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Fin whales and microplastics: The Mediterranean Sea and the Sea of Cortez scenarios

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ABSTRACT

The impact that microplastics have on baleen whales is a question that remains largely unexplored. This study examined the interaction between free-ranging fin whales (*Balaenoptera physalus*) and microplastics by comparing populations living in two semi-enclosed basins, the Mediterranean Sea and the Sea of Cortez (Gulf of California, Mexico). The results indicate that a considerable abundance of microplastics and plastic additives exists in the neustonic samples from Pelagos Sanctuary of the Mediterranean Sea, and that pelagic areas containing high densities of microplastics overlap with whale feeding grounds, suggesting that whales are exposed to microplastics during foraging; this was confirmed by the observation of a temporal increase in toxicological stress in whales. Given the abundance of microplastics in the Mediterranean environment, along with the high concentrations of Persistent Bioaccumulative and Toxic (PBT) chemicals, plastic additives and biomarker responses detected in the biopsies of Mediterranean whales as compared to those in whales inhabiting the Sea of Cortez, we believe that exposure to microplastics because of direct ingestion and consumption of contaminated prey poses a major threat to the health of fin whales in the Mediterranean Sea.

1. Introduction

Litter enters the sea from land-based sources, maritime activities and sea-based infrastructure, among other sources, and can travel long distances (Eriksen et al., 2014). At the global scale, the highest percentage (~80%) of marine litter consists of plastic (Thompson et al., 2009). As larger pieces of plastic debris fragment into smaller pieces, the abundance of microplastics (plastic fragments smaller than 5 mm; Thompson et al., 2004) in marine habitats increases, outweighing larger debris. Plastic debris accumulates in semi-enclosed basins, such as the Mediterranean Sea, to a greater degree than in the open oceans. The Mediterranean Sea has been considered for centuries as “the cradle of civilization” and a medium for cohesion among different cultures. Over the past century, however, it has also become a dumping ground for the anthropogenic waste generated by the 22 countries (and 450 million people) bordering its shores. As a result of one of the highest levels of per-capita solid-waste production annually (208–760 kg/year), the Mediterranean Sea has become highly polluted with litter (Eriksen et al., 2014; Cózar et al., 2015). It has been estimated that 62 million items of macro-litter are floating on

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the surface of the Mediterranean Basin at any given time (Suaria and Aliani, 2014). The mean densities of floating microplastics in the Mediterranean Sea (more than 100,000 items/km^2) demonstrate the importance of this threat to the health of the basin (Collignon et al., 2012). Despite the ratification of the Marine Litter Action Plan by the Barcelona Convention in the 2013 Conference of the Parties, production trends, improper waste management, and the lack of mitigation actions and governance on the basin scale may lead to greater hazards for both marine wildlife and seafood safety (Seltenrich, 2015). Conversely, little data have been reported on the average density of microplastics in Mexico's Sea of Cortez (Gulf of California), a semi-enclosed body of water that is considered to be one of the more pristine areas in the world's oceans.

Research on the impact of microplastics on the biota of semi-enclosed marine ecosystems (Deudero and Alomar, 2015) and their potential toxicological effects on the large filter-feeding species that inhabit these environments, such as baleen whales, is still in its infancy (Fossi et al., 2012); to date, this research has been performed solely on a single stranded organism from Atlantic Ocean (Besseling et al., 2015). Cózar et al. (2014) estimated the global load of plastic at the ocean surface to be on the order of tens of thousands of tons, a level far lower than expected; these data were later confirmed by an assessment of plastic pollution in the world's oceans (Eriksen et al., 2014). The difference between the estimates and what was expected may be due to a combination of nano-fragmentation of microplastics along with their transfer to the ocean biomass through food webs. Intriguingly, the missing proportion of microplastics has about the same size interval as that of zooplankton (Cózar et al., 2014; Collignon et al., 2014). Zooplanktivorous predators, such as mesopelagic fish and large filter-feeding species (including baleen whales and some sharks), represent an important trophic component in the oceans and seas and they are at risk of exposure to microplastics in the water column. It is known that accidental ingestion of plastic occurs directly during feeding activities and indirectly via the consumption of zooplankton that previously ingested microplastics (Desforges et al., 2015; Farrell and Nelson, 2013; Setälä et al., 2014; Besseling et al., 2015; Romeo et al., 2015). Zooplankton and zooplanktivores may play a major role in capturing plastic at the millimeter scale (Cózar et al., 2014; Cole et al., 2013). Moreover, marine organisms may bioaccumulate toxic chemicals through the consumption of contaminated prey, large plastic debris and even microplastics. The major toxicological impact related to microplastic ingestion by filter-feeding organisms is the role that microplastics may play in persistent, bioaccumulative, and toxic (PBT) chemicals and in the leaching of plastic additives. Because PBT chemicals have low solubility in seawater, they tend to concentrate in the sea-surface microlayer, where they can be absorbed by microdebris, and thus may bioaccumulate in organisms that can ingest microplastic particles (Engler, 2012). However, modelling studies indicate that microplastics may act as a cleansing mechanism for PBT chemicals with log KOW between 5.5 and 6.5 (Gouin et al., 2011).

The more direct toxicological effects of microplastics are related to the leaching of plastic additives, such as bisphenol A, brominated flame retardants and phthalates, that enhance the performance of the plastic (Teuten et al., 2009). Phthalates, in particular, are a class of chemicals commonly used to soften rigid plastics. Di-(2-ethylhexyl) phthalate (DEHP), the most abundant phthalate in the environment, is rapidly metabolized in organisms to its primary metabolite MEHP (mono-(2-ethylhexyl) phthalate) (Barron et al., 1989). MEHP can be used as a marker of exposure to DEHP. Most of the chemicals that are absorbed by (PBTs) or added to (phthalates) plastics can negatively affect marine organisms through such means as endocrine disruption and subsequent population viability (Teuten et al., 2007). As such, organochlorines and phthalates are used in this paper as indirect (absorbed contaminants) and plastic-related (constituent contaminants) tracers of the microplastics in the baleen whale food chain.

This paper focus on the fin whale (*Balaenoptera physalus*), the second-largest filter feeder inhabiting in two semi-enclosed marine basins, the Mediterranean Sea and Mexico's Sea of Cortez (or Gulf of California). Despite its global distribution, fin whales are listed as “Endangered” worldwide (including in the Sea of Cortez) and “Vulnerable” in the Mediterranean Sea on the IUCN Red List of Threatened Species. Fin whales forage on dense aggregations of krill in the water column during the daytime and near the surface during both the day and night in some areas in the Mediterranean (Croll et al., 2005), engulfing an average of 71 m^3 of water per mouthful (Goldbogen et al., 2007). As a result, fin whales are exposed to a high potential risk of microplastic ingestion in their feeding grounds, both at the sea surface and throughout the water column.

