to those of commercial mixtures, indicating that in birds the isomer specific accumulation of the *syn*-isomer does not occur. For example, Muñoz-Arnanz et al. (2011a) reported f_{anti} values ranging from 0.55 to 0.73 and from 0.40 to 0.87 in white storks from Madrid and Doñana National Park, respectively. These values are similar to those found in our white stork eggs, with values ranging from 0.44 to 0.81.

The number of studies reporting dechlorane levels in bird eggs is still quite limited, and most of them are focused only on DP. Besides, the different species studied usually complicate comparisons. Muñoz-Arnanz et al. (2011a) reported DP values in white stork eggs from Madrid (collected in 2005) and Doñana National Park (collected between 1999 and 2000) ranging from 0.79 to 19.5 and 0.04 to 6.39 ng/g lw respectively. These values are considerably lower than those observed for our white stork samples (from nd to 102 ng/g lw), suggesting that the use of DP is increasing in the Doñana area. Another study analyzed peregrine falcon eggs from Spain with levels of Dec-602, Dec-603 and DP of 8.36 ng/g lw, 3.98 ng/g lw and 1.78 ng/g lw respectively (Guerra et al., 2011). These values were consistent with our low level range levels.

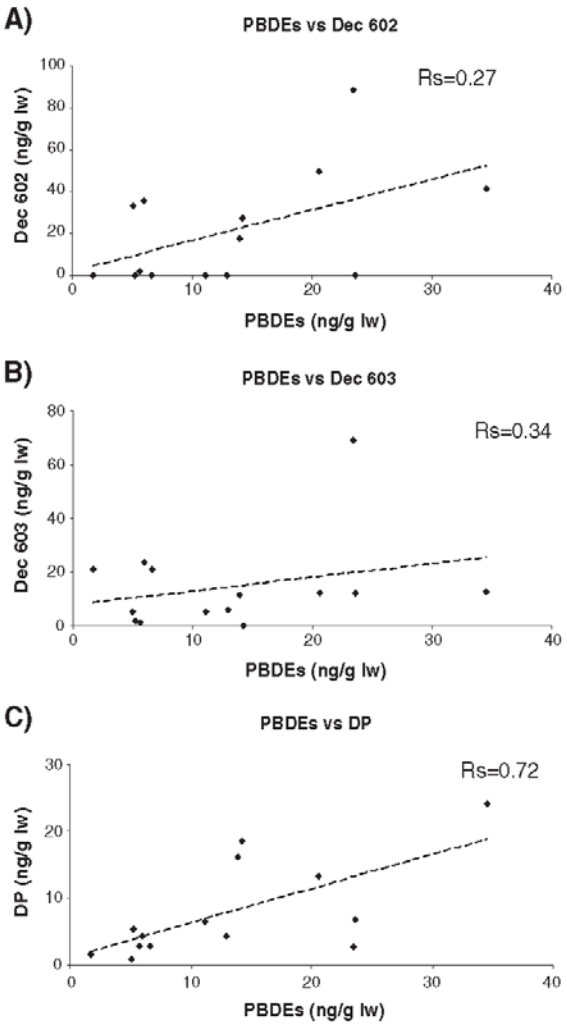

Fig. 2. Correlations between PBDEs and Dec-602 (A), Dec-603 (B) and DP (C), with the Spearman's coefficient (Rs).

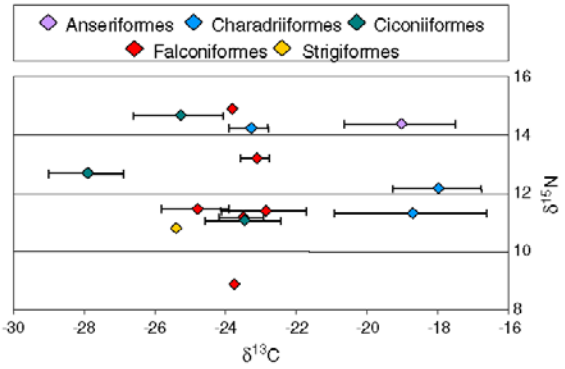

Fig. 3. Plot of the mean $\delta^{15}N$ and $\delta^{13}C$ values (±SD) from stable isotope analysis of bird eggs analyzed in this study.

Finally, DP was analyzed in eggs from two gull species from the Chafarinas Islands, in the south-western Mediterranean Sea. Muñoz-Arnanz et al. (2012) found DP levels in yellow-legged gull and Audouin's gull ranging from 0.52 to 6.05 and 0.06 to 1.75 ng/g lw, respectively. These species have an aquatic-based diet, similar to the slender-billed gull. As a result, the values found for this specie were similar (from 0.28 to 1.53 ng/g lw) to the other two gull species, while values from black-headed gull were clearly higher (0.79 to 15.2 ng/g lw) possibly due to the fact that it follows a more opportunistic diet.

Other authors have focused their research in the Great Lakes area. Guerra et al. (2011) analyzed dechloranes in peregrine falcon eggs from Canada, and found levels of Dec-602, Dec-603 and DP of 73.0, 35.6 and 36.4 ng/g lw, respectively. Gauthier and Letcher (2009) analyzed DP in eggs from herring gulls and reported total DP levels ranging from 5.10 to 67.9 ng/g lw, which are higher than our values in gulls. It is worth mentioning that one of the two known production sources of DP is located in the Great Lakes, which explains why the values are often higher than in other places of the world (Sverko et al., 2011). In China, Sun et al. (2013) reported levels of DP in eggs of light-vented bulbul (*P. sinensis*), yellow-bellied prinia (*P. flaviventris*), plain prinia (*P. inornata*), and dark green white-eye (*Z. japonicus*) ranging from 4.60 to 268 ng/g lw, which are in general higher than our values for DP.

4.3. PBDEs vs dechloranes

Since the three commercial mixtures of PBDEs have already been banned in Europe, it is to be expected that their environmental levels will decrease and, consequently, levels of alternative FRs such as dechloranes will rise. In this study we compared the total concentration levels of both FR families (Tables 2 and 3). In general, levels of PBDEs and dechloranes were similar and, in some cases, dechlorane levels were even higher. For example, in western marsh harrier the ΣPBDEs were 23.4 ng/g lw while ΣDechloranes were 161 ng/g lw. The same situation was observed for white stork, where ΣPBDEs were 27.7 ng/g lw and ΣDechloranes were 62.8 ng/g lw. Further studies should be done in order to confirm this trend, which might confirm the increasing uses and applications of dechloranes as a result of legal restrictions on PBDEs.

Fig. 2 shows the mean concentration values of ΣDechloranes in each species studied versus the corresponding mean concentration of ΣPBDEs. As can be seen, Dec-602 and Dec-603 were slightly correlated with the ΣPBDE values (Spearman's test, Rs = 0.27 for Dec 602 and Rs = 0.34 for Dec-603) while for DP a stronger correlation was found (Spearman's test, Rs = 0.72). This could indicate that DP and PBDEs come from the same sources. In fact, the positive correlation was maintained when BDE-209 and DP were compared (Rs = 0.73), which could show the use of DP as a substitute of BDE-209. Muñoz-Arnanz et al. (2011a) found a positive correlation between PBDE and total DP concentration in white stork eggs from Doñana National Park, which was attributed to the fact that the contamination sources in the area are quite scattered.

4.4. Biomagnification study through stable isotope analysis

Stable isotope analysis has become a powerful tool to study dietary exposure and biomagnification of contaminants in wild animal populations. The trophic position is defined with $\delta^{15}N$, based on the enrichment of ^{15}N throughout the food web. On the other hand, $\delta^{13}C$ is related to the food sources, providing information about the average diet of individuals over a long period. $\delta^{13}C$ values provide information regarding the source of dietary carbon, i.e. aquatic or terrestrial (Jardine et al., 2006).

Inputs of nutrients from exogenous sources, both natural (such as nitrification or denitrification) and anthropogenic (such as human sewage and agriculture), are common in many ecosystems. These nutrients often have distinct isotopic ratios and, as a result, can cause changes in

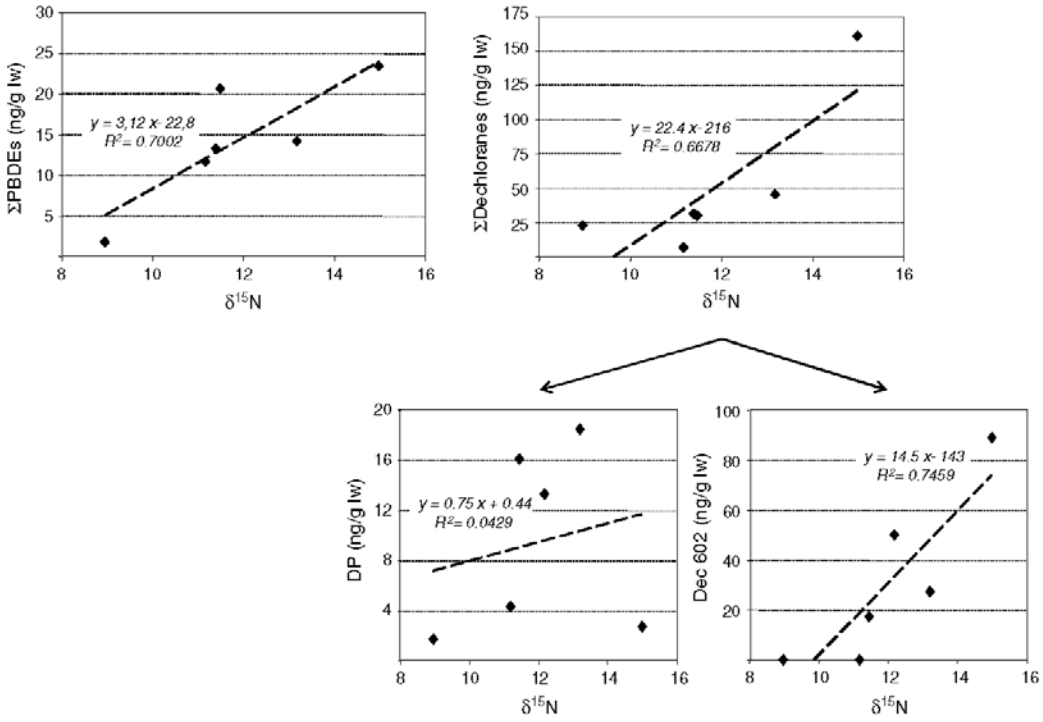

Fig. 4. Plot of the PBDE and dechlorane concentrations in falconiformes eggs (ng/g lw) versus $\delta^{15}N$. Data points are mean values. The dotted line represents linear regression.

baseline isotope ratios. Fluctuating baseline $\delta^{15}N$ have the potential to confound interpretation of trophic differences within species when one compares across systems (Jardine et al., 2006). Precisely, water that flows from the Doñana National Park are susceptible of NO_3^- contamination from small urban areas in the surroundings of the park and agricultural practices allowed in the ecotone, where farming of strawberries and rice is common (Tortosa et al., 2011). That is why we must be cautious in assessing trophic levels from our $\delta^{15}N$ values.

Fig. 3 illustrated mean values of $\delta^{13}C$ and $\delta^{15}N$ in the different species studied. As regards $\delta^{15}N$ data, it is surprising, for instance, the high values observed for gadwall. These high values may be due to the high levels of nitrogen in the aquatic system where this species inhabits. Therefore, we cannot compare $\delta^{15}N$ data from all the species inhabiting different systems with a different baseline $\delta^{15}N$. If we now look at the $\delta^{13}C$ values, we can see significant differences between species, with data ranging from −16.6 for slender-billed gull (*Chroicocephalus genei*) to −29.1 for purple heron (*A. purpurea*). Thus, and based on $\delta^{13}C$ values, bird species could be assigned to different habitats. However, if you look at the falconiformes, the different species presented similar values of $\delta^{13}C$ (between −21.40 and −26.32), indicating a similar behavior with regard to the diet. On the other hand, these species presented $\delta^{15}N$ dissimilar values (between 8.95 and 14.98) enough to evaluate biomagnification processes of contaminants along trophic chain.

Fig. 4 showed the representation of total PBDE and dechlorane values in front $\delta^{15}N$. A positive correlation was found ($R^2 = 0.70$), which indicates that PBDE levels increase as the trophic position increases, showing biomagnification capacity. This has been shown in other studies (Losada et al., 2009). Furthermore, a positive correlation was also found for dechloranes when the total concentration was represented against $\delta^{15}N$ ($R^2 = 0.67$). As discussed above, this might show possible biomagnification behavior. However, since only one sample was available for two species of the group these results should be interpreted with caution.

Furthermore, it is important to note the different behaviors showed by different dechloranes. As shown in Fig. 4, DP levels were not linearly correlated with $\delta^{15}N$ ($R^2 = 0.04$), while a good correlation was observed for Dec-602 ($R^2 = 0.75$). We can conclude that Dec-602, similar to PBDEs, biomagnifies along the food chain, while this behavior is not clear for DP. In fact, previous studies showed similar conclusions (Sverko et al., 2010, 2011). Unfortunately, there is still a lack of information about the environmental behavior of dechloranes in the environment. However, our results show that more attention should be given to these compounds since they show similar biomagnification behavior as historically shown for PBDEs.

5. Conclusions

PBDEs and dechloranes were detected in bird eggs of several different species from Doñana Natural Space and surrounding areas, some of them of high conservation concern (e.g. the extinction-threatened red kite). Our dechloranes data are interesting because the number of studies reporting dechlorane levels in bird eggs is limited, and over-focused on DP only. Our results showed that it is important to include also Dec-602 and Dec-603, because Dec-602 was the predominant among dechloranes, followed by Dec-603, and finally DP. Moreover, levels of PBDEs and dechloranes are similar and even higher for dechloranes, probably indicating the increasing use of dechloranes as a result of legal restrictions on PBDEs. Further studies should be implemented in order to evaluate time trends.

In addition to dechlorane occurrence, we reported correlational evidence of biomagnification capacity for Dec-602 and Dec-603. However, biomagnification was not clear for the case of DP. These results indicate the need for further studies focused on the ecological impact of these emerging contaminants. More attention should be paid to dechloranes since they show similar environmental behavior as PBDEs.

Taking into account the potentially toxic effects of dechloranes and the exposure to these pollutants by all the bird species analyzed, further investigation of dechloranes effects on avian biota are urgently needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2014.03.013.

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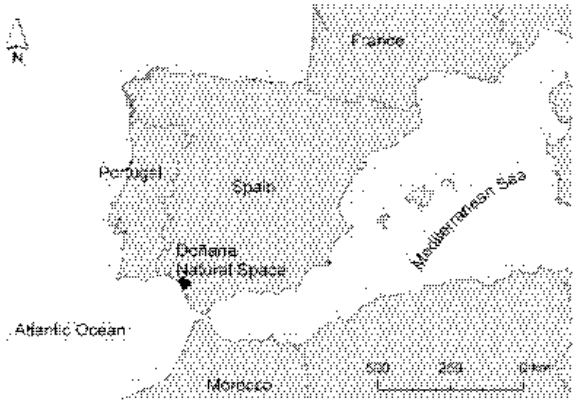
Location of Doñana National Park



Common and scientific name, date of sampling, sample position, nest substrate, egg larger diameter and weight, % of lipid content and % of H_2O for each bird egg.

Species	Scientific name	Date	Coord. X	Coord. Y	Nest substrate	Larger diameter (mm)	Egg weight	% Lipid	% H_2O
Black kite	Milvus migrans	25-05-11	N 36.208	W 06.189	Holm oak	51.90	47.50	35.61	79.76
Black kite	Milvus migrans	03-06-11				58.5	50.5	29.34	73.4
Black kite	Milvus migrans	16-06-11				55.60	8.50	18.55	86.72
Black kite	Milvus migrans	16-06-11				49.00	37.00	31.57	75.55
Black kite	Milvus migrans	05-07-11				51.60	36.50	31.49	79.93
Black kite	Milvus migrans	12-05-11				56.20	48.00	34.99	81.12
Black kite	Milvus migrans	15-06-11				54.80	42.00	35.92	77.92
Black kite	Milvus migrans	06-06-11				51.20	43.00	22.87	10.95
Black kite	Milvus migrans	31-05-12	N 37.128	W 06.447	Cork oak	58.00	54.50	27.86	79.95
Black kite	Milvus migrans	11-06-12	N 37.121	W 06.450	Cork oak	50.60	33.00	36.78	76.18
Black kite	Milvus migrans	06-07-12	N 37.145	W 06.432	Stone pine	53.00	36.50	27.35	79.15
Black kite	Milvus migrans	12-06-12					39.67	29.92	79.05
Black kite	Milvus migrans	24-06-12					29.13	33.07	76.35
Black kite	Milvus migrans	06-07-12					36.95	30.76	80.30
Black kite	Milvus migrans	12-06-12					26.45	8.11	85.97
Black kite	Milvus migrans	12-06-12					31.19	32.96	76.47
Black kite	Milvus migrans	25-06-12					29.94	39.27	76.72
Black kite	Milvus migrans	24-06-12					34.53	20.29	85.03
Black kite	Milvus migrans	13-06-12					31.54	29.11	83.29
Black kite	Milvus migrans	12-06-12					34.30	24.03	82.71
Black kite	Milvus migrans	23-06-12					31.88	25.19	84.91
Black kite	Milvus migrans	28-06-12					34.40	26.84	75.64
Red kite	Milvus milvus	21-05-12					35.83	37.37	80.91
Red kite	Milvus milvus	30-04-12					43.22	34.32	82.21
Western marsh harrier	Circus aeruginosus	02-06-11	N 36.946	W 06.231	Common Reed	44.60	21.50	36.23	75.59
Booted eagle	Aquila pennata	23-07-12	N 37.143	W 06.435	Stone pine	54.40	40.00	32.31	76.78

Booted eagle	Aquila pennata	25-05-10				42.10	47.60	32.92	69.15
Booted eagle	Aquila pennata	25-05-10				46.50	47.90	22.15	46.24
Booted eagle	Aquila pennata	24-06-12					31.40	30.87	72.20
Booted eagle	Aquila pennata	24-06-12					54.13	26.42	82.28
Booted eagle	Aquila pennata	25-06-12					41.96	21.47	80.15
Common Kestrel	Falco tinnunculus	14-06-11	N 37.030	W 06.294	Abandoned Building	40.00	16.50	25.30	81.32
Common Kestrel	Falco tinnunculus	14-06-11	N 37.030	W 06.294	Abandoned Building	38.50	13.00	34.80	77.76
Common Kestrel	Falco tinnunculus	30-06-11	N 37.129	W 06.537	Building	39.20	5.50	35.51	80.04
Common Kestrel	Falco tinnunculus	29-05-12	N 37.126	W 06.573	Building	39.85	22.00	22.04	81.73
Common Kestrel	Falco tinnunculus	29-05-12	N 37.141	W 06.571	Building	36.60	17.00	32.77	82.05
Common Kestrel	Falco tinnunculus	29-05-12	N 37.049	W 06.305	Nest box in turret	38.80	19.00	37.10	80.72
Common Kestrel	Falco tinnunculus	29-05-12	N 37.066	W 06.301	Nest box in turret	39.45	17.50	30.91	78.56
Common Kestrel	Falco tinnunculus	23-07-12	N 37.049	W 06.305	Nest box in turret	39.60	17.50	10.87	80.04
Common Kestrel	Falco tinnunculus	02-07-12	N 37.128	W 06.537	Nest box in building	40.00	11.00	23.80	79.35
Common Kestrel	Falco tinnunculus	02-07-12	N 37.128	W 06.537	Nest box in building	39.25	17.00	10.62	76.68
Black-winged kite	Elanus caeruleus	17-05-12	N 37.251	W 06.265	Cork oak	41.35	19.00	29.42	80.82
Glossy ibis	Plegadis falcinellus	27-05-10	N 37.072	W 06.378	Southern cattail	36.60	30.10	38.07	63.34
Glossy ibis	Plegadis falcinellus	27-05-10	N 37.072	W 06.378	Southern cattail	36.40	20.90	32.45	63.75
Glossy ibis	Plegadis falcinellus	27-05-10	N 37.072	W 06.378	Southern cattail	38.90	20.30	39.68	78.89
Glossy ibis	Plegadis falcinellus	27-05-10	N 37.072	W 06.378	Southern cattail	35.00	27.70	40.09	69.47
Purple heron	Ardea purpurea	27-05-10	N 37.072	W 06.371	Southern cattail	38.00	25.40	31.99	79.18

Purple heron	Ardea purpurea	27-05-10	N 37.072	W 06.371	Southern cattail	39.60	30.20	35.54	78.63
Purple heron	Ardea purpurea	27-05-10	N 37.072	W 06.371	Southern cattail	41.20	42.90	32.48	56.10
White stork	Ciconia ciconia	24-05-11	N 37.218	W 06.167	Nest in turret	75.20	100.00	39.21	-
White stork	Ciconia ciconia	24-05-11	N 37.210	W 06.191	Holm oak	66.40	76.00	35.41	83.05
White stork	Ciconia ciconia	24-05-11	N 37.207	W 6.170	Wild olive tree	71.10	79.50	35.64	79.09
White stork	Ciconia ciconia	24-05-11	N 37.208	W 6.169	Wild olive tree	74.05	89.00	38.13	75.89
White stork	Ciconia ciconia	24-05-11	N 37.208	W 6.169	Wild olive tree	71.50	92.00	39.20	76.16
White stork	Ciconia ciconia	24-05-11	N 37.209	W 6.172	Wild olive tree	67.45	85.50	8.10	77.69
White stork	Ciconia ciconia	24-05-11	N 37.209	W 6.172	Wild olive tree	69.40	76.50	36.22	78.47
White stork	Ciconia ciconia	24-05-11	N 37.208	W 6.172	Wild olive tree	73.05	85.50	37.88	77.14
White stork	Ciconia ciconia	25-05-11	N37.208	W 6.189	Holm oak	74.15	89.50	35.37	73.87
White stork	Ciconia ciconia	25-05-11	N37.208	W 6.189	Holm oak	71.10	64.50	39.27	77.24
White stork	Ciconia ciconia	31-05-11	N37.237	W 6.269	Holm oak	72.60	88.50	37.08	78.54
White stork	Ciconia ciconia	01-06-11	N37.123	W 6.452	Cork oak	66.10	77.00	36.71	76.96
White stork	Ciconia ciconia	07-06-11	N37.125	W 6.453	Cork oak	76.40	110.00	36.61	80.26
White stork	Ciconia ciconia	12-06-12	N 37.239	W 06.129	Stone pine	71.60	88.00	35.48	77.56
White stork	Ciconia ciconia	12-06-12	N 37.239	W 06.129	Stone pine	71.05	81.50	30.77	76.54
White stork	Ciconia ciconia	12-06-12	N 37.239	W 06.129	Stone pine	70.60	81.00	35.78	76.70
White stork	Ciconia ciconia	12-06-12	N 37.239	W 06.129	Stone pine	71.90	76.00	31.57	76.30
White stork	Ciconia ciconia	05-06-12	N 37.210	W 06.192	Holm oak	65.45	70.50	35.90	76.83
White stork	Ciconia ciconia	05-06-12	N 37.210	W 06.192	Holm oak	68.45	78.50	33.81	76.26
White stork	Ciconia ciconia	05-06-12	N 37.210	W 06.192	Holm oak	65.90	78.50	27.75	80.75
White stork	Ciconia ciconia	05-06-12	N 37.208	W 06.188	Holm oak	70.00	82.00	39.89	76.78
White stork	Ciconia ciconia	07-06-12	N 37.198	W 06.210	Eucalyptus	69.50	72.00	22.23	77.53
White stork	Ciconia ciconia	13-06-12	N 37.237	W 06.269	Holm oak	75.50	76.50	34.88	77.96
Barn owl	Tyto alba	31-05-12	N 37.135	W 06.407	Abandoned building	36.90	15.00	33.78	78.25
Slender-billed gull	Chroicocephalus genei	28-06-12	N 36.920	W 06.235	Island ground	54.60	36.50	39.56	73.36
Slender-billed gull	Chroicocephalus genei	28-06-12	N 36.920	W 06.235	Island ground	56.00		40.74	67.63
Slender-billed gull	Chroicocephalus genei	28-06-12	N 36.920	W 06.235	Island ground	54.80		39.07	72.20
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	51.10	27.50	29.06	69.61

Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	50.45	17.00	47.76	54.20
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	46.85	29.50	40.23	72.71
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	48.50	28.50	43.72	72.38
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	47.35	25.00	46.64	70.83
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	53.30	31.5	37.59	74.17
Black-headed gull	Chroicocephalus ridibundus	11-07-12	N 37.912	W 06.236	Ground or palustre vegetation	49.50	25.00	43.52	70.41
Black-headed gull	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	47.90	23.50	39.56	71.39
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	49.40	21.50	37.73	67.07
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	45.50	10.50	32.03	37.43
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	46.90	23.00	18.63	72.27
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	47.45	21.50	41.62	63.77
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	44.60	23.50	47.10	73.62
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	45.60	21.00	40.40	70.13
Gull-billed tern	Gelochelidon nilotica	11-07-12	N 37.912	W 06.236	Ground	47.40	23.50	41.89	69.34
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	51.70	34.00	35.75	65.16
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	51.30	33.50	32.47	62.68
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground		35.50	33.32	61.37
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	53.25	36.00	32.77	61.94
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	53.40	38.50	34.87	66.19
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	54.40	37.50	33.17	67.57
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	53.50	39.00	33.24	65.91
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	55.10	36.00	32.99	62.34

Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	52.40	36.50	32.47	62.81
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	55.20	36.50	37.49	61.17
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	54.55	37.50	32.37	62.97
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	56.10	36.50	35.02	70.86
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	55.10	38.50	27.54	61.38
Gadwall	Anas strepera	11-07-12	N 37.912	W 06.236	Ground	53.70	26.00	34.48	52.04

Publicación científica #9

Temporal trends in classical and alternative flame retardants in bird eggs from Doñana Natural Space and surrounding areas (south-western Spain) between 1999 and 2013

E. Barón, C. Bosch, M. Máñez, A. Andreu, F. Sergio, F. Hiraldo, E. Eljarrat, D. Barceló

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Temporal trends in classical and alternative flame retardants in bird eggs from Doñana Natural Space and surrounding areas (south-western Spain) between 1999 and 2013

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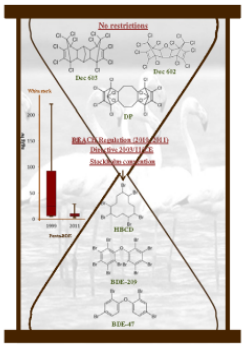
HIGHLIGHTS

- HFRs detected in bird eggs corresponding to two different time periods.
- Decrease of the contribution of the components of Penta-BDE in birds from South Spain.
- Ratio between HNs and PBDEs in each sample was higher in contemporary samples.
- Presence of PBDEs is still higher than the presence of HNs.
- Percentage contribution of α-HBCD increased between the two time periods.

