Biomonitoring Atlantic deep waters through the assessment of shark biomarkers

Luís Miguel Fonseca Alves

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Resumo

Os ecossistemas marinhos estão continuamente a ser sobrecarregados com contaminantes derivados de atividades humanas resultando numa dimunuição dos recursos marinhos. A exposição crónica a contaminantes, como metais pesados e poluentes orgânicos persistentes (POPs), pode afetar negativamente o ambiente marinho e, eventualmente, também os seres humanos. Grandes predadores pelágicos, como tubarões, são particularmente afetados pela poluição, principalmente através de processos de bioacumulação e biomagnificação.

A fim de resolver o problema acima mencionado, são necessários estudos de avaliação de risco ambiental para prever os padrões de contaminação e evitar efeitos adversos que muitas vezes só são visíveis quando é tarde demais para tomar ações preventivas.

Análises de concentração de químicos fornecem-nos informações sobre o nível de contaminação no ambiente; no entanto, podem não ser suficientes para entender como os organismos estão a ser afetados e há uma necessidade de relacionar essas quantificações com parâmetros biológicos. A avaliação de parâmetros bioquímicos, como a atividade enzimática, pode fornecer uma visão mais sensível e precisa sobre os níveis de contaminação. Tubarões como *Prionace glauca* são predadores de topo e portanto extremamente importantes nos ecossistemas marinhos. A sua grande distribuição, juntamente com o fácil acesso a amostras, fornecidas por barcos de pesca comercial, tornou-as um alvo favorável para utilização em ensaios toxicológicos.

Este estudo teve como objetivo avaliar o potencial de *P. glauca* como uma espécie sentinela para pesquisas de monitorização de poluição, através do desenvolvimento e da aplicação de biomarcadores apropriados. As amostras de tecidos foram recolhidas de vinte tintureiras na costa de Portugal, a bordo de um barco comercial de pesca de espadarte. Níveis de POPs, assim como parâmetros bioquímicos relacionados com destoxificação, stress oxidativo e funções neuronais, foram medidos. A caracterização prévia da atividade das colinesterases no músculo e cérebro de *P. glauca* foi feita, já que não havia dados disponíveis sobre esta matéria. Esta caracterização foi essencial devido à existência de três classes de ChE conhecidas em peixes, acetilcolinesterase (AChE), butirilcolinesterase (BChE) e propionilcolinesterase (PChE), todas bastante suscetíveis a agentes anticolinérgicos, e outros contaminantes, tornando-as biomarcadores relevantes em estudos de monitorização de poluição. Os

resultados obtidos indicaram que o cérebro de *P. glauca* aparenta possuir ChEs atípicas, revelando propriedades mistas de AChE e BChE e, que o músculo aparentemente possui maioritariamente AChE. A exposição *in vitro* a chloropyrifos-oxon provocou inibição de ChE das tintureiras em ambos os tecidos, com o cérebro sendo o tecido mais sensível e, por isso, o mais adequado para a detecção de compostos anticolinérgicos no ambiente. Este estudo indica que a actividade de ChE em tintureiras tem potencial para ser usada como um biomarcador sensível e fiável em programas de biomonitorização marinha.

O fígado apresentou níveis mais elevados de POP, quando comparado com músculo. Foram encontradas correlações positivas e negativas entre os parâmetros de contaminação e de stresse oxidativo.

Este estudo destaca a importância da caracterização de Che antes de a usar como um biomarcador em estudos ecotoxicológicos, e demonstra o grande potencial de *P. glauca* como espécie modelo e como sentinela de poluição marinha, através do uso de biomarcadores adequados.

Palavras chave: Poluição; Ecotoxicologia; Biomarcadores; Stress oxidativo; Acetilcolinesterase; Poluentes orgânicos persistentes; Tubarões

Abstract

Marine ecosystems are being continuously loaded with contaminants derived from human activities resulting in a decline of the marine resources. The chronic exposure to contaminants like heavy metals and persistent organic pollutants (POPs) can cause negative impacts to marine environment and, eventually, to humans. Big pelagic predators, like sharks, are particularly affected by pollution, mainly through bioaccumulation and biomagnification.

Given the aforementioned problem, environmental risk assessment studies are needed to predict contamination patterns and prevent injurious effects that are often only visible when it is too late to take preventive actions.

Chemical analysis of the pollutants' concentrations provide information about the contamination level in the environment, but may not however be enough to fully understand how organisms are being affected, and there is a need to link these measurements with biological endpoints. Assessment of biochemical endpoints, like enzymatic activity, can provide a more sensible and accurate view on the effects of contamination and risk levels. Sharks like *Prionace glauca*, are apex predators and therefore extremely important parts of marine ecosystems. Their large distribution along with the fairly easy access to samples, provided by commercial fishing boats, has made them a favorable target for use in biomonitoring studies.

This study aimed to evaluate the potential of *P. glauca* as a sentinel species for pollution monitoring surveys, through the development and application of suitable biomarkers. Tissue samples were collected from twenty blue sharks of the coast of Portugal aboard a commercial swordfishing boat. POPs concentrations, as well the levels of biochemical parameters related with detoxification, oxidative stress and neuronal functions, were measured. As a first part of this work, a characterization of the cholinesterases present in muscle and brain tissues of *P. glauca* was made, as there was no data available on this matter. This characterization was essential due to the existence of three known types of ChE in fish, acetilcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE), all very susceptible to anticholinergic agents, and to other contaminants, making them relevant biomarkers in pollution monitoring studies. The results suggest that the brain of *P. glauca* seems to contain atypical ChEs, revealing mixed properties of AChE and BChE, and that the muscle tissue seems to contain mostly AChE. *In vitro* exposures to chloropyrifos-oxon

inhibited blue shark's ChE in both tissues, the brain being the most sensitive tissue and therefore the most suitable for detection of exposure to low concentrations of anticholinergic compounds in the environment. This study indicates that ChE activity in blue sharks has the potential to be used as a sensitive and reliable biomarker in marine biomonitoring programs.

Liver tissue presented higher POP levels, when compared to muscle. Both positive and negative correlations were found between physiological parameters and POPs accumulation levels. DNA damage was the main consequence of contamination and the inhibition of ChE activity the most strongly correlated effect.

This study highlights the relevance of ChE characterization before using it as a biomarker in ecotoxicology and biomonitoring studies, and demonstrates the great potential of *P. glauca* to be used as a model species and as a sentinel of marine pollution, through the use of suitable biomarkers of effect.

Keywords: Pollution, Ecotoxicology, Biomarkers, Oxidative stress, Acetylcholinesterase, persistent organic pollutants; Sharks

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List of abbreviations

AChE – acetilcholinesterase
ANOVA – analysis of variance
ATCh – acetylthiocholine iodide.
BChE – butyrylcholinesterase
BTCh = S-butyrylthiocholine iodide.
CAT – catalase
CCA – canonical correspondence analysis
CDNB – 1-chloro-2,4-dinitrobenzene
DDTs – dichlorodiphenyltrichloroethanes
DNA – Deoxyribonucleic acid
DTPA – diethylene triamine pentaacetic acid
EDTA – ethylenediamine tetraacetic acid
GPx – glutathione peroxidase
GR – glutathione reductase
GSH – glutathione
GST – glutathione S-transferase
GSSG – glutathione disulfide
H2O2 – hydrogen peroxide
ICCAT – international commission for the conservation of atlantic tunas
IOC – Intergovernmental Oceanographic Commission
LPO – lipid peroxidation
NADPH – nicotinamide adenine dinucleotide phosphate-oxidase
NRC – National Research Council
$O_2^{\bullet -}$ – superoxide radical
$OH^{\bullet}$ – hidroxyl radical
PCFs – perfluorochemicals
PCBs – polychlorinated biphenyls
PCDFs – polychlorinated dibenzofurans
PCDDs – polychlorinated dibenzo-p-dioxins
PCFs – perfluorochemicals
PChE – propionylcholinesterase
POPs – persistent organic pollutants

PPM – parts per million

PTCh – Propionylthiocholine iodide.

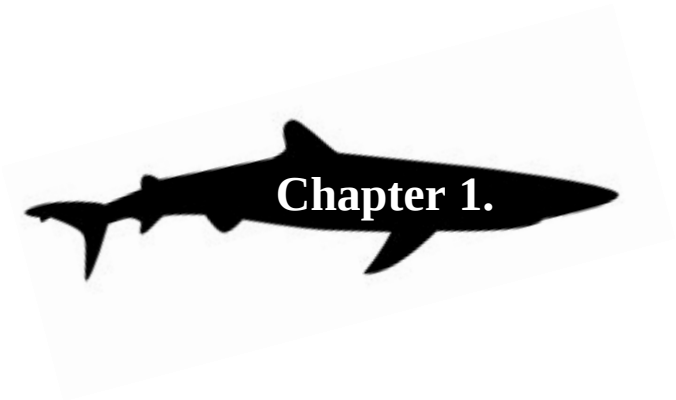
ROS – reactive oxygen species

SOD – superoxide dismutase

TG – total glutathione

UNESCO – United Nations Educational, Scientific and Cultural Organization

WHO – World Health Organization



Chapter 1.

General Introduction

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1.1. Pollution of marine ecosystems – an overview

Comprising over 71% of our planet's surface, the oceans are home to a great percentage of the World's biodiversity (IOC/UNESCO et al., 2011). According to Lalli and Parsons (1993), besides having an essential role in the regulation of the planet's climate, the oceans are also responsible for over 35% of the primary production of the planet. Furthermore, the importance of marine waters is not only ecological but also economical, with 60% of the total economic value of the biosphere being attributed to the oceans (Costanza et al., 1997).

The environment is continuously burdened with xenobiotic contaminants released by urban communities and industries and, most of the times, the final destination for many of these contaminants is the aquatic environment, either due to direct discharges or to hydrologic and atmospheric processes (Stegeman and Hahn, 1994; Van der Oost, et al., 2003). Human activities are responsible for the great majority of the contamination of marine environments, and consequently, for the decline of their resources (Derraik, 2002; Matthiessen and Law, 2002).

Xenobiotic compounds like persistent organic pollutants (POPs), organochlorine pesticides (DDTs), and heavy metals pose a serious threat to both marine fauna and humans because they are highly toxic and persistent (Gramatica and Papa, 2007). Being strongly bound to particulate material and accumulating in sediments, they are taken up by benthic organisms, entering this way the marine food web, becoming concentrated as the trophic levels rise (Kalay et al., 1999; Farombi et al., 2007; Storelli et al. 2011). Bioaccumulation happens when the intake of a substance by an organism is faster than its excretion. Since these contaminants accumulate in the tissues, predators tend to amass the contaminants present on their prey. In marine environments, bioaccumulation occurs mainly in fish, especially in large long-lived predators like sharks (Gomes et al. 2004).

Along with bioaccumulation, persistent hydrophobic chemicals may accumulate in aquatic organisms through different mechanisms, like the direct uptake from water by gills or skin (bioconcentration) and via uptake of suspended particles (ingestion) (Van der Oost et al., 2003). Because of the aforementioned facts, although the presence of a xenobiotic compound in a segment of an aquatic ecosystem may not indicate, *per se*, a deleterious effect, its accumulation/biomagnefication can eventually cause injurious effects (Franke et al., 1994; Tillitt et al., 1992; Wang, 2002; Dautremepuits et al., 2004).

Some of the most well studied POPs include polychlorinated biphenyls (PCBs), perfluorochemicals (PFCs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), all of them having negative effects on the living organisms present in the water such as growth impairment and effects resulting from endocrine disruption (Jones and de Voogt, 1999; Storelli *et al.* 2011). PCBs and DDTs usually form complex mixtures, and their presence is a clear indication of anthropogenic pollution (Storelli et al., 2005). PCDDs and PCDFs, unlike the remaining POPs, are not intentionally produced by industries but are unwanted by-products of industrial and thermal processes (Stevens et al., 2000).

Heavy metals like lead (Pb), nickel (Ni), cadmium (Cd), and mercury (Hg) can all be found in aquatic environments and once accumulated by marine organisms, can induce the formation of reactive oxygen species, causing excessive oxidative stress which can lead to damage on important macromolecules like proteins, lipids or DNA (Bryan, 1976; Mart et al., 1982; Stohs and Bagchi, 1995; Canli and Atli, 2003). The organic form of Hg can bind to sulfhydryl groups of fish enzymes and proteins and can easily cross both the placental and blood brain barrier (Boening, 2000; Castro-Gonzalez and Mendez-Armenta, 2008; Guzzi and La Porta, 2008). High and ecologically alarming concentrations of these metals have been found in marine organisms, particularly in those higher in the food chain, like sharks (Branco et al., 2007; Barrera-García et al., 2013; de Carvalho et al., 2014).

1.2. Ecotoxicology and biomarkers: Nature's early-warning signals

Ecotoxicology aims to understand how different xenobitotics can disturb the ecosystems to be able to protect it and prevent those effects from becoming permanent (Newman, 2008). This is not a simple task given the numerous xenobiotics that coexist in the environment, along with biotic and abiotic stress factors (eg. temperature and predation), which can have combined and complex effects (Calabrese, 1991; Lemos et al., 2010). In order to efficiently understand how xenobiotics affect organisms in the ecosystems, ecotoxicologists cannot rely solely in environmental levels of contaminants and need have the need to develop and apply reliable and accurate tools for pollution biomonitoring studies (Bucheli and Fent, 1995; Newman, 2008).

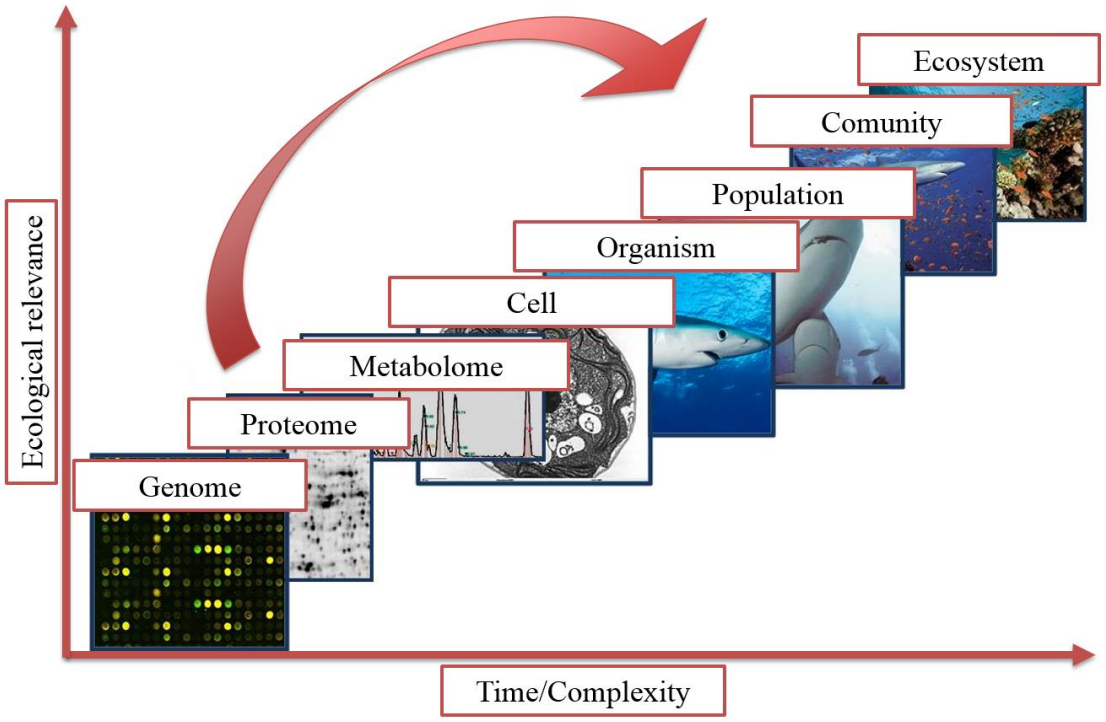

Figure 1 - Relationship between ecological relevance, time of response and the different levels of biological organization after stress exposure.

