Temporal trends of flame retardants and non-targeted analysis of halogenated contaminants in striped dolphins from the Mediterranean Sea

Aznar-Alemany Ò[1], Sala B[1], Jobst KJ[2], Reiner EJ[2], Borrell A[3], Aguilar À[3], Barceló D[1,4], Eljarrat E[1]

[1]Institute of Environmental Assessment and Water Research (IDAEA-CSIC); Barcelona (Spain), oaaqam@cid.csic.es; [2]Ontario Ministry of the Environment and Climate Change (MOECC); Toronto, (Canada); [3]Institute of Biodiversity Research (IRBio) and Department of Evolutionary Biology, Ecology and Environmental Sciences, Universitat de Barcelona; Barcelona (Spain); [4]Catalan Institute for Water Research (ICRA); Girona (Spain)

Introduction

Flame retardants (FRs) effects on human health and the environment have been a growing concern through time. Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are included in the Stockholm Convention on Persistent Organic Pollutants (POPs). POPs are often halogenated and have high lipid solubility, leading to their bioaccumulation in fatty tissues. The use of PBDEs and HBCD has also been restricted or banned: Regulation (EC) No 1907/2006 REACH; Directive 2011/65/EU; Directive 2013/39/EU.

Alternative FRs include decabromodiphenyl ethane (DBDPE), pentabromoethylbenzene (PBEB) and hexabromobenzene (HBB). DBDPE is the marketed alternative to Deca-BDE: their structures and flame retardant properties are similar. Additionally, Dechlorane Plus (DP) and dechloranes 602, 603 and 604 (Dec 602, Dec 603, Dec 604) are chlorinated alternatives to Mirex, which was banned in the United States due to its toxicity.

A growing alternative to halogenated FRs (HFRs), are organophosphorus FRs (OPFRs). They represented 20 % of all FRs used in 2006 in Europe —roughly twice the quantity of brominated FRs— and the ban on PBDEs has increased their popularity[1]. OPFRs are also released from materials and access environmental matrices through washout, infiltration, deposition, etc. Moreover, OPFRs are used as plasticisers; so they leak from the tones of plastic that reach seas and oceans. Their presence has been reported in sediments, water and fish[2-4]. OPFRs show toxic effects on the reproductive and endocrine systems, as well as systemic and carcinogenic effects[1].

Not all halogenated organics originate from anthropogenic sources. Methoxylated PBDEs (MeO-PBDEs) are natural analogues to PBDEs that are synthesized by some marine sponges, algae and their associated cyanobacteria. They have been found in cetaceans and seafood and can be detected in marine mammals at similar levels to manufactured halogenated organic compounds.

There are over 100,000 chemicals in use, but few have been investigated for deleterious effects to the environment and human health. High resolution mass spectrometry (HRMS), performed using time-of-flight (TOF) and Orbitrap mass analysers, can generate measurements of thousands of compounds from a single sample. Interpretation of such information rich datasets may reveal the identities and occurrence of contaminants that are not routinely analysed or regulated. In this work, HRMS data is visualized constructing a Kendrick mass defect plot. This enables efficient characterisation of compounds in the complex environmental samples of this study by displaying the large number of spectral peaks is a scatter plot with the mass defect of each peak (y axis) plotted against the nominal mass (x axis)[5]. Visual inspection of the plot results in facile identification of compounds as each Br/Cl congener series appears as a line of peaks parallel to the x-axis. Isotope patterns, like those of bromine and chlorine, are also easily recognizable.

This study assesses the occurrence of the aforementioned compounds in striped dolphin (*Stenella coeruleoalba*) from the Mediterranean Sea through three time periods. This is the first report about OPFRs in marine mammals. A search for non-targeted halogenated contaminants was also performed.

Materials and methods

1. Sampling

Dorsal muscle samples were collected from 42 striped dolphins (*Stenella coeruleoalba*) stranded on the northwestern Mediterranean coasts. The samples were collected in three different periods, including 15 samples from 1990, 15 samples from 2004-2009 and 12 samples from 2014-2018. All individuals were adult males

ranging from 164 to 224 cm of length. Samples were kept frozen at −20 °C and were freeze-dried prior to analysis. Lipid content referenced to dry weight (dw) was between 0.3-10.7%.

