FIRST DETERMINATION OF ORGANOPHOSPHOROUS FLAME RETARDANTS AND PLASTICIZERS IN MARINE MAMMALS

G. Santín[1], Ò. Aznar-Alemany[1], J. Giménez[2], R. de Stephanis[2], E. Eljarrat[1*], D. Barceló[1,3]

[1]Water and Soil Quality Research Group, Dep. of Environmental Chemistry (IDAEA-CSIC) Barcelona, Spain
[2]Department of Conservation Biology, EBD-CSIC, Sevilla, Spain
[3]Catalan Institute for Water Research (ICRA), Girona, Spain
E-mail: gsgqam@cid.csic.es

Introduction

The plastic industry is one of the most important nowadays. Unfortunately, even though plastics have made our lives easier, the contamination produced by them is one of the most known, and not only for the material itself, but for the chemicals used for its manufacturing. In order to give stability to these polymers, some chemicals called plasticizers are added into the mixture as well as flame retardants (FRs) to satisfy the safety standards. These chemicals are not really bonded to the polymer, which increases their release into the environment. These FRs are used to increase the fire resistance of a wide variety of materials, not only plastics. If we put together the fact that plastics are used everywhere and they are contaminated with plasticizers and FRs, and at the same time these FRs are used in all kind of materials, we have a big and wide source of contaminations for the ecosystems and for humans. One of these chemicals used as plasticizers or FRs are organophosphorous FRs (OPFRs). Non-halogenated OPFRs are used as FRs and plasticizers while halogenated are just used as FRs. It is expected that due to the ban of polybrominated diphenyl ethers (PBDEs, the previous most used FRs), the production of alternative FRs such as OPFRs will rise in the coming years.

The chemical structure of OPFRs is pretty similar to that of organophosphorous insecticides, which are designed to affect the nervous system of insects[1]. This led us to wonder if all OPFRs can cause this effect on other animals, including humans. In fact, some of them can actually cause a delayed neuropathy, which can lead to irreversible paralysis, which is known as OPIDN (organo-phosphate-induced delayed neuropathy). This effect has been seen in cats, dogs, monkeys and chickens[2] and humans (see Jamaica Ginger poisoning). One of the most famous OPFRs that cause this neuropathy is tricresyl-phosphate (TCP), mainly by the *ortho*-TCP isomer, so nowadays the mixtures of TCP contain lower amounts of *ortho*-TCP[2]. Besides neurotoxicity, OPFRs are also known for being endocrine disruptors[3] (tris(chloroethyl)phosphate (TCEP), tris(2-butoxyethyl)phosphate (TBEP) and tris(chloroisopropyl)-phosphate (TCPP)) and carcinogenic (TCEP and tris(1,3-dichloro-2-propyl)phosphate (TDCPP)).

OPFRs have been found in environmental samples (indoor air, house dust, drinking water, river water and sediment) as well as in some biota (fish, mussels and bird eggs) and human samples to date. The range of K_{ow} for OPFRs is wide, from 1.47 for TCEP to 9.49 for tris(2-ethylhexyl)phosphate (TEHP). There are not enough data to conclude a specific way of bioaccumulation, and having into account the differences among the different OPFRs certainly it is not going to be the same for all of them.

The purpose of this study was to assess for the first time the occurrence and levels of OPFRs in dolphin liver samples from the coast of the Alboran Sea in Spain. Analyses include 17 different OPFRs: tributyl phosphate (TBP), triphenyl phosphate (TPhP), 2-ethylhexyldiphenyl phosphate (EHDP), diphenylcresylphosphate (DCP), diphenylphosphate (DPP), isodecyldiphenyl phosphate (IDPP), TCP, trihexyl phosphate (THP), triphenylphosphine oxide (TPPO), TBEP, TCEP, TCPP, TDCPP, TEHP, tris(isopropyl-phenyl)phosphate (IPPP), tris(tribromoneopentyl)phosphate (TBNPP), tetrekis(2-chlorethyl)dichloroisopentyl-diphosphate (V6).