In order to detect contamination before it hinders a community or even an entire ecosystem, and because effects at higher hierarchical levels are preceded by variations in biological processes at lower levels of organization (Fig. 1), early-warning signalling biomarkers can be created (Bayne et al., 1985). In an environmental context, these early-warning signals, also known as biomarkers, can help scientists to better understand which contaminants the organism has been exposed to, in which tissues they have been accumulated and if they are causing a toxic effect at critical targets (McCarthy and Shugart, 1990).

Numerous candidate biomarkers can be used in order to assess if an organism has been exposed to a pollutant and to evaluate the effects caused by environmental pollutants on aquatic ecosystems. Many studies have successfully used the activity of biotransformation enzymes involved in detoxification mechanism (Gorbi et al., 2004), oxidative stress parameters like lipid peroxidation (Barrera-García et al., 2012), hormones involved in reproductive and endocrine mechanisms (Koenig, 2012) and neuromuscular enzymatic activities (Solé et al., 2008) as biomarker of effect to contamination.

A pollutant stress situation usually provokes a cascade of biological responses and, initially, McCarty et al. (1991) stated that each of those responses could serve as a

biomarker. A few years later, in 1996, Van Gastel and Van Brummelen proposed a definition that is widely accepted today, stating that a biomarker can be *"any biological response to an environmental chemical at the subindividual level, measured inside an organism or in its products"* (eg. blood, liver, urine, faeces, etc.), demonstrating an deviation from the normal status, that cannot be detected in the intact organism.

Biomarkers may be grouped in different classes, but their classification can vary depending on the way they are used (Suter, 1993). A generally accepted description is the one used by the National Research Council (NRC) (1987) and WHO (1993), according to which biomarkers of exposure are used to detect and measure a xenobiotic or its metabolites, or the product of an interaction between a xenobiotic a target molecule or cell within an organism; biomarkers of effect allow to measure alterations in an organism (biochemical, physiological, etc.) that are associated with health impairment; lastly, biomarkers of susceptibility were described as indicators of the ability of an organism to respond to the effects of exposure to a xenobiotic (eg. genetic factors) which affect the vulnerability to that contamination. Increased activities of antioxidant enzymes and detoxification agents, oxidation of proteins and lipids, DNA damage and alterations in hormonal pathways, among others, have the potential to be used as biomarkers (Winston and Di Giulio, 1991; Filho, 1996; Elia et al., 2006; Labrada-Martagón et al., 2011).

Biomarkers can be useful tools in several steps of the risk assessment process allowing an evaluation of effect, exposure and hazard caused by the contaminant, providing means to monitoring the environmental quality of the ecosystems (Van der Oost et al., 2003). Particularly in aquatic ecosystems, fish species have attracted considerable interest for studies assessing biological and biochemical responses to environmental contaminants (Powers, 1989). Certain species can operate as sentinels, demonstrating the presence of bioavailable contaminants and how they affect the organisms, giving us insights on long-term effects on the health of populations or the integrity of the ecosystem (McCarthy and Shugart, 1990). Although a great deal of variation among fish species can occur, concerning both the basic physiological features and the responsiveness of certain biomarkers, fish have been the group par excellence for pollution monitoring in aquatic systems (Lopes et al., 2001; Van der Oost et al., 2003; Dautremepuits et al., 2004) since they are abundant and vastly distributed in all aquatic environments, being very susceptible to environmental pollutants. Even though most of the general biomarker criteria appear to be directly transferable to certain fish

biomarkers, monitoring fish species should be chosen taking some practical considerations: the test species must be part of the exposed community and have an important ecological role in the ecosystem (Stegeman et al., 1992; Suter, 1993).

Van der Oost and co-workers (2003), based upon the work made by Stegeman et al. (1992), proposed the following criteria to evaluate the strength and weaknesses of fish biomarkers objectively: 1) the methodology to assess the quantity/activity of the biomarker should be reliable; 2) the biomarker response should be sensitive to stressors; 3) the baseline data of the biomarker should be clear and the impacts of confounding factors to biomarker response should be well understood; 4) the underlying mechanism of the relationships between biomarker response and pollutant exposure should be well-known; 5) the toxicological significance of the biomarker should be established.

Since it remains hard to accurately predict bioaccumulation in fish, even with highly sophisticated models, analyses of tissue contaminant levels are required.

1.3. Organisms' responses to contamination

When an exogenous harmful compound enters a living organism, a detoxification process is initiated, comprised by two main phases: phase I and phase II (Liska, 1998; Chen, 2012). During phase I, or functionalization phase, the organism first response is to facilitate the excretion of the contaminant and, in order to do that, a functional group is introduced in the chemical structure of the lipophilic xenobiotic, usually by oxidizing or hydrolysing it (Schlichting et al., 2000; Chen, 2012). A common Phase I oxidation involves conversion of a C-H bond to a C-OH. Although this process assists the organism to eliminate hazardous compounds, it can sometimes convert a nontoxic molecule into a toxic one, generating reactive oxygen species (ROS) that are injurious and cause stress to the cells (Di Giulio et al., 1989). In phase II, the second phase of detoxification, the metabolites generated in phase I are conjugated with small molecules like, for instance, glutathione (GSH) (Jakoby and Ziegler, 1990). This process increases the molecular weight of these xenobiotics and increases their polarity, thus making them less harmful and easier to excrete (Liska, 1998) (Fig.2).

It is not easy to define stress, but for this work purposes, the definition proposed by Brett in 1958 will be used, where he states that stress is *"a physiological state produced by an environmental factor that extends the normal adaptive responses of the animal, or disturbs the normal functioning to such an extent that the chances for survival are significantly reduced".* Oxidative stress specifically, or oxygen toxicity, is

defined as injurious effects due to cytotoxic reactive oxygen species (ROS) (Di Giulio et al., 1989; Halliwell and Gutteridge, 1999; Winzer, 2001).

Cells are constantly suffering some degree of oxidation, either by anaerobiosis, spontaneous mutagenesis and/or self defense mechanisms (Imlay, 2003; Valko et al., 2007). The oxygen metabolism in mitochondria originates a great amount of ROS, such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 2001). Due to this, when animals practice some kind of intense physical activity, like hunting prey or escaping predators, or try to eliminate harmful xenobiotics, higher rates of ROS production occur (Bejma and Ji, 1999; Best et al., 1999; Ji, 1999). The reduction products of molecular oxygen are capable of reacting with critical cellular macromolecules, potentially leading to enzyme inactivation, lipid peroxidation, DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991; Lucas and Szweda, 1998; Ambrosio and Tritto, 1999; Semenza, 2000).

Many pollutants, or the metabolites deriving from them, can induce oxidative stress by increasing the production of ROS, disturbing the natural balance in the organism (Lackner, 1995; Pandey et al., 2003; Di Giulio and Meyer, 2008). These pollutants can be detoxified, and the effects of ROS minimized, through the activity of several enzymes and cofactors (Fig. 2). Variations in activity rates of biotransformation and antioxidant enzymes are extremely sensitive biomarkers of effect, and xenobiotics have been proven to cause alterations on these enzymes (Bucheli and Fent, 1995).

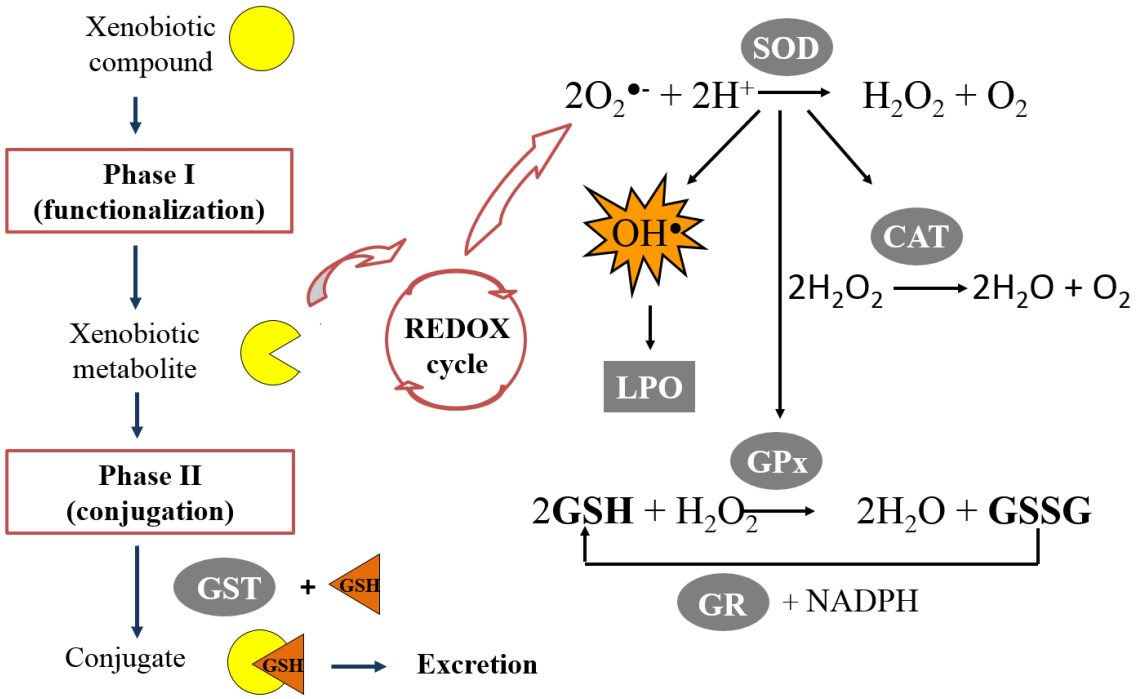

Figure 2 - Main enzymatic mechanisms involved in reactive oxygen species neutralization (adapted from Howcroft et al., 2009). GST – glutathione S-transferase; SOD – superoxide dismutase; CAT – catalase; LPO – lipid peroxidation; GPx – Glutathione peroxidise; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione.

The metalloenzyme superoxide dismutase (SOD) is usually one of the most important agents in the process of preventing and counteracting the negative effects of ROS, being responsible for the transformation of $O2^{\bullet-}$ into H_2O_2. The role of this enzyme is so important that it can be found in all aerobic organisms studied so far (Stegeman et al., 1992). The H_2O_2 molecules resulting from the action of SOD can still cause harmful effects and need to be removed from the organisms.

Catalase (CAT) and glutathione peroxidase (GPx) are two enzymes responsible for the elimination of H_2O_2 and preventing its accumulation. Catalase is a hematin-containing enzyme that facilitates the elimination of H_2O_2 by metabolizing it to molecular oxygen and water. Catalase specifically reduces H_2O_2, unlike some peroxidases that can reduce different peroxides (Stegeman et al., 1992; Filho, 1996). CAT uses one of H_2O_2 molecule as donor in the reduction another, while peroxidases like GPx need to use other reductant molecules. Glutathione peroxidase aids in the conversion of H_2O_2 to H_2O, involving a concomitant oxidation of reduced glutathione (GSH) to its oxidized disulfide form (GSSG).

The enzyme glutathione reductase (GR), as its name suggests, is involved in the transformation of the GSSG to the reduced form (GSH) (Worthington and Rosemeyer,

1974). GSH can act directly to neutralize oxidizing molecules and also as a cofactor used by GST, increasing the water solubility of xenobiotic compounds, facilitating their excretion (Egaas et al., 1995; Halliwell and Gutteridge, 2001; Livingstone, 2001; Valko et al., 2007).

Aside from the enzymes involved in oxidative stress responses, pollutants can also affect the activity of enzymes like acetylcholinesterase (AChE). Acetylcholinesterase is part of a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, an essential process for both neuronal and motor capabilities (Pohanka, 2011). ChE inhibition is a serious impairment as it can cause behaviour alterations and even death to the organisms (Fulton and Key, 2001),

Because of their high sensitivity to organophosphate and carbamate pesticides, and also to other contaminants often present in marine ecosystems, the measurement of ChE activity has already been widely used in pollution monitoring studies as a biomarker of effect (Galgani et al., 1992; Payne et al, 1996; Kirby et al., 2000; Fulton and Key, 2001; Chambers et al., 2002; Van der Oost et al., 2003; Galloway et al., 2002; Arufe et al., 2007). Since usually there is more than one type of ChE in different fish tissues, to use these enzymes in ecotoxicology and biomonitoring studies, it is often necessary to perform a previous characterization of these enzymes in the target sampling tissue (Sturm et al., 1999a; Sturm et al., 2000; Kirby et al., 2000; Solé et al., 2008). The three known types of ChE in fish are acetilcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE) (Solé et al.; 2010). AChE is essential to neuronal function but the roles of BChE and PChE, often named pseudocholinesterases, are still unclear (Chambers et al., 2002).

1.4. Sharks as tools for biomonitoring studies

Sharks are elasmobranchs, an ancient group of fish, existing for more than 400 million years (Ballantyne, 1997). These animals have always attracted the interest of scientists and oceanographers, like the Portuguese King Carlos I who reigned between 1889-1908 and published a pioneering study of portuguese sharks, including deepwater species not known to science at the time (Bragança, 1904).

Scientists estimate that over 90% of all marine predators have been lost and the decline of these animals can cause potentially irreversible effects in the ecosystems, by increasing prey populations and altering the normal interactions between the mentioned

prey and other members of the ecosystem (Schindler et al., 2002; Myers et al., 2007). The majority of shark species are at the top of their respective food chains and, as all large predators, play a very important role in the oceans, by maintaining the ecosystems in balance.

Being apex predators with long life spans, these animals are more susceptible to environmental contamination via bioaccumulation and biomagnification through the food web (Al-Yousuf et al., 2000; Escobar-Sánchez et al., 2011). A very good example of the aforementioned is the higher concentration of mercury that sharks tend to have in their bodies when compared to other fish species (figure 3), usually in the form of monomethylmercury, the most toxic form of the metal, which accounts for more than 95% of organic mercury in fish muscle tissues (Bloom, 1992; Porcella, 1994; Hueter et al., 1995). Extensive accumulation of heavy metals and POPs makes sharks' immune system particularly affected, leading to a population decrease (Safe, 1994, Ross et al. 1995; Jones and de Voogt, 1999). Additionally, as a result of the previously stated, the consumption of shark meat makes it a potential entrance route of heavy metals and other pollutants into the human food web (Castro-Gonzalez and Mendez-Armenta, 2008).

