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Assessment of current dietary intake of organochlorine contaminants and polycyclic aromatic hydrocarbons in killer whales (*Orcinus orca*) through direct determination in a group of whales in captivity

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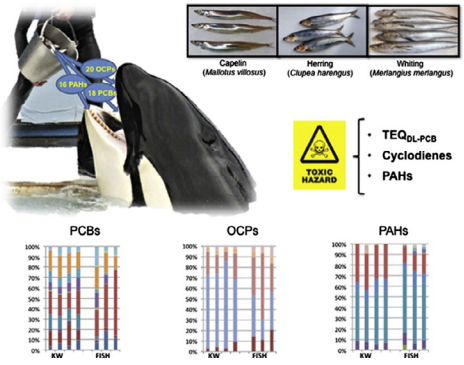
HIGHLIGHTS

- We assess the daily dietary intake of pollutants in captive orcas.
- We gain insight on the current magnitude of the exposure of wild killer whales.
- Very high estimated daily dietary intake of dioxin like PCBs
- Calculated intake of PAHs could be related with an increase in the risk of cancer.
- Proportionality between the intake of pollutants and the blood levels was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

We determined the levels of 16 polycyclic aromatic hydrocarbons (PAHs), 19 organochlorine pesticides (OCPs) and 18 polychlorinated biphenyls (PCBs) in the plasma of captive adult killer whales and in their food. The goal of this research was the assessment of the dietary exposure of killer whales to these pollutants to gain insight on what is the actual magnitude of the exposure in this species, which is considered among the most contaminated in the planet. Plasma median $\sum$OCP and $\sum$PCB contents were 3150.3 and 7985.9 ng g^{-1} l.w., respectively. A total of 78.9% of the PCBs were marker-PCBs, and 21.1% were dioxin-like PCBs (6688.7 pg g^{-1} l.w. dioxin toxic equivalents). This is the first report of the blood levels of PAHs in killer whales, and their median value was 1023.1 ng g^{-1} l.w. In parallel, we also determined the levels of these contaminants in the fish species that are used to feed these animals to estimate the orcas' average daily dietary intake of pollutants. All the contaminants in the fish were detected in the plasma of the killer whales, and proportionality between the intake and the blood levels was observed in all the animals. The calculated intake was extremely high for certain contaminants, which is a concern, giving a glimpse of what possibly occurs in the wild, where exposure to these contaminants can be even higher. Therefore, although many of these chemicals have been banned for decades, even today, the levels of these chemicals could reach very toxic concentrations in the tissues of these endangered animals because of their diet.

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1. Introduction

Persistent organic pollutants (POPs) are toxic chemicals that are resistant to degradation in the environment and biota. It is a well-known fact that these anthropogenic pollutants easily reach aquatic environments and that contaminants in water may dissipate. Thus, marine life serves as a living record of the pollutants discharged into the environment. Due to their fat solubility and resistance to chemical and biological degradation, ingestion of certain classes of POPs by animals leads to bioaccumulation throughout their lives, generally in the fatty tissues, and to biomagnification in the food chain (Safe, 1994). Among the POPs, organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) are highly prevalent in marine vertebrates. Because of their efficient metabolization, strictly speaking, polycyclic aromatic hydrocarbons (PAHs) cannot be considered as POPs, but due to their high prevalence in the environment and their lipophilicity, PAHs are usually considered as POPs. The majority of POPs, such as PCBs and OCPs, are currently banned from use and are no longer produced or used around the world; therefore, their levels have been constantly declining through the years. Nevertheless, relevant amounts of these pollutants still persist in the environment, and certain foodstuffs, such as fish, are especially contaminated (EFSA, 2008, 2010, 2012).

Various studies have shown that several aquatic mammal species are sensitive to the toxicological effects of certain xenobiotic compounds, including the large class of endocrine disrupting chemicals (such as PCBs, OCPs and PAHs) (Fossi and Marsili, 2003; Hamlin and Guillette, 2010). In some species of marine mammals it has been suggested that POPs may provoke adverse health effects, such as reproductive impairment, immune-toxicity, immune suppression, endocrine disruption and skeletal abnormalities (Fossi and Marsili, 2003). Cetaceans, particularly odontocetes, are considered among the most contaminated vertebrates on earth, and recent studies have shown that the killer whale (*Orcinus orca*) is probably the most contaminated marine mammal species in the world (Ross et al., 2000). This high contamination is probably due to their condition of being both, mammals and carnivores, as well as their long life span and large size (Ross et al., 2000; Ylitalo et al., 2001). Due to the metabolic recalcitrance of organohalogenated compounds, it has been estimated that certain POPs will be threatening populations of killer whales until 2069 (Hickie et al., 2007), and chemical contamination is one of the major threats for these animals that has caused them to be included in the IUCN Red List of Threatened Species (Taylor et al., 2013).