Materials and methods

Extraction and clean up

Samples of dolphin liver (37 samples corresponding to 27 males and 10 females of different ages) were collected from striped dolphin (*Stenella coeruleoalba*) from the coast of Andalusia (Alboran Sea), south of Spain. From these samples, 7 belong to dolphins found stranded between 2003 and 2005 and the remaining 30 were found between 2008 and 2010. The liver was removed and stored at -20 °C, once in the lab it was defrosted, crushed, frozen, lyophilized, and stored at −20 °C until analysis. Sample (0.1 g dry weight) was extracted with 15 mL of a

mixture of acetone:hexane (1:1) using an ultrasound system. The extraction was carried out twice and both extracts were combined in a vial. The 30 mL extract was dried with a purified nitrogen stream and then reconstituted with 60 mL of acetonitrile. Then it underwent a SPE tandem with cartridges of 5 g of basic alumina and 2 g of C18. The collected extract was evaporated under a purified nitrogen stream. Labeled standards of TCEP-d_{12}, TDCPP-d_{15}, TBP-d_{27}, TPhP-d_{15} and $^{13}C_2$-TBEP (10 ng) were added as internal standards and, finally, the sample was reconstituted to 200 µL with methanol.

Lipid weight (lw) was determined as following. One gram of sample was extracted using the same methodology described above. The solvent was evaporated using a nitrogen stream and, after that, dried in an oven at 100 °C. Lipid weight was then determined gravimetrically.

Instrumental analysis
Instrumental analysis was performed by LC, using a Symbiosis™ Pico Pico (SP104.002, Spark, Holland), connected in series with a 4000 QTRAP Hybrid Triple Quadrupole – Linear Ion Trap-MS equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Target compounds were separated on a Purospher Star RP-18 end-capped column (125 mm × 2.0 mm, particle size 5 µm) with a C18 guard column (4 × 2.0 mm), both supplied by Merck (Darmstadt, Germany). The optimized separation conditions were as follows: solvent (A) water (0.1 % formic acid) and (B) methanol (10 mM ammonium acetate) at a flow rate of 0.25 mL/min. The gradient elution was: 50 % (B) for initial and hold for 1 min; 80 % (B) at 3 min and hold for 1 min; 90 % (B) at 9 min and hold for 8 min; 100 % (B) at 22 min and hold for 9 min. 50 % (B) at 32 min and hold for 5 min to return to initial mode. Total chromatographic time was 37 min. Sample injection was 10 µL.

Recoveries were between 48.5 and 124 % with relative standard deviations below 13 %. Limits of detection (LODs) defined as three times the noise level were in the range of 0.75 to 13.3 ng/g lw (with the exception of TBNPP and IPPP that had 29.0 and 48.0 ng/g lw, respectively) and limits of quantification (LOQs) defined as 10 times the noise level were in the range of 2.36 to 44.33 ng/g lw (with the exception of TBNPP and IPPP that had 96.0 and 160 ng/g lw, respectively). We followed a strict internal analytical quality assurance protocol by measuring procedural blanks in each batch of measurements. Seven blank methods were carried out and detected signals were subtracted for quantification of real samples.

Results and discussion
OPFRs were detected in all samples. Total OPFR concentrations were between not quantifiable (NQ) and 55819 ng/g lw (Table 1). Fourteen out of 17 tested analytes were detected. Only V6, THP and TBNPP were not detected in any sample. TDCPP, TCEP and TPPO were detected, but their levels were below the LOQs. The most abundant OPFR was TCPP followed by TPhP, EHDP, DPP, DCP, IPPP, IDPP, TCP, TBP, TBEP and TEHP. TBP was the most frequent with 87 % detection frequency and levels ranging from ND to 2676 ng/g lw. The most contaminated sample was a juvenile male; TCPP was the most abundant OPFR in this sample, contributing 29641 ng/g lw to the total (55819 ng/g lw).

As far as we know, this is the first time that IDPP, DCP, IPPP and DPP were found in biota samples. The presence of these contaminants confirms the fact that analytical methods should include more compounds into the list of OPFRs. It is important to notice also that in Europe there are a few companies that produce these chemicals, which might be the reason for what we found.

Moreover, this is also the first time that OPFRs are detected in marine mammals. There are a few studies dealing with OPFRs in fish published to date. If we compare our results with those obtained in fish studies we can find some similarities. A study carried out in the Manila Bay in the Philippines[4] also found that TBP was one of the most frequent OPFR (83 %). Another study in lakes and coastal areas of Sweden[5] found that the most abundant OPFRs were TCPP (23-750 ng/g lw), TPhP (4.2-180 ng/g lw) and EHDP (3.0-14000 ng/g lw) and the less abundant were TBEP (ND-1000), TCP (ND-137) and TBP (1.6-4900).