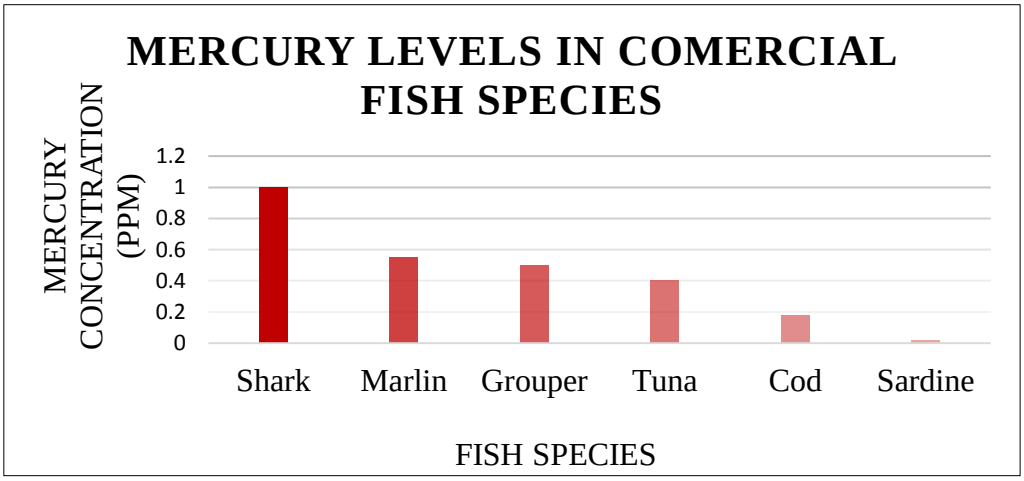

Figure 3 - Levels of mercury in some commercial fish species, according to the 2010 US Food and Drug Administration report (1990-2010).

Given their susceptibility to pollutant accumulation, their wide distribution and their importance to the ecosystems, sharks are ideal candidates to be used in marine pollution monitoring studies.

There are over 470 known species of sharks in the world and about 30 can be found in Portuguese waters (Compagno, 1999). The blue shark (*Prionace glauca* L.1758) is the most frequently caught shark species in the planet, with an under-

estimation of 20 million individuals caught annually as target or by-catch by fishing fleets such as the Portuguese longline swordfish fishing fleet (Bonfil, 1994; Santos et al., 2002; Stevens, 2009). A typical blue shark (Figure 4) has a slender body that can measure over 350 cm. These animals hunt mainly squids and small teleost fish, but they also consume other small sharks, birds, and whale carcasses (Compagno, 1999).

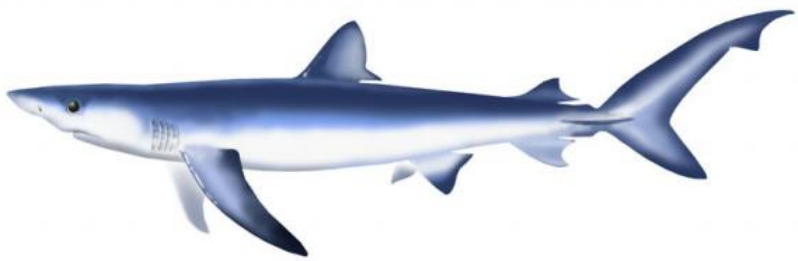

Figure 4 - Drawing of a blue shark by A. López, "Tokio", as seen in ICCAT Manual (2009).

Being known for making long migrations, usually due to prey distribution, water temperature and reproduction, these animals segregate by size and gender (Stevens, 1976; Nakano and Seki, 2003; Aires-da-Silva et al., 2009; Stevens et al., 2010; Campana et al., 2011).

Pollutants quantification in these animals have already been done by several authors, mainly focusing on heavy metals (Branco et al., 2004; 2007; De Carvalho et al., 2014) and POPs (Storelli and Marcotrigiano, 2001; Storelli et al., 2005; 2006; 2011; Strid et al., 2007). Other sharks have been object of the same type of studies (Maz-Courrau et al., 2012; Shanshan et al., 2013), but contamination levels alone can't explain the effects caused by xenobiotics (Van der Oost et al., 2003).

Recently some authors have establish correlations between contaminant trace elements body burden and detoxification mechanisms and antioxidant responses in blue sharks (Barrera-García et al., 2012; 2013), with the results indicating that this species has the potential to be used as biomonitor. Studying enzymes like cholinesterase is also of interest when trying to understand the consequences of pollutant contamination but to characterize the different forms of cholinesterases present in each tissue is imperative. Up until now the characterization of ChE in sharks have only been done for the muscle of *Scyliorhynus canicula* and *Galeus melastomus* (Solé et al., 2008).

1.5. Aims of the study

The main purpose of this work was to assess if *Prionace glauca* could be used as a sentinel species in pollution monitoring studies recurring to biomarker analysis. Potential biomarkers, involved in detoxification, oxidative stress response and neurological functions were evaluated as potential tools for use in environmental effect assessment.

To fulfil the main objective, the following tasks were completed:

1) Characterization of cholinesterases' activity in muscle and brain tissues of *P. glauca* in order to use it as a biomarker of effect in future studies (Chapter 2);

2) Measurement of contaminant body burden (persistent organic pollutants) in muscle and liver tissues of *P. glauca* and relate them with physiological responses (Chapter 3);

3) Development and optimization of sample preparation procedures and biomarker protocols to better address the blue shark as a sentinel species for marine pollution assessment (Chapters 2 and 3).

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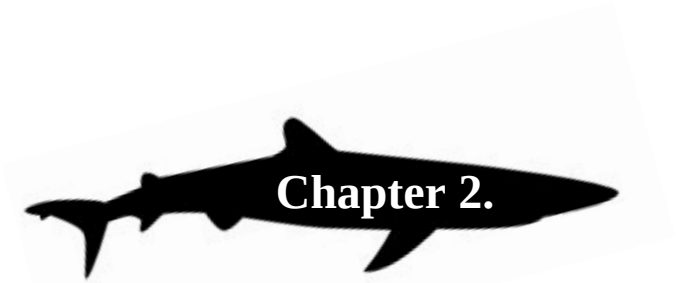

Chapter 2.

The potential of cholinesterases as tools for biomonitoring studies with sharks: biochemical characterization in brain and muscle tissues of *Prionace glauca*

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The potential of cholinesterases as tools for biomonitoring studies with sharks: biochemical characterization in brain and muscle tissues of *Prionace glauca*

Abstract

Cholinesterases (ChE) are a family of enzymes that play an essential role in neuronal and motor functions. Because of the susceptibility of these enzymes to anticholinergic agents and to other contaminants, their activity is frequently used as biomarker in pollution monitoring studies. The three known types of ChE in fish are acetilcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE), for which the presence in each tissue tends to differ between species, thus demanding enzyme characterization before using them as biomarkers. Sharks, mostly acting as apex predators, help maintain fish population's balance, performing a key role in the ecosystem. Blue sharks (*Prionace glauca*) are one of the most abundant and heavily fished sharks in the world, thus being good candidate organisms for ecotoxicology and biomonitoring studies. The present study aimed to characterize the ChE present in the brain and muscle of the blue shark using different substrates and selective inhibitors, and to assess the *in vitro* sensitivity of these sharks' ChE to chlorpyrifos-oxon, a metabolite of a commonly used organophosphorous pesticide, recognized as a model anticholinesterase contaminant. The results suggest that the brain of *P. glauca* seems to contain atypical ChEs, displaying mixed properties of AChE and BChE, and that the muscle tissue seems to contain mainly AChE. *In vitro* exposures to chloropyrifos-oxon inhibited blue shark's ChE in both tissues, the brain being the most sensitive tissue and therefore the most suitable for detection of exposure to low concentrations of anticholinergic compounds in the environment. This study indicates that ChE activity in blue sharks has the potential to be used as a sensitive and reliable biomarker in marine biomonitoring programs.

Keywords: biomarker, blue shark, pollution, chlorpyrifos-oxon

2.1. Introduction

Cholinesterases (ChE) are a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, an essential process for both neuronal and motor capabilities (Pohanka, 2011). The measurement of ChE

activity is widely used in pollution monitoring as a biomarker of effect, mainly due to their high sensitivity to anticholinergic chemicals, such as organophosphate and carbamate pesticides, (Kirby et al., 2000; Fulton and Key, 2001; Chambers et al., 2002; Van der Oost et al., 2003; Galloway et al., 2002; Arufe et al., 2007) but also to other contaminants often simultaneously present in marine environments (Galgani et al., 1992; Payne et al., 1996).

Additionally, ChEs are among the less variable biomarkers, making them suitable for environmental pollution biomonitoring studies (Solé et al., 2008).

Currently there are three known types of ChE in fish: acetilcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE) (Sturm et al., 1999a; Sturm et al., 2000; Kirby et al., 2000; Solé et al., 2008). AChE is a key enzyme of the nervous system, and a well-accepted biomarker of exposure to neurotoxic chemicals, existing predominantly in brain tissue but also,for example, in muscle, liver and blood (Stien et al., 1998; Bresler et al., 1999; Burgeot et al., 2001; Lionetto et al., 2004; Solé et al., 2008). BChE and PChE, the other types of cholinesterases frequently named pseudocholinesterases, have also been found, for example, in plasma, liver and muscle (Sturm et al., 1999a, Sturm et al., 2000; Kirby et al., 2000; Chambers et al., 2002; Solé et al.; 2010). The roles and physiological functions of pseudocholinesterases are still not completely understood but in humans they seem to be involved in detoxification processes, cell regeneration, lipid metabolism, neurogenesis, and neural development (Mack and Robitzki, 2000).

Activity levels, and types of ChE present in each tissue, vary among species (Chuiko, 2000). Given the lack of information on the forms of ChE present in several organisms, studies reporting screening for AChE may, in fact, be dealing with other ChE types that may have distinct sensitivities to contaminants. Different types of cholinesterases can be distinguished using both the aid of different substrates and specific inhibitors (Silver, 1974).

In recent years, fish have become very useful for the quality assessment of aquatic environments, acting as bioindicators of environmental pollution (Lopes et al., 2001; Dautremepuits et al., 2004).

Like all top of the food chain animals, sharks play a very important role in the oceans population dynamics, by maintaining other fish populations in balance. Thus, if the number of predators is significantly altered, the consequences for the ecosystem structure, functioning, and resilience can be significant (Paine, 1996; Duffy, 2002;

Baum and Worm, 2009). According to Bonfil (1994) and Stevens (2009), blue sharks are one of the most abundant and heavily fished sharks in the world, with an estimated 20 million individuals caught annually as target or by-catch species, making them suitable organisms for ecotoxicology and biomonitoring studies. To our knowledge, and to date, ChE of shark species have only been characterized in the muscle tissue of *Scyliorhinus canicula* and *Galeus melastomus* (Solé et al., 2008).

This research had three main goals: 1) to characterize the ChE present in the brain and muscle of blue shark (*Prionace glauca*), using different substrates and specific inhibitors, in order to optimize tissue selection and experimental procedure; 2) to assess the *in vitro* sensitivity of these sharks' ChE to chlorpyrifos-oxon, a metabolite of a vastly used organophosphorous pesticide, recognized as a model anticholinesterase contaminant; and 3) to address the blue shark ChE as a tool for future biomonitoring studies in marine ecosystems.

2.2. Materials and methods

2.2.1. Test Organisms

Muscle and brain tissues of eight juvenile blue sharks (*P. glauca*) were sampled aboard a commercial fishing boat on November 2013, off the Atlantic coast of Portugal at 36°43'11.2"N 13°09'30.0"W. The organisms used in this study consisted of four males and four females ranging 105 to 157 cm and 113 to 167 cm, respectively. Tissues from each shark were collected immediately after capture and landing on the vessel, after which all samples were stored on ice until they were deep-frozen in the lab at −80° C for further biochemical measurements.

2.2.2. Chemicals

The substrates acetylthiocholine iodide (ATCh), S-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh), as well as the inhibitors eserine hemisulfate, 1,5-bis[4-allyl dimethyl ammonium phenyl] pentan-3-one dibromide (BW284C51), tetra[monoisopropyl]pyrophosphortetramide (iso-OMPA), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chlorpyrifos-oxon (CPF-oxon) was obtained from Greyhound Chromatography (Birkenhead, Merseyside, UK).

2.2.3. Tissue preparation

Brain and muscle tissues from each shark were homogenized in potassium phosphate buffer (0.1 M, pH 7.2) in a 1:5 proportion. The homogenates were centrifuged at 3000 g, for 3 minutes (4º C), and the supernatant of each sample was transferred to new microtubes and stored at -80º C.

2.2.4. Cholinesterase characterization

Before the enzymatic assays, the total protein concentration in the supernatant was quantified according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford microassay set up in a 96 well flat bottom plate and using bovine γ-globuline protein standard. The ChE activity of each muscle and brain sample was determined in quadruplicates in the previously diluted supernatant (final protein concentration of 0.8 mg/mL) by the method proposed by Ellman (1961) adapted to microplate (Guilhermino et al., 1996). For the determinations, 250 µl of the reaction solution [30ml potassium phosphate buffer (0.1M, pH 7.2), 1 ml of reagent 5,5-dithiobis-(2-nitrobenzoic acid) 10mM (DTNB) and 200 µl of substrate] was added to 50 µl of the diluted supernatant. The absorbance was measured every 20 seconds for 5 min at 414 nm (25º C).

All spectrofotometric measurements were performed using a microplate reader Synergy H1 Hybrid Multi-Mode (BioTek® Instruments, Vermont, USA).

2.2.4.1 Substrates

Cholinesterases substrate preferences were assessed in both muscle and brain tissues by determining the enzyme activity at 12 increasing concentrations of the substrates ATCh, BTCh, and PTCh: 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24 and 20.48 mM. Cholinesterases activity in the presence of these substrates was determined as described in the previous section, with 200 µl of each substrate being dissolved in the reaction buffer (Howcroft et al., 2011). Blank reactions were made for each substrate concentration using the same volume of potassium phosphate homogenization buffer (0.1 M, pH 7.2) instead of sample.

2.2.4.2 Inhibitors

Eserine sulfate, BW284C51, and iso-OMPA were used as selective inhibitors of total ChE, AChE, and BChE, respectively. Cholinesterases activities were measured in all muscle and brain samples as described in section 2.4, using 200 µl of ATCh 0.075M solution as substrate, at six increasing concentrations of each inhibitor dissolved in the reaction buffer (Howcroft et al., 2011). Final concentrations of the inhibitors were 0.781, 3.125, 12.5, 50, 200 and 800 µM for eserine sulfate and BW284C51, and 0.0156, 0.0625, 0.25, 1, 4 and 16 mM for iso-OMPA.