The bioaccumulation of POPs in biota is a complex process that not only depends on the lipophilicity of the compounds but is also influenced by different factors, including diet, age, gender, genetic variation and metabolism, which occur in human beings (Henriquez-Hernandez et al., 2011; Zumbado et al., 2005). Thus, studies performed in other species of odontocetes such as *Tursiops truncatus* have demonstrated higher levels of POPs in their tissues than in top predator fishes such as tuna, probably due to a different elimination potential between water- and air-breathing animals. Furthermore, cetaceans show lower enzymatic activity compared with other marine vertebrates such as polar bears, marine birds and pinnipeds (Fossi et al., 2007). Note that in killer whales, adult males are much more subjected to the toxic effects of these compounds because they accumulate higher amounts than reproductively active females, who pass significant amounts of these compounds to their offspring during gestation and lactation (Ross et al., 2000). In addition, it is obvious that bioaccumulation and biomagnification processes mainly depend on the exposure level to which the animals are subjected. This level may vary significantly from animal to animal; although one of the characteristics of man-made endocrine disrupters is their capability of spreading across all continents and oceans, it is also known that their distribution is not homogeneous, and there are some geographic areas that are potentially more threatened than others due to their higher levels of contamination. Although the different levels of contamination that have been observed between resident and transient communities of killer whales have been partially attributed to differences in their dietetic pattern (fish-eaters vs. mammal-eaters) (Ross et al., 2000), the influence of the geographic distribution of the contaminants could also magnify these differences. Considering this inhomogeneity, specimens that live in a controlled environment and that are fed with known commercially available foodstuffs (that are considered safe for human consumption) should be less exposed to these environmental contaminants.

To date, no controlled tests have been conducted to assess which is currently the real source of exposure of killer whales to POPs, almost four decades after the banning of most of these chemicals. Due to ethical, legal and logistical reasons, it is practically impossible to perform such controlled studies in free-ranging killer whales. Thus, aquarium specimens (especially those that have been born and bred in captivity) that have their blood drawn in a regular pattern to monitor their health status represent a valuable source of data for this species. As we have had the exceptional opportunity of regularly sampling blood from four captive killer whales, we have performed a study in which we monitored their blood profile of contamination by POPs and determined the levels of these contaminants in their diet. The goal of this research was the assessment of the dietary exposure of killer whales to OCPs, PCBs and PAHs to gain insight on what is the actual magnitude of the exposure in this species, which is located on top of the food chain. In addition, to our knowledge, this is the first time in which the levels of PAHs are described in killer whales.

2. Material and methods

2.1. Animals and diet

A total of four killer whales, two males and two females, were included in the study. Orca #1 was captured in Icelandic waters in the 1980s, and in 1996, she successfully gave birth to her only live calf (orca #3). The other two killer whales were all born from wild parents caught in the same waters as orca #1 and raised in a French marine park (Marineland, Antibes, France).

The animals have been fed a diet of wild-caught herring (*Clupea harengus*), whiting (*Merlangius merlangus*) and capelin (*Mallotus villosus*).

The characteristics of the animals and their feeding patterns are summarized in Table 1.

2.2. Sampling

Blood samples from killer whales were taken from the ventral vein of the tail using a Venoject needle and a BD Vacutainer with lithium heparin (LH 68 I.U.). Plasma was obtained from the entire blood sample after a centrifugation program of 10 min at 22 °C × 4000 rpm (Rotofix 32 A, Hettich Zentrifugen, Germany). Blood was collected during periodical veterinarian health controls throughout January and June 2011 and the plasma was stored at −18 °C until analysis. A total of 16 samples were collected: five samples from animals #1 and #2 and three samples from animals #3 and #4.

To evaluate the dietary intake of POPs, the amount of fish of each species that was given to the each orca was recorded daily. A sample of 500 g of the fish of each species was taken from each delivery, homogenized in a blender and taken as an individual subsample of that species. Six determinations were performed on each one of the fish species that were kept frozen at −18 °C until the analysis.

2.3. Analytes of interest

In this study, we determined the levels of 19 OCPs: methoxychlor, *p,p′*-DDT and its metabolites *p,p′*-DDE and *p,p′*-DDD; hexachlorobenzene (HCB); four isomers of hexachlorocyclohexane (α-, β-, γ-, δ-HCH); aldrin; endrin; dieldrin; heptachlor; cis- and trans-chlordane; α- and β-endosulfan; endosulfan-sulfate; and mirex. We also determined the

Table 1
Characteristics of killer whales and the foodstuff included in the study.

	Killer whale			
	#1	#2	#3	#4
Killer whales				
Gender	Female	Male	Male	Female
Name	Freya	Inouk	Val	Wikie
Age of birth	1982	1999	1996	2001
Origin	Wild	Captive	Captive	Captive
Length (cm)	610	470	600	470
Estimated weight (kg) [a]	2621	2174	3304	2019
Foodstuff				
Ingested diet (kg) [b]	33	37	25	42
Capelin	7	–	10	5.5
Herring	26	27	25	31.5
Merlan	–	10	10	5

[a] Values are the mean of 9 individual weightings.
[b] Values are mean calculated of the 4 month of study.

levels of seven markers and 12 dioxin-like PCBs (IUPAC numbers # 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180,189), and we also included the 16 EPA priority PAHs that are often targeted for measurement in environmental samples (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[k]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c*,*d*]pyrene, dibenz[*a*,*h*]anthracene and benzo[*g*,*h*,*i*]perylene).