2. Sample preparation

The extraction of OPFRs was carried out by ultrasound assisted extraction according to an existing method[4]. Freeze-dried sample (0.5 g) was extracted by sonication. The extract was reconstituted and centrifuged and an aliquot of 200 μl was used for the instrumental analysis. Purification was performed on-line at the beginning of the instrumental analysis. Labelled OPFRs standards were added prior to analysis by turbulent flow chromatography coupled to LC-MS/MS (TFC-LC-MS/MS).

For the other compounds, sample extraction was carried out according to previous works. Freeze-dried sample (1.5 g) was spiked with the labelled standards. Pressurized liquid extraction (PLE) was used, lipid content was determined gravimetrically, the extract underwent an acid attack to remove the fat, the organic phase was cleaned by solid phase extraction (SPE) and the extract was reconstituted with toluene.

3. Instrumental analysis

For OPFRs, online sample purification and analysis was performed with a Thermo Scientific TurboFlow™ system[5]. CycloneTM-P and C18-XL columns were used in combination for purification. Chromatographic separation was achieved with an analytical column Purosphere Star RP-18. Mobile phase was a gradient of water and methanol, both 0.1% formic acid, at 0.75 ml min^{-1}. Spectrometric analysis was performed with a triple quadrupole with a heated-electrospray ionization source. RSDs were 2.4-16%. LOQs and LODs were, respectively, 0.97-24.8 ng g^{-1} lipid weight (lw) and 0.19-19.3 ng g^{-1} lw. HFRs were analysed by GC-MS/MS using an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer and a DB-5ms column. Brominated compounds were analysed using electronic ionization (EI). The instrumental conditions[6] and the spectrometric determination[7] are described in previous publications. Due to their low sensibility with GC-EI-MS/MS, BDE-209 and DBDPE were analysed by GC-MS with the same chromatographic conditions in an Agilent 5975A mass spectrometer using negative chemical ionization (NCI). The analysis of dechloranes was performed by NCI as described in a previous article[8]. RSDs were 1.1-22 %, LODs were 0.0023-10.6 ng g^{-1} lw and LOQs were 0.0077-35.4 ng g^{-1} lw. After analysis of the previous HFRs, extracts were redissolved in methanol and d$_{18}$-HBCD was added. HBCD was analysed using an Agilent HP 1100 binary pump LC system coupled to a hybrid triple quadrupole/linear ion trap 4000QTRAP[9]. RSDs for α-, β- and γ-HBCD were 3.1-8.3 %. LOQs and LODs were 0.4-4.4 ng g^{-1} lw and 0.2-2.0 ng g^{-1} lw.

2.4. Non-targeted analysis

GC×GC/HRQTOFMS analysis was performed with an Agilent 7890B gas chromatograph fitted with a Zoex ZX2 GC×GC thermal modulator and interfaced to a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer. The first-dimension column was an Rtx-5 (60 m) followed by a Restek Siltek deactivated guard column (1 m) in the modulator loop. The second-dimension column was an Rtx-17 SIL (1 m) and was placed in a secondary oven. The secondary column was then connected to a Custom MXT tubing, which was inserted into the transfer line. Helium was used as the carrier gas, and the flow was 1.5 ml min^{-1}. The modulation period was of 4 s. The transfer line and ion source temperatures were 340 °C and 150 °C. The acquisition range was 50–1200 amu and the acquisition rate was 30 Hz. The MS was operated at a resolving power of > 20,000 (FWHM).

A composite mass spectrum was generated by combining all mass spectra recorded during the chromatographic separation. Mass measurements, recorded using the IUPAC mass scale were converted to the H/Cl mass scale, which is defined by the substitution of a hydrogen atom by a chlorine atom being equal to 34.000 Da. H/Cl mass is calculated by applying a 34/33.96102 factor to IUPAC mass. The H/Cl mass defect plot enables the efficient and comprehensive characterisation of halogenated compounds[10] and helped guide the extraction of mass chromatograms from the GCxGC contour plot (not shown).

