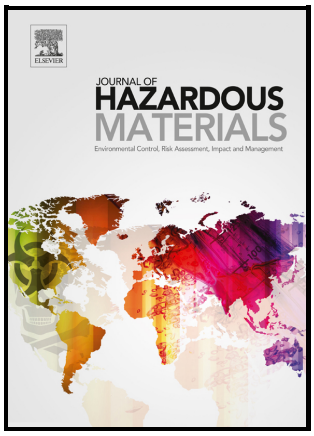

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Discriminative influence of persistent organic pollutants on nesting green sea turtles through genotoxicity, oxidative stress and reproductive related markers

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Abstract

Persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and organochloride pesticides (OCPs) affect biodiversity by bioaccumulating through food webs, impacting marine organisms like endangered sea turtles. This study represents the first evaluation of these contaminants in sea turtles nesting in São Tomé and Príncipe. The main goal was to evaluate PCBs, PBDEs and OCPs levels in sea turtles' blood and investigate their potential effects on erythrocytes' nuclear abnormalities (ENAs) and oxidative stress and reproduction-related gene expression. The relative mean abundance for contaminants was ΣPCBs > ΣOCPs > ΣPBDEs. Contaminants such as PCB-28, PCB-138, PCB-153, PCB-180 and *p,p'*-DDE were associated with ENAs, suggesting potential genetic instability and cellular disruption. PCB-126, PBDE-100, and *o,p'*-DDD correlated with antioxidant and detoxification genes (*glrx3*, *gst*, *txnip*, *txnrd2*, and *gclc*), suggesting oxidative stress responses. The reproduction-related gene est17 was correlated with α and β-HCH, potentially affecting ovary development. Correlations between *ace2* and various PCBs, PBDEs, and *o,p'*-DDD suggest disruptions in follicular development and egg transport. Embryo development genes (*hoxA1* and *tuba1*) were associated with PBDE-154, PBDE-100 and *o,p'*-DDD, suggesting possible embryonic alterations. These findings highlight the impacts of POPs on nesting female green turtles in São Tomé, threatening this endangered population.

Keywords: PCBs, PBDEs, OCPs, TEQ values, oxidative stress, reproduction

Environmental Implications

Persistent organic pollutants (POPs) are hazardous contaminants that can disrupt key physiological functions in wildlife. This study shows that different groups of POPs bioaccumulate in nesting green sea turtles from São Tomé and can be linked to genotoxicity and altered gene expression related to antioxidant, detoxification, reproduction, and embryo development processes. These changes may disrupt critical metabolic and reproductive pathways, representing an additional risk to this endangered population. The findings underscore the distinct impacts of various POPs chemicals and highlight the urgent necessity for targeted monitoring of POPs in the Gulf of Guinea to ensure the protection of biodiversity in the area.

1. Introduction

Human activities exert significant pressure on the environment, resulting in the release of various contaminants, including persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs) which pose serious threats to global biodiversity (Lionetto et al., 2021; Muñoz et al., 2021). PCBs had an extensive use in a range of applications, most notably as dielectric fluids in electric transformers and capacitors. These versatile compounds also serve as plasticizers and have been integrated into various industrial products (Erickson & Kaley, 2011; Vagi et al., 2021). On the other hand, PBDEs used as flame retardants, find application in diverse polymeric materials like furniture foam, rigid plastics, and textiles. Flame retardants play a crucial role by being added to flammable materials to reduce the risk of fire and ensure adherence to fire safety regulations (Yogui & Sericano, 2009). As for OCPs, they have been applied to protect plants from pests, weeds, and diseases, with their primary usage concentrated in agriculture and, to a lesser degree, extended to non-agricultural and urban applications (Jayaraj et al., 2016; Vagi et al., 2021).

POPs have been extensively used and are known for their ability to travel over long distances, leading to their ubiquitous presence in the environment, thanks to their chemical, physical and biological stability that confers them an extended persistence (Muñoz & Vermeiren, 2018). Some research has been made to understand the impacts of the accumulation of these contaminants on marine organisms. Given their lipophilic properties, scientists are exploring molecular tools like the analysis of gene and protein expressions associated with lipid metabolism as potential indicators for this exposure

(Olivares-Rubio & Vega-López, 2016). Moreover, further studies have focused on the oxidative pathways of chemical toxicity and the use of oxidative stress biomarkers in marine organisms, highlighting the involvement of reactive oxygen species (ROS) in response to these environmental contaminants, with POPs having been found to cause damage to chromosomes and DNA (Espinosa-Reyes et al., 2019; Regoli & Giuliani, 2014). For these reasons, genotoxicity biomarkers have been incorporated into biomonitoring programs aimed at assessing this type of chemical exposure (Casini et al., 2018; Rajput et al., 2021).

It has been established that POPs have the potential to overall interfere with endocrine systems, reproduction, initiate genotoxic effects and induce immunosuppression (Bonefeld-Jørgensen et al., 2014; Gaur et al., 2022). For example, it was found that exposure to dichlorodiphenyltrichloroethane (DDT) and its metabolites, had adverse effects on the androgen and corticosteroid balance in dolphins, potentially influencing their overall health and well-being (Galligan et al., 2019). Additionally, the exposure of dolphin cell lines to PBDEs indicated that the extended accumulation of these compounds in dolphin organs/tissues could result in genotoxic effects and ultimately lead to apoptosis (Rajput et al., 2021). Furthermore, the exposure of seals to PCBs resulted in immune suppression, contributing to population-wide consequences (Sonne et al., 2020). While reptiles are phylogenetically distant from mammals, studies on chelonian species, such as freshwater turtles, have shown that even at low concentrations, PCBs can have significant effects in inducing sex reversal in various species, possibly influencing their future behaviour (Bergeron et al., 1994; Ford, 2017). OCPs have demonstrated the ability to modify turtles' sex and trigger developmental abnormalities (De Solla et al., 2011; Willingham & Crews, 1999). Regarding flame retardants, their transfer from mother to offspring was observed, yet this transference did not reveal apparent instances of sex reversal or developmental abnormalities at lower concentrations (Ford, 2017). These findings highlight the broad biological impacts of POPs across taxonomic groups.

Sea turtles tend to accumulate POPs in their tissues over their long-life spans and through their migratory behaviour (Muñoz et al., 2021). POPs have been detected in tissues and organs across all seven species of sea turtles, and their presence has been linked to various health problems, including immune, endocrine, and reproductive disruptions (Muñoz & Vermeiren, 2018). There has been increased interest in understanding the effects of these pollutants on these threatened animals; for example, in loggerhead sea turtles, researchers found a relationship between the presence of

chlordanes in the blood and lower red blood cell counts, hemoglobin, and hematocrit levels, which could suggest potential anaemia (Keller et al., 2004). Additionally, this previous study revealed associations between various organochlorine contaminants and increased white blood cell count, along with potential changes in protein, carbohydrate, and ion regulation, suggesting possible modifications in the immune system. Similar results were observed for the same species and between higher levels of PCBs and a decrease in Packed Cell Volume, indicating a potential link between PCB exposure and anaemia in loggerhead sea turtles (Camacho et al., 2013). Also, changes in the activity of enzymes such as catalase, glutathione reductase and cholinesterase have been linked to OCP presence in hawksbill sea turtles (Salvarani et al., 2018; Tremblay et al., 2017). Furthermore, a correlation between the levels of DDTs and a decline in T-cell proliferation was found in Kemp's ridley sea turtles, suggesting that these contaminants may have the potential to disrupt their immune system (Swarthout et al., 2010). Currently, there is only one study providing evidence of PBDEs' effects on sea turtles (Bianchi et al., 2022). This study, found that exposure to PBDE-47 in whole blood aliquots led to an increase in serum hemolytic complement activity, suggesting potential immune system impairment and increased susceptibility to infections in turtles. Additionally, some authors have suggested a possible association between PBDEs and reproductive failures in seabirds (Zeeman, 2004).

Five out of the seven existing endangered sea turtle species are present in the São Tomé and Príncipe archipelago. Among these, the green sea turtle (*Chelonia mydas*) population stands out for its genetic singularity, contributing significantly to the archipelago genetic pool (Formia et al., 2006; Hancock et al., 2019). This population exhibits notable levels of genetic diversity and distinctiveness (Hancock et al., 2019; Ferreira-Airaud et al., 2024). A recent study indicated potential effects of metal exposure on gene expression markers in nesting females of the green sea turtle population of São Tomé Island (Morão et al., 2022). Still, the effects and the threatening degree of POPs on these females remain unexplored, including the potential consequences for future generations.

Therefore, the main goal of this study was to provide insights into the levels and effects of these substances on green sea turtles, addressing signs of oxidative stress, genotoxicity and reproductive effects at the cell and molecular levels. To accomplish this, the present study is structured around three specific objectives: firstly, to evaluate the concentrations of different POPs, specifically PCBs, PBDEs, and OCPs, in the blood of nesting female green sea turtles; secondly, to establish potential correlations between erythrocytic

nuclear abnormalities (ENA assay) and these contaminants; and finally, to investigate potential associations between the levels of the analysed contaminants and the expression of genes related with oxidative stress, lipid metabolism and transport, reproduction and embryo development, and immune response.

2. Material and Methods

2.1 Sampling collection

Under CITES permission (18ST000001/AC, 18PTLX00159I) and with ethical approval from the "Direcção Geral do Ambiente (DGA)" of São Tomé and Príncipe (STP) and the "Instituto da Conservação da Natureza e das Florestas (ICNF)" of Portugal, female green sea turtles (*Chelonia mydas*) were sampled at Jalé beach (0º03′16.6″N, 6º30′54.5″E), in the southern region of São Tomé Island (Fig.1, 859 km^2), during the nesting season of 2017/2018 (Morão et al., 2022) and subsequently imported to Portugal (Polytechnic University of Leiria).

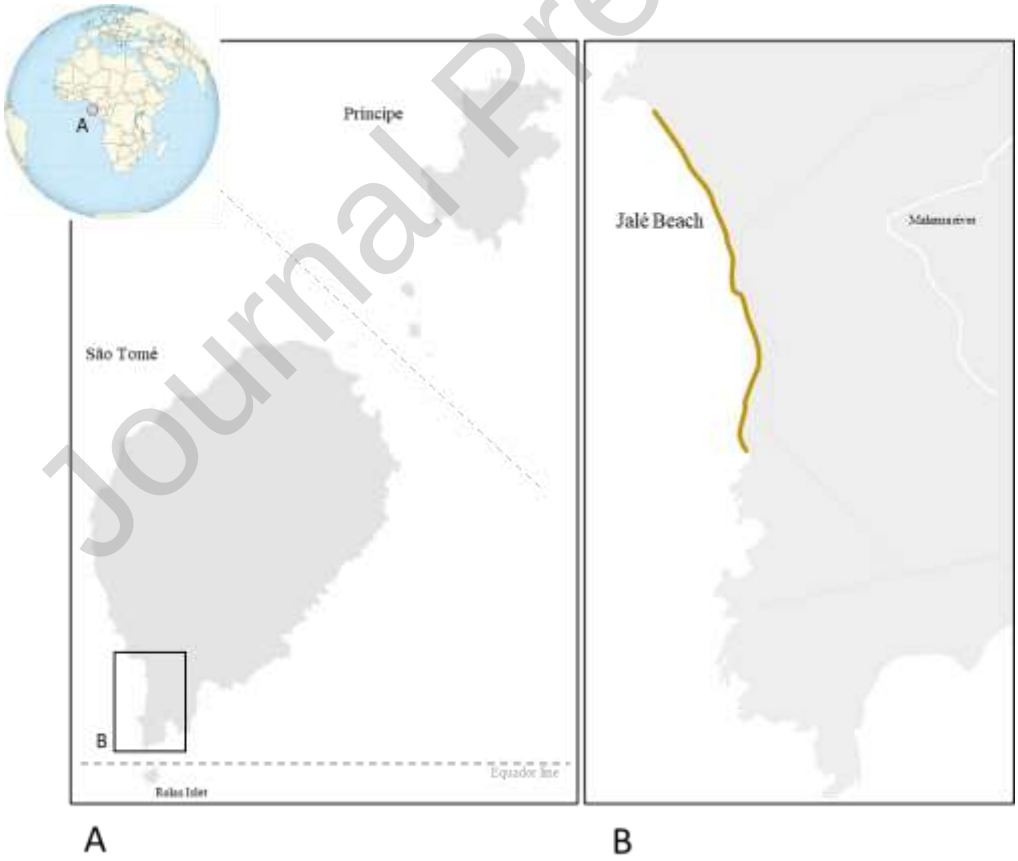

Figure 1 – Geographical localization of Jalé beach (São Tomé and Príncipe archipelago) in relation to Africa, indicating the green sea turtle sampling site.

