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Blue sharks (*Prionace glauca*) as bioindicators of pollution and health in the Atlantic Ocean: Contamination levels and biochemical stress responses

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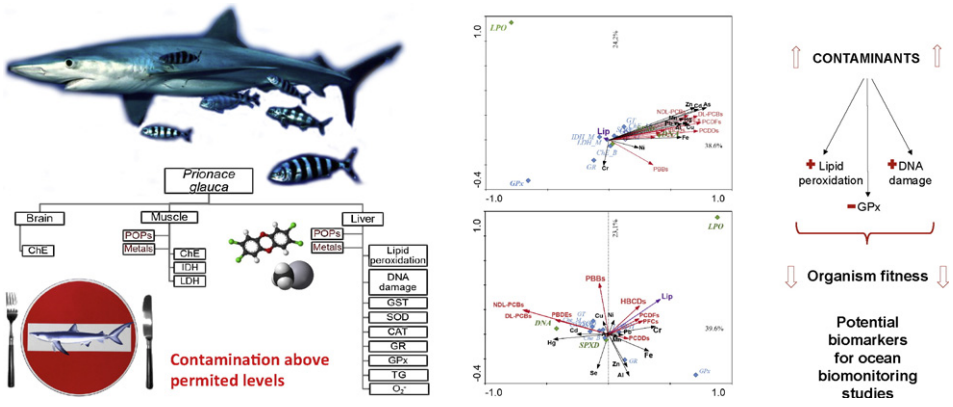
HIGHLIGHTS

- Metals and POPs were quantified in the muscle and liver of blue sharks.
- Biochemical stress responses were addressed in the same sharks.
- Correlations between contaminant levels and biochemical responses were found.
- Suitable biomarkers for future pollution biomonitoring studies were proposed.
- Sharks presented contamination values above regulatory limits for human consumption.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine ecosystems are constantly being threatened by contaminants produced by human activities. There is an urge to better understand their impacts on marine organisms and develop reliable tools for biomonitoring studies, while also assessing their potential impacts on human health. Given their position on top of food webs, sharks are particularly susceptible to bioaccumulation, making them potential sentinel species of marine contamination. The main objective of this study was to find suitable biomarkers for future marine pollution biomonitoring studies by correlating biochemical responses with tissue contaminant body burden in blue sharks (*Prionace glauca*), a species heavily caught and consumed by humans, while also addressing their general health. The chemical contaminants analysed comprised different persistent organic pollutants (POPs) families from polychlorinated compounds to brominated flame retardants (BFRs) and perfluorinated compounds (PFCs) and different trace and heavy metals. Concentrations of some contaminants in sharks' tissues were found to be above the legally allowed limits for human consumption. A canonical correspondence analysis (CCA) was performed and some strong associations were found between biochemical responses and contaminants' accumulation levels. DNA damage and lipid peroxidation levels, as well as the inhibition of the antioxidant enzyme glutathione peroxidase, were the main effects and consequences of contamination. The impact of contamination on these vital macromolecules underlines the suboptimal conditions of the sampled *P. glauca*, which can ultimately lead to the degradation of

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core ecological aspects, such as swimming, feeding, and reproduction. It can be concluded that *P. glauca* demonstrates great potential to be used as environmental sentinel and suitable biomarker candidates were identified in this work. Moreover, this study also highlights the risks that the consumption of blue shark derived products can pose to human health, which is of upmost interest as the sampled organisms were still juveniles and already presented values above regulatory limits.

1. Introduction

Marine ecosystems are being continuously loaded with xenobiotics produced by human activities, very often affecting aquatic organisms (Van der Oost et al., 2003). Persistent organic pollutants (POPs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs) and perfluorinated compounds (PFCs), or metals such as arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) can negatively affect marine fauna and human health given their high toxicity and persistence in the environment, leading to bioaccumulation processes (Heath, 1995; Storelli et al., 2002; Gramatica and Papa, 2007; Storelli et al., 2011; Skomal and Mandelman, 2012; Barrera-García et al., 2013). Bioaccumulation of these xenobiotics is a growing concern and has been shown to cause injurious effects on biodiversity (Franke et al., 1994; Marchettini et al., 2001; Wang, 2002).

The changes caused by pollutants in the ecosystem usually have an earlier effect at lower levels of biological organization, such as at the organism level or even at the gene and cellular level, allowing the development of biomarkers to monitor changes caused by the contaminants, before they cause an effect at higher complexity levels – communities or ecosystems (Bayne et al., 1985; Lemos et al., 2010). In order to assess the health of marine ecosystems, different biological parameters with the potential to be used as biomarkers may be of use, such as oxidative stress related enzymatic activities, DNA damage and lipid peroxidation (LPO) as measurements of oxidative damage, and indicators of neuromuscular activity and energy expenditure (Winston and Di Giulio, 1991; Filho, 1996; Van der Oost et al., 2003).

Usually, when a contaminant enters a living organism, a two-phase detoxification process is initiated in order to facilitate its elimination (Chen, 2012). Glutathione-*S*-transferase (GST) plays a role in the second phase of the detoxification process, where it facilitates the excretion of xenobiotics (Van der Oost et al., 2003). This process is essential but increases the already naturally occurring cellular oxidation (Valko et al., 2007). Furthermore, the presence of POPs and metal contaminants further increases the amount of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), forcing the cells to fight the harmful effects of these oxidizing molecules (Buet et al., 2006; Kumar et al., 2014). Defence mechanisms to eliminate ROS, and prevent oxidative damage, include enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD is an enzyme responsible for the transformation of $O_2^{\bullet-}$ into H_2O_2 that is then eliminated by the enzymes CAT and GPx, both acting to prevent its accumulation. GR ensures that reduced glutathione (GSH) is available to act as an antioxidant itself, or as a cofactor for GPx and GST (Egaas et al., 1995; Halliwell and Gutteridge, 2001; Livingstone, 2001; Richardson et al., 2008; Valko et al., 2007).

All the internal responses of the organisms to cope with pollution stress are highly energy-costly. Examples of biomarkers that can be used to assess effects related with changes in the energy metabolism are the enzymes lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH), which are involved in the anaerobic and aerobic metabolism, respectively (Bernal et al., 2003; Walsh et al., 2006), and have been successfully applied in studies addressing effects of marine contamination by metals (e.g. Antognelli et al., 2003; Vieira et al., 2009), or by different organic pollutants (e.g. Monteiro et al., 2006; Greco et al., 2007).