GRAPHICAL ABSTRACT



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ABSTRACT

Several halogenated flame retardants were detected in black kite, white stork and greater flamingo unborn eggs from Doñana Natural Space (Spain) collected in 1999, 2003, 2011 and 2013. The main components of Penta-BDE commercial mixture (BDE-47, -99 and -100) showed a decrease in the studied time interval, concurring with the ban of this mixture in the European Union (EU) in 2006. On the other hand, BDE-209, the main component of Deca-BDE mixture showed a clear trend in black kites but further monitoring is needed since its production ceased at the end of 2013. Besides, even if Dechlorane Plus (DP) was proposed by the EU as an alternative to BDE-209 no time trends were observed. Furthermore, total concentrations of PBDEs (classical FRs) are still higher than concentrations of hexabromocyclododecane (HBCD) and alternative FRs halogenated norbornenes (HNs), which are theoretically substitutes of the already banned PBDEs.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been widely applied in a wide range of materials and represent one of the main groups of halogenated flame retardants (HFRs) in the environment

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(Alaee et al., 2003). Its production increased considerably after polybrominated biphenyls (PBBs) were banned and were first detected in the environment in 1970 (DeCarlo, 1979). Back then, three commercial mixtures were available: Penta-BDE, Octa-BDE and Deca-BDE. PBDEs are highly lipophilic, resistant to degradation and can lixiviate from the products that contain them (Hites, 2004). Thus, they are ubiquitous in the environment and have been found both in environmental samples such as sediment (Barón et al., 2014c), air (Piazza et al., 2013) or soil (Covaci et al., 2005), and in biological matrices such as fish (Labandeira et al., 2007), birds (Muñoz-Arnanz et al., 2011a,b), cetaceans (Alonso et al., 2014) and humans (Norén and Meironyté, 2000). Thus, they were classed as persistent organic pollutants (POPs) under the Stockholm Convention. In addition Penta-BDE and Octa-BDE mixtures were banned in the European Union (EU) in 2004, their production was ceased by manufactures in the United States the same year and also banned in Canada in 2006 (Muñoz-Arnanz et al., 2011a,b). Furthermore, Deca-BDE mixture was banned in the EU in 2008 and its production was restricted and supposed to phase out production by the end of 2013 in the USA (Wäger et al., 2012). To our knowledge, except for EU and North America, there are no other restrictions on the use of PBDEs.

Consequently, restrictions over PBDEs resulted in a market demand of alternative and non-regulated FRs to replace them (Covaci et al., 2011). For example, the hexabromocyclododecane (HBCD) technical mixture, with three main diasteroisomers (α-, β- and γ-HBCD) has been produced for many years and was considered a serious alternative to Penta- and Octa-BDE after they were banned (Al-Odaini et al., 2015). Its physicochemical properties are similar to those of PBDEs and it was first detected in the environment in 1995 (Sellström et al., 1998). Since then, it has been detected in a wide range of environmental and biological matrices, proving its ubiquity in the environment (Covaci et al., 2006; Guerra et al., 2008; Law et al., 2012). HBCD was included in the list of global elimination compounds under the Stockholm Convention in May 2013 (Al-Odaini et al., 2015). However, its use is still permitted in expanded polystyrene and extruded polystyrene in buildings, as long as materials are clearly labelled indicating that they contain HBCD (UN, 2013). Other new compounds such as hexabromobenzene (HBB), pentabromoethyl benzene (PBEB), decabromodiphenyl ethane (DBDPE), and halogenated norbornenes (HNs) or dechloranes: Dechlorane 602 (Dec 602), 603 (Dec 603), 604 (Dec 604) and Dechlorane Plus (DP) have been considered as further alternatives to classical FRs. HNs have been produced for about 40 years and at the beginning they were used as replacements of Mirex, banned as FR in 1976 (Sverko et al., 2011). In addition, DP was recently proposed as an alternative to the mostly used PBDE mixture, Deca-BDE mixture (Xian et al., 2011). DP is considered a high production volume chemical in USA and a low production volume chemical in the EU, and has been included in Canada's domestic substance list, while Dec 602, Dec 603 and Dec 604 have been included in Canada's non domestic substance list (Sverko et al., 2011). This shows, at least for North America, that attention is being put over them. To date, no restrictions over dechloranes have been proposed. Nonetheless, dechloranes have proved to be present in the environment and, since their first detection in 2006 (Hoh et al., 2006), dechloranes have been found in several environmental samples such as sediment (Sverko et al., 2008), sludge (De la Torre et al., 2010b), air (De la Torre et al., 2010a), fish (Sverko et al., 2010), birds (Muñoz-Arnanz et al., 2012), cetaceans (De La Torre et al., 2012) and also in humans (Siddique et al., 2012). This means that they can lixiviate out the materials and enter various environmental compartments, and some studies have shown that they might have biomagnification potential, similarly to PBDEs (Barón et al., 2014b). Even though there is still a lack of information regarding their behaviour in the environment, restrictions over other FRs might cause a raise of their production and use, thus their environmental impact is expected to expand. All these compounds alter bird behaviour, malformations in chicks and reduce egg shell thickness (Morales et al., 2012). Furthermore, they reduce reproductive viability and thus having a critical effect on bird populations (Helander et al., 2002).

Given the above, the aim of this work was to evaluate possible time trends in the concentrations of classical and alternative FRs in three bird species from Doñana Natural Space (south-western Spain). This protected area is classified as World Heritage Site for UNESCO and is a renowned bird sanctuary hosting more than 300 different species. Thus it is relevant to evaluate if legal restrictions over PBDEs have had an effect on local environmental concentrations. On the other hand, since alternative FRs are supposed to become substitutes of PBDEs, it is important to see if their levels have increased since PBDEs were banned. Previous studies have proved the bioaccumulation and possible biomagnification of FRs in birds from Doñana Natural Space (Barón et al., 2014b; Guerra et al., 2011, 2012; Muñoz-Arnanz et al., 2011a,b), which shows the need of further surveillance studies since FRs have been found in more than 10 different bird species.

2. Materials and methods

2.1. Sampling

Several bird eggs that had failed to hatch were collected in 1999, 2003, 2011 and 2013. Egg samples were collected opportunistically during nest checking and chick ringing operations, so that the number of samples per species depended on local abundance in each sampling year (Fig. 1). Eggs were frozen and sent to the laboratory in individual and protected containers. Three different species of three different families were sampled: one falconiiform, the black kite (*Milvus migrans*), one circoniiform, the white stork (*Ciconia ciconia*) and one phoenicopteriform, the greater flamingo (*Phoenicopterus roseus*). In 1999, 10 samples of black kites and 10 of white storks were collected; 10 samples of greater flamingos were collected in 2003; while 39 and 29 samples were collected in 2011 for black kites and white storks, respectively. Finally, 10 samples of greater flamingos were collected in 2013.

Black kite is a predator and scavenger. It feeds young and sick rabbits, birds (especially young), reptiles, amphibians, fish (usually dead, dying or diseased), insects, carrions, remains of human food, and even road killed animals. Their clutch size is normaly of 2 or 3 eggs and its length does not exceed 50–60 cm. White stork feeds on red-swamp crayfish, large insects, rodents, snakes, lizards, snakes, frogs, fish, bird eggs and nestlings and remains of human food. Normally they lay four eggs and their size averages 120 cm. Greater flamingo feeds on small shrimp, seeds, blue–green algae, microscopic organisms and mollusks. They lay only one egg and have a size of 110–150 cm.

2.2. Sample treatment

Egg samples were measured and broken and their content was weighed, homogenized and freeze-dried afterwards. Lyophilized samples were weighed and homogenized again and stored at −20 °C until analysis.

The sample extraction method applied was based on our previous work (De La Cal et al., 2003; Eljarrat et al., 2007) and it is described in detail in the SI. Briefly, 1 g of sample was spiked with internal standards and extracted by pressurized liquid extraction (PLE). After lipid content determination, extracts were treated with

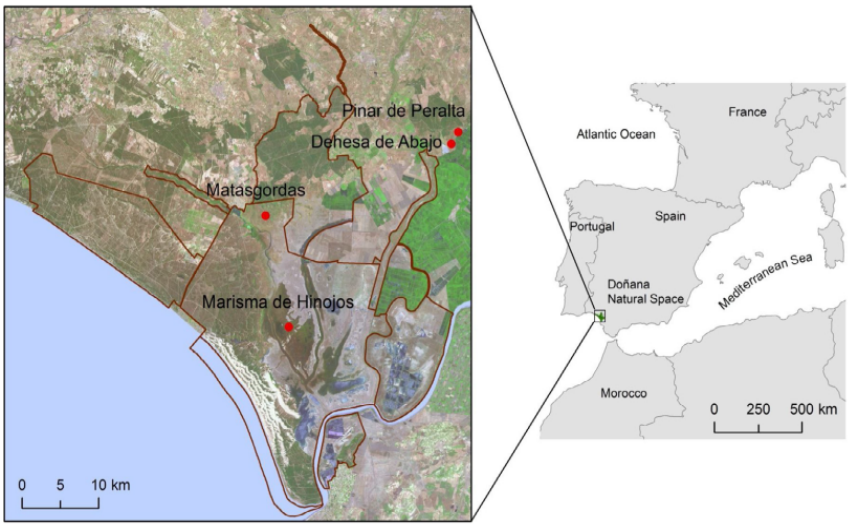

Fig. 1. Location of Doñana Natural Space and sampling sites. Great flamingos: Marisma de Hinojos (20 eggs); Black kites: Matasgordas (39 eggs); White storks: Dehesa de Abajo and Pinar de Peralta (49 eggs). Red line: Doñana Natural Space limits. (Map generated by LAST-EBD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

H_2SO_4 and clean-up was performed using solid phase extraction (SPE). Resulting extracts were evaporated to incipient dryness and concentrated to a final volume of 40 μL.

2.3. Instrumental analysis

Analysis of PBDEs and HNs were performed by gas chromatography coupled to tandem mass spectrometry following previously optimized methodologies, described in detail in the SI (Barón et al., 2012, 2014a). PBDEs were analysed using GC–MS–MS using electron ionization (EI) and HNs were analysed by negative chemical ionization (NCI) using CH_4 as reagent gas. HBCD was analysed by liquid chromatography coupled to tandem mass spectrometry using electrospray ionization (ESI), following the protocol optimized by Guerra et al. (2008) (Table S1). In all cases selecting reaction monitoring (SRM) was used, monitoring the two most intense transitions. First transition was used for quantification and second transition was used for confirmation.

2.4. QA/QC

Recoveries, method detection limits (MDLs) and method quantification limits (MQLs) are given in Table S1. Recoveries were evaluated by spiking 6 replicate samples of a pool of several commercial eggs with 10 ng of PBDEs, HBB, PBEB, DBDPE, HNs and HBCD (α-, β- and γ-isomers) and extracted following the procedure described in Section 2.3. Three procedural blank samples were made and the concentration in these samples never exceeded the 4% of the spiked concentration. MDL was defined as the concentration which gave a signal to noise ratio (S/N) of 3, while MQL was defined as the concentration which gave a S/N of 10.

2.5. Data analysis

Data were tested for normality and homogeneity of variances using the Shapiro–Wilks test of normality and an *F* test. Data were log-transformed to meet normality criteria when they were not normally distributed. Differences between time periods and species were tested using a *t*-test or an ANOVA combined with Tukey's test, using p-value $\leqslant 0.05$ to determine statistical difference. Statistical analyses were conducted using the open-source statistical programming language R v.3.1.1 (http://cran.r-project.org).

3. Results and discussion

3.1. Classical and alternative FR levels

Seven PBDEs (BDE-47, BDE-99, BDE-100, BDE-154, BDE-153, BDE-183 and BDE-209) were detected in the three species. ∑-PBDEs ranged from 32.2 to 311 ng/g lw and from 2.05 to 251 ng/g lw in white storks from 1999 and 2011, respectively. In black kites, values ranged from 7.80 to 259 ng/g lw and from 1.80 to 172 ng/g lw in 1999 and 2011, respectively. Finally, in greater flamingos values ranged from nd to 78.9 ng/g lw and from nq to 25.9 ng/g lw in 2003 and 2013, respectively (Table 1). No significant differences were found between concentrations in white stork and black kites or between concentrations in black kites and greater flamingo, whereas concentrations in white storks were significantly higher than in greater flamingo (ANOVA and post hoc Tukey's test, $p < 0.05$). This profile was the same for the two time periods. Interestingly, BDE-209 was the most abundant compound in all cases. Even though the bioaccumulation capacity of BDE-209 was considered low, recent studies have shown that it can dominate PBDEs contamination profile in animals with a terrestrial based diet (Barón et al., 2014b; Muñoz-Arnanz et al., 2011a,b). Our results for white storks are within the same range of values previously reported for this species from Doñana Natural Space, *i.e.* 2.92 ng/g lw to 129 ng/g lw (Muñoz-Arnanz et al., 2011a,b) and nq to 90.7 ng/g lw (Barón et al., 2014b). Regarding black kites, previous work in Doñana Natural Space reported levels ranging from nd to 39.7 ng/g lw (Barón et al., 2014b), which are in the lower portion of the range reported in this study. To our knowledge, this is the first report of PBDEs in greater flamingos.

HBB, PBEB and DBDPE were not detected in any sample. Dec 602, Dec 603 and both isomers of DP were detected in all three species while Dec 604 was not detected in any sample. ∑HNs in white storks ranged from 41.7 to 121 ng/g lw and from 0.92 to 260 ng/g lw in 1999 and 2011, respectively. In black kites, values

Table 1
Levels (Median [range]) of PBDEs, HNs and HBCD (ng/g lw) found in the three species between 1999 and 2013.

	White stork		Black kite		Greater flamingo	
	1999	2011	1999	2011	2003	2013
BDE-47	16.3 (nd–26.6)	4.29 (nd–21.3)	10.6 (nq–63.9)	4.02 (nd–28.0)	28.2 (nq–28.6)	–
BDE-99	56.2 (nd–148)	3.32 (nd–16.9)	16.0 (nd–35.4)	11.5 (nd–20.2)	4.45 (nq–20.6)	7.68 (nd–22.7)
BDE-100	14.9 (nd–49.1)	5.25 (nd–22.9)	5.82 (nq–58.2)	7.11 (nd–48.5)	–	–
BDE-154	3.12 (nd–7.65)	2.70 (nd–2.8)	8.82 (nd–21.8)	5.25 (nd–30.4)	–	–
BDE-153	8.16 (nd–10.8)	1.64 (nd–4.82)	6.03 (nq–36.7)	6.77 (nd–43.8)	4.81 (nd–6.82)	2.72 (nd–7.72)
BDE-183	4.00 (nd–5.11)	5.26 (nd–6.97)	2.29 (nq–2.77)	5.11 (nd–8.46)	–	–
BDE-209	57.0 (nq–98.5)	31.6 (nq–235)	184 (nq–254)	15.3 (nq–47.4)	11.8 (nq–51.1)	8.18 (nq–10.5)
∑-PBDEs	79.0 (32.2–311)	41.4 (2.05–251)	55.0 (7.80–259)	19.5 (1.80–172)	11.0 (nd–78.9)	8.95 (nq–25.9)
Dec 602	48.7 (nd–64.2)	34.5 (nd–138)	31.1 (nq–74.9)	14.9 (nd–32.7)	29.2 (8.98–204)	29.2 (5.24–115)
Dec 603	20.2 (nd–40.4)	6.57 (nq–54)	1.95 (1.00–15.9)	9.95 (0.71–29.3)	5.51 (2.09–44.6)	9.42 (nq–47.5)
syn-DP	24.5 (nq–79.7)	5.92 (0.44–45.3)	3.77 (nq–5.04)	3.67 (nd–10.6)	–	–
anti-DP	4.90 (nq–25.6)	11.4 (0.48–73.1)	1.00 (nd–9.48)	5.21 (nq–24.3)	–	1.12 (nq–1.12)
∑-DP	23.5 (nq–79.7)	18.7 (0.92–102)	1.00 (nq–11.9)	7.33 (nq–34.4)	–	1.12 (nq–1.12)
f_{anti}	0.42 (0.13–1.00)	0.68 (0.02–1.00)	1.00 (0.47–1.00)	0.71 (0.57–1)	–	–
∑-HNs	90.3 (41.7–121)	56.2 (0.92–260)	16.9 (1.44–75.9)	18.7 (5.38–74.4)	33.3 (8.98–249)	37.6 (5.24–162)
α-HBCD	6.59 (3.82–13.6)	3.23 (nd–27.8)	4.63 (1.32–6.79)	4.47 (nd–18.8)	2.00 (nd–4.73)	3.25 (nd–5.32)
β-HBCD	1.20 (nd–4.76)	0.93 (nd–10.5)	0.79 (nq–2.20)	0.69 (nd–1.18)	–	–
γ-HBCD	3.24 (1.32–17.6)	1.35 (nd–0.97)	3.51 (nq–7.05)	1.39 (nd–1.88)	1.45 (nd–1.50)	–
∑-HBCDs	10.8 (6.89–35.9)	3.19 (nd–36.7)	7.66 (4.87–14.2)	4.42 (0.18–19.5)	2.51 (nd–5.23)	3.25 (nd–5.32)

PBDEs: polybromodiphenyl ethers. HNs: halogenated norbornenes. HBCD: hexabromocyclododecane. nd: not detected (below MDL). nq: not quantified (below MQL).

ranged from 1.44 to 75.9 ng/g lw and from 5.38 to 74.4 ng/g lw in 1999 and 2011, respectively; while in greater flamingos, ∑-HNs ranged from 8.98 to 249 ng/g lw and from 5.24 to 162 ng/g lw in 2003 and 2013, respectively (Table 1). No significant inter-species differences were observed in 1999, while white storks presented higher values than black kites in 2011 (ANOVA and post hoc Tukey's test, $p < 0.05$). Dec 602 was the most abundant compound in all cases, with contributions between 32% and 100% of the total HN burden. Besides, DP was only detected in one greater flamingo egg from 2013. Levels found in white storks and black kites are in the same range of those reported by Barón et al. for the same area: nd-260 ng/g lw and 7.51–74.4 ng/g lw for white storks and black kites, respectively (Barón et al., 2014b). Muñoz-Arnanz et al. reported DP values ranging from 0.35 to 6.39 ng/g lw in white storks from this area, which is in the lower range of the concentrations found in this study for DP (Table 1) (Muñoz-Arnanz et al., 2011a,b). As for PBDEs, there are no other published levels of HNs in greater flamingo.

Ratio between BDE-209 and DP was evaluated when both compounds were detected (range [median]). Values ranged from 0.99 to 63 (2.17) and from 0.10 to 50 (1.88) in white storks from 1999 and 2011, respectively. In black kites values ranged from 0.02 to 33 (1.52) and from 1.28 to 25.5 (17.5) in 1999 and 2011, respectively. No differences were found in white storks ($t = 1.234$, d.f = 43, $p > 0.05$) whereas values in black kites were higher in 1999 than in 2011, due to the decrease on the levels of BDE-209 ($t = 2.14$, d.f = 30, $p > 0.05$). Ratio between these two compounds is used to evaluate if restrictions over BDE-209 have caused an increase in the use of DP (Barón et al., 2014c; Yang et al., 2012).

Moreover, f_{anti} ([*anti*-DP]/[∑-DP]) was evaluated in black kites and white storks (DP was not detected in greater flamingo) (Table 1). In the commercial mixture, a f_{anti} value between 0.65 and 0.75 has been described, but this ratio can change in the environment due to the different behaviour of *syn*- and *anti*-isomers (Xian et al., 2011). Thus, f_{anti} gives information of the behaviour of DP isomers in the environment and it is used to characterise differences in metabolisms within different species, or the different behaviour of DP isomers in sediment and biota (Sverko et al., 2011). No significant differences were found in samples from the same specie between 1999 and 2011 but, interestingly, f_{anti} values in white storks were significantly lower than values in black kites, both in samples from 1999 ($t = 2.399$, d.f = 13, $p < 0.05$) and 2011 ($t = 2.965$, d.f = 63, $p < 0.05$). Since these species belong to different families and may differ in diet, physiology and location of their wintering areas, this difference could be attributed to their habitat use and diet, similarly to what Chen et al. reported for different bird species from China (Chen et al., 2013). Other values of f_{anti} in white stork and black kite have been reported in this area: Baron et al. reported values ranging from 0.44 to 0.81 (mean 0.65) in white storks and from 0.59 to 0.77 (mean 0.68) in black kites (Barón et al., 2014b) where no significant difference was observed, while Muñoz-Arnanz et al. reported values ranging from 0.40 to 0.87 (median 0.68) also in white storks (Muñoz-Arnanz et al., 2011a,b). This was not surprising since behaviour of DP isomers is likely to be consistent through time.

The three main isomers of HBCD (α-, β-, γ-) were detected in white stork and black kite, while in greater flamingo only α-HBCD was detected. White stork was the most contaminated species, with values ranging from 6.89 to 35.9 ng/g lw and from nd to 36.7 ng/g lw in 1999 and 2011, respectively. No inter-species differences were found in the archived eggs (ANOVA and post hoc Tukey's, $p > 0.05$) but, on the other hand, in the contemporary eggs levels in white stork and black kite were similar between them and higher than in greater flamingo in both cases (ANOVA and post hoc Tukey's, $p < 0.05$). ∑-HBCD levels in black kites ranged from 4.87 to 14.2 ng/g lw in 1999 and from 0.18 to 19.5 ng/g lw in 2011. Finally, the less contaminated species was the greater flamingo, with concentrations ranging from nd to 5.23 ng/g lw and from nd to 5.32 ng/g lw in 2003 and 2013, respectively. Our values were considerably lower than the values reported for peregrine falcon eggs from Spain, which ranged from 0.90 to 1600 ng/g lw (Guerra et al., 2012). However, the peregrine falcon is mainly specialized in hunting other birds and different values are thus expectable. In fact, the aquatic-based diet of greater flamingo might explain that only α-HBCD was detected. Different profiles of the three isomers of HBCD have been associated to terrestrial or aquatic diet (Guerra et al., 2012). To date, no other data of HBCD are available for this area.

