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Monk seals (*Monachus monachus*) in the Mediterranean Sea: The threat of organochlorine contaminants and polycyclic aromatic hydrocarbons

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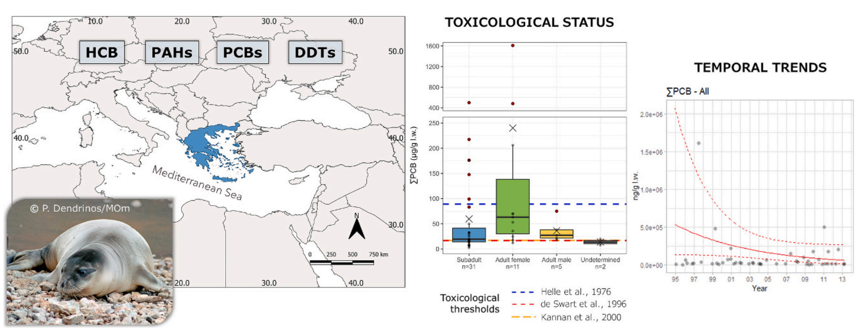
HIGHLIGHTS

- Assessment of PCBs, DDTs, HCB and PAHs in monk seals from Greece from 1995 to 2013
- All pollutants groups were detected in all samples with PCBs > DDTs ≫ PAHs > HCB
- Organochlorine levels exceeded common toxicological thresholds
- First assessment of PAHs in Mediterranean monk seals
- Monitoring studies to better address the pollution threat to monk seals are needed

GRAPHICAL ABSTRACT



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ABSTRACT

The Mediterranean monk seal (*Monachus monachus*) is an emblematic species of conservation concern. Anthropogenic pressures have led to severe population declines and significant fragmentation of the remaining populations. Because of their close relationship with coastal areas, Mediterranean monk seals may be potentially exposed to pollution from agricultural sources, as well as from oil tanker spills. Although monitoring of pollution has long been considered a priority for this species, data on monk seal contamination levels are scarce. In this study, 55 blubber samples of all genders and age classes collected during necropsies (1995–2013) from seals of the Eastern Mediterranean subpopulation were analyzed for organochlorine compounds (OCs), i.e., hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDTs), and polycyclic aromatic hydrocarbons (PAHs). Overall, PCBs > DDTs ≫ PAHs > HCB in all samples. Results showed a significant downward trend over the 19-year period for DDTs and HCB. No marked pattern was found for PAHs, even though relative abundance of cancerogenic PAH fraction rose in recent years. PCB levels in subadult specimens increased noticeably over time despite worldwide ban. Our findings did not suggest recent releases of

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DDT or new pesticides (e.g., Dicofol) in the environment, but may indicate an improper disposal of ancient storages of PCBs or a remobilization from reservoirs. OC levels exceeded thresholds that are commonly associated with immunosuppression and reproductive impairment in other pinnipeds. Hence, OCs could be responsible of potential toxicological effects in this subpopulation. This study represents the first report on PAH and one of the few reports on OC bioaccumulation in Mediterranean monk seals. Based on the levels found in the blubber of monk seals from the Eastern Mediterranean, and given the known harmful effects associated to the prolonged exposure to these substances and the reduced long-term expectations of species recovery, regular monitoring is advocated.

1. Introduction

The Mediterranean Sea represents a unique ecosystem, characterized by high levels of biodiversity. However, it is particularly susceptible to anthropogenic pressures, due to its unique geomorphology and the various human activities carried out within its coastal zone. Therefore, it has long been considered a sink for many environmental contaminants, such as persistent organic pollutants (POPs), including organochlorine contaminants (OCs) and polycyclic aromatic hydrocarbons (PAHs) (Marsili et al., 2018). These contaminants compose a large group of natural and xenobiotic substances that ubiquitously occur in the marine environment and are known for their persistency and toxicity. POPs have been monitored and regulated in most parts of the world for the last five decades (European Environment Agency, 2021; Stockholm Convention, 2001). However, they still represent a serious threat to the Mediterranean Sea, and especially to its marine mammals, because of their persistency, their continuous input into the marine ecosystem, and/or remobilization from long-term reservoirs or disposal loss (Dron et al., 2022; Jepson et al., 2016). As long-living apex predators, marine mammals are particularly vulnerable to POP pollution, as a consequence of bioaccumulation and biomagnification (Marsili et al., 2018). Although direct effects of POPs are difficult to assess, several detrimental health effects have been recorded in marine mammals (Bergman and Olsson, 1985; Desforges et al., 2018), including carcinogenicity (Baines et al., 2021), immunotoxicity (Desforges et al., 2018), endocrine disruption (Troisi et al., 2020), adverse effects on reproduction, offspring survivorship rates and population growth (Helle et al., 1976; Robinson et al., 2018; Roos et al., 2012) and an increased susceptibility to epizootics (Aguilar and Borrell, 1994; Hall et al., 1992).

The Mediterranean monk seal (*Monachus monachus*) is the only pinniped in the Mediterranean Sea. With a total population of <800 individuals the species had been considered to be “Endangered” by the IUCN since 2015 (Karamanlidis and Dendrinos, 2015), an improvement from its former designation as “Critically Endangered” back in 2008 (Aguilar and Lowry, 2008). Following years of concerted conservation actions, the species has recently shown signs of range expansion and population growth. Recent assessments place the count of mature individuals between 450 and 600, prompting a re-categorization to “Vulnerable” under the Red List criterion D1 in 2023 (Karamanlidis et al., 2023). The species once occurred throughout the entire Mediterranean Basin, including the Marmara and Black Sea, and in the Atlantic Ocean, along the northwest coast of Africa, as far south as Cabo Blanco, and around the Cape Verde, Canary, Madeira and Azores Islands. Nowadays Mediterranean monk seals have been reduced to three main, disjunct subpopulations located in the eastern Mediterranean Sea, in Cabo Blanco and in the Archipelago of Madeira with no genetic flow between them (Karamanlidis et al., 2019). The main threats to the species have historically been and remain strictly related to anthropogenic activities, including alteration and fragmentation of suitable habitat, continued mortality due to deliberate killing, and fisheries by-catch (Karamanlidis et al., 2016, 2021). These factors are the primary drivers of population decline, compounded by a significant reduction in genetic variability (Pastor et al., 2004, 2007). In Greece, an area with intense human activity and one of the last strongholds of the Mediterranean monk seal, deliberate killing and other sources of mortality that in the past were significant have declined in the last decades (Androukaki et al., 1999) and pollution may currently represent an additional potential threat to the recovery of the species and therefore monitoring of it has been identified as one of the national research and conservation priorities (Dendrinos et al., 2020). However, to date only limited information are available on the effects of OCs (Borrell et al., 1997, 2007), while no information exists about the levels of PAHs in Mediterranean monk seal, although oil spills have been recorded in important monk seal habitats (Kiraç, 1998).

The aims of the present study were: a) to assess the levels according to age, sex and location of stranding and evaluate the temporal trends of four groups of POPs (i.e., polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane and its isomers and metabolites (DDTs), hexachlorobenzene (HCB) and polycyclic aromatic hydrocarbons (PAHs)), on the largest population worldwide of the vulnerable Mediterranean monk seal, in Greece; b) check for variation in the contamination fingerprints of Mediterranean monk seals in Greece linked with age, sex, location or year of sampling; c) identify the potential source of pollution affecting Mediterranean monk seals in Greece; d) evaluate the toxicity and carcinogenic potency related to PAH exposure through the application of benzo(*a*)pyrene toxic equivalents.

2. Materials & methods

2.1. Sampling

Blubber samples were collected during necropsies, performed between 1995 and 2013 on 55 Mediterranean monk seals from the Aegean and Ionian Seas (Greece) by the MOm/Hellenic Society for the Study and Protection of the Monk seal (Fig. 1). Based on external morphological criteria (Samaranch and González, 2000) seals were identified as males ($n = 25$) or females ($n = 28$) and classified as pups ($n = 12$), juveniles ($n = 24$) and adults ($n = 17$) (Table S1). Sex and age could not be determined for 2 individuals.