Acetylcholinesterase (AChE), a vital enzyme for good neuronal functions in vertebrates, is highly sensitive to anti-cholinergic compounds and other contaminants often present in marine ecosystems, and has already demonstrated great potential to be used in pollution monitoring studies as a biomarker of effect (Payne et al., 1996; Kirby et al., 2000; Van der Oost et al., 2003; Arufe et al., 2007; Solé et al., 2008; Alves et al., 2015).

Due to their role as apex predators, elasmobranchs such as sharks end up being more exposed to environmental contamination through bioaccumulation and biomagnification processes through the food web (Serrano et al., 2000; Strid et al., 2007). Additionally, their wide distribution and importance to the ecosystem makes them ideal sentinel organisms for marine pollution biomonitoring studies (Marcovecchio et al., 1991; Vas, 1991). Sharks are known to accumulate high concentrations of metals (Storelli et al., 2002; Pethybridge et al., 2010; Storelli and Marcotrigiano, 2004; Turoczy et al., 2000). Some of them are essential for physiological processes (e.g., Fe and Zn), while others do not have any recognized physiological purpose (e.g., Hg and Pb) (Barrera-García et al., 2013). To know the concentrations of these metals present in fish muscle, and particularly Hg, is of utmost importance since in most cases this is the main route of exposure to these elements in humans (Järup, 2003; Khayatzadeh and Abbasi, 2010). Furthermore, Hg can be found in different forms and one of the most toxic, methylmercury (meHg), accounts for >95% of the total Hg found in the muscle of fishes (Krystek and Ritsema, 2005; Piraino and Taylor, 2009; Payne and Taylor, 2010). Sharks have also been found to accumulate large amounts of POPs in their muscle and liver tissues (e.g., Johnson-Restrepo et al., 2005; Storelli et al., 2005, 2011). These compounds have even been found in sharks from remote areas as the Artic (Strid et al., 2007), and they can severely impair the health of both marine organisms and humans (Mandal, 2005; Foster et al., 2012).

The blue shark (*Prionace glauca*, L. 1758) is one of the most frequently caught shark species all over the world and in particular by the Portuguese longline swordfish fishing fleet as bycatch (Bonfil, 1994; Santos et al., 2002; Stevens, 2009). Some recent studies have demonstrated the potential of this species to be used as a biomonitor of marine contamination (Storelli et al., 2011; Barrera-García et al., 2012, 2013), but very little is known about the mechanisms involved in these sharks' detoxification and antioxidant processes, or other mechanisms of response to chemical pollutants. Understanding these relations is of upmost importance to public health since almost 122,000 t of shark meat were imported worldwide in 2011, with *P. glauca* increasingly becoming the preferential source of both fins and meat (Dent and Clarke, 2015; Eriksson and Clarke, 2015).

The main objective of the present study is two-folded: 1) Do Atlantic blue sharks present high levels of POPs and metals in their tissues that can pose a risk for these organisms as well as for humans health?; and 2) Are the contaminant body burden levels correlated with biochemical responses of stress in the organisms?

With this approach, reliable biomarkers for *P. glauca* can be addressed as prospect tools for biomonitoring Atlantic waters. Also, to our knowledge, this is one of the most comprehensive studies of its kind, attempting to integrate the levels of different contamination sources (e.g. metals and POPs) with detoxification pathways, oxidative

stress response mechanisms, and energetic and neuronal parameters in sharks.

2. Materials and methods

2.1. Organisms

Twenty *P. glauca* individuals were captured at a depth of approximately 20 m, southwest of Portugal (36°43′11.2″N, 13°09′30.0″W), aboard a commercial swordfishing vessel as bycatch. Captured sharks were sacrificed by the fisherman after landing on the vessel, and the procedure of capture and handling was performed as fast as possible and in a similar way between individuals. Immediately after capture, tissues (liver, muscle, and brain) were collected from each individual taking the appropriate measures to minimize cross-contamination – dissections were performed in a closed space inside the fishing vessel (specially dedicated for this work), and all material and working station were cleaned with ethanol before each dissection. Samples were preserved on ice and transported to the laboratory, where they were stored at −80 °C until further analysis. Size (total length, cm) and gender were recorded for all sampled individuals. All sharks in this study were classified as juveniles according to Pratt (1979).

2.2. Contaminant chemical analysis

2.2.1. POPs

Different POP families were determined in the tissues of each individual. Prior to analysis, muscle and liver tissues were freeze-dried, ground and homogenized. Regarding polychlorinated compounds, approx. 3 g (dry weight) of liver and muscle samples were analysed for the following congeners: 17 PCDDs and PCDFs, 12 dioxin-like polychlorinated biphenyls (DL-PCBs) and 6 non dioxin-like PCBs (NDL-PCBs) (Table S1 – supplementary data). Identification and quantification of these compounds were performed by gas chromatography (GC; 7890 A; Agilent Technologies, CA, USA) coupled to a double electromagnetic sector high resolution mass spectrometer (HRMS; JMS-700D and 800D, Jeol, Japan). The analysis of BFRs was performed using approx. 3 g (dry weight) of liver and muscle tissues and focused on 8 polybrominated diphenyl ethers (PBDEs), 3 polybrominated biphenyl (PBBs) and 3 diastereomeric pairs of enantiomers of 1,2,5,6,9,10-hexabromocyclododecane (HBCDs). Identification and quantification of PBDEs and PBBs were also achieved by GC-HRMS, while HBCDs were determined by liquid chromatography (LC; Agilent 1200 series) coupled with tandem mass spectrometry (MS/MS; Agilent 6410 triple quadupole). The analysis of PFCs was done using approx. 1 g (dry weight) of liver and muscle samples and included 9 perfluoroalkyl carboxylic acids (PFCAs) and 5 perfluoroalkyl sulfonic acids (PFSAs). Identification and quantification were performed by LC (Agilent 1200 series) coupled to MS/MS (LTQ-Orbitrap™, Thermo Fisher Scientific Inc., MA, USA). Further details can be found as supplementary data (Text S1). All the POP analyses were performed at the French national reference laboratory for PCDD/F and PCB analysis in food and feed, according to validated and accredited methods (ISO/IEC 17025:2005 standard).