3.2. PBDE time trends

Although the intra-specific variability was high, this is a common factor in this type of assessment. This fact has been reported in other studies about lipophilic contaminants and is usually

attributed to differences in diet composition (Voorspoels et al., 2007). In birds, factors such as age, body condition and habitat may affect the contaminants accumulated by the female, which are transferred to the egg (Herzke et al., 2002). The fact that there is no information regarding the parents' diet or age makes it difficult to control for such factors, so the results must be interpreted with caution. Total PBDEs levels were similar in the two sampling periods for the three species studied, with no significant differences observed (Table 1). However, some trends involving the main components of the Penta-BDE mixture (BDE-47, BDE-99 and BDE-100) could be observed (Fig. 2). In white storks, ∑-Penta-BDEs ranged from 5.23 to 220 ng/g lw and from 1.73 to 28.5 ng/g lw in samples from 1999 and 2011, respectively, which represented a significant decrease ($t = 3.665$, d.f = 37, $p < 0.05$). In black kites, values ranged from 2.94 to 149 ng/g lw and from 0.54 to 67.2 ng/g lw in 1999 and 2011, respectively. Whereas in this case the decrease was not significant ($t = 1.959$, d.f = 32, $p = 0.06$), it might be obscured by the statistical noise given by a high variability observed in this species. Finally, in greater flamingos values ranged from 3.64 to 49.2 ng/g lw and from 2.33 to 22.7 ng/g lw in 2003 and 2013, respectively. No trend was observed in this case, with rather similar values ($t = 0.543$, d.f = 10, $p > 0.05$).

Moreover, some trends were observed for BDE-209 as well. BDE-209 represents up to 99% of Deca-BDE mixture (Alaee et al., 2003) which has been the most used BDE mixture for many years. In white storks, BDE-209 values ranged from nq to 98.5 ng/g lw and from nq to 235 ng/g lw in 1999 and 2011, respectively, but no clear trend was observed ($t = 0.183$, d.f = 45, $p > 0.05$). On the other hand, BDE-209 levels in black kites from 1999 were significantly higher than the ones found for individuals from 2011, with values ranging from nq to 254 ng/g lw and from nq to 47.4 ng/g lw, respectively ($t = 10.2$, d.f = 29, $p < 0.05$). Finally, values found in the greater flamingo ranged from nq to 51.1 ng/g lw and from nq to 10.5 ng/g lw in 2003 and 2013, respectively, and as for black kites levels in archived eggs were higher than in contemporary eggs ($t = 3.324$, d.f = 10, $p > 0.05$). This decrease could indicate that the use of BDE-209 has also been reduced, as has been documented by other authors (Sun et al., 2015; Yu et al., 2009).

Restrictions in the use of Penta-BDE mixture seem to yield a general decrease in the concentrations of its components in the environment (Law et al., 2014). In particular, Crosse et al. (2012) reported a decrease in the levels of BDE-47, BDE-99 and BDE-100 in bird eggs collected offshore the U.K. between 1994 and 2002. Furthermore, the increase of higher brominated PBDEs was also reported and considered a cause of concern (Crosse et al., 2012). This is in agreement with this study, where only the low-brominated congeners showed a significant decrease. The same trend was observed by Miller et al. (2014), who analysed eggs of two seabird species from the Pacific coast of Canada collected from 1990 to 2011. PBDEs presented a high peak in the year 2000 and then started to decline, especially the components of the Penta-BDE mixture. Interestingly, this trend was observed in eggs sampled close to the coast of North America but not in eggs collected in a population close to Asia, where no legal restrictions over PBDEs have been applied yet. This could be evidence of the effectiveness of the legal restrictions over Penta-BDE mixture (Miller et al., 2014). Sutton et al. reported a decrease in BDE-47 in bivalves and aquatic birds in San Francisco bay between 2002 and 2012, with declines up to 80%. However, there was not a clear trend regarding levels of BDE-209 (Sutton et al., 2014). Finally, a study on barn owls (*Tyto alba*) from Belgium and France reported that PBDE concentrations decreased between 2003/2004 and 2008/2009, corresponding with the EU bans on Penta-BDE and Octa-BDE mixtures (Eulaers et al., 2014). Our results suggest that this trend is also occurring in birds breeding in Doñana Natural Space and to the best of our knowledge this is the first study of this kind carried out in Southern Europe.

3.3. HNs time trends

As shown in Section 3.1, levels of HNs did not change significantly from 1999 (2003 for greater flamingo) and 2011 (2013 for greater flamingo). Underlying trends may have been obscured by high intra-specific variability, which was high even within years. Nonetheless, when looking at each compound separately, two different trends for Dec 603 were found. Levels of this compound in white storks were higher in 1999 than in 2011 ($t = 2.026$, d.f = 44, $p < 0.05$), whereas the reverse was true for black kites, whose levels of Dec 603 were higher in 2011 than in 1999 ($t = 2.960$, d.f = 38, $p < 0.05$). Moreover, ratio between HNs and PBDEs concentration in each sample was compared (Fig. 3). Despite the high variability, values in the contemporary eggs were higher than in archived eggs, although these differences were not statistically significant ($t = 1.382$, d.f = 45 for white stork; $t = 1.087$, d.f = 37 in black kite; $t = 0.844$, d.f = 16 in greater flamingo, $p > 0.05$ in all cases). In particular, median values in white storks were 0.63 and 1.31 in 1999 and 2011, respectively; for black kites, values were 0.34 and 0.95 in 1999 and 2011, respectively; and for greater flamingos values were 3.34 and 1.80 in 2003 and 2013, respectively. This might be due to the slight increase of HN levels and the decrease of PBDE levels and consequently, due to the legal restrictions over PBDEs, this ratio should become even higher in the future.

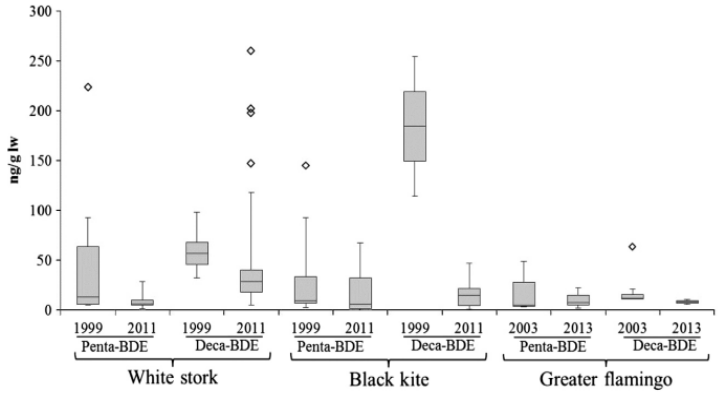

Fig. 2. Box plot of the levels (ng/g lw) of Penta-BDE and Deca-BDE mixtures in the three species studied, for the two time periods.

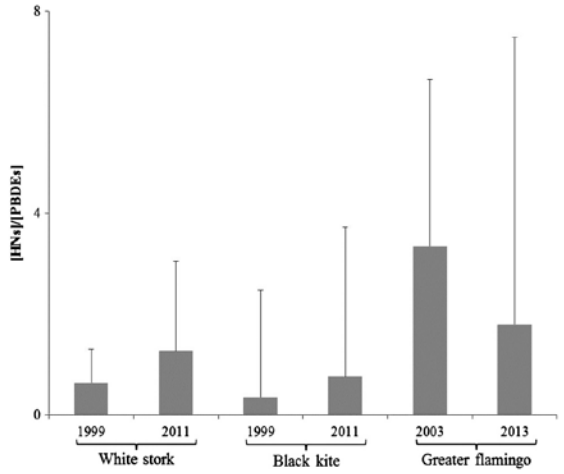

Fig. 3. Ratio of lipid normalized concentrations of HNs and PBDEs for the three species in the different sampling years (median values + standard deviation).

Even if HNs have been used for many years in North America, the fact that they are considered a low production volume chemical in Europe would explain why the levels in this 10 year gap were similar in almost all the cases and always lower than levels reported in bird eggs from Canada through these years (Gauthier et al., 2007; Guerra et al., 2011). Since DP has been proposed as an alternative to BDE-209, the main component of Deca-BDE mixture, its levels are expected to increase in the near future.

Our results are in agreement to what Zhu et al. reported in Indo-Pacific humpback dolphins (*Souse chinensis*) and finless porpoises (*Neophocaena phocaenoides*) from the South China Sea. Even if the comparison with this study must be done with caution since the species studied are considerably different, it is interesting to see that Zhu et al. found a positive temporal trend of the ratio of DP to BDE-209 between 2003 and 2012. This was assumed as evidence of a use-shift from PBDEs to other alternatives such as DP in China, even if there are not restrictions or regulations on the use of PBDEs in Asia (Zhu et al., 2014). In this study BDE-209/DP ratio was not significantly different between the two time periods neither for white stork nor black kite, despite BDE-209 concentrations decreased considerably in black kites. To our knowledge this is the first study of HNs time trends in birds from Europe.

3.4. HBCD time trends

No significant differences were found for $\sum$-HBCD between 1999 and 2011 (white stork and black kite) or between 2003 and 2013 (greater flamingo). Thus, no time trend was observed for HBCD, which is in contrast to what Miller et al. reported for storm petrel and rhinoceros auklet eggs from the northern Pacific, where a positive time trend between 1990 and 2011 was found. However, they did not find a clear time trend in eggs of storm petrel from a different sampling site from the northern Pacific (Miller et al., 2014), which shows that evaluation of time trends must be done with caution since lots of factors such as food sources, different feeding ecology or migration can affect the concentrations accumulated in the eggs (Lavoie et al., 2010). On the other hand, levels of HBCD decreased in ivory gulls (*Pagophila eburnea*) between 1976 and 2004 (Braune et al., 2007). Overall, time trends for HBCD present both increases and decreases depending on the matrix and location, although levels seem to be increasing in predatory birds (Law et al., 2014). Thus, more data are needed in order to gain a clear picture of the real behaviour of HBCD through time.

Furthermore, contribution of α-HBCD to the total HBCD burden was evaluated. Despite α-HBCD represents only the 10% of the commercial HBCD mixture, which is dominated by γ-HBCD with a contribution of about the 75%, it is the isomer most frequently detected in biota (Guerra et al., 2012). Interestingly, values of α-HBCD contribution varied between the two years. In white storks, values were significantly higher ($t = 6.421$, d.f = 36, $p < 0.05$) in 2011 (72–100%) than in 1999 (37–77%). Similarly, for black kites, values in 2011 (69–100%) were significantly higher ($t = 6.501$, d.f = 33, $p < 0.05$) than values in 1999 (27–100%). On the other hand, values of greater flamingo were slightly but not significantly higher ($t = 1.357$, d.f = 16, $p > 0.05$) in 2013 than in 1999 (66–99% and 40–90%, medians of 88–80%, respectively). Since a different metabolic and/or degradation capacity in the same specie is not expected, these results might suggest that the percentage contribution of α-HBCD has increased in the commercial mixture. However, no evidence of this fact has been reported in the literature and other factors (e.g. a change on the diet) could affect this comparison.

4. Conclusions

Several classical and alternative FRs were detected in black kites, white storks and greater flamingos from Doñana Natural Space. PBDEs, HNs and HBCD were detected in archived eggs from 1999 (black kite and white stork) and 2003 (greater flamingo), and also in contemporary eggs from 2011 (black kite and white stork) or 2013 (greater flamingo). Other alternative FRs (PBEB, HBB and DBDPE) were not detected in any sample. Even if total PBDE levels did not show a significant trend in most cases, our results show a decrease of the contribution of the main components of the Penta-BDE commercial mixture to the total PBDE burden. This trend agrees with the ban of this mixture in 2008. Data regarding Deca-BDE mixture showed a decrease in black kites but, since restrictions regarding the production and use of Deca-BDE mixture only started in recent years, further monitoring is needed. Furthermore, HNs levels were similar in both periods. Since DP has been proposed as a substitute of BDE-209, its levels are expected to increase in the near future. Moreover, the presence of PBDEs is still higher than the presence of HNs. However, it is interesting to note that the ratio between HNs and PBDEs in each sample was higher in the contemporary samples, probably due to a slightly increase of HNs and a concomitant decline in PBDEs. Furthermore, whereas HBCD did not show a clear time trend, the percentage contribution of α-HBCD to the total HBCD burden increased between the two time periods, which may be due to a variation of the isomeric composition of HBCD in the commercial mixture, although other factors might affect α-HBCD concentrations.

Even though Penta-BDE levels seem to have declined in the last 10 years, other FRs are still present in high amounts in bird species breeding in an extensive protected area. In addition, HNs levels might increase in the near future, which might result in higher environmental levels. Thus, continued eco-toxicological monitoring of local species seems fundamental in the future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.06.013.

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Chemicals and reagents

Standard PBDE mixture (containing BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209), HBB, DBDPE and PBEB were purchased from Wellington Laboratories (Guelph, ON, Canada). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical (Toronto, ON, Canada). α-, β- and γ-HBCD and its deuterated congeners, together with *syn*- and *anti*-isomers of DP were obtained from Cambridge Isotope Laboratories (Andover, MA). Mass labelled PBDE mixture (containing ^{13}C-BDE-28, ^{13}C-BDE-47, ^{13}C-BDE-99, ^{13}C-BDE-100, ^{13}C-BDE-153, ^{13}C-BDE-154, ^{13}C-BDE-183 and ^{13}C-BDE-209) was purchased from Wellington Laboratories, while ^{13}C-*syn*-DP was purchased from Cambridge Isotope Laboratories. Mass labelled compounds were used as surrogate standards. Sulphuric acid was purchased from Sigma-Aldrich (St. Louis, USA). Al-N cartridges were obtained from Biotage (Uppsala, Sweeden). Dichloromethane (DCM), hexane and toluene were purchased from Merck (Darmstadt, Germany).

Sample treatment

Sample (1.5 g dry weight (dw)) was spiked with 5 ng of ^{13}C-PBDEs (50 ng of ^{13}C-BDE-209), 5 ng of d_{18}-HBCD (α-, β-, γ-), and 5 ng of ^{13}C-*syn*-DP and kept overnight to equilibrate. Extraction was carried out by pressurised liquid extraction (PLE) using an Accelerated Solvent Extraction system (ASE 350, Dionex). Hexane and DCM (1:1) were used as extraction solvents in 2 static cycles of 10 min at 100 ºC and 1500 psi. Afterwards, lipid content was determined gravimetrically and the resulting extracts were re-dissolved in hexane and treated with H_2SO_4 (conc.) to remove fat. Then, the organic phase was cleaned by solid phase extraction (SPE) using Al-N (5 g) cartridges, previously conditioned with 20 mL of hexane and eluted with 20 mL of hexane:DCM (1:2). Extracts were evaporated to incipient dryness and reconstituted in 40 µL of toluene prior to the instrumental analysis.

Instrumental analysis

Analysis of PBDEs, HBB, PBEB and DBDPE were performed by gas chromatography coupled to tandem mass spectrometry using an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quadrupole, except for HBCD. A DB-5MS (15 m, 0.1 i.d, 0.1 µm) was used for the chromatographic separation. In order to enhance

sensitivity and selectivity, selected reaction monitoring (SRM) was applied in all cases, monitoring the two most intense transitions for each compound. The most intense transition was used for quantification purposes and the second one was used for confirmation criteria (Table S1). Brominated flame retardants (PBDEs, PBEB, HBB and DBDPE) were analysed using electron ionization (EI) mode. The temperature program started at 140 ºC, held for 1 min, then ramped to 310 at 10 ºC/min and held for 10 min, for a total run time of 36.5 min. The source temperature was set at 250 ºC. Quantification was done by isotope dilution for PBDEs and using similar surrogate standards for HBB (^{13}C-BDE-153), PBEB (^{13}C-BDE-28) and DBDPE (^{13}C-BDE-209). Halogenated norbornenes were analysed using negative chemical ionization (NCI) mode with CH_4 as reagent gas. Temperature program started at 80 ºC, was held for 2 min and then ramped to 300 ºC in 10 ºC/min. The final temperature was maintained for 10 min for a total run time of 34 min. The source temperature was set at 175 ºC.

On the other hand, HBCD was analysed by liquid chromatography using an Agilent HP 1100 binary pump LC system (Agilent Technologies, Palo Alto, CA, USA), coupled to a hybrid triple quadrupole/linear ion trap (4000QTRAP, Applied Biosystems, Foster City, CA, USA) equipped with an electrospray (ESI) Turbospray interface. Analytical separation was performed using a Symmetry C_{18} column (2.1 × 150 mm, 5 µm) preceded by a C_{18} guard column (2.1 × 10 mm) supplied by Waters (Milford, MA, USA). The elution program started at an initial composition of 100% A (H_2O:MeOH 3:1 v/v) and was ramped to 10% A in 17 min. Solvent B was MeOH. The initial conditions were reached again in 3 min and maintained for an additional 10 min for a total run time of 30 min. The source temperature was set at 350 ºC.

Table S1: Recoveries, reproducibility, method detection limit (MDL, ng/g lw), method quantification limit (MQL, ng/g lw) and the two transitions used for each compound (SRM_1 and SRM_2).

Compound	R (%)	RSD (%)	MDL	MQL	SRM1	SRM2
BDE-28	82	6.2	0.03	0.09	408>246	408>248
BDE-47	72	5.7	0.03	0.10	486>326	488>328
BDE-100	67	15	0.12	0.41	406>297	564>404
BDE-99	73	9.7	0.13	0.45	406>297	564>404
BDE-154	57	1.9	0.35	1.17	486>377	644>484
BDE-153	53	13	0.31	1.02	486>377	644>484
BDE-183	61	4.7	1.51	5.02	721>562	721>564

BDE-209	57	4.7	3.20	10.7	298>220	361>280
HBB	75	14	0.12	0.39	468>308	468>310
PBEB	70	9.7	0.14	0.47	500>485	485>406
DBDPE	78	7.2	3.54	11.8	485>406	325>165
Dec 602	79	6.2	0.08	0.26	612>35	612>37
Dec 603	85	11	0.06	0.21	638>35	638>37
Dec 604	65	13	0.23	0.78	460>79	504>79
syn-DP	81	5.4	0.03	0.10	654>35	654>37
anti-DP	79	5.9	0.02	0.05	654>35	654>37
α-HBCD	79	4.4	0.15	0.48	639>79	639>81
β-HBCD	81	5.3	0.16	0.53	639>79	639>81
γ-HBCD	74	7.9	0.39	1.30	639>79	639>81

R: Recovery. **RSD**: Relative standard deviation. **MDL**: Method detection limit. **MQL**: Method quantification limit.

4.6. Discusión

En este apartado se discuten los resultados obtenidos en las diferentes especies estudiadas, la información que se ha obtenido sobre el comportamiento de los diferentes HFRs en las diferentes cadenas tróficas, los diferentes perfiles de contaminantes observados y las diferencias temporales observadas.

4.6.1. Resultados en bivalvos y peces

Se detectaron tanto PBDEs como HNs en las diferentes especies estudiadas en la Bahía de Concepción, así como los MeO-PBDEs. Como se ha explicado anteriormente, es sabido que los PBDEs tienen una presencia global, pero este fue el primer estudio donde se determinaron en diferentes especies de moluscos y crustáceos en América del Sur, y el segundo en hacerlo en peces. Por otro lado, la presencia de los HNs aún no había sido estudiada en ninguna zona o especie del continente con la excepción de un estudio en delfines de Brasil (De La Torre *et al.*, 2012) y por lo tanto se aportaron nuevos datos sobre su presencia en diferentes especies de menor nivel trófico. Las diferencias entre especies se discuten en los apartados 4.6.4 y 4.6.5. La presencia de HNs en la zona era muy baja, posiblemente debido a su poco uso en el país chileno.

Los resultados obtenidos en las muestras de biota de Chile fueron utilizados para un estudio de desarrollo y validación de un modelo de evaluación del riesgo de POPs en organismos acuáticos, basado en la lógica difusa. Este trabajo se encuentra publicado, aunque no ha sido incluido en la tesis (Seguí *et al.*, 2013: *Fuzzy model for risk assessment of persistent organic pollutants in aquatic ecosystems*). El modelo se mostró capaz de predecir la situación de riesgo real del ecosistema, utilizando datos obtenidos experimentalmente en combinación con los disponibles en la bibliografía. La principal ventaja que presenta es que permite realizar una evaluación de riesgo en zonas donde es difícil obtener datos experimentales suficientes, comunicando los resultados en un lenguaje comprensible para las instituciones con el poder de tomar medidas. El modelo indicó un riesgo moderado para el caso de la zona muestreada en Chile.

Por otro lado, en el marco del proyecto SCARCE introducido en el capítulo 3 también se dispuso de muestras de peces recolectados en diferentes puntos del Ebro y Llobregat. El trabajo se encuentra publicado aunque el artículo no ha sido incluido en esta tesis (Santín *et al.*, 2013: "*Emerging and historical halogenated flame retardants in fish samples from Iberian rivers*"). Se encontraron por primera vez diferentes HNs en peces

de la Península Ibérica y además en concentraciones a tener en cuenta. A modo de comparativa, en la figura 4.5 se muestran las concentraciones de HNs encontradas en peces, donde se puede ver claramente la diferencia entre Chile (bilagay, castañuela y rollizo) y España (barbo, carpa y siluro). El Dec 602 suele dominar los perfiles de HNs, y además los niveles de Dec 603 parecen ser significativamente más altos en el Llobregat, mientras que para los demás las concentraciones en ambas cuencas son parecidas. Al tratarse de unas diferencias tan significativas no se cree que el hecho de comparar diferentes especies afecte a la comparación entre diferentes países.

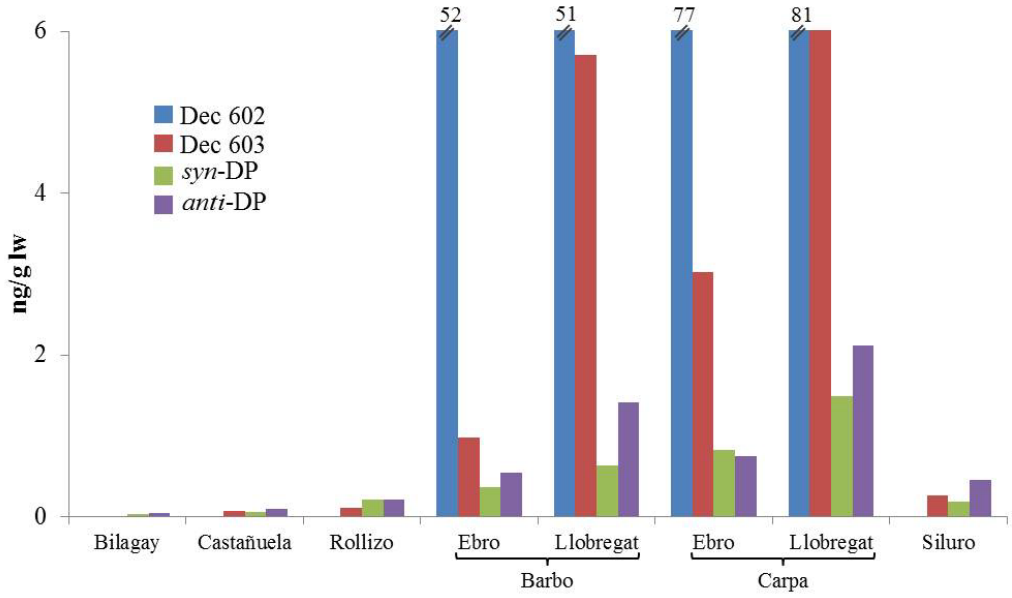

Figura 4.5. Concentraciones de HNs encontradas en peces procedentes de Chile y España.

Así pues, estos estudios parecen indicar la capacidad de bioacumulación de estos nuevos retardantes de llama, con frecuencias de detección entre el 69% y el 100%, con niveles de concentración inferiores a los de los clásicos y ya prohibidos PBDEs en ambas zonas estudiadas, aunque estas diferencias no fueron tan pronunciadas en España posiblemente debido a un mayor uso de esta familia de compuestos. Por otro lado, cabe destacar que prácticamente todos los peces analizados en las 4 cuencas de ríos españoles presentaban concentraciones de PBDEs superiores al límite establecido en la Directiva 2013/39/EU (0,0085 ng/g ww).

4.6.2. Resultados en delfines

En los artículos #6 y #7 se encuentran detallados los 2 estudios llevados a cabo en la zona sur-oeste del Mar Mediterráneo y que aportaron los primeros datos sobre PBDEs,

HBCD, HNs y HNPs en la zona, con la excepción de los aportados por Fossi *et al.*, (2013) en delfines listados, resumidos también en la figura 4.6. Dada la especial importancia de la zona estudiada, con varias demarcaciones de protección marina, el estudio de la contaminación por HFRs aporta datos útiles que pueden ser usados para reclamar más protección en el área o ampliar las demarcaciones ya existentes. Se determinaron elevadas concentraciones tanto de PBDEs como de HNPs y concentraciones relativamente altas de HNs, especialmente del Dec 602, en muestras de grasa obtenidas ya fuera por biopsia o varamientos. Fijándose en los niveles totales de HFRs pueden observarse algunas diferencias entre las diferentes zonas o especies, aunque el hecho de que las muestras del mar de Alborán provengan de varamientos puede afectar a la comparación. De hecho, las concentraciones en esa zona fueron superiores a las encontradas tanto en el Golfo de Cádiz como en el estrecho de Gibraltar en delfines comunes, y superiores a las del estrecho de Gibraltar en el caso del calderón. De todos modos es evidente que la problemática por la contaminación por HFRs existe en las 3 zonas estudiadas y, pese a que los niveles no son de los más elevados documentados mundialmente, sí son considerables. De hecho, varios individuos superaron el umbral establecido a partir del cual existe riesgo de daños en el sistema endocrino de las focas grises, concretamente en la hormona tiroidea (1500 ng/g lw).