Fin whales, the only resident mysticete in the Mediterranean, aggregate during the summer months on the feeding grounds of the Pelagos Sanctuary for Mediterranean Marine Mammals (hereafter referred to as “Pelagos Sanctuary”; Notarbartolo di Sciara et al., 2003) and presumably migrate to the southern Mediterranean Sea during winter (Panigada et al., 2011). The Pelagos Sanctuary, which is located in the northwestern Mediterranean Sea and encompasses 87,500 km^2 (Fig. 1a), is one of the Special Protected Areas of Mediterranean Interest (SPAMI) under the Barcelona Convention. This area is characterized by high offshore primary productivity, which attracts a variety of predators, including eight cetacean species and many large marine vertebrates (Notarbartolo di Sciara et al., 1993; Coll et al., 2012). This remarkable biodiversity coexists with extremely high human pressure (e.g. coastal tourism, recreational/commercial fishing, maritime traffic) and, consequently, is subject to a considerable amount of pollution (Fossi et al., 2013), including large amounts of plastic debris and microplastics (Collignon et al., 2012; Cózar et al., 2015).

Mexico's Sea of Cortez (Fig. 1b) presents a different scenario. It covers approximately 260,000 km^2, is extraordinarily productive (Carvajal et al., 2010), and features a high endemic biodiversity (857 endemic species, including the endangered vaquita, *Phocoena sinus*). However, the impact of pollution in the Sea of Cortez stemming from human coastal activities amplifies the conservation priorities in this coastal ecosystem, where marine debris and microplastic impact have not yet been investigated. Fin whales are resident in the Sea of Cortez and are genetically isolated from other populations (Bérubé et al., 1998).

In order to shed light on the under explained impact of microplastics on baleen whales, we followed up on previous studies on the use of phthalates as tracers of microplastic ingestion in stranded fin whales (Fossi et al., 2012) and the first evidence of direct ingestion of microplastics in a stranded humpback whale (Besseling et al., 2015) by investigating the potential toxicological effects of microplastics and their related contaminants on free-ranging fin whale populations in two separate basins with different levels and forms of human pressure and abundance of plastic debris. The study consists of two experimental steps: 1) counting microplastics, and mapping and detecting phthalates via zooplankton/microplastic sampling in two areas of the Pelagos Sanctuary and in the Sea of Cortez (preliminary sampling); and 2) performing genetic analysis and detection of phthalates, PBT chemicals and biomarker responses via biopsies of skin samples collected from fin whales, collected at three different times (July, August and September) in the Pelagos Sanctuary and in the Sea of Cortez, to investigate the temporal and geographical differences in microplastic-related pollutants.

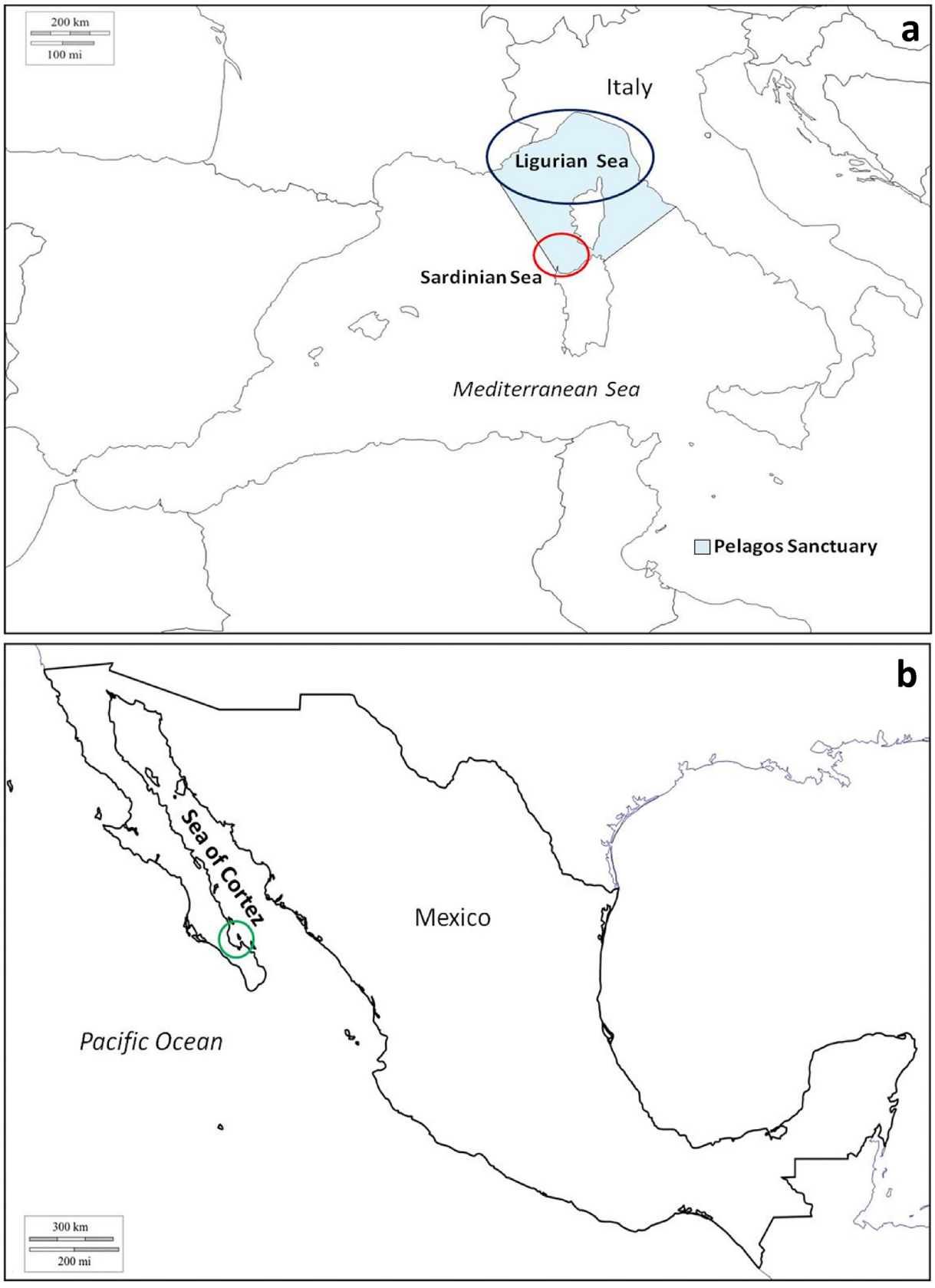

Fig. 1. a) The sampling sites for microplastics and fin whale skin biopsies in the Pelagos Sanctuary (Mediterranean Sea); sites in the Ligurian Sea are shown in blue, sites in the Sardinian Sea are shown in red. b) The sampling area in La Paz Bay (green), in the Sea of Cortez (Mexico). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. Sampling of microplastics