2.2.2. Metals

Liver and muscle samples from each individual were used for trace and heavy metals quantification. In each sample, the concentrations of the following metals were analysed: Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Ag, Cd, Pb, and Hg. Digestion of the tissues was performed by adding 3 mL of HNO_3 to 200 mg of tissue. Samples were digested in a microwave oven for 10 min, at 175 ± 5 °C. After cooling to room temperature, 0.1 mL of H_2O_2 were added to the samples and a new digestion was performed for 5 min at 175 ± 5 °C. From each digested tissue sample, the different metal concentrations were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a Thermo X-Series ICP-MS spectrometer (Thermo Fisher Scientific Inc., MA, USA).

2.3. Biochemical responses

2.3.1. Tissue preparation

After defrosting, tissue samples were homogenized and separated for different biochemical measurements, depending on the tissue. Approximately 300 mg of liver from each organism were homogenized, in a 1:10 proportion, in K-phosphate buffer (0.1 M, pH 7.4). Part of the tissue homogenate was transferred to a microtube containing BHT (2.6-dieter-butyl-4-metylphenol) 4% in methanol to prevent tissue oxidation for further determination of LPO. Another portion was separated for DNA strand breaks quantification and the rest was centrifuged at 10,000 g for 20 min (4 °C) to isolate the post mitochondrial supernatant (PMS), which was then separated into different microtubes and stored at −80 °C for posterior protein and total levels of glutathione (TG) quantification, as well as for the activity measurements of GST, SOD, CAT, GPx, and GR. An additional 100 mg of liver tissue were used for the quantification of $O_2^{\cdot-}$ as a measurement of ROS.

A sample of muscle tissue from each organism (200 mg) was homogenized in K-phosphate buffer (0.1 M, pH 7.2), in a 1:5 proportion. The homogenate was then centrifuged at 3000 g for 3 min (4 °C) and the supernatant was separated into different microtubes and stored at −80 °C, for posterior protein quantification and measurements of LDH, IDH, and cholinesterases (ChE) activities.

Regarding the brain, the preparation was similar to that used for muscle analysis with the exception that only protein concentration and ChE activity measurements were performed with this tissue's supernatant.

In all biochemical assays, blanks were made using K-phosphate buffer instead of the sample. All spectrophotometric measurements were performed, in quadruplicates and at 25 °C, in a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

2.3.2. Protein quantification

Before the enzymatic assays, the soluble proteins were quantified according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford microassay set up in a 96-well flat bottom plate, using bovine γ-globuline as protein standard. Absorbance was read at 600 nm and results were expressed in mg of protein mL^{-1}.

2.3.3. Detoxification and antioxidant mechanisms

For the assessment of GST (EC 2.5.1.18) activity, an adaptation of the procedure described by Habig et al. (1974) was used. The formation of the thioether glutathione dinitrobenzene, a product of the reaction between GSH and CDNB, was followed at 340 nm for 3 min. GST activity was calculated, using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$, and expressed in nmol $min^{-1}\ mg^{-1}$ of protein. The activity of SOD (EC 1.15.1.1) was measured performing an adaptation of the method described by McCord and Fridovich (1969), using the xanthine/xanthine oxidase mediated reduction of cytochrome C. The decrease of the cytochrome C reduction was followed at 550 nm and SOD activity was expressed in U mg^{-1} of protein using a SOD standard of 1.5 U ml^{-1}, where 1 U represents the amount of enzyme in the sample that causes 50% inhibition of cytochrome C reduction. CAT (EC 1.11.1.6) activity was determined by following the decay in the H_2O_2 concentration at 240 nm, adapting the method described by Claiborne (1985). Absorbance was read every 10 s for 1 min. CAT activity was expressed in nmol $min^{-1}\ mg^{-1}$ of protein, using a molar extinction coefficient of 40 $M^{-1}\ cm^{-1}$. GR (EC 1.8.1.7) catalytic activity was measured by following the decrease in absorbance during NADPH oxidation, according to Cribb et al. (1989). Absorbance was read at 340 nm for 1 min. The enzymatic activity was calculated using a molar extinction coefficient of $6.2 \times 10^3\ M^{-1}\ cm^{-1}$, and expressed in nmol $min^{-1}\ mg^{-1}$ of protein. GPx (EC 1.11.1.9) activity was measured by

monitoring the oxidation of NADPH, when GSSG is reduced back to GSH by GR, using H_2O_2 as a substrate (Mohandas et al., 1984). The activity was measured following the absorbance at 340 nm for 1 min and expressed in nmol min^{-1} mg^{-1} of protein, using a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1}. Total glutathione (TG) levels were measured using the recycling reaction of GSH with DTNB in the presence of GR excess (Tietze, 1969). The absorbance was read at 421 nm and TG levels were calculated using the molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}. Results were expressed in nmol min^{-1} mg^{-1} of protein.

2.3.4. Oxidative stress and oxidative damage parameters

For evaluating the effects of ROS, the superoxide anion radical (O_2^-) was quantified by following the method described by Drossos et al. (1995). Krebs buffer was added to the 100 mg of liver tissue from each animal, and the samples were left resting for 5 min. After the addition of cytochrome C (15 μM), the samples were incubated at 37 °C in a shaking water bath for 15 min. To stop the reaction, *N*-ethylmaleimide (3 mM) was added. Samples were finally centrifuged for 10 min at 1509 g and the supernatant was transferred to a microplate. The presence of O_2^- was determined by the capacity of the radicals to reduce cytochrome C, which can be measured at 550 nm, with an extinction coefficient of 19×10^3 M^{-1} cm^{-1}. The amount of O_2^- produced was calculated and expressed in nmol O_2^- mg^{-1} of protein. LPO levels were assessed by measuring the content of thiobarbituric acid reactive substances (TBARS), using the method described by Ohkawa et al. (1979) and Bird and Draper (1984) with the modifications made by Wilhem et al. (2001) and Torres et al. (2002). TBARS were measured at 535 nm and the results were calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as nmol TBARS g^{-1} of wet weight (ww). The DNA strand breaks were measured using the DNA alkaline precipitation assay (Olive, 1988), adapted from LaFontaine et al. (2000). Fluorescence was measured using an excitation/emission wavelength of 360/450 nm and the results were expressed as mg of DNA g^{-1} of ww, using calf thymus DNA as standard.