2.4. Extraction and clean-up procedure

Plasma samples were subjected to solid-phase extraction using Chromabond® C18ec cartridges (Macherey-Nagel, Germany) that yielded recoveries in the range of 89–107% for the analytes studied. Before application of the plasma samples, the cartridges were cleaned and conditioned with three 1-ml volumes of methanol followed by four 1-ml volumes of ultrapure water. The samples were then passed through the cartridge by gravity flow. The plasma tubes were rinsed with four 1-ml aliquots of ultrapure water, and each aliquot was applied to the corresponding cartridge by gravity flow. The cartridges were rinsed with three 1-ml portions of ultrapure water and dried for 20 min under gentle vacuum (~15 mm Hg). The adsorbed analytes were eluted with 1 ml of methylene chloride by gravity flow. Gentle vacuum was then used to elute the residual methylene chloride from the cartridges. Solvent of the extracts was then evaporated under gentle nitrogen stream, and the extracted analytes were solubilized in 200 μl of cyclohexane that was transferred to 1.8-ml GC vials with 250 μl inserts (Chromatographic Research Supplies, Inc., USA). No additional purification was necessary for the plasma samples that were subjected to chromatographic analysis.

Because the contaminants included in this study are totally lipid-soluble and therefore found bound to the lipid fraction, when we extracted contaminants from the fish, we first extracted the fat of the fish. The mean fat content was 17.8% for Pacific herring, 0.7% for whiting and 17.9% for capelin. A total of 10 g of the homogenated fish were spiked with the 10-ppm surrogate's mix in acetone to yield a final concentration of 100 ppb and mixed with 30 g of diatomaceous earth to absorb all the humidity. The method of extraction and purification followed that recommended by the European Standard for the determination of pesticides and PCBs in fatty food (EN, 1996a,b), whose validity has been previously proven in our laboratory for fatty foods (Almeida-Gonzalez et al., 2012; Luzardo et al., 2012). This method combines an automated Soxhlet extraction method (FOSS Soxtec Avanti 2055) with a purification step using gel permeation chromatography (GPC). This method gives acceptable recoveries that ranged between 74.5% and 104.7%. No additional purification steps were required, and the 1-ml extracts in cyclohexane obtained at the end of the GPC were used for the gas chromatography/triple quadrupole mass spectrometry (GC-MS/MS) analysis.

2.5. Procedure of chemical analysis

Gas chromatography analyses of 53 contaminants, three surrogates and two ISs were performed in a single run on a Thermo Trace GC Ultra equipped with a TriPlus Autosampler and coupled to a Triple Quadrupole Mass Spectrometer Quantum XLS (Thermo Fisher Scientific Inc., Waltham, MA, USA), using the appropriate internal standards (ISs) as previously described for the plasma of sea turtles in our laboratory (Camacho et al., 2012, 2013; Luzardo et al., 2013). A fused silica capillary column BPX5 (Crosslinked 5% phenyl methylpolysiloxane, SGE Inc., USA) with a length of 30 m, a 0.25 mm i.d. and a film thickness of 0.25 μm was used as the stationary phase. Helium (99.999%) at a constant flow rate of 1.0 ml/min was used as the carrier gas. The temperatures were programmed as follows: the initial oven temperature of 60 °C was maintained for 1 min, ramped at 12 °C/min to 210 °C, then raised at 8 °C/min to 320 °C with a 6 min hold time. The total run time was 61 min. The injector and transfer line were set to 270 °C and 310 °C, respectively. The standards and samples were injected (1 μl) in the splitless mode. ThermoFisher Xcalibur Software (Ver. 2.0.1) was used for the instrument control, data acquisition, and data analysis. After the retention times were determined in full scan mode (range m/z 45–650), a timed selected reaction monitoring (SRM) method was developed to analyze the 53 target compounds plus four surrogates and three internal standards in one single run. The mass spectrometry settings of this method are presented in Supplementary Table 1. A calibration curve was constructed from 0.05 to 100 ng/ml with all the compounds, with the exception of the surrogates and internal standards, contained in each calibration standard mixture. Argon (99.99%) was used as the collision gas, and the collision cell pressure was set to 0.2 Pa. The triple quadrupole mass spectrometer was operated under the following conditions: ionization with electron impact at 70 eV in MRM with an emission current of 50 μA. The ionization source temperature was set to 220 °C. A filament multiplier delay of 5 min was established to prevent instrument damage. The electron multiplier voltage was set to 1500 V. The scan width was 0.15, and the scan time was 0.05 s. Peak widths of m/z 0.7 Da were set for both the first quadrupole (Q1) and third quadrupole (Q3). The analytical performance of this method (confirmation criteria, precision, linearity, limits of quantification (LOQs), and repeatability) has been previously studied and published (Luzardo et al., 2013). We added 20 μl of the IS mixture, which was prepared at 1 ppm in cyclohexane, immediately before the GC–MS/MS analysis. Because no matrix effects have been observed with this method, all quantifications were performed against a 10-point calibration curve using cyclohexane (0.05 to 40 μg l^{-1}).

2.6. Dietary intake estimates and calculations

The exposure assessment was calculated by multiplying the respective concentrations of the contaminants in the fish fat (mean values) by the amount of fat contained in the average daily fish consumption by each animal and divided by the body weight of each killer whale. The exposures were assessed for all the contaminants, both individually considered and also grouped in different forms. For the calculations, when the concentration of a given contaminant was below the limit of detection (LOD), the value was assumed to be that LOD (upper bound approach). The data of fish consumption were daily recorded by the trainers.