If we compare our results with those obtained for some BFRs, such as PBDEs, in some dolphin species, we can appreciate that OPFRs levels are higher than PBDEs levels. Published PBDE levels in liver dolphins collected from 1990 to 2008[6] ranged from 7 to 5532 ng/g lw. If we compare more recent levels found in blubber and brain

in stripped dolphins[7], we can also see that OPFRs levels are higher than PBDEs levels. OPFRs levels in liver are five times higher than PBDEs levels in blubber and ten times higher than PBDEs levels in brains.

Table 1. Frequency of detection, mean and range of the levels found in stripped dolphin livers.

	Frecuency (%)	Mean (ng/g lw)	Range (ng/g lw)
TCEP	76	NQ	ND-NQ
TPPO	62	NQ	ND-NQ
V6	0	ND	ND
TCPP	62	5801	ND-29641
TDCPP	51	NQ	ND-NQ
TPhP	73	5270	ND-26374
TBP	87	506	ND-2676
DCP	30	1741	ND-10192
TBEP	78	279	ND-728
TCP	41	635	ND-1110
EHDP	73	3510	ND-6804
DPP	65	1776	ND-5105
IDPP	51	1319	ND-6671
TBNPP	0	ND	ND
IPPP	5	1435	ND-1625
THP	0	ND	ND
TEHP	62	267	ND-410
∑OPFRs	100	5953	NQ-55819

Dolphin samples where used to assess bioaccumulation. Previous studies showed that an increase of pollutant levels with the length of the specimen (which is directly related to age) is an indication of bioaccumulation. Figure 1 shows the measured total OPFR concentrations versus dolphin length. According to these results, there is no evidence of OPFR bioaccumulation in male dolphins.

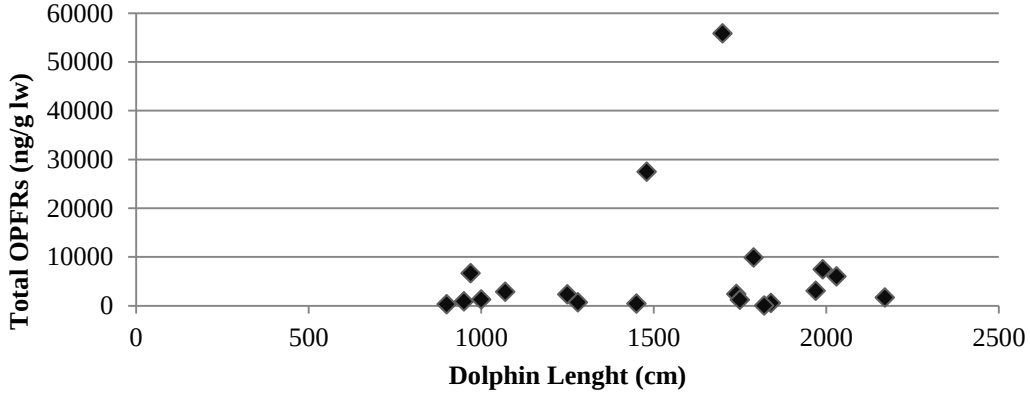

Figure 1. Individual total OPFR levels (ng/g lw) for male dolphin livers according to dolphin length (cm).

Usually, POP concentrations in females are lower than in males as a result of maternal transfer. However, in our dolphin samples, we did not see any significant difference between OPFR levels in males and females (K-W X^2= 0.0209 p= 0.8851) (Figure 1a). These findings could indicate that mothers do not transfer OPFRs to their newborns, or at least that this transfer is not as significant as for other POPs. This is consistent with a recent study in human milk samples from Asia[8], in which the amount of OPFRs in human milk is independent of the number of gestations, and contamination was related to the lifestyle of the mothers.

In order to study temporal trends, data from samples collected in 2003-2005 were compared with those collected 5 years later, in 2008-2010 (Figure 2b). No significant differences were observed between both sampling periods (K-W X^2= 0.0593 p= 0.8077). Figure 2c shows the box plots comparing concentration levels for calves, juveniles and adults. There are no significant differences between the three maturity stages (K-W X^2= 0.9527 p= 0.621). All these results agree with the absence of correlation between sex or age of fish and OPFR concentrations observed by Kim *et al.* in Philipines[4]. Further studies are necessary in other cetacean species and other areas to understand the behavior of these contaminants and the possible health effects in these organisms.