2.3.5. Energy metabolism and neuronal related parameters

LDH (EC 1.1.1.27) activity was assessed using the method described by Vassault (1983) and later adapted by Diamantino et al. (2001), following the oxidation of NADH when pyruvate is converted to lactate. The absorbance was read at 340 nm for 5 min and the results were expressed as nmol min^{-1} mg^{-1} of protein, using a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1}. IDH (EC 1.1.1.42) activity was measured following the protocol described and adapted by Ellis and Goldberg (1971) and Lima et al. (2007), respectively. The activity of IDH was determined by following the increase in NADPH, which is concomitant with the decarboxylation of isocitrate by IDH. The change in absorbance was measured at 340 nm for 3 min and results expressed as nmol min^{-1} mg^{-1} of protein, using a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1}. The ChE activities were measured in muscle and brain samples of each organism following the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). Absorbance was followed for 5 min, at 414 nm. ChE activity was calculated using the molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1} and expressed in nmol min^{-1} mg^{-1} of protein.

2.4. Statistical analysis

All data were checked for normality and homoscedasticity. To compare gender and size, as well as metal concentrations between tissues, independent sample Student *t*-test or Mann-Whitney non-parametric test were conducted (depending on the violation or not of the normality and homogeneity of variance assumptions). Correlations between biochemical parameters were assessed using Pearson Correlation tests. For all statistical tests, the significance level was set at $p \leq 0.05$. Where applicable, results are expressed as mean ± standard-deviation. A Canonical Correspondence Analysis (CCA) (Ter Braak, 1986) was performed to evaluate the pattern of distribution and correlation based on the contaminants' concentrations and biochemical biomarkers. The resultant ordination biplot approximated the weighted average of enzymatic and non-enzymatic biomarkers (points) measured with regard to each of the organic pollutants (POPs) and metals quantified, which were represented as arrows. The lengths of these arrows indicated the

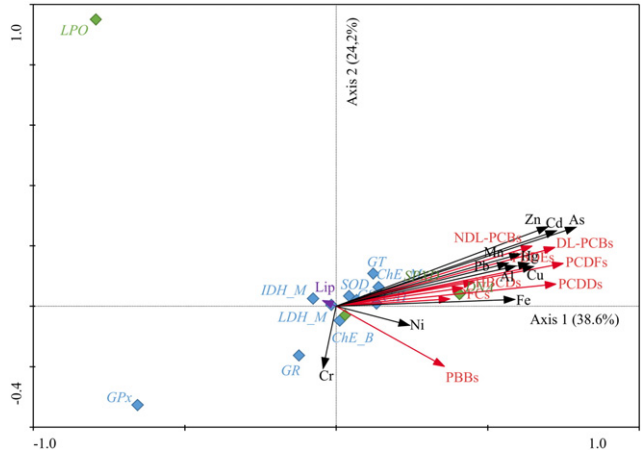

Fig. 1. Biplot of axes 1 and 2 of the Canonical Correspondence Analysis (CCA) on contaminants and biochemical parameters data, on liver samples. Arrows indicate the different types of persistent organic pollutants (POPs) and metals quantified, blue diamonds represent enzymatic biomarkers measured, and green diamonds represent non-enzymatic biomarkers measured. *POPs abbreviations*: PCDDs = polychlorinated dibenzo-*p*-dioxins; PCDFs = polychlorinated dibenzofurans; NDL-PCB = non-dioxin-like polychlorinated biphenyls; HBCDs = hexabromocyclododecanes; DL-PCBs = dioxin-like polychlorinated biphenyls; PBDEs = Polybrominated diphenyl ethers; Lip = lipid percentage; PBBs = Polybrominated biphenyls; PFCs = perfluorinated compounds. *Biomarkers abbreviations*: LDH = lactate dehydrogenase; IDH = isocitrate dehydrogenase; TG = total glutathione; GPx = glutathione peroxidase; GST = glutathione *S*-transferase; GR = glutathione reductase; SOD = superoxide dismutase; CAT = catalase; ChE_M = cholinesterase measured in muscle; ChE_B = cholinesterase measured in brain; LPO = lipid peroxidation; SPXD = superoxide anion radical; DNA = DNA damage. *Colour code*: red – POPs; black – metals; Purple – Lipids; Green – non-enzymatic parameters; Blue – enzymatic parameters.

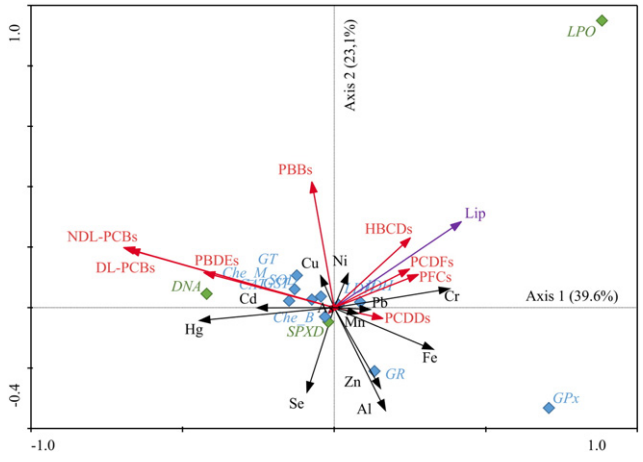

Fig. 2. Biplot of axes 1 and 2 of the Canonical Correspondence Analysis (CCA) on contaminants and biochemical parameters data, on muscle samples. Arrows indicate the different types of persistent organic pollutants (POPs) and metals quantified, blue diamonds represent enzymatic biomarkers measured, and green diamonds represent non-enzymatic biomarkers measured. Abbreviations, symbols and colour codes are as in Fig. 1.

Table 1
Comparison of Persistent Organic Pollutant (POP) concentrations (ng g^{-1} lipid weight) found in muscle and liver samples of different pelagic shark species. Only the most abundant compounds per POP family, which had data from the literature to compare, are shown. Data is presented as mean, mean ± standard deviation or range of values (minimum-maximum), as found on the literature.