This sampling site (Fig.1) lies adjacent to the Obô Natural Park and is part of a rich ecological zone that encompasses coastal mangroves, marine habitats, and tropical rainforest (Brito et al., 2017).

Blood samples from twenty-one nesting females were extracted from the dorsal cervical sinus of the green sea turtles, following all the strict ethical guidelines in place and using the methodology described in detail in Morão et al. (2022). To minimize stress and reduce the risk of nest abandonment, sampling was conducted during the nesting trance phase, and blood collection was ceased once the full sample was obtained or egg-laying terminated (Perrault et al., 2011). Additionally, all approaches to the turtles were performed in silence and under red light, following the guidelines of the local NGO Programa Tatô to minimize disturbance to nesting females. The collected blood was immediately transferred into 6 mL tubes containing EDTA for the analysis of persistent organic pollutants. A small portion of blood was transferred to a microtube containing RNA later to prevent RNA degradation, for gene expression analysis by quantitative real-time PCR (qPCR). At the same time as the blood collection, two blood smears per female were prepared on slides, fixed with hair lacquer, and stored for the analysis of erythrocytic nuclear abnormalities (ENA) assay.

2.2 Organic pollutant analysis

2.2.1 Sample processing

Previously freeze-dried whole blood (300 mg) was homogenised and mixed with anhydrous sodium sulphate (Na_2SO_4), spiked with a known amount of ^{13}C-labeled standards as surrogates, and put into a falcon tube with 15 mL of cyclohexane:acetone extraction mixture (3:1). Pollutants' extraction was ultrasound-assisted by means of an ultrasonic water bath (Ultrasons-H, Model 3000838, J.P. Selecta, s.a.) for 15 min at room temperature. After extraction, the mixture was centrifuged (3858 g for 5 min), the supernatant collected, and the pellet placed with 15 fresh mL of the extraction mixture to undergo a second extraction cycle. After three cycles, the total volume of extraction mixture (~45 mL) was reduced using a TurboVap® system (Zymarck Inc., Hopkinton, MA, USA), and exchanged to 5 mL of a cyclohexane:ethyl acetate (1:1). This mixture underwent a purification step using gel permeation chromatography (GPC, LC Tech Uno GmbH, Dorfen, Germany) that rendered two fractions per sample, one containing the lipid content and the other containing the bulk of POPs. The first fraction was used to determine the lipid content gravimetrically, while the second fraction underwent

additional purification using open columns with modified acidic silica (70-230 mesh, Merck, 44% H_2SO_4 w:w) using n-hexane:dichloromethane mixture (9:1) as eluent. After all purification processes, each sample was transferred to vials and concentrated using a multiple Pasvial evaporator system under a gentle N_2 stream. The samples were then reconstituted by adding few microliters of ^{13}C-labeled injection standards of PCBs, PBDEs and OCPs in nonane. Details about sample processing are comprehensively described in the supplementary material.

2.2.2 Instrumental procedure

Eighteen PCB congeners (six non dioxin-like (NDL): # 28, 52, 101, 138, 153, 180 and twelve dioxin-like (DL) congeners: #77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) and twenty-six PBDEs (#7, 15, 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, 156, 183, 184, 191, 196, 197, 206, 207, 209) were analysed by gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) on a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Milan, Italy) coupled to a high-resolution mass spectrometer (DFS, Thermo Fisher Scientific, Bremen, Germany). Thirteen organochlorine pesticides (pentachlorobenzene (PeCB), hexachlorobenzene (HCB), alfa-, beta- and gamma-hexachlorocyclohexane (α-, β-, γ-HCH), α-endosulfan, β-endosulfan, *o,p'*- and *p,p'*-DDE, -DDD, and-DDT) were analysed by GC (7890B, Agilent, Palo Alto, CA, USA) coupled to tandem mass spectrometry with a triple quadrupole as analyser (Agilent 7010B). Quantitation of all target analytes was carried out by the isotopic dilution technique. A full description of the instrumental parameters can be found in Muñoz-Arnanz et al. (2022, 2024) and Roscales et al. (2016) and in the supplementary material. All concentration values are provided in $ng.g^{-1}$ of dry weight (d.w.) and of lipid weight (l.w.) basis in order to maximise comparability with other studies.

2.2.3 Quality assurance/quality control (QA/QC)

Metal and glass materials underwent a series of three cleaning cycles utilizing solvents with different polarities: acetone, dichloromethane, and n-hexane. Additionally, within each batch of five samples, a procedural blank was included. When needed, concentration values were blank subtracted. Full details in relation to QA/QC including limits of detection (LOD) and surrogate recoveries are provided in the supplementary material (section 1.1.5). The procedures followed were based on the Guidance on the Global

Monitoring Plan for Persistent Organic Pollutants (Secretariat of the Stockholm Convention, UNEP).

2.3 RNA extraction and quantitative real-time PCR

Total RNA from whole blood was extracted using the RiboPure™ Blood Kit (Ambion, Life Technologies), following Morão et al. (2022). The concentration and quality of RNA samples were assessed with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Additionally, the Qubit 3 fluorometer (Invitrogen, USA) was used to check for DNA contamination, and sample integrity was confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide.

A reverse transcription reaction was performed using the iScript™ cDNA Synthesis Kit (Biorad) to convert mRNA into cDNA for qPCR. The qPCR was conducted using iTaq™ Universal SYBR® Green Supermix (Biorad) on the CFX Connect™ Real-Time System (Biorad, USA). For this study, sixteen target genes were selected, along with six potential housekeeping genes. These genes were selected based on their roles in oxidative stress and detoxification pathways, immune response and reproduction, which have been used in previous studies in sea turtles and in other species (Labrada-Martagón et al., 2011; Rivas-Hernández et al., 2023; Sah et al., 2021; Chen et al., 2012; Klein et al., 2011; Hubert & Wellik, 2023; Schroeder & Cavacini, 2010). Housekeeping genes were chosen for their reported stability in all tissues across organisms (Thorrez et al., 2008). Primers for these genes were designed using Oligo Explorer software (version 1.1.2, Gene Link™) based on gene sequences sourced from the National Center for Biotechnology Information (NCBI) database and all the information regarding primer sequences and properties are showed in Table 1.

Table 1 - Primer properties for the housekeeping and target genes of green sea turtles (*Chelonia mydas*): NCBI accession number, primer sequence (5'-3'), primer efficiency (%) and R squared of standard curve. fw: forward primer, rv: reverse primer.

	Gene abbrev.	Gene name	Accession	Primer sequence (5'-3')	efficiency (%)	R squared
Housekeeping	*rps15*	Ribossomal protein S15	XM_007072177.1	fw: ATACAACGGCAAAACCTTC rv: TAAGTGATGGAAAACTCGC	95.80	0.999

Target						
	rps13	Ribossomal protein S13	XM_007057128.1	fw: GTCAGCCTTGCCGTATAGAC rv: GGGAGTCAGACCTTTCTTAGC	90.60	0.999
	rps2	Ribossomal protein S2	XM_007058569.1	fw: ATGCTCCAAAGAAGTCGC rv: TGCCAATCTTGTTACCCC	90.40	0.996
	eef1a1	Eukaryotic translation elongation factor 1 alpha 1	XM_007061501.1	fw: TGCGTGACATGAGACAGAC rv: GACTTTGTGACCTTGCCAG	92.00	0.995
	actb	actin beta	XM_027825165.3	fw: AGCAAGCAGGAGTACGATG rv: CAAAGGTGGGATGTGGTAAC	95.4	0.998
	rpl4	ribosomal protein L4	XM_007059493.4	fw: TGCTGTATTTAAGGCTCCTA rv: GGTTGTCTATTGTTCTTGCG	97.9	0.997
	cat	Catalase	XM_007067965.1	fw: CTCAGCATTTCATCCAGAAG rv: CAGCATTGTATTTGTCCAGC	93.80	0.996
	sod1	Superoxide dismutase 1, soluble, transcript variant X1	XM_007070714.1	fw: GGTCCATGAGAAAGAAGATG rv: CAGACGACTACCAGCATTG	93.80	0.998
	gsr	Glutathione reductase	XM_007062837.1	fw: AGGATGTGACGAAATGCTG rv: TGATGAAGTCGGGTGAATG	92.30	0.993
	glrx3	Glutaredoxin 3	XM_007053035.1	fw: GCAAAAGAGGACCTCAACG rv: CTGCTGAAACCACAACGAG	94.00	0.997
	gclc	Glutamate-cysteine ligase, catalytic subunit	XM_007055062.1	fw: GATGGAGAAGCAGCAAAAG rv: AAGCCTGGAATGTTACCTG	91.50	0.997

gst	Glutathione S-transferase Mu 1-like	XM_007053489.1	fw: CTACCTGCTGACCCTTATGAG rv: GAGTCCCCACCATAGAACAC	94.40	0.989
txnip	Thioredoxin interacting protein	XM_007064572.1	fw: TAATGTCGCCAGTTGCTG rv: CCTTTTCGGTCAATCCTG	100.00	0.998
txnrd2	Thioredoxin reductase 2	XM_007054943.1	fw: CCCACTACAGTTTTCACTCC rv: TCCAGTCCATAATGTTCCAC	98.30	0.996
tuba1	tubulin alpha-1B chain	XM_007064711.4	fw: CTGTGGACTATGGCAAGAAG rv: GGTCAGGATGGAGTTGTAGG	101.1	0.995
ace2	angiotensin converting enzyme 2	XM_007070499.3	fw: AGGGTATTCTTCCAACACTG rv: GACAATGACTCCAATCACAG	89.8	0.693
srebf2	sterol regulatory element binding transcription factor 2	XM_007053325.4	fw: CCACATCACAGGTAAACTTCC rv: ATTCACCAGCCACAAGAG	102.3	0.991
est17	estradiol 17-beta-dehydrogenase 11	XM_007066636.4	fw: GAAATGTGCCTTGGTCTTG rv: TCTGGTCTGAGCCTGTGAA	98	0.97
nav3	neuron navigator 3	XM_007057255.4	fw: GACAGCGGAAGAGAAGATG rv: GAGCCACCACTATTTAGCC	103.3	0.939
hoxA1	homeobox protein Hox-A1	XM_037884237.2	fw: AGGCTAACCCAATGAAAGG rv: GGCTGTTGAAGAAGAATGC	91.9	0.924
ig	immunoglobulin Y heavy chain	KT698944.1	fw: CCTCGGTTCTGAATGCTG rv: ACCCCTTGTGCTTTGGAG	98.5	0.979

agpat5	1-acylglycerol-3-phosphate O-acyltransferase 5	XM_007055053.4	fw: GTCAGCAAGCCTATGGTTC rv: GCCTATTTCCTGGACAAGAG	96.4	0.998

To ensure reliability, the efficiency and specificity of all primer sets were evaluated through standard and melting curve analyses, respectively. Amplification reactions were carried out in triplicate for all samples and different technical controls were conducted, including non-template controls (NTC) to confirm the absence of primer dimers and -RT controls (cDNA synthesis without reverse transcriptase) to ensure that no amplification occurs due to eventual genomic DNA contamination. Additional details regarding the qPCR reaction conditions are provided in the supplementary material (section 1.2). The software CFX Connect™ Real-Time System (Biorad, USA) was used to determine the relative expression of each target gene in the turtle samples using the formula:

$$\Delta\Delta\ \mathrm{Cq} = \frac{\text{Target Efficiency}^{[\text{Cq (min target)} - \text{Cq (value target)}]}}{\text{mean HKs Efficiencies}^{[\text{Cq (min mean HKs)} - \text{Cq (value mean HKs)}]}}$$

where “Target Efficiency” refers to the efficiency of the target genes' amplification, “Cq (min target)” is the minimum value of quantification cycle (Cq) for the target gene across all samples, “Cq (value target)” is the specific Cq value of the target gene in the analysed sample, “mean HKs Efficiencies” represents the average amplification efficiency of the housekeeping (HK) genes, “Cq (min mean HKs)” is the minimum Cq mean value of the housekeeping genes across all samples, “Cq (value mean HKs)” is the specific Cq mean value of the housekeeping genes in the analysed sample. Ribosomal protein S15 and ribosomal protein L4 (*rps15* and *rpl4*) were selected as housekeeping genes.