Results and discussion:

1. Temporal trends

PBDEs levels decreased from 5120±2271 ng g^{-1} lw in 1990 to 2737±2437 ng g^{-1} lw in 2004-2009 (t-test without outliers, $t = 5.03$, df = 25, $p < 0.01$) and have remained constant since (Table 1). The bans in Europe and North America in the early 2000s and the inclusion of PBDEs in the Stockholm Convention in 2011 may account for a decrease in the emission of these pollutants and, therefore, the amount detected in biota. An annual

decrease of PBDE concentrations in Canada over the last decade has also been reported (ECCC, 2016), These findings could validate the success of the prohibition measures if the trend continues in the future. However, the estimated risk threshold for thyroid endocrine disruption in grey seals from the United Kingdom was estimated at $1500 \, \mathrm{ng \, g^{-1}} \, \mathrm{lw}$[11]. Conceding certain parallelism between marine mammals, there is still a strong potential risk for Mediterranean dolphins ($1350\text{-}10700 \, \mathrm{ng \, g^{-1}} \, \mathrm{lw}$). BDE-28, -47, -99, -100 and -154 were found in all the samples. The others were BDE-153 (98 %), BDE-183 (88 %) and BDE-209 (95 %), still with high frequencies of detection. BDE-47 was the main contributor to the PBDEs profile ($\approx$40 %) followed by and BDE-154 ($\approx$20 %) and BDE-99 and -100 ($\approx$12 %).

Table 1 Sampling information and contaminant concentrations ($\mathrm{ng \, g^{-1}}$ lw)

period and samples	dolphin size (cm)		$\sum$PBDEs	$\sum$Decs[a]	$\sum$MeO-PBDEs	$\sum$OPFRs
1990	181-216	mean	5120	14.1	62.6	376
n=15		SD	2271	12.8	25.0	468
2004-2009	166-224	mean	2737	18.4	59.2	1253
n=15		SD	2437	18.5	60.2	1689
2014-2018	164-210	mean	2061	20.4	38.1	436
n=12		SD	2653	41.5	28.3	539
frequency of detection (%)			100	100	100	100
		LOD[b]	0.04	0.002	0.43	0.35
		LOQ	0.12	0.008	1.42	1.30

[a]Dec 602 accounted for 95 % of the total dechloranes.
[b]LOD and LOQ of the compound with the lowest values of the group.

Only α-HBCD, not β nor γ, was detected in a third of the samples from different periods and no temporal trend could be seen.

Regarding the brominated emerging FRs, PBEB and HBB were detected in 7 % and 21 % of the samples around their LOQs and DBDPE was detected in 88 % of them ranging from 1.24 to 370 $\mathrm{ng \, g^{-1}}$ lw. Concentrations of dechloranes in Mediterranean dolphins also seem to have been steady for three decades ($t = 0.67$, df $= 25$, $p > 0.1$) at $17.5 \pm 25.6 \, \mathrm{ng \, g^{-1}}$ lw (Table 1). Dec 602 was detected in all the samples and accounted for 95 % of the total dechloranes contanimantion. Dec 603 and *anti*-DP were detected in 19 % of the samples and Dec 604 and *syn*-DP in just 5 % and 7 % of them.

Dolphins from the same and different species sampled from the south of Spain between 2004 and 2012 showed levels of PBDEs, HBCD and dechloranes in the same range as in our dolphins from those periods[12,13].