Tissue	N	Size (cm)	Species	Sampling site	PFCs		BFRs	
					PFSAs	PFCAs		
					PFOS	PFUnA	PBDE 47	PBDE 100
Muscle	**20**	**112–167**	***P. glauca***	**NE Atlantic**	**0.15 ± 0.10***	**0.37 ± 0.19***	**48.51 ± 16.93**	**171.54 ± 137.30**
	15	112 ± 20	*P. glauca*	Pacific			0.64	3.59
	7		*Isurus oxyrinchus*	Pacific			0.40	0.41
	13		*A. pelagicus*	Pacific			0.66	0.20
	3	82.3–97.1	*S. tiburo*	NE Atlantic	<0.1–0.4*	<0.1–0.1*		
	10	355–480	*Somniosus microcephalus*	NE Atlantic				
Liver	**20**	**112–167**	***P. glauca***	**NE Atlantic**	**1.46 ± 0.81***	**6.88 ± 3.89***	**1260.54 ± 414.15**	**4815.79 ± 2317.27**
	22	105–124	*P. glauca*	SE Mediterranean				
	44	80.5–212.0	*P. glauca*	Mediterranean				
	3	82.3–97.1	*S. tiburo*	NW Atlantic	0.2–6.3*	0.1–2.6*		
	10	355–480	*S. microcephalus*	NE Atlantic				
	64	84–102	*Dalatias licha*	Mediterranean				

[1] Present work; [2] (Lee et al., 2015); [3] (Storelli et al., 2011); [4] (Kumar et al., 2009); [5] (Strid et al., 2007); [6] (Storelli et al., 2005).
PFCs = perfluorinated compounds; PFSAs = perfluoroalkane sulfonates; PFCAs = perfluorinated carboxylic acids; PFUnA = perfluoroundecanoic acid; PFOS = perfluorooctane sulfonate; BFRs = brominated flame retardants; PCBs = polychlorinated biphenyls; DL-PCB = dioxin like polychlorinated biphenyls; NDL-PCBs = non-dioxin like polychlorinated biphenyls; PCDDs = polychlorinated dibenzo-*p*-dioxins; PCDFs = polychlorinated dibenzofurans; PBDEs = Polybrominated diphenyl ethers.
* Values presented in ng g^{-1} wet weight.
** Values presented in pg g^{-1} lipid weight.

relative importance of POP and metals, explaining variations in the enzymatic and non-enzymatic biomarkers' profiles, whereas the angle between the arrows and the axis reflected the degree to which they were correlated. Thus, the length of the arrow is proportional to the change in that direction. Those contaminants factors that have long arrows are more closely correlated in the ordination than those with short arrows, and are more important in influencing biomarkers variation. Contaminant data were standardized and the biomarkers data were log (x + 1) transformed (Legendre and Legendre, 1979). Downweighting of biochemical biomarkers were performed.

All univariate statistical tests were performed with Sigma plot 11.0 (Systat Software, Inc. Chicago, IL, USA). CCA was performed with CANOCO version 4.5 package (Ter Braak and Smilauer, 1998).

3. Results

The 20 sharks sampled in the present study consisted of 12 females and 8 males, and ranged from 112 to 167 cm total length (TL). Although all individuals were considered juveniles, samples were also grouped according to sex and size, in order to assess eventual effects of these physiological characteristics. Size groups were defined as: 1) individuals with <130 cm TL (lower than the average TL); and 2) individuals over 130 cm TL (higher than the average TL).

3.1. Contaminant chemical analysis

The concentrations of POPs and metals measured in muscle and liver of the sampled sharks are summarized in Tables S1 and S2 (supplementary data).

Regarding the POP concentrations the results showed that NDL-PCBs were the dominant chemicals in muscle tissue, with 1.12 ± 1.04 ng g^{-1} ww, followed by PFCs and DL-PCBs, with 0.62 ± 0.32 ng g^{-1} ww and 0.10 ± 0.08 ng g^{-1} ww, respectively (Table S1 – supplementary data). In liver tissue, it is possible to observe that, although in higher levels, the dominant contaminants are mostly in accordance with the ones in muscle, with the concentrations being 328.44 ± 273.32 ng g^{-1} ww for NDL-PCBs, 45.97 ± 43.97 ng g^{-1} ww for DL-PCBs and 12.03 ± 6.16 ng g^{-1} ww for PFCs (Table S1 – supplementary data). In general, liver samples showed higher concentrations of POPs when compared with the respective contaminants in muscle. No statistically significant differences were found between genders in terms of POP accumulation ($p > 0.05$), although a positive correlation with size was observed for NDL-PCBs in muscle ($r = 0.497$, $p = 0.026$) and both DL- and NDL-PCBs in liver ($r = 0.511$, $p = 0.025$; and $r = 0.536$, $p = 0.018$, respectively).

The metal analysis showed that As and Hg concentrations were significantly higher ($p < 0.001$) in the muscle tissue, whereas the metals Mn, Fe, Cu, Zn, and Cd were more concentrated (Table S2 – supplementary data). Concentrations of Ag, Ni, Se, and Cd in the muscle were below the minimum detection level (MDL) in >50% of samples; the same was observed for Ni, Se, and Ag concentrations in liver. Hg levels in both muscle and liver tissues proved to be positively correlated with the size of the individuals ($r = 0.454$, $p = 0.044$; and $r = 0.538$, $p = 0.047$, respectively), meaning that as the size of the animals increases, so does the concentration of Hg in their tissues.

3.2. Biochemical responses

Considering the measured enzymatic activities of the whole group of 20 individuals, CAT, showed the highest mean activity (18.45 ± 5.55 μmol min^{-1} mg^{-1} protein), followed by LDH (3.55 ± 1.37 μmol min^{-1} mg^{-1} protein), SOD (0.17 ± 0.06 μmol min^{-1} mg^{-1} protein), and GST (0.11 ± 0.03 μmol min^{-1} mg^{-1} protein).

The biochemical responses did not seem to be influenced by gender or size since none of the Student *t*-tests performed individually for the different biomarkers showed any statistical significant differences between male and female responses or between the two different size groups ($p > 0.05$).

However, significant correlations were found between the several biochemical responses quantified in the different tissues of the organisms (Table S3 – supplementary data). Activities of GST and CAT were positively correlated, meaning that whenever the activity of one of these enzymes is induced/inhibited, the other enzyme followed the same pattern of response. The other pair of variables with a high significant correlation ($p < 0.01$), albeit negative, was IDH and O_2^- (Table S3 – supplementary data).