2.2. Analysis of POPs

2.2.1. Organochlorine contaminants

PCBs, DDTs and HCB were identified and quantified by high resolution capillary gas chromatography, using an Agilent 6890 N chromatograph coupled with a ^{63}Ni Electron Capture Detector (ECD), following the U.S. Environmental Protection Agency (EPA) 8081/8082 protocol with minor modifications (Marsili and Focardi, 1997). Briefly, approximately 0.75 g of lyophilized subcutaneous blubber samples were spiked with 100 ng of CB30 as surrogate recovery standard and subjected to Soxhlet extraction with 200 mL of *n*-hexane for 9 h. A two-step purification procedure was adopted, involving sulfuric acid purification and Florisil-packed columns chromatography. The purified extracts in *n*-hexane (5–150 mL) were spiked with 10 ng of CB209 as an internal standard and analyzed using GC-ECD equipped with an SBP-5 bonded phase capillary column (30 m, 0.2 mm i.d.). Nitrogen was used as the carrier gas, and argon/methane (95/5) served as the scavenger gas. External standard calibration was applied for the identification and quantification of individual PCB congeners and DDT isomers. The calibration standard was a mixture of Arochlor 1260, HCB, and pp.’- and op'-DDT, DDD and DDE. Total PCBs were quantified as the sum of 30 PCB (penta- to nona-CB) congeners (IUPAC #95, 101, 99, 151, 144, 135, 149, 118, 146, 153, 141, 138, 178, 187, 183, 128, 174, 177, 156, 171, 202, 172, 180, 199, 170, 196, 201, 195, 194, 206). Total DDTs were calculated as the sum of *p,p'*- and *o,p'*-DDT, and its metabolites: *p,p'*- and *o,p'*-DDD, and *p,p'*- and *o,p'*-DDE. The extracted organic material (EOM %; lipid content) was calculated gravimetrically following extraction with *n*-hexane.

To test for variations in the fingerprint of PCB contamination among groups, the relative percentage of PCB homologous groups (penta-, hexa-, epta, −octa and nonaCB) was calculated. Similarly, for DDTs the relative contribution of the 6 isomers of DDT and its metabolites was considered. In order to identify the main source of pollution (agricultural versus industrial input) and the potential recent release of new pesticides, such as Dicofol, in the environment the diagnostic ratios ∑DDT/∑PCB, *o,p'*-DDT/*p,p'*-DDT and ∑*o,p'*-DDT (*o,p'*-DDT + *o,p'*-DDD + *o,p'*-DDE)/∑DDT were calculated. The *p,p'*-DDE/*p,p'*-DDT and *p,p'*-DDE/∑DDT ratios were considered as an indicator of degradation of DDT in the environment. A full description of all analytical procedures and instrumental parameters, as well as the estimated extracted organic material (EOM %) for each sample, is provided in the Supplementary data.

2.2.2. PAHs

PAHs were extracted from ~0.7 g of lyophilized blubber samples following the procedures described elsewhere (Griest and Caton, 1983; Holoubek et al., 1990) with some modifications (Marsili et al., 1997), using 100 mL of a mixture of KOH/methanol in a Soxhlet apparatus for 4 h. To obtain the PAH fraction, a liquid/liquid separation was performed with cyclohexane, followed by a chromatographic column cleanup. PAHs were analyzed by high-performance liquid chromatography (HPLC) using a reversed-phase column (Supelcosil™ LC-18, 250 × 4.6 mm, 0.5 μm particle size) installed on a liquid chromatogram (Waters™ 600) with a scanning fluorescence (Waters™ 474 Scanning Fluorescence Detector) and UV detector (Waters 2487 Dual λ Absorbance Detector). Individual peaks obtained from the HPLC chromatograms were identified and quantified by comparison with the standard (EPA610, Supelco). Results were expressed as the sum of 14 of the 16 PAHs included in the US EPA priority pollutant list: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*ah*]anthracene, benzo[*g,h,i*]perylene. The carcinogenic PAH fraction was calculated as the sum of benzo[*a*]anthracene (B[*a*]A), chrysene (Chry), benzo[*b*]fluoranthene (B[*b*]F), benzo[*k*]fluoranthene (B[*k*]F), benzo[*a*]pyrene (B[*a*]P), dibenzo[*a,h*]anthracene (D[*a,h*]A) (International Agency for Research on Cancer, 2010). Besides, PAH concentrations were converted to benzo[*a*]pyrene toxic equivalent (B[*a*]PTEQ) toxicity using the toxic equivalency factor (TEF) approach (Nisbet and LaGoy, 1992). In order to compare PAH contamination fingerprints of different groups, the relative proportion of carcinogenic and non-carcinogenic compounds was assessed and compared among groups. Moreover, ∑c-PAH/∑PAH was considered to infer the main source of PAH. A full description of all analytical procedures and instrumental parameters is provided in the Supplementary

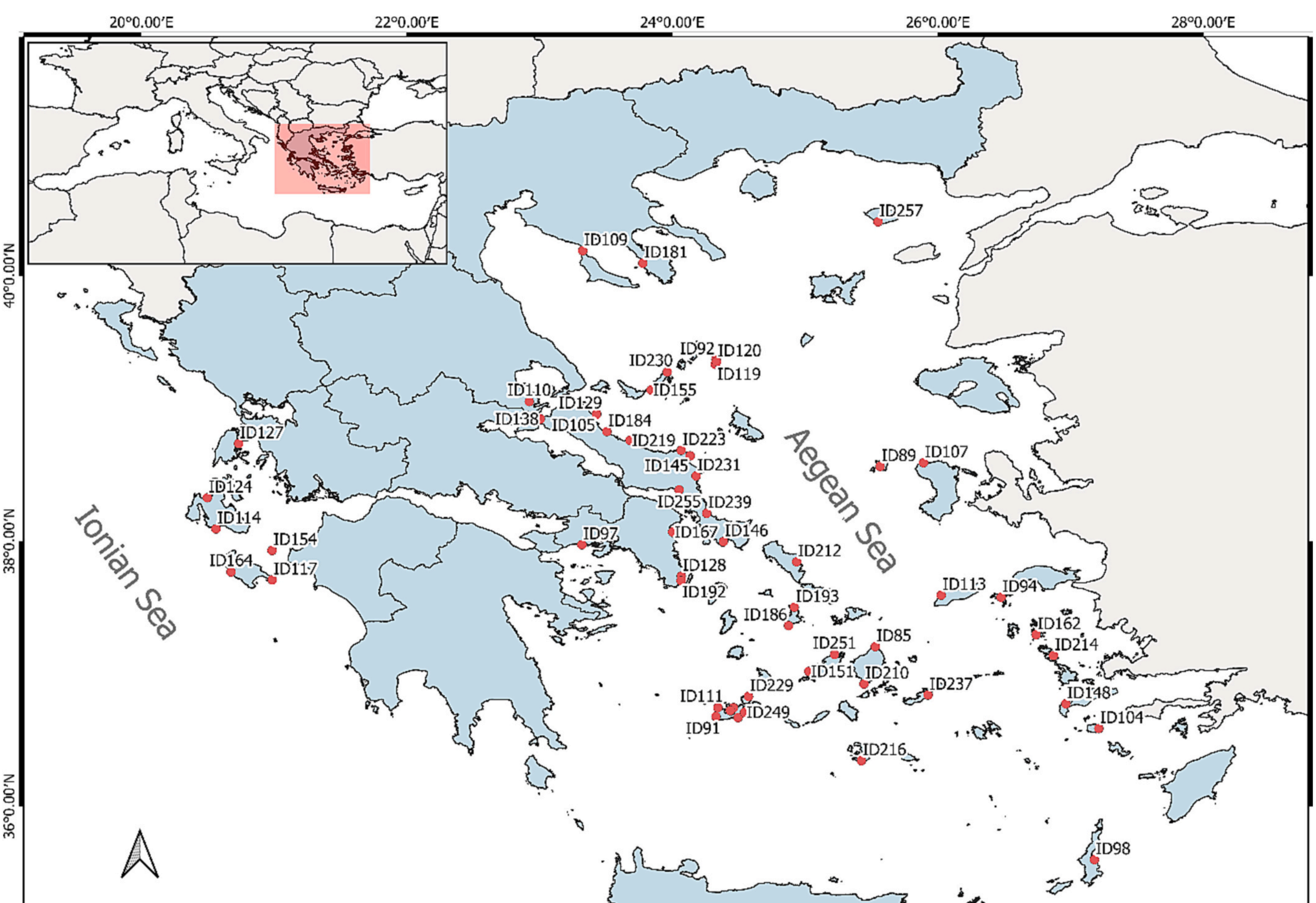

Fig. 1. Map of Greece indicating the stranding location of Mediterranean monk seals (*Monachus monachus*) sampled during this study.

data.