In the Pelagos Sanctuary, 36 zooplankton/microplastic samples were collected from the Gulf of Asinara and the Sardinian Sea over the course of three expeditions in the summer of 2011 (n = 9), 2012 (n = 13) and 2013 (n = 14), and 34 samples were collected from the Ligurian Sea during three expeditions in the summer of 2011 (n = 14), 2012 (n = 4) and 2013 (n = 16) (Fig. 1a). In 2013, three samples were collected from La Paz Bay in the Sea of Cortez, as a preliminary sampling (Fig. 1b). All zooplankton/microplastic samples were collected during daylight hours, and under calm weather and sea conditions. The samples were collected with a Neuston net (200-μm mesh size) equipped with a flowmeter to measure the volume of filtered water (m^3). The net was towed horizontally along the surface layers of the water at a speed of approximately 1.5 knots for 20 min. The net was washed on board, and each 2-l sample was split with a Folsom splitter into two separate aliquots of 1-l each. One 1-l aliquot was filtered through a 200-μm mesh sieve and immediately frozen in liquid N_2 for subsequent analysis of phthalates, whereas the second aliquot was preserved in a 4% formaldehyde-seawater buffered solution for subsequent analyses of plastic particles and zooplankton.

2.2. Counting and characterization of microplastics

As commonly defined by most of the scientific literature and also within the European Marine Strategy Framework Directive protocols, in this work we considered as microplastics the plastic particles less than 5 mm. However, it needs to be considered that recent works refer to plastics less than 1 mm as microplastic and those between 1 and 5 mm as mesoplastic, this would adhere to the standard empirical units (SI units) (Browne, 2015).

Samples were observed under a Leica Wild M10 stereomicroscope, and the plastic particles (microplastics) were counted and grouped into five size categories: 0.2–0.5 mm, 0.51–1 mm, 1.01–2.5 mm, and 2.51–5 mm. All data were normalized to the total volume filtered and expressed as items/m^3. A blank analysis was performed for each set of samples as contamination control during the analytical procedure.

2.3. GIS (geographic information system) mapping

Data and observation points for each sampling were georeferenced in the Gauss Boaga geographic projection (Monte Mario Italy 1, EPSG 3003) and incorporated as shape-files in ArcGIS v. 9.2 (ESRI). To obtain the spatial distribution (raster files) of each of the observed variables, the values of each variable were interpolated via the inverse weighted distance (IDW) method using the Spatial Analyst and 3D Analyst extension tools of ArcGIS. The raster files of the spatial distribution of the variables featured cells of equal size for each survey area, with each cell value corresponding to the value of the observed variable.

2.4. Fin whale skin biopsy

Skin biopsies were collected in three different periods (J: July, A: August and S: September) in the Pelagos Sanctuary in 2012 and 2013, corresponding respectively to early permanence (J), permanence (A) and late permanence (S) in the summer feeding grounds and in two different areas, the Ligurian Sea (n = 19) and the Sardinian Sea (n = 11). In the Sea of Cortez, skin biopsies were collected from February through March 2013 (n = 10) (Fig. 1). Skin samples were obtained using biopsy darts launched with a crossbow and immediately stored in N_2 (CITES permit Nat. IT025IS, Int. CITES IT 007), as described by Fossi et al. (2008).

2.5. Detection of plastic additives: phthalates

Both DEHP and MEHP were extracted from zooplankton/microplastic and fin whale skin biopsy samples, and were subsequently analysed following the method described by Takatori et al. (2004), with modifications (Fossi et al., 2012; see Supplementary material).

2.6. Detection of organochlorine compounds (OCs)

Blubber from the biopsy samples was analysed for the presence and concentration of HCB, DDTs (DDT and its metabolites) and PCBs (30 congeners; see Supplementary material) using a high resolution capillary gas chromatograph equipped with an electron capture detector (63Ni ECD) (AGILENT 6890/N), in accordance with a modified EPA 8081/8082 protocol (Marsili and Focardi, 1996).

2.7. Biomarkers analysis: CYP1A1, CYP2B and LPO

CYP1A and CYP2B have been previously detected in the skin of cetaceans via biopsy using WB analysis (see Supplementary material). Semi-quantitative analysis was performed for each WB using Quantity One software (Bio-Rad, 1-D Analysis Software), in accordance with the methods described by Fossi et al. (2008).

LPO was determined using the procedure of Ohkawa et al. (1979) and Bird and Draper (1984). The absorbance of each aliquot was measured at 535 nm and the rate of LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed/mg protein ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

2.8. Genetic analyses

DNA was extracted from 20 to 30 mg of fin whale skin and homogenised with a Tissue Lyser (Qiagen); then, it was isolated using a Wizard® Genomic DNA Purification Kit (Promega) in accordance with the manufacturer's instructions. Gender was determined by following the approach described by Bérubé and Palsbøll (1996).

The genotype was determined at nine microsatellite loci (see Supplementary material), which were amplified using the methods described in Palsbøll et al. (1997) and Bérubé et al. (2000). The nucleotide sequence at the 5′ end of the mitochondrial (mt) control region was determined through direct sequencing (Saiki et al., 1988) of PCR products, as described by Bérubé et al. (1998). Direct sequencing was conducted using standard ddNTP-based cycle sequencing (BigDye ver. 3.1, Applied Biosystems Inc.), following the manufacturer's instructions. The order of the sequencing products was resolved on a 3730 DNA Analyzer™ under standard conditions.

Table 1
Concentrations of microplastics (Items/m^3 ± SD) and MEHP (ng/g f.w. ± SD) in the Pelagos Sanctuary (Mediterranean Sea) in the Sardinian Sea and the Ligurian Sea.

	Items/m^3	MEHP ng/g
Sardinian Sea 2011 (n = 9)	0.13 ± 0.27	40.30 ± 41.55
Sardinian Sea 2012 (n = 13)	0.10 ± 0.12	93.37 ± 57.54
Sardinian Sea 2013 (n = 14)	0.24 ± 0.43	29.17 ± 7.78
Average Sardinian Sea (n = 36)	**0.16 ± 0.31**	**55.14 ± 49.21**
Ligurian Sea 2011 (n = 14)	0.94 ± 2.55	61.93 ± 124.26
Ligurian Sea 2012 (n = 4)	0.30 ± 0.36	48.52 ± 24.76
Ligurian Sea 2013 (n = 16)	0.19 ± 0.39	37.26 ± 17.05
Average Ligurian Sea (n = 34)	**0.49 ± 1.66**	**48.75 ± 80.05**