3.3. Contaminants versus biochemical responses

A multivariate analysis was performed in order to evaluate the relation between environmental contaminants present in the liver tissues of each shark and their biochemical stress responses. Therefore, CCA was

PCBs				PCDDs		PCDFs		REFs
DL-PCBs		NDL-PCBs						
PCB 105	PCB 118	PCB 138	PCB 153	1.2.3.6.7.8 - HxCDD	1.2.3.7.8 - PeCDD	2.3.4.7.8 - PeCDF	2.3.7.8 – TCDF	
876.42 ± 764.3	**4097.75 ± 3669.1**	**17.92 ± 20.47**	**30.63 ± 34.6**	**0.15 ± 0.07****	**0.08 ± 0.04****	**0.17 ± 0.08****	**0.16 ± 0.12****	[1]
								[2]
								[2]
								[2]
								[4]
110	470	1100	1200	0.67**	0.58**	1.3**	5.8**	[5]
18.47 ± 22.65	**68.36 ± 78.37**	**198.63 ± 195.91**	**353.07 ± 315.88**	**1.95 ± 2.63****	**1.37 ± 2.15****	**5.80 ± 10.33****	**6.86 ± 11.17****	]1[
3.07	52	193	307	56	15	26	179	[3]
	229 ± 95	616 ± 260	915 ± 383					[6]
								[4]
130	500	1200	1300	20**	24**	73**	280**	[5]
	136 ± 27	519 ± 128	605 ± 140					[6]

performed and its ordination results produced the biplot presented in Fig. 1.

Although the analysis was performed for all axes, only the first factorial plane (axes 1 and 2) is shown, as they retain most of the variability in the data analysis (i.e., 62.8% of the total explained variability; see Fig. 1). Beyond that, the results showed that POPs and metals are correlated with both axes; however the highest correlations are with the first axis. Namely, along the first axis the biochemical stress responses accounted for 38.6% of the total variation, followed by axis 2 which explained 24.2% of the variability. Thus biplot diagram resulting from the CCA (Fig. 1) revealed that all POPs presented a positive correlation with each other. Moreover, this result demonstrated that the activities or levels of the enzymatic and non-enzymatic biomarkers could be influenced by POPs and metals.

The results showed that axis 1 is primarily driven by PCDFs, PCDDs, DL-PCBs, As, Cd, and Zn, while axis 2 is mainly influenced by Cr (although less expressive) (Fig. 1). The CCA analysis indicated that low GPx activity, as well as high DNA damage levels, were the biomarker responses more strongly characterized by high levels of most contaminants (POPs and metals). LPO levels can also be associated with PBBs levels. Note that the variables HBCD, PFCs, Ni, and Cr have a lower importance in determining the observed default pattern.

Additionally, a CCA was performed in order to evaluate the relation between environmental contaminants present in muscle tissue and biochemical stress responses. Ordination resulting from the CCA produced the biplot showed in Fig. 2.

Similarly to the results obtained with liver, only the first factorial plane (axes 1 and 2) is shown, as they retain most of the variability in the data analysis (i.e., 62.7% of the total explained variability; see Fig. 2). Also, this indicates that the environmental contaminants factors considered here accounted for most of the variation in the biomarkers measured. Namely, along the first axis the biochemical stress responses accounted for 39.6% of the total variation, followed by axis 2 which explained 23.1% of the variability.

More specifically, axis 1 is mainly influenced by Cr, Fe, DL-PCBs, NDL-PCBs, PBDEs and Hg, while axis 2 is mainly influenced by PBBs, Al, Se and Zn (Fig. 2). From this CCA analysis, it is also possible to identify groups of contaminants, namely the ones composed by: 1) HBCDs, PCDFs, PFCs, and Cr; 2) DL-PCBs, NDL-PCBs, and PBDEs; and 3) Hg and Cd (Fig. 2). The accumulation of contaminants from the group 1 in the muscle samples is positively correlated with lipid levels. The plot analysis indicates that high LPO levels are associated with high levels of contaminants from group 1, while high DNA damage levels and low GPx activities are associated with high levels of contaminants from groups 2 and 3. The DL- and NDL-PCBs are the most influential pollutants to the data variability, and As, Mn, Pb, and PCDDs are less important to the determination of the observed default pattern.

4. Discussion

Marine apex predators are known to accumulate higher levels of pollutants in their bodies than the majority of other animals in their food chain (Storelli et al., 2005, 2007, 2008; Storelli and Marcotrigiano, 2006). It is therefore of upmost importance to understand if and how these pollutants might affect the organisms, as all changes, even if seemingly inconsequential, might affect their physiology and natural behaviour which can have repercussions in the entire ecosystem. This study intended to give a step forward in that knowledge of pollution effects on sharks by quantifying a vast array of chemical contaminants in shark tissues and trying to correlate those levels with important and diverse metabolic responses as a strategy to predict physiological and ecological consequences and find biomarkers for future biomonitoring studies.

The present results with *P. glauca* showed that concentrations of POPs were higher in the liver than in the muscle (Table S1 – supplementary data), which was expected since it is known that these contaminants have a tendency to accumulate in tissues with high lipid content, such as the liver (Wu et al., 2001; Korhonen et al., 2001). Sharks are known for having high lipid contents in their livers, as demonstrated in the present work, and these contaminant accumulation patterns have already been observed in other elasmobranchs, as reviewed by Gelsleichter and Walker (2010).

POP chemical analysis in the present study also revealed that NDL-PCBs were the most abundant contaminants in *P. glauca* tissues. Although NDL-PCBs were for some time seen as harmless non-active compounds, it is presently known that they can be toxic and affect fish behavioural responses (Fischer et al., 1998; Péan et al., 2013). Moreover, some of the individuals sampled in this work even presented levels of NDL-PCBs in the liver above the ones legally permitted for human consumption. The majority of liver samples showed NDL-PCB concentrations higher than the 200 ng g^{-1} ww maximum established by the European Commission

(European Commission, 2011). Although shark liver is not usually directly consumed by humans, liver-derived products frequently are, and have already been found to contain and induce accumulation of POPs on consumers (Akutsu et al., 2006; Kakimoto et al., 2008).

There are already some studies about the concentrations and accumulation patterns of POPs in sharks sampled in different oceans (Storelli and Marcotrigiano, 2001; Gelsleichter et al., 2005; Strid et al., 2007; Storelli et al., 2011Lee et al., 2015), although information on the impacts of some of the POPs analysed in the present study, like BFRs and PCDDs/Fs, is still scarce.

When looking at the studies available in the literature about the contaminants' levels in sharks, in general, concentrations seem to fluctuate not only among different species of sharks, but also among animals of the same species and captured in the same geographical area (e.g. Branco et al., 2007; Gelsleichter et al., 2008; Storelli et al., 2011; Barrera-García et al., 2013; De Carvalho et al., 2014. Some of these differences are summarized in Table 1.

