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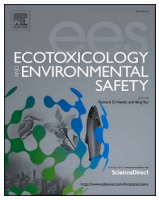

Comparative study of polycyclic aromatic hydrocarbons (PAHs) in salt gland and liver of loggerhead turtle *Caretta caretta* (Linnaeus, Cheloniidae) stranded along the Mediterranean coast, Southern Italy

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ABSTRACT

The levels of polycyclic aromatic hydrocarbons, PAHs, were determined in the liver and salt gland of 19 loggerhead turtles (*Caretta caretta* Linnaeus, Cheloniidae) stranded along the coasts of the south Tyrrhenian Sea, Italy, from 2019 to 2021. The 16 EPA's priority PAHs were determined by gas chromatography coupled with mass spectrometry (GC-MS). The average values of ΣPAHs in liver, 139 ± 55.0, were exceptionally high and up to one hundred times those of literature and appeared even more worrying in salt gland, 320 ± 97.6 ng/g, w.w. Naphthalene was the predominant contributor to PAHs richness and accounted for 90.0% and 93.7% of ΣPAHs in the two matrices, highlighting the petrogenic source of exposure. An overall higher bioaccumulation of NAP, more than two-fold, was detected in salt gland and especially in female and adults. Data of PAHs richness highlighted a potential risk of neoplastic disease development and that anthropogenic activities may seriously impair healthy state conditions of *C. caretta.* populations in south Tyrrhenian Sea.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants that can be detected quite often in marine and coastal environments (Nácher-Mestre et al., 2010). The literature reports their occurrence in marine benthic invertebrates as mussels and clams (Kannan and Perrotta, 2008) as well as in higher trophic level predators, especially under continuous exposure (Kannan and Perrotta, 2008; Camacho et al., 2013; Vilca et al., 2018; Cocci et al., 2018). Main sources have been reported to be industrial discharge and urban runoff, vessel operation, tanker accidents, atmospheric deposition, exploration production and plastic ingestion (Marsili et al., 2001; Kannan and Perrotta, 2008; Arienzo et al., 2017; Athey et al., 2016; Arienzo et al., 2019; Ferrara et al., 2020).

Being very hydrophobic (Varanasi, 1989), they have great potential to affect the biology of many resident and migratory marine species. In the Mediterranean Sea, PAHs hot spots have been individuated in the Port of Milazzo in Sicily (Orecchio and Sammartano, 2006), Port Marghera in Veneto (Manodori et al., 2006) and gulf of Pozzuoli in Campania (Arienzo et al., 2017; Esposito et al., 2020). In the gulf of Pozzuoli, including a former steel making facility, PAHs levels were thirty eleven thousandfold higher than those of other industrial impacted sites (Arienzo et al., 2017). PAHs are highly toxic, mutagenic, carcinogenic, teratogenic, and immunotoxicogenic to various life forms. One of the main PAHs targets in marine environments are sea turtles (Meador et al., 1995). They place at the top of the marine food chain and, despite being migratory, are good indicators of marine contamination (Perugini et al., 2006). The loggerhead turtle *C. caretta* (Linnaeus, Cheloniidae) is the most common sea turtle species in the Mediterranean Sea and it often uses the Italian coasts for nesting and feeding ground (Esposito et al., 2023). At the juvenile life stage, they live in oceanic areas and behave as opportunistic predators feeding of planktonic biota (Esposito et al., 2022). Adults instead feed closer to the coast of benthic organisms (Snape et al., 2020) in the form of crustaceous, crabs, fish (Cammilleri et al., 2017).