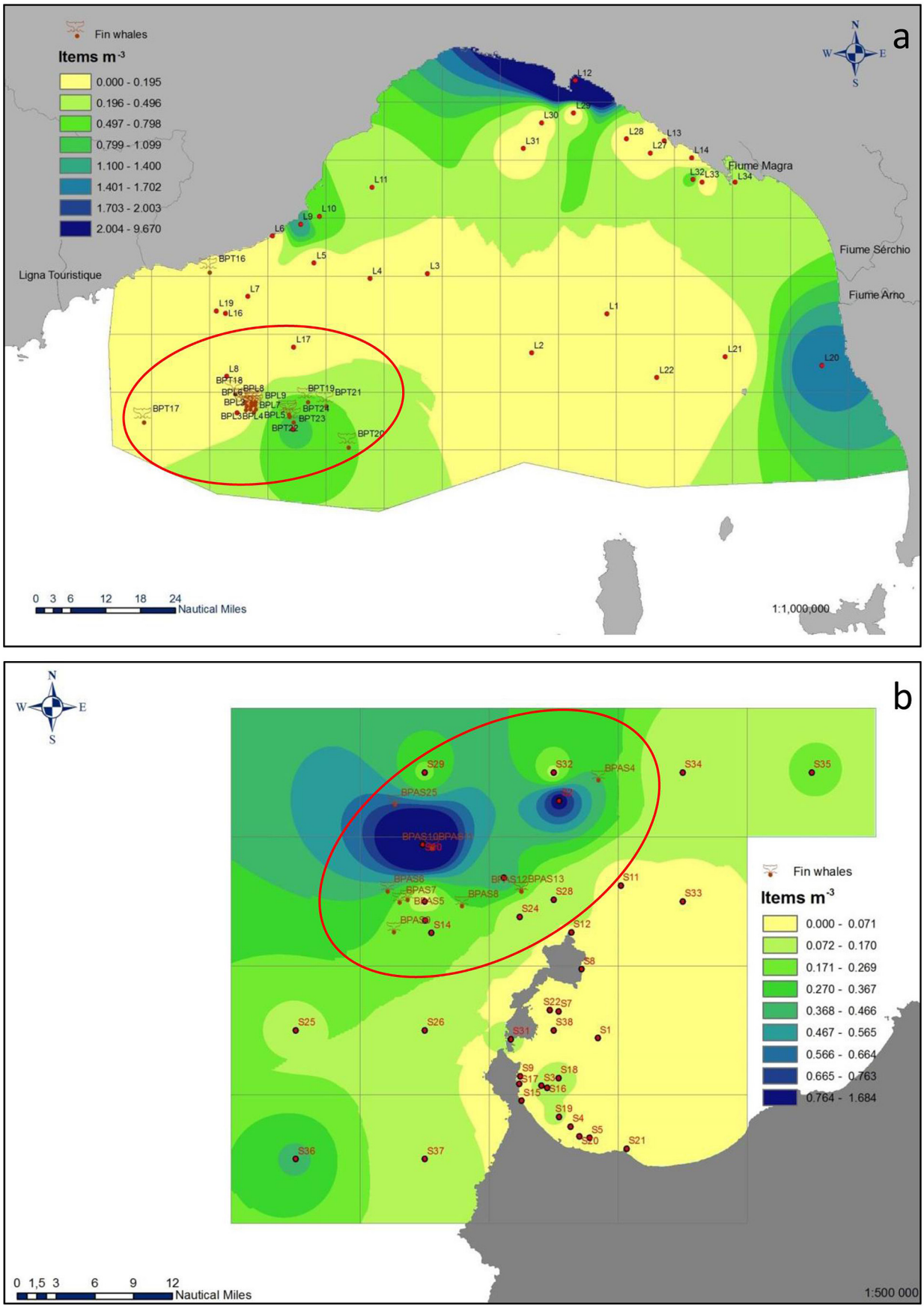

Fig. 2. Microplastic density (items/m^3) in the Pelagos Sanctuary (Mediterranean Sea) and Mediterranean fin whale sampling site/feeding grounds. a) Ligurian Sea: microplastic samples L1-L36 (expressed as items/m^3), fin whale sampling points (BPL-BPT); b) Sardinian Sea: microplastic samples S1−S34 (items/m^3); fin whale sampling points (BPA). The red circle represents where high-microplastic-density areas and fin whales sampling sites overlap. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Both strands of DNA were sequenced in all samples. The level of variation at the nuclear loci was estimated as the expected heterozygosity, and the probability of identity, *I* (Paetkau and Strobeck, 1994), was estimated using Cervus 3.0.3 (Marshall et al., 1998). The degree of genetic divergence between sample partitions, F_{ST} (Weir, 1990), was estimated using Genepop (ver. 4.0; Raymond and Rousset, 1995). The STRUCTURE (ver. 2.0) software was used to estimate the most likely number of panmictic clusters, *K*, present in the sampled multi-locus genotypes by using the default parameter values (Pritchard and Wen, 2003; for details see Supplementary material). The degree of variation within samples for the mt control-region nucleotide sequences was estimated as the nucleotide diversity, π (Nei, 1987). The degree of genetic divergence for the mt control-region between sample partitions was estimated as either F_{ST} or Nei's *D* (Nei, 1987) using the software package DnaSP (ver. 5; Librado and Rosas, 2009).

2.9. Statistical analysis of biomarkers and levels of contaminants

Hierarchical cluster analysis by the minimum energy (E) distance method was used to define clusters on the basis of area and temporal variables, and canonical discriminant analysis on PCA factors was performed to reveal clustering variables. The goodness of discrimination was determined using a Monte Carlo simulation (a non-parametric version of Pillai's test) with 999 permutations. All statistical analyses were performed using the "ade4" (Dray and Dufour, 2007) and "energy" (Rizzo and Szekely, 2010) packages of R software (R Core Team, 2012).

3. Results

3.1. Abundance of microplastics and phthalate concentrations

The concentration of microplastics in the Pelagos Sanctuary, as determined by the zooplankton/microplastic samples, ranged from 0 to 9.67 items/m^3 (mean: 0.31 items/m^3, SD ± 1.17 items/m^3), with the average concentration of MEHP − used in this study as a tracer of plastic additives − ranging from 29.17 ng/g to 93.37 ng/g (Fossi et al., 2012). Moreover, microplastic density differed between the Ligurian and Sardinian seas, with a higher abundance of microdebris detected in the Ligurian Sea (Table 1, Fig. 2); this difference was not statistically significant. However, DEHP levels were below the limits of detection (<LOD) in all of the samples we analysed.

Preliminary data on the average density of microplastics in the superficial zooplankton/microplastic samples collected from the Sea of Cortez (La Paz Bay) showed that the values ranged from 0.00 items/m^3 to 0.14 items/m^3; furthermore, concentrations of MEHP ranged from 13.08 ng/g to 13.69 ng/g, that is, they were nearly 4 times lower than the mean values detected in the Pelagos Sanctuary samples. These findings suggest that microplastics are far less abundant in the Sea of Cortez sampling sites than in the Mediterranean sampling sites.