Blank reactions were specifically made for each inhibitor concentration using the same volume of potassium phosphate buffer (0.1 M, pH 7.2) instead of sample. Controls of ChE activity in the absence of inhibitors in the reaction buffer were also made.

2.2.5. In vitro effects of chlorpyrifos-oxon on ChE activity

An *in vitro* test was performed using the organophosphate insecticide chlorpyrifos-oxon. The ChE activity was measured as described for the inhibitors using ATCh 0.075M as substrate and dissolving the different pesticide concentrations in the reaction buffer. A stock solution of the insecticide was prepared in ethanol, and reactions were done using pesticide final concentrations ranging from 0.0365 to 2400 nM.

Blank reactions were made for each contaminant concentration using the same volume of potassium phosphate buffer (0.1 M, pH 7.2) instead of sample. A control of ChE activity without chlorpyrifos in the reaction buffer was also done as well as an extra solvent control (same solvent concentration as in the maximum tested pesticide concentration).

2.2.6. Statistical analysis

To calculate the catalytic efficiency of the enzyme with each substrate, experimental curves were fitted (monotonic increase part of the curve) using the Michaelis-Menten equation, in order to determine the ChE kinetic parameters: maximal velocity (Vmax), Michaelis-Menten constant (Km), and their ratio (Vmax/Km), indicating the catalytic efficiency of the enzyme.

Data from the ChE activity with the specific inhibitors, as well as from the *in vitro* exposures, were analyzed using one-way analysis of variance (ANOVA) followed

by Dunnett's multicomparison test to evaluate significant differences between tested concentrations and the control/solvent control at a significance level of 0.05. *In vitro* inhibition concentration values for chlorpyrifos oxon (IC_{50}) were calculated using a nonlinear four parameter logistic curve. To address effects of gender and size on the inhibitory capacity of chlorpyrifos-oxon *in vitro* at the different concentrations, a two-way ANOVA was performed, followed by Holm-Sidak test to discriminate statistical significant differences between groups.

All the referred tests were made using the Sigma Plot software for Windows, Version 11.0 (Systat Software, 2008).

2.3. Results

2.3.1. Cholinesterase characterization

The results of ChE substrate preference in the brain and muscle tissues are shown in Figure 5.

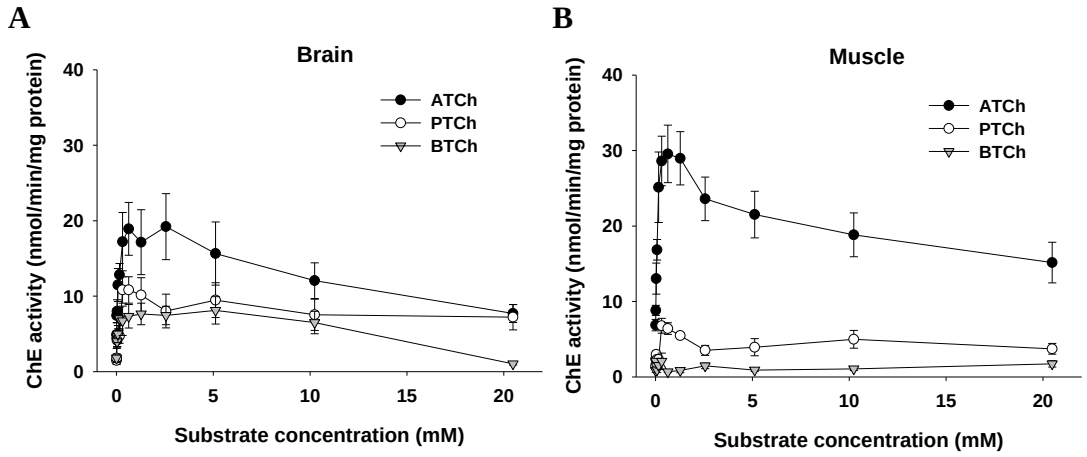

Figure 5 – Cholinesterase substrate preferences in the brain (A) and muscle (B) of *Prionace glauca*. Cholinesterases (ChE) activity is expressed as mean values ± standard error. ATCh = Acetylthiocholine iodide. BTCh = S-Butyrylthiocholine iodide. PTCh = Propionylthiocholine iodide.

In brain tissue (Fig. 5A), the substrate with higher hydrolysis rate was ATCh (19.2 nmol/min/mg protein), followed by PTCh (10.8 nmol/min/mg protein) and BTCh (8.1 nmol/min/mg protein). The enzymatic catalytic efficiency indicated by the parameters of the Michaelis–Menten equation (Tab. 1) also demonstrates the preference for the substrate ATCh (higher Vmax/Km values). Furthermore, a decrease in ChE

activity caused by excess of the substrates ATCh and BTCh was also verified with concentrations higher than 2.56 mM and 5.12 mM, respectively.

Table 1 - Values of the Michaelis–Menten constant (Km), maximal velocity (Vmax) and the catalytic efficiency (Vmax/Km) of *Prionace glauca* cholinesterases for the three tested substrates. Values of the Michaelis-Menten equation are expressed as the mean ± standard error.

	Km (mM)	**Vmax (nmol/min/mg protein)**	**Vmax/Km**
Brain			
ATCh	0.045±0.0181	18.82±1.6499	422.90
PTCh	0.056±0.0257	9.09±0.7340	163.26
BTCh	0.027±0.0120	7.50±0.5637	279.76
Muscle			
ATCh	0.053±0.0140	31.62±2.0914	598.87
PTCh	0.034±0.0140	4.97±0.3371	147.36
BTCh	-	-	-

In muscle tissue (Fig. 5B), there was a clear preference for the substrate ATCh, as seen by the higher hydrolysis rates and greater catalytic efficiency of ChE with this substrate (Tab. 1). Indeed, there were higher hydrolysis rates in the muscle than in the brain tissue with maximum ChE activities of 29.5 nmol/min/mg protein at 0.64 mM of ATCh when compared with the 19.2 nmol/min/mg protein maximum activity in brain at 2.56 mM of the same substrate. Almost no ChE activity was observed in muscle when using the substrate BTCh. In this tissue there was also an inhibition of hydrolysis by excess of ATCh at concentrations higher than 0.64 mM.

Regarding the results with the specific inhibitors, incubation with eserine, a generic inhibitor of ChE, significantly inhibited ChE activity in brain already at the lowest concentration tested of 0.781 µM ($F_{6,49}$ = 115.1, p<0.001) whereas in muscle significant inhibitions only occurred at concentrations higher than 12.5 µM ($F_{6,49}$ = 179.5, p<0.001) (Fig. 6A). However, at concentrations higher than 50µM almost complete inhibitions were observed in both tissues (over 95% inhibition). Concerning the specific inhibitor for AChE (Fig. 6B), incubation with BW284C51 in brain only

significantly inhibited the enzyme activity at concentrations higher than 50 µM ($F_{6,49}$ = 35.8, p<0.001) and inhibitions above 90% were observed only at 800µM, whereas in muscle a significant inhibition of 93% occurred already in the lowest concentration tested ($F_{6,49}$ = 122.8, p<0.001). No effects on enzyme activity were observed with iso-OMPA incubations, a specific inhibitor of BChE, either in brain ($F_{6,49}$ = 9.69, p = 0.138) or muscle ($F_{6,49}$ = 0.47, p = 0.825) tissues (Fig. 6C). However, there was a dose-response inhibition in the brain, albeit non-significant, reaching 40% inhibition in the highest iso-OMPA concentration tested.

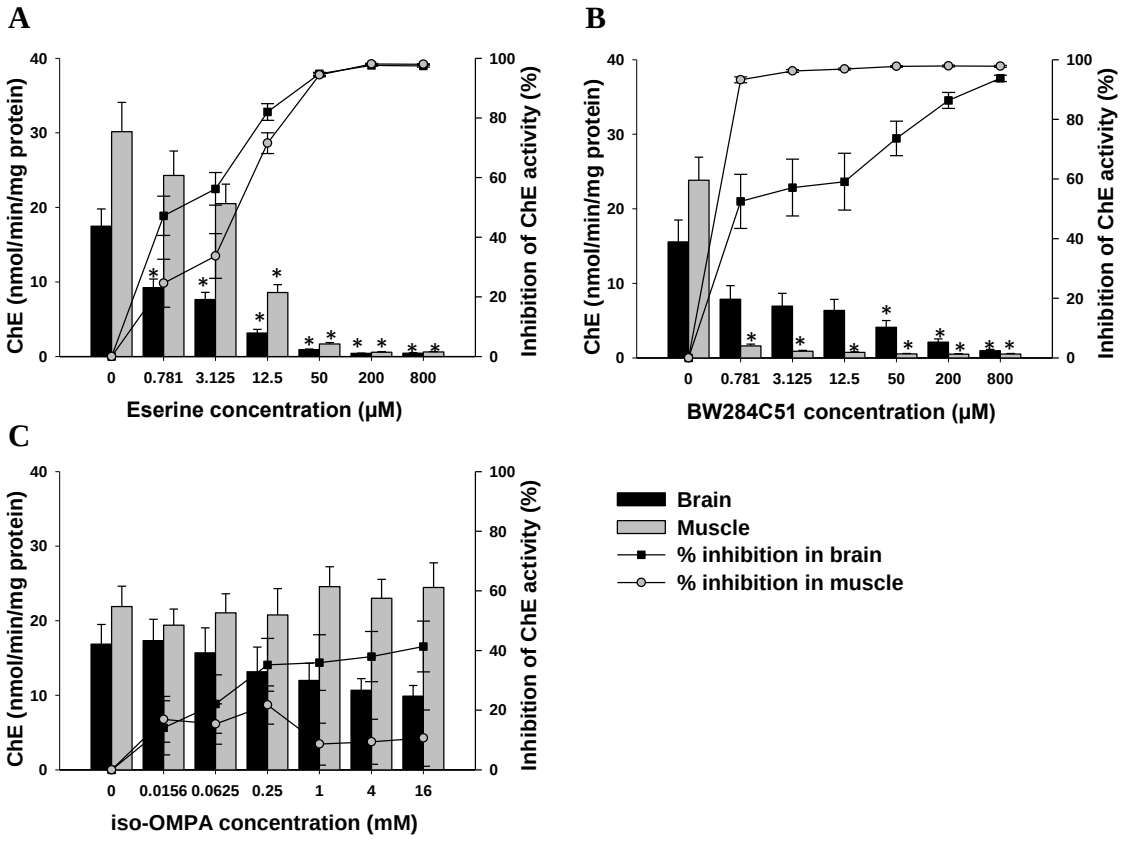

Figure 6 – Effect of the inhibitors eserine (A), BW284C51 (B) and iso-OMPA (C) on *Prionace glauca* cholinesterase (ChE) activities in brain and muscle tissues (expressed as mean values ± standard error) using acetylthiocholine as substrate. Bars correspond to ChE activities and lines correspond to the percentage of ChE inhibition. An asterisk indicates a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's test).

2.3.2. In vitro effects of chlorpyrifos-oxon

The effect of ethanol, the solvent used for chlorpyrifos stock solution, in the ChE activity was tested and compared with control and no statistical difference was observed either in brain ($t_{(14)}$ = 0.403, p = 0.693) or muscle ($t_{(14)}$ = 0.420, p = 0.681) tissues.

Regarding the effects of the *in vitro* exposure to chlorpyrifos-oxon, there was a dose-response pattern showing lower ChE activities with increasing pesticide concentrations, in both tissues tested, with almost complete inhibitions (over 97%) with the highest pesticide concentration (Fig. 7).

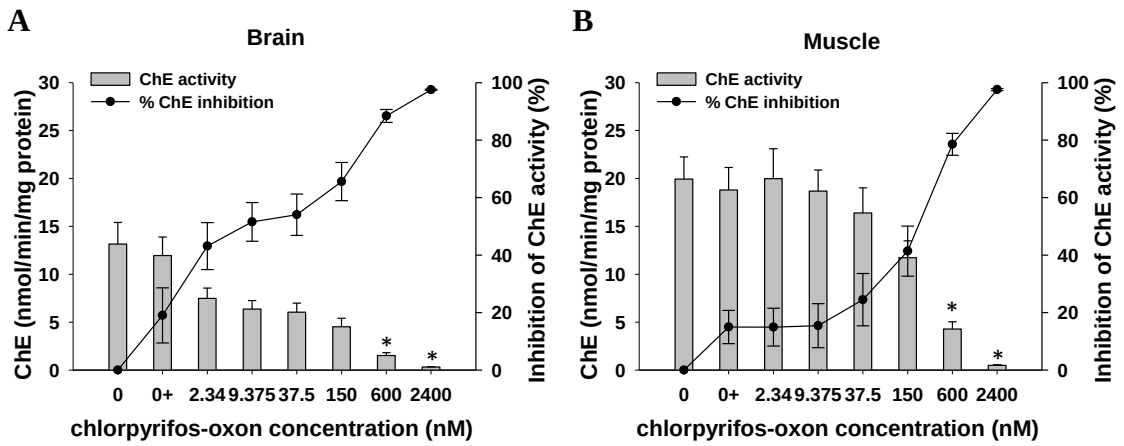

Figure 7 – Cholinesterase (ChE) activity values and percentage of activity inhibition (expressed as mean values ± standard error) in the brain (A) and muscle (B) of *Prionace glauca* exposed *in vitro* to chlorpyrifos-oxon. An asterisk indicates a significant difference from the solvent control (0+) at $p \leq 0.05$ (ANOVA, Dunnett's test).

Although significant inhibitions in relation to control were only detected at 600 nM of pesticide in both tissues (brain: $F_{6,49} = 40.38$, $p<0.001$; muscle: $F_{6,49} = 37.77$, $p<0.001$), the lower concentrations of chlorpyrifos (until 150nM) caused statistically higher ChE inhibitions in the brain than in the muscle (two-way ANOVA, $p < 0.001$). This higher sensitivity to chlorpyrifos in the brain tissue can also be seen by the estimated IC_{50} (±SE) values of 48.97 ± 3.79 nM (i.e. 16.39 µg/L) in brain and 204.97 ± 94.32 nM (i.e. 68.57 µg/L) in muscle.

In order to address effects of gender and size on the susceptibility to chlorpyrifos-oxon, samples were divided in two groups of four according to gender (males and females) and size (larger or smaller than 130 cm). The IC_{50} values for these separate groups, in brain and muscle tissues, were calculated and the results can be seen in Table 2.

Table 2 – *In vitro* inhibition concentrations (IC_{50}) of chlorpyrifos-oxon in *Prionace glauca*, depending on gender and size of the organisms.

	IC50 ± SE (nM)	
	Brain	Muscle
Gender		
Males	86.04 ± 6.38	136.49 ± 11.44
Females	21.99 ± 4.57	291.93 ± 73.37
Size		
<130 cm	69.98 ± 6.80	206.36 ± 111.56
>130 cm	29.93 ± 3.69	215.96 ± 40.04

According to the IC_{50} values, the higher sensitivity of brain tissue when compared to the muscle is visible independently of the organisms' gender or size, and the differences between tissues are even more pronounced within females and in larger individuals (Tab. 2).