2.2.3. Quality assurance/quality control

Quality control and quality assurance measures included the analysis of spiked blank samples covering the entire analytical procedure. A procedural blank was analyzed within each batch of 10 samples. Blanks contained undetectable amounts of PCBs and PAHs, other than internal standards. Assay reproducibility was assessed using three homogeneous replicates of three samples, and the coefficient of variation (CV) was calculated for the obtained results. CV values remained consistently below 7 % for all analyzed compounds. Recoveries of standard for OCs were calculated by adding known quantities of CB #30 and #209 to all samples and blanks. Recoveries for PAHs were assessed through repeated analyses of the certified EPA 610 Polynuclear Aromatic Hydrocarbons standard mixture. The recoveries for the OC and PAH analyses ranged from 85 to 95 % and from 80 to 98 %, respectively. The detection limit was calculated as the mean of the background noise +2 standard deviation (SD) of 20 replicates of blank samples and was found to be 0.1 ng/kg for all OCs and 0.1 ng/g for all PAHs analyzed.

2.2.4. Data analysis

Concentrations of organochlorine contaminants were expressed in ng/g on a lipid weight basis (l.w.). ∑PAH were expressed in ng/g on a dry weight basis (d.w.). Concentrations that were below the detection limits were considered as LOD (limit of detection) values. ∑PCB, ∑DDT, HCB and ∑PAH concentrations were checked for conformation to a normal distribution using Shapiro-Wilk's normality test. All variables showed a significant deviation from normality and were therefore log10 transformed. Differences between the mean levels of contaminants by age class, sex, location and year were assessed by analysis of variance (ANOVA) using R version 4.0.3 (R Core Team, 2020) and RStudio version 2022.7.1.554 (RStudio Team, 2022). A statistical significance level of $p < 0.05$ was used for all analyses. For statistical purposes, pups and juveniles of both sexes were merged into a single group termed "Subadult", as comparison of all contaminants found no differences between these groups. Outliers in our data were identified based on values outside of 1.5 * the interquartile range (IQR) for the sum of each contaminant class (∑PCB, ∑DDT, HCB and ∑PAH) within each group of samples. In order to preserve the comprehensiveness and accuracy of our dataset, we made the decision to retain these outliers and not exclude them from our statistical analysis. This choice was made after thorough examination and reanalysis of specific borderline samples (ID97, ID107, ID230), which confirmed the absence of analytical issues or potential contamination during the analytical procedure. General Linear Regression Models (GLM) with a gamma error distribution and log link fit were used to analyze variations in lipid-normalized concentrations of ∑PCB, ∑DDT, HCB and ∑PAH. Temporal trends including all data were fit, as well as distinct models for subadult and adult female groups. There were insufficient observations in the adult male and undetermined groups to create a model. Using ∑PCB, ∑DDT, HCB or ∑PAH as the response variable, several multiple linear regression models were fitted. Variables included in the full model were year, age (adult, subadult, undetermined), sex (male, female, undetermined), location (Aegean Sea, Ionian Sea). All possible variable combinations were tested to obtain several candidate models, which were ranked according to their AIC (Akaike's Information Criterion) values. Graphics and figures were generated using R version 4.0.3 (R Core Team, 2020) and RStudio version 2022.7.1.554 (RStudio Team, 2022), and QGIS 3.0 Girona (QGIS Development Team, 2018).

3. Results

3.1. Age, sex and location-related POP levels and temporal patterns

All POP groups studied were detected in all blubber samples analyzed. Overall, PCBs > DDTs ≫ PAHs > HCB, with PCBs and DDTs showing on average levels of two orders of magnitude higher than PAHs and HCB. Average lipid weight contaminant concentrations across all samples are reported in Table 1. One DDT metabolite (*o,p'*-DDE) and two PCB congeners (#99, 199) were below LOD in >91 % of all samples. In two adult females [ID97 and ID107, Table S1] we recorded extremely high levels of organochlorine contaminants: total PCBs were 1610,000 ng/g l.w. and 481, ng/g l.w.; total DDTs were 568,000 ng/g l.w. and 893,000 ng/g l.w.; HCB was 1060 ng/g l.w. and 978 ng/g l.w. in the two samples, respectively. Similarly, in a juvenile female [ID230, Table S1] we recorded extreme levels of PAHs (7890 ng/g d.w.).

The results of the study indicated a high interindividual variability and no significant difference linked to age and gender (Table S5). However, the relative contamination profiles of adult males, adult females and subadults were not consistent when considering different groups of contaminants.

In general, adult females demonstrated the highest mean levels across all OC groups (Table 2, Fig. 2). The extreme levels recorded for ID97 and ID107, both adult females, heavily skewed upwards this statistical descriptor. By looking at median values, adult females showed the greatest levels only in PCBs, while DDTs and HCB were the highest in adult males (Table 2, Fig. 2). Regarding PAHs, subadults exhibited the highest concentrations on average of total PAHs, followed by adult females (Table 2, Fig. 2). Again, the elevated mean levels found in subadult specimens are influenced by ID230, a juvenile female demonstrating extreme levels of PAHs. Considering only the carcinogenic PAH fraction, the highest levels were reported in adult males (Table 2).

Some differences were found based on geographic location although not being statistically significant (i.e., most likely due to the limited number of samples from the Ionian Sea). In general, samples from the Ionian Sea showed a greater relative abundance of ∑PAH, while samples from the Aegean displayed a larger relative abundance of OCs (Table 1, Table S5).

Model results for temporal trends are reported in Tables S6–17 and displayed in Fig. 3. Time was shown to be a significant predictor of pollutant levels only for ∑DDT (pvalue = 0.0013; Fig. 3D, Table S9) and HCB (*p*-value = 0.0042; Fig. 3G, Table S12), when considering the whole dataset of monk seal contamination. Both pollutants had a declining trend during the 19-year period. ∑PCB and ∑PAH did not reveal significant trends over time, even though an overall tendency toward decrease was observed as well (Fig. 3A, J). Pollutant levels showed a significantly different behavior over time in adult females and subadult specimens for all pollutant groups (Tables S6, S9, S12, S15). Adult females exhibited a decreasing trend for all contaminant groups (Fig. 3B, E, H, K) which resulted to be statistically significant for total DDTs (p-value = 0.0052; Table S11, Fig. 3E) and HCB (p-value = 0.0084; Table S14, Fig. 3H). Conversely, subadult specimens showed no tendency in ∑DDT, HCB and ∑PAH (Fig. 3F, I, L) but displayed a significant increasing trend of PCB levels over time (p-value = 0.0342; Table S7, Fig. 3C). It is crucial to remember that the highly contaminated individuals ID97 and ID107 were adult females who were sampled between 1997 and 1999. These individuals may have had an impact on the relatively high levels of organochlorine contaminants found in the first time window of the period considered. Additionally, due to the limited sample size, their presence may have contributed to the declining patterns observed in the model for the group of adult females. ID230, on the other side, was a juvenile specimen sampled in 2011, but it did not appear to have an influence on subadults' trends most likely due to the larger sample size of the subadults' group.