3.2. Genetic analysis of fin whales

The first 454 base pairs at the 5′ end of the mtDNA control region were sequenced from samples collected from 26 whales (20 from the Mediterranean Sea, 6 from the Sea of Cortez). A total of 14 polymorphic sites defining 4 unique haplotypes were detected. No insertion/deletion events were observed. Whales from the Ligurian Sea had mtDNA sequences of haplotypes Bp01, Bp02 or Bp03, whereas whales from the Sardinian Sea were defined by the mtDNA haplotype Bp01. All whales from the Sea of Cortez (used as an outgroup) were of haplotype Bp04 (See Supplementary material, Table S1). For the combined samples of whales in the Mediterranean Sea, the probability of identity (*I*) was estimated at a very low 7.11×10^{-12} for unrelated individuals. At this level of *I*, the expected number of samples from different individuals matching at all 10 microsatellite loci by chance is very small (8×10^{-10}). The low degree of genetic structuring among the Mediterranean Sea samples at the microsatellite loci was also made evident by the Bayesian clustering analysis.

3.3. Phthalates, PBT chemicals and biomarkers in fin whales

Plastic additives (phthalates), organochlorine compounds (OCs) and biomarker responses [cytochrome P450 1A (CYP1A1), 2B (CYP2B), and lipid peroxidation (LPO)] were detected in 40 integument biopsies (epidermis, dermis and blubber) of fin whales. The toxicological data pertaining to both the two sub-groups of Mediterranean fin whales and the two whale populations (i.e. Mediterranean Sea and the Sea of Cortez) are reported in Table 2. There was no statistical difference between genders (males n = 13; females n = 17) in terms of the parameters CYP1A1, CYP2B, LPO, phthalates and HCB in the Mediterranean fin whale population, but the parameters PCBs, DDTs and total OCs were significantly different ($p < 0.001$, Mann–Whitney U test) between males and females.

No significant difference (Monte Carlo test: RV = 0.038, p = 0.378) was found between the two Mediterranean sub-groups, that is, between whales from the Ligurian and Sardinian seas. The canonical variables chosen do not discriminate between the two sub-groups from a geographical perspective (see Supplementary material, Fig. S2). With regard to the sample size, the whales analysed in this study constitute approximately 20% of the whale population (a total of approximately 150 individuals) residing in the Pelagos Sanctuary during summer (Panigada et al., 2011).

An additional Monte Carlo simulation with 999 permutations was performed assuming the areas of the Mediterranean Sea and Sea of Cortez as response variables (Table 2) and resulted in the detection of a significant difference between the two basins (RV = 0.052, p = 0.011). In addition, significant differences were found for the canonical variables between the fin whale populations in the two basins (Fig. 4). In particular, the analysis revealed that the parameters CYP2B and, albeit to a lesser extent, HCB were higher in whales sampled in the Sea of Cortez, whereas the values for PCBs, DDTs, total OCs, MEHP, LPO and CYP1A1 were

Table 2
Contaminant levels and biomarker responses in Mediterranean and Sea of Cortez fin whale sub-groups and populations.

	CYP1A1	CYP2B	LPO	MEHP	HCB	DDTs	PCBs	OCs
Sardinian Sea July 2012 (n = 11)	64.5 ± 37.0	34.6 ± 15.6	7.0 ± 5.5	54.8 ± 26.9	40.3 ± 18.2	11074.2 ± 6079.9	13103.4 ± 5512.1	24217.9 ± 11326.1
Ligurian Sea September 2012 (n = 9)	78.7 ± 13.2	55.5 ± 16.8	8.9 ± 4.5	65.5 ± 20.6	32.8 ± 8.2	16061.7 ± 11388.8	15302.6 ± 9702.7	31397.1 ± 20961.4
Ligurian Sea August 2013 (n = 10)	73.4 ± 23.9	33.3 ± 27.2	14.5 ± 5.5	40.2 ± 34.1	24.7 ± 9.1	8508.7 ± 3718.5	15739.1 ± 10258.6	24272.5 ± 13160.4
Average Mediterranean Sea (n = 30)	**71.9 ± 25.9**	**41.5 ± 22.9**	**11.0 ± 6.4**	**54.8 ± 27.7**	**29.9 ± 12.0**	**10477.3 ± 7477.4**	**13327.3 ± 8548.3**	**23834.5 ± 15057.2**
Average Sea of Cortez (n = 10)	**61.4 ± 28.4**	**52.9 ± 23.4**	**6.7 ± 3.8**	**40.0 ± 23.2**	**38.5 ± 33.6**	**3109.9 ± 2245.9**	**8753.8 ± 6542.6**	**11897.4 ± 6763.0**

CYP1A1 pmol/mg protein, CYP2B pmol/mg protein, LPO nmol TBARS/mg protein, MEPH ng/g f.w., HCB ng/g l.b., DDTs ng/g l.b., PCBs ng/g l.b., OCs ng/g l.b. All values are mean ± SD.

higher in whales sampled in the Pelagos Sanctuary. These results suggest that whales in the Pelagos Sanctuary are exposed to greater toxicological risk than are whales in the Sea of Cortez.

4. Discussion

The results of our study are examined in greater detail in the form of addressing four main questions, in order to explore the interaction between fin whales and microplastic pollution in the whale living/feeding grounds.

4.1. Do fin whales feed in areas affected by microplastics?

The estimates of the density of microplastics in the Pelagos Sanctuary are consistent with those of previous studies for the Mediterranean Sea (Collignon et al., 2012, 2014; Fossi et al., 2012; de Lucia et al., 2014), indicating the occurrence of this threat in this area of the Mediterranean. According to Ivar do Sul and Costa (2014) and Cózar et al. (2015), microplastic density in the Mediterranean is at about the same order of magnitude as in the North Pacific Ocean, a region that contains one of the highest concentrations of microplastic debris.

The GIS data from the study areas highlights how microplastics are primarily distributed in the pelagic environment (Fig. 2b) rather than the neritic environment, suggesting the presence of transient convergence areas during summer in the Sardinian Sea. Conversely, in the Ligurian Sea, significant accumulation areas were identified adjacent to two large harbours, Genoa and Livorno (Fig. 2a), although the pelagic areas of the Ligurian Sea exhibit the same level of microplastic abundance as in the pelagic areas of the Sardinian Sea. Circulation and current patterns can create regions of convergence (gyres) where floating debris accumulate at all depths, as a function of their composition and specific weight (Maximenko et al., 2012). Because the Mediterranean basin is characterized by a net inflow of surface waters from the North Atlantic Ocean and a negative outflow of surface water through the Strait of Gibraltar, floating litter tends to remain within the basin (Galgani et al., 2014). Unlike the open ocean, the oceanographic features of the Mediterranean Sea generally do not facilitate the creation of permanent gyres, but seasonal formations occur that may concentrate floating litter in specific areas at specific times of the year (Galgani et al., 2014). The geographic areas that we examined may encompass some of these convergence zones, but data on currents were not collected during the sampling periods to verify the potential formation of accumulation zones.