2.4 Erythrocytic Nuclear Abnormalities (ENA) assay

The methodology used for the Erythrocytic Nuclear Abnormalities (ENA) assay is described in detail in Morão et al. (2024). Briefly, the two slides with blood smears from each female turtle were subsequently stained with Diff-Quick stain and total of 1000 mature erythrocytes per sample were examined. The nuclear lesions were categorised into micronuclei, lobed nuclei, segmented nuclei, and kidney-shaped nuclei. The findings

were presented as the mean value (‰) for each abnormality and the total sum of all observed lesions.

2.5 Data and statistical analysis

Contaminant concentrations falling below the limits of detection were estimated using the methodology proposed by Hites (2019), in the cases where the percentage of censored data was below 50% as recommended for little bias. In this approach, values were estimated by calculating the mean between zero and the LOD value (i.e., LOD/2). This correction method was specifically applied to PCB congeners -114, -123, and -189; PBDE congeners -99, -100, -153, and -154; as well as pesticides HCB, α-HCH, β-HCH, γ-HCH, *o,p'*-DDT, and *p,p'*-DDT. Congeners presenting more than 50% censoring across turtle samples were excluded from statistical analyses to avoid biasing statistical outcomes and are reported together with their corresponding sample sizes (i.e. PCB-81, PCB-169, PBDE-17, PBDE-28, PBDE-85, PBDE-126, PBDE-138, PBDE-183, PBDE-197, PBDE-209 and *p,p'*-DDD). Details on detection frequency and data censoring are provided in Table S2.1 of the supplementary material (SM).

More information on the average recovery percentages and LOD values for each congener within the turtle samples are reported in Table S1.1.5 and Table S1.1.6, respectively (SM). Toxic equivalent quantities (TEQ) for dioxin like-PCBs (DL-PCBs) were obtained using the World Health Organization (WHO)-1998 toxic equivalency factors (TEF) for birds (Van Den Berg et al., 1998). TEQs are reported in upper bound (i.e. substitution of non-detected compounds for detection limit values).

Statistical analyses were conducted using R software (version 4.2.3), along with the RStudio user interface (version 4.2.3) (R Core Team, 2023), setting a significance level of 0.05. Box plots were generated using Statistica (version 14.0.0.15, TIBCO, California, United States). Canonical Correspondence Analysis (CCA) was performed with CANOCO version 4.5 package 5 (ter Braak & Šmilauer, 2002). The Shapiro-Wilk test, implemented through the "shapiro.test ()" function, was employed to assess the normality of variances in the data. As most of the variables here analysed were not normally distributed (Table S1.3 in supplementary material), non-parametric correlations were followed.

The relationship between the levels of the groups of POPs and the ENAs frequency or the gene expression responses was analysed through CCA. For this analysis, the data on POPs concentration were standardised and transformed log (x + 1). Downweighing of

ENAs frequency and gene expression responses was performed to take into consideration the less representative variables (Lepš & Šmilauer, 2003). Additionally, to discern the specific congeners exerting more influence on both ENA amounts and target gene expressions, a Spearman correlation heatmap was constructed using the "corrplot ()" function and the "corrplot" package in R. These integrative analyses were performed for 19 blood samples, as two of the 21 samples collected did not pass the quality criteria after RNA extraction. However, these two excluded samples were still included in the independent analyses of POP concentrations and ENA frequency, ensuring that the overall dataset for those endpoints remained complete.

3. Results

3.1 Levels of POPs

From all the organic pollutants analysed, 13 PBDEs and 2 pesticides were not quantifiable in the blood of any of the turtle samples (values below the LOD) and were therefore excluded from the analyses (PBDE-7, PBDE-15, PBDE-49, PBDE-66, PBDE-71, PBDE-77, PBDE-119, PBDE-156, PBDE-184, PBDE-191, PBDE-196, PBDE-206, PBDE-207, endosulfan α and β). All mean, median and range values for the overall concentrations of the quantified PCBs, NDL-PCBs, DL-PCBs, PBDEs, OCPs, DDTs, HCHs, and CBs, as well as the concentrations of their respective congeners can be found in Table S2.1 (supplementary material), while a summary of the data used in the statistical analyses (censorship below 50%) is displayed in Fig. 2.

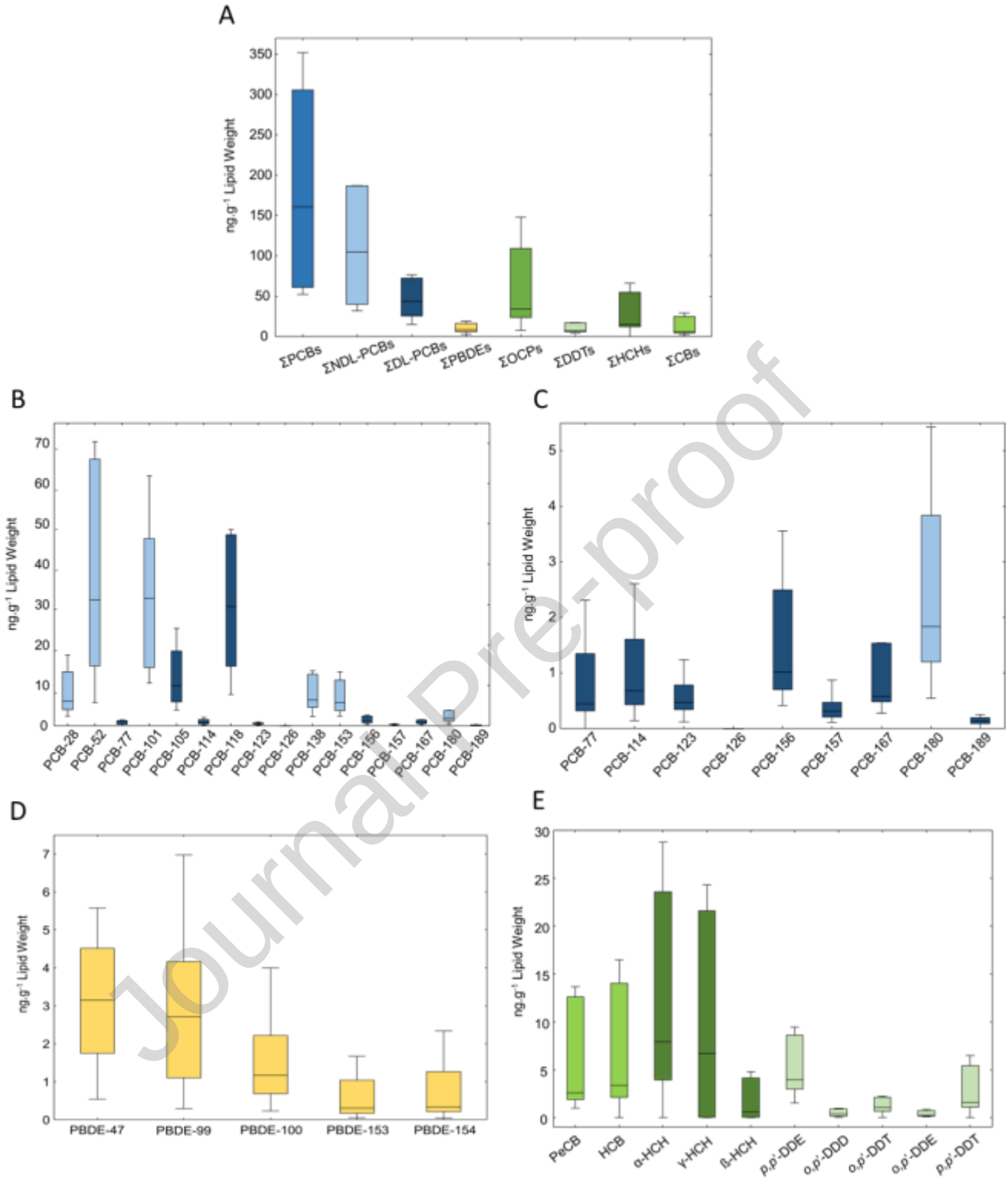

Figure 2 – Box plots representing persistent organic pollutants (POP) values measured in ng.g^{-1} of lipid weight of the blood of green sea turtles (*Chelonia mydas*), namely: A) total PCBs (blue), including NDL (light blue) and and DL-PCBs (dark blue), as well as total PBDEs (yellow) and total OCPs in green (DDTs light green, HCHs dark green, CBs; medium green); B) PCB congeners; C) PCB congeners under 5 ng.g^{-1} of lipid weight; D) PBDE congeners; E) OCPs. Each box in the plot provides information about the median,

quartiles and min/max values. Values exceeding 1.5 times the interquartile range were considered outliers and excluded from the visual analysis.

The relative mean abundance of target contaminants followed the sequence PCBs (293 $ng.g^{-1}$) > OCPs (90.2 $ng.g^{-1}$ > PBDEs (12.4 $ng.g^{-1}$) (Fig. 2A and Table S2.1). Within the PCBs, non-dioxin-like congeners (NDL-PCBs) exhibited a higher relative abundance than dioxin-like PCBs (DL-PCBs), with mean values of 194 $ng.g^{-1}$ and 98.9 $ng.g^{-1}$, respectively (Fig.2A). Among NDL-PCBs, congener -101 had the highest mean value (64.4 $ng.g^{-1}$), followed by -52 (61.4 $ng.g^{-1}$) and -153 (24.4 $ng.g^{-1}$), while for DL-PCBs, congener -118 had the highest mean value (60.3 $ng.g^{-1}$), followed by -105 (23.9 $ng.g^{-1}$) and -77 (5.36 $ng.g^{-1}$) (Fig.2B).

Regarding PBDEs, congener -47 exhibited the highest mean value (4.83 $ng.g^{-1}$), followed by -99 (3.67 $ng.g^{-1}$) and -100 (1.51 $ng.g^{-1}$) (Fig.2D). Among pesticides, HCHs displayed the highest mean value (39.6 $ng.g^{-1}$), followed by chlorobenzenes (CBs) (28.9 $ng.g^{-1}$) and DDTs (21.7 $ng.g^{-1}$) (Fig.2A). Within HCHs, α and γ isomers were the most representative, with 18.1 $ng.g^{-1}$ and 17.4 $ng.g^{-1}$, respectively (Fig.1E). Both CBs, PeCB and HCB, exhibited similar mean values (13.1 $ng.g^{-1}$ and 15.8 $ng.g^{-1}$, respectively) (Fig.2E). For DDTs, the metabolite *p,p'*-DDE displayed the highest mean value with 10.3 $ng.g^{-1}$ (Fig.2E).