3.2. Pollutant profile

Overall, the PCB congener profile was dominated by hexa- and hepta- congeners, which on average accounted for 86.0 % of the total levels (Fig. S1a); the most abundant were marker congeners CB #153 (median 28.4 %), #138 (14.9 %) and #180 (13.0 %). The relative

Table 1
Organochlorine compound (∑PCB, ∑DDT and HCB) (ng/g l.w.) and ∑PAH (ng/g d.w.) levels in blubber samples of monk seals from Greece grouped by location of stranding (i.e., Ionian vs. Aegean Sea). Number of samples, arithmetic mean, median and range, together with detection frequencies (% > LOD) are reported for each contaminant group. c-PAHs are carcinogenic compounds, nc-PAHs are non-carcinogenic compounds, according to IARC.

	Ionian Sea				Aegean Sea				All				
Compounds	N.	Mean	Median	Range	N.	Mean	Median	Range	N.	Mean	Median	Range	>LOD (%)
∑Penta-CB	5	1290	895	596–2190	44	4300	1510	265–43,000	49	4000	1490	265–43,000	100
∑Hexa-CB	5	14,200	11,400	5410-29,900	44	51,600	14,100	1200-742,000	49	47,800	13,900	1200-742,000	100
∑Hepta-CB	5	10,300	6570	2400-25,900	44	39,300	8880	724–686,000	49	36,300	8560	724–686,000	100
∑Octa-CB	5	2290	795	447–7070	44	7730	2000	199–130,000	49	7180	1870	198–130,000	100
∑Nona-CB	5	1070	54	39.4–5110	44	482	108	<LOD-7470	49	542	106	<LOD-7470	85.7
∑PCB	**5**	**29,200**	**19,400**	**9100-70,200**	**44**	**103,000**	**26,000**	**2380-500,000**	**49**	**95,800**	**24,900**	**2380-1610,000**	**100**
o,p'-DDE	5	191	0.0001	<LOD-953	44	10.3	0.0001	<LOD-424	49	28.7	0.0001	<LOD-953	6.12
p,p'-DDE	5	24,500	19,700	8160-56,800	44	80,400	23,300	2040-874,000	49	74,700	23,200	2040-874,000	100
o,p'-DDD	5	213	121	59.7–610	44	391	145	43.6–2680	49	373	142	43.6–2680	100
p,p'-DDD	5	559	507	195–1200	44	1650	776	87.4–17,100	49	1540	733	87.4–17,100	100
o,p'-DDT	5	39.1	41.9	<LOD-66.3	44	139	89.2	<LOD-725	49	121	77	<LOD-725	77.6
p,p'-DDT	5	842	520	259–2180	44	2520	941	96.1–20,400	49	2350	868	96.1–20,400	100
∑DDT	**5**	**26,400**	**20,400**	**8830-61,700**	**44**	**85,100**	**24,500**	**2280-893,000**	**49**	**79,100**	**24,500**	**2280-893,000**	**100**
HCB	**5**	**189**	**132**	**96.2–343**	**44**	**256**	**150**	**<LOD-1060**	**49**	**216**	**149**	**<LOD-1060**	**91.8**
∑c-PAH	6	25.6	23.2	3.35–55.7	49	18.9	10.7	1.77–121	55	19.7	10.8	1.77–121	100
∑nc-PAH	6	636	653	61.9–1140	49	615	345	121–7830	55	617	345	61.9–7830	100
∑PAH	**6**	**662**	**701**	**69.1–1160**	**49**	**634**	**363**	**126–7890**	**55**	**637**	**363**	**69.1–7890**	**100**

proportion of homologous PCB groups differed between subadult and adult specimens. Lower chlorinated compounds (i.e., penta- and hexa-congeners) showed the highest percentage in subadults, while congeners with >6 Cl were higher in adults (Fig. S1a). Pairwise comparisons (Wilcoxon rank sum exact test with Bonferroni p-value adjustment) revealed significant differences between pups and adult females for pentaCBs (p.adj = 0.048), hexaCBs (p.adj = 0.037) and heptaCBs (p.adj =0.0007), and between juveniles and adult females for hexaCBs (p.adj = 0.0002), heptaCBs (p.adj = 0.017), octaCBs (p.adj = 0.0002) and nonaCBs (p.adj = 0.027). DDT contamination fingerprints were similar in all samples, with *p,p*'-DDE representing the most abundant compound of total DDTs (median 92.3 %) (Table 1), followed by *p,p*'-DDT and *p,p*'-DDD (3.40 % and 2.64 %, respectively) (Fig. S1b). The non-carcinogenic compounds pyrene (51.5 %), naphthalene (16.9 %) and fluoranthene (13.8 %) were the most abundant PAHs; in contrast, carcinogenic PAHs accounted for only 4.8 % (range = 0.5–39.4 %) of total PAHs (Fig. S1c). The mean residual percentages were found to be in the order B[*b*]F (1.10 %) > Chry (0.383 %) > B[*k*]F (0.282 %) > B[*a*]P (0.148 %) > D[*a*,*h*]A (0.133 %) > B[*a*]A (0.0802 %). According to age class, higher proportions of nc-PAHs were recorded in subadult monk seals than in adult individuals of both sexes, though the differences were not statistically significant (Fig. S1c). Overall, adult females showed the lowest percentage of lower-molecular-weight (LMW) PAHs (2–3 benzene rings) (median 24.1 %) as compared to adult males (36.1 %) and subadults (33.1 %). No substantial geographical variations in the fingerprints of contamination emerged from the analysis (Fig. S2).

3.3. Potential source of pollution

Relative concentrations of *p,p*'-DDE were significantly higher than the relative concentrations of the active substance *p,p*'-DDT (pairwise Wilcoxon rank sum exact test with Bonferroni *p*-value adjustment, p.adj <0.01) (Table 3). The ratio of *p,p*'-DDE/ΣDDT around the value of 1 further indicate the prevalence of *p,p*'-DDE compound. Overall, *o,p*' isomers showed low percentage concentrations (median 1.04 % of total DDTs), not exceeding the threshold of Σ*o,p*'-DDT/ΣDDT = 0.20 which indicate a non-insecticidal source of DDT in the environment (Nowell et al., 1999). The median ratio *o,p*'-DDT/*p,p*'-DDT was 0.0764, below the set value of 0.25 for Dicofol-type pollution (Qiu et al., 2005). ΣDDT/ΣPCB ratio showed a substantial inter-individual variability, ranging from 0.101 to 3.12 and averaging around 1. A significant reduction of ΣDDT/ΣPCB in the 19-year period was found (p-value<0.0001), mainly driven by Aegean Sea samples (Fig. 4A, Table S18). Overall, the value of this ratio decreased from a median of 1.55 in the period 1995–2004 to 0.665 in 2005–13. No significant differences were found in organochlorine contaminant ratios between adult males, adult females and subadults or linked to location of stranding (Table S19).

Regarding PAHs, a significant increase of the relative proportion of the carcinogenic fraction to total PAHs with time was visible (p-value<0.0001; Fig. 4B, Table S20).

3.4. Toxicity assessment

Total B[*a*]P-TEQ ranged from 1.26 to 61.5 ng /g d.w. (Table 4).

4. Discussion

This study investigated OC and for the first time PAH levels in 55 Mediterranean monk seals from Eastern Mediterranean subpopulation. Blubber samples were collected in Ionian Sea and in several localities in the Aegean Sea, over a temporal period of 19 years from 1995 to 2013.