In this work, we expressed the total value of the OCP residues ($\sum$ OCPs) as the sum of the 19 OCPs and metabolites measured; the total value of the DDTs ($\sum$ DDT) was expressed as the sum of the measured values of *p*,*p*′-DDT, *p*,*p*′-DDE and *p*,*p*′-DDD; the total value of the HCH residues ($\sum$ HCH) was expressed as the sum of the 4 HCH isomers measured (α-, β-, δ- and γ-HCH); and the total value of the cyclodiene

residues (∑cyclodienes) was expressed as the sum of aldrin, dieldrin, endrin, cis-chlordane, trans-chlordane and heptachlor. Similarly, we expressed the total value of the PCB residues (∑PCBs) as the sum of the 18 PCB congeners measured; in addition, the seven congeners considered as markers of environmental contamination by PCBs (#28, 52, 101, 118, 138, 153 and 180) were also considered as a group (∑M-PCBs), and the total value of dioxin-like PCBs (∑DL-PCBs) was also considered as the sum of the measurements of the 12 individual dioxin-like congeners (#77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189). In addition, we estimated the potential toxicity (in terms of the toxic equivalence to dioxins; TEQs) for the DL-PCBs using the toxic equivalency factors (TEFs) as revised by the World Health Organization (WHO) in 2005 (Van den Berg et al., 2006). We expressed the total TEQs (∑TEQs) as the sum of the TEQs individually obtained from the DL-PCBs. Finally, we considered the total content of the PAHs (∑PAHs) as the sum of the values of the 16 US-EPA compounds included in this study.

2.7. Statistical analysis

Database management and statistical analysis were performed using PASW Statistics v 18.0 (SPSS Inc., Chicago, IL, USA). Because the POP levels (individually or grouped) did not follow a normal distribution, the results were expressed with the median and the 5th and 95th percentiles of the distribution. Differences in the concentrations of POPs between two groups or more were tested with the non-parametric Mann–Whitney U-test and the Kruskal–Wallis test. The categorical variables were presented as percentages and were compared between variables with the chi-square test. The correlations between the POPs and the continuous variables were analyzed by the Spearman correlation test. P values of less than 0.05 (two-tail) were considered to be statistically significant.

2.8. Quality control

The recoveries of the 53 analytes and surrogates were acceptable using this method because in all of the cases, the recoveries were above 74%. All the individual measurements were corrected by the recovery efficiency for each analyte. All the measurements were performed in triplicate, and the values used for calculations were the mean of the three values. In each batch of samples, two controls were included every 12 samples: a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of melted butter spiked at 20 ng g^{-1} of each of the analytes that was processed with the same method as used for the samples. The batch analyses were considered valid when the values of the analytes in the QC were within 15% of the deviation of the theoretical value.

3. Results and discussion

The levels of 53 POPs were determined in the plasma of four killer whales living in captivity. Most of the published studies are performed on blubber and liver samples from stranded whales or on fat biopsy samples from free-ranging killer whale individuals. To our knowledge, until now, only one study has been conducted on the blood of an orca, which was from a captive female (Bennett et al., 2009). However, a close relationship between the concentrations of POPs in both the blubber and the blood has been reported in cetaceans, and with the exception of PCB209 (not measured in the present study), the lipid-normalized concentrations of POPs in blubber and plasma are well correlated (Yordy et al., 2010). Thus, blubber may be used to estimate the blood concentrations and vice versa, and to facilitate such comparisons, we normalized our results and expressed them on a lipid weight basis (Table 2).

3.1. Levels of OCPs in the plasma of killer whales and in the fish used to feed them

We detected nine out of 23 OCPs in the plasma of these animals. There were no differences in the compounds that were detected among the individuals, and those that were present at the highest levels were *p,p′*-DDE and dieldrin in all the cases. The median values of ∑DDT ranged from 827.4 ng g^{-1} l.w. to 3853.3 ng g^{-1} l.w., the median values of ∑cyclodienes ranged from 325.1 ng g^{-1} l.w. to 1161.4 ng g^{-1} l.w., and the median values of ∑OCPs ranged from 1266.1 ng g^{-1} l.w. to 5077.6 ng g^{-1} l.w. (Table 2). Compared with another killer whale living in captivity, we observed lower levels of ∑DDT and higher levels of dieldrin (Bennett et al., 2009). These values are similar to those reported by Bennett et al. (2009) in herring; that was the only fish species that was analyzed in that work. In our study, herring was the least contaminated fish, and the highest contamination values were found in capelin and whiting.

Because captive specimens (three of them born in captivity) were used, it is not surprising that our normalized results are lower than those described for dead animals from the wild. Thus, the levels of OCPs of this study were lower than those observed in stranded killer whales at the Oregon coast (Hayteas and Duffield, 2000) and the west coast of North America (Jarman et al., 1996) and lower than those in "Southern residents" from the west coasts of the USA and Canada (Krahn et al., 2009).