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This species is listed as endangered on the Red List of the IUCN (International Union for Conservation of Nature) (IUCN, 2012). The deleterious effect of marine pollutants is included among the top 20 research topics for sea turtle conservation (Hamann et al., 2010). The Mediterranean Sea represent an area where conservation efforts are of paramount relevance due to historic release from former industrial sites, and current commercial release from cargo, tanker, and passenger transportation (Pasanisi et al., 2022). *C. caretta*, diving into deep and cold water and returning in surface for breathing, rest or rewarm are highly exposed to hydrocarbon pollution from both seabed and surface (Pasanisi et al., 2022). However, literature on PAHs accumulation by loggerhead turtles in the Mediterranean Sea is very limited with a substantial lack of data on the mortality effects (Varanasi, 1989; Alam et al., 2020, Athey et al., 2016; Camacho et al., 2013). PAHs may have wide mechanisms of toxicity including modulation in target gene expression and DNA methylation (Cocci et al., 2018). Chronic or high-level exposure to PAHs or crude oil may cause serious health and reproductive problems (Alam and Brim, 2000). Toxicity varies between species and largely depends on route of exposure, sex, and life stage. PAHs generally accumulate into lipidic tissues, and their metabolites can be found in most tissues (Meador et al., 1995). In fish, liver accumulate the highest levels of parent PAHs and metabolites; hence, this is the best tissues to analyse when determining PAH exposure. However, there are contrasting opinions on the fate and risk of exposure to PAHs in liver: for some authors biomagnification risk is insignificant due to intense metabolization (Rossi et al., 2023), whereas for others (Hall, 1980; Varanasi et al., 1989) enzymatic activities, are capable to metabolize PAHs into more toxic compounds. In loggerhead turtles, PAHs bioaccumulation was also evidenced in matrixes suitable for the determination of recent exposure to PAHs as blood plasma (Camacho et al., 2013; Cocci et al., 2018) liver, (Vilca et al., 2018), pectoral muscle, and adipose tissue (Perugini et al., 2006). Cocci et al. (2018) reported detectable levels of PAHs in plasma of juvenile individuals rescued along the Italian coast and significant correlation between gene biomarkers and PAHs congeners, with significative level of DNA methylation. Athey et al. (2016) investigated the PAHs in fluids of the stomach, small intestine, and large intestine of adult loggerhead turtles and found significant PAHs concentration in gastro-intestinal fluids. However, there are other potential tissues and organs that might accumulate PAHs. One of this is the bioaccumulation in salt gland, which is a gland placed near turtle eyes, which allow them to drink sea water and excrete salt in high concentration (Camiñas et al., 2020). This represents an important adaptation system playing important physiological function in turtles as osmotic, electrolyte, mineral, and hydration balance (Innis et al., 2017). In addition to its physiological function, salt gland can accumulate but also excrete inorganic contaminants as outlined by Perrault (2012). Perrault et al. (2019) revealed that concentrations of Cd and Hg in salt gland of leatherback (*Dermochelys coriacea*) increased with curved carapace length. The authors hypothesized that salt gland could be a potential organ for assessing blood inorganic contaminant levels in sea turtles. On the other hand, there are no studies on the accumulation of organic contaminants in this organ. Harms et al. (2019) assayed PAHs exposure in hatchling loggerhead sea turtles following the explosion of the deep-water horizon oil rig in 2010 in the Gulf of Mexico and evidenced that combination of crude oil and dispersant increase organism exposure to oil hydrocarbons and affects salt gland function. Increasing the number of studies on sea turtle PAHs bioaccumulation routes and target organs appears extremely demanding. To fill this gap a comparative study of the concentrations of 16 PAHs in the liver and salt gland of loggerhead turtles stranded on the Southern Tyrrhenian coast was performed. Thus, response and bioaccumulation data of these two organs can help to determine how these two different organs respond to PAHs and represent in the meantime the baseline to be used for regular monitoring.

2. Materials and methods

2.1. Sample collection

As established by the Regional Decree n. 231/2015, the local health authority transfers sea turtle carcasses to the Istituto Zooprofilattico Sperimentale del Mezzogiorno to ascertain the cause of animal death as soon as of the stranding notification.