All POP groups (PCBs, DDTs, HCB, PAHs) investigated were detected in the specimens. To date, the only available studies reporting the contamination status of this subpopulation related to OC exposure refers to few individuals sampled in the '90s (Borrell et al., 1997, 2007). Mean PCB, DDT and HCB levels found here are in accordance with those previously reported. In particular, Borrell et al. (1997) described the highest PCB mean values of 64.3 μg/g l.w. (as the sum of 18 congeners) in two specimens in 1991. Later, between 1995 and 1999 lower PCB mean levels were reported in 11 specimens by Borrell et al. (2007) ($\sum_{25}$PCB = 24.7 ± 32.2 μg/g l.w.) and in this study ($\sum_{30}$PCB = 23.2 ± 18.0 μg/g l.w., *n* = 10 – excluding ID97 and ID107 considered as extreme values) in the same period. Conversely, DDTs show an inverse trend, with lower levels reported in the early nineties ($\sum_4$DDT = 19.0 μg/g l.w.; Borrell et al., 1997) as compared to 1995–99 ($\sum_3$DDT = 36.2 ± 51.7 μg/g l.w., Borrell et al., 2007; $\sum_6$DDT = 48.3 ± 53 μg/g l.w., this study, n = 10 – excluding ID97 and ID107 considered as extreme values). The data presented above confirmed the heavier pollutant load of Eastern Mediterranean as compared to Western Sahara monk seal subpopulation (Borrell et al., 1997, 2007). Moreover, these data resulted 1–2 orders of magnitude higher as compared to Hawaiian monk seal (*Monachus schauinslandi*) for both DDTs and PCBs (Lopez et al., 2012; Willcox et al., 2004; Ylitalo et al., 2008).

Toxicological implications of OC body levels found in this study can be inferred by comparison with thresholds from which body concentrations have been observed to elicit physiologic effects in various

Table 2
Mean, median and range of HCB, ∑DDT, ∑PCB (ng/g l.w.) and ∑PAH (ng/g d.w.) levels presented by sex and age class of the Mediterranean monk seals (*Monachus monachus*) sampled in Greece between 1995 and 2013. c-PAHs are carcinogenic compounds, nc-PAHs are non-carcinogenic compounds according to IARC.

Compound	Subadults				Adult females				Adult males				Undetermined			
	N.	Mean	Median	Range	N.	Mean	Median	Range	N.	Mean	Median	Range	N.	Mean	Median	Range
∑PCB	31	59,500	19,300	2380-500,000	11	240,000	63,000	11,800-1610,000	5	36,000	27,200	18,400-74,900	2	13,700	13,700	7500-19,800
∑DDT	31	55,900	24,500	2280-541,000	11	171,000	26,800	4010-291,000	5	47,500	42,000	121,600-75,600	2	14,300	14,300	8630-20,000
HCB	31	239	149	<LOD-823	11	324	139	<LOD-1060	5	180	162	<LOD-374	2	158	158	82.5–234
∑c-PAH	36	17.3	11	1.77–62.3	12	25.8	13	3.46–121	5	27.1	34	3.16–50.5	2	7.23	7.23	5.48–8.97
∑nc-PAH	36	707	393	61.9–7830	12	524	338	121–1100	5	367	227	154–1040	2	191	191	168–213
∑PAH	36	724	413	69.1–7890	12	549	366	126–1130	5	394	231	173–1090	2	198	198	174–222

marine mammal species (Kannan et al., 2000), including immunological depression in harbor seals, *Phoca vitulina* (de Swart et al., 1994, 1996), and pathological stenosis in the uteri of ringed seals, *Pusa hispida* (Helle et al., 1976).>70 % of monk seal samples from the Ionian and Aegean Seas exhibited PCB levels above the advised threshold of 17 μg/g l.w. above which adverse toxic effects (including immunosuppression) have been described by a series of toxicological experiments on seals, otters and minks (Kannan et al., 2000).

Moreover, the levels found herein were above the mean PCB (16.5 μg/g l.w.) and DDT (2.4 μg/g l.w.) blubber levels detected in harbor seals with impaired cellular immune response after a period of two-and-a-half years of feeding with contaminated herrings from the Baltic Sea (de Swart et al., 1996). These results suggest immunological effects due to OCs for the Eastern Mediterranean monk seal subpopulation. Regarding potential effects on reproduction, Helle et al. (1976) reported pathological changes in the uterus for females ringed seals with mean values of PCBs higher than 89 ± 11 μg/g l.w. and DDTs >100 ± 15 μg/g l.w. Three out of 11 adult females investigated herein exceeded these thresholds, including ID97 and ID107 previously highlighted as extremely contaminated individuals, which showed values 5 to 20 times greater than proposed thresholds (∑PCB = 1610 μg/g l.w. and 481 μg/g l.w.; ∑DDT = 568 μg/g l.w. and 893 μg/g l.w., respectively). Thus, it seems reasonable to suppose that in these animals, organochlorines may have exerted their toxicological activity and may have had a role in the alteration of the immunity and the reproductive rate (Mauritsson et al., 2022).

As for other OCs, HCB levels found in the current study (0.134 μg/g l.w., $n = 10$ – excluding ID97 and ID107 considered as extreme values) are in accordance with previous data published in the Mediterranean Sea by Borrell et al. (2007) (0.242 ± 0.217 μg/g l.w.) and exceed by far values from West Sahara monk seal subpopulation and Hawaiian monk seal (Borrell et al., 2007; Lopez et al., 2012).

PAH comparisons are limited by the paucity of data on the contamination status of this pinniped. Indeed, this is the first assessment of PAH in *Monachus* spp. Our results are in line with values reported by Marsili et al. (1997) for southern sea lions (*Otaria flavescens*) males sampled in the colony of Punta Bermeja (Argentina) (578 ± 263 ng/g d.w., $n = 3$), considered as a "control area" with respect to the Mar de Plata colony for which higher levels were noted (2790 ± 1680 ng/g d.w., n = 3). Conversely, Stimmelmayr et al. (2018) found much lower levels (<50 ng/g, wet weight, $n = 4$) in oiled ringed (*Phoca hispida*) and spotted seals (*Phoca largha*) from the Bering Strait region, Alaska. Authors hinted that a rapid metabolization of these substances has occurred (Stimmelmayr et al., 2018). Based on in-vitro studies, benzo[*a*]pyrene has been suggested to alter T cell function in harbor seals (*Phoca vitulina*) and impaired cell-mediated immunity against viral pathogens (Neale et al., 2002). PAH concentrations have been converted to B[*a*]P equivalent toxicity using the TEF approach (Nisbet and LaGoy, 1992) to provide a more accurate risk assessment from environmental exposure to PAH. However, to the best of authors knowledge, no toxicological threshold neither for PAH levels nor for B[*a*]P TEQ has been proposed so far for marine mammals. Therefore, it is difficult to determine whether the levels found can be considered toxic for this subpopulation. In general, PAH fingerprints were dominated by LMW congeners which represent the most water-soluble and bioavailable fraction for the uptake by marine biota (Marsili et al., 2001).