On the other hand, OPFRs appear to have had experienced a slightly significant growth from 1990 to 2004-2009 ($t = 2.23$, df $= 27$, $p < 0.05$) just to decrease back in the following decade ($t = 2.20$, df $= 24$, $p < 0.05$). An increase would make sense as in 2006 they already doubled the amount of PBDEs used in Europe and in 2009 the Stockholm Convention included PBDEs. However, a proper management of this contaminants or the important variability of levels amongst individual dolphins — which are high in the food web and experience important biomagnification — could prevent from observing a more significant growth. Additionally, TPPO showed unusually high concentrations (1398 to 4794 $\mathrm{ng \, g^{-1}}$ lw) in four dolphins from 2008-2009, while it was only detected in 12 other samples below 4 $\mathrm{ng \, g^{-1}}$ lw. This could be due to a specific abnormal exposure of these individuals to this compound. Were this an isolated incident that allowed not considering the TPPO component of these samples in the time trend, the levels of OPFRs would be reported as stable through the three decades. TBOEP was found in all but two samples making 20 ± 32 % of the total OPFRs contamination, but showing a constant decrease through time ($t = 4.01$, df $= 22$, $p < 0.01$). This decrease was not reflected in the total OPFRs levels as TBP was found in 69 % of the samples accounting for 82 ± 30 % of the total contamination. However, that is not enough to talk about a change on OPFRs profiles yet.

MeO-PBDEs also showed stable levels through time, as expected, since they are natural compounds independent from human activity. Agreeing with the most occurring congeners in our published studies, 2-MBDE-68, 6-MBDE-47 and 5-MBDE-100 were the three quantifiable congeners. Their mean concentrations and frequencies of detection were 44.0 $\mathrm{ng \, g^{-1}}$ lw and 100 %, 4.55 $\mathrm{ng \, g^{-1}}$ lw and 95 % and 6.69 $\mathrm{ng \, g^{-1}}$ lw and 81 %, respectively.

2. Non-target analysis

Three groups of peaks showing chlorine isotope patterns and separated 34 mass units from each other showed in most samples' mass defect plots (Figure 1).

With $C_{18}H_{14-x}Cl_x$ ($x=5-7$) as a formula, compound group A may well correspond to polychlorinated terphenyls (PCTs). PCTs are similar to PCBs, in terms of chemical properties and toxicity. Few publications address the occurrence of PCTs in the Mediterranean Sea. They were detected in shellfish collected between 1989 and 1991 from the Catalan coast in levels lower than PCBs'[14,15].

Compound group B was present in a few samples of each period. Its likely formula would be $C_{14}H_{12-x}Cl_x$ ($x=5-8$). This might be a higher chlorinated form of dichlorodiphenyldichloroethylene (DDE) or some related compound. DDTs have regularly been found in striped dolphins sampled between 1992 and 2009 from the western Mediterranean Sea in several studies[16].

Compound group C was identified as having the formula $C_{12}H_{10-x}OCl_x$ ($x=4-8$), and are most probably polychlorinated diphenyl ether (PCDE). PCDEs due to their higher log K_{ow} values bioaccumulate in organisms at a much higher level than hydroxyl PCBs[17]. Despite their connection with PCBs and PBDEs, PCDEs are not as well documented as the others. They do not seem to be documented in the Mediterranean Sea yet.

A published article reported that the homologous series of PCTs, DDTs and PCDEs show in the same area of the H/Cl mass defect plot where A, B and C showed in this study[10].
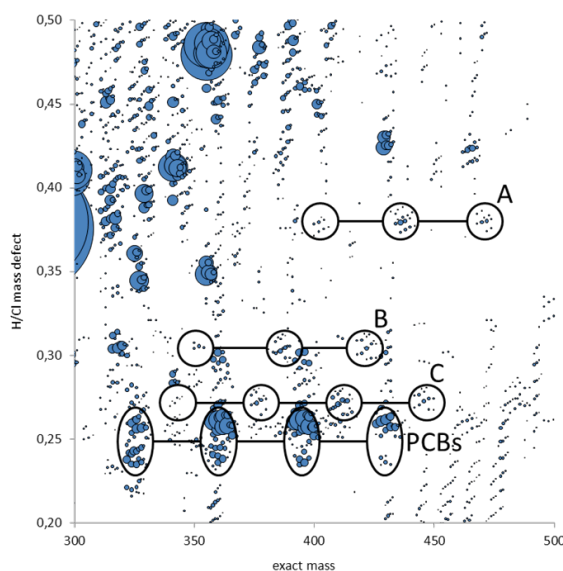

Figure 1. H/Cl mass defect plot of a sample

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