Regarding the quantification of PFCs in the present *P. glauca* samples, PFOS was found in similar concentrations to those previously reported for *Sphyrna tiburo* also from NE Atlantic, but PFUnA was present in higher amounts in both muscle and liver tissues (Table 1). Other compounds that were also considerably higher in the muscle of these sampled *P. glauca* were the BFRs when comparing with other sharks from the Pacific Ocean, including the same species (Lee et al., 2015). The aforementioned compounds have been proven to cause severe negative effects on both marine organisms and humans, impairing organs like the brain and the thyroid (De Wit et al., 2010; Gelsleichter and Walker, 2010). On the other hand, PCBs, PCDDs, and PCDFs were generally lower than the values found for the other shark species or the same *P. glauca* species from different sampling sites. These differences can be due to many factors (e.g. state of maturation of the organisms, feeding ecology, characteristics of the sampling site, etc.) but it is reasonable to assume that the POP concentrations measured in the present study (juvenile individuals) would have been higher in older organisms, as bioaccumulation has been demonstrated to increase with size and age (Fernandes et al., 2007).

Regarding the metal analysis, the results obtained in this work showed that, contrary to what was observed for POPs, some of the metals accumulated preferably in the muscle while others had higher concentrations in the liver (Table S2 – supplementary data). The analysis showed different accumulation patterns between the tested tissues but, in general, both hepatic and muscular metal concentrations are within the ranges previously described for this species, in different parts of the globe (Table 2). Surprisingly, the biggest differences in metal concentrations can be observed when comparing with a study performed with samples from older animals collected in the same ocean (Vas, 1991): our *P. glauca* organisms presented higher values of Mn and Cd in the liver and higher levels of Fe and Cu in both tissues, whereas the levels of Ni were lower (Table 2). The previous stated study was done in the same ocean, but more than two decades ago, and still, bigger organisms presented an overall lower contaminant burden. This evidence should flag the possibility of an historical increase of the contamination of these waters, which deserve to be further studied as it may represent a worrying fact.

The levels of As were also found to be higher than the ones from other studies, in both tissues, which might be attributed to different feeding habits since As accumulation in fish is known to occur mainly through feeding (De Gieter et al., 2002). However, these As levels should not constitute a reason for concern given that is known that the majority of As found in fish is in its less toxic organic form (Olmedo et al., 2013). The Hg concentrations determined in our muscle samples were similar to the ones recorded by other authors for the same species (Table 2), but it is important to highlight that they are higher than 1.0 μg Hg g^{-1} ww, which is the limit established for human consumption by international agencies, such as the European Commission (European Commission, 2006). This is of special concern given the fact that the sampled animals were small juveniles and Hg concentration is known to increase with the size of the organisms, a behaviour well described in the literature (De Pinho et al., 2002; Branco et al., 2007; Barrera-García et al., 2012). Both POP and heavy metal concentrations found in these juvenile organisms should place and highlight this topic as priority concerning human health risk for this highly consumed species.

Marine organisms are constantly in contact with foreign contaminants, which may induce physiological alterations in response to the stress, namely regarding their detoxification and antioxidant systems, as well as in terms of their energetic status (Heath, 1995; Carson, 2013). It has been described that LPO and DNA damage can cause several pathologies in different organisms, and over the last decade authors have observed that exposure to contaminants and to the resulting reactive oxygen species, such as O_2^-, lead to the increase of these and other injurious conditions (Thomas and Wofford, 1993; Baker et al., 1997; Steinberg, 1997; Berntseen et al., 2003; Valavanidis et al., 2006).

The correlation analysis performed between the different biochemical parameters (Table S3 – supplementary data) indicates that GST and CAT are significantly ($p < 0.001$) and positively correlated, and therefore may work together to minimize the effects of contaminants: when GST is being induced to biotransform the xenobiotics, CAT is also being induced to cope with the resulting ROS. IDH and O_2^- have also demonstrated a significantly negative correlation ($p < 0.001$), which might indicate an inhibition of the enzyme with the increase of these free radicals or can be related indirectly via intermediate mechanisms, like for example through the consumption of the enzyme substrate (NADP) in other oxidative stress response processes. The negative correlation between GPx and DNA damage suggests that this enzyme can act to prevent oxidative damage in these macromolecules and whenever the enzyme is induced or inhibited there are less or more harmful effects on DNA, respectively.

Although some studies have already been successful in correlating metal concentrations and biochemical responses of oxidative stress in sharks (e.g. Barrera-García et al., 2012, 2013), no information is available on the effects of POPs in the enzymatic activities of these fish. The Canonical Correspondence Analysis (CCA) was performed with the purpose of linking POP and metal concentrations with the biochemical parameters. The components derived from this analysis allowed a better interpretation of the relations between the environmental and biochemical variables and some strong correlations could be identified. Two separated statistical analysis were performed, one for the liver (Fig. 1) and one for the muscle (Fig. 2), given that bioaccumulation of the measured compounds were shown to be different on each tissue.

From the analysis of the liver CCA (Fig. 1) it is possible to observe a similar pattern of response towards all contaminants, except PBBs, Ni and Cr. This might indicate that these three types of compounds have different accumulation patterns and toxicity pathways than others, or simply that their concentrations in the tissues are too low to affect these animals. It is also possible to observe that the levels of As, Cd and Zn are very closely and positively correlated. As and Cd are heavy metals with known toxic effects in fish (Kumar and Singh, 2010; Magellan et al., 2014; Pandey and Madhuri, 2014). The fact that Zn is found in high concentrations in these samples, although being an essential trace metal, might be explained by the fact that this element has a protective action against heavy metals like Cd, in the liver (Hidalgo et al., 1985). The CCA plot of the liver also revealed an apparent positive association between practically all contaminants and DNA damage which suggests that when the organisms are exposed to most of the analysed POPs and metals, they are not able to effectively counter the effects of ROS and prevent damage in the DNA. This is in accordance with the work of González-Mille et al. (2010) where it was demonstrated that contaminants like POPs have the capacity to induce DNA damage in fish through the formation of ROS.

The CCA performed with muscle results (Fig. 2) show that HBCDs, PCDFs, and PFCs, along with Cr, were positively correlated with lipid content. These classes of POPs have already been described as having

Table 2
Comparison of metal concentrations (μg g^{-1}) found in muscle and liver samples from different studies with *Prionace glauca*. Data is presented as mean or as mean ± standard deviation, as found on the literature.