Among the individuals in our study population, we found statistically significant differences when we applied the Kruskal–Wallis test in the levels of ∑cyclodienes ($p = 0.004$), ∑DDT ($p = 0.003$) and ∑OCPs ($p = 0.003$). Age, and to a lesser extent, length (a parameter closely related to age) were the two factors that determined the residue levels between individuals, being higher in older and longer animals ($p = 0.002$ for ∑cyclodienes, ∑DDT and ∑OCs). As expected, orca #1, which is the largest and oldest animal, exhibited the highest levels of OCPs and of all the contaminants included in this study. On the contrary, the youngest orca (#4) had the lowest levels. This result suggests the existence of a close relationship between the age and the bioaccumulation of these persistent contaminants. The influence of age has been reported for killer whales in relation to ∑HCHs and HCB (Krahn et al., 2009), but it had not been described until now for ∑cyclodienes or ∑DDT. Note that the age is not the only factor influencing the accumulation of these contaminants; however, as occurs in human beings and other species (Henriquez-Hernandez et al., 2011; Luzardo et al., 2006; Zumbado et al., 2005), the natural history of the individuals, together with their geographic distribution and diet habits are key factors to understand the inherent contamination of killer whales, especially when studies with small number of subjects are conducted.

3.2. Levels of PCBs in the plasma of killer whales and in the fish used to feed them

The levels of 18 toxicologically relevant PCB congeners were determined in the plasma samples of all of the killer whales. The observed level of ∑PCBs was similar to that found in the plasma of other captive killer whales fed with herring homogenate (Bennett et al., 2009). As in the case of the rest of organohalogens included in this work, it is difficult to compare our results with other studies because most of them have been performed on tissues from dead animals and not in plasma. Nevertheless, if we extrapolate from the results of Yordy et al. (2010) that reported a good correlation between the PCB levels in blood and blubber, our results would be similar to those found in wild fish-eater killer whales from the west coast of North America (Jarman et al., 1996; Krahn et al., 2009) but lower than those observed in free-ranging pacific killer whales from waters of British Columbia, Canada, and the US states of Alaska and Washington (Ross et al., 2000). Other killer whales tested have shown very high levels of PCBs, often over 200,000 ng g^{-1} l.w. (Ylitalo et al., 2001). As in the case of OCPs, it seems logical that these

Table 2
Concentrations of POPs in plasma samples of killer whales and in whole fish homogenates. All the results are presented as median and percentiles 5th–95th, and expressed in ng g^{-1} lipid weight.

	Killer whales				Food supply		
	(#1)	(#2)	(#3)	(#4)	Capelin	Herring	Merlan
% lipid	0.47 (0.44–0.49)	0.46 (0.42–0.48)	0.47 (0.44–0.49)	0.44 (0.41–0.47)	17.92 (16.31–18.45)	17.83 (16.89–18.74)	0.54 (0.49–0.63)
OCPs							
HCB	131.9 (108.5–174.4)	123.9 (104.3–134.7)	89.3 (80.1–108.5)	120.5 (93.2–140.9)	26.8 (17.1–46.9)	13.1 (0.7–28.3)	31.1 (29.4–39.2)
∑ Cyclodienes	1161.4 (984.9–1233.7)	562.8 (475.9–591.1)	346.7 (238.2–363.7)	325.1 (277.3–347.8)	37.45 (0.8–227.5)	7.4 (0.4–294.3)	0.0 (0.0–133.3)
∑ DDTs	3835.3 (3316.3–4173.6)	2349.1 (2092.6–2583.7)	2822.5 (2803.4–3099.1)	827.4 (811.5–934.2)	81.7 (0.5–248.5)	1.9 (0.0–127.1)	101.8 (1.1–151.9)
∑ OCPs	5077.6 (4422.4–5469.1)	3016.1 (2785.8–3281.2)	3241.6 (3167.1–3556.3)	1266.1 (1231.9–1416.1)	210.5 (20.6–390.2)	17.5 (10.5–449.7)	131.2 (32.2–324.4)
PCBs							
∑ M-PCBs	10618.9 (9763.8–12912.1)	6718.9 (5280.4–6823.2)	6329.9 (6104.5–6636.3)	4709.6 (4045.9–5023.3)	61.4 (11.5–113.1)	8.1 (0.0–107.0)	57.9 (19.2–95.6)
∑ DL-PCBs	4041.3 (3717.4–5254.2)	2303.4 (1738.4–2651.1)	2058.9 (2041.9–2184.5)	1963.9 (1909.3–2045.7)	27.7 (0.0–72.5)	0.0 (0.0–73.1)	44.5 (8.5–59.0)
∑ PCBs	11.946.4 (10933.8–13954.4)	7292.6 (5845.4–7866.3)	7240.3 (7082.9–7487.1)	5464.3 (4864.2–5750.7)	62.2 (11.5–162.1)	8.03 (0.0–122.0)	57.94 (19.2–97.6)
∑ TEQs[a]	9459.7 (6058.3–11076.4)	4002.7 (2716.3–7138.3)	6559.7 (5626.9–7495.5)	6732.6 (4445.9–7278.2)	1.8 (0.7–241.7)	2.8 (0.8–71.3)	1.9 (9.2–73.5)
PAHs							
∑ lwPAHS	638.16 (448.8–765.8)	582.4 (525.9–786.6)	446.7 (146.7–512.6)	572.8 (461.4–600.1)	90.8 (40.6–128.9)	117.8 (64.1–162.7)	63.4 (55.8–112.2)
∑ hwPAHS	340.4 (219.1–833.9)	656.2 (117.4–1332.1)	348.8 (129.7–512.6)	384.2 (118.2–1393.4)	52.7 (2.0–99.6)	1.5 (0.2–141.8)	47.0 (2.1–98.2)
∑ PAHs	989.2 (875.3–1354.6)	1262.5 (651.9–1910.1)	861.4 (257.4–961.4)	979.6 (590.9–1409.3)	140.1 (89.9–187.4)	119.2 (64.6–304.6)	114.3 (110.4–154.3)

∑ lwPAHs (low molecular weight PAHs): sum of naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene.
∑ hwPAHs (high molecular weight PAHs): sum of fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d] pyrene, benzo[g,h,i]perylene, and benzo[a,h]anthracene.
[a] Expressed in pg g^{-1} lipid.

captive orcas should have lower levels than free-ranging animals because they live in a pristine, controlled environment. However, these comparisons should be taken with caution because the correlations of Yordy et al. (2010) have been made on other cetacean species (*T. truncatus*).