Regarding the effects of gender or size within each tissue, there were some differences in the IC_{50} values, e.g. lower IC_{50} for females or larger organisms in the brain (not the same trend in muscle). These differences were however not statistically significant and therefore the response to chlorpyrifos-oxon in brain or muscle was not affected by either gender or size (two-way ANOVA, $p > 0.05$).

2.4. Discussion

2.4.1. Cholinesterase characterization

To use ChE as a biomarker of effect to pollutants in a particular species, it is vital to characterize this enzyme on different target tissues, as they may have several non-specific esterases that can mislead ecotoxicological studies (Gomes et al., 2014; Pestana et al., 2014).

Regarding the brain tissue, incubation with eserine sulfate, an organophosphorus compound well-known as a general inhibitor of ChE at low concentrations, resulted in an almost complete enzyme inhibition (Fig. 6A), meaning that the measured enzymatic activity is mostly due to ChE, and not to other nonspecific esterases (Eto, 1974; Pezzementi et al., 1991). The characterization of the brain's ChE was performed by

testing its affinity to different substrates, and response to specific inhibitors. As typical with AChE, ChE in the brain showed a preference for the substrate ATCh, presenting higher hydrolysis rates and greater catalytic efficiencies with this substrate (Tab. 1). Also, there was an inhibition of hydrolysis by excess substrate (Fig. 1), which is another characteristic of AChE (Toutant, 1989), and was previously reported for the brain of other fish species (e.g. Sturm et al., 1999a; Monteiro et al., 2005; Rodríguez-Fuentes et al., 2013). Moreover, ChE from the brain were sensitive to BW284C51, a specific inhibitor of AChE, although significant inhibitions only occurred at concentrations higher than 50 µM (Fig. 6B). Despite all these typical responses of AChE, brain ChE were also able to hydrolyze BTCh (although at a lower rate and with lower catalytic efficiency than with ATCh; Tab. 1) and showed some sensitivity to iso-OMPA, a specific inhibitor of BChE, at high concentrations (Fig. 6C). Therefore, considering the responses to the different substrates and inhibitors, these findings suggest that the brain of *P. glauca* seems to contain atypical ChE, displaying mixed properties of AChE and BChE. According to the literature, the majority of fish have almost exclusively AChE in their brains, such as *Limanda limanda*, *Platichthys flesus*, and *Serranus cabrilla*, described in a study by Sturm et al. (1999a) or several others described by Solé et al. (2008). However, similar results to the ones obtained in our study were also described for the reef fish *Haemulon plumieri* (Alpuche-Gual and Gold-Bouchot, 2008).

Regarding the muscle tissue, the enzyme preferred the substrate ATCh, presenting much higher hydrolysis rates and catalytic efficiency in relation to other substrates (Fig. 1 and Tab. 1). Similarly to what was seen in the brain, the incubation with eserine sulfate in muscle caused an almost complete inhibition of enzymatic activity (Fig. 6A) and therefore suggests that most of the measured activity is related to ChE and not other esterases. The preference for the substrate ATCh and the observed inhibition of activity by excess of this substrate (Fig. 5), along with the high sensitivity to BW284C51 and insensitivity to iso-OMPA (Fig. 6B,C), leads to the conclusion that the ChE present in the muscle of *P. glauca* have characteristics of true AChE and this seems to be the main form present. Although most marine species express both AChE, and pseudocholinesterases, in their muscle tissues at different levels depending on the species - such as demonstrated by Sturm et al. (1999a) for three teleosts species and by Solé et al. (2008) for two sharks - Garcia and co-authors (2000) observed that, like in the present blue shark samples, the muscle of *Poecilia reticulate* contains mainly AChE.

Considering the maximum enzymatic activity levels in both brain and muscle tissues observed for blue sharks, they were lower than those usually found in marine fish (Sturm et al., 1999a; Arufe et al., 2007; Oliveira et al., 2007; Alpuche-Gual and Gold-Bouchot, 2008; Rodríguez-Fuentes et al., 2013), which is in accordance with the study by Solé et al. (2008) where the authors have also reported lower enzymatic activities in the sharks *Scyliorhynus canicula* and *Galeus melastomus.*

To our knowledge, there is scarce information regarding the enzymatic pathways of sharks, specifically concerning ChE, but generally existing data suggests that activity levels might oscillate seasonally, due to size, sex, and dietary composition (Chuiko et al., 1997; Sturm et al., 1999b; Kirby et al., 2000; Beauvais et al., 2002; Flammarion et al., 2002; Chuiko et al., 2003). These correlations between ChE activity rates and physiological characteristics are, however, not always observed (Solé et al., 2006; Tortelli et al., 2006). Also in the present work, no correlations were found between the enzymatic activity levels and the physiological parameters sex and size, although it is important to refer that the sampling group was limited and more information could be taken from a larger sampling, preferably using both mature and immature individuals.

2.4.2. In vitro effects of chlorpyrifos-oxon

Given the important role of AChE in the neuromuscular system and the fact that it is often the main target of toxicity for organophosphate or carbamate insecticides, and might also be affected by other pollutants, the activity of this enzyme can be used as a biomarker of effect after exposure to contaminants, providing extensive applicability for both laboratory and field studies (Whitehead et al., 2005; Alpuche-Gual and Gold-Bouchot, 2008).

Chlorpyrifos-oxon is a metabolite of the organophosphate insecticide chlorpyrifos, widely used for pest control (Sparling and Fellers, 2007). The presence of chloropyrifos in marine ecosystems, as well as other pesticides of similar nature, is well documented making it a suitable candidate for (Lund et al.; 2000; Barakat et al.; 2002; Garcia et al.; 2014).

The results of the present study show that chloropyrifos-oxon inhibited blue shark's ChE *in vitro*, with brain tissue showing higher sensitivity towards the compound when compared to muscle tissue. Although some authors state that ChE inhibition in muscle is a better predictor of induced mortality than inhibitions in brain, with brain tissue inhibition being a better tool to foresee alterations in behaviour (Fulton and Key,

2001), it is generally assumed that, in order to monitor pesticide accumulation in aquatic environments, one should perform measurements in the tissue proven to be the most sensitive to these compounds (Whitehead et al., 2005). Therefore, as in the present study brain tissue in *P. glauca* showed higher sensitivity to chloropyrifos-oxon than muscle, one may infer that the brain has a greater potential for detection of exposure to low concentrations of this and other similar compounds in the environment.

The lack of statistically significant differences in sensitivity to chlorpyrifos-oxon between genders and sizes gives strength and robustness to this tool. Nevertheless, in order to complement and validate these findings, a greater number of samples should be collected and analyzed, preferably of adult individuals, as the increased sizes and different metabolic responses might influence ChE activity and sensitivity.

When comparing the present *in vitro* results with the ones of vertebrate fish (Carr et al., 1997), it is possible to see that the blue shark has similar IC_{50} values in the brain, but considerable higher values in muscle. To our knowledge, so far, there are no studies addressing the effects of chloropyrifos, or other pesticides, in elasmobranchs. However, there are reports of pesticide accumulation in different tissues of several shark species (Schlenk et al., 2005; Shanshan et al., 2013), which enforce the need for studies that provide better understanding on how these compounds affect such marine predators.

After addressing the ChE characterization in both tissues of blue shark, and given the high sensitivity of ChE in the *in vitro* study, the foundation work has been set to further study this enzyme and its great potential to be used as a biomarker to assess the effects of anticholinergic contaminants and for the use of this prospect tool in the oceans biomonitoring using the widespread and easy to obtain blue shark.

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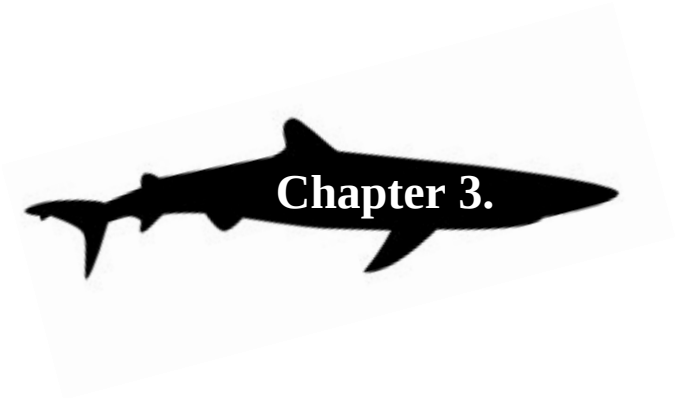

Chapter 3.

Biochemical responses in Blue sharks (*Prionace glauca*) exposed to persistent organic pollutants (POPs) – an Atlantic survey

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Biochemical responses in Blue sharks (*Prionace glauca*) exposed to persistent organic pollutants (POPs) – an Atlantic survey

Abstract

Marine ecosystems are constantly being threatened by the accumulation of xenobiotic contaminants produced by human activities. There is a need to better understand the impacts caused by pollutants on marine organisms and the development of reliable methods for biomonitoring studies is imperative. Persistent organic pollutants (POPs) are organochlorinated compounds highly toxic to humans and wildlife. Sharks are particularly susceptible to bioaccumulation of high levels of these and other pollutants, given their position on top of food webs, therefore being potential sentinel species to biomonitor such contaminants in the marine environment. The main objective of this study was to correlate biochemical responses, related with detoxification, oxidative stress or neuronal processes, with tissue contaminant body burden in blue sharks (*Prionace glauca*), and find suitable biomarkers for future pollution biomonitoring studies. No significant differences were found between shark size nor gender and POPs concentrations nor biochemical parameters studied, giving an indication of robustness of the parameters to be used as biomarkers. POPs levels were higher in the liver tissue than in the muscle. A canonical correspondence analysis (CCA) was performed and some interesting correlations were found between physiological parameters and POPs accumulation levels. DNA damage was the main consequence of contamination and the inhibition of ChE activity the most strongly correlated effect.

It can be concluded that *P. glauca* demonstrates great potential to be used as a pollution sentinel and some suitable biomarker candidates were identified with this work.

Keywords: Pollution, xenobiotics, biomarkers, oxidative stress.

3.1. 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), and perfluorochemicals (PFCs), negatively affect marine fauna or human health because of their high toxicity and persistence in the environment, which leads to bioaccumulation (Gramatica and Papa, 2007; Skomal and Mandelman, 2012). Bioaccumulation of these xenobiotics is a growing concern and has been proven to cause injurious effects on biodiversity (Franke et al., 1994; Tillitt et al., 1992; Wang, 2002; Dautremepuits et al., 2004; Storelli et al. 2011).

The changes caused by pollutants in the ecosystem usually have an earlier effect at the lower levels of biological organization, allowing the development of biomarkers to monitor changes caused by the contaminants before they cause an effect at higher complexity levels (Bayne et al., 1985; Lemos et al., 2009). Among other biological parameters, oxidative stress related enzymatic activities, DNA damage and lipid peroxidation (LPO) levels have the potential to be used as biomarkers (Winston and Di Giulio, 1991; Filho, 1996).

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 conjugates reduced glutathione (GSH) with xenobiotics, increasing their polarity and facilitating their excretion (Van der Oost et al., 2003). This process is essential but due to the redox-cycles involved it also produces oxygen radicals. Moreover, most living cells are normally undergoing some degree of oxidation, due to natural biochemical processes like respiration (Valko et al., 2007). However, the presence of POPs and other xenobiotics tends to further increase the amount of reactive oxygen species (ROS), like superoxide radical ($O2^{\bullet-}$) and hydrogen peroxide ($H2O2$), 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 on macromolecules (DNA, lipids or proteins), include enzymatic and non-enzymatic antioxidants, such as: superoxide dismutase (SOD), an enzyme responsible for the transformation of $O2^{\bullet-}$ into H2O2; the enzymes catalase (CAT) and glutathione peroxidase (GPx), both acting to eliminate H2O2 and prevent its accumulation; and glutathione reductase (GR), which reduces oxidized glutathione, ensuring that 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; Valko et al., 2007; Richardson et al., 2008).

Enzymes like acetylcholinesterase (AChE), which is responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, in a vital process for neuromuscular functions, can also be affected by exposure to contaminants (Payne et al, 1996; Kirby et al., 2000). Because of the high sensitivity of cholinesterases (ChE) to anti-cholinergic and other contaminants often present in marine ecosystems, the different ChE forms have been characterized in many different fish tissues, showing great potential to be used in pollution monitoring studies as a biomarker of effect (Van der Oost et al., 2003; Arufe et al., 2007; Solé et al., 2008)

Most sharks are top predators, ending up being more exposed to the hazardous effects of environmental contamination trough bioaccumulation (Serrano et al., 2000; Strid et al., 2007). The blue shark (Prionace glauca, L.1758) is one of the most frequently caught shark species all over the world and consequently by the Portuguese longline swordfish fishing fleet, mainly due to 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; Barrera-García et al., 2013), but very little is known about the mechanisms involved in these sharks' detoxification and antioxidant processes.

The main objective of the present study was to develop reliable biomarkers in blue sharks as tools for biomonitoring Atlantic deep waters by linking the biochemical responses with tissue contaminant body burden. To our knowledge, this study is the first attempt to correlate POPs levels with detoxification pathways, oxidative stress response mechanisms and neuronal parameters in sharks.

3.2. Materials and methods

3.2.1. Organisms

Liver, brain and muscle samples from 20 blue sharks were collected southwest of Portimão (36°43'11.2"N, 13°09'30.0"W) in December 2013, aboard a commercial swordfishing vessel. Size (total length, cm) and gender were recorded for all sampled individuals. Samples were kept on ice for transport to the laboratory, where they were stored and frozen at −80 °C until further analysis. All sharks in this study were classified as juveniles.

3.2.2. POPs chemical analysis

Liver and muscle samples were separated for POPs quantification.

Regarding polychlorinated compounds, the following congeners were analyzed: 17 polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), 12 dioxin-like polychlorinated biphenyls (dl-PCBs) and 6 indicator PCBs (i-PCBs) (Tab. 1). A fraction of each sample was transferred into Accelerated Solvent Extraction (ASE) cells. The extraction was performed in a Dionex ASE 300 device, using as solvent a mixture composed of toluene and acetone at 70:30 (v/v), and with pressure and temperature set to 100 bar and 120 °C, respectively. A following purification procedure included three consecutive chromatographic steps with silica, Florisil and celite/carbon columns. Identification and quantification of PCDD/Fs, dl-PCBs and i-PCBs were performed by gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS) using a Hewlett–Packard 6890 gas chromatograph (CA, USA) equipped with an Agilent J&W DB-5MS column (CA, USA) and a JEOL JMS-800D double sector mass spectrometer (Tokyo, Japan). The HRMS was operated in electron ionization mode at 38-40 eV and the ion source temperature was set to 280 °C.