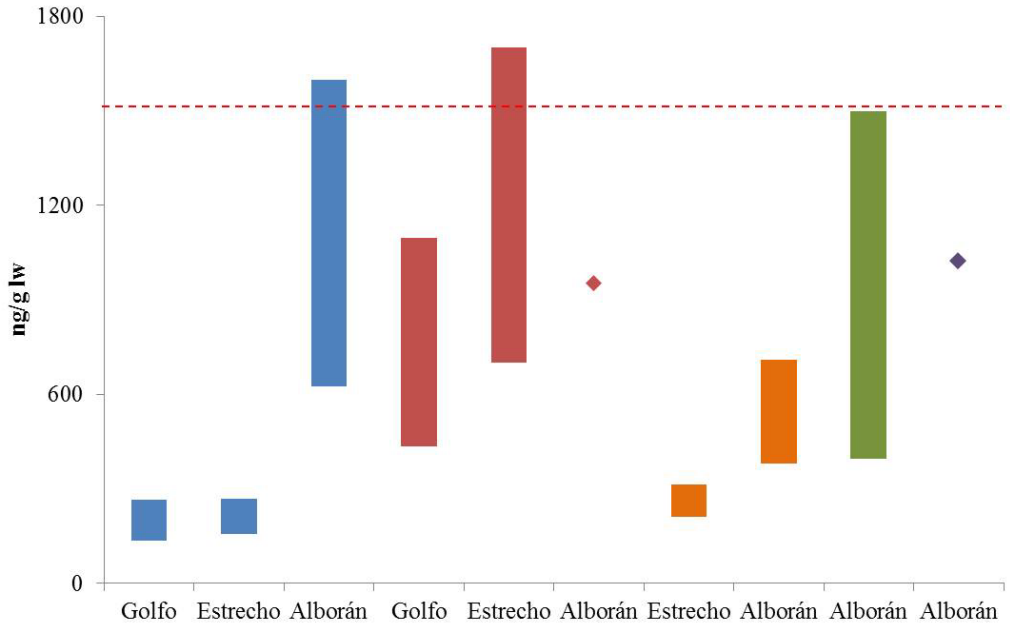

Figura 4.6. Concentraciones determinadas a lo largo de todo el Mediterráneo sur (entre el 1er y 3er cuartil). Azul: delfín común. Rojo: delfín nariz de botella. Naranja: calderón común. Verde: delfín listado. Lila: calderón gris. La línea roja marca el umbral de 1500 ng/g lw a partir del cual puede haber alteraciones en el sistema endocrino.

Los resultados obtenidos en los delfines del Golfo y del Estrecho se integraron posteriormente en un estudio multidisciplinar que tenía como objetivo definir correctamente las unidades de gestión medioambiental de los delfines nariz de botella en el sur de la Península Ibérica. Este trabajo forma parte de la tesis doctoral de Joan Giménez, estudiante de la Estación Biológica de Doñana, y por tanto sólo se hará una breve mención. Utilizando diferentes marcadores genéticos, análisis de isótopos estables, técnicas de identificación fotográfica, así como las concentraciones mostradas en el artículo #6, se concluyó que los delfines de ambas zonas debían ser incluidos en una única unidad de gestión o zona especial de conservación (ZEC). Esto supone un paso adelante en la protección de los delfines nariz de botella, especie listada como vulnerable en el catálogo español de especies amenazadas así como en el anexo II de la directiva de hábitats de la UE. Actualmente el artículo, que también integra concentraciones de otros contaminantes como los PCBs, se encuentra en preparación.

Por otro lado, la mayoría de contaminantes antropogénicos así como compuestos de origen natural se detectaron también en muestras de cerebro de las 5 especies de delfines. Este tipo de datos es muy escaso debido a la dificultad en la obtención de muestras de cerebro, y es muy interesante disponer de ellos para poder valorar la capacidad de estos compuestos para traspasar la barrera hematoencefálica (BBB, del inglés blood-brain barrier) ya que varios de ellos son probados neurotóxicos (ver capítulo 5). En la figura 4.7 se resumen las concentraciones encontradas en cerebro de las 5 especies de delfines incluidas en esta tesis, así como en otras especies analizadas en otros estudios de la literatura. Es evidente que los HNs pueden atravesar la BBB, al igual que los PBDEs, lo que tiene implicaciones considerables en términos de impacto ambiental ya que sus efectos en un órgano tan importante como el cerebro podrían ser críticos. Los PBDEs ya están prohibidos pero no así los HNs, por lo que esta información adicional sobre su presencia en cerebro de un mamífero superior puede contribuir a establecer medidas más estrictas sobre su uso. Además, el ratio PBDEs/HNs presentó un valor muy inferior en cerebro que en grasa, lo que parece indicar que los HNs tienen mayor capacidad para atravesar la BBB que los PBDEs. Como se puede ver en la figura 4.7, esta misma capacidad se muestra al observar las concentraciones de HNs en pollos y peces cercanos a estaciones de e-waste en China, que son realmente muy altas (Corsolini *et al.*, 2014; Zheng *et al.*, 2015).

Por tanto, queda claro que los HNs, especialmente el Dec 602, también están presentes en el Mediterráneo, habiendo sido acumulados por 5 especies diferentes de delfines. En

grasa, el Dec 602 se detectó entre el 50-100% de las muestras (dependiendo de la especie), el Dec 603 entre el 50-92%, el *syn*-DP entre el 70-100% y el *anti*-DP entre el 70-100%. El Dec 604 no fue detectado en ninguna muestra. Además, se demuestra por primera vez que el Dec 602 y Dec 603 son capaces de traspasar la BBB y se aportan los primeros datos sobre este hecho en delfines sobre el DP, aunque tanto las concentraciones como la frecuencia de detección fueron inferiores a las detectadas en grasa.

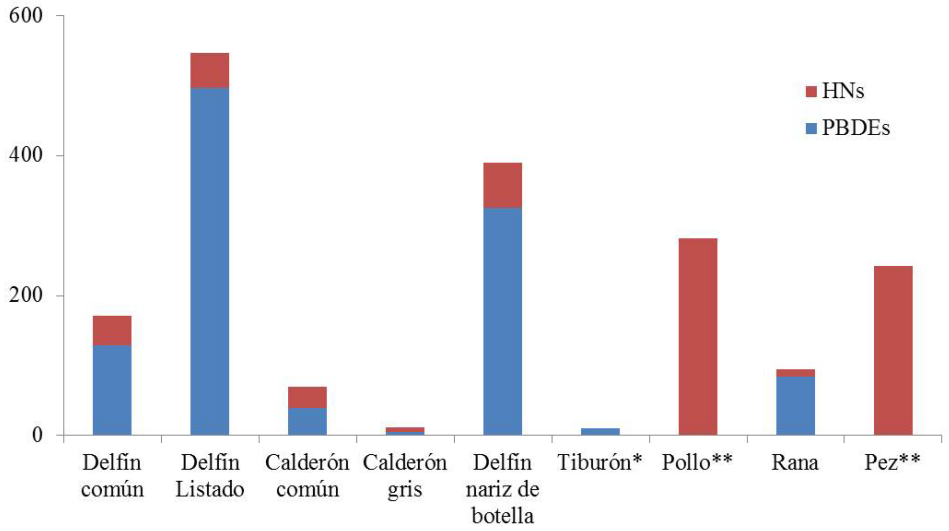

Figura 4.7. Concentraciones medianas en cerebro de varias especies. *: Sólo analizados PBDEs. **: Sólo analizados HNs. Tiburón: Corsolini *et al.* 2014. Pollo: Zheng *et al.* 2014. Rana: Li *et al.* 2014. Pez: Peng *et al.* 2014.

Por último, no se debe olvidar a los HNPs, que son compuestos producidos de manera natural y cuyos efectos en el organismo de las diferentes especies marinas aún no están muy claros. No obstante, son compuestos que siempre estarán ahí y no se puede hacer nada para evitarlo, a diferencia del caso de los HFRs que son contaminantes antropogénicos cuya presencia se debe por completo a la actividad humana. Las concentraciones de HNPs encontradas en el mar de Alborán son superiores a las de los HFRs al completo, cosa que presumiblemente sería el caso también en el Golfo de Cádiz y el Estrecho de Gibraltar si se hubieran incluido todos los HNPs estudiados en el artículo #7. De hecho, sólo con la aportación de los MeO-PBDEs las concentraciones de ambos eran prácticamente parejas. Por tanto, a un medio con una elevada presencia de compuestos halogenados posiblemente nocivos en algún grado se le está añadiendo una elevada cantidad de compuestos adicionales, cosa que además no parece tener fin ya que el uso de los HNs no está prohibido y los PBDEs tienen una elevada persistencia. Pese a

que es difícil llevar a cabo estas comparaciones dado el gran número de compuestos que no son incluidos por ambos bandos, queda claro que los HFRs acumulados en delfines lo hacen en organismos que ya soportan una elevada carga de HNPs.

4.6.3. Resultados en huevos de aves

Los artículos #8 y #9 recogen los trabajos llevados a cabo en la zona del Parque Nacional y Natural de Doñana. Se identificaron tanto PBDEs, HBCD y HNs en conjunto en prácticamente todas las especies, demostrando la presencia de estos contaminantes en el Parque. Este hecho es bastante significativo, ya que es una zona con muy poca actividad humana y por tanto la presencia de HFRs en la zona debería ser residual. De hecho, al analizar sedimentos y diferentes especies de peces y crustáceos pequeños de la misma zona no se detectaron más que concentraciones muy bajas de PBDEs (datos no publicados). Tanto el sedimento como las especies que habitan en los riachuelos de Doñana sólo podrían haber acumulado HFRs mediante el transporte atmosférico (considerando la aportación humana como residual) y por ello las concentraciones eran bajas. En cambio, las aves estudiadas pueden desplazarse para obtener alimento y algunas como las gaviotas o cigüeñas incluso se alimentan de basura. Por otro lado, muchas de ellas son especies migratorias que pasan periodos de su vida tanto en el Norte de Europa como en África, por lo que podrían haber acumulado estos contaminantes en otras zonas y ser ellas el vector de entrada de los mismos al Parque, por lo menos en parte. En muestras de medio acuático las concentraciones de PBDEs eran superiores a las de HNs, mientras que en aves los niveles hallados mostraban una mayor igualdad. Se observó además que el DP y los PBDEs presentaban una buena correlación, cosa que podría indicar una fuente común, mientras que no ocurría así con el Dec 602 o Dec 603.

4.6.4. Bioacumulación y biomagnificación

En este apartado se estudia en detalle la capacidad de bioacumulación y biomagnificación de los HFRs y HNPs, basándose en los datos obtenidos en los diferentes trabajos presentados en el capítulo y ampliando con algunos datos hallados también en la bibliografía. La bioacumulación se expresa de manera cuantitativa mediante el BSAF, y la biomagnificación mediante el BMF o la correlación con la $\delta^{15}N$.

BSAF

El hecho de encontrar cualquier compuesto en biota ya implica que tiene capacidad de bioacumulación, aunque sea a baja escala. No obstante, la manera de cuantificar esta capacidad es mediante el BSAF. Este factor se calculó cuando fue posible en el Ebro y el Llobregat, aunque el bajo número de muestras de pez limitó el estudio. En la tabla 4.1 se resumen los valores obtenidos para los HNs y se comparan con los disponibles de otros estudios en otras regiones. Para hacerse una idea, los valores calculados en Ebro y Llobregat en PBDEs fueron de 7 (carpa) y 17 (barbo) para el BDE-47 y 0,02 (barbo) para el BDE-209. Al comparar estos valores con los calculados para los HNs vemos como la capacidad de bioacumulación del Dec 602 es comparable a la del BDE-47, mientras que la del Dec 603 y DP es superior a la del BDE-209 pero igualmente baja. Este hecho también ha sido observado en los grandes lagos de Canadá (Shen *et al.*, 2011; Tomy *et al.*, 2008) y en China, donde se observaron BSAFs hasta 6 veces más altos para el Dec 602 que para el DP (Jia *et al.*, 2011; Wu *et al.*, 2010; Zhang *et al.*, 2011). Esto confirma la tendencia vista en biota donde casi siempre el Dec 602 es más abundante que el DP, cosa que podría indicar que el Dec 602 presenta mayor capacidad de bioacumulación (Wang *et al.*, 2012), aunque sería necesario conocer también las cantidades emitidas al medio ambiente de cada uno de ellos así como evaluar otros factores, como la dieta o la capacidad metabólica de las especies, que pueden crear gran variabilidad.

Tabla 4.1. BSAF reportados en bibliografía y comparados con los calculados utilizando los resultados obtenidos en esta tesis.

	Ebro y Llobregat		**Dailing (China)[a]**	**Grandes Lagos[b]**	**Huanghai (China)[c]**	**Canadá[d]**	**Pearl (China)[c]**
	Barbo	Carpa	Viruelas	Trucha	Ostras	Trucha**	Pez gato
Dec-602	0,02 - 11	9,51	4,7	270	2,1 – 12	n.d	n.d
Dec-603	0,69	-	n.d	12	n.d	n.d	n.d
Dec-604	-	-	n.d	4,5	n.d	n.d	n.d
syn-DP	0,14 - 0,23	0,27	0,88	0,8	1 – 8*	5,2	0,1*
anti-DP	0,04 - 0,09	0,1	0,33	0,3	1 – 8*	1,9	0,1*

[a]Wang *et al.* (2012). [b]Shen *et al.* (2011). [c]Jia *et al.* (2011). [d]Tomy *et al*. (2008). *Calculado para ∑DP. ** Experimento. -: No fue posible calcularlo. n.d: No disponible

Los valores de BSAFs que han ido apareciendo durante el trascurso de esta tesis varían bastante debido a las condiciones de equilibrio entre los sedimentos y especies (con capacidades metabólicas variadas, por ejemplo) muestreadas. No obstante, dos cosas

parecen claras: el Dec 602 presenta mayor capacidad de bioacumulación que el DP, y los datos de este último suelen ser menores que 1 (He *et al.*, 2014; Wang *et al.*, 2012). De todos modos, este hecho no descarta al DP como compuesto a tener en cuenta en biota, ya que pese a tener una acumulación baja en algunas especies éstas pueden acumularlo a lo largo del tiempo, llegándose a concentraciones igualmente altas.

BMF

El único de nuestros estudios donde se disponía de parejas de muestras presa-predador es el realizado en Chile. En él se calcularon los BMFs para diversos HFRs y para los MeO-PBDEs. Curiosamente, el predador utilizado (loco) presentaba niveles de BDE-47 inferiores a sus presas (picoroco y piure), tal vez debido a su capacidad para metabolizarlo. En la otra pareja de predador-presa, el bilagay y la lapa, sí se encontró un valor de BMF para el BDE-47 superior a la unidad, cercano a 14 de hecho, superando con mucho a los determinados para otros PBDEs. Esto no es nuevo (aunque sí en la zona estudiada) ya que varios estudios anteriores habían encontrado BMFs > 1 tanto para PBDEs como para MeO-PBDEs, aunque el caso del BDE-209 fue sorprendente. No obstante, los BMFs > 1 del BDE-209 se encontraron usando el loco como predador, mientras que en el bilagay el BMF > 1 correspondió al BDE-47. Esto podría ser debido a las diferentes características de la especie.

Los HNs no se detectaron en el loco, aunque sí en el bilagay. El BMF calculado fue inferior a 1 para el Dec 602, Dec 603 y ambos isómeros del DP. En la tabla 4.2 se resumen los BMFs existentes en bibliografía para los HNs. En los estudios donde se analizaron también PBDEs, los BMFs fueron del mismo orden, demostrando de nuevo que los HNs tienen un comportamiento parecido en biota a los clásicos HFRs.

Por otro lado, se observó una correlación positiva entre los valores de $\delta^{15}N$ y los niveles de contaminación, tanto en las 2 especies de delfines disponibles en el golfo de Cádiz y estrecho de Gibraltar como en diferentes aves de la familia de las falconiformes de Doñana. Si bien es cierto que no se disponía en ninguno de los casos de muestras de especies en niveles tróficos inferiores, los estudios se consideran indicativos de la capacidad de biomagnificación de PBDEs, HBCD, MeO-PBDEs y Dec 602. Pese a no poder cuantificar esta capacidad utilizando el TMF (ver capítulo 1) debido a este hecho, los resultados demuestran que incluso en un mismo nivel trófico se puede observar un incremento entre especies. De hecho, las pendientes no son muy pronunciadas probablemente por este hecho. Algunos estudios sí pudieron evaluar este parámetro para

los HNs al estar realizados en especies con diferente nivel trófico. Sin embargo, los datos son un tanto ambiguos. Por un lado, algunos estudios parecen indicar que el DP no tiene una capacidad alta de biomagnificación mientras que el Dec 602 sí parece tenerla, presentando TMFs < 1 y > 1 respectivamente (Tomy *et al.*, 2007).

Tabla 4.2. BMFs disponibles en bibliografía para los HNs.

Compuesto	Zona	Predador	Presa	BMF	Referencia
Dec 602	China	Gaviota	Anchoa	1,7 – 3	Peng 2014
Dec 603	China	Gaviota	Anchoa	0,08 – 1	Peng 2014
syn-DP	China	Gaviota	Anchoa	0,05 – 0,34	Peng 2014
		Cernícalo	Gorrión	0,31	Yu 2013
		Cernícalo	Rata	0,06	
		Búho	Gorrión	12	
		Búho	Rata	2,4	
	Lago Ontario	Trucha	Pinchagua	1	Tomy 2008
			Capellán	12	
	Lago Winnipeg	Sander	Ojo de luna	0,4	
anti-DP	China	Gaviota	Anchoa	0,08 – 0,33	Peng 2014
		Cernícalo	Gorrión	0,35	Yu 2013
		Cernícalo	Rata	0,1	
		Búho	Gorrión	6,8	
		Búho	Rata	1,9	
	Lago Ontario	Trucha	Pinchagua	0,9	Tomy 2008
			Capellán	12	
	Lago Winnipeg	Sander	Ojo de luna	0,8	

Sin embargo, otros autores han documentado lo contrario: un mayor potencial de bioacumulación del DP que el Dec 602, aunque en este caso ambos compuestos presentaron TMFs mayores que 1 (Wang *et al.*, 2015). Por otro lado, el análisis de isótopos de nitrógeno reveló que los delfines comunes y los delfines nariz de botella estaban en posiciones tróficas diferentes, mientras que no se observaron diferencias significativas en el $\delta^{13}C$, cosa que indica que seguían una alimentación similar. Del mismo modo, de todas las especies de aves estudiadas se seleccionaron las especies de la misma familia con valores de $\delta^{13}C$ similares pero con $\delta^{15}N$ diferentes. Como se discute en el artículo, esta se consideró la mejor manera de evaluar la capacidad de biomagnificación de estos compuestos en aves de dieta terrestre. Al margen de las diferencias obvias en el tipo de dieta, las concentraciones o valores de $\delta^{15}N$, es interesante ver como tanto en individuos con dieta acuática como terrestre se repite la misma tendencia: el Dec 602 presenta capacidad de biomagnificación mientras que

tanto el Dec 603 como el DP no lo hacen. Por último, es importante remarcar que, tal y como se ha comentado en el capítulo 1, se han descrito algunos productos fruto de la decloración del DP, tales como el DPMA, aunque el metabolismo de los HNs sigue siendo una incógnita. El hecho de que el DP pueda metabolizarse podría ser una de las causas de que no encontremos la capacidad de biomagnificación en nuestros estudios. Sería necesario poder determinar también los niveles de DPMA para poder evaluar mejor el comportamiento del DP.

La información sobre la capacidad de biomagnificación es clave para caracterizar realmente el peligro que supone la presencia de estos compuestos en diferentes ecosistemas. De hecho, uno de los factores claves cuando se evalúa la peligrosidad de los PBDEs o el HBCD es la capacidad de algunos de ellos para biomagnificarse, comportamiento observado también en el trabajo de esta tesis y reportado previamente por otros autores (Covaci *et al.*, 2006; Law *et al.*, 2006; Losada *et al.*, 2009; Wan *et al.*, 2008). Además, la capacidad de biomagnificación de los MeO-PBDEs también concuerda con lo descrito previamente en bibliografía (Kelly *et al.*, 2008; Losada *et al.*, 2009). Por tanto, a la vista de los resultados obtenidos se intuye que los HNs se comportan de manera similar, biomagnificándose a lo largo de la cadena trófica, y por tanto deberían ser puestos al mismo nivel que los contaminantes clásicos a la hora de evaluar su peligrosidad en este sentido.

4.6.5. Perfiles de contaminación

En este apartado se muestran los congéneres más abundantes encontrados en las diferentes matrices estudiadas, y se discuten las posibles causas e implicaciones.

PBDEs

Hace años estaba aceptado, y había sido documentado por numerosos estudios, que los PBDEs mayoritarios en biota y matrices ambientales como sedimentos, suelos o lodos de depuradora eran el BDE-47 y el BDE-209, respectivamente. Esto se atribuía a una mayor biodisponibilidad del primero, mientras que el segundo no se acumulaba debido a su gran masa molecular y $\log K_{ow}$. No obstante, en estos últimos años existen evidencias de que el BDE-209 no solo se bioacumula sino que puede convertirse en el PBDE más abundante (Gentes *et al.*, 2012; Gentes *et al.*, 2015; Moon *et al.*, 2007; Morales *et al.*, 2012; Muñoz-Arnanz *et al.*, 2011; Sutton *et al.*, 2014). Este hecho puede explicarse ya sea a causa de una cantidad puntual elevada a la que hayan sido expuestos

los organismos, o por una acumulación continuada unida a una incapacidad para metabolizarlo. De hecho, se ha sugerido que la aportación mayoritaria del BDE-209 en aves alimentándose en vertederos viene causada precisamente por este hecho (Gentes *et al.*, 2015). En la figura 4.8 se muestran las contribuciones de los diferentes PBDEs en todas las especies estudiadas.

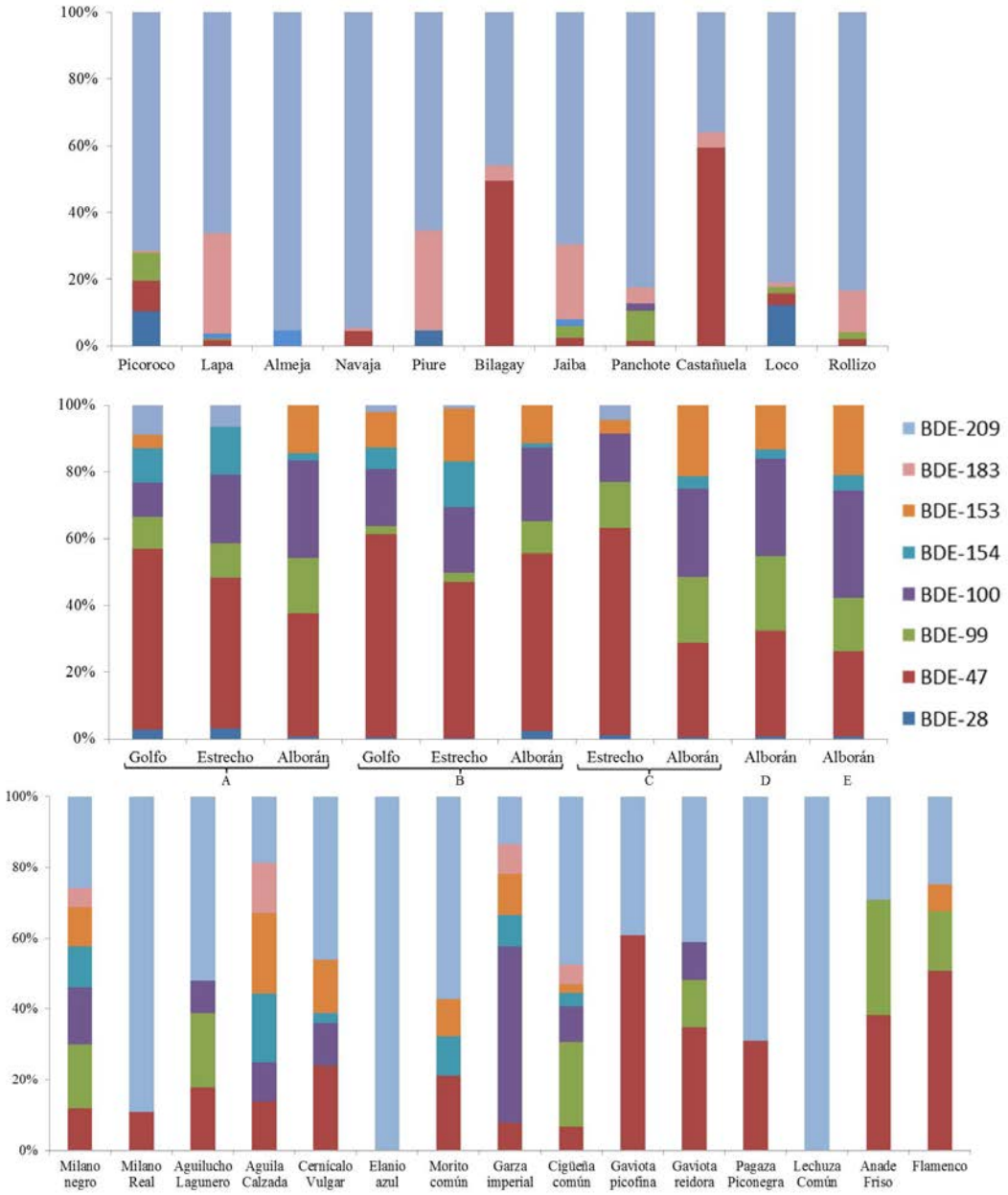

Figura 4.8. Contribución de los diferentes PBDEs al valor total en todas las especies estudiadas en esta tesis.