To evaluate the possible harmful effects that DL-PCBs may exert on the sea turtles, the concentration of each congener was calculated also as 2,3,7,8-TCDD toxic equivalent (TEQ) concentration (Table 2).

Table 2 - Toxic equivalent quantities (TEQs) for DL-PCBs present in blood of green sea turtles (*Chelonia mydas*), calculated using toxic equivalency factors (TEFs) for birds (Van den Berg et al., 1998), are presented in units of femtograms per gram of dry weight (pg $TEQ.g^{-1}$ d.w), picograms per gram of wet weight (pg $TEQ.g^{-1}$ w.w) and lipid weight (pg $TEQ.g^{-1}$ l.w), with data reported as mean values, median and ranges (minimum and maximum).

	pg $TEQ.g^{-1}$ d.w.				**pg $TEQ.g^{-1}$ w.w.**				**pg $TEQ.g^{-1}$ l.w.**			
Compound	**mean**	**median**	**min**	**max**	**mean**	**median**	**min**	**max**	**mean**	**median**	**min**	**max**
ΣDL-PCBs	**5.73**	**1.71**	**0.0629**	**88.4**	**22**	**8.64**	**0.211**	**292**	**392**	**92**	**2.39**	**4840**

Non-ortho	PCB-77	4.73	0.474	0	88.2	16	1.97	0	292	270	29.1	0	4830
	PCB-81	0.316	0.396	0	0.958	1.98	2.16	0	4.18	39.1	19.1	0	194
	PCB-126	0.626	0.805	0	2.29	3.95	4.05	0	8.12	79.2	40.5	0	416
	PCB-169	0.00187	0.00248	0	0.00596	0.0127	0.0127	0	0.0253	0.245	0.131	0	1.29
Mono-ortho	PCB-105	0.034	0.018	0.00422	0.26	0.172	0.0633	0.01461	1.8	2.44	1.22	0.39	24
	PCB-114	0.00156	0.00079	0	0.0109	0.00697	0.00379	0.0021	0.0557	0.122	0.0427	0.017	1.01
	PCB-118	0.00826	0.00492	0.000779	0.0653	0.0422	0.0163	0.00497	0.4513	0.618	0.298	0.0775	6.04
	PCB-123	0.000132	0.0000721	0	0.00101	0.000616	0.000326	0.00018	0.00516	0.0109	0.00384	0.0015	0.0933
	PCB-156	0.00432	0.00198	0.000530	0.028	0.02001	0.007	0.00221	0.175	0.286	0.104	0.0412	2.34
	PCB-157	0.0012	0.000487	0.0000973	0.0082	0.00559	0.00213	0.00032	0.0478	0.0775	0.0314	0.0112	0.639
	PCB-167	0.000339	0.000144	0.0000340	0.00273	0.00147	0.000494	0.00013	0.0105	0.0211	0.0073	0.0027	0.141
	PCB-189	0.00008333	0.00002650	0	0.00117	0.000313	0.0000941	0	0.00389	0.00468	0.00143	0	0.0268

The average toxic equivalent (TEQ) concentration for DL-PCBs in blood of green sea turtles was 392 pg TEQ.g^{-1}. Among the 12 congeners, -77 exhibited the highest mean value at 270 pg TEQ.g^{-1}, followed by -126 with a significantly lower mean value of 79.2 pg TEQ.g^{-1}.

In terms of TEQ distribution, the observed pattern in the blood of green sea turtles was as follows: -77 (68.9%) > -126 (20.2%) > -81 (9.97%) followed by -105, -118, -156, -169, -114, -157, -167, -123 and -189 (<0.62%). Remarkably, the sum of the four non-ortho congeners accounted for ~99.1% of the total DL-PCBs TEQ value, while the eight mono-ortho congeners represented only ~0.9%.

3.2 Gene expression

In this study, the expression of the target genes related with antioxidant defence (*cat*, *sod1*, *gsr*, *glrx3*, *gclc*, *gst*, *txnip*, and *txnrd2*), lipid metabolism and transport (*agapt5* and

srebf2), reproduction and embryo development (*est17, hoxA1, nav3, ace2,* and *tuba1*) and immune response (*ig*) was analysed in the turtle's blood samples. Among the potential housekeeping genes, *rps15* and *rpl14* exhibited the least variation across all samples (CVs of 2.15% and 5.50%, respectively), and consequently, were selected as the housekeeping genes for calculating the relative expression of the target genes.

The mean relative expression (ΔΔ Cq) of each target gene between the different sea turtles sampled, along with the coefficient of variation (CV), is presented in Table S2.2 (supplementary material). The highest mean expression values were obtained for *sod1* (ΔΔ Cq = 1.080) and *gsr* (ΔΔ Cq = 0.885) followed by *glrx3* (ΔΔ Cq = 0.822) and *srebf2* (ΔΔ Cq = 0.725). The genes with the lowest mean expressions were *hoxA1* (ΔΔ Cq = 0.11) and *ace2* (ΔΔ Cq = 0.17) followed by *est17* (ΔΔ Cq = 0.217) and *txnip* (ΔΔ Cq = 0.249). However, the genes that varied the most in their expression between the sampled sea turtles were *hoxA1* (CV = 231 %), followed by *ig* (CV= 166 %), *ace* (CV = 133 %) and *txnip* (CV = 104 %).

3.3 Erythrocytic Nuclear Abnormalities (ENA) assay

The results of ENA assay in the blood of the green sea turtles sampled for this study have been previously described in Morão et al. (2024), and a summary is showed in Table S2.3 (supplementary material). In general, lobed nuclear abnormalities were the most frequent with 19.08 ‰ ± 14.31, followed by micronucleus formation with 8.65 ‰ ± 5.36, kidney-shaped with 0.50 ‰ ± 0.70, and segmented with 0.08 ‰ ± 0.27.

3.4 Integrative analysis of POP levels versus biological responses and effects

The integrative analysis between the POP levels and the different endpoints measured can be seen in the Canonical Correspondence Analysis (CCA) plot from Fig.3. Overall, it can be observed that PCBs and PBDEs are more closely associated with the overexpression of three genes related with embryo development (*hoxA1*), albumin secretion (*ace2*) and estradiol balance (*est17*), while OCPs seem to be more associated with the expression of oxidative stress genes (cat, *gclc, glrx3,* and *txnrd2*) and genotoxic effects on the turtles' red blood cells (nuclear abnormalities).

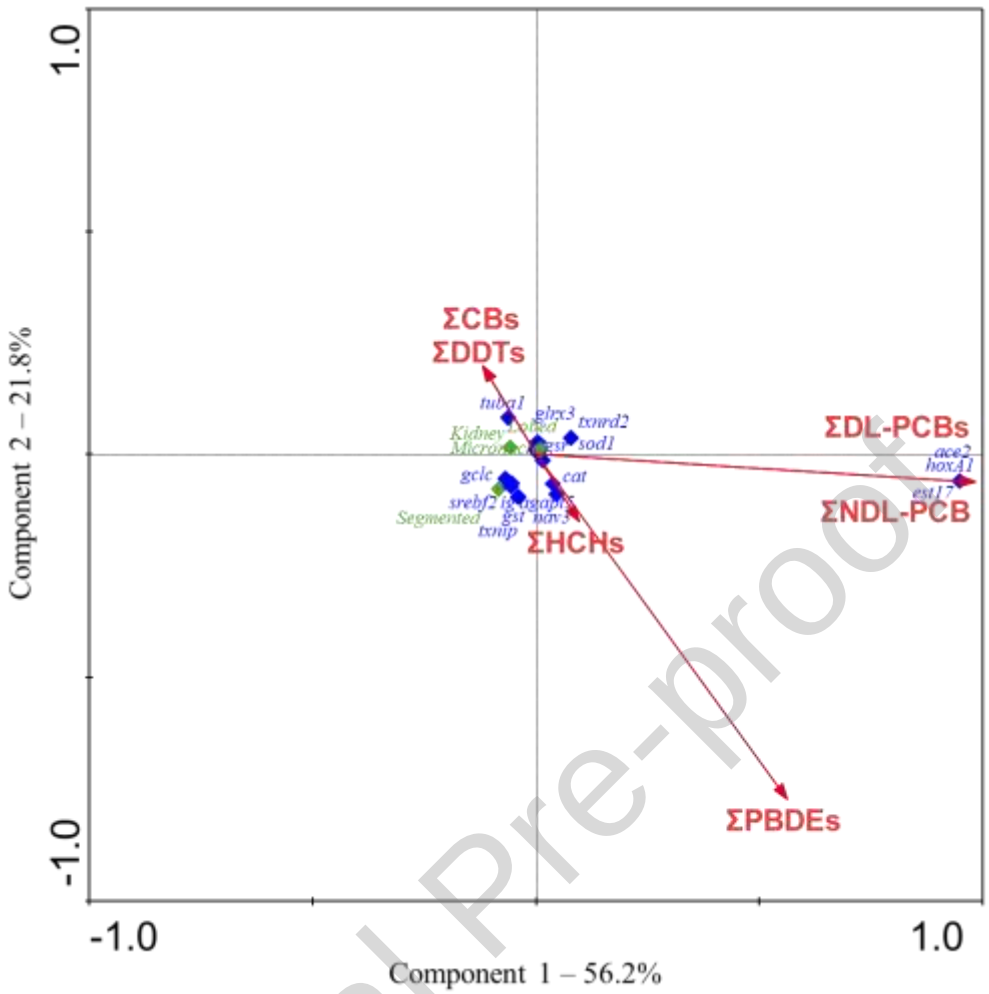

Figure 3 – Biplots for axes 1 and 2 of the Canonical Correspondence Analysis (CCA) between each group of POPs in red (NDL-PCBs, DL-PCBs, PBDEs, HCHs, CBs, and DDTs) analysed in blood of female green sea turtles (*Chelonia mydas*) and the presence of both ENAs in green - encompassing total ENAs, lobed, kidney, segmented, and micronuclei - and target gene expression (*cat, sod1, gsr, glrx3, gclc, gst, txnip, txnrd2, agapt5, srebf2, est17, hoxA1, nav3, ace2, ig* and *tuba1*) in blue.

To gain deeper insights into how the contamination profiles of individual POPs may be contributing to the frequency of nuclear abnormalities or to the expression patterns of each tested gene, a graphical heatmap was created to visualise these individual correlations (Fig.3). The correlation analysis between each POP congener and the addressed biological parameters showed an overall agreement with previous data from Fig.3, but it enables the observation that the congeners are having stronger and more significant correlations with the responses.

More specifically, lobed and kidney shaped nuclei presented significant positive correlations with several of those contaminants (Fig. 3), namely with PCB-28 (r_s = 0.51,

P = 0.03), PCB-138 (r_s = 0.56, *P* = 0.02), PCB-153 (r_s = 0.51, *P* = 0.03), PCB-180 (r_s = 0.50, *P* = 0.04), and *p,p'*-DDE' (r_s = 0.54, *P* = 0.02) in the case of lobed nuclei and with *o,p'*-DDD (r_s = 0.49, *P* = 0.04) and *o,p'*-DDE (r_s = 0.49, *P* = 0.04) for kidney-shaped nuclei (Table S2.4.1 and S2.4.3).