However, our data show that there is clear overlap between pelagic areas with high densities of microplastics and the feeding grounds of fin whales in both the Ligurian and Sardinia seas (Fig. 2), indicating that whales are exposed to high concentrations of microplastics in their summer feeding grounds. The analysis of the 70 zooplankton/microplastic samples collected in the Pelagos Sanctuary show that 49.7% and 37% of the items measured 1–2.5 mm and 2.5–5 mm, respectively (Fig. S1, Supplementary material). This is about the same size range of the primary zooplanktonic taxa (Wright et al., 2013; Cole et al., 2013), and thus represents a potential threat for zooplanktivorous species. Moreover, the same currents that concentrate plankton may also act to concentrate microplastic debris in the same convergence zones during the summer season, which may pose a potential risk for large filter feeders preying on plankton, as they ingest microplastics along with their prey.

Due to the selective filtering of small particles and their skimming action during feeding, baleen whales are more likely to ingest microplastics than larger plastic debris. Very few studies have reported the ingestion of large plastic debris by baleen whales in comparison to toothed whales and other marine vertebrates, focusing primarily on entanglement events (Kühn et al., 2015). Due to the porosity of the baleen, suspended particles do not remain on baleen fringes and fall onto the tongue upon water expulsion (Werth, 2013). Given that the average filtration rate of a fin whale is approximately 5800 m^3 of seawater daily, it is likely that thousands of pieces of microplastic debris, along with their associated toxic chemicals, may be ingested on a daily basis by an actively feeding fin whale in the Pelagos Sanctuary (Fossi et al., 2014).

In addition to direct intake, fin whales may also indirectly ingest microplastics through the consumption of large quantities of euphausiids and small schooling fish contaminated with microplastics (Fossi et al., 2012).

Evidence of ingestion by and the impact of microplastics on zooplankton have been described (Cole et al., 2013) for several species. Recently, Desforges et al. (2015) reported the first evidence of microplastic ingestion by the euphausiid *Euphausia pacifica* and the copepod *Neocalanus cristatus* in the wild, demonstrating that the organisms at the lowest trophic levels of the marine food web may occasionally mistake plastic particles for food. Similarly, zooplanktivorous schooling fish, such as *Sardina pilchardus* and *Engraulis encrasicolus*, and other zooplanktivorous pelagic fish, such as *Trachinotus ovatus*, have been shown to ingest microplastics as part of their food intake (Collard et al., 2015; Battaglia et al., 2015). Fin whales in the Mediterranean Sea feed preferentially and predominantly on the euphausiid *Meganyctiphanes norvegica*, although a wider spectrum of marine organisms, ranging from copepods and other euphausiid species (such as *Thysanoessa inermis*, *Euphasia krohnii* and *Nyctiphanes couchii*) to small schooling fish, are known to be part of their diet (Notarbartolo di Sciara et al., 2003). Samples reveal that concentrations of MEHP average 36.92 ng/g in *E. krohnii* (Fossi et al., 2014). Moreover, preliminary analysis of specimens collected in the Ligurian Sea during our study show concentrations of MEPH ranging from 8.87 ng/g to 21.79 ng/g in *M. norvegica*, indicating the presence of plastic additives in the main prey species of fin whales. The presence of MEPH in *M. norvegica* is most likely a result of exposure to phthalate-contaminated water and food, and direct consumption of microplastics. It is exceptionally difficult to obtain samples from the carcasses of stranded baleen whales to confirm the assumption of microplastic by whales but a recently published study on humpback whales reported that microplastics were found in the guts of stranded baleen whales (Besseling et al., 2015), an observation that supports the previous hypothesis by Fossi et al. (2012, 2014).

Finally, the gut passage time, the retention time and the excretion of microplastics by baleen whales are largely unknown. Retention time may depend on the size and type of plastic, the composition of the stomach contents, and features of the gastrointestinal tract, which may vary among whale species, as they do in birds (Ryan 2015). Further research on these factors in cetaceans is needed.

4.2. Are there two Mediterranean fin whale sub-groups?

Skin biopsies are used for genetic analysis and for assessing the impact that multiple anthropogenic stressors have on free-ranging cetaceans (Fossi et al., 2010; Godard-Codding et al., 2011). The genetic analysis, which focused on the mtDNA control region and nuclear markers (microsatellites), was undertaken on the two potential sub-groups of Mediterranean fin whales in order to exclude any genetic influence on the biomarker responses and contaminants burden (Supplementary material, Table S1).

One possible interpretation of the genetic results is that Mediterranean fin whale samples constitute a unique panmictic population, displaying maternally directed site fidelity (Clapham and

Seipt, 1991). However, the higher degree of genetic divergence at maternally inherited loci, such as in the mtDNA, compared to that of Mendelian inherited markers, such as microsatellite loci, are indicative of male-mediated gene flow (Palumbi and Baker, 1994). Moreover, the difference in the divergence rates (all other factors being equal) between the two markers may be due to differences in the effective population size for each of the two genomes, for which nuclear loci are four times larger than mtDNA loci. Finally, the genetic divergence may also be due to the fact that the two sub-groups have diverged only recently, which would account for the lower rate of genetic divergence observed at nuclear loci, given the larger effective population size of this genome (Bérubé et al., 1998). In addition, toxicological analyses (Table 2) did not differentiate between the two sub-groups living in the Pelagos Sanctuary (Fig. S2, Supplementary material).

In conclusion, the genetic (Tables S1 and S2, Supplementary material) and ecotoxicological data (Fig. S2, Supplementary material) suggest that the panmictic fin whale population reflects the state of microplastic contamination within the entire SPAMI, despite the level of genetic variation between the two Mediterranean groups being as high as that detected between the eastern and the western coasts of the North Atlantic (Bérubé et al., 1998).

4.3. Does the sampling period affect the toxicological responses?

The time variable is another factor that was taken into account in the analysis of the Mediterranean fin whale data. The data were subdivided into three sampling periods: July (J), August (A) and September (S), which correspond to early permanence (J), permanence (A) and late permanence (S) in the summer feeding grounds of the Pelagos Sanctuary. Fin whales begin to arrive in the Ligurian Sea in April and leave for the winter feeding grounds beginning in October, as confirmed by telemetry data (Panigada et al., 2011). Fig. 3 shows the results of the discriminant analysis when assuming that the time variable corresponds to these three periods (i.e. J, A and S). Toxicological analyses demonstrated that these three sampling/foraging periods differed significantly (Monte Carlo test: RV = 0.125, p = 0.005). Notably, phthalate concentrations were found to be higher in July and LPO (an indicator of oxidative stress) levels were high in August, but most intriguingly, the highest values of the other variables (OCs and biomarkers) occurred in September, towards the very end of the summer foraging season. This trend suggests that exposure to microplastic contamination increases over the span of permanence in the summer feeding grounds in the Mediterranean.