Se puede ver como incluso entre organismos con mecanismos de alimentación parecidos (filtradores) existen diferencias evidentes. En cambio, entre las diferentes

especies de delfines no se observan variaciones significativas, al igual que en las diferentes zonas. Esto es de esperar ya que las presas de las que se alimentan son similares, como indican los estudios de $\delta^{13}C$, aunque el perfil parece ligeramente diferente en los individuos del mar de Alborán. Por último, existe también una gran variabilidad en las diferentes especies de aves, también de esperar ya que se trata de especies con hábitos migratorios y alimentarios muy variados.

HNs

La mayoría de estudios publicados sobre los HNs se han centrado en el estudio del DP, por lo que no existe mucha información sobre la distribución de los diferentes compuestos de esta familia en biota. En la figura 4.9 se muestra la contribución de los diferentes compuestos de la familia de los HNs al total. A diferencia del caso de los PBDEs, donde la presencia de algunos congéneres puede ser debida a la debrominación de congéneres de mayor grado de bromación, en el caso de los HNs la presencia de los compuestos sólo puede ser debida a una acumulación desde el medio ambiente. Esto explicaría la nula contribución del Dec 602 en Chile, donde tal vez no era utilizado en aquel momento, mientras que es el compuesto mayoritario en prácticamente todas las especies de delfines de nuestro país. De nuevo se puede ver como existe una gran variabilidad en aves, que se explica también a causa de las diferentes dietas y las probables diferencias entre los metabolismos de las mismas. La contribución del Dec 603 en aves es otro dato interesante ya que incluso es el compuesto que mayor contribuye al valor total en algunas especies aunque, curiosamente, este hecho sólo se da en los casos donde el Dec 602 no fue detectado. Los estudios sobre la bioacumulación del Dec 602 sugieren que presenta una mayor capacidad de bioacumulación que el resto de HNs, como se ha visto en el apartado anterior, y tanto los resultados obtenidos en esta tesis como los disponibles en bibliografía (De La Torre *et al.*, 2012; Feo *et al.*, 2012; Guerra *et al.*, 2011; Shen *et al.*, 2010; Sühring *et al.*, 2014; Sühring *et al.*, 2015; Wang *et al.*, 2012) en cadenas tróficas acuáticas y terrestres parecen confirmarlo. Este hecho pone de manifiesto que, pese a que el DP no debe ser despreciado ya que parece ser que su uso es mucho mayor que el del Dec 602, este último presenta mayor presencia en biota y por tanto se debería poner más atención sobre él.

Fanti: Uno de los parámetros utilizados habitualmente para evaluar el diferente comportamiento de los dos isómeros del DP es la f_{anti}, cuyo valor puede ser comparado además con el de las mezclas comerciales. En la figura 4.10 se agrupan los rangos de valores obtenidos en las diferentes familias de especies incluidas en esta tesis, así como en las 2 matrices ambientales cuyos estudios ya han sido descritos en el capítulo 3. Se puede ver como en sedimento y lodo, las matrices ambientales, el f_{anti} está en el rango descrito en las mezclas comerciales (entre 0.65 y 0.75).

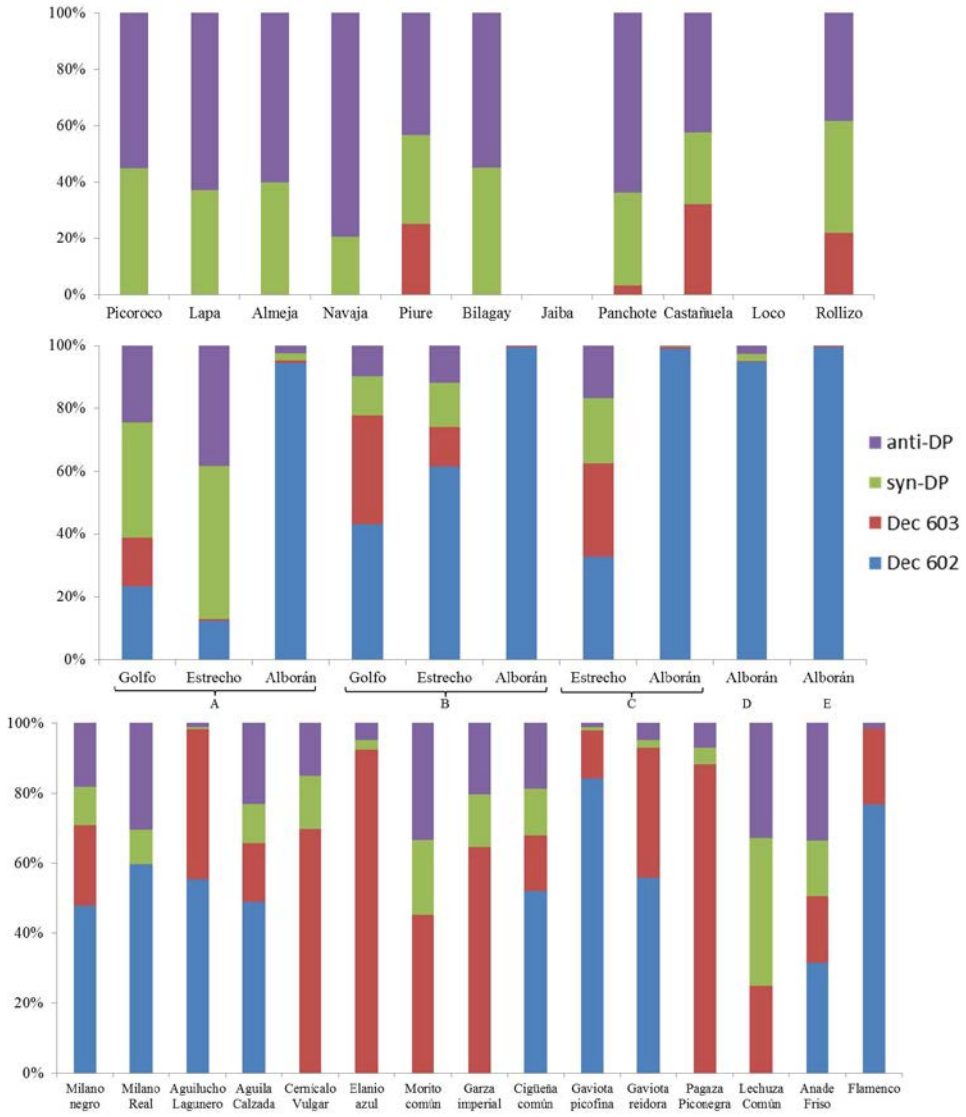

Figura 4.9. Contribución de los diferentes HNs al valor total en todas las especies estudiadas en esta tesis.

En organismos “simples” como los bivalvos ya se observa una disminución del valor, mientras que en crustáceos el único valor disponible está justo en el mínimo descrito en mezclas comerciales. En organismos más complejos como peces y luego los delfines se

ve una clara disminución de la f_{anti} respecto al valor en mezclas comerciales. Por último, en aves los valores son ligeramente inferiores a los de la mezcla comercial, aunque no significativamente. Estos datos evidencian el diferente comportamiento de los 2 isómeros del DP en el medio ambiente, ya sea por su diferente capacidad de bioacumulación (como también parecen indicar los BSAF) o la diferente capacidad metabólica de las diversas especies. De esto último se tiene poca información, y si los 2 isómeros se comportasen de manera diferente, podría afectar a la f_{anti}. Por ejemplo, si alguno de los 2 fuera convertido a DPMA con mayor facilidad, el valor de f_{anti} se vería distorsionado (Guerra *et al.*, 2011). Además, la diferencia entre el medio acuático y medio terrestre podría indicar que el diferente comportamiento de los 2 isómeros del DP tiene lugar en medio acuático pero no tanto en el terrestre.

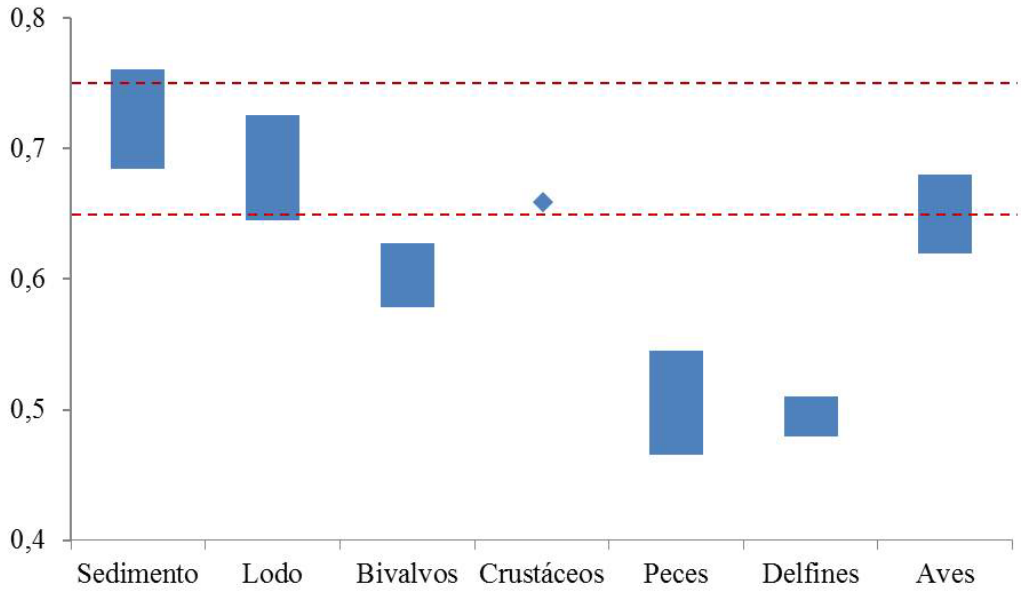

Figura 4.10. Valores de f_{anti} obtenidos en las diferentes matrices estudiadas en esta tesis. Las líneas rojas marcan el rango de valores reportado en las mezclas comerciales.

HFRs clásicos y emergentes

Como se ha explicado anteriormente ya existen medidas reguladoras sobre los PBDEs y el HBCD, pero no sobre los HNs o los BFRs emergentes. Al margen de las tendencias temporales observadas, discutidas en el apartado 4.6.6, es interesante estudiar las diferencias entre ambos tipos de HFRs en los diferentes estudios realizados. Pese a que en algunas zonas como Chile o el Mediterráneo no existían datos previos, las concentraciones encontradas durante esta tesis son un buen punto de partida para

estudiar las tendencias temporales que se darán en el futuro. Esto es de vital importancia para poder evaluar si las restricciones legales han dado sus frutos o no.

En todas las especies analizadas en Chile los niveles de PBDEs superaban ampliamente a los de los HNs, siendo estos últimos muy bajos, lo que podría indicar que por el momento sólo son utilizados excepcionalmente. De cara al futuro estas concentraciones servirán de referencia para estudiar el aumento del uso de los HNs, especialmente el DP, en la zona. Por otro lado, en los delfines analizados en el Mar Mediterráneo también se observó el mismo hecho, aunque las diferencias no fueron tan grandes como en Chile. Es muy difícil llegar a ninguna conclusión ya que son diferentes zonas, especies y dietas, pero parece claro que la presencia de los HNs en el medio acuático europeo es más elevada que en América del Sur.

Curiosamente, la relación entre PBDEs y HNs en las aves de Doñana es diferente a la observada en los delfines. Incluso en algunos casos las concentraciones de HNs eran superiores a las de PBDEs o HBCD. La capacidad de acumulación de los HNs en especies de dieta terrestre ha sido considerada superior a la de las especies con dieta acuática en aves de otras partes del mundo (Chen *et al.*, 2013; Guerra *et al.*, 2011) y podría explicar esta diferencia con el resto de especies estudiadas en esta tesis, de dieta acuática y con mayores diferencias entre HFRs clásicos y alternativos. Las relaciones entre PBDEs y HNs se muestran agrupadas en la figura 4.11 (dejando de lado el HBCD ya que no está incluido en todos los trabajos realizados).

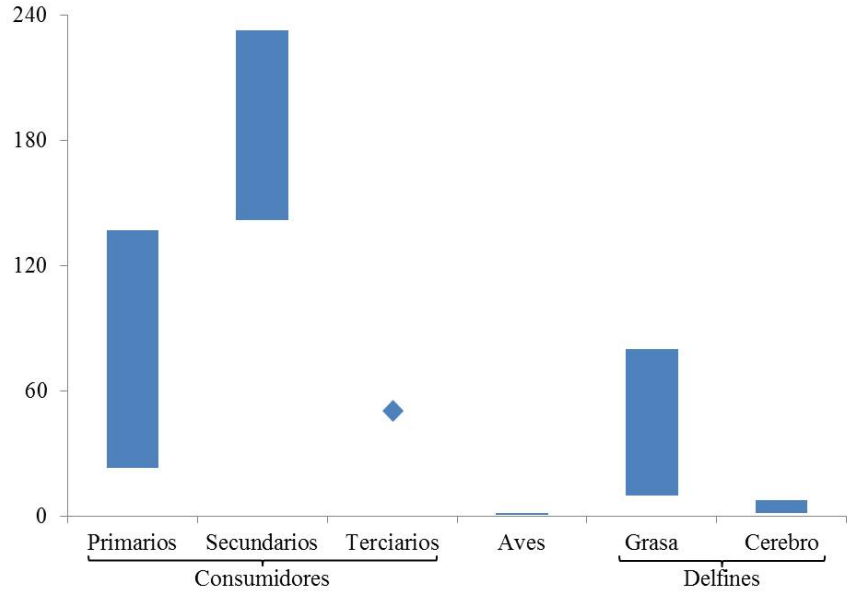

Figura 4.11. Ratio entre los PBDEs y los HNs en los diferentes grupos de biota estudiados en esta tesis.

HNPs

A pesar de que el número de posibles compuestos organohalogenados de origen natural es inmenso, aquí nos centraremos en evaluar los perfiles entre los compuestos incluidos en nuestros análisis. Pero hay que tener presente que pueden existir otras familias de compuestos cuya contribución sea aún mayor, pero que a día de hoy se desconoce su presencia en biota marina. Se vio como el Q1 y sus derivados dominan el perfil de los HNPs, representando entre el 50% y el 90%. Este hecho ya ha sido descrito con anterioridad (Gribble 1998; Melcher *et al.*, 2007; Vetter *et al.*, 2000; Vetter *et al.*, 2002) y permite hacerse una idea de la importancia de estos compuestos. Los MeO-PBDEs son analizados debido a que comparten estructura con los PBDEs, y por tanto podrían suponer el mismo peligro para los organismos y tener un comportamiento similar en el medio ambiente. Son, con diferencia, los HNPs más analizados, especialmente en delfines (Alonso *et al.*, 2014; Gribble 2010; Vetter 2006). Pero como se puede ver la contribución del Q1 y resto de PMBPs siempre triplica, como poco, la contribución de los MeO-PBDEs al valor total, por lo que una evaluación de sus posibles efectos en los organismos marinos sería recomendable. Aunque la comparación se ve afectada por el diferente número de compuestos analizados en cada estudio, normalmente los HNPs están presentes en concentraciones superiores a las de los HFRs (Covaci *et al.*, 2008; Hauler *et al.*, 2014; Losada *et al.*, 2009).

Distribución en tejidos

En los estudios llevados a cabo en biota normalmente se analiza el individuo entero (bivalvos, crustáceos, peces…) o un tipo de muestra concreto (sangre, grasa, huevos, heces…). No obstante, en algunos estudios se tiene la oportunidad de trabajar con diferentes tejidos, ya sea por planificación previa (por ejemplo, en peces) o en el caso de especies más complejas porque se dispone de individuos enteros (delfines varados, aves, etc.). Por desgracia, en esta tesis sólo se tuvo la ocasión de realizar una comparativa entre grasa y cerebro de delfín. El hecho de que los HFRs tengan la capacidad de desplazarse por el organismo y acumularse en diversos tejidos les confiere una mayor peligrosidad ya que pueden tener un efecto mucho más directo en el tejido u órgano en cuestión. Como se discute en la sección “blubber and brain distribution” del artículo, el flujo sanguíneo es el principal responsable de la movilización de los contaminantes hacia los diferentes tejidos. Es más, épocas que exigen el consumo de grandes cantidades de energía, y por tanto del uso de grasa, afectarán al estudio ya que pueden

influir en los niveles en tejidos sensibles a la contaminación como el hígado. Una cosa muy diferente es la afinidad que mostraron algunos compuestos como el HBB, BDE-153 o el TetraBHD al cerebro, encontrándose en mayores concentraciones en éste. Como se explica en el apartado correspondiente del artículo, esto podría ser debido a la afinidad con algunas moléculas transportadoras. El HBB sólo fue encontrado de manera residual tanto en bivalvos, peces, aves o grasa de delfín, pero sin embargo su frecuencia de detección en cerebro estaba en torno al 70%, y a niveles de concentración superiores a los hallados en grasa. Esto pone de manifiesto que el análisis de tejidos clásicos, como la grasa en mamíferos marinos, podría no ser suficiente para caracterizar correctamente su problemática en el medio ambiente.

En un estudio en esturiones de China se detectaron el Dec 602, Dec 603 y ambos isómeros del DP en 13 órganos (gónadas, tejido adiposo, hígado, corazón, músculo, intestino, estómago, agallas, riñones, páncreas, vesícula biliar y bazo). Se detectaron en todos los órganos excepto el páncreas, donde sólo fue detectado el DP. Es el estudio con mayor número de tejidos estudiados disponible y demuestra que estos compuestos tienen la capacidad de distribuirse por todo el organismo del animal. Además también se observaron diferencias entre los congéneres más abundantes en los tejidos: el Dec 602 y Dec 603 se acumularon mayoritariamente en intestino y estómago, mientras que el DP se acumuló preferentemente en tejidos de elevado contenido lipídico como el tejido adiposo o el hígado (Peng *et al.,* 2012). Del mismo modo, se ha detectado el DP en músculo e hígado de diferentes especies de aves rapaces, también en China, lo que demuestra que también pueden distribuirse en diferentes tejidos en animales de dieta terrestre (Chen *et al.,* 2013). De igual modo el DP se ha encontrado en diferentes tejidos de gallinas en China (Zheng *et al.,* 2014). Ordenados por presencia de DP en ellos, los tejidos fueron: grasa (56% del contenido total), gónadas, riñones, corazón, hígado, pulmones, cerebro y músculo. De nuevo, existe una evidencia clara de que los HNs se comportan de manera similar a los PBDEs, cuya distribución en diferentes tejidos ha sido documentada con anterioridad (Wan *et al.,* 2013).

También existe el fenómeno conocido como transferencia materna y que afecta a un gran número de contaminantes. Su importancia radica en que la contaminación acumulada por la madre a lo largo de su vida es transferida a los huevos, en el caso de especies ovíparas, o al feto, en el caso de los mamíferos. Es un fenómeno de gran relevancia en delfines y muy bien caracterizado para compuestos como los PBDEs, aunque aún no para los HNs (Alonso *et al.,* 2012). No obstante, ya se ha descubierto que

este fenómeno afecta también a los HNs en aves (ya que han sido detectados en huevos, tanto en los estudios comprendidos en esta tesis como en trabajos anteriores) y en peces (Peng *et al.*, 2012; Sühring *et al.*, 2015).

4.6.6. Tendencias temporales

El hecho de disponer de muestras de huevos de 1999, 2003, 2011 y 2013 permitió evaluar las tendencias temporales de diversos HFRs acontecidas en Doñana (artículo #9). Se observó una disminución con el tiempo de los componentes de la mezcla Penta-BDE, así como una disminución pronunciada del BDE-209 en milanos negros. Este hecho parecería indicar que las restricciones sobre el uso de estos compuestos han tenido un efecto en las concentraciones encontradas. Por el contrario, no se apreció una tendencia clara para los HNs, lo cual puede ser debido a que aún es demasiado pronto para poder evaluar alguna tendencia en una zona cuyos principales inputs se suponen externos y, por tanto, de aparición más tardía. La documentación de estas tendencias no se limita solamente a aves. Por ejemplo, se advirtió una disminución significativa de los niveles de BDE-47 en sedimentos y bivalvos de la bahía de San Francisco entre 2002 y 2012 (Sutton *et al.*, 2014) (Figura 4.12). Otros autores han documentado esta tendencia a la baja de los niveles de otros PBDEs como el BDE-99 o BDE-153, pero sin embargo esta tendencia general no es tan clara para el BDE-209 o el HBCD (Law *et al.*, 2014; Lee y Kim 2015; Li *et al.*, 2015; Murtomaa-Hautala *et al.*, 2015; Venier *et al.*, 2015). Debido a que ha sido producido hasta hace relativamente poco, es de esperar que no se pueda observar una tendencia temporal consistente hasta dentro de algunos años. Además, la debrominación del BDE-209 puede dar lugar a PBDEs de menor grado de bromación como el BDE-47, componente de la mezcla Penta-BDE, representando un factor de variabilidad importante en el estudio de las tendencias temporales (Law *et al.*, 2014). No obstante, sí se ha observado una posible transición desde el BDE-209 al DP o el DBDPE, como por ejemplo en el estudio realizado por Zhu *et al.* (2014) en marsopas y delfines rosados del sur de China. Se apreció un incremento significativo de la relación DP/BDE-209 y DBDPE/BDE-209 entre 2003 y 2013, asumiéndose como una evidencia de la substitución del BDE-209 por dos de sus substitutos (Figura 4.12). Es interesante observar como en zonas tan alejadas entre ellas, y que tradicionalmente presentan niveles de contaminación muy elevados, se estén dando tendencias temporales similares.