Biological factors such as age and sex, are known to play an important role in the pollutant levels of phocids (Lehnert et al., 2018), and, in general, of marine mammals (Aguilar et al., 1999; Reijnders et al., 2009, 2018). Reproductive discharge, resulting in lower levels of lipophilic pollutants in adult females as compared to males of the same age has been described for Hawaiian monk seals (Lopez et al., 2012; Willcox et al., 2004; Ylitalo et al., 2008). Nevertheless, in our study, no statistically significant differences in OC or PAH levels between age classes or gender were found, in line with previous observation on Mediterranean monk seal subpopulation by Borrell and colleagues (Borrell et al., 1997,

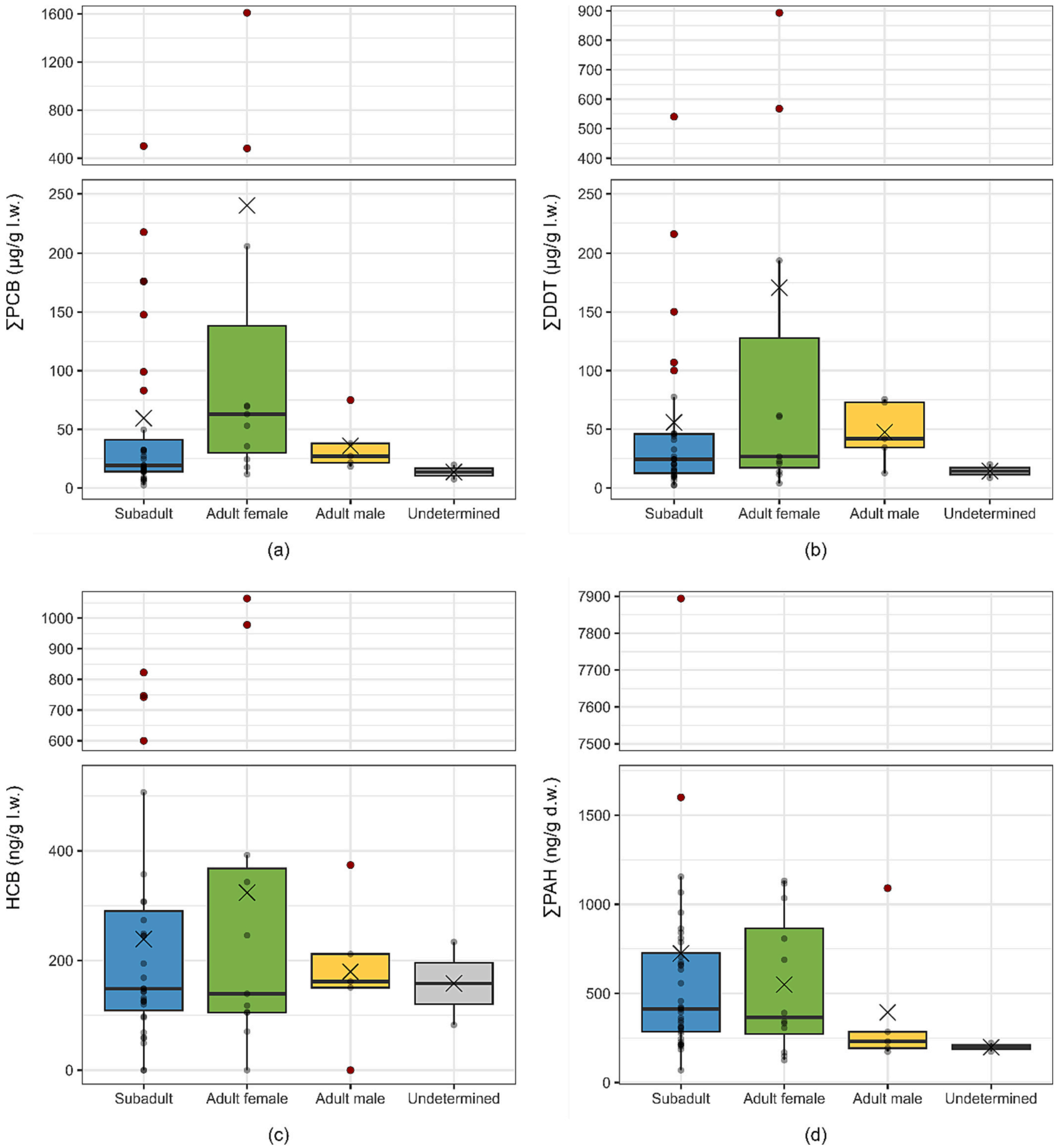

Fig. 2. Box and whisker plots of ∑PCB (a) and ∑DDT (b) (µg/g l.w.), HCB (ng/g l.w.) (c) (n = 49) and ∑PAH (ng/g d.w.) (d) (n = 55) concentrations by sex and age class of the monk seals (*Monachus monachus*) sampled in Greece between 1995 and 2013. The dark horizontal line indicates the median, × symbol indicates the mean and outliers are highlighted by red circles.

2007). This may be inconsistent with the fact that phocid seals rapidly transfer high fat milk from mother to pup during lactation, losing up to 40 % of their initial body mass, by mobilizing blubber and muscle and all substances associated with these biological tissues (Atkinson, 1997); nonetheless this result can be explained by several other factors. Specifically, their continuous foraging behavior while breastfeeding can reduce detoxification of females (Gazo and Aguilar, 2005). In addition, a low reproductive rate has been observed for *Monachus monachus*, both in Eastern Mediterranean (Gucu et al., 2004) and Cabo Blanco (Gazo et al., 1999) colonies, and this would result in a reduction in the intensity of any potential mother-pup transfer (Hassrick et al., 2014; Ross et al., 2000; Tanabe et al., 1987). This situation may be worsened by the loss of genetic diversity and subsequent inbreeding depression, and the consequent reduced fertility (Karamanlidis et al., 2021).

Pollutant profile analysis revealed that subadult seals exhibited higher concentrations of lower chlorinated PCBs as compared to higher