Regarding the congeners that are considered as the markers of environmental contamination by PCBs, we found detectable levels of all of them in the four killer whales. PCBs 118 and 153 were those detected at the highest concentrations except for subject #3, where PCBs 52, 153 and 118 were the highest, in that order. In the diet, as occurred with the rest of contaminants, the highest levels of PCBs were found in capelin homogenates, also with congeners 118 and 153 being of higher concentration. Nevertheless, it is remarkable that in the case of fish, we did not observe PCB 101 in any of the homogenates of the three species used to feed the orcas. These results suggest that other factors probably exist in addition to the usual diet that influence the profile of contamination detected in the plasma from killer whales. Note that the accumulation of individual xenobiotics occurs dynamically throughout life. The mobilization of lipid from blubber under certain physiological and even pathological circumstances would modify the profile of xenobiotics detected in plasma at a given moment (Yordy et al., 2010).

Serum and feed toxic equivalent quantity (TEQ) levels for dioxin-like PCBs were calculated using the toxic equivalency factors (TEFs) determined in 2005 (Van den Berg et al., 2006). Data are scarce on the concentrations and the accumulation profiles of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (DL-PCBs) in the tissues of cetaceans and even more scarce in the case of data in blood. In any case, when compared with the available data, our results seem to be higher than those found in finless porpoises, common dolphins and minke whales (Moon et al., 2010a,b,c). This difference could be related to the presence of PCB 126 in the diet of the group of killer whales of this study because this congener was present in all the fish homogenates that we analyzed. PCB 126, along with PCB 169, has the highest toxicity in terms of dioxin potency (TEF = 0.1). Nevertheless, note that in studies in cetaceans, the TEQ approach has failed to predict the toxicity of OCs, and its applicability in the risk assessment process for these species has been questioned (Levin et al., 2007).

The levels of ∑ PCBs, ∑ M-PCBs and ∑ DL-PCBs (and correspondingly TEQs) were significantly different among the four killer whales. Again, age and length were the factors affecting the levels of the residues, with the oldest and largest animals being those that had the highest levels. Other authors have observed that PCB concentration increased with age in males but were reduced in reproductively active females (Ross et al., 2000). This reduction in the females that have given birth has not been observed in our study. However, note that this reduction has mainly been related with the breast-feeding of calves, and in our study, such breast-feeding occurred only once (orca #1).

3.3. Levels of PAHs in the plasma of killer whales and in the fish used to feed them

Regarding the content of PAHs in the analyzed samples (plasma and food supply), we included those 16 PAHs initially considered by the Environmental Protection Agency (EPA) as priority contaminants due to their potential toxicity, mainly mutagenicity and carcinogenicity because some of these PAHs were shown to be capable of inducing toxic effects on whales and could act as procarcinogens in these animals (Wilson et al., 2005). In addition, data on the circulating levels of PAHs in marine mammals are scarce, and to our knowledge, this is the first time that their levels have been determined in *O. orca*.

Phenanthrene and pyrene were the compounds detected at the highest concentrations and were present in all the animals. The other detected PAHs in the plasma of the orcas were fluoranthene and fluorene. No other PAHs were detected in the killer whales of this study. In addition to these compounds, levels of chrysene, benz[b]

fluoranthene, benz[k]fluoranthene and benz[a]pyrene were detected in capelin samples, which was the food with the higher PAH levels (Table 2), whereas the profile of contamination by PAHs in the herring and whiting samples was very similar to that found in the orcas. As shown in Table 2, the low-molecular-weight PAHs (di- and tri-cyclic PAHs) were the most frequently measured chemicals of this group in our samples, both in the plasma and the food. This pattern has also been reported in other species of marine mammals (Kannan and Perrotta, 2008), whereas in other reports, completely different profiles of contamination have been found. This is the case of southern sea lions (*Otaria flavescens*) from Argentina, which exhibited both a higher level of PAHs and a larger number of compounds (Marsili et al., 1997). However, these differences can be easily explained because, unlike most organohalogenated compounds, PAHs are efficiently metabolized, and the levels present in a given moment reflect only the recent exposure. Thus, if we take into account that these studies usually rely on unique sampling and are not prolonged along a period of time, these reports should be considered as cross-sectional studies, where the data reflects only the exposure at a given moment. In our case, the plasma and food samples were collected over six months (median values are shown in Table 2), so we can be sure that the exposure to these contaminants is continuous in some extent.