Carcasses which did not show any evidence of decomposition were subjected to necropsy and biometric parameters assessment as biomass weight BW, straight line carapace length SCL, curved carapace length CCL, straight line width SCW, curved carapace width CCW, head length HL, head width HW. In the meantime, individual sex was determined by examination of the reproductive apparatus (Wyneken, 2001). The growth stage was assayed based on the CCL value, discriminating juveniles, if CCL was $<$ 64 cm, and adult if $>$ of 64 cm (Broderick and Godley, 1996). For each loggerhead turtle it was calculated, as an indicator of the health status, Fulton's Body condition Index (BCI) based on body mass and carapace length (Lamont and Darren, 2021). Nineteen specimens of *C. Caretta,* stranded along the south Tyrrhenian coast, were recovered in the years 2019–2021. Liver and salt gland were sampled and both specimens were kept frozen at − 20 °C until analysis.

2.2. Sample preparation and chemical analysis

The 16 EPA's priority PAHs listed by the U.S. Environmental Protection Agency (EPA) in 1970 (Mumtaz and George, 1995) were investigated: acenaphthene (ACE), acenaphthylene (ACY), anthracene (ANT), benzo(a)anthracene (BaA), benzo(b) fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(ghi)perylene (BgP), benzo(a)pyrene (BaP), chrysene (CHR), dibenz(ah)anthracene (DhA), fluoranthene (FLT), fluorine (FLR), indeno(1,2,3-cd)pyrene (IPY), naphthalene (NAP), phenanthrene (PHE), pyrene (PYR). 40–60 g of liver and salt gland were homogenized and dried in an oven at 35 °C. A sample of 5 g was weighed and placed in an automatic extractor, under reflux, at 100 °C for 2 h with a 2 M KOH solution in methanol. After extracting with 40 mL of cyclohexane and 20 mL of a mixture methanol/water (4:1), the organic phase was extracted with 25 mL of cyclohexane for three times. The extract was purified on a column of sodium sulphate and on a column of activate silica, dried in a rotary evaporator, and redissolved with 1 mL of mixture hexane/acetone (1:1 v/v). The extract was analysed by a gas chromatograph coupled to a mass spectrometer (GC-MS).

10 μL of the internal standard were added to extracts (mixture of Naphthalene D8, Acenaphthene D10, Phenanthrene D10, Chrysene D12, Perylene D12 at the concentration of 10 mg/L) and injected to a gas chromatograph (Shimadzu 2010 Plus, Japan) coupled with a mass spectrometer (MS-TQ8030- Shimadzu, Japan). The instrumental conditions adopted are: fused silica HP5-MS capillary column (30 m × 0.25 mm i.d.) with film thickness of 0.25 μm (Agilent Technologies, US). The separation was conducted with oven temperature programmed as follows: initial setting at 80 °C (2 min hold) ramped to 180 °C at 20 °C min− 1 and finally to 300 °C at 5 °C min (9 min hold). The injector was held at 280 °C. (Prigioniero et al., 2022). The LOD and LOQ were calculated by method of prediction to 95% of linear regressions, for each investigated PAH. The calculated average values in the matrix of liver and salt gland, were 0.09 ng/g and 0.3 ng/g respectively.

The precision and accuracy were ensured by standard reference materials (SRM 1974 C-NIST) and the recovery percentage was estimated by analysing in triplicate the SRM and the range was 70–110%. Samples were analysed in triplicate for each studied organ and basic statistical functions, mean, median, standard deviation, correlation coefficients, were determined by STATISTICA 5 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

Table 1 reports the main biometric features of loggerhead turtles, BW, CCL, SCL, SCW, CCW, HW. The mean CCL value was of 65.0 cm with a range of variation of 43.0–80.2 cm. Individual class discrimination allowed to report no turtles as of small size, 20–40 cm, 7 individuals as medium, 40–60 cm, and 12 individuals as large, > 60 cm (Esposito et al., 2022). Only 7 individuals can be considered as sexually mature based on the reference minimum CCL threshold of 70 cm for Mediterranean turtles (Piovano et al., 2011). Based on the CCL threshold of 64 cm proposed by Broderick and Godley (1996), 13 specimens were adults, and 6 juveniles. Sex-based discrimination showed that 63.1% of the individuals were females and 36.9% males. Table 1 also displays the BCI values whose mean was 1.23 ± 0.20, with peaks of 1.80 for adult and female. No significant differences were recorded between life stages, 1.12 in juvenile vs. 1.28 in adult, and sexes, 1.23 in female vs. 1.23 in male. Compared with the literature (Lamont and Darren, 2021; Esposito et al., 2022) these BCI values reveal that the specimens do not manifest problematic health status.