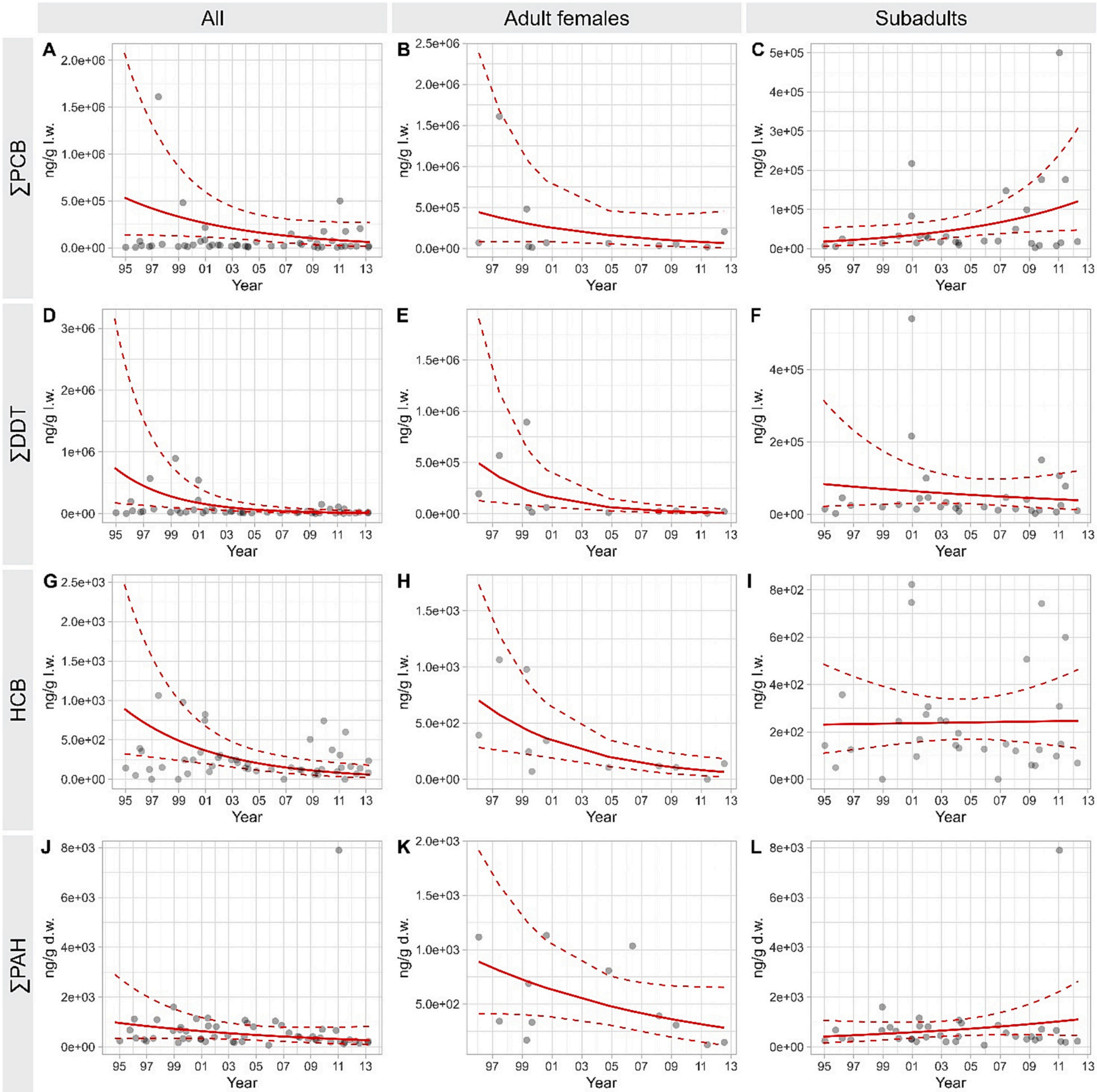

Fig. 3. Temporal trends of the levels of ∑PCB (A, B, C), ∑DDT (D, E, F), HCB (G, H, I) in ng/g l.w. and ∑PAHs (J, K, L) in ng/g d.w., found in Mediterranean monk seals (*Monachus monachus*) sampled in Greece between 1995 and 2013. Generalized linear model predictions (red solid line) with 95 % confidence intervals (red dashed lines) are provided for all specimens (left panels; N = 49 for OCs and N = 55 for PAHs), as well as for adult female (medium panels; N = 49 for OCs and N = 55 for PAHs) and subadult (right panels; *N* = 49 for OCs and *N* = 55 for PAHs) groups.

chlorinated ones. The reason may lie in the selective transfer of congeners during pregnancy and lactation, which has been previously described as an inverse correlation between log K_{ow} and mother-fetus ratio of these contaminants' levels (Brown et al., 2016; Greig et al., 2007; Zhang et al., 2021). Regarding PAHs, subadult specimens showed higher percentages of LMW compounds than adult females, but comparable to those of adult males. This can be explained by the rapid assimilation, metabolism, and excretion of PAHs, leading to lower bioaccumulation compared to PCBs (Lourenço et al., 2021). Nevertheless, adult seals exhibited higher levels of carcinogenic PAH fraction as compared to subadults, suggesting different metabolic capacity linked to age. Similar findings were reported in Indo-Pacific humpback dolphins (*Sousa chinensis*) by Zhang et al. (2021), where fetal tissues had higher PAH concentrations than maternal tissues, and the fetal-to-mother ratio decreased with increasing PAH molecular weight. These results provide evidence for the concept of PAH partition through the placental barrier, and might be associated with an underdeveloped biotransformation system in fetal bodies, as proposed by the authors (Zhang et al., 2021).

This study revealed minor geographical differences in the contamination levels found in the blubber of Mediterranean monk seals, mostly because of the large difference in sample sizes between the two groups. In a purely descriptive analysis, samples from the Ionian islands demonstrated overall higher mean values with respect to Aegean Sea regarding PAHs but showed the lowest values regarding OCs. This may be linked with the fact that eastern Ionian Sea is a well-known region of oil and gas shale reservoirs, and that it has already been the scene of oil and natural gas exploration and extraction campaigns (Anastasopoulou et al., 2022). The Aegean Sea exhibited relatively higher levels of

Table 3
Mean and range of *p,p*'-DDE/*p,p*'-DDT, *p,p*'-DDE/ΣDDT, Σ*o,p*'-DDT/ΣDDT, *o,p*'-DDT/*p,p*'-DDT, ΣDDT/ΣPCB ratios in Mediterranean monk seals from Greece ($n = 49$) analyzed in this study, grouped by location of stranding and age-sex classes.

		p,p'-DDE/*p,p*'-DDT	*p,p*'-DDE/ΣDDT	Σ*o,p*'-DDT/ΣDDT	*o,p*'-DDT/*p,p*'-DDT	ΣDDT/ΣPCB
	N.	Median (range)	Median (range)	Median (range)	Median (range)	Median (range)
Aegean Sea						
Adult females	10	27.3 (6.5–91.4)	0.912 (0.683–0.979)	0.0166 (0.00152–0.108)	0.0717 (2.53E-07 – 0.895)	0.574 (0.101–2.79)
Adult males	5	45.8 (30.7–56.8)	0.957 (0.907–0.970)	0.00579 (0.00293–0.0110)	0.0791 (1.12E-07 – 0.144)	1.55 (0.687–1.99)
Subadults	27	26.3 (14.6–89.2)	0.923 (0.830–0.964)	0.0103 (0.00177–0.0520)	0.0681 (5.45E-09 – 0.601)	1.06 (0.214–3.12)
Undetermined	2	37.6 (26.4–48.9)	0.938 (0.917–0.959)	0.0104 (0.0104–0.0105)	0.200 (0.153–0.247)	1.08 (1.01–1.15)
All	44	26.9 (6.5–91.4)	0.923 (0.683–0.979)	0.0103 (0.00152–0.108)	0.07364 (5.45E-09 – 0.895)	1.04 (0.101–3.12)
Ionian Sea						
Adult females	1	26.0	0.920	0.0253	4.59E-08	0.879
Subadults	4	30.2 (25.0–51.2)	0.930 (0.917–0.966)	0.00962 (0.00541–0.0179)	0.106 (0.0764–0.143)	0.969 (0.821–1.05)
All	5	28.8 (25.0–51.2)	0.924 (0.917–0.966)	0.0106 (0.00541–0.0253)	0.0807 (4.59E-08 – 0.143)	0.968 (0.821–1.05)
All	49	27.5 (6.5–91.4)	0.923 (0.683–0.979)	0.0104 (0.00152–0.108)	0.0764 (5.45E-09 – 0.895)	0.998 (0.101–3.12)