In our study, unlike OCs and PCBs, we found no differences between the levels of PAHs of individual killer whales, and there was no influence of gender, age, length or reproductive status on the levels of PAHs. This observation is similar to that reported by other authors, who published that the hydrocarbon concentrations in sea otters (*Enhydra lutris*) from British Columbia were similar between areas and among age and sex classes, thus suggesting that metabolism dominates the fate of these compounds in sea otters (Harris et al., 2011). In any case, the levels of PAHs in killer whales seem to be even lower than those observed in humans (Moon et al., 2012), which may be due to more efficient metabolism of these compounds by these animals. Note that no dose–response relationship exists for PAHs; thus, it is not possible to assign a safe level of PAH exposure (Gaga et al., 2012).

3.4. Estimation of the daily dietary intake of POPs by killer whales

Dietary exposure calculations are performed by combining data on consumption with the concentrations of the contaminants found in the food samples. In this study, the consumption data used were directly obtained from the daily records of the marine park trainers, and the dietary intake was calculated by multiplying the concentration value of each contaminant in each of the three classes of fish by the daily consumption of this food (in g fish fat/day) by each one of the killer whales and then dividing by the average body weight of each animal. Some indirect calculations through the content in food and feces or quantitative structure-activity relationships techniques have been performed in terrestrial and aquatic mammals (Christensen et al., 2013; Mansouri et al., 2012), but to our knowledge, this is the first study in which a direct estimation through experimental data in food and a controlled population of mammals has been performed. In Table 3, we summarized the estimated dietary intakes of all the contaminants included in this study.

Our results show that the estimated daily intake (EDI) of OCPs for killer whales is relevant, especially in the case of cyclodienes. There are no data considered to be the tolerable daily intakes (TDIs) of these contaminants in marine mammals, but if we compare the EDIs with the TDIs that have been established for humans by the World Health Organization for these contaminants (JMPR, 2000), the dietary exposure to dieldrin could exceed as much as five times the tolerable level (100 ng kg^{-1} b.w.). For the rest of the OCPs, while keeping in mind the human thresholds, the recommended levels were not exceeded in this group of orcas, but the intake of DDT would reach 60% of the value established as the maximum for humans (JMPR, 2000). In all the cases, all the OCPs that were consumed daily by these animals appeared in their plasma, and the amount of contaminants ingested was proportional to their corresponding amounts in the plasma in all the animals (Fig. 1).

As shown in Table 3, PCB intake estimates were also very relevant. The importance of PCBs is not a surprising result because fish has been shown by many studies to be the main dietary source of organohalogenated contaminants (EFSA, 2008, 2010, 2012). As in the case of OCPs, all the PCB congeners that were consumed by means of the fish intake appeared in the plasma of killer whales, and in this case, the proportionality was also observed between the intake and plasma levels (Fig. 1). Note that in the plasma, there were certain contaminants detected, such as PCB 101, that were not detected in the fish samples, thus suggesting that, even in captive animals, there are other sources of these chemicals apart from the digested food. One possible source of the xenobiotics not found in the regular diet is the food-gifts that trainers give to whales, such as fish-jelly and salmon, which were not included in this study.

When we consider the intake of the most toxic PCBs, the dioxin analogs (DL-PCBs), expressed in terms of equivalency to dioxin toxicity, these orcas were found to have extremely high exposure through fish consumption (mean = 125.42 pg kg^{-1} b.w.). Again, there are no direct data on marine mammals for comparison, but taking as a reference the TDI of 2 pg kg^{-1} b.w. d^{-1} that has been established for $TEQ_{DL\text{-}PCBs}$ for humans, this limit was greatly surpassed in the orcas (by 50 to 80 times). Despite the failure of the TEQ approach to predict the toxicity of mixtures of PCBs in various species (Levin et al., 2007), our data are of significant concern because it is well known that the toxicological properties of DL-PCBs are similar to those exhibited by polychlorodibenzodioxins (PCDDs) and polychlorodibenzofurans (PCDFs) (Van den Berg et al., 2006), and some evidence suggests that even low doses of DL-PCBs can cause subtle effects during prolonged exposure, especially if exposure occurs during prenatal and postnatal development and the levels exhibited by these top predator animals are extremely high.

Finally, as shown in Table 3, the daily intake of PAHs greatly depended on the mixture of the fish used to feed the animals because those orcas consumed a higher proportion of capelin and herring, which were the more contaminated species of the fish used. As shown in Fig. 1, proportionality was also observed between the intake and the plasma levels of these contaminants. Until now, no estimates have been performed regarding the intake of PAHs coming from the diet in marine mammals; therefore, this study represents the first evidence that a diet exclusively based on fish represents a relevant source of these contaminants. Note that although a TDI value for these compounds has not been established, some authors have described that an acceptable daily intake would be 0.71 ng kg^{-1} b.w. d^{-1}, which is associated with a $1/10^6$ increase in risk of cancer (Voutsa and Samara, 1998). Taking this reference value into account, although it has not been estimated for marine mammals, our results are to some extent of concern

Table 3
Mean values of daily dietary intakes of POPs (ng kg^{-1} b.w. day^{-1}) for each of the adult killer whales included in this study.