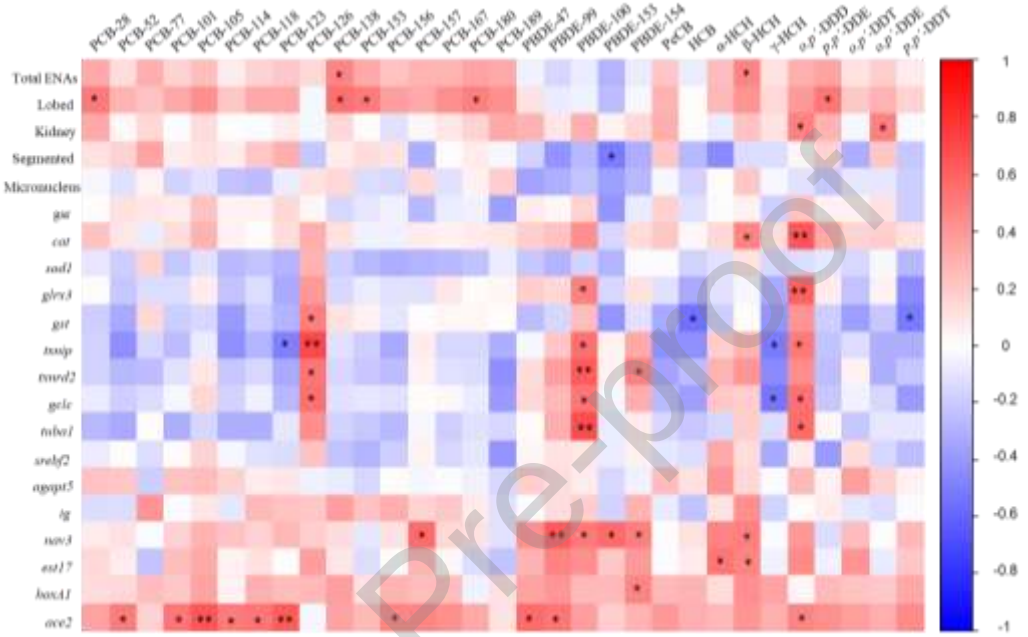

Figure 4 – Correlation heatmap illustrating Spearman correlation coefficients between specific POPs congeners present in green sea turtles (*Chelonia mydas*) females' blood and its influence on both ENA amounts and target gene expressions. The colour gradient ranges from -1 in blue to 1 in red, and the corresponding *P* values are identified (* < 0.05 and ** < 0.01). The columns represent individual POPs (upper part), and the rows display nuclear abnormalities presence and target gene expression (left side).

The genes related with antioxidant and detoxification responses (*cat*, *glrx3*, *gst*, *txnip*, *txnrd2*, and *gclc*), and also in specific the *tuba1* gene related with embryo development, have also demonstrated a clear pattern of overexpression response when higher levels of PCB-126, PBDE-100, or *o,p'*-DDD were present (Fig.4). Specifically, for PCB-126 significant positive correlations were observed with the expressions of *gst* (r_s = 0.48, *P* = 0.04), *txnip* (r_s = 0.72, *P* < 0.001), *txnrd2* (r_s = 0.53, *P* = 0.02) and *gclc* (r_s = 0.54, *P* = 0.02) (Table S2.4.1). In the case of PBDE-100, significant correlations were found with

the expressions of *glrx3* (r_s = 0.49, *P* = 0.04), *txnip* (r_s = 0.55, *P* = 0.02), *txnrd2* (r_s = 0.64, *P* < 0.001), *gclc* (r_s = 0.54, *P* = 0.02) and *tuba1* (r_s = 0.68, *P* < 0.001) (Table S2.4.2). Lastly, for *o,p'*-DDD positive and significant correlations were noted in the expressions of *cat* (r_s = 0.65, *P* < 0.001), *glrx3* (r_s = 0.61, *P* = 0.01), *txnip* (r_s = 0.52, *P* = 0.03), *gclc* (r_s = 0.56, *P* = 0.02) and *tuba1* (r_s = 0.57, *P* = 0.01) (Table S2.4.3).

In relation to the genes *nav3* and *hoxA1* linked to embryonic development, to *ace2* associated with albumin secretion, and to *est17* associated with estradiol balance, positive correlations were observed with other congeners (Fig.4). Specifically, the higher expression levels of *nav3* exhibited stronger correlations with higher PBDE levels, namely with -99 (r_s = 0.62, *P* = 0.01), -100 (r_s = 0.51, *P* = 0.03), -153 (r_s = 0.56, *P* = 0.01) and -154 (r_s = 0.48, *P* = 0.04) (Table S2.4.2). Moreover, the expression of this gene also displayed a positive correlation with PCB-157 (r_s = 0.57, *P* = 0.01) and pesticide β-HCH (r_s = 0.50, *P* = 0.04) (Table S2.4.1 and S2.4.3). The expression of *ace2* appear to be primarily influenced by PCBs, particularly congeners -52 (r_s = 0.51, *P* = 0.03), -101 (r_s = 0.53, *P* = 0.02), -105 (r_s = 0.66, *P* < 0.001), -114 (r_s = 0.51, *P* = 0.03), -118 (r_s = 0.53, *P* = 0.02), -123 (r_s = 0.64, *P* < 0.001) and -156 (r_s = 0.48, *P* = 0.04) (Table S2.4.1), although PBDEs -47 (r_s = 0.56, *P* = 0.01) and -99 (r_s = 0.53, *P* = 0.02) and pesticide *o,p'*-DDD (r_s = 0.48, *P* = 0.04) also seem to induce the expression of this gene. As for *hoxA1* positive correlations were verified with PBDE-154 (r_s = 0.47, *P* = 0.05), whereas *est17* showed positive correlations with α (r_s = 0.54, *P* = 0.02) and β-HCH (r_s = 0.48, *P* = 0.04).

Lastly, the immune response (*ig*) did not present any significant correlation with the diverse contaminants analysed.

4. Discussion

POPs contamination in sea turtles has emerged as a critical concern, impacting their physiology and ecology (Muñoz & Vermeiren, 2018). Understanding the extent and consequences of POP exposure in sea turtles is crucial for effective conservation strategies, aiming to safeguard these iconic marine species and preserve the global sensitive environmental balance. This is the first time that these contaminants, PCBs, PBDEs, and OCPs are being examined in the Gulf of Guinea region, particularly in São Tomé and Príncipe archipelago.

4.1 Contaminant levels in perspective with other studies

A compilation of studies analysing different POPs in blood of green sea turtles are summarised in Table 3 for comparative purposes. This table presents the values found for different contaminants, developmental stages, and geographical areas around the globe where other green sea turtle populations have been analysed. The present study data are also reported in wet weight for comparison with other studies. From the studies' compilation, this current study was the only that presented and analysed a broader range of POPs and their respective congeners.

Generally, ΣPCBs values in the current work were higher than those from the studies reported in Table 3, except for Barraza et al. (2020), with values exceeding the present ones. Barraza's study, however, focused on green turtles from an industrialised location in the USA. Thus, the fact that congeners -138, -153, -167, and -180 were lower in São Tomé turtles than those in USA sub-adults and adults (Barraza et al., 2020), can likely be explained by the higher industrialisation of Californian sampling sites. Additionally, PCB-118 and -180, in particular, were higher in Cape Verde juveniles (Camacho et al., 2014) than in the adult turtles from the present study. The varied omnivorous diet of juvenile turtles involving a broader range of food sources compared to the strictly herbivorous diet in the adult stage (Arthur et al., 2008), place them higher in the food chain and, thereby, more susceptible to POPs accumulation.

Regarding ΣPBDEs, values in São Tomé turtles were lower than those reported in the Gulf of Mexico (Swarthout et al., 2010) Malaysia (van de Merwe et al., 2010), and Australia (van de Merwe et al., 2010b) - regions known to have elevated concentrations of persistent pollutants (Kaw & Kannan, 2016; Prange et al., 2002; Kennicutt, 2017). This pattern is consistent with the observed tendency for higher POPs bioaccumulation from more industrialised areas (Williams et al., 2023). PBDEs are generally categorised into three main groups: penta-BDEs, octa-BDEs, and deca-BDE. Of these, penta-BDEs, specifically PBDE-47 and PBDE-99, considered more toxic (Zhao et al., 2018), had lower values in São Tomé turtles than in Gulf of Mexico and Californian studies, attributed to varied diets and higher contamination levels in the latter populations.

Consistently with the other POP groups, ΣOCPs levels in turtles from the present study were also lower than those observed in Cape Verde (Camacho et al., 2014), but higher than the green sea turtles from Brazil (Filippos et al., 2021) and from the USA western coast (Barraza et al., 2020) reports, reflecting the different industrialisation degree in the study areas and the different diets. Even though DDT is still in use for malaria control in some developing countries like São Tomé and Príncipe (Zhang et al., 2021), *p,p'*-DDE

(dichlorodiphenyldichloroethylene) values (one of its most toxic metabolites (Harada et al., 2016)) were lower than those of the green turtle population in the Gulf of Mexico (Swarthout et al., 2010) and the USA (Komoroske et al., 2011). Despite being banned in most countries (Sun et al., 2022), its persistence is high and observable in these sea turtles' populations, confirming its extensive past use in the region of south America and Mexico. Lindane (γ-HCH), the most toxic HCH isomer (Jackovitz & Hebert, 2015) mostly used in insecticide formulations, was also banned in most countries (Humphreys et al., 2008); however, it was found in lower values than in most studies, suggesting that São Tomé's population may be less contaminated with this hazardous substance.

Table 3 – Concentration of Persistent Organic Pollutants in whole blood of green sea turtles (*Chelonia mydas*) in $ng.g^{-1}$ of wet weight from different sampling sites. N = number of individuals sampled, A = adult, F = female, M = male, J = juveniles, S = sub-adult, NA = not applicable, N.A = not analysed, PA = Punta Abreojos, BM = Bahía Magdalena, HB = Hervey Bay, PG = Port of Gladstone, MB = Moreton Bay, SDB = San Diego Bay, SBNWR = Seal Beach National Wildlife Refuge, SD = standard deviation, SE = standard error, * = contaminants analysed in the plasma of green turtles, a) = data in pg.g converted to ng.g, b) = data in ng.mL converted to ng.g, c) = samples pool.

Reference	**Present study**	(van de Merwe et al., 2010) (mean ± SE) a)	(Filippos et al., 2021)* (mean ± SD)	(Camacho et al., 2014)* (mean ± SD) b)	(Swarthout et al., 2010) (geomean ± SD) a)	(Komoroske et al., 2011)* (mean ± SE) c)	(Labrada-Martagón et al., 2011)* (median) b), c)		(van de Merwe et al., 2010) (mean ± SE) a)	(Chaousis et al., 2023)* (mean ± SD) a), b)			(Barraza et al., 2020)* (mean ± SE)	
N	**21**	11	31	21	9	20	39	13	16	25	24	23	16	23
Developmental Stage	**A**	A	A	J	J&S	J&S	J&A		J&S	S			S&A	
Sex	**F**	F	F	NA	NA	NA	NA		6 M and 10 F	NA			6 M and 10 F	NA
Sampling site	**STP**	Malaysia	Brazil	Cape Verde	Gulf of Mexico	USA	Mexico		Australia	Australia			USA	
							PA	BM		HB	PG	MB	SDB	SBNWR
ΣPCBs	**0.91 ± 0.38**	0.58 ± 0.09	0.28 ± 0.25	0.50 ± 1.00	0.33 ± 0.70	N.A	N.A	N.A	0.68 ± 0.15	N.A	N.A	N.A	8.31 ± 7.25	0.85 ± 0.33
ΣNDL-PCBs	**0.61 ± 0.3**	N.A	0.10 ± 0.08	0.45 ± 0.93	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
ΣDL-PCBs	**0.3 ± 0.16**	N.A	0.02 ± 0.02	0.25 ± 0.57	0.01 ± 0.04	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-52	**0.18 ± 0.09**	N.A	0.01 ± 0.01	0.09 ± 0.11	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-77	**0.03 ± 0.01**	N.A	0.004 ± 0.001	0.002 ± 0.006	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-81	**0 ± 0**	N.A	< LOD	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-101	**0.19 ± 0.07**	N.A	0.03 ± 0.03	0.003 ± 0.01	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-105	**0.08 ± 0.04**	N.A	0.008 ± 0.006	0.02 ± 0.01	N.A	N.A	N.A	N.A	0.02 ± 0.008	N.A	N.A	N.A	0.01 ± 0.04	0