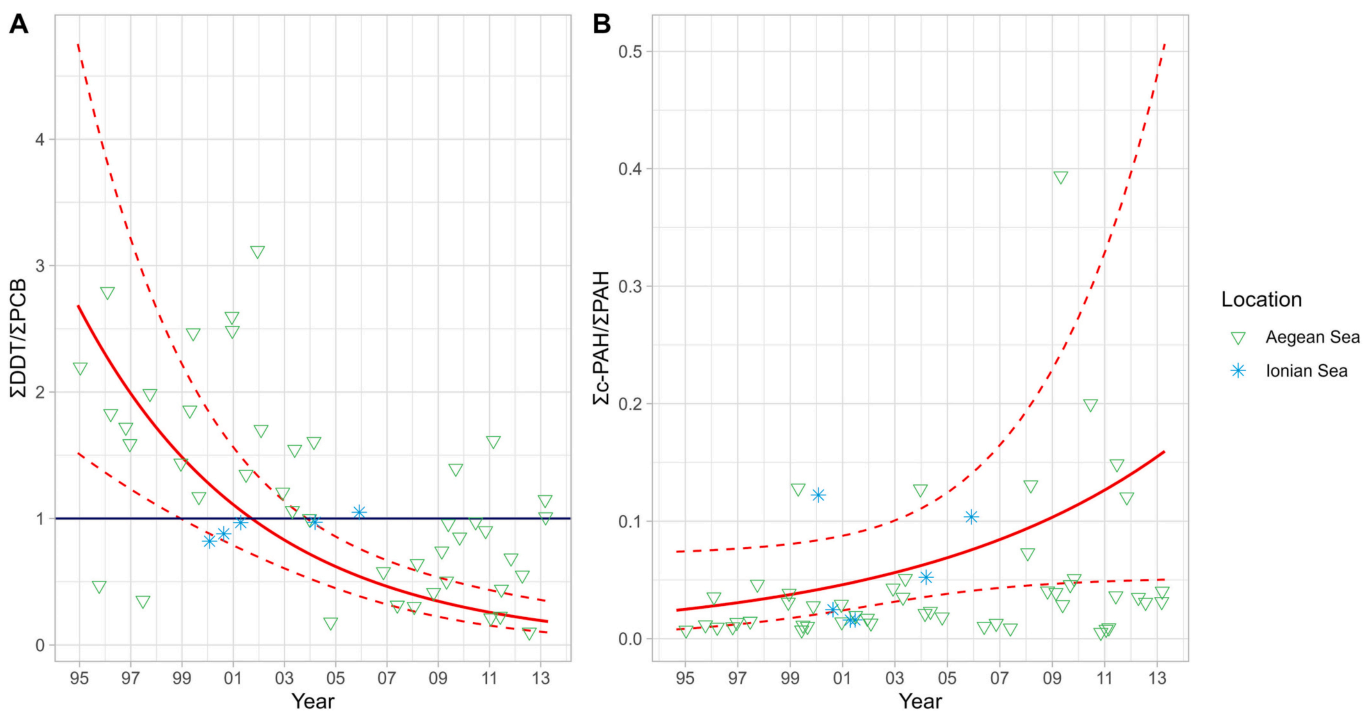

Fig. 4. Temporal trends of the ΣDDT/ΣPCB (A) and Σc-PAH/ΣPAH (B) ratios, calculated for Mediterranean monk seals (*Monachus monachus*) sampled in Greece between 1995 and 2013. Generalized linear model predictions (red solid line) with 95 % confidence intervals (red dashed lines) are provided for all specimens. Values obtained from the analysis of samples from the Ionian Sea are shown with a blue asterisk; values for samples from Aegean individuals are shown with green triangles. The blue line on the left panel (A) indicates a ratio of 1.

contamination by OCs, which may be the result of direct discharges from coastal urban activities, main rivers outflow and intense vessel traffic (Berrojalbiz et al., 2011; Hatzianestis et al., 2020; Lammel et al., 2015). The results presented are particularly important in the light of the fact that the coastal and marine environment of Greece is the most important habitat for the Mediterranean monk seal worldwide and hosts several important areas for its conservation, such as the National Marine Park of Alonissos, Northern Sporades, established in 1992 (Karamanlidis et al., 2010), the marine protected areas at Northern Karpathos - Saria and the Astakida islets and the Natura 2000 site of the Island of Gyaros and its surrounding 3-mile marine area (Karamanlidis et al., 2016). Despite much effort has been put in the conservation of the species, pollution threat may have been underestimated and may need greater attention in the future.

Over time, different trends have been highlighted for the investigated contaminant groups. OC exposure levels in *M. monachus* from the Eastern Mediterranean Sea subpopulation appeared to have declined since the early nineties, as previously reported for other Mediterranean marine mammals (Aguilar and Borrell, 2005; Dron et al., 2022). In particular, DDTs and HCB demonstrated significant downward trends over time, while PCBs showed a less pronounced tendency. Consequently, the ∑DDT/∑PCB ratio decreased by half in in 2007–13 as

Table 4
PAH TEQs (ng /g d.w.) in blubber samples of Mediterranean monk seals sampled in Greece between 1995 and 2013. Number of samples, arithmetic mean, median and range are reported. c-PAHs are carcinogenic compounds, nc-PAHs are non-carcinogenic compounds according to IARC.

		B[*a*]P-TEQ (ng/g d.w) [a]			
Compound	N.	Mean	Median	Range	% total TEQ
∑c-PAH	55	11.0	4.77	0.888–61.2	90.0
∑nc-PAH	55	0.64	0.36	0.0878–7.88	10.0
∑PAH	**55**	**11.6**	**5.05**	**1.26–61.5**	–

[a] TEF values from Nisbet and LaGoy (1992).

compared to 1995–2006, demonstrating a shift in the source pollution from primarily agricultural to primarily industrial. When adult males and females were excluded from the study to limit the influence of age-related variability, (Williams et al., 2023), subadult specimens did not show clear downward trends, and indeed exhibit rising PCB blubber levels in recent years. Given the small samples size over such a wide time span it is difficult to accurately interpret these results. The overall descending trends may be a result of the globally imposed restrictions, but they are undoubtedly influenced by the two outlier adult females (ID97 and ID107) sampled in 1997 and 1999. The increasing tendency of PCB in subadult specimens may be explained by the remobilization of PCB from sediment and soil, or the improper disposal of old stockpiles, rather than stemming from recent usage. This persistence of PCBs in Mediterranean marine fauna, as highlighted in recent literature (Bartalini et al., 2020; Dron et al., 2022; Marsili et al., 2018), underscores the limitations of mitigation strategies and the limited success of global initiatives to address PCB contamination thus far (Jepson et al., 2016; Jepson and Law, 2016; Law and Jepson, 2017; Stuart-Smith and Jepson, 2017). Regarding PAHs, no trend was found.

A comparable ∑DDT/∑PCB ratio value (mean = 1.77) to the one found in this study was reported by Borrell et al. (2007) for seals analyzed in the same area between 1995 and 1999. Similarly *p,p'*-DDE/*p,p'*-DDT of 0.93 reported for those seals (Borrell et al., 2007) is almost equal to the mean value of the ratio found here, suggesting no recent DDT inputs in Greece (Aguilar, 1984). Additionally, high *p,p'*-DDE/∑DDT, confirmed high metabolic capacity of this species toward these xenobiotics (Borrell and Aguilar, 1987). Mean Σ*o,p'*-DDT/ΣDDT<0.20 and *o,p'*-DDT/*p,p'*-DDT < 0.25 excluded the possibility of a non-insecticidal (or industrial) (Nowell et al., 1999) or Dicofol-type source of DDT in the environment (Qiu et al., 2005). Ratio between carcinogenic PAH fraction and total PAHs remained almost stable in the first 10 years considered. From 2006 to 08 a significant increment of c-PAHs/∑PAH occurred, possibly connected with a shift to pyrogenic sources of contamination, linked to industrial activities, which mainly release high molecular PAHs such as benzo[*a*]pyrene (Wang et al., 1999). The relative increase of carcinogenic fraction in recent times cause concerns for Mediterranean monk seals from a toxicological point of view. These high-molecular-weight compounds are known to be activated into carcinogenic metabolites by the cytochrome P450 system, causing mutagenic and genotoxic effects, as well as affecting the immune and endocrine systems (Gauthier et al., 2002; Marsili et al., 2014; Villeneuve et al., 2002). In literature, various studies describe a linkage between PAHs exposure and cancer in marine mammals (Martineau et al., 2002).

The relatively low sample size force to consider the results with caution, but since levels found could be causing harmful effects on seal health, monitoring pollution threat should be considered a priority for the conservation of the species.

5. Conclusions

This study represents the first assessment of PAH exposure in the Eastern Mediterranean subpopulation of the vulnerable Mediterranean monk seal (*Monachus monachus*) and one of the few studies on the contamination status by PCBs, DDTs and HCB of this species in Greece over an extended period of time (1995–2013). The paucity of data regarding some of these persistent organic pollutants in pinnipeds make it difficult to evaluate the relevance of pollution threat accurately. Nevertheless, our data revealed that Mediterranean monk seals exhibit PAH and OC levels that may affect health of this species, inducing alteration of the immunity and its reproductive rate. As previously confirmed in this subpopulation, no significant age and sex-related differences were found in contamination loads. This likely reflects the reduced fecundity rate at which the population is subjected and the resulting low discharge of pollutants from mothers to pups. Blubber levels of HCB and DDTs showed significant downward trends over the 19-year time period. Conversely, PCBs did not show a marked reduction and even rose in subadult specimens. Given the recognized detrimental effects linked to prolonged exposure to persistent organic pollutants, and the reduced long-term expectations of species recovery, monitoring programs and conservation actions are mandatory to better address and mitigate all anthropogenic threats affecting monk seals.

CRediT authorship contribution statement

Francesca Capanni: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Alexandros A. Karamanlidis:** Conceptualization, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **Panagiotis Dendrinos:** Investigation, Project administration, Writing – review & editing. **Annalisa Zaccaroni:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Costanza Formigaro:** Formal analysis, Writing – review & editing. **Antonella D'Agostino:** Formal analysis, Writing – review & editing. **Letizia Marsili:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169854.

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