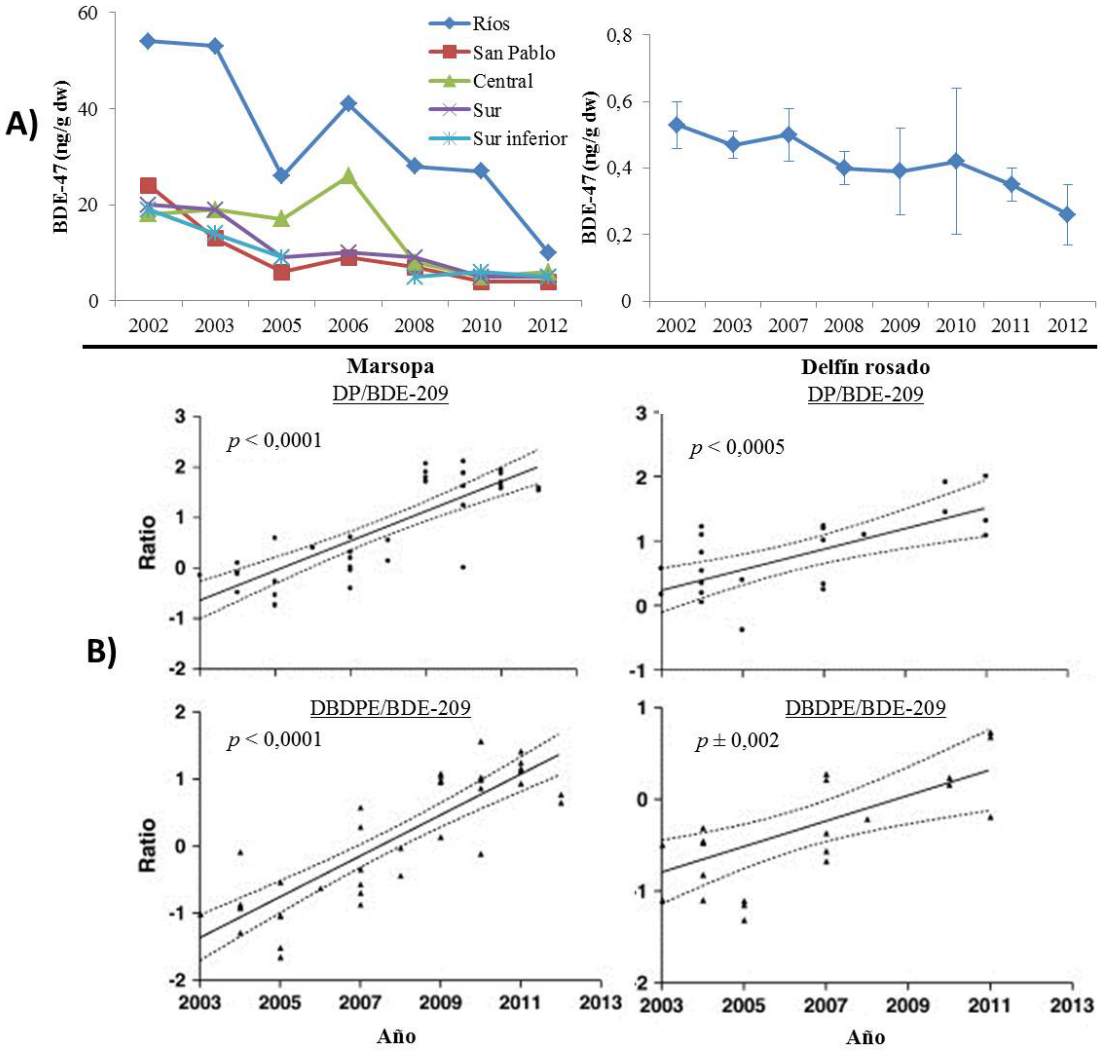

Figura 4.12. Diferentes tendencias temporales observadas en bibliografía. A): datos de Sutton *et al.* 2014, mejillones (izquierda) y sedimentos (derecha). B): adaptada de Zhu *et al.* 2014.

4.7. Conclusiones

A través de todo lo expuesto anteriormente, se ha llegado a las siguientes conclusiones en lo que respecta a la presencia y comportamiento de los HFRs en biota:

- Los nuevos datos aportados confirman la presencia global de los HNs, siendo encontrados en zonas donde hasta ahora no se había detectado su presencia. Pese a no haberse tenido en consideración hasta hace relativamente poco, se han venido usando durante años. Las evidencias aportadas desde entonces ponen de manifiesto que no se debe subestimar su presencia en el medio ambiente ya que han sido hallados en zonas, matrices y especies muy diferentes entre sí.
- A través de los BSAF y los BMF se ha podido ver como los HNs presentan propiedades comparables a las de los PBDEs. Concretamente, Dec 602 y BDE-47

presentan valores similares, al igual que el DP y BDE-209. Estos datos pueden ser importantes en un futuro a la hora de tomar medidas contra la producción y uso de estos compuestos, además de poner el foco sobre el Dec 602, compuesto que normalmente no es tenido en cuenta.

- Los HNs han mostrado, al igual que PBDEs, HBCD y MeO-PBDEs, capacidad para biomagnificarse a lo largo de cadenas tróficas tanto acuáticas como terrestres. El hecho de que este patrón sea observado en cadenas tróficas tan diferentes entre sí es especialmente de interés.
- Se ha visto como los HNs tienen la capacidad de distribuirse en diferentes tejidos e incluso acumularse en un órgano tan vital como el cerebro. El hecho de que estos compuestos sean capaces de llegar directamente a órganos como cerebro o hígado les confiere una mayor peligrosidad ya que estos órganos juegan un papel muy importante en las funciones vitales de los organismos. Además queda claro que, si existe la posibilidad de ello, varios tejidos han de ser analizados para poder llevar un seguimiento completo de todos los POPs.
- El estudio de las tendencias temporales en los últimos años parece indicar que el uso de los PBDEs está disminuyendo mientras que el uso de HFRs emergentes como los HNs o el DBDPE va en aumento.

CAPÍTULO 5

EVALUACIÓN DE LOS EFECTOS TOXICOLÓGICOS

5.1. Introducción

En los capítulos 3 y 4 de esta tesis se ha visto como los HFRs son prácticamente omnipresentes en el medio ambiente, encontrándose tanto en matrices ambientales como en animales pertenecientes a todos los niveles de cadenas tróficas acuáticas y terrestres. Como se ha discutido, este hecho ya era conocido para HFRs clásicos como los PBDEs y otros como el HBCD. En cambio, se ha ido haciendo evidente sólo en los últimos años en el caso de los HNs. Sin embargo, los estudios sobre su toxicidad son escasos en comparación. Con la intención de comprender mejor la toxicidad del DP, se realizaron exposiciones *in vivo* en mejillones y se compararon los resultados con los obtenidos para el BDE-209 en las mismas condiciones. Este trabajo se llevó a cabo durante la estancia pre-doctoral realizada en la Universidad de Plymouth, bajo la supervisión del Dr. Awadhesh Jha y el Profesor James Readman, y el artículo resultante se encuentra enviado a la revista Environmental Science & Technology bajo el título "*Evaluation of the genetic and physiological effects of decabromodiphenyl ether (BDE-209) and dechlorane plus (DP) flame retardants in Mytilus galloprovincialis by in vivo exposure*" (Artículo #10). Se eligió el DP ya que es el compuesto de la familia de los HNs de mayor volumen de producción y el BDE-209 para poder comparar los resultados con un compuesto ya prohibido, además de por el hecho de que el DP ha sido propuesto directamente como su substituto. En ambos casos se utilizaron mezclas comerciales con el fin de realizar un estudio más realista. Si bien es cierto que hubiera sido interesante llevar a cabo estos estudios con un número mayor de compuestos, como por ejemplo el Dec-602, la mezcla Penta-BDE, MeO-PBDEs o Q1, la estancia sólo duró 3 meses y por tanto existía una limitación de tiempo que impidió ampliar el estudio.

5.2. Exposiciones *in vivo*

5.2.1. Mejillones

La estancia pre-doctoral realizada en la Universidad de Plymouth permitió el aprendizaje de algunas técnicas nuevas, que serán descritas a continuación. Se pudo trabajar además con la técnica de exposición *in vivo*, que consiste en la evaluación de los efectos de una o varias substancias en organismos vivos y por tanto proporciona resultados más realistas que las exposiciones *in vitro*, aunque por motivos obvios presenta un mayor número de factores que pueden afectar a los resultados. En este caso se utilizaron individuos de mejillón mediterráneo (*Mytilus galloprovincialis*) que fueron

expuestos a diferentes concentraciones de BDE-209 y DP. Los mejillones se dispusieron individualmente en vasos de precipitados de 1,8 L y fueron expuestos a los contaminantes durante 6 días, cambiando el agua diariamente y limpiando los vasos para evitar un aumento de la concentración de exposición (Figura 5.1). Se usó un método de exposición a través de la dieta, por lo que se dopaba el alimento de los mejillones (*Isochrysis galbana*) la noche anterior y ésta era suministrada 2 h después del cambio de agua. Se comprobó visualmente que las células del alga no eran dañadas por los contaminantes o la acetona usada como vector (Figura 5.1).

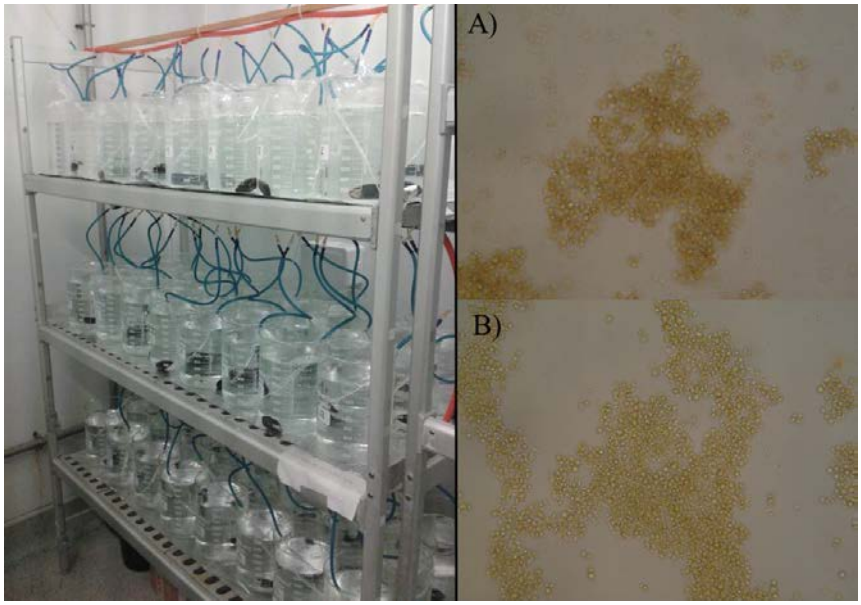

Figura 5.1. Diseño del experimento (izquierda) y células del alga utilizada vistas con microscopio (derecha) expuestas a A) Agua B) Acetona, DP y BDE-209.

Estudios previos han demostrado que la biodisponibilidad de los PBDEs en mejillones aumentaba en presencia del alga usada para alimentarlos en comparación con exposiciones directas en medio acuoso debido a un proceso de adsorción (Gustafsson *et al.*, 1999). Además, este escenario se considera más realista ya que los mejillones obtienen su alimento mediante filtración del agua, que en el medio marino presenta una gran cantidad de particulado donde potencialmente se encontrarán adsorbidos los HFRs.
Al finalizar el experimento se evaluaron los efectos a nivel fisiológico, mediante el "clearance rate" (CR) y genético mediante el test del cometa (CA, del inglés comet assay) y el test de micronúcleos (MN, del inglés micronucleus assay). Estas metodologías se encuentran descritas con detalle en el artículo #10.

5.2.2. Invertebrados estuarinos

En los inicios de esta tesis se tuvo la ocasión de colaborar en un estudio multidisciplinar en el que se expusieron peces cebra o zebrafish (*Danio rerio)* a sedimentos contaminados con 12,5 μg/g de BDE-209 durante 8 días, evaluándose los efectos provocados. Este estudio se encuentra publicado (García-Reyero *et al.,* 2014: *Effects of BDE-209 contaminated sediments on zebrafish development and potential implications to human health*). Dado que se han encontrado concentraciones elevadas de BDE-209 en sedimentos (artículos #3 y #4) este estudio pone en contexto estas concentraciones con los efectos observados en el zebrafish.

Por otro lado, cuando se estaba concluyendo esta tesis se realizó una colaboración que tenía como objetivo evaluar los efectos en poliquetos (*Laeonereis acuta*) y cangrejo de las rocas (*Cyrtograpsus angulatus*) tras una exposición a BDE-47 a través de la dieta. En un primer experimento tanto los poliquetos como los cangrejos se pusieron en contacto con sedimento previamente dopado con BDE-47, mientras que en un segundo experimento los cangrejos fueron alimentados con los propios poliquetos del experimento anterior. El artículo se encuentra actualmente en preparación, y sirve como complemento a las concentraciones encontradas en Chile en 2 especies de cangrejos (artículo #5).

5.2.3. Mamíferos y aves

Pese a que en ningún momento de esta tesis se ha trabajado con mamíferos o aves a la hora de evaluar posibles efectos toxicológicos de los HFRs, sí se han analizado un gran número de muestras de estas especies. Por tanto, se exponen aquí algunos de los efectos descritos en bibliografía que la exposición a HFRs ha causado en ellas.

Mamíferos

Un grueso importante de los estudios sobre los efectos toxicológicos de los HFRs constituye el ensayo en ratones de laboratorio. El BDE-209 indujo hiperglicemia en ratones expuestos a 0,05, 1 y 20 μg/g durante 8 semanas, causando además más de 1.000 alteraciones en los procesos de transcripción de genes del hígado y favoreciendo mecanismos asociados a enfermedades autoinmunes y diabetes de tipo I. Además, por lo visto los machos son más sensibles al BDE-209 que las hembras (Zhang *et al.,* 2013). En otro estudio donde se administró BDE-209 en 10 y 30 μg/g a ratones recién nacidos se observó como el desarrollo neuronal se veía afectado, ralentizando la adquisición de

la visón espacial así como un retraso en la apertura de los ojos (Reverte *et al.,* 2014). En general, ni el BDE-209 ni otros PBDEs de menor grado de bromación presentan efectos mortales en ratones, pero sí son capaces de generar trastornos tanto metabólicos como fisiológicos (Kodavanti y Ward 2005). Del mismo modo, aunque no existen estudios en ratones sobre el Dec 602, Dec 603 o Dec 604 (lo cual debería cambiar a corto plazo, ya que como se ha visto en el capítulo 4 el Dec 602 tiene una gran presencia en biota) sí los hay sobre el DP. Se advirtió una disminución de los niveles de expresión de cadenas de mRNA de algunos genes y un aumento de la actividad enzimática de CYP 2B2 tras 90 días de exposición continuada a 1, 10 y 100 μg/g de una mezcla comercial de DP (Li *et al.,* 2012). En el estudio de Li *et al.* se puede ver además como el DP tiene tendencia en acumularse en mayores cantidades en el hígado que en el músculo, lo que es de interés ya que existen estudios sobre los efectos de este compuesto en hígado de ratones. Concretamente, tras una exposición durante 10 días a concentraciones muy altas de DP (500, 2000 y 5000 μg/g) se vio como la expresión de genes que regulan procesos tan importantes en el hígado, como el metabolismo de carbohidratos o lípidos, era alterada, además de producir un estrés oxidativo (Wu *et al.,* 2012).

El estudio de los efectos de los HFRs en organismos más complejos como los mamíferos marinos mediante exposiciones *in vivo* es muy complicado, tanto por razones logísticas como éticas. Algunos autores han relacionado las concentraciones de PBDEs encontradas en focas capuchinas con alteraciones en procesos del sistema tiroideo (Villanger *et al.,* 2013). Del mismo modo, Hall *et al.* relacionó las concentraciones de PBDEs encontradas en grasa y sangre de focas grises con trastornos tiroideos en cachorros con menos de 1 año de vida, estableciendo una concentración de 1500 ng/g lw a partir de la cual podían empezar a observarse los efectos (Hall *et al.,* 2003). Varios individuos de delfines analizados en los artículos #6 y #7 superaban este valor, aunque probablemente lo alcanzaron en su vida adulta y se trata de una especie diferente. Hasta la fecha no se han estudiado los efectos del resto de los HFRs estudiados en esta tesis en mamíferos marinos.

Aves

El único estudio evaluando los efectos causados por el BDE-209 en aves, y no sólo su acumulación, fue llevado a cabo en cernícalos americanos (Letcher *et al.,* 2014). Se expusieron 22 machos a 116 μg/día durante 21 días, seguido de un periodo de 25 días de depuración. Se observó una alteración en la actividad de algunos enzimas como el

citocromo P450, que pueden llegar a afectar a procesos como la apoptosis celular y estrés oxidativo. Estas concentraciones son muchísimo más altas que las encontradas tanto en esta tesis (artículos #8 y #9) como en el resto de bibliografía. Además, se ha visto como los PBDEs pueden retrasar la puesta de huevos y afectar al grosor de su cáscara, lo que compromete la viabilidad del mismo (Fernie *et al.*, 2009).

Publicación científica #10

Evaluation of the genetic and physiological effects of decabromodiphenyl ether (BDE-209) and dechlorane plus (DP) flame retardants in Mytilus galloprovincialis by in vivo exposure

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Evaluation of the genetic and physiological effects of decabromodiphenyl ether (BDE-209) and dechlorane plus (DP) flame retardants in *Mytilus galloprovincialis* by *in vivo* exposure

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17 **Abstract**

18 Dechlorane plus (DP) is a proposed alternative to the legacy flame retardant
19 Decabromodiphenyl ether (BDE-209), a major component of Deca-BDE formulations.
20 In contrast to BDE-209, toxicity data for DP are scarce and often focused in mice.
21 Validated dietary *in vivo* exposure of the marine bivalve (*Mytilus galloprovincialis*) to
22 both flame retardants did not induce effects at the physiological level (algal clearance
23 rate), but did induce oxidative DNA damage, as determined by the comet assay, at all
24 the concentrations tested. Micronuclei formation was induced by both DP and BDE-209
25 at the highest exposure concentrations (100 and 200 µg/L, respectively, at 18% above
26 controls). DP caused similar effects to BDE-209 but at lower exposure concentrations
27 (5.6, 56 and 100 µg/L for DP and 56, 100 and 200 µg/L for BDE-209). Moreover,
28 bioaccumulation of DP was shown to be concentration dependent, in contrast to BDE-
29 209. The results described suggest that DP poses a greater genotoxic potential than
30 BDE-209.

Introduction

Polybromodiphenyl ethers (PBDEs) were one of the most used halogenated flame retardants (HFRs) worldwide, available as three main commercial mixtures: Penta-BDE, Octa-BDE and Deca-BDE.[1] However, nowadays this situation has changed due to recent restrictions over PBDEs. Within the European Union (EU), Penta- and Octa-BDE mixtures were banned in 2004, while Deca-BDE mixture was banned in 2008.[2] PBDEs have been found in a wide range of environmental matrices such as sediment, water, fish or cetaceans, and also in humans.[3-8] Nonetheless, knowledge of environmental behavior and effects of BDE-209 is still scarce compared to the lower brominated PBDEs. This might be due to the limitations in the analytical methodologies for the analysis of this compound in the past, due to its high $\log K_{ow}$ and molecular weight.[9] Despite the bioaccumulation potential being lower than other low brominated PBDEs such as BDE-47, BDE-209 has been found in different vertebrate and invertebrate species worldwide.[2, 10, 11] In fact, BDE-209 was the main PBDE found in species with terrestrial diet[2, 10, 12] and also in mussels.[11, 13] BDE-209 has shown thyroid and endocrine disruption properties[14, 15] and it could affect the liver of fish and mice.[15, 16] Most of the studies are focused in vertebrate models such fish or mice,[17] thus studies in invertebrates such as mussels are scarce.

Dechlorane plus (DP) was selected as an alternative to Mirex when it was banned as a FR, and nowadays it has been proposed as an alternative to the Deca-BDE mixture. It is considered a novel HFR and is still barely regulated.[18-20] Similar to BDE-209, DP has been found a wide range of biological matrices such as fish, mussels or cetaceans and also in humans, proving its bioaccumulation capacity.[18-21] Toxicity data for DP are still very scarce. In fish, DP affected protein responses in the liver and induced apoptosis,[22]

while it showed genotoxical potential in bacteria[23] as well as histopathological changes in mice liver.[24]

Mussels have proven to be a good tool to evaluate the environmental behavior of organic pollutants.[25] Furthermore, effects of organic pollutants in mussels have been correlated with effects of the same pollutants in humans[26] which shows that these contaminants can affect the whole food chain. Thus, the study of the effects of FRs in mussels could provide useful information concerning the potential for effects of these contaminants in other biota including humans. Consequently, the aim of this study was to evaluate the genetic and physiological effects of one classical FR (BDE-209, which represents about the 98% of Deca-BDE commercial mixture) and one alternative FR commercial mixture (DP) in *Mytilus galloprovincialis* through an *in vivo* exposure via the dietary pathway. To our knowledge, this is the first time that the toxicity of DP has been evaluated in this way.

Materials and methods

Sample collection

M. galloprovincialis (5-6 cm length) were collected during the last week of July 2014 from Trebarwith Strand (North Cornwall, UK) and were immediately transported to the laboratory, rinsed with sea water and acclimatised in an aerated tank with 50 L of filtered seawater (<0.8 µm) maintained at 15 ºC ± 1 ºC with a photoperiod of 12h L:12h D for 10 days and fed every two days with *Isochrysis galbana* (Liquifry, Interpet, Dorking, UK). Stocking density was 3 mussels per L. Water was changed 2-3 hours after feeding. Any spawning animals were removed from the holding conditions and were excluded from the experiments.

Chemicals and reagents

Triton X-100, Sodium chloride, Normal Melting point Agarose (NMPA), Low melting point agarose (LMPA) and N-lauryl sarcosine were purchased from Sigma-Aldrich (UK). BFR-PAR solution, containing BDE-28, BDE-47, BDE-99, BDE-100, BDE-154, BDE-183 and BDE-209, together with *syn*- and *anti*- DP were purchased from Wellington Laboratories (Guelph, ON, Canada), as well as the internal standard ^{13}C-BDE-209. ^{13}C-*syn*-DP, used also as internal standard, was obtained from Cambridge Isotope Laboratories (Andover, MA).

Experiment

To assess whether the feeding route was a valid exposure pathway for filter-feeding organisms when exposed to high Log K_{ow} organic contaminants, a preliminary experiment using B(α)P was performed. Individual mussels were placed in 2 L beakers containing 1.8 L of filtered seawater and exposed to B(α)P at either 100 or 200 μgL^{-1} for six days dosed either by spiking algae *Isochrysis galbana* or directly into the aqueous media (n=6 per treatment, including a solvent carrier (acetone, 0.05 % v/v) control with only acetone). B(α)P was either added to the algal feed (dietary pathway) or directly in the aqueous phase. Both exposure pathways were conducted following a semi-static model where water was changed every day and mussels fed daily. B(α)P was chosen as a model organic contaminant as it is relatively insoluble in water (Log K_{OW} = 6.04), is known to cause genetic damage and is a priority pollutant.[27]

After the dietary pathway proved to be a valid exposure route, mussels were exposed to three different concentrations of BDE-209 (56, 100 and 200 μgL^{-1}) and DP (5.6, 56 and 100 μgL^{-1}), following the procedure described above. Concentrations of DP found in the environment and specifically in mussels are considerably lower than concentrations

found for BDE-209. Thus, exposure concentrations of DP were settled in lower scale, although 2 common concentrations were maintained for comparisons. A B(α)P exposure at 100 μgL^{-1} as positive *in vivo* control together with a negative control (acetone, 0.05 %, v/v final volume) were also performed (n=7 per treatment). H_2O_2 was also used as positive *in vitro* control (1 mM and 30 min of exposure time).

In both experiments, after the six days of exposure mussel haemolymph was extracted from the posterior adductor muscle using an ice-chilled 1 mL syringe and 21G needle and transferred into individual Eppendorf tubes held on ice.

Water quality

Water quality (temperature, salinity, dissolved oxygen and pH) was measured every day for each beaker and three water samples of each treatment were taken immediately after dosing and prior to water change (*i.e* after 23 h of exposure).

Clearance rate

Clearance rate (CR) was determined prior to haemolymph collection as described previously.[28] Mussels were placed in separate 400 mL beakers containing 350 mL seawater (filtered to 0.8 µm) and a stirring bar. They were allowed to acclimatise at 15 °C for 15 min. *Isochrysis galbana* were added in a concentration of 10,000 cells/mL including several procedural blanks (beaker plus 300 mL of seawater). Aliquots of 20 mL were removed immediately after the addition and after 10, 20 and 30 minutes. These aliquots were analysed on a Beckman Coulter Particle Size and Count Analyser set to count particles between 4 and 10 µm. Three separate counts per mussel were made. CR was calculated using the equation $CR = V(\log C_1 - \log C_2)/t$, where V is the volume of

129 water, C_1 and C_2 are the cell concentrations at the beginning and end of each increment,
130 and t corresponds to the time interval.[29]
131
132 *Comet assay*
133 Determination of DNA strand breaks using haemocytes was evaluated following a
134 previously optimized protocol.[30, 31] Slides were pre-coated with normal melting point
135 (NMP) agarose and kept overnight at 20 ºC to dry. 150 µL of haemolymph were
136 centrifuged at ~350 g at 4 ºC for 2 min and then mixed with 150 µL of molten low
137 melting point (LMP) agarose. Two separate drops of 75 µL were placed on the slide and
138 immediately covered with a coverslip. Prior to performing the comet assay, cell viability
139 was determined using Eosin Y staining;[32] viability was deemed >95 %. Slides were kept
140 at 4 ºC and in the dark for one hour to allow the gel to solidify. In the case of the H_2O_2
141 *in vitro* positive control, after one hour 1 mL of H_2O_2 (1 mM) was added dropwise and
142 incubated at 4º C for 30 min. Slides were incubated in lysis solution for one hour and in
143 the dark at 4 ºC, placed in the electrophoresis chamber, filled with electrophoresis
144 buffer, and incubated for 20 min to unwind. Afterwards, the chamber was turned on (25
145 V, 400 mA) and electrophoresis performed for 20 min. Following on, slides were
146 neutralised with cold neutralization buffer. All the steps in the electrophoresis procedure
147 were performed at 4 ºC and in the dark. Slides were stained with ethidium bromide (20
148 µL of a 20 µg/mL solution in each drop) and scored under an epifluorescense
149 microscope (Leica, DMR) using the Komet 5 software (Kinetic Imaging, Nothingam).
150 50 cells in each drop, thus a total of 100 cells per slide, were scored and % tail DNA
151 was used for the evaluation of DNA strand breaks, since it has been validated through
152 inter-laboratory comparisons.[33, 34]
153

Mn assay

Induction of micronuclei (Mn) in haemocytes was evaluated as described by Jha *et al.*[31] Slides were previously coated with 10% poly-L-lysine solution and dried overnight. 200 µL of haemolymph was spread gently onto the slide and left at 15 ºC for 30 min and then fixed with MeOH for 15 min. Afterwards, slides were stained using Giemsa stain (5%, v/v) for 20 min; excess stain was removed with Milli-Q water and once the slides were air dried, a coverslip was mounted using DPX. Slides were scored randomly under the microscope for the induction of Mn. Approximately 1000 cells from each slide were scored following the criteria described in previous works.[31]

Chemical analysis

Regarding water and algae analysis, the methodology described by Di *et al.* was adopted.[27] Hexane (1 mL) was added to 9 mL of the exposure water samples and internal standards (^{13}C-BDE-209 and ^{13}C-*syn*-DP) were added. Samples were manually shaken and then centrifuged at 3500 rpm for 10 min. The aqueous phase was discarded and the organic phase was evaporated to dryness and was reconstituted to a final volume of 500 µL with toluene.