The concentrations of PAHs, expressed in ng/g of wet weigh, w.w., were reported in Table 2. They were not normalized for the lipid content as this approach is not usual as it is done for other organic pollutants (PCB, Dioxins and Dioxins like). The data show how compounds as PYR, CHR, IPY and DhA were not detected in any of the samples whereas BaA, BbF, BkP and BaP were identified only in liver. The rate of samples with levels above the LOQ were 28.8% and 18.6% in liver and salt gland, respectively, whereas there is one compound, i.e., NAP, that was detected at levels above the LOQ in eighteen samples over nineteen, 94.7% of the specimens. Based on mean values, PAHs scaled in liver as follows: NAP>ACE>BgP>BaP>PHE>FLR>ANT>ACY>BkF>BaA>FLT and in salt gland as: NAP>ACE>ACY>ANT>PHE>FLR>BgP>FLT. Relating the individual PAHs concentrations to the ∑PAHs, NAP represented the most abundant compound with presences of 90.0% and 93.7% in liver and salt gland, respectively. Other compounds contributed to PAHs summation at lower extent, as ACE, 2.85%, PHE, 2.06% and BgP, 1.71% in liver, and ACE, 3.0% and ACY, 1.49%, in salt gland. NAP mean levels, 125 ng/g, (range 19.6–226 ng/g) appeared exceptionally high and up to 10–20 times those, 1.08 and 12.21 ng/g, found in liver of juvenile green sea turtles, *Chelonia mydas,* from Brazil, (Vilca et al., 2018). In the same species but in a different matrix as plasma, Camacho et al. (2013), reported higher NAP means of 1.94 and 0.86 ng/mL in specimens from the Canary Island and Cape Verde, respectively. In another study on PAHs presence in *Chelonia mydas* and hawksbill sea turtles, *Eretmochelys imbricata*, Camacho et al. (2014) showed that the PAHs profile was dominated by anthracene>phenanthrene>naphthalene with mean levels of 4.97, 2.81 and 1.7 ng/mL in green turtles and of 0.84, 1.28 and 0.53 ng/mL in the hawksbill individuals.