	N	Size (cm)	Sampling site	Al	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Ag	Cd	Pb	Hg	Ref
Muscle	**20**	**112–167**	**NE Atlantic**	**23.77 ± 47.01**	**2.58 ± 3.27**	**0.63 ± 0.58**	**28.21 ± 26.17**	**0.34 ± 0.57**	**1.15 ± 0.55**	**24.61 ± 15.51**	**78.19 ± 21.98**	**0.29 ± 0.93**	**0.0**	**0.01 ± 0.03**	**0.12 ± 0.11**	**1.36 ± 0.83**	[1]
	44	117–269	Pacific				27.39 ± 3.57		1.64 ± 0.13	6.10 ± 0.37	6.66 ± 0.55	0.22 ± 0.02	<Mdl	0.2 ± 0.12	<Mdl	1.03 ± 0.08	]3[
	21	206.2 ± 52.8	Pacific									0.10 ± 0.05				1.96 ± 1.48	[4]
	38	113–287	Pacific									0.084–0.30				1.39 ± 1.58	[5]
	37	84–239	NE Atlantic								7.20 ± 3.05					0.22–1.3	[6]
	23	80.5–212.0	Mediterranean			1.55	6.34	2.58	0.24					0.45	<0.02		[7]
	5	203–219	N Atlantic														[8]
Liver	**20**	**112–167**	**NE Atlantic**	**24.42 ± 40.10**	**1.61 ± 0.12**	**2.46 ± 1.14**	**99.85 ± 55.81**	**0.04 ± 0.15**	**6.81 ± 3.89**	**43.99 ± 39.65**	**39.98 ± 27.76**	**0.0**	**0.0**	**4.52 ± 3.60**	**1.30 ± 4.35**	**0.28 ± 0.35**	[1]
	35	117–269	Pacific				195.678 ± 95.57		9.28 ± 8.39	49.94 ± 27.1	10.62 ± 4.76	1.67 ± 0.58		34.66 ± 29.61	0.37 ± 0.37	0.22 ± 0.35	[2]
	37	84–239	NE Atlantic									0.47–3.0				0.032–0.96	[6]
	23	80.5–212.0	Mediterranean								5.95 ± 2.67						[7]
	5	203–219	N Atlantic			0.37	4.02	3.23	0.65					0.25	1.14		[8]

[1] Present work; [2] (Barrera-García et al., 2013); [3] (Barrera-García et al., 2012); [4] (Maz-Courrau et al., 2012); [5] (Escobar-Sánchez et al., 2011); [6] (Branco et al., 2007); [7] (Storelli and Marcotrigiano, 2004); [8] (Vas, 1991).
Al = aluminium; Cr = chromium; Mn = manganese; Fe = iron; Ni = nickel; Cu = copper; Zn = zinc; As = arsenic; Se = selenium; Ag = silver; Cd = cadmium; Pb = lead; Hg = mercury. Mdl = minimum detection level.

a tendency to accumulate in highly lipid tissues (Korhonen et al., 2001; Wu et al., 2001), and Cr is known, not only for accumulating preferably in tissues with high lipid content, but also for being necessary for lipid metabolism itself (Halver and Hardy, 2002). Cr has also recently shown to have beneficial effects for fish health, preventing the proliferation of parasites (Fujimoto et al., 2010; Mehrim, 2014). In the muscle samples, it was also possible to observe a positive association between LPO and HBCDs, PCDFs and PFCs, suggesting that the higher the concentration of these compounds in the muscle, the greater will be the level of lipid damage in the tissue. Additionally, levels of DNA damage were positively associated with concentrations of DL-PCBs, PBDEs, Cd, and Hg. This increase in oxidative damage to the DNA molecules, and its relation with contaminant levels, has been described in the literature (González-Mille et al., 2010; Tchounwou et al., 2012) and it was a pattern observed when analysing levels of contaminants in both muscle and liver samples. The levels of the same group of POPs and metals have also shown a strong negative association with GPx activity, suggesting that high concentrations of these contaminants may inhibit this enzyme, which usually acts to prevent the effects of ROS. The neuronal and energetic biomarkers assessed do not seem to be influenced by the contaminants' concentration, leading us to conclude that these parameters are not the most appropriate for future studies of similar nature. Thus, from the CCA analyses performed between contamination levels and biochemical responses, it can be drawn that LPO, DNA damage, and GPx inhibition seem to be the main consequences of *P. glauca* exposure to the measured POPs and metals, representing therefore the best candidates to be used as biomarkers for future biomonitoring studies. These results indicate that the higher the contaminants values, the higher the inhibition of this antioxidant enzyme and the levels of damage in important macromolecules such as lipids and DNA, which might have impacts in the organisms' regular metabolism and general well-being. These impacts can ultimately lead to the degradation of core ecological aspects in *P. glauca* (e.g. swimming, feeding and reproduction), something already extensively described for many other marine taxa (e.g. Van der Oost et al., 2003; Valavanidis et al., 2006; Benedetti et al., 2015; Mearns et al., 2015).

In summary, the present results represent a first insight into the cellular and physiological effects caused by POPs and metals on sharks. However, it should be noted that the organisms have most definitely accumulated some other contaminants and an integrated analysis with, for example, polycyclic aromatic hydrocarbons (PAHs) would improve the interpretation and provide additional knowledge on the mechanisms involved in the sharks' response to xenobiotics. Nevertheless, the results suggest some interesting associations between biochemical parameters and POPs and metals accumulation levels, being LPO and DNA damage the main consequences of certain types of these contaminants, along with the inhibition of GPx activity. DNA damage was the most positively associated biomarker with contamination, in both muscle and liver tissues, even if with slightly different profiles depending on the contaminant. Although liver presented higher contamination levels, the results obtained in the muscle allowed a much clearer understanding of the responses associated to each particular contaminant, suggesting that this should be the tissue preferably used in future studies of similar nature. Evaluating stomach content would also add a valuable insight into feeding habits of these animals and how they can affect contaminant accumulation. In order to complement and validate the findings of this work to have a better overall assessment of these species health status, it would be of upmost value to have a larger sampling area of the Atlantic as well as of other oceans, including size-normalized time series as well as adult individuals, as the increased size/age may induce different metabolic responses when in the presence of xenobiotics. However, the fact that no statistical differences were found between gender or size (within juveniles) and the biochemical parameters, already gives an indication of robustness of the parameters to be used as biomarkers for biomonitoring studies.

In conclusion, *P. glauca* demonstrates great potential to be used as a pollution sentinel, and some suitable biomarker candidates were identified with this work. With this study, we also intend to highlight the human health risks of consuming *P. glauca* meat and liver derived products, given that some of the contaminants here quantified presented higher concentrations than the legally allowed for human consumption, an issue for special concern particularly since the sampled organisms were still juveniles.

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