The analysis of brominated flame retardants (BFRs) was 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 (HBCD). In short, muscle and liver samples were extracted in an ASE as described previously, followed by purification in solid-phase using silica gel. For PBDEs and PBBs, additional procedures of purification in solid-phase were conducted, the first one using

Florisil® and the second using Florisil®, carbon and celite. Identification and quantification were achieved by GC-HRMS using an Agilent 7890A gas chromatograph and equipped with a DB-5MS or a Restek Rtx-1614 column (PA, USA), depending on the congeners, and coupled to a JEOL JMS-800D double sector mass spectrometer. The HRMS was run in the same conditions as described for PCDD/Fs and PCBs. For HBCD, a liquid-liquid purification was performed, followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). It was used an HPLC system 1200 series Agilent equipped with a reverse phase column Thermo Scientific Hypersil Gold C18 (MA, USA) and an Agilent 6410 triple quadrupole mass spectrometer. The MS was operated in negative electrospray ionization mode.

3.2.3. Tissue preparation

Blue shark tissue samples were homogenized after defrosting. From each sample, approximately 300 mg of liver was 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 10,000g, for 20 minutes (4 ºC). The resulting post mitochondrial supernatant (PMS) was then separated into different microtubes and stored at -80 ºC for posterior protein quantification and total levels of glutathione (TG) as well as for the activity measurements of SOD, GPx, GR, GST and CAT.

From each brain and muscle sample approximately 200 mg of tissue was homogenized, in a 1:5 proportion, in K-phosphate buffer (0.1 M, pH 7.2), followed by a centrifugation at 3,000 g, for 3 minutes (4ºC). After this, the supernatant was separated into different microtubes and stored at -80 ºC for posterior protein quantification and measurements of the ChE activity. In all assays, blanks were made using K-phosphate buffer instead of the sample. All of the spectrofotometric measurements were made at 25ºC in a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

3.2.4. 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. In each well of the microplate 10 µL of each sample (diluted 40x) was added along with 290 µL of Bradford reagent (in quadruplicates). After 15 minutes of agitation at 150 revs/min, absorbance was read at 600 nm and results were expressed in mg of protein/mL.

3.2.5. Oxidative stress parameters

3.2.5.1. Lipid Peroxidation

Lipid peroxidation levels was 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 Wilhelm et al. (2001) and Torres et al. (2002). The samples were deproteinized in a mixture containing 12% trichloroacetic acid (TCA), followed by the addition of 0.73% 2-thiobarbituric acid (TBA). The tubes were left incubating at 100 ºC for one hour and later centrifuged at 11,000g for 5 minutes. The supernatant was then used to measure TBARS (in quadruplicates), at 535 nm and the results were calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$. Results were expressed as nmol TBARS/g of wet weight.

3.2.5.2. DNA damage

The DNA strand breaks were measured using the DNA alkaline precipitation assay (Olive, 1988), adapted from Lafontaine et al. (2000). Tissue homogenates (50 µL) were incubated with 500 µL of a SDS solution (2%) containing 50 mM NaOH, 10 mM Tris, and 10 mM EDTA plus 500 µL of 0.12 M KCl at 60ºC for 10 minutes. Samples were cooled on ice for 15 minutes to induce the precipitation of SDS associated nucleoproteins and genomic DNA, and were finally centrifuged for 5 minutes, at 8,000g (4 ºC) to enhance precipitation. Finally, to measure the levels of single and double-stranded DNA remaining in the supernatant, 50 µL of this supernatant was mixed and labelled with 200 µL of Hoesch dye (1µg/mL, bisBenzimide, Sigma-Aldrich) in each microplate well (in quadruplicates). Fluorescence was measured using an excitation/emission wavelength of 360/450 nm. Results were expressed as mg of

DNA/g of wet weight, using calf thymus DNA as standard to extrapolate DNA concentration.

3.2.5.3 Glutathione S-transferase activity

For the assessment of the glutathione S-transferase (GST; EC 2.5.1.18) activity, an adaptation of the procedure described by Habig et al. (1974) was used. In each microplate well, 275 µL of reaction buffer [4950 µL of K-Phosphate 0.2 M, 900 µL of GSH (10 mM) and 150 µL of CDNB (60 mM)] were added to 25 µL of previously diluted sample to a protein concentration of 0.8mg/mL (in quadruplicates).

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 $9.6x10^3$ $M^{-1}cm^{-1}$, and expressed in nmol/min/mg of protein.

3.2.5.4 Superoxide dismutase activity

The activity of superoxide dismutase (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. A stock solution of xanthine (0.7 mM) with 0.001 M NaOH was prepared and later diluted, in a 1:5 proportion, in K-Phosphate 0.05 M (pH 7.0), to obtain the required xanthine concentration (0.14 mM). A stock solution of superoxide dismutase standard, containing 30 U/mL, was also made and then diluted in K-Phosphate 0.05M (pH 7.0) to a concentration of 1.5 U/mL. Samples were diluted 200x in buffer K-phosphate (0.1M), pH 7.4. The reaction mixture in each microplate well contained 50 µL of superoxide dismutase standard or sample (in quadruplicates), 100 µL of K-Phosphate 0.05 M (pH 7.0), 50 µL of xanthine (0.14 mM), 50 µL of cytochrome c (0.06 M) and 50 µL of xanthine oxidase (0.01 U/mL). The decrease of the cytochrome c reduction was followed at 550 nm and SOD activity was expressed in U/min of protein using a SOD standard of 1.5 U/ml where 1U represents the amount of enzyme in the sample that causes a 50% inhibition of cytochrome c reduction.

3.2.5.5 Catalase activity

Catalase (CAT; EC 1.11.1.6) activity was estimated by following the decay in the H_2O_2 concentration at 240 nm, adapting the method described by Clairborne (1985). Samples were diluted to a protein concentration of 0.8 mg/mL and to each microplate well 15 µL of sample were added, along with 135 µL of K-Phosphate 0.05M (pH 7.0) and 150 µL of H_2O_2 0.03 M (in quadruplicates). Absorbance was read every 10 seconds for 1 minute. CAT activity is expressed in nmol/min/mg of protein, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

3.2.5.6 Glutathione reductase activity

Glutathione reductase (GR; EC 1.8.1.7) catalytic activity was measured by following the decrease in absorbance during NADPH oxidation. Samples were diluted to a protein concentration of 0.8 mg/mL and a reaction buffer containing K-Phosphate with pH 7.0 (0.05 M), NADPH (0.2 mM), GSSG (1 mM) and DTPA (0.5 mM) was made. In the microplate wells, 20 µL of sample and 280 µL of reaction buffer were added (in quadruplicates). Absorbance was read at 340 nm for 1 min. The enzymatic activity was calculated using a molar extinction coefficient of $6.2x10^3$ $M^{-1}cm^{-1}$, and expressed in nmol/min/mg of protein.

3.2.5.7 Glutathione peroxidase activity

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was measured by monitoring the oxidation of NADPH at 340 nm, when GSSG is reduced back to GSH by GR, using H_2O_2 as a substrate (Mohandas et al., 1984). The reaction mixture contained K-Phosphate 0.05M (pH 7.0) with EDTA (1mM), Sodium Azide (1mM), GR (0.13U/mL), GSH (4mM), NADPH (0.8mM) and H_2O_2 (0.5mM). Samples were diluted to a protein concentration of 0.8 mg/mL and each well of the microplate was loaded with 50 µL of sample and 250 µL of reaction mixture (in quadruplicates). GPx activity was measured during 1 min. Activity was calculated using a molar extinction coefficient of $6.2x10^3$ $M^{-1}cm^{-1}$ and expressed in nmol/min/mg of protein.

3.2.6 Total glutathione activity

To measure TG activity, liver tissue samples were previously diluted in a 1:2 proportion. In each microplate well, 250 µl of the reaction solution [2 mL of NADPH (6mM), 4 mL of DTNB (50 mM), 1 mL of GR (14.2U/mL) and 12 mL of NaK-

Phosphate (0.2M)] were added to 50 µl of sample (in quadruplicates), and the absorbance was read at 340 nm. TG activity was calculated using the molar extinction coefficient of DTNB ($13.6x10^3$ $M^{-1}cm^{-1}$) and expressed in nmol/min/mg of protein.

3.2.7 Cholinesterase activity

The ChE activity was measured in supernatant of each sample, with a final protein concentration of 0.8 mg/mL, from both muscle and brain tissues, following the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). In each microplate well 250 µl of the reaction solution [30ml potassium-phosphate buffer (0.1 M, pH 7.2), 1ml of reagent 5,5-dithiobis-(2-nitrobenzoic acid) 10mM (DTNB) and 200 µl of acetylcholine (0.075 M)] was added to 50 µl of sample (in quadruplicates), and the absorbance was followed for 5 min, at 414 nm. ChE activity was calculated using the molar extinction coefficient of $13.6x10^3$ $M^{-1}cm^{-1}$ expressed in nmol/min/mg of protein.

3.2.8 Statistical analysis

All data were checked for normality and homoscedasticity. To compare averages between sexes and sizes, independent sample t-tests or Mann-Whitney non-parametric tests were conducted (depending on the violation or not of the normality and homogeneity of variance assumptions). For all statistical tests, the significance level was set at $p \leq 0.05$. Correlations between oxidative stress parameters were assessed using Pearson Correlation tests. A Canonical Correspondence Analysis (CCA) was performed to evaluate distribution and correlation patterns based on the POPs concentrations and the oxidative stress biomarkers. POPs data were standardized and the oxidative stress biomarkers were log (x + 1) transformed (Legendre and Legendre, 1979). Downweighting of oxidative stress biomarkers were performed. A forward selection procedure was performed on the set of POPs variables.

Only the significant variables ($p \leq 0.05$, Monte Carlo test) were included in the model (ter Braak and Smilauer, 2002). All statistical tests were performed with Sigma plot 11.0 (Systat Software, Inc. Chicago, IL, USA). CCA were performed with CANOCO version 4.5 package (ter Braak and Smilauer, 1998).

3.3. Results

The 20 blue sharks sampled for 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. Individuals with less than 130 cm TL were classified as small (S) and the ones over 130 cm TL were classified as large (L).

3.3.1 POPs chemical analysis

The concentrations of POPs measured in the liver and muscle of the blue sharks are summarized in Table 3.

Table 3 - Lipid percentage and concentrations of quantified POPs (ng/g wet weight) in muscle and liver from 20 blue sharks (*Prionace glauca*) captured in the Atlantic Sea.

	Muscle			**Liver**		
	Min	**Max**	**Average**	**Min**	**Max**	**Average**
Lipids (%)	1.3609	2.6452	**1.7984**	15.884	58.5538	**44.8628**
2.3.7.8 – TCDD	0.0006	0.0018	**0.0011**	0.0320	0.5823	**0.1449**
1.2.3.7.8 – PeCDD	0.0005	0.0029	**0.0014**	0.1235	2.0483	**0.4943**
1.2.3.4.7.8 – HxCDD	0.0007	0.0024	**0.0014**	0.0236	0.6486	**0.1589**
1.2.3.6.7.8 – HxCDD	0.0008	0.0062	**0.0027**	0.1123	2.3924	**0.7439**
1.2.3.7.8.9 – HxCDD	0.0008	0.0032	**0.0016**	0.0239	0.4511	**0.1290**
1.2.3.4.6.7.8- HpCDD	0.0074	0.1168	**0.0185**	0.0960	0.9086	**0.3252**
OCDD	0.0126	0.2256	**0.0445**	0.0545	0.9348	**0.2353**
Total PCDDs	0.0251	0.3129	**0.0712**	0.5406	7.9660	**2.2315**
2.3.7.8 – TCDF	0.0009	0.0114	**0.0029**	0.7538	10.7056	**2.4622**
1.2.3.7.8 – PeCDF	0.0009	0.0095	**0.0024**	0.1864	2.6830	**0.7173**
2.3.4.7.8 – PeCDF	0.0015	0.0065	**0.0029**	0.4163	9.8109	**2.0221**
1.2.3.4.7.8 – HxCDF	0.0015	0.0046	**0.0024**	0.0775	0.9234	**0.2889**
1.2.3.6.7.8 – HxCDF	0.0008	0.0038	**0.0021**	0.0601	1.0696	**0.3051**
1.2.3.7.8.9 – HxCDF	0.0005	0.0016	**0.0011**	0.0035	0.0272	**0.0168**
2.3.4.6.7.8 – HxCDF	0.0005	0.0032	**0.0014**	0.0624	1.8499	**0.4457**
1.2.3.4.6.7.8 –HpCDF	0.0027	0.0099	**0.0049**	0.0291	0.3598	**0.1105**
1.2.3.4.7.8.9 –HpCDF	0.0008	0.0039	**0.0018**	0.0060	0.0378	**0.0228**
OCDF	0.0040	0.0333	**0.0116**	0.0092	0.0769	**0.0441**
Total PCDFs	0.0181	0.0563	**0.0336**	1.7838	26.8555	**6.4355**
OMS-TEQ$_{2005}$ PCDD/F / fresh weight	0.0031	0.0102	**0.0052**	0.4298	7.4168	**1.7271**
OMS-TEQ$_{2005}$ PCDD/F - incertitude	0.0025	0.0084	**0.0043**	0.3549	6.1240	**1.4261**
PCB 77	0.0459	0.2900	**0.1044**	24.3814	333.3864	**87.6985**
PCB 81	0.0046	0.0250	**0.0117**	1.1243	16.8506	**5.1215**
PCB 126	0.0077	0.2195	**0.0468**	13.2526	255.4532	**70.7059**
PCB 169	0.0119	0.1208	**0.0316**	3.7824	146.4000	**30.9622**
Total coplanar	0.0855	0.6098	**0.1945**	43.4218	620.6693	**194.4881**
PCB 105	3.3250	46.6195	**14.912**	1010.538	33847.489	**7564.7556**
PCB 114	0.2365	5.5752	**1.5593**	55.7470	1918.8458	**463.6749**
PCB 118	14.4592	188.541	**69.176**	4064.472	110391.63	**27609.247**
PCB123	0.1597	1.5174	**0.6507**	39.3213	1071.9972	**280.3289**
PCB 156	1.4167	13.8913	**6.1635**	609.8411	15257.222	**4133.4432**
PCB 157	1.0667	16.0312	**5.0351**	297.3476	7565.8815	**2017.7113**
PCB 167	1.0442	10.1388	**4.1780**	430.8727	12103.195	**2991.5649**
PCB 189	0.1913	2.1505	**0.8536**	70.6880	2584.9119	**717.0053**
Total non-coplanar	23.2250	277.520	**102.529**	6578.827	184741.17	**45777.731**
OMS-TEQ$_{2005}$ PCB DL / fresh weight	0.0023	0.0309	**0.0087**	1.6653	35.5048	**9.3831**