On the other hand, our high NAP levels seem to be of the same order of magnitude than those reported for eggs of loggerhead sea turtles, 60–328 ng/g (Alam and Brim, 2000). In liver the maximum peak of PAHs exceeded the mean level up to 2-fold for NAP, 226 vs 125 ng/g and 20-fold for FLT, 4.26 vs 0.22 ng/g. The maximum peak of NAP in liver, 226 ng/g, and salt gland, 955 ng/g, were quite outstanding being two to three times higher than their relative mean levels. Even the maximum peak of ACY was on the overall more than 10-fold higher than the means in liver, 0.281 ng/g and salt gland 66.2 ng/g, highlighting a potential health risk for this compound. Thus, it appears quite evident that the hepatic and salt gland PAHs profile is markedly dominated by the abundancies of di-cyclic compounds suggesting the petrogenic origin rather than urban sources of PAHs. The examination of the comparative PAHs accumulation in liver and salt gland revealed, except for BgP and PHE, higher richness of the hydrocarbons in salt gland respect to liver, with ~2-to-20-fold higher accumulation rates for NAP, 300 vs. 125 ng/g, ACY, 4.79 vs. 0.281 ng/g, ACE, 9.69 vs. 3.90 ng/g, and ANT, 1.85 vs. 0.41 ng/g. The exception to this trend was evidenced for PHE and BgP that showed higher richness, and up to three-fold, in liver than in salt gland. It was also noteworthy that in both organs the ∑PAHs practically coincided with that of the PD PAHs with a slight deviation of the formers from the latter, ~5.5%. The mean ∑PAHs in liver, 139 ng/g, was from 13 to more than one hundred times higher than ∑PAHs range of 1.48–17.35 ng/g reported for green sea turtle by Vilca et al. (2018). Even more exceptional appeared mean salt gland ∑PAHs, 320 ng/g, but no literature comparison is allowed due to lack of reference data. The Wilcoxon test, Fig. 1, confirmed the existence of significant differences in ∑PAHs richness between liver and salt gland when all individuals were taken in consideration, Fig. 1a, and with a similar trend unlike sex, Fig. 1b, and life stage, Fig. 1c. It was interesting to note how female seem to accumulate more significant levels of PAHs in salt gland respect to liver with differences becoming larger for adults, due likely to longer exposure time. No significant differences seem to emerge by the comparison of PAHs richness of liver. These data seem to highlight that PAHs levels in *C. caretta* can pose serious toxic risk since they are extraordinary higher, up to 20 times, than the mean ∑PAHs values, 7.35 ng/g, reported in *Chelonia mydas* by Vilca et al. (2018) causing development of fibropapillomatosis neoplastic disease. The same study also reports how individuals with no evidence of neoplasy display mean load of ΣPAHs of 1.48 ng/g. Moreover, our data fall inside the acute toxic range for aquatic organisms of 0.2–10 μg/g and are in large excess of the deleterious sublethal range of 0.005–0.01 μg/g (Neff, 1985).

What it appears evident from the comparison of our data with those of the literature is that, beyond the data scarcity on PAHs presence in

Table 1
Morphometric parameters (BW; SCL; CCL; SCW; CCW; HL; HW) and Fulton's Body Condition Index (BCI) of *C. caretta.* Data (n = 19) were expressed as mean ±standard deviation, SD, median and minimum and maximum value, Min-Max.

	Mean ± SD	Median	Min-Max
BW (kg)	37.1 ± 18.0	34.5	7.60–64.1
CCL (cm)	65.0 ± 10.1	65.7	43.0–80.2
SCL (cm)	60.9 ± 10.6	60.9	39.5–78.4
SCW (cm)	48.4 ± 7.58	48.8	32.0–60.2
CCW (cm)	60.5 ± 9.92	62.5	40.2–75.0
HW (cm)	11.5 ± 1.75	11.5	7.2–14.0
BCI Tot	1.23 ± 0.20	1.2	1.0–1.80
BCI J	1.12 ± 0.15	1.13	1.0–1.36
BCI A	1.28 ± 0.20	1.26	1.1–1.80
BCI F	1.23 ± 0.23	1.15	1.0–1.80
BCI M	1.23 ± 0.13	1.24	1.0–1.39

Table 2
Mean concentrations, mean±standard deviation, SD, and range, minimum and maximum value, Min-Max, of PAHs in liver and salt gland of C. caretta expressed as ng/g w.w.

	Liver		Salt gland	
NAP	125 ± 57.2	19.6–226	300 ± 233	7.0–955
ACY	0.281 ± 0.87	0.00–3.31	4.79 ± 15.1	0.00–66.2
ACE	3.90 ± 3.14	0.00–12.8	9.69 ± 6.96	0.00–26.9
FLR	0.74 ± 1.02	0.00–2.58	0.92 ± 1.51	0.00–4.55
ANT	0.41 ± 0.87	0.00–2.69	1.85 ± 2.21	0.00–6.42
PHE	2.87 ± 6.32	0.00–22.8	1.03 ± 1.77	0.00–6.20
FLT	0.22 ± 0.98	0.00–4.26	0.27 ± 0.89	0.00–3.70
PYR	0	0	0	
BaA	0.271 ± 1.19	0.00–5.18	0	
CHR	0	0	0	
BbF	1.49 ± 6.48	0.00–28.2	0	
BkF	0.273 ± 1.20	0.00–5.22	0	
BaP	1.09 ± 4.74	0.00–20.7	0	
IPY	0	0	0	
DhA	0	0	0	
BgP	2.38 ± 5.95	0.00–19.7	1.10 ± 3.26	0–13.27
∑PAHs	139 ± 55.10	23.3–250	320 ± 233	5.78–976
*PD PAHs	131 ± 55.76	0.00–249	303 ± 234	1.76–969

* PD PAHs: priority dangerous PAHs: NAP+ANT+BbF+BkF+BaP+BgP as outlined by the of the law nr. 219/2010 (Esposito et al., 2017).