4.4. Is the toxicological pressure different for Mediterranean and Mexican fin whales?

The final factor taken into account in the toxicological analysis was the sea/basin variable. The discriminant analysis (Fig. 4), in conjunction with the analysis of hierarchical clusters based on the contaminant level and biomarker responses (Figs. 4 and 5), shows a clear distinction between the fin whales in the Mediterranean Sea and the fin whales in the Sea of Cortez. The higher concentrations of PCBs, DDTs, total OCs, MEHP, LPO and CYP1A1 detected in whales sampled in the Pelagos Sanctuary (Table 2, Fig. 4) compared to whales in the Sea of Cortez demonstrates that the Mediterranean whales inhabit waters in which the concentrations of microplastic and PBT compounds are much higher than those in the more pristine Sea of Cortez. Concentrations of microplastics appear to be much lower in the Sea of Cortez, and this is reflected in the lower MEHP levels observed in samples collected from whales in the Sea of Cortez. In addition to the low level of diversity in the mtDNA

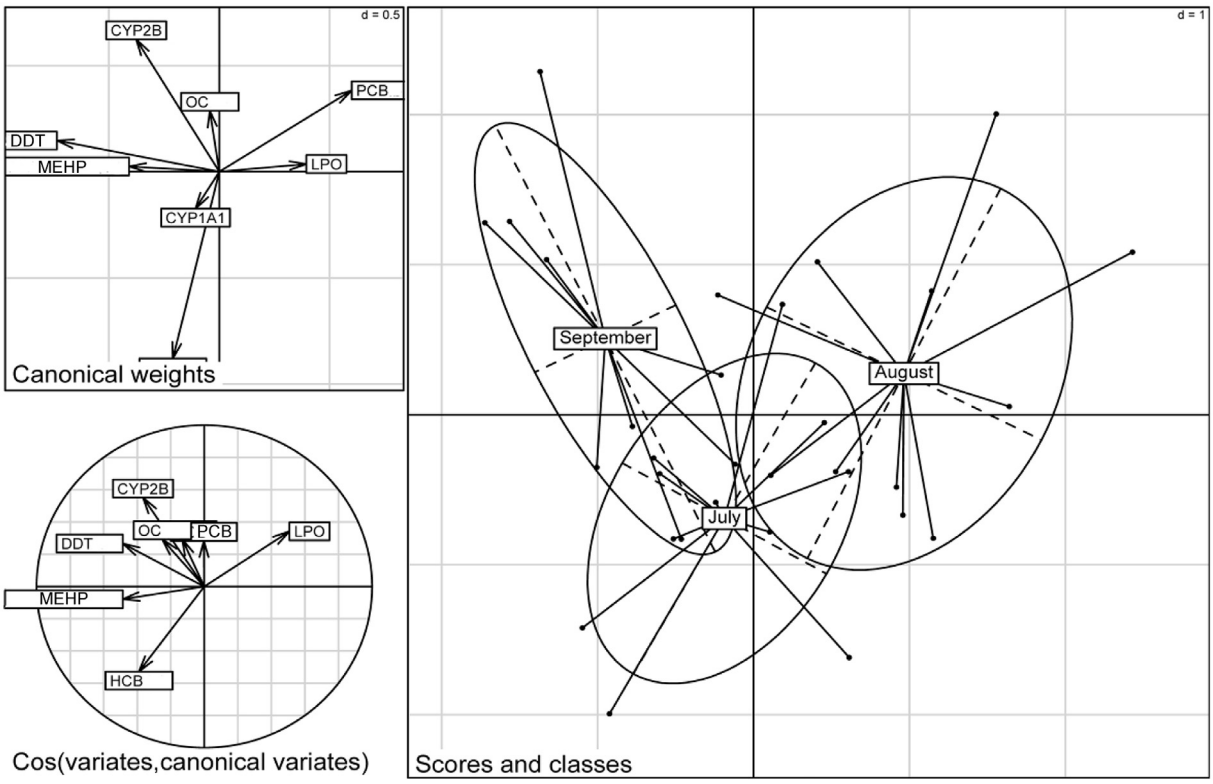

Fig. 3. Discriminant analysis on the PCA factors applied to the three sampling periods (July, August and September) of Mediterranean fin whales, biomarkers (CYP1A, CYP2B, LPO) and contaminants (HCB, DDT, PCB, OCs and MEHP).