PCB-114	**0.01 ± 0.01**	N.A	< LOD	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	0.02 ± 0.08	0
PCB-118	**0.18 ± 0.1**	0.03 ± 0.006	0.013 ± 0.011	0.20 ± 0.49	N.A	N.A	N.A	N.A	0.03 ± 0.07	N.A	N.A	N.A	0.21 ± 0.19	0.01 ± 0.01
PCB-123	**0.01 ± 0.01**	N.A	< LOD	0.009 ± 0.03	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-126	**0.01 ± 0**	N.A	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-138	**0.07 ± 0.03**	N.A	0.032	0.02 ± 0.04	N.A	N.A	N.A	N.A	N.A	0.004 ± 0.002	0.004 ± 0.002	0.005 ± 0.002	2.92 ± 2.61	0.32 ± 0.12
PCB-153	**0.1 ± 0.03**	N.A	0.025	0.09 ± 0.34	N.A	N.A	N.A	N.A	N.A	0.004 ± 0.04	0.004 ± 0.003	0.006 ± 0.003	2.79 ± 3.01	0.18 ± 0.08
PCB-156	**0.01 ± 0.01**	N.A	0.005 ± 0.001	0.006 ± 0.01	N.A	N.A	N.A	N.A	< LOD	N.A	N.A	N.A	0.01 ± 0.02	0
PCB-157	**0.01 ± 0.01**	N.A	0.003 ± 0.000	0.005 ± 0.02	N.A	N.A	N.A	N.A	< LOD	N.A	N.A	N.A	0.01 ± 0.02	0
PCB-167	**0.01 ± 0.01**	N.A	N.A	0.003 ± 0.006	N.A	N.A	N.A	N.A	< LOD	N.A	N.A	N.A	0.06 ± 0.07	0
PCB-169	**0 ± 0**	N.A	N.A	0.001 ± 0.006	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PCB-180	**0.04 ± 0.01**	N.A	0.009 ± 0.010	0.06 ± 0.24	N.A	N.A	N.A	N.A	N.A	0.002 ± 0.002	0.002 ± 0.001	0.002 ± 0.001	0.38 ± 0.41	0.05 ± 0.01
PCB-189	**0.01 ± 0.01**	N.A	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	0.001 ± 0.003	0
ΣPBDEs	**0.05 ± 0.05**	0.12 ± 0.01	0.003 ± 0.001	N.A	0.08 ± 0.22	N.A	N.A	N.A	0.08 ± 0.01	N.A	N.A	N.A	N.A	N.A
PBDE-28	**0.01 ± 0.01**	N.A	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PBDE-47	**0.02 ± 0.01**	0.01 ± 0.001	< LOD	N.A	0.06 ± 0.14	0.2	N.A	N.A	0.017 ± 0.002	NA	NA	0.005 ± 0.003	N.A	N.A
PBDE-99	**0.02 ± 0.02**	0.021 ± 0.004	0.007 ± 0.00	N.A	0.03 ± 0.07	0.2	N.A	N.A	0.02 ± 0.005	N.A	N.A	N.A	N.A	N.A
PBDE-100	**0.01 ± 0.01**	N.A	< LOD	N.A	0.03 ± NA		N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PBDE-153	**0.01 ± 0.01**	0.09 ± 0.01	< LOD	N.A	0.02 ± NA	0.2	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
PBDE-154	**0.01 ± 0.01**	N.A	< LOD	N.A	0.03 ± NA	0.2	N.A	N.A	0.020 ± 0.004	N.A	N.A	N.A	N.A	N.A
PBDE-183	**0.02 ± 0.02**	N.A	0.006 ± 0.006	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
ΣOCPs	**0.29 ± 0.13**	N.A	0.06 ± 0.06	0.31 ± 0.71	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	0.25 ± 0.16	0.20 ± 0.05
∑DDTs	**0.07 ± 0.04**	N.A	0.005 ± 0.004	N.A	0.08 ± 0.11	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
∑HCHs	**0.14 ± 0.07**	N.A	0.027 ± 0.027	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
***p,p'*-DDE**	**0.04 ± 0.02**	N.A	0.009 ± 0.004	0.57 ± 0.11	0.07 ± 0.11	0.736 ± 0.097	N.A	N.A	N.A	N.A	N.A	N.A	0.02 ± 0.07	N.A
***o,p'*-DDE**	**0.01 ± 0.01**	N.A	< LOD	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
***o,p'*-DDD**	**0.01 ± 0.01**	N.A	< LOD	0.0009 ± 0.004	N.A	N.A	0.71	N.A	N.A	N.A	N.A	N.A	N.A	0.05 ± 0.05
***p,p'*-DDD**	**0.02 ± 0.01**	N.A	< LOD		N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A

***o,p'*-DDT**	**0.01 ± 0.01**	N.A	0.007 ± 0.007	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
***p,p'*-DDT**	**0.02 ± 0.01**	N.A	< LOD	N.A	0.06 ± 0.01	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
α-HCH	**0.07 ± 0.03**	N.A	< LOD	N.A	N.A	N.A	0.15	1.26	N.A	N.A	N.A	N.A	N.A	N.A
ϒ-HCH	**0.06 ± 0.03**	0.50 ± 0.06	0.034 ± 0.026	N.A	N.A	0.915 ± 0.092	0.74	2.82	N.A	N.A	N.A	N.A	N.A	N.A
β-HCH	**0.02 ± 0.01**	N.A	< LOD	< LOD	N.A	N.A	1.11	4.37	N.A	N.A	N.A	N.A	N.A	N.A
HCB	**0.05 ± 0.02**	N.A	0.003 ± 0.001	0.11 ± 0.19	N.A	N.A	N.A	N.A	N.A	0.003 ± 0.002	0.004 ± 0.003	0.003 ± 0.002	N.A	N.A

Regarding TEQ values for DL-PCBs, a comparison was made between the values obtained using two reference WHO TEFs (mammals from 2022 *versus* birds from 1998). It was found that TEQ values calculated with 2022 TEFs were overall lower than the ones calculated with 1998 TEFs. In fact, overall TEQ values using bird TEFs were around 10-fold higher than the ones using mammal TEFs (see Table S3.1 in supplementary material) suggesting higher toxicity. Being reptiles more evolutionarily related to birds than mammals, and lacking reference values for reptiles, we believe that the 1998 TEFs are more suitable for use. In fact, in a few studies analysing the same topic, there is no consensus in which TEFs are to be used when applied to sea turtles. For example, Lambiase et al. (2021) and Storelli & Zizz (2014) used the same TEF birds' values from 1998, but Miao et al. (2001) used TEF mammals' values from 1998. Thus, in the present study a more conservative approach was adopted based on their phylogenetic proximity by using the 1998 reference TEF bird values.

To the best of our knowledge, this study is the first to report TEQ values in sea turtle blood, which is particularly relevant because TEQs provide toxicity information on chemical mixtures, offering more meaningful insights than total contaminant concentrations alone (Van Den Berg et al., 1998). Although no previous studies have provided blood TEQ values, three studies have measured TEQ values in the liver or fat of loggerheads and green sea turtles (Lambiase et al., 2021; Miao et al., 2001; Storelli & Zizzo, 2014). Since Miao et al. (2001) used TEF values from humans/mammals, present findings will be compared with the other two studies that used the same 1998 reference TEF bird values as the present study. Thus, the present values of ΣTEQ in blood presented in Table 2, were higher than those in liver of loggerheads turtles from the Tyrrhenian Sea (Mediterranean Sea, 8.72 $pg.g^{-1}$ w.w. (mean)) in Lambiase et al., (2021) *versus* present 22 $pg.g^{-1}$ w.w. (mean) and from

the Adriatic Sea (Mediterranean Sea, 27.02 $pg.g^{-1}$ l.w. (mean)) in Storelli & Zizz (2014) *versus* present 392 $pg.g^{-1}$ l.w. (mean).

It is important to note that blood can serve as an indicator of internal organ contamination, as reported for green sea turtles (van de Merwe et al., 2010). Therefore, it is possible that similar or higher contamination levels might be present in the livers of São Tomé green sea turtles. This suggest liver contamination could be comparable to or exceed the levels observed in other studies, such as those involving loggerhead turtles, highlighting the potential for even greater differences in contamination levels between these species. Additionally, it should be considered that the accumulation and distribution of POPs in different tissues can be influenced by chemical properties, such as lipophilicity (Kow), as well as metabolic processes and tissue-specific affinities (Muñoz & Vermeiren, 2020, 2023). While this study focused on blood as a transport tissue, future research should examine tissue-specific accumulation to better understand how these compounds may exert biological effects across different organs.

Despite the existing law prohibiting the consumption and capture of sea turtles for human consumption, such practices persist in São Tomé and Príncipe, although with a reported decay comparatively to previous decades (Ferreira-Airaud et al. 2022; Ferreira et al., 2019; Graff, 1996). Considering the levels of contaminants found in this study, the consumption of turtle meat and eggs may also be posing a potential health risk to humans. Therefore, these results are not only relevant for sea turtle conservation but may also serve as an important tool for raising public awareness and supporting outreach efforts that highlight both environmental and public health concerns in the country.

Additionally, and despite the presence of POPs levels in São Tomé green sea turtles, it is important to note that blood, as a transport tissue, reflects recent exposure to contaminants, typically within a window of a few weeks to three months (Keller et al., 2004; Komoroske et al., 2011). Given that female green sea turtles stay in São Tomé only during the nesting season, which lasts on average 2 to 3 months (Ferreira-Airaud et al., 2024), the contaminant levels found in this study likely represent bioaccumulation from foraging areas - such as Guinea-Bissau, Angola or Mauritania (ONG Programa Tatô GPS unpublished data) - rather than local exposure at the nesting beaches (Ceriani et al., 2012; Filippos et al., 2021). While some green turtles have been observed feeding during the nesting period, as reported by our partner NGOs (Ferreira-Airaud et al., 2024), it remains unclear whether the sampled turtles were among those observed.

This temporal aspect should be considered when interpreting the results, particularly for migratory species like sea turtles that occupy various ecological niches throughout their life cycle. To gain a more comprehensive understanding of contamination sources, future studies should adopt a broader approach—examining not only nesting females or sites but also foraging areas and other life stages, such as juveniles, post-incubation eggs, or hatchlings, which are more closely linked to specific environments. Such ecological differentiation is not only important for identifying exposure pathways but also crucial for conservation, as it enables the development of targeted mitigation strategies targeted to each habitat type. Ultimately, a holistic understanding of turtles' habitat use across their migratory range is essential to guide effective protection efforts and ensure the long-term viability of these populations.

4.2 POP levels versus genotoxicity indicators

Genotoxic effects can be assessed using different blood parameters, including the evaluation of erythrocytic nuclear abnormalities in cell blood. This technique allows finding valuable insights into organism health (Casini et al., 2018; Morão et al., 2022), and helps identifying genotoxic impacts like chromatin fragmentation causing micronucleus formation (Zapata et al., 2016). However, the origin of lobed and kidney shaped nuclei and other abnormalities remains to be clarified (Casini et al., 2018; Morão et al., 2022). Some authors mentioned that lobed nuclei can be formed when tangled chromosomes or amplified genes are not properly separated, as the cell tries to remove extra DNA (Ergene et al., 2007), whereas segmented nuclei can happen due to problems during cell division, especially if the spindle is formed incorrectly, leading to irregular distribution of genetic material (Amorim et al., 2024). Furthermore, the formation of micronuclei is closely linked to chromosomal mis-segregation during cell division, often aggravated by the stabilization of kinetochore-microtubule attachments, which can interfere with proper chromosome separation and lead to the accumulation of genetic material as micronuclei (Cimini et al., 2004; Gomes et al., 2022). Microtubules are composed primarily of β-tubulin and α-tubulin in eukaryotic cells (Wade, 2007) which are important in several cellular processes, including cell motility and division (Moores, 2008). Proper tubulin dynamics are essential for accurate chromosome alignment and segregation during cell division. Disruptions in these dynamics can cause errors in chromosome distribution, leading to micronuclei formation and genomic instability. This highlights the importance of tubulin in maintaining genomic stability and preventing

micronuclei formation (Gomes et al., 2022). However, no correlations between the expression of *tuba1* gene and nuclear abnormalities in green sea turtles were found in the present study (MN: $r_s = 0.34$, $P = 0.16$; segmented: $r_s = 0.31$, $P = 0.19$; kidney: $r_s = -0.09$, $P = 0.72$; lobed: $r_s = -0.32$, $P = 0.18$) but other genes may be involved.