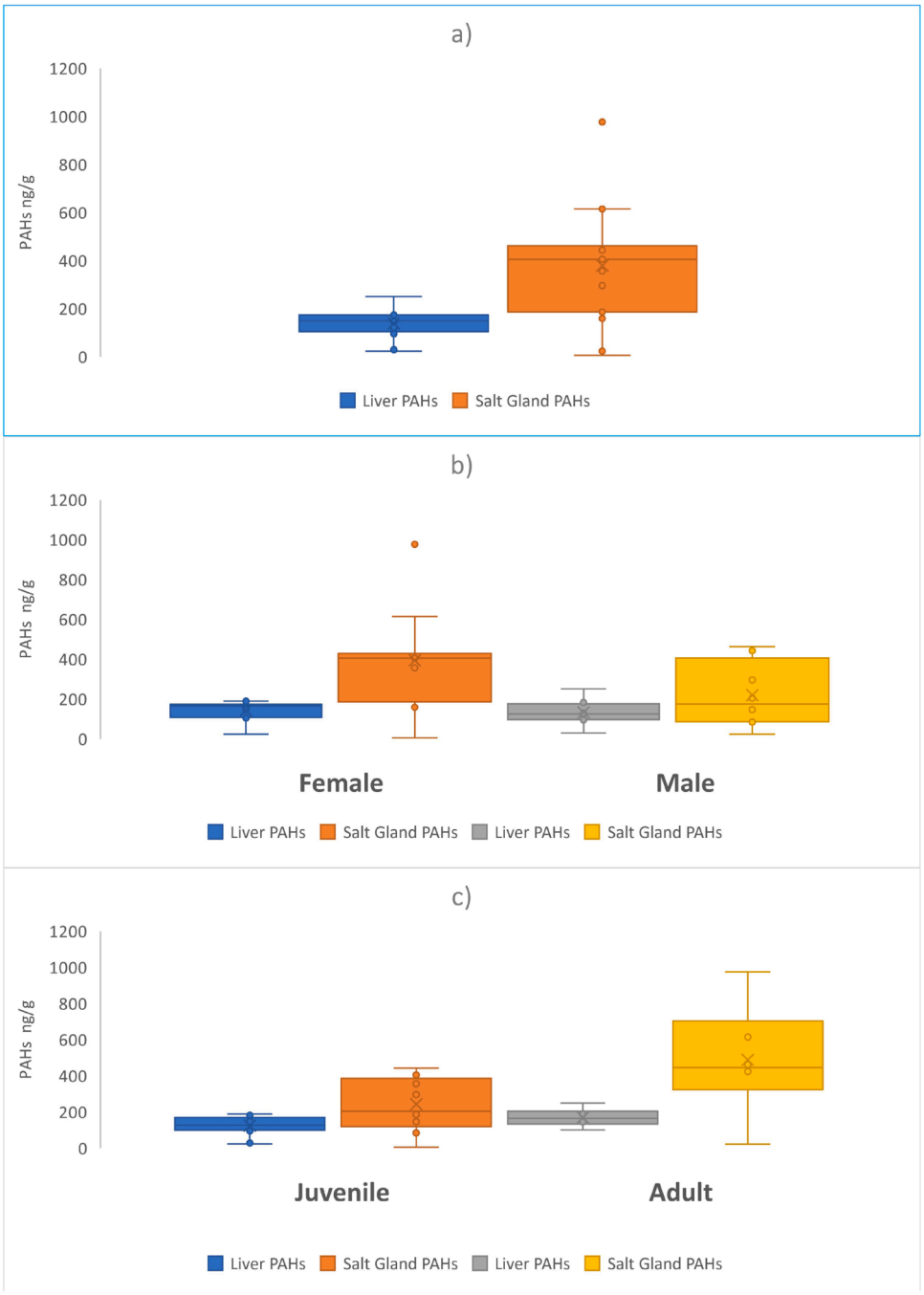

Fig. 1. Boxplot of the PAHs (ng/g w.w) relative to liver, and salt gland: a) all turtles; b) sex, female and male; c) life stage, juvenile and adult. First, third quartile, median, and whiskers (minimum and maximum) and weak (∘) outliers are drawn.

turtles, there is a large variation of data in terms of frequency, concentration and compositions of PAHs. Many factors can drive this phenomenon as their migratory nature, age, sex, trophic level, dietary differences, and reproductive state (Perugini et al., 2006). Orós et al. (2009) considered sea turtles as individuals that diving through areas with different levels of pollution have different bioaccumulation and exposure levels. Data also highlight that the studied individuals have encountered foraging areas in the south Tyrrhenian Sea affected by high pollution by PAHs and more than any other population reported in the literature.

No significant correlation was found between CCL and ∑PAHs in liver as well in salt gland, $r = 0.18$ and 0.27, $p < 0.05$, highlighting that PAHs accumulation is not driven by life stage and that the levels of PAHs in the two matrixes appeared positively interrelated, $r = 0.45$, $p < 0.05$. A negative correlation exists between BCI and the ∑PAHs in liver and salt gland, $r = -0.36$ and -0.14 $p < 0.05$, which reveals a worsening of the organism's health condition with the richness of PAHs, especially in the liver. The negative correlation was even more evident when ∑PAHs was replaced by NAP, $r = -0.44$ for liver and -0.20 for salt gland, $p < 0.05$, suggesting the strong and the dominant role of NAP among all the sixteen priority hydrocarbons on driving health condition.

4. Conclusions

The study evidenced how *C. caretta* is significantly exposed to the presence of PAHs in south Tyrrhenian Sea. Turtles, in fact, accumulated