Mussel samples were extracted using a previously described methodology.[35, 36] Briefly, samples were spiked with 100 ng of ^{13}C-BDE-209 and ^{13}C-*syn*-DP and kept overnight to equilibrate prior to extraction by pressurized liquid extraction (PLE). Afterwards, lipid content was determined gravimetrically and re-dissolved in hexane prior to acid treatment ($H_2SO_{4(c)}$). A solid phase extraction (SPE) using Al-N cartridges was performed to complete the clean-up and resulting extracts were concentrated to a final volume of 40 µL.

178 Instrumental analysis was carried out using gas chromatography coupled to mass
179 spectrometry working in negative chemical ionization mode (GC-NCI-MS) using an
180 Agilent Technologies 7890A GC system coupled to 5890A GC/MS Single Quadrupole,
181 following previously optimized protocols.[37, 38] BDE-209 was analysed using NH_4 as
182 reagent gas, whereas DP was analysed using CH_4 as reagent gas. Selected ion
183 monitoring (SIM) was used to enhance sensitivity. Two ions were monitored for each
184 compound: the most intense was used for quantification and the second for
185 confirmation. Ions monitored were *m/z* 487 and 489 for BDE-209 (497 and 499 for ^{13}C-
186 BDE-209) and *m/z* 654 and 656 for DP (664 and 666 for ^{13}C-*syn*-DP). Recoveries,
187 method detection limits (MDLs) and method quantification limits (MQLs) are shown in
188 Table 1.
189
190 *Statistical analysis*
191 Data were tested for normality and homogeneity of variances using the Shapiro–Wilks
192 test of normality and an F test. Statistical significance between different treatments was
193 determined using analysis of variance (ANOVA), post-hoc Tukey's test and t-test; a p
194 value ≤ 0.05 was used to determine significant differences. Statistical analyses were
195 conducted using the open-source statistical programming language R v.3.1.1
196 (http://cran.r-project.org).
197
198 **Results and discussion**
199 *H_2O_2 in vitro control validation*
200 Various concentrations (0.2, 0.5 and 1 mM) and time points (10 and 30 min) were
201 explored in order to validate H_2O_2 doses to promote DNA damage. Results show that
202 DNA damage due to H_2O_2 exposure *in vitro* is time-dependant with significantly more

DNA damage apparent at the longer time point (ANOVA, $p < 0.001$). Based on these data, both in the pathway validation and in the main experiment *in vitro* controls were performed using a concentration of 1 mM and 30 min of exposure time.

Dietary pathway validation

DNA damage was observed in all B(α)P-exposed mussels, irrespective of exposure route (diet or aqueous), and was significantly different from control mussels (ANOVA, $p < 0.001$), (Fig. 1). The solvent control exhibited a small amount of DNA damage (<10 %) and DNA damage levels of B(α)P were similar in all B(α)P-exposed mussels (*ca.* 30 ± 3 %), approximately 20 % higher than in controls. DNA damage observed in the positive *in vitro* control, H_2O_2, was fivefold greater than observed in the controls (at 50 ± 6 %). DNA damage was not concentration-dependent. Results showed that the dietary pathway and the direct aqueous exposure did not affect the results. B(α)P is a known genotoxin and our results are in agreement with previous studies.[27, 39, 40] For instance, Di et al. report 60% damage following a 12 days *in vivo* exposure *Mytilus edulis*.[27] However, DNA strand breaks in control mussels were 30% and thus, DNA relative damage induced by B(α)P was up to 30%, similar to our reported values.

Water quality parameters

Water temperature during the exposure was 16.0 ± 0.2% ºC, salinity was 36.3 ± 0.8% ‰, dissolved oxygen was 7.93 ± 0.07% mg/L, and pH was 7.92 ± 0.03%. No intra- or inter-day variations among treatments were observed (ANOVA and post-hoc Tukey's test) and these values were considered optimal for the exposures.

Clearance rate

It has been previously demonstrated that CR in mussels can be affected by several chemical contaminants.[28] In this experiment, CR ranged from 0.49 to 0.90 L/h in the first time increment (10 min) both for BDE-209 and DP, while it was 0.46 and 0.69 L/h for B(α)P and control treatments, respectively. No statistical differences were found among the treatments, although all of them were significantly different than the seawater control (ANOVA, $F_{2,78}$=3.196, p <0.05). The same scenario occurred in the second time increment (20 min), where the CR value increased to 1.64-2.16 for BDE-209 and DP, to 1.29 for B(α)P and to 1.57 in controls. Even though values for BDE-209 (100 µg/L) and DP (56 µg/L) increased faster than other treatments, differences were not significant with any treatment with FR. Finally, after 30 min CR reached values ranging from 1.98 to 2.92 L/h both for BDE-209 and DP, 1.77 L/h for B(α)P and 2.09 for control mussels. Again, even if BDE-209 (100 µg/L) and DP (56 µg/L) showed higher values than the other treatments, these differences were not significant (Figure 2). Thus, we can summarize that mussels are not significantly affected by these FRs at a physiological level. This fact was described for B(α)P in a similar experiment[27] and suggests that mussels can take up these types of compounds without showing significant physiological changes.[32]

Comet assay

In all cases, DNA strand breaks observed were significantly higher than the negative control (ANOVA and Tukey's test, p < 0.001) (Figure 3). *In vivo* positive control, B(α)P, caused an effect of 35 ± 2%, while the *in vitro* positive control, H_2O_2, resulted in 56 ± 4%. Damage induced by BDE-209 was 13 ± 1%, 21 ± 1% and 21 ± 2% for 56, 100 and 200 µg/L exposure concentrations, respectively. Damage induced by DP was 13 ± 2%, 23 ± 1% and 18 ± 2% for 5.6, 56 and 100 µg/L exposure concentrations,

253 respectively. For BDE-209, DNA damage displayed a significant increase from 56 to
254 100 µg/L treatments, but no increase was observed from 100 to 200 µg/L treatments.
255 Concerning DP, DNA damage induced by the 56 µg/L was higher than the 5.6 µg/L
256 treatment. However, damage induced by the highest concentration (100 µg/L) was less
257 than that induced by 56 µg/L. It has been described that DNA repair mechanisms can
258 affect the response of the mussels to organic contaminants, since the simple breaks
259 mainly produced by these compounds might be repaired by base excision (BER).[41]
260 Furthermore, reduction of the DNA damage in the most concentrated treatments could
261 be caused by the exclusion of the apoptotic cells of the cell count.[42] Comparison
262 between BDE-209 and DP exposures at 56 and 100 µg/L showed that DP at 56 µg/L
263 induced oxidative damage at the same level as BDE-209 at 100 µg/L (23% and 21%,
264 respectively), while DNA strand breaks induced by BDE-209 at 56 µg/L were in the
265 same level as the low level of DP (13% and 13%, respectively). Surprisingly, oxidative
266 damage induced by DP at 100 µg/L (18%) was lower than at 56 µg/L (or following
267 BDE-209 exposure at 100 and 200 µg/L). This difference might be attributed to possible
268 differences in BDE-209 and DP metabolization. DP is known to produce de-chlorinated
269 analogues in some species[43] which could have lower toxicity. In contrast, de-
270 bromination products of BDE-209 are often more toxic than parent BDE-209.[44]
271 Furthermore, BDE-209 presents a more complex metabolism since low-brominated OH-
272 PBDEs could also be formed.[45]
273 Hence, results presented demonstrate that BDE-209 and DP can both induce DNA
274 strand breaks in mussels. This is in agreement with what previously reported effects in
275 zebra mussel (*Dreissena polymorpha*) where, similar to this study, BDE-209 caused
276 non-dose dependant DNA damage after an *in vivo* exposure of 7 days to 0.1, 2 and 10
277 µg/L.[46] *In vivo* exposures of BDE-47, BDE-100 and BDE-154, also in zebra mussel,

278 caused significant DNA damage up to 5, 11 and 12% respectively (expressed as % tail
279 DNA; controls up to 5%). These values are lower than those reported in this study, but
280 exposure concentrations (0.1, 0.5 and 1 µg/L) and exposure time (4 days) were also
281 lower.[47] To our knowledge, this is the first study reporting the oxidative capacity of DP
282 in mussels.
283
284 *Mn assay results*
285 Mn induced in the negative control were 1.7 ± 0.3%, while in the positive B(α)P control
286 it was 2.9 ± 0.5%, representing a significant 2 fold increase (ANOVA and post-hoc
287 Tukey's test, $p<0.05$). Concerning BDE-209, inductions were 1.6 ± 0.4%, 1.7 ± 0.3%
288 and 2.7 ± 0.4% for 56, 100 and 200 µg/L treatments, respectively. The first two
289 concentrations did not cause significant Mn induction compared to controls, but Mn
290 induced by 200 µg/L exposure was significantly higher (ANOVA and post-hoc Tukey's
291 test, $p<0.05$). Furthermore, DP caused Mn inductions of 2.0 ± 0.4%, 2.0 ± 0.6% and 2.5
292 ± 0.4% at 5.6, 56 and 100 µg/L treatments, respectively (Figure 4). In this case, BDE-
293 209 and DP showed the same pattern, i.e, Mn induction was only significant at the
294 highest level of exposure. Consequently, DP showed an effect at a lower concentration
295 than BDE-209 (100 and 200 µg/L, respectively) which implies that DP is more capable
296 of causing this kind of damage. However, no other studies are available to corroborate
297 this statement.
298 Mn induced by BDE-47, BDE-100 and BDE-154 in zebra mussel were up to 2, 2 and
299 2.5, respectively, but inductions were not significantly different than negative controls.[47]
300 Furthermore, both exposure concentrations (0.1 µg/L, 0.5 µg/L and 1 µg/L) and
301 exposure time (4 days) were lower than our conditions. This is in agreement with our
302 study, where Mn induction was only found at the highest exposure concentrations. Riva

et al. (2007) also reported that BDE-209 can induce DNA strand breaks, but not Mn induction.[46] Oxidative stress induced by reactive oxygen species (ROS) has been described as one the most plausible mechanism of the toxicity of BDE-209.[48] As a result, de-bromination of BDE-209 was also considered, since less brominated BDEs present higher oxidative capacity.[49] In this case, no other brominated congeners were detected (see results below), probably because metabolic/enzymatic capacity of mussels is not as high as in fish. Comet and Mn assay results were not correlated either for BDE-209 or DP. This might indicate that these compounds induce primary and repairable lesions rather than permanent ones.[46] However, this topic still requires further work in order to truly understand how these pollutants induce oxidative DNA damage.

Chemistry results

Water analysis: Concentrations found in water samples taken immediately after dosing were 0.02 ± 0.01%, 0.03 ± 0.01% and 0.3 ± 0.2% µg/L in BDE-209 treatments (56, 100 and 200 µg/L, respectively). Compared to values found after 23 h of exposure, concentrations in water decreased 92, 97 and 90%, respectively; in all cases concentrations after 23 h were lower (One-way ANOVA, $p<0.05$). Similarly, concentrations of DP immediately after dosing were 0.4 ± 0.2%, 0.3 ± 0.1% and 0.7 ± 0.4% µg/L in 5.6, 56 and 100 µg/L treatments, respectively. These concentrations decreased significantly (one-way ANOVA, $p<0.05$) up to 77%, 79% and 86%, respectively, after 23 h. Levels in control water were below MDL for both compounds in all cases (Figure 5A). Concentrations used in this study exceeded the estimated solubility of these compounds (< 1 µg/L).[50] However, it has been demonstrated that presence of dissolved organic matter enhances solubility.[27] BDE-209 and DP rapidly

distributes between particulates and mussels, thus concentrations in the aqueous phase are expected to be low.

Mussel analysis: Levels of BDE-209 found in the exposed mussels at the end of the treatment were always higher than the controls, proving that mussels bioaccumulated BDE-209 through the *in vivo* exposure (ANOVA and post-hoc Tukey's test, $p < 0.05$). Values were 1.9 ± 0.5%, 1.7 ± 0.4% and 1.6 ± 0.6% µg/mussel, corresponding to the 56, 100 and 200 µg/L exposures. No differences were observed between the three exposures (ANOVA and Tukey's test, $p>0.05$). This could be due to BDE-209 de-bromination, but while it has been described in fish[51] to the best of our knowledge there are no studies in mussels. During the instrumental analysis no other peaks were observed. On the other hand, values found in mussels exposed with DP were 4.7 ± 0.9%, 8.8 ± 0.96% and 21 ± 4% µg/mussel corresponding to the 5.6, 56 and 100 µg/L treatments, respectively. BDE-209 and DP values were significantly higher than control in all cases (ANOVA and post-hoc Tukey's test, $p<0.05$). Furthermore, in the case of DP a concentration dependant increase was found (ANOVA and post-hoc Tukey's test, $p<0.05$). These results show that DP is bioaccumulated by mussels, as has been previously reported.[11, 52] Moreover, the ratio between the anti-isomer and the total DP burden was also evaluated. F_{anti} is defined as the concentration of anti-DP with respect to the total DP concentration, both lipid-normalized. It has been described as a good indicator of the different behaviour of the two isomers in the environment, since the initial F_{anti} in the commercial mixture (~0.7) can change when analysing complex organisms such as dolphins.[18] F_{anti} values found in mussels from the three different exposures (0.74 ± 0.01%, 0.69 ± 0.01% and 0.73 ± 0.01% for low, medium and high levels, respectively) were similar and significantly lower than values found in the control mussels, which

were up to 0.79 ± 0.02% (ANOVA and post-hoc Tukey's test, $p<0.05$). The commercial mixture of DP used in the exposure was also analysed (n=3, 0.72 ± 0.01%). Even if values of the exposed mussels were different than controls, values are still in the range described for commercial DP mixtures. Thus, no *syn*-DP enrichment was observed, which is in agreement with other studies of DP in mussels.[11] It has been described that the particulate matter in the gastro-intestinal tract can affect BDE-209 determinations in mussels.[53] However, since mussels were sampled 24 h after the last feeding, influence of ingested food in BDE-209 analysis was considered to be minimal, as has been suggested previously.[46]

Overall, these data confirm the use of *M. galloprovincialis* as a suitable biological model for *in vivo* exposures to FRs. In addition, data for DP represents the first evidence of a genotoxic capacity of this compound in mussels. Both FRs induced significant DNA damage even at the lowest selected concentrations, whereas Mn induction was only significant in the highest doses. Other factors such as the timeframe needed to induce micronuclei require further investigation. In contrast, the feeding rate was not significantly altered by exposure to either compound.

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Notes

The authors declare no competing financial interest

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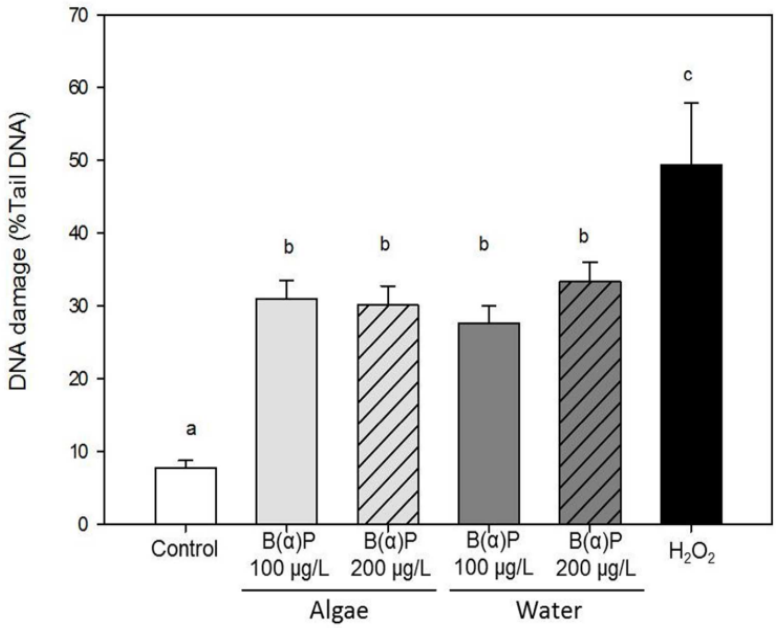
558
559

560 **Table 1**: Recoveries (%), RSD (%), MDL and MDL of BDE-209 and DP in water

561 (ng/mL) and mussel (pg/g lw)

Compound	Water				Mussel			
	R	RSD	MLOD	MLOQ	R	RSD	MLOD	MLOQ
BDE-209	75	11	0.40	1.30	68	6	200	330
syn-DP	67	8	0.60	2.00	85	7	5.50	18.3
anti-DP	73	12	0.10	0.30	88	5	4.30	14.3

562



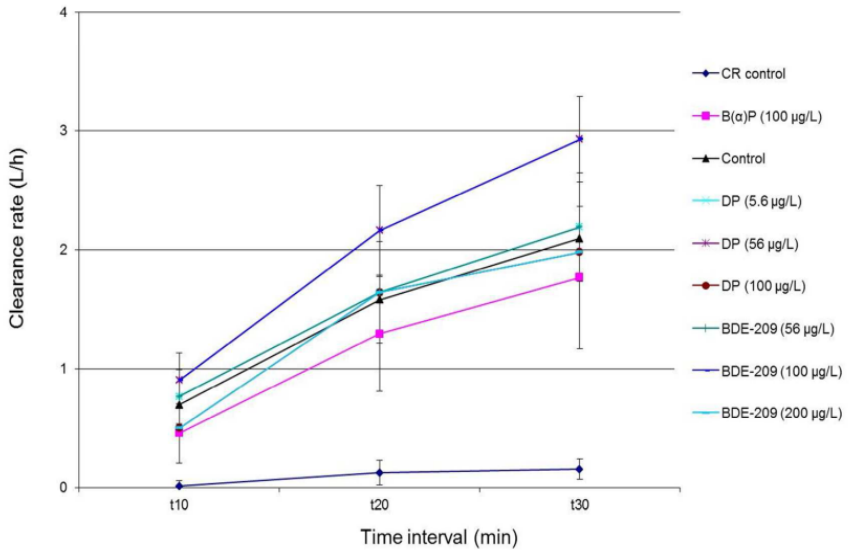
563

564 **Figure 1**: DNA damage (mean ± SE; n = 6 per treatment) in benzo(α)pyrene-exposed

565 mussels. Treatments with the same letter are not significantly different; where

566 significant differences occur between treatments, $p < 0.001$.

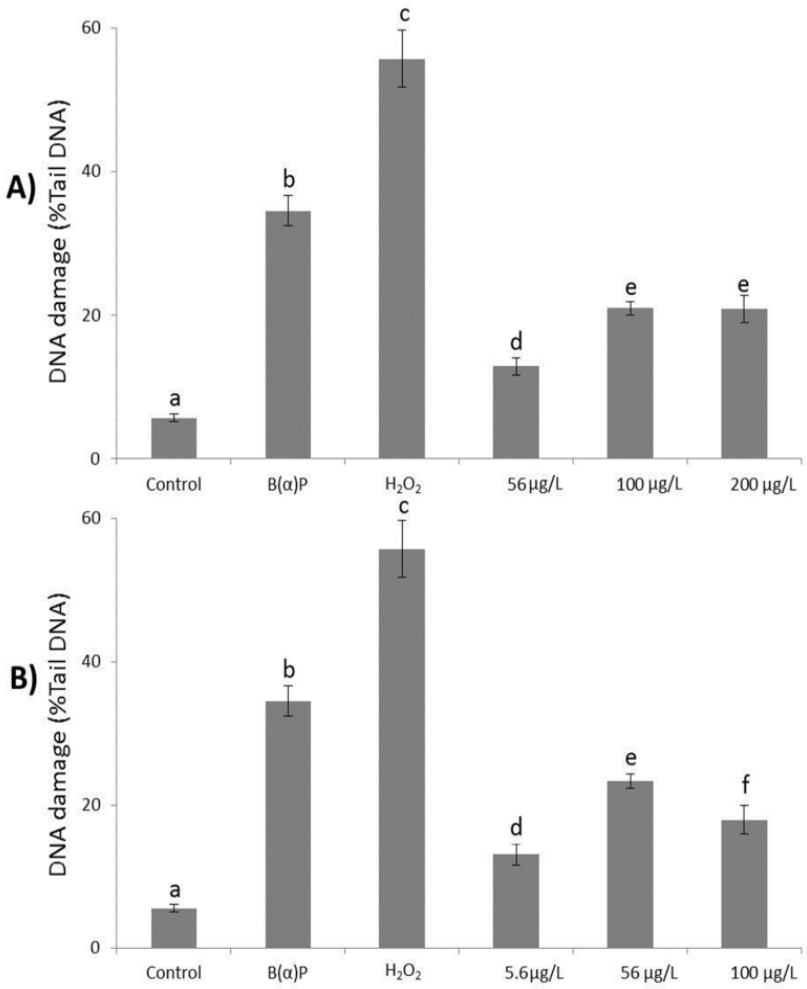
567



568

569 **Figure 2:** Clearance rate (L/h) of the different treatments. Error bars represent SE. n=7

570 CR control = seawater. Control = control mussel exposed to acetone (0.05 %, v/v)



571

572 **Figure 3**: Induction of DNA strand breaks (represented as % Tail DNA) in *Mytilus*
573 *galloprovincialis* haemolymph after 6 days of exposure to FRs compared to control
574 mussels, exposure to B(α)P (100 µg/L) and H_2O_2 (1 mM, *in vitro*). **A**) BDE-209. **B)** DP.
575 Treatments with the same letter are not significantly different; where significant
576 differences occur between treatments, $p < 0.05$.