	(#1)	(#2)	(#3)	(#4)
OCPs				
HCB	40.88	32.82	37.36	56.27
∑ Cyclodienes	314.18	326.78	260.79	464.89
∑ DDTs	171.49	142.69	154.36	238.15
∑ OCPs	506.85	512.67	426.59	743.74
PCBs				
∑ M-PCBs	116.60	110.50	100.71	167.85
∑ DL-PCBs	82.11	82.11	69.72	120.10
∑ PCBs	149.11	136.09	129.98	212.30
$\sum TEQ_{S_{PCBs}}$[a]	114.91	100.98	122.81	164.06
PAHs				
∑ lwPAH	251.42	261.91	208.82	372.23
∑ hwPAH	125.85	127.12	106.01	184.59
∑ PAHs	376.74	390.23	313.97	556.83

[a] Values expressed in pg kg^{-1} b.w. day^{-1}.

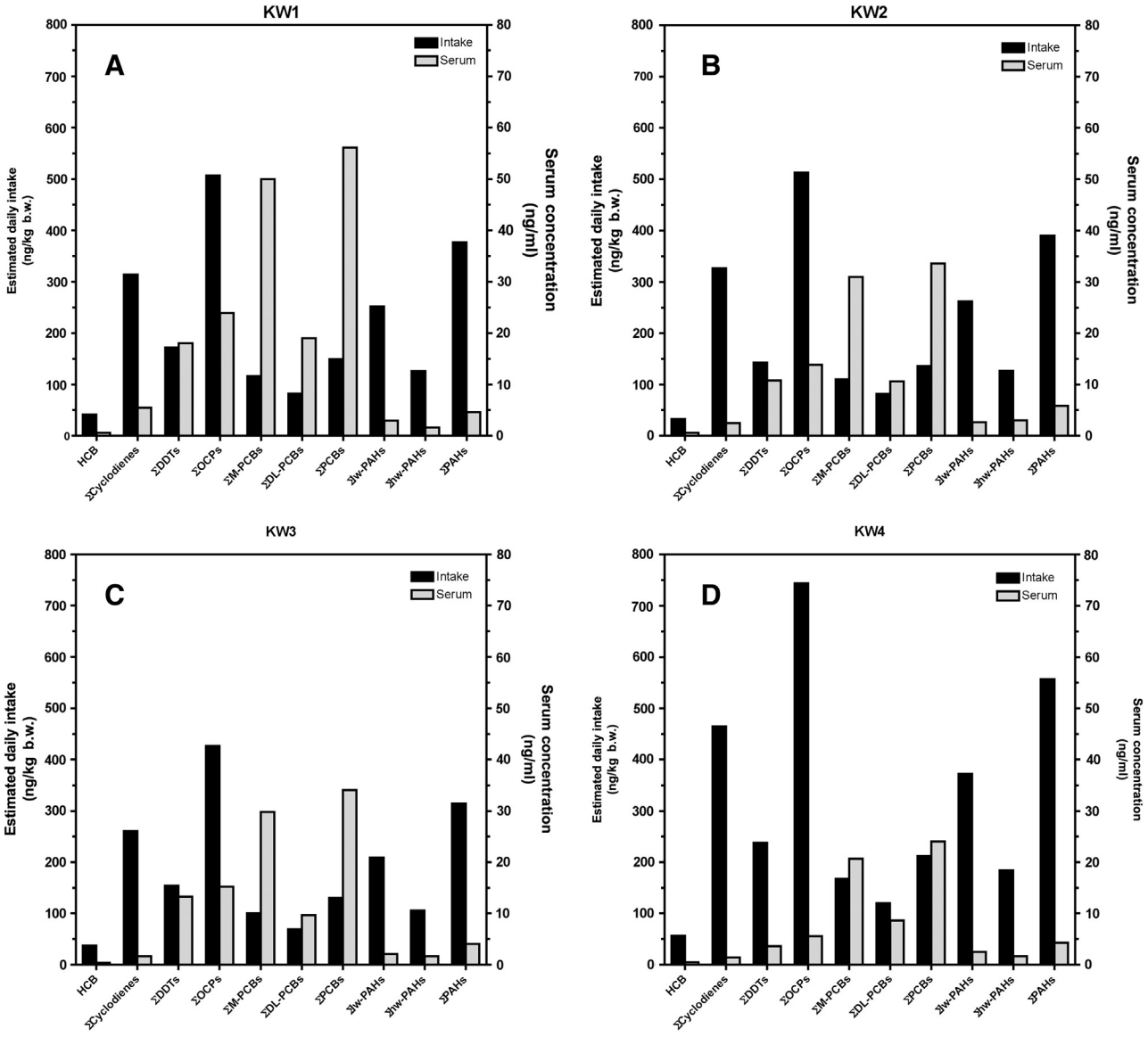

Fig. 1. Comparison between daily intake of the contaminants and their plasma levels in the four killer whales. Dietary intakes are plotted against left Y axis and plasma values against the right Y axis.

because the diet of fish-eating orcas would have exceeded by several hundred times the acceptably daily intake, thus leading to an increase in the risk of cancer by as much as $794/10^6$ in these animals.

Although diet seems to be the main route of entry of these contaminants, other factors exist to explain the profile of pollutants detected in killer whales because some of these contaminants were not detected in the food. The fact that captive animals exhibit such high levels of contamination is related with the ubiquity of these pollutants.

Although this study was performed in captive orcas, our results provide a glimpse into the current level of contamination in free-living animals, which remains quite high despite the steady decline in the levels of many of these pollutants in the environment due to their being banned from use.

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Conflict of interest

The authors declare no conflict of interest.

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