in liver and especially in salt gland, a matrix so far known as target of inorganic contaminants, exceptional levels of hydrocarbons and among the other of NAP. The study also highlighted how sea turtles have been exposed to levels of contaminants of several order of magnitude higher that those causing fibropapillomatosis, and hence they are exposed to significant toxicological and conservation risk from PAHs. Detected PAHs levels were of several order of magnitude higher than those of other geographic areas unlike polychlorinated biphenyls, whose levels were recently been reported to be much lower than those elsewhere determined (Esposito et al., 2022). To this end, further studies are currently being conducted to assay the role of PAHs on an extended number of tissues and organs, as target of bioaccumulation, the development of histopathological alterations, the potential role of salt gland an effective PAHs pollution indicator and the role of NAP as cofactor of neoplastic diseases. Because of the rapid metabolism of PAHs in many sea organisms, further studies will examine PAHs association with health effects and hydroxylated metabolites levels in liver and salt gland. Data of our study and especially those related to salt gland represent an added value to the literature to understand the impact of environmental change and anthropogenic activities on sea turtles' population and take protective action and manage habitats of the Tyrrhenian Sea.

Ethical approval

No approval of research ethics committees was required to accomplish the goals of this study because experimental work falls within the fields of activity of the Department of Chemical Science and Istituto Zooprofilattico Sperimentale del Mezzogiorno.

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CRediT authorship contribution statement

Michele Arienzo: Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Maria Toscanesi:** Conceptualization, Supervision. **Mauro Esposito:** Conceptualization, Supervision. **Fabio Di Nocera:** Conceptualization. **Silvia Canzanella:** Formal analysis, Investigation. **Luciano Ferrara:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Marco Trifuoggi:** Conceptualization, Formal analysis, Investigation, Data curation, Supervision.

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.

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