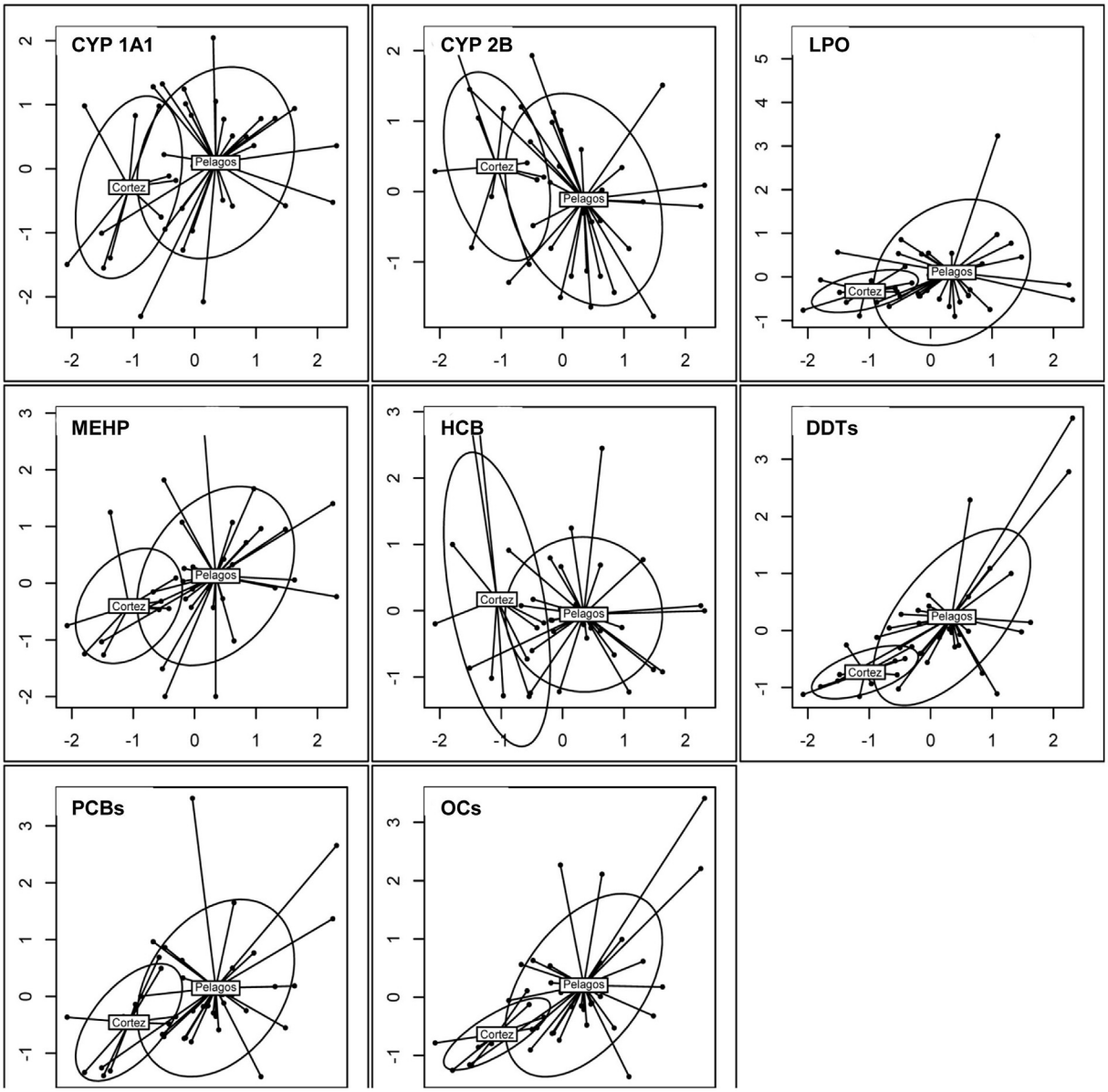

Fig. 4. Discriminant analysis on the PCA factors applied to the variables: basin (the Mediterranean Sea and the Sea of Cortez), biomarkers (CYP1A, CYP2B, LPO) and contaminants (HCB, DDT, PCB, OCs and MEHP). The canonical discriminant scores are plotted on the horizontal axis and the values of variables processed by PCA are on the y-axis.

control region (Supplementary material, Table S2), these results confirm the high degree of divergence between the two populations (Bérubé et al., 1998). Analyses of contaminant levels provide important information about exposure of whales to both PBT chemicals accumulated through the food chain and pollutants related to input from microplastics (plastic additives and OCs); these data, when correlated with biomarker responses, provide the ecotoxicological status of the two populations.

Due to a general lack of knowledge about the toxicological effects of plastic on marine mammals, the biomarkers previously used to evaluate the exposure and effects of microplastics, plastic additives and adsorbed contaminants on fish in laboratory studies (Rochman et al., 2013; Oliveira et al., 2013) were chosen. To evaluate the exposure to and effects of PBT chemicals, however, we used the same biomarkers that were used in previous studies on cetaceans (Fossi et al., 2008; Godard-Codding et al., 2011; Wilson et al., 2007).

In Mediterranean fin whales, CYP1A1 induction is a marker of particular importance for detecting exposure to planar PBT chemicals. Moreover, exposure to toxic chemicals, such as phthalates, may overwhelm antioxidant defences and other mechanisms that prevent cell damage (Halliwell and Gutteridge, 1999; Mathieu-Denoncourt et al., 2015). Given that the Mediterranean fin whale population displayed LPO values twice those of the Sea of Cortez whales (Table 2), we can assume that tissues in the Mediterranean whales might be exposed to higher oxidative stress due to the presence of plasticisers.

Despite the level of microplastic pollution in the two basins, it remains to be determined to what extent PBT chemicals are absorbed from direct microplastic ingestion in comparison to the amount ingested indirectly from feeding on contaminated prey. However, the higher levels of plastic additives detected in the Mediterranean whale population appear to be more linked to leaching from directly ingested plastic debris (Fossi et al., 2012).

In view of (a) the presence of high concentrations of microplastics in the Mediterranean Sea, especially in the SPAMI Pelagos

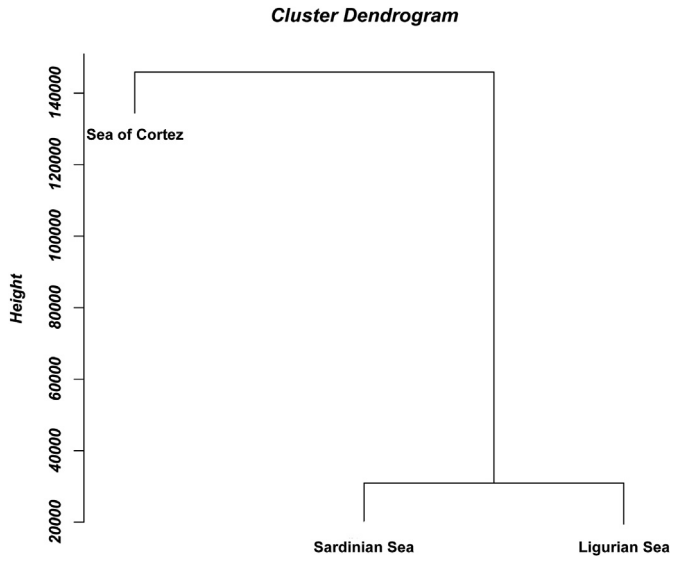

Fig. 5. Cluster dendrogram (analysis of hierarchical clusters) of classification of the Sardinian and Ligurian seas (Pelagos Sanctuary, Mediterranean Sea) and the Sea of Cortez (Mexico). The x-axis represents the sampling areas and the y-axis represents the distances gradually calculated. Fin whale sub-groups and populations are grouped according to level of contamination (HCB, DDT, PCB, OCs and MEHP) and biomarker responses (CYP1A, CYP2B, LPO).

Sanctuary; (b) the detection of high concentrations of PBT chemicals and plastic additives in the blubber of Mediterranean fin whales; (c) the high level of biomarker responses; and (d) the long lifespan of the species, Mediterranean fin whales appear to be exposed to absorbed and constituent contaminants of plastic, as result of direct and indirect ingestion of microplastic, macroplastic and contaminated prey. These results represent a warning for the vulnerable Mediterranean fin whale population. Although this species may use adjacent areas for feeding purposes, the decline in the fin whale population in the Pelagos Sanctuary by a factor of six (Panigada et al., 2011) raises concerns about the status of this species in the Mediterranean Sea.

Finally, the data show that there is a clear overlap between areas with high levels of microplastic pollution and the feeding grounds of fin whales in the Mediterranean Sea, an indication that fin whales are subjected to a high level of exposure to microplastic ingestion during feeding in the areas. These data also support the hypothesis that zooplanktivorous predators (Cózar et al., 2014), including baleen whales, play a relevant role in capturing plastic at the millimeter scale. Fin whales, which consume 900 kg of plankton daily, including microplastic debris incorporated into the marine food chain, can contribute to the removal of microplastics from marine waters and facilitate their transport to different oceanic regions.

In conclusion, in the present study temporal and regional ecotoxicological differences in fin whales were identified, suggesting that the Mediterranean Sea, particularly the Pelagos Sanctuary, is exposed to the risk of microplastics in comparison to other basins.

Future studies on the impact of microplastics on the biota of the Mediterranean Sea, in conjunction with mitigation efforts, are mandatory under the auspices of the European Marine Strategy Framework Directive and the Marine Litter Action Plan of the Barcelona Convention.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.022.

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