In a previous work where the results of ENA in green turtles were first described (Morão et al., 2024), some correlations could be seen between these abnormalities and some of metals analysed, being especially strong between Hg and lobed nuclei. However, as verified in the present study, the green sea turtles are being exposed to a myriad of other contaminants including the POPs analysed here, which can also be contributing to the formation of the observed nuclear abnormalities. In fact, strong positive correlations were also verified between lobed and kidney shaped abnormalities and different congeners of PCBs and DDTs.

These results are in accordance with the work by Sula et al. (2020), where PCBs and OCPs were found to be related to ENA formation in crucian carp, particularly with PCB-153 and DDD, although the type of abnormalities were not specified. The present findings correlating the presence of lobed nuclei with PCBs -28, -138, -153, and -180, as well as with *p,p'*-DDE, and the formation of kidney nuclei with *o,p'*-DDD and *o,p'*-DDE metabolites, represent the initial insights into congener specificity for distinct erythrocyte nuclear abnormalities.

Although specific congeners responsible for the formation of nuclear lesions were not identified, overall PCB have been associated with elevated levels of micronuclei and nuclear abnormalities in other teleost fish species (Al-Sabti, 1986). Moreover, increased occurrences of micronuclei and nuclear buds were observed in amphibian species sampled in agricultural regions with the presence of DDT and its metabolites (Cruz-Esquivel et al., 2017). Other pesticides such as bifenthrin, temephos, cyclophosphamide, and glyphosate, have also been linked to a higher incidence of general nuclear abnormalities in African common toad (Osman et al., 2022).

These results highlight the need for further research to elucidate the specific mechanisms and congener specificity involved in the formation of erythrocytic nuclear abnormalities in higher organisms, such as sea turtles.

4.3 POP levels versus gene expression

A major aim of this study was to relate the different levels of POPs analysed with gene expression responses, associated with antioxidant defence, and embryo development and

reproduction, thus exploring the potential consequences for the general fitness of female turtles and future populations, considering their crucial role as reproductive assets.

POPs in the marine environment can induce oxidative stress (Kumar et al., 2014; Regoli & Giuliani, 2014). Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the organism's antioxidant defences. Normally, organisms manage oxidative stress through natural antioxidant mechanisms such as the enzymes catalase, superoxide dismutase, or glutathione peroxidase. However, excess ROS or a compromised defence system can lead to oxidative stress which in turn can result in damage to proteins, lipids, and DNA, potentially impacting cellular structures and functions (Lemos, 2021).

The relationship between the induction of oxidative stress and increasing concentrations of organochloride pesticides has been shown in previous studies with sea turtles (Labrada-Martagón et al., 2011; Rivas-Hernández et al., 2023; Tremblay et al., 2017). However, there is a lack of research on the effects of other contaminants like PCBs or PBDEs on sea turtles, concerning oxidative stress-related effects or particularly addressing reproductive features. To our knowledge, this study provides the first approach to understanding sea turtle's responses to these contaminants.

PCBs are known to disrupt antioxidant defence-related enzymes and signalling pathways, thereby inducing oxidative stress (Liu et al., 2020). In the present study, three compounds – DL-PCB-126, PBDE-100, and *o,p'*-DDD – showed a positive correlation with most oxidative stress genes (*glrx3*, *gst*, *txnip*, *txnrd2*, and *gclc*). These results suggest that the sampled green sea turtles may be able to cope with oxidative stress caused by these contaminants, under the detected concentrations, by regulating key genes and activating the metabolic antioxidant pathways (Alberts et al., 2002).

Among the various PCB congeners, PCB-126 is recognised as one of the most toxic (Van den Berg et al., 2006). In this study, PCB-126 exhibited the most significant positive correlations with oxidative stress-related genes (namely, *gst*, *txnip*, *txnrd2*, and *gclc*), indicating that higher levels of contamination lead to an upregulation of these genes, at least until the maximum concentration detected. This upregulation may result in increased activity of enzymes that respond to the stress caused by this highly toxic congener. Consistent with present findings, a study on zebrafish revealed that PCB-126 elevates the expression of oxidative stress genes, such as orthologous *gclc*, *gpx* (glutathione peroxidase), and *gstp1* (glutathione S-transferase Pi 1) (Liu et al., 2015).

The PBDE-47, PBDE-99, and PBDE-100 are frequently detected in wildlife (Karpeta & Gregoraszczuk, 2010). Research has highlighted their varied impact on oxidative stress, mitochondrial membrane potential (MMP) response, cellular calcium levels, and the expression of apoptosis-associated genes (Xue et al., 2023). As mentioned by Bartalini et al. (2022), each congener has the potential to disrupt vital systems in various ways and at different levels, making it essential to conduct species-specific investigations and acquire a comprehensive understanding of toxicity effects induced by diverse substances and congeners. Notably, PBDE-47 stands out in the literature as the most cytotoxic among these congeners (Bartalini et al., 2022; Rajput et al., 2021) and is known to cause oxidative stress (He et al., 2008). In the context of this study, while PBDE-47 showed the highest average value, PBDE-100 exhibited more positive correlations with several oxidative stress genes, thus suggesting a greater impact of PBDE-100 and a higher level of toxicity to this species of sea turtles.

In relation to pesticides, several studies have reported the connection between oxidative stress and the presence of pesticides in the blood of sea turtles (Labrada-Martagón et al., 2011; Rivas-Hernández et al., 2023; Tremblay et al., 2017). Despite focusing on antioxidant enzyme activity, these studies have findings that align with the present ones. Labrada-Martagón et al. (2011) found a positive correlation between catalase enzyme activity and the sum of DDTs. Similarly, the present study showed that the expression of the gene encoding catalase was positively influenced by the presence of *o,p'*-DDD, suggesting that this metabolite may promote its upregulation. In Tremblay et al. (2017), an increase in the catalase activity was associated to HCHs which is also in agreement with the results of the green sea turtles studied here where β-HCH appear as the congener primarily influencing catalase upregulation.

Considering that the sampled individuals are nesting females and that endocrine and reproduction systems are largely regulated by lipids and their derivatives (Hari Kumar, 2018), along with the previously mentioned lipophilicity of POPs, a major objective of this study was to investigate whether these compounds could be impacting the expression of genes involved in reproduction and embryo development (*ace2, est17, tuba1, hoxA1,* and *nav3*).

The *ace2* gene is crucial for regulating cardiovascular and renal functions, along with fertility in humans (Fan et al., 2021; Pan et al., 2013) and in mice (Hagaman et al., 1998). Studies of laying hens have demonstrated that this gene is also involved in the secretion of albumen and the transportation of eggs through the oviduct (Sah et al., 2021). In this

work, positive correlations were found between the *ace2* gene and different PCBs and PBDEs, along with *o,p'*-DDD. Existing studies suggest that PCB exposure may affect the renin-angiotensin system (RAS), involving the *ace2* gene, potentially linking PCB exposure to hypertension through the influence on RAS-related gene expression (Perkins et al., 2016). In fact, in this work, PCBs were the family of POPs with a higher number of congeners (PCB-52, -101, -105, -114, -118, -123, and -156) showing significant correlations with the expression of this gene. While research on the connection between *ace2* expression and PBDE levels is lacking, PBDE contaminants are reported to negatively affect the reproductive system (Yu et al., 2015). The present findings suggest that certain PBDE congeners (-47 and -99) may influence *ace2* gene expression, possibly compromising the RAS system. Regarding OCPs, evidence indicates that perinatal DDT exposure can elevate the expression and activity of the RAS system (La Merrill et al., 2016). Here, the metabolite *o'p*-DDD (Kaushik & Kaushik, 2007), showed a positive correlation with *ace2,* suggesting its possible impact on this system as well. Overall, this link of *ace2* with RAS which plays a role in reproductive processes like embryo development, underscores that disruptions in the expression of this gene by these contaminants may impact follicular development, ovulation, and egg transport (Sah et al., 2021). Although direct evidence in reptiles is lacking, given the conserved role of ACE2 in cardiovascular, renal, and reproductive functions across vertebrates (Nishimura, 2016), it is plausible that these contaminants could similarly affect ACE2 expression in sea turtles, potentially disrupting RAS-mediated reproductive processes and posing a risk to the reproduction of this endangered population.

Additionally, it is important to highlight other genes that have shown significant positive correlations with the detected contaminants. One such gene is *est17*, which is essential for steroid synthesis and facilitates the transfer of vitellin from the pancreas to promote ovary development (Chakraborty et al., 2022; Fu et al., 2022). For this gene, the specific compounds α-HCH and β-HCH appear to have the most substantial impact by inducing its expression, suggesting that as disrupting chemicals, they may impair ovary development. Similar mechanisms have been observed in other reptiles exposed to estrogenic contaminants. For example, Marquez et al., (2011) reported that elevated hepatic estrogen receptor alpha (ERα) in adult and juvenile painted turtles exposed to dioxin-like contaminants suggested the presence of estrogen-like compounds, which could potentially interfere with vitellogenesis by altering the hepatic ER-mediated yolk deposition process. Furthermore, Hale et al., (2018) showed that developmental exposure

to estradiol in alligators led to persistent changes in ovarian gene expression, including dysregulation of ERα, estrogen receptor beta (ERβ), aryl hydrocarbon receptor (AHR) isoforms, and anti-müllerian hormone (AMH). Altered ESR2 expression, in particular, has been linked to reduced follicular development and impaired ovarian responsiveness to gonadotropins (Moore, et al., 2010), while disrupted AMH expression may further compromise ovarian function (Urushitani et al., 2011). Together, these findings support the hypothesis that HCHs and other POPs detected in São Tomé green sea turtles may interfere with ovarian maturation and vitellogenesis through similar estrogenic pathways, with potential consequences for reproductive success.

Regarding genes related to embryo development, *tuba1* encodes an intermediate filament protein, serving as a crucial element in the microtubule cytoskeleton during embryonic development (Casano et al., 1996; Chen et al., 2012). This gene exhibited positive correlations with PBDE-100 and *o,p'*-DDD, suggesting that these compounds are inducing its expression. However, contrasting evidence has shown that *tuba1* expression was significantly downregulated in zebrafish larvae exposed to PBDEs, linking this downregulation to adverse effects on neurodevelopment in the offspring (Chen et al., 2012). Furthermore, exposure to PBDE-47 in zebrafish embryos has been reported to cause abnormal neurobehavioral changes, affecting the expression of genes involved in central nervous system development, early neurogenesis, and axonal growth, including tubulin genes (Zheng et al., 2022). In reptiles, such as the lizard *Podarcis sicula*, α-tubulin its essential in ovarian follicle cells for the proliferation, migration, and transport of important components - including yolk proteins - to the oocyte during follicle differentiation critical for reproductive success (Maurizii et al., 2004). In fact, disrupted tubulin expression, which can lead to impaired microtubule function, has been linked to altered estrogenic biosynthesis and signalling pathways following exposure to POPs across various organisms (Amir et al., 2021; Díaz-Martín et al., 2021; Pan et al., 2023; Qiu et al., 2016; Wu et al., 2024; Zhang et al., 2020), highlighting the potential of these contaminants to act as endocrine disruptors. Taken together, these observations suggest that the induction of *tuba1* expression by PBDE-100 and o,p'-DDD in green sea turtles could have important implications for ovarian function, oocyte maturation, and early embryo development, even though direct studies in turtles are still lacking.