OMS-TEQ$_{2005}$ PCB DL - uncertainty	0.0019	0.0246	**0.0069**	1.3253	28.2547	**7.4671**
TOTAL-TEQ$_{2005}$ (PCDD/F + PCB DL) / fresh weight	0.0058	0.0372	**0.0140**	2.1391	39.2087	**11.1102**
TOTAL-TEQ$_{2005}$ (PCDD/F + PCB DL) - uncertainty	0.0047	0.0298	**0.0113**	1.7165	31.3130	**8.8931**
PCB 28	0.0023	0.0125	**0.0049**	0.1510	3.2817	**0.9355**
PCB 52	0.0039	0.0436	**0.0143**	0.3120	10.9271	**3.3262**
PCB 101	0.0132	0.2549	**0.0588**	1.7509	62.4268	**15.3901**
PCB 138	0.0545	1.2085	**0.2970**	11.9020	331.6049	**83.1821**
PCB 153	0.0883	2.0576	**0.5087**	18.9865	487.3271	**148.3582**
PCB 180	0.0346	0.8075	**0.2376**	10.1000	268.4717	**77.2515**
Total 6 Nd-I_PCB / fresh weight	0.1970	4.3845	**1.1214**	43.2025	1164.0393	**328.4436**
Total 6 Nd-I_PCB / - uncertainty	0.1523	3.3897	**0.8670**	33.3999	899.9188	**253.9198**
PBDE 28	0.0003	0.0025	**0.0008**	0.0375	0.6603	**0.1551**
PBDE 47	0.0056	0.0696	**0.0208**	0.8005	13.8874	**4.1090**
PBDE 99	0.0004	0.0072	**0.0019**	0.0490	1.9052	**0.4270**
PBDE 100	0.0014	0.0248	**0.0061**	0.2230	5.7280	**1.3827**
PBDE 153	0.0003	0.0040	**0.0012**	0.0400	1.5189	**0.2992**
PBDE 154	0.0011	0.0146	**0.0047**	0.1738	5.2560	**1.1917**
PBDE 183	0.0002	0.0062	**0.0018**	0.0051	0.0458	**0.0151**
PBDE 209	0.0014	0.0465	**0.0171**	0.0097	0.1872	**0.0512**
Total 7 PBDE_Ind	0.0090	0.1154	**0.0354**	1.3239	28.9813	**7.5672**
Total 8 PBDE_Ind	0.0175	0.1314	**0.0543**	1.3455	28.9943	**7.6309**
PBB 52	0.0001	0.0018	**0.0007**	0.0045	0.1734	**0.0459**
PBB 101	0.0002	0.0015	**0.0008**	0.0082	0.2763	**0.0780**
PBB 153	0.0002	0.0018	**0.0008**	0.0065	0.3447	**0.0996**
Total 3 PBB	0.0006	0.0052	**0.0023**	0.0200	0.6596	**0.2234**
Alpha	0.0000	<0.0061		0.0000	5.3159	
Béta	0.0000	<0.0026		0.0000	0.0119	
Gamma	0.0024	<0.0079		0.0000	0.3200	
Total 3 HBCD (Low)	0.0000	0.0030	**0.0003**	0.2967	11.1709	**2.3461**
Total 3 HBCD (Upp)	0.0031	0.0132	**0.0063**	0.2990	11.1839	**2.3561**

PCDDs = polychlorinated dibenzo-p-dioxins; PCDFs = polychlorinated dibenzofurans; PCBs = polychlorinated biphenyls; Nd-I_PCB = non-dioxin like polychlorinated biphenyls; PBDE_Ind = Polybrominated diphenyl ether indicators; PBB = Polybrominated biphenyls; HBCD = hexabromocyclododecanes.

The results showed (Tab. 3) that non-coplanar PCBs were the dominant chemicals in liver tissue, with an average of around 46,000 ng/g wet weight, (ww) followed by non-dioxin like PCBs and coplanar PCBs, with around 328 ng/g ww and 194 ng/g ww, respectively. Regarding the POPs concentrations in muscle tissue, it is possible to observe that, although in lower levels, the dominant contaminants are in accordance with the ones in liver, following the same order with 103 ng/g ww for non-coplanar PCBs, 1.12 ng/g ww for non-dioxin like PCBs and 0.2 ng/g ww for coplanar PCBs.

3.3.2 Oxidative damage

Concerning DNA damage and LPO levels, no statistically significant differences were found between genders or between the two different groups of sizes (Fig. 8; Student t-test, $p > 0.05$). Also, the levels of damaged DNA and LPO were not statistically correlated with the size of the individuals (Pearson's correlation, $p > 0.05$).

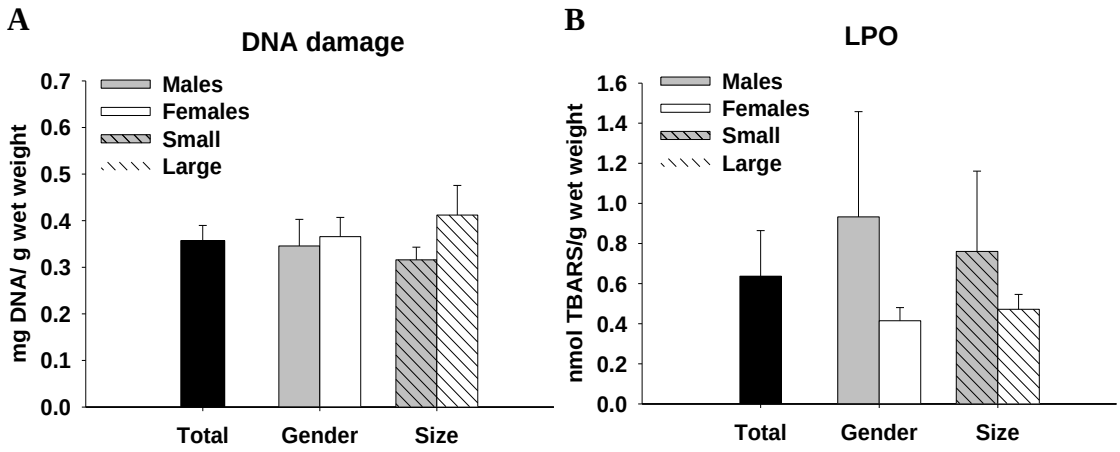


Figure 8 – Oxidative damage measured in the liver of juvenile blue sharks (*Prionace glauca*) by means of A) DNA damage levels and B) Lipid peroxidation (LPO) levels. Results are presented as mean ± standard error (SE) and are divided by total number of samples (n = 20), by gender (males, n = 8; females, n = 12) and by size (<130cm, n = 12; >130cm, n = 8).

3.3.3 Detoxification/antioxidant and neurotoxicity related enzymes

Considering the enzymatic activities of the whole group of 20 individuals (Total, Fig. 9), catalase showed the highest activity values (12,917 nmol/min/mg protein), followed by SOD (169.36 nmol/min/mg protein) and GST (107.64 nmol/min/mg protein), whereas total glutathione showed the lowest activity values (2.20 nmol/min/mg protein).

As it was observed for the non-enzymatic oxidative stress parameters, also the enzymatic activity levels results showed no statistically significant differences between genders or between the two different groups of sizes (student t-tests, $p > 0.05$).

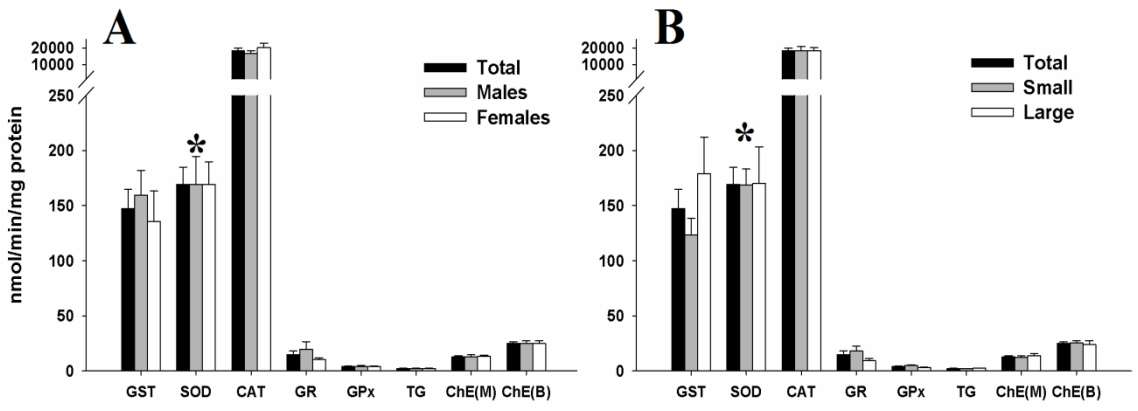


Figure 9 - Distribution of enzymatic activity values of the total juvenile blue sharks (*Prionace glauca*) samples (n= 20) when comparing to values of: A – male and female group of individuals; B – small and large group of individuals. GST = Glutathione S-transferase; SOD = Superoxide dismutase; CAT = catalase; GR = Glutathione reductase; GPx = Glutathione peroxidase; TG = Total glutathione; ChE(M) = Acetycholinesterase in muscle; ChE(B) = Acetycholinesterase in brain. Results are presented as mean ± SE. * - SOD activity is expressed in U/min.

Correlations between all tested biomarkers were assessed and the results are described in Table 4.

Table 4 - Correlation analysis (Pearson Correlation) between all oxidative stress parameters assessed in this study. The pair of variables with significant positive or negative correlation coefficients ($p < 0.05$) are underlined and highlighted in bold.

	DNA damage	***LPO***	***GST***	***SOD***	***CAT***	***GR***	***GPx***	***TG***	***ChE (M)***	***ChE (B)***
DNA damage										
LPO	-0.40									
GST	0.31	-0.21								
SOD	0.27	0.16	-0.12							
CAT	0.23	-0.08	**0.78**	-0.22						
GR	-0.12	-0.22	-0.22	-0.24	-0.30					
GPx	**-0.57**	0.08	-0.33	0.08	-0.33	0.15				
TG	0.42	0.11	0.00	0.42	0.02	-0.08	-0.38			
ChE (M)	-0.01	-0.04	0.12	**-0.54**	0.24	-0.10	-0.36	0.08		
ChE (B)	**-0.57**	-0.02	-0.23	0.01	-0.01	0.12	0.32	-0.16	-0.14	

LPO = lipid peroxidation; GST = Glutathione S-transferase; SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; GPx = Glutathione peroxidase; TG = Total glutathione; ChE(M) = Acetycholinesterase in muscle; ChE(B) = Acetycholinesterase in brain.

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. DNA damage correlated negatively both with GPx and ChE_B, as well as SOD and ChE_M.

3.3.4 Multivariate analysis

CCA was performed, in order to evaluate the relation between environmental contaminants present in liver tissue and biological stress responses. The results of the CCA are shown in Table 5 and Figure 10.

Table 5 - Eigenvalues for CCA axes and correlation coefficients between environmental factors and CCA ordination axes.

	Axis 1	***Axis 2***	***Axis 3***	***Axis 4***
Eigenvalues	0.0024	0.008	0.006	0.003
Biological stress responses – environmental contaminants correlations	0.399	0.788	0.306	0.682
Cumulative percentage variance				
of biological stress responses	9.9	13.1	15.4	16.9
of biological stress responses – environmental contaminants relation	54.7	72.4	85.2	93.1
Sum of all unconstrained eigenvalues		0.240		
Sum of all canonical eigenvalues		0.044		

Although the analysis was performed for all axes, only the first factorial plane (axes 1 and 2) will be interpreted, as they retain most of the variability in the data analysis. (i.e., 72.4% of the explained variability; see Tab. 5). Biological stress responses accounted for 9.9% of the total explained variability (axis 1). Physic and chemical variables explain 18.33% of the total explained variability. Of these 18.33%, 54.7% correspond to axis 1 and 17.7% to axis 2.

The clustering of data resulting from CCA produced the biplot presented in Fig. 10, which reveals that all POPs presented a positive correlation (since the vectors that represent such variables have an acute angle.).

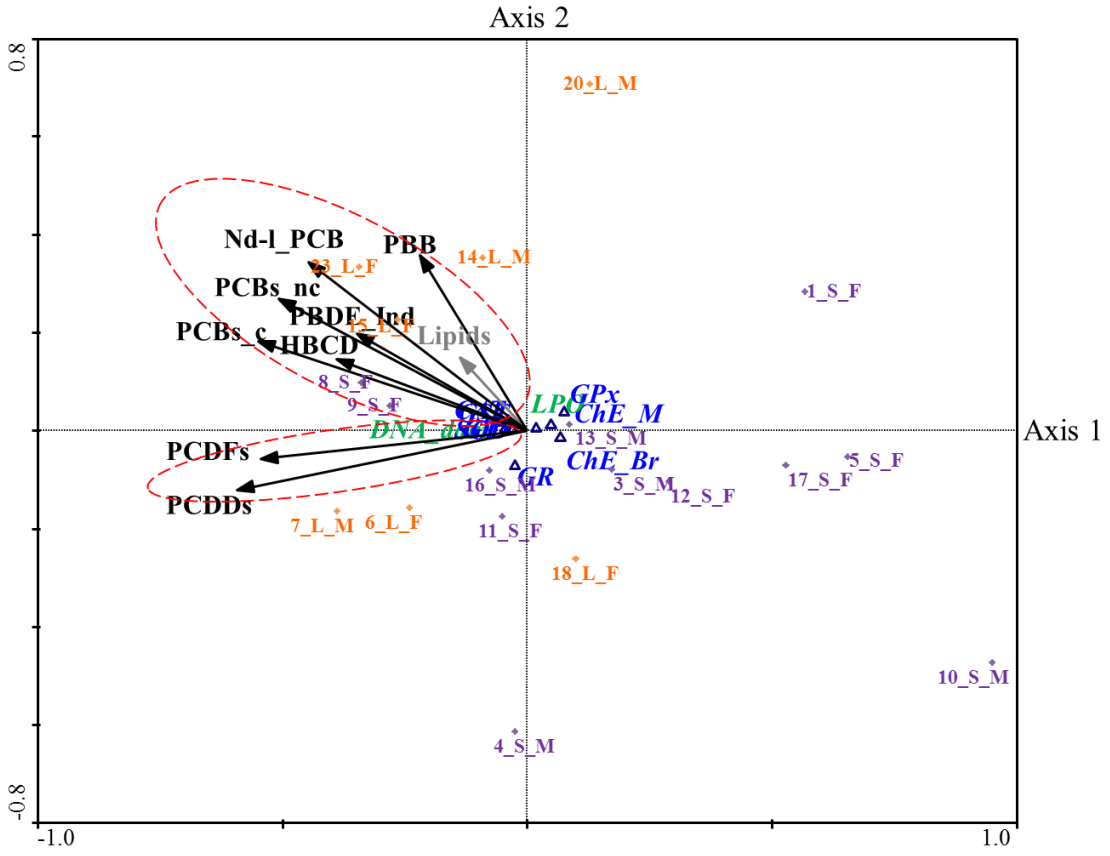


Figure 10 - Triplot of axes 1 and 2 of the Canonical Correspondence Analysis (CCA) on contaminants and oxidative stress parameters data. Arrows indicate the different types of persistent organic pollutants (POPs) quantified, triangles represent enzymatic and non-enzymatic biomarkers measured and dots indicate each individual sample numbered from 1 to 20 (M = males; F = females). POPs abbreviations: PCDDs = polychlorinated dibenzo-p-dioxins; PCDFs = polychlorinated dibenzofurans; PCBs_c = coplanar polychlorinated biphenyls; HBCD = hexabromocyclododecanes; PCBs_nc = non-coplanar polychlorinated biphenyls; PBDE_Ind = Polybrominated diphenyl ether indicators; Nd-I_PCB = non-dioxin like polychlorinated biphenyls; Lipids = lipid percentage; PBB = *Polybrominated biphenyls.* Biomarkers abbreviations: 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_Br = cholinesterase measured in brain. Colour code: black – POPs; Grey – Lipids; Green – oxidative damage parameters; Blue – enzymatic parameters; Purple – Small animals (S); Orange – Large animals (L).