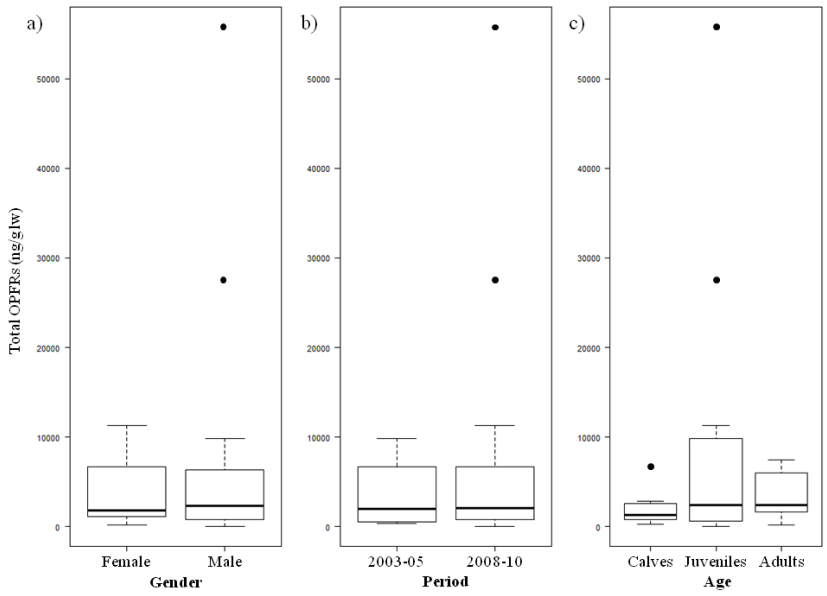

Figure 2. Box plots for total OPFR concentrations in liver dolphin according to (a) gender, (b) sampling period and (c) age.

Acknowledgments

This work has been supported by the Spanish Ministry of Economy and Competitiveness through the projects Consolider-Ingenio 2010 CSD2009-00065 and EcoCet (CGL2011-25543), by the Generalitat de Catalunya (Consolidated Research Groups "2014 SGR 418 - Water and Soil Quality Unit"), Loro Parque Foundation, and CEPSA. R. de Stephanis and J. Giménez were supported by the Spanish Ministry of Economy and Competitiveness, through the Severo Ochoa Programme for Centres of Excellence in R+D+I (SEV-2012-0262) and by the "Subprograma Juan de la Cierva.". Thanks are due to the "Consejería de Agricultura, Pesca y Medio Ambiente" and the "Agencia de Medio Ambiente y Agua" of the "Junta de Andalucía" especially to M.S. Vivas, C. Fernández and E. Fernández and all the people who helped in sample collection. Biotage is acknowledged for SPE cartridges.

References

1. Pope CN. (1999); J Toxicol Environ Health, Part B: Critical Reviews. 2: 2, 161-181.
2. Smith MI and Lillie RD. (1931); *Arch Neurol Psychiatry*. 26: 976-992.
3. Liu X, Ji K, Choi K. (2012); *Aquat Toxicol.* 114-115: 173-181.
4. Kim JW, Isobe T, Chang KH, Amano A, Maneja RH, Zamora PB, Siringan FP, Tanabe S. (2011); *Environ Poll.* 159: 3653-3659.
5. Sundkvist AM, Olofsson U, Haglund P. (2010); *J Environ Monitor.* 12: 943-951.
6. Alonso MB, Azevedo A, Torres JPM, Dorneles PR, Eljarrat E, Barceló D, Lailson-Brito J, Malm O. (2014); *Sci Total Environ.* 481: 619-634.
7. Barón E, Eljarrat E, Barceló D. Natural and anthropogenic halogenated compounds in dolphin blubber and brain from southern Mediterranean Sea. Displayed in the current 34th International Symposium on Halogenated Persistent Organic Pollutants.
8. Kim J, Isobe T, Muto M, Tue NM, Katsura K, Malarvannan G, Sudaryanto A, Chang K, Prudente M, Viet PH, Takahashi S, Tanabe S. (2014); *Chemosphere.* In Press.