Additionally, the *nav3* gene has been associated with hepatocyte migration and heart development in zebrafish (Klein et al., 2011). It has also been linked to cerebellar development, cell migration, and axon growth (Accogli et al., 2022), suggesting its

crucial role in the development and morphogenesis of various cell types, particularly in neural development (Powers et al., 2023). The deletion and loss of function of the *nav3* gene resulted in deficiencies in cardiac morphology and structure in zebrafish (Lv et al., 2022). Furthermore, knocking down *nav3* impaired neurological growth (Ghaffar et al., 2024) and hepatocyte movement (Klein et al., 2011). Here, the results suggest that its expression may be induced by PCB-157, most PBDE congeners analysed (except for PBDE-47), and by the organochloride β-HCH, potentially indicating disruptions. However, the consequences of these changes for neuronal development remain uncertain.

Lastly, *hoxA1*, a member of the *Hox* gene family, actively contributes to hindbrain development and segmentation, shaping patterns throughout embryonic development (Hubert & Wellik, 2023). While PBDEs have been studied for their potential toxic effects (Reddam et al., 2023), specific information on their impact on the *hoxA1* gene is lacking. The present findings suggest that PBDE-154 is influencing *hoxA1* expression, as indicated by the positive correlation, suggesting potential alterations in embryonic development.

Regarding the immune response-related gene, *ig* encodes for a major antibody found in birds, amphibians and reptiles and primarily defends against pathogens by binding to and disabling them (Schroeder & Cavacini, 2010; Warr et al., 1995). Although this gene did not show significant correlations with any of the contaminants analysed, it exhibited a higher variation coefficient, as did the *hoxA1* gene. The lack of significant correlations and the high variation coefficient could indicate that these variations are due to other contaminants (e.g. metals) and/or factors, such as diseases, infections or climate stressors (Schroeder & Cavacini, 2010), not analysed here.

Overall, these results indicate how these specific compounds can differently disrupt bottom-line gene expression. The present findings suggest a potential link between POPs exposure and disruptions in gene expression related to processes controlling reproduction and embryonic development in nesting females, thereby possibly affecting both egg and embryonic development. Such risks are particularly concerning for São Tomé green sea turtles rookery, a genetically unique and conservation-relevant population with relatively high levels of genetic diversity and distinct characteristics (Formia et al., 2006; Hancock et al., 2019, Ferreira-Airaud et al. 2024).

These findings offer new insights into POP exposure in green sea turtles, though they should be interpreted having into consideration some limitations. The study was based on a relatively small number of individuals (n=21), which may limit statistical power and the

ability to detect subtle effects, all sampled during the nesting season, and included only adult females, which limits the ability to generalise to other demographic groups or capture seasonal variability in contaminant exposure. In addition, turtles' age was only estimated, which introduces some uncertainty into age-related comparisons. Addressing these aspects in future studies, by increasing sample size, including males and juveniles, extending sampling across seasons, and using molecular age markers such as DNA methylation to refine age estimation, would allow for more robust interpretation. A more integrative approach combining POPs with other contaminants (e.g., metals and PAHs) would also provide a more comprehensive understanding of multiple stressor effects, ultimately supporting more effective conservation strategies. Despite these limitations, the present study presents important insights into the potential molecular impacts of POP exposure on a critical life stage of green sea turtles, highlighting possible risks to their reproductive success and long-term viability of this endangered population.

5. Conclusions

This study presents the first attempt to evaluate POPs in green sea turtles nesting in the Gulf of Guinea region and relate them with expression profiles of biomarker genes indicative of oxidative stress and reproductive functions. Specific correlations between target genes and particular PCBs, PBDEs, and OCPs were identified and described in the whole blood of female green sea turtles nesting on São Tomé Island.

Lobed-shaped nuclei were the nuclear abnormality most strongly positively correlated with contaminants, particularly PCBs, mainly congeners -28, -138, -153, and -180. Regarding oxidative stress-related genes (*glrx3*, *gst*, *txnip*, *txrnd2*, and *gclc*), PCB-126, PBDE-100, and *o,p'*-DDD showed the most positive significant correlations with the expression of such genes, despite the general negative association trend observed across most congeners and metabolites analysed. For reproduction and embryo development genes, *nav3* was positively related to all PBDEs except -47, as well as PCB-157 and β-HCH. Lastly, *ace2* expression was mostly positively influenced by PCBs, specifically PCB-52, -101, -105, -114, -118, and -123.

These results highlight the importance of analysing the individual effects of each congener and metabolite, as distinct compounds may exert different biological effects. The detected levels of POPs in the blood of green sea turtles could lead to molecular and cellular changes, suggesting potential damage and disruptions to the reproductive fitness

of female individuals. Further detailed data on specific biomarkers of exposure and effect, as well as long-term monitoring of contaminant accumulation, is essential.

This study contributes with specific information on POPs, required to enhance environmental quality standards and safety thresholds for this and other migratory species. In sum, the findings may enable a promising strategy for future monitoring strategies of POP exposure in sea turtles and help identify contaminant-related risks to reproductive success—ultimately informing conservation actions.

Building on these findings, and as discussed, contamination likely occurs primarily at foraging grounds, given the short duration females spend at nesting sites in São Tomé Island. Nonetheless, future studies should expand to include life stages more closely tied to reproductive habitats—such as juveniles, post-incubation eggs, and hatchlings—to better assess local exposure. Adopting this broader ecological perspective will be key to developing effective, habitat-specific conservation strategies.

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Abbreviations list

2,3,7,8-TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
ace2	Angiotensin-converting enzyme 2
actb	Actin beta
agpat5	1-acylglycerol-3-phosphate O-acyltransferase 5
AHR	Aryl Hydrocarbon Receptor

AMH	Anti-Müllerian Hormone
cat	Catalase
CB	Chlorobenzenes
CCA	Canonical Correspondence Analysis
cDNA	Complementary DNA
CITES	Convention on International Trade in Endangered Species
Cm	*Chelonia mydas*
Cq	Quantification cycle
CV	Coefficient of variation
DDT	Dichlorodiphenyltrichloroethane
DGA	Direção Geral do Ambiente
DL	Dioxin-Like
DL-PCB	Dioxin-Like Polychlorinated Biphenyls
DNA	Deoxyribonucleic acid
e2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
eef1a1 -	Eukaryotic translation elongation factor 1 alpha 1
ENA	Erythrocyte Nuclear Abnormalities
est17	Estradiol 17-beta-dehydrogenase 11
fer	Ferritin
fg	Femtogram
Fw	Forward primer
GC	Gas Chromatography
GC-HRMS	Gas Chromatography-High Resolution Mass Spectrometry
gclc	Glutamate-cysteine ligase, catalytic subunit
gDNA	Genomic DNA
glrx3	Glutaredoxin 3
gpx	Glutathione Peroxidase
gr	Glutathione Reductase
GRx	Glutaredoxin
GSH	Glutathione
gsr	Glutathione Reductase
GSSG	Glutathione Disulfide
gst	Glutathione S-transferase Mu 1-like
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid

HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HCl	Hydrochloric acid
hoxA1	Homeobox Protein Hox-A1
ICNF	Instituto da Conservação da Natureza e das Florestas
ig	Immunoglobulin Y Heavy Chain
IQR	Interquartile Range
IUCN	International Union for Conservation of Nature
LOD	Limit of Detection
LOQ	Limit of Quantification
MN	Micronuclei
N	Sample number
NA	Not applicable
Na_2SO_4	Sodium sulphate
NaCl	Sodium Chloride
nav3	Neuron navigator 3
NCBI	National Center for Biotechnology Information
NDL	Non-Dioxin-Like
NDL-PCB	Non-Dioxin-Like Polychlorinated Biphenyls
NGO	Non-governmental organisation
NTC	Non-Template Controls
O_2^-	Peroxide ion
OCPs	Organochlorine Pesticides
o,p'-DDD	ortho,para-Dichlorodiphenyldichloroethane
o,p'-DDE	ortho,para-Dichlorodiphenyldichloroethylene
o,p'-DDT	ortho,para-Dichlorodiphenyltrichloroethane
PAHs	Polycyclic Aromatic Hydrocarbons
PBDEs	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated Biphenyls
PCR	Polymerase chain reaction
PeCB	Pentachlobenzene
POPs	Persistent Organic Pollutants
p,p'-DDD	para,para-Dichlorodiphenyldichloroethane
p,p'-DDE	para,para-Dichlorodiphenyldichloroethylene
p,p'-DDT	para,para-Dichlorodiphenyltrichloroethane
p-value	Statistical Significance Indicator

QA/QC	Quality Assurance/Quality Control
qPCR	Quantitative Real-Time PCR
R^2	Coefficient of determination
RNA	Ribonucleic Acid
RAS	Renin-Angiotensin System
ROS	Reactive Oxygen Species
rpl4	Ribosomal Protein L4
rps13	Ribosomal Protein S13
rps15	Ribosomal Protein S15
rps2	Ribosomal Protein S2
Rv	Reverse primer
SE	Standard Error
selp	Selenoprotein P
SOD	Superoxide Dismutase
sod1	superoxide dismutase 1
srebf2	Sterol regulatory element binding transcription factor 2
STP	São Tomé and Príncipe
tdx	Thioredoxin
TEF	Toxic Equivalency Factors
TEQ	Toxic Equivalent Quantities
tuba1	Tubulin alpha 1
txnip	Thioredoxin interacting protein
txrnd2	Thioredoxin reductase 2
α-HCH	Alpha-Hexachlorocyclohexane
β-HCH	Beta-Hexachlorocyclohexane
γ-HCH	Gamma-Hexachlorocyclohexane (Lindane)
ΔΔ Cq	Quantification Cycle
ΔΔ CT	Relative Quantification

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Declaration of interests

☒ 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.

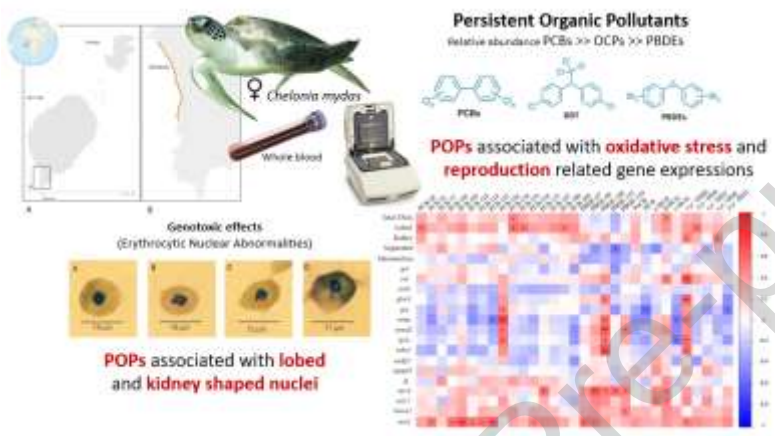
☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Environmental Implications

Persistent organic pollutants (POPs) are hazardous contaminants that can disrupt key physiological functions in wildlife. This study shows that different groups of POPs bioaccumulate in nesting green sea turtles from São Tomé and can be linked to genotoxicity and altered gene expression related to antioxidant, detoxification,

reproduction, and embryo development processes. These changes may disrupt critical metabolic and reproductive pathways, representing an additional risk to this endangered population. The findings underscore the distinct impacts of various POPs chemicals and highlight the urgent necessity for targeted monitoring of POPs in the Gulf of Guinea to ensure the protection of biodiversity in the area.

Graphical abstract



Highlights

- First assessment of POP effects on green sea turtles in the Gulf of Guinea.
- First report TEQ of POPs values in blood of nesting female green sea turtles.
- PCBs and OCPs correlated with erythrocyte nuclear abnormalities in nesting females.
- Blood POP levels linked to antioxidant and detoxification related gene expressions.
- POPs exposure in nesting females may impact their reproductive capacity.