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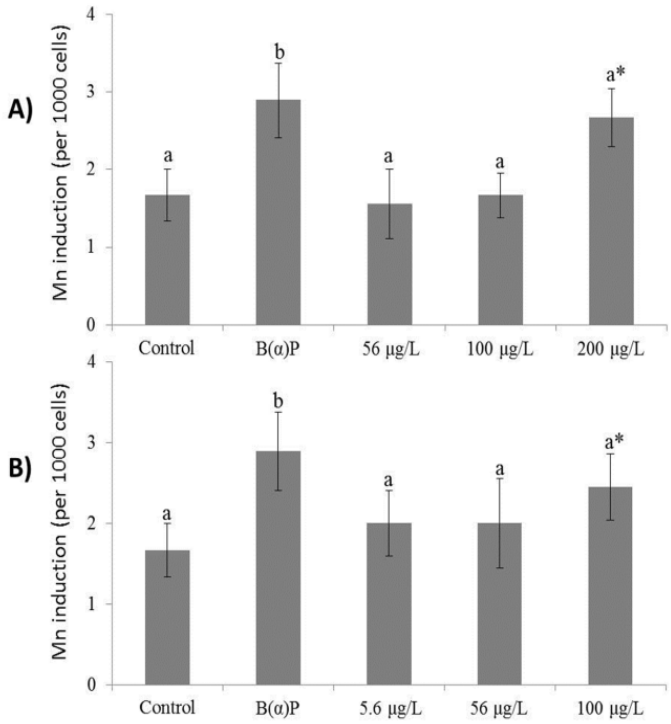
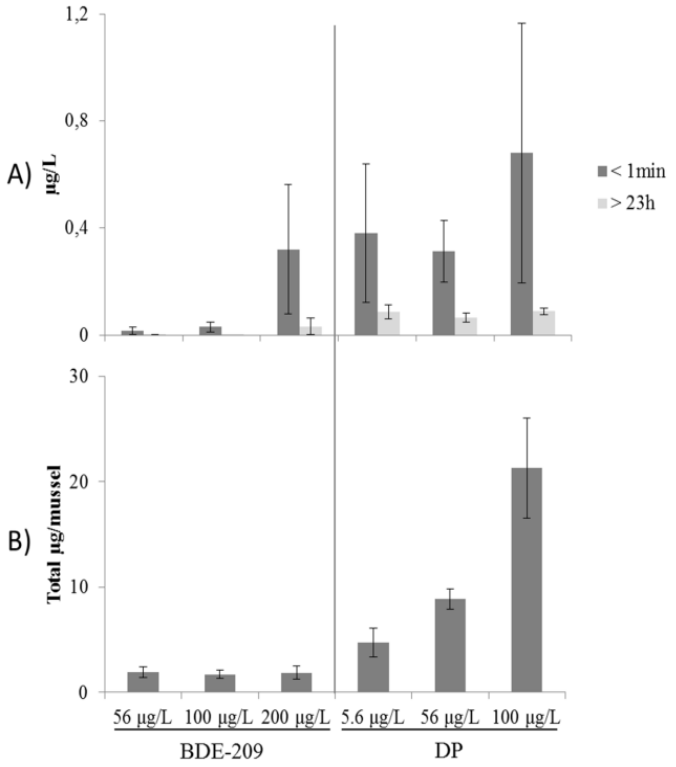


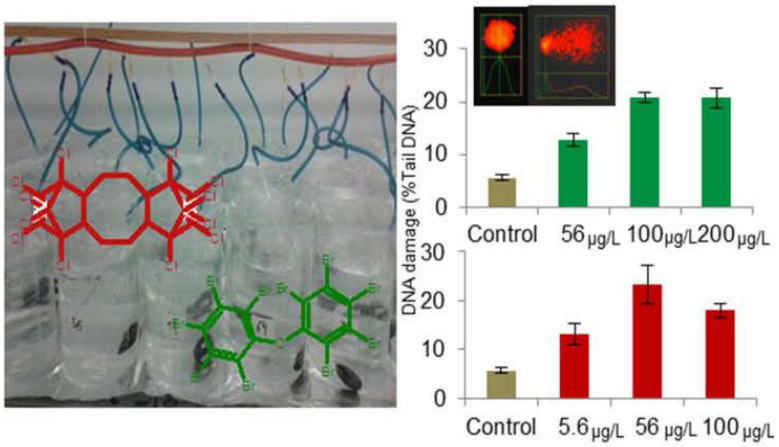
578

579 **Figure 4:** Mn induction (represented as mean ± SE) in *Mytilus galloprovincialis*
580 haemolymph after 6 days of exposure to FRs compared to control mussels and exposure
581 to B(α)P (100 µg/L). **A**) BDE-209. **B)** DP. Treatments with the same letter are not
582 significantly different; where significant differences occur between treatments, $p < 0.05$.



583

584 **Figure 5**: **A)** Concentrations of BDE-209 and DP found in water samples corresponding
585 to the exposures (n=3 per treatment) after dosing and immediately before the water
586 change. Concentrations in control samples were below the MDL in both cases. **B)**
587 Levels of BDE-209 and DP found in exposed mussels (n=7). Control levels were 0.04 ±
588 0.01% µg for BDE-209 and 0.11 ± 0.02% µg for DP.



106x59mm (150 x 150 DPI)

5.3. Discusión

Mejillones

Las exposiciones *in vivo* a BDE-209 y DP demostraron que ambos compuestos pueden causar daños oxidativos en la cadena del ADN e inducción de micronúcleos, mientras que el comportamiento fisiológico del mejillón no se vio alterado: los valores del CR no variaron respecto al grupo control. Con el objetivo de ver si estos contaminantes causaban un retraso en la obtención del alimento se midieron los valores a 10, 20 y 30 min., cuando lo normal es hacerlo sólo a los 30 min. Esto es debido a que un ligero retraso en el ritmo de alimentación puede afectar a la obtención de nutrientes por parte del individuo en situaciones en las que estos no abundan, comprometiendo su desarrollo. Sin embargo, no fue el caso y todos los individuos mostraron valores normales para la época del año. Algunos autores sugieren que la época en la que se muestrearon los mejillones (agosto) no es la adecuada ya que acaba de pasar su etapa de reproducción y se encuentran debilitados (Canty *et al.*, 2007). De hecho, se consideró que con más motivo aún es importante evaluar los efectos que el BDE-209 o el DP causarán en los mejillones cuando están en este estado de vulnerabilidad, ya que existe la posibilidad de que en otras épocas del año sí sean capaces de resistir la acción de estos compuestos mientras que en esta sí sean vulnerables. No existen otros datos en bibliografía referentes al efecto del BDE-209 o DP en el CR, y de hecho ni siquiera para otros HFRs, pero por lo visto en este estudio no parece ser un parámetro relevante. Es de destacar que a las concentraciones estudiadas no se observaron tampoco efectos letales.

Donde sí se observaron efectos, para todas las concentraciones estudiadas, fue a nivel genético. Mediante el CA se observaron daños significativos en la cadena del ADN, observando que en los niveles más altos el daño no sólo no aumentaba, sino que disminuía ligeramente. Esto podría explicarse debido a la completa destrucción de las células, no observándose entonces daño alguno ya que el CA necesita un mínimo de núcleo central intacto para calcular la relación cabeza-cola, y afectando por tanto a la cuantificación de los resultados (Figura 5.2).

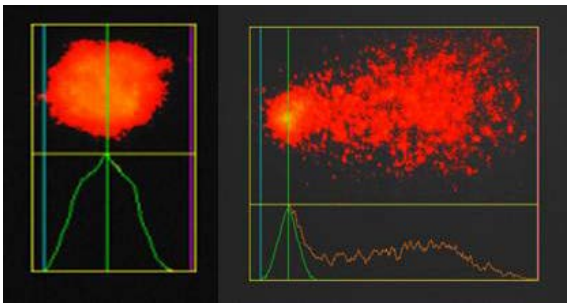


Figura 5.2. "Scores" finales de la técnica CA. Izquierda: célula correspondiente a un control. Derecha: célula correspondiente a una exposición por HFR.

La información existente para el BDE-209, y en realidad para los PBDEs en general, es escasa en lo que respecta a sus efectos genotóxicos en mejillones. En un estudio *in vivo* en mejillones cebra expuestos al BDE-47, BDE-100 y BDE-154 (Figura 5.3) se observó como el BDE-100 y BDE-154 inducían daños en el DNA a concentraciones entre 10 y 100 veces inferiores a las utilizadas en el artículo #10 (Parolini y Binelli 2012). Ignorando el hecho de que se trata de especies diferentes, se podría concluir que el BDE-100 y BDE-154 tienen más potencial genotóxico que el BDE-209.

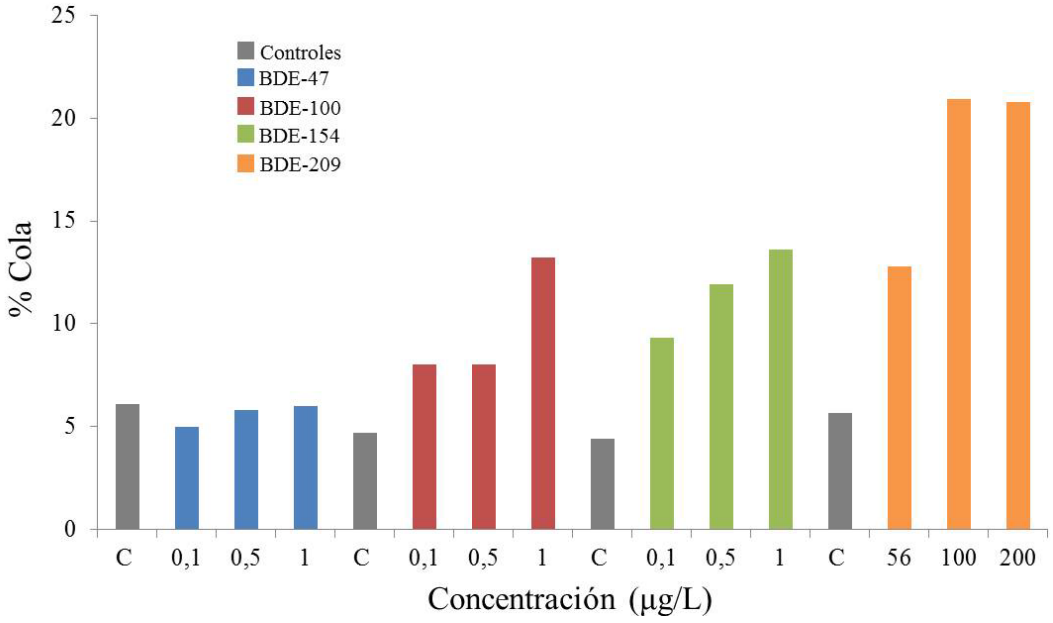


Figura 5.3. Daños oxidativos en el ADN inducidos por diferentes congéneres de los PBDEs. BDE-47, BDE-100 y BDE-154: mejillón cebra (Parolini y Bineli 2012). BDE-209: mejillón mediterráneo (Artículo #10).

Los efectos del BDE-209 en mejillones cebra mediante exposición *in vivo* a 0,1, 2 y 10 µg/L también han sido estudiados. Lamentablemente están expresados en base a la relación entre la longitud y el diámetro de la cabeza (LDR, del inglés length diameter ratio) por lo que es difícil establecer una comparación con los resultados obtenidos en

mejillones mediterráneos. Los valores de % de cola reportados, basados en la equivalencia: % cola <10% es daño inexistente, % cola 10-25% será daño bajo, % de cola 25-50% daño moderado, 50-75% daño alto, y finalmente >75% será daño extremo, permitieron concluir que tras 7 días los 3 niveles de concentración habían causado un daño mayoritariamente moderado. Aplicando estas equivalencias en los valores del artículo #10 los daños observados en mejillones mediterráneos serían bajos. El estudio en cebras duró 7 días, 1 día adicional (Riva *et al.*, 2007). En el caso del DP sólo otro estudio ha evaluado sus efectos en el ADN mediante el CA, y en una especie completamente diferente: el protozoo ciliado *Tetrahymena thermophila* (Dou *et al.*, 2014). Tras una exposición durante 30 min a 2,4, 12, 60, 300 y 1500 µg/L se observaron colas de entre 4% y 18%, siendo los 2 niveles de concentración más altos los que produjeron un mayor daño.

Por lo que respecta a la inducción de micronúcleos, tanto el BDE-209 como el DP sólo la provocaron en los valores más altos de concentración (200 y 100 µg/L, respectivamente). El caso del BDE-209 concuerda con un estudio en mejillones cebra donde no se observó inducción de micronúcleos para una concentración de 2 µg/L suministrada durante 11 días (Riva *et al.*, 2007). Por el contrario, tanto el BDE-47, BDE-100 y BDE-154 han demostrado ser capaces de provocar esta inducción a concentraciones muy inferiores a las utilizadas en el ensayo con BDE-209 (Figura 5.4)

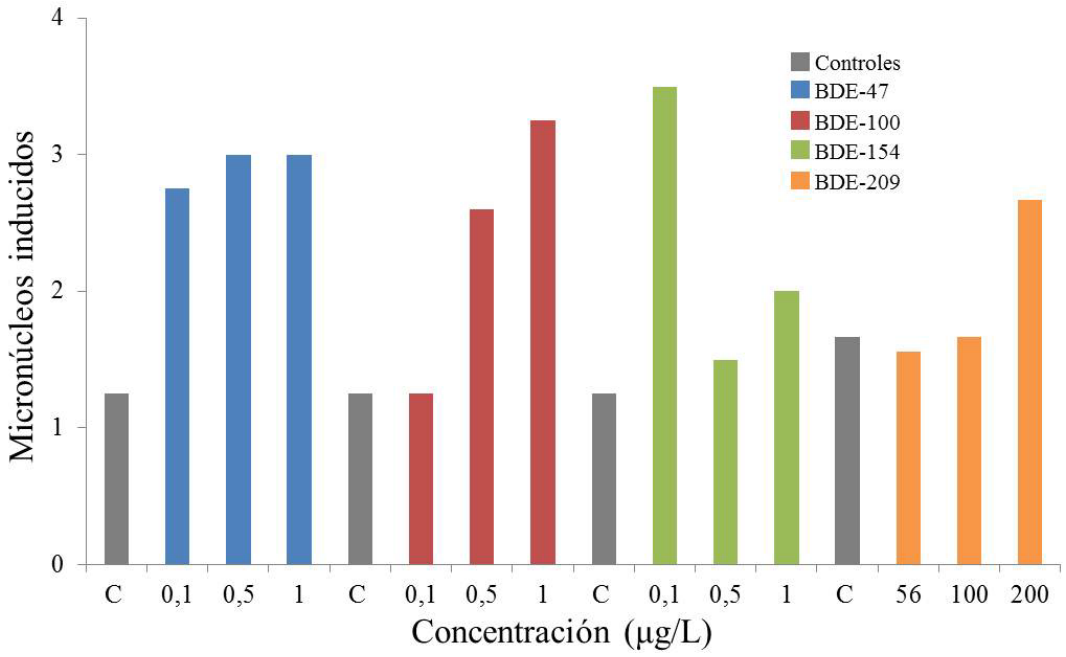


Figura 5.4. Mn inducidos por diferentes PBDEs. BDE-47, BDE-100 y BDE-154: mejillón cebra. BDE-209: mejillón mediterráneo.

De nuevo la acción del DP sólo ha sido estudiada en la *T. thermophila,* donde sólo se produjo una inducción significativa a la concentración de 300 μg/L (Dou *et al.*, 2014), pero por lo visto en este estudio de nuevo parece causar efectos similares al BDE-209 a menor concentración.

Otros invertebrados

En Garcia-Reyero *et al.* (2014) se vio como el BDE-209 era biodisponible y bioacumulado en larvas de zebrafish, lo que reafirma lo discutido en el capítulo 3. Además, causó alteraciones en el comportamiento y por tanto se generaron nuevas evidencias de que el BDE-209 puede provocar alteraciones neuronales, algo también visto en ratones (Buratovic *et al.*, 2014). La metodología propuesta, basada en exposiciones en larvas de peces cebra, análisis *in vitro* y programas de predicción, dio buenos resultados y sería interesante aplicarla en un futuro a los HNs. Del mismo modo la metodología de exposición utilizada en poliquetos y cangrejos también dio buen resultado ya que se observó la acumulación del BDE-47 tanto desde el sedimento a ambas especies, como desde el poliqueto al cangrejo. En el capítulo 2 se ha visto como el potencial de acumulación del Dec 602 es similar, o incluso superior al del BDE-47, por lo que esta ruta de exposición podría utilizarse para comprender mejor los mecanismos de incorporación de los HNs en pequeños invertebrados.

Pese a que existen varios mecanismos propuestos sobre la hidroxilación de los PBDEs (Gross *et al.*, 2015; Xu *et al.*, 2015; Zhai *et al.*, 2014), algunos autores han sugerido que los OH-PBDEs también podrían proceder de fuentes naturales a través de la substitución del grupo metilo de los MeO-PBDEs (Fan *et al.*, 2014). De todos modos, la principal problemática que presentan estos compuestos reside en su elevada toxicidad, que la mayoría de estudios revelan superior a la de los propios PBDEs. Por tanto su determinación, especialmente en especies de metabolismo avanzado, permite completar el estudio sobre el impacto ambiental de los PBDEs. Con este objetivo se desarrolló un método para su análisis (Feo *et al.*, 2013) pero la falta de tiempo y el hecho de que sólo aproximadamente el 1-5% de PBDEs parece ser metabolizado a OH-PBDEs hicieron que se decidiera no seguir por esta vía. No obstante, fue aplicado a los poliquetos y cangrejos expuestos a BDE-47, detectando tres congéneres diferentes (3-OH-BDE-47, 5-OH-BDE-47 y 6-OH-BDE-47) que podrían ser los principales causantes de algunas actividades enzimáticas anómalas observadas.

5.4. Conclusiones

El trabajo realizado en este capítulo tenía como objetivo obtener información de los efectos que algunos de los compuestos estudiados en esta tesis podían causar, habiendo demostrado previamente su omnipresencia en todo tipo de biota. Tanto parte del trabajo realizado como el disponible en bibliografía demuestra que el peligro que representan estos compuestos en especies que van desde pequeños invertebrados hasta mamíferos marinos es real, habiéndose observado alteraciones en distintos procesos enzimáticos y daños causados por estrés oxidativo. La importancia de este hecho radica en que, pese a no causar un efecto mortal, las alteraciones causadas pueden afectar seriamente la vida normal de las especies en sus hábitats naturales.

En el estudio concreto que pretendía comparar el BDE-209, ya prohibido, con uno de sus substitutos posibles, el DP, se vio como el DP producía los mismos efectos que el BDE-209 e incluso parece presentar un mayor potencial genotóxico, ya que las alteraciones provocadas fueron a una concentración inferior a la del BDE-209. No obstante, los estudios sobre la toxicidad del DP son muy escasos y claramente se necesita aumentar el conocimiento sobre sus propiedades toxicológicas. Además, las evidencias presentadas en el capítulo 4 en cuanto a la mayor presencia del Dec 602 hacen aún más evidente que no debe ser despreciado a la hora de llevar a cabo estudios sobre la toxicidad de los HNs.

CAPÍTULO 6

CONCLUSIONS

The results obtained in this thesis, exposed and discussed in previous chapters, result in the following conclusions:

Development of analytical methodologies

A new methodology for the analysis of HNs by GC-NCI-MS-MS, with mLODs between 0.1 and 2.9 pg/g dw in environmental samples, between 10 and 150 pg/g lw in biota samples, and with recoveries (%) between 57 and 114%, was developed. This methodology proved to be suitable for the chemical analysis of these contaminants. It was the first methodology using MS-MS for HNs analysis and, to date, it is the only one using NCI as the ionization technique. NCI-MS-MS provides better mLODs than EI-MS-MS for all HNs and, in addition, allows the detection of HNs in areas far away from production sources, where their levels are considerably low. Furthermore, a methodology for the analysis of PBDEs and emerging BFRs by GC-EI-MS-MS was also developed, as an improvement of the methodology previously available in the laboratory which was based on GC-NCI-MS. Unlike the previous method, GC-EI-MS-MS allows quantitative analysis by isotopic dilution. As a result, mLODs between 0.01 and 2.78 ng/g dw in environmental matrices and between 0.01 and 3.59 ng/g lw in biota were obtained. This method also included BDE-209 and DBDPE, which are not normally analyzed by MS-MS. Recoveries ranged from 51% to 105% with sediment as the matrix providing the best values, whereas sludge and bird eggs gave the worst recoveries. Both methods provided solid reproducibility, never exceeding a RSD (%) of 15% (intra-day) or 20% (inter-day) in any compound or matrix. In short, both methodologies allow a sensitive and selective analysis of HFRs in several environmental and biological matrices.

Presence and behaviour in the environment

First evidence of the environmental presence of DP dates from 2006, and from 2010 in the case of Dec 602, Dec 603 and Dec 604. However, its production and use began in the 70s after Mirex was banned as FR. Therefore, they have been used for decades, even though their production volume was lower than PBDEs or HBCD. During this thesis numerous matrices and species from different areas and even different continents have been analysed, such as sediment, sludge, filtering species, crustaceans, fish, dolphins or birds. Sediment and sludge represent a first entrance, or re-entrance, point into the various trophic chains. It was the first time that the presence of HNs is reported in

Spanish sediments, with levels between 0.01 and 3.74 ng/g dw, as well as Dec 603 presence in sludge worldwide. More precisely, concentrations of HNs in sludge ranged from 2.66 to 19.0 ng/g dw. All these results expose the differences between different geographical regions: while in South America PBDEs represented almost the 100% of HFR contamination, in Spain HNs concentrations were in the same order, or even higher, than concentrations of PBDEs. This fact shows its ubiquity and the need to include these compounds in future studies aiming to characterize the contamination caused by HFRs, especially in areas with a high industrial activity. Furthermore, it also shows a variable global use of the different HFRs. Moreover, regarding emerging BFRs, DBDPE was detected both in sludge and sediments in concentrations up to 124 and 31.5, respectively, suggesting that this compound might be used as a replacement of BDE-209. On the contrary, PBEB and HBB were not detected in almost any sample.

HNs were also detected in filtering animals, crustaceans and fish from Chile, in concentrations between nd and 9.8 ng/g lw. These are the first data reporting the presence of HNs in marine organisms from the pacific coast of South America. Likewise, HNs levels found in birds from Doñana natural space represent the first evidence of the presence of these compounds in birds from this region, with the exception of white stork, gulls and peregrine falcon. Moreover, top predators like dolphins presented the highest HNs burden (from nd to 506 ng/g lw), similarly to PBDEs and other organic contaminants. These results represented the first evidence of the accumulation of Dec 602 and Dec 603 in dolphin blubber and brain, and it is only the third study describing the presence in dolphins of DP worldwide (first one in Mediterranean Sea).

Bioaccumulation and biomagnification capacity was evaluated in different trophic chains. These processes have been characterized previously for classic FRs such as PBDEs and HBCD, and results show that HNs have similar capacities. Regarding bioaccumulation, BSAFs of Dec 602 were up to 11 indicating that its bioavailability is comparable to BDE-47. On the other hand, DP presented a bioavailability closer to BDE-209. As regards to biomagnification, Dec 602 displayed biomagnification capacity among terrestrial and aquatic trophic chains, while this capacity was not observed for DP. Again, it is showed that HNs should be taken into account in future studies and regulations, paying more attention to Dec 602.

Moreover, diet seems to play an important role concerning HNs bioaccumulation, as it has been described for PBDEs or HBCD. Species with an aquatic diet presented PBDEs

concentrations considerably higher than HNs concentrations, whereas in birds (terrestrial diet) levels of both families were closer. Furthermore, different behavior of isomers *syn*-DP and *anti*-DP was noticed, which might be diet dependent: f_{anti} was around 0.5 in aquatic organisms, but in birds it was around 0.7, closer to the value in commercial mixtures.

It was found that PBDEs, emerging BFRs, HNs and HNPs were capable of surpassing BBB and accumulate in the brain. PBDEs are proved to be neurotoxic, and this potential capacity should be studied for the other contaminants found in brain. Special attention needs to be paid to compounds such as HBB, which is normally absent in blubber but was found in concentrations up to 84 ng/g lw in brain, while in blubber maximum value was 8.1 ng/g lw. This case evidences that using only blubber in the evaluation of the environmental risk that poses a new lipophilic compound might not be enough, and other tissues should be analyzed if possible. Furthermore, PBDEs and HNs concentrations in blubber and brain show that HNs might have a greater capacity than PBDEs to pass through the BBB: PBDEs/HNs ratio ranged from 0.65 and 130 in blubber, while in brain this ratio ranged from 0.10 and 10. This was consistent across the 5 species studied. Thus, evaluation of the effects of these compounds in brain should be evaluated.

Based on the results obtained, it seems that environmental concentrations of classic and alternative HFRs recently found are different than the ones reported in last years. DBDPE might be replacing BDE-209, as suggested by the ratio BDE-209/DBDPE in sediment (between 0.1 and 2.4) and sludge (between 1.3 and 6.4). Besides, a clear decrease in the PBDEs levels in birds, especially of the main components of Penta-BDE mixture, was observed in the last decade. However, the time frame chosen was reduced and this trend should be confirmed in the future, when more years have passed since the regulations over PBDEs came out.

Evaluation of the toxicological effects

It was demonstrated through *in vivo* exposures in mussels that DP has a similar behavior than BDE-209 both at the physiological and genetic level. While no effects concerning filtering capacity of the mussels were observed after separate exposures to both chemicals, they produced oxidative DNA damage and Mn induction, showing the genotoxic capacity of these FRs. In addition, DP had similar or higher effects than BDE-209 at lower concentrations (5.6, 56 and 100 μg/L for DP and 56, 100 and 200

μg/L for BDE-209). Therefore, DP might have higher genotoxic potential than BDE-209. As future work, it would be interesting to carry out these experiments with the rest of HNs, especially Dec 602 due to its high concentrations found in biota.

Overall, studies performed during this thesis reflect that HNs meet some of the criteria stipulated in Stockholm Convention to be included in the list of banned compounds, such as ubiquity, bioaccumulation and biomagnification capacity, and toxicological effects. These data, as well as long-range transport capacity, should be expanded as soon as possible. In particular, high concentrations of Dec 602 in biota prove its bioaccumulation and biomagnification capacity, but there are no studies evaluating its toxicological effects. Moreover, DP has also shown bioaccumulation capacity comparable to BDE-209 and a higher toxicity in mussels. Thus, it could be included in the list to prevent its environmental concentrations to increase in the near future.

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