Along the first two axes of the biplot it is possible to identify two main clusters, namely one of composed by PCDFs and PCDDs, and other by the remaining contaminants. The plot analysis indicates that, although minimal, there is a positive correlation between the concentration of POPs and the biochemical parameters CAT, SOD, GST, GT and DNA damage. The enzymes GPx, ChE and GR, along with the lipid peroxidation levels, tend to have a negative correlation with contaminants. The PCDDs are the most influential pollutants to the data variability (given the size of the

vector associated with it) and HBCD and PBDE_Ind are less clear in explaining the variability in the data analysis (vectors with smaller longevity). DNA damage is the most positively correlated parameter and ChE activity in brain is the least.

3.4. Discussion

Marine apex predators are known to accumulate pollutants in their bodies in a more severe way than the majority of other animals in their food chain (Storelli et al., 2005; Storelli and Marcotrigiano, 2006; Storelli et al., 2007; 2008). Due to the placement of top predators in the trophic chain, their immune system have been shown to be particularly affected by contamination (Ross et al., 1995) and a correlation between the concentration of contaminants, like POPs in a given site, and the number of these animals present has also been observed (Safe, 1994; Jones and Voogt, 1999).

The results show there was a higher concentration of POPs in the liver when compared with muscle (Tab. 3), which was expected since the liver is the main detoxification organ in most organisms, including sharks. POPs chemical analysis in shark tissues also showed that dioxin-like PCBs were the most abundant contaminants, especially non-coplanar PCBs. Although non-coplanar 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). There are very few studies about the concentrations and accumulation patterns of POPs in sharks, and information concerning Atlantic waters is scarce (Storelli and Marcotrigiano, 2001; Storelli et al., 2005; 2011; Strid et al., 2007). Some of the POPs analysed in the present study, to our knowledge and to date, have never been quantified in sharks, which is the case for polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) and hexabromocyclododecanes (HBCDs).

When looking at the results available in the literature about the concentration of contaminants in sharks, and although there are some differences in the studies in terms of sampled tissues, species used or contaminants quantified, in general concentrations seem to fluctuate not only among different species of sharks, but also between animals of the same species (Hueter et al., 1995; Serrano et al., 2000; Storelli and Marcotrigiano, 2001; Branco et al., 2004; Storelli et al., 2005; Branco et al, 2007; Storelli et al., 2011; Barrera-García et al, 2012; Maz-Courrau et al., 2012; Barrera-García, 2013; Shanshan et al., 2013; De Carvalho et al., 2014). This difference is also

influenced by the state of maturation of the organisms and most certainly the POPs concentrations measured in the present study, all from juvenile individuals, would have been higher if the organisms sampled were older, as bioaccumulation has been proven to increase with size and age (Fernandes et al., 2007).

The lipid percentage measured in the liver and muscle tissues was, as expected, higher in liver samples (Tab. 3). It is known that PCDDs/Fs in fish have a tendency to accumulate in tissues with high lipid content, like the liver (Wu et al., 2001; Korhonen et al., 2001). This tendency was also seen for the blue shark in the present study for these particular POPs and also for all the others measured, presenting higher concentrations in liver then in muscle. Sharks are known for having high lipid contents in their bodies, especially in livers, and this contaminant accumulation patterns have already been observed for Greenland sharks (Somniosus microcephalus) (Strid et al., 2007).

Marine organisms are persistently in contact with foreign contaminants, causing them to suffer physiological alterations which can be followed and studied, for example, by measuring the activity of antioxidant enzymes and indicators of oxidative damage (Heath, 1995; Carson, 2013). It has been described that LPO and DNA damage can both cause several pathologies and, over the last decade, researches have observed that exposure to contaminants, and the resulting exposure to reactive oxygen species 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 levels of LPO here quantified for the blue sharks was similar to what has been seen for other blue sharks from the pacific ocean (Barrera-García et al., 2013) but lower than what was observed in mako sharks (Vélez-Alavez et al., 2013). Most of the studies addressing enzymatic activities in sharks have been focused in relating the antioxidant defence system with trace elements concentrations and the present results on LPO levels are in accordance with these previous studies where the authors have verified that, due to their hunting habits, more active sharks, like mako (Isurus oxyrinchus) and silky sharks (Carcharhinus falciformis), have higher levels of free radicals than species such as blue (Prionace glauca), smooth hammerhead (Sphyrna zygaena) and small-spotted catsharks (Scyliorhinus canicula) (Gorbi et al., 2004; López-Cruz et al., 2010; Barrera-García et al., 2013; Vélez-Alavez et al., 2013).

The present results showed that, amongst the enzymes tested, CAT presented the highest activity rates, followed by SOD and GST (Fig. 9). These results are similar to the ones observed in other studies with blue sharks, although related with other types of contamination (Barrera-García et al., 2012; 2013), and may be explained by the essential role that GST, SOD and CAT play in the detoxification process and oxidative stress response. Similarly to what was observed by Barrera-García and colleagues (2012, 2013), GR and GPx showed very low activity rates when compared with SOD, CAT and GST.

The correlation analysis performed between the different biochemical parameters (Tab. 2) indicates that GST and CAT are positively correlated and therefore may work together to minimize the effects of contaminants. Likely, when GST is being induced to biotransform the xenobiotics, CAT is also being induced to cope with the resulting ROS. The negative correlation between GPx and DNA damage suggests that this enzyme acts to prevent oxidative damage in these macromolecules and whenever the enzyme is induced there are less harmful effects on DNA or vice-versa. The same type of correlation was observed between DNA damage and ChEB and between SOD and ChEM, suggesting that the contamination levels of the organisms can negatively affect them through these different pathways (oxidative stress and neuronal dysfunction) by inhibiting ChEs and at the same type inducing SOD and causing oxidative damage in DNA.

The Canonical Correspondence Analysis (CCA) was performed with the purpose of trying to link persistent organic pollutant concentrations with the biochemical biomarkers and physiological parameters (Tab. 5). The components derived from this analysis allow a better interpretation of the relations between the environmental and the physiological variables. The statistical analysis was performed using the POPs data found in the liver given that this was the tissue where higher concentrations were found, and also because bioaccumulation effects are usually greater in this tissue.

Although some studies have already been successful in correlating trace element concentrations and biochemical markers of oxidative stress in sharks (e.g. Barrera-Garcia et al., 2012; 2013) no information is available on the effects of POPs in the enzymatic activities of these fish. PCDFs and PCDDs differ from the rest of the POPs quantified in this study in their origin, being by-products formed in some industrial

processes and not intentionally created products for industrial uses (Altarawneh et al., 2009). The fact that these two pollutants formed a separate cluster in the CCA (Fig. 10) may indicate that they have different and specific bioaccumulation patterns and toxicity pathways.

CCA plot also reveals an apparent positive correlation between the contaminants and the variables DNA damage, CAT, SOD, GST and GT (Fig. 10). These results suggest that when the organisms are exposed to POPs, GST is induced along with levels of glutathione (TG) which are needed as substrate to increase the polarity of the xenobiotics and facilitate their excretion (Lenártová et al., 1997; Van der Oost et al., 2003). In turn, with the increase in the formation of ROS resulting from this xenobiotics´ biotransformation process, the activity of SOD and CAT seem to also be induced, which can be explained by the role of SOD in converting $O2^{\bullet-}$ into H2O2, which is then eliminated by CAT. Although the induction of these enzymes might have prevented LPO (negative correlation), it seems that they were not able to effectively counter the effects of ROS and prevent DNA damage, which is in accordance with the work of González-Mille and co-authors (2010) where it was demonstrated that POPs have the capacity to induce DNA damage in fish through the formation of ROS.

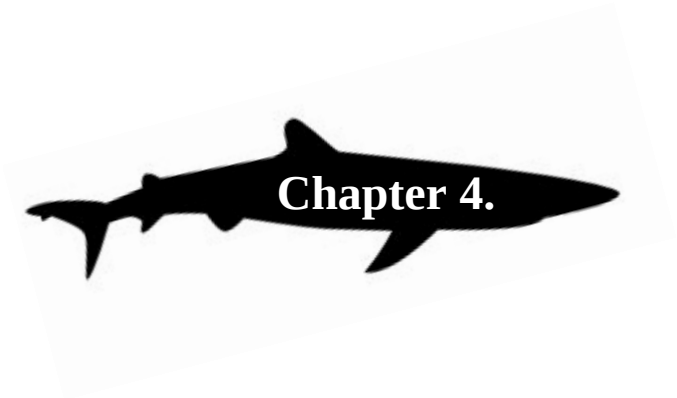
Cholinesterases do not have a role in POPs detoxification or in preventing their harmful effects, but the role of these enzymes are vital for the organism's neuronal and muscular functions (Solé et al., 2006). The negative correlation between POPs concentrations and the activity of these enzymes indicates that the organochlorinated contaminants can impair their action. The inhibition of ChE can have particularly severe implications causing nervous impulses to become continuous and this constant nervous stimulation can lead to the organism's death (Payne et al., 1996). Being the most strongly correlated parameter, ChE stands as a suitable biomarker to assess the exposure to POPs in blue sharks.

In sum, the present results represent a first insight into the physiological effects caused by POPs on sharks. However, it should be noted that the organisms have most definitely accumulated some other contaminants besides POPs and an integrated analysis with, for example, trace metals or/and polycyclic aromatic hydrocarbons (PAHs) would improve the interpretation and provide additional knowledge on the mechanisms involved in the sharks' response to xenobiotics. This information can then

be very useful to better discriminate and suggest potential biomarkers for biomonitoring studies with blue sharks.

Nevertheless, the results suggest some interesting correlations between physiological parameters and POPs accumulation levels, being DNA damage the main consequence of contamination and the inhibition of ChE activity the most strongly correlated effect. In order to complement and validate these findings, a similar study should be performed, although using adult individuals instead, as the increased size may cause 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 POPs accumulation or the biochemical parameters, already gives an indication of robustness of the parameters to be used as biomarkers.

As a conclusion, *P. glauca* demonstrates great potential to be used as a pollution sentinel and some suitable biomarker candidates were identified with this work.



Chapter 4.

General discussion and concluding remarks

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General discussion and concluding remarks

Marine ecosystems are constantly being threatened by contaminants derived from human activities, causing the decline of our oceanic resources (Stegeman and Hahn, 1994; Matthiessen and Law, 2002). The list of endangered marine species rises every year, along with the number of human illnesses caused by the consumption of contaminated fish (Gochfeld, 2003). The injurious conditions usually seen on the organisms are caused by combined action of the many xenobiotic compounds present in the oceans, like POPs, DDTs and heavy metals (Gramatica and Papa, 2007). The simultaneous presence of the contaminants makes it difficult to understand how each one hinders marine organisms, and only through a comprehensive environmental risk assessment can scientists evaluate the consequences that these compounds have to the ecosystem (Van Leeuwen and Hermens, 1995).

Bioaccumulation makes large predators like sharks prone to be affected by pollution in a more severe way that other organisms, with these animals being known to amass high quantities of POPs and heavy metals in their bodies (Branco et al., 2004; Storelli et al., 2011; Barrera-García et al., 2013)

In this study it is reported, for the first time, the characterization of the ChE forms present in both brain and muscle tissues of the blue shark. The test species, P. glauca, seems to contain atypical ChEs in the brain, displaying mixed properties of AChE and BChE, and its muscle tissue seems to contain mainly AChE. In vitro exposures to chloropyrifos-oxon inhibited blue shark's ChE in both tissues. The brain was particularly affected, therefore being the most suitable tissue for detection of exposure to low concentrations of anticholinergic compounds in the environment. This enzyme showed great potential to be used as a biomarker to measure the effects of anticholinergic contaminants.

Persistent organic pollutants were found in all sampled individuals and the multivariate analysis indicated the existence of both positive and negative correlations with the physiological parameters tested. DNA damage demonstrated the most positive correlation with POPs and the activity of ChE appeared to be impaired by the presence of these contaminants. No significant differences were found between gender or size and the POPs levels or the several physiological parameters measured. This may be due to the fact that all sampled individuals were small and immature.

The test species P. glauca revealed to be a good pollution sentinel and the assessment of physiological parameters in this shark, when combined with pollutant body burden determinations, can be promising tools to be applied in routine monitoring programs to infer about the health of the oceans.

In this study, persistent organic pollutants, detoxification pathways, oxidative stress response mechanisms and neuronal parameters in sharks were assessed, but there is still much to understand and many potential biomarkers to test and develop. Heavy metals quantification on the tissues of blue sharks could be of great interest as well, as it has been proven to strongly accumulate in sharks' tissues, and has been correlated with negative effects to organisms (Berntseen, et al., 2003; Barrera-García, et al., 2013). An integrated study with several types of contaminants would most certainly provide better insights on the physiological responses and their relations with the environmental levels of contaminants. Parameters related with energy expenditure (e.g. cellular energy allocation, lactate dehydrogenase, isocitrate dehydrogenase), histopathological alterations and endocrine disruption could also be interesting to evaluate and complement the present results, allowing for a better understanding of how these sharks are dealing with exposure to hazardous contaminants (Van der Oost, et al., 2003). The effects of pollutants on the endocrine system may be particularly important in environmental risk assessment programs, since impairments in reproductive capability may have a serious impact on fish populations.

It has already been pointed out that older, bigger sharks have the potential to concentrate higher concentrations of xenobiotics in their bodies (Maz-Courrau, et al., 2012) and because of this, additional studies including both juvenile and adult individuals would be important to understand how bioaccumulation affects the metabolic responses of P. glauca. However, the absence of significant differences between sharks size or gender and POPs concentrations or biochemical parameters studied already give an indication of the reliability of these parameters to be used as biomarkers.

Finally, an optimization of the sampling methods is mandatory, as procedure inconsistencies during this critical phase will increase the variability of the results and reduce the accuracy of posterior assays. One of the possibilities for future studies is to develop sampling methods that can be used on live animals in the least invasive way

possible. Being able to perform these biomarker tests and contaminant chemical analysis using blood tissue would allow for much easier samplings and most importantly would make possible the use of live animals – with further release and potential recapture. In theory, it would be conceivable to collect tissue samples during tagging campaigns, follow the tagged animal and make a posterior collection of tissues, allowing this way the assessment of some behavioral parameters, something which otherwise would be impossible to do on big pelagic predators like blue sharks.

In sum, with this work it is possible to say that blue sharks can offer ecotoxicologists an easy, accurate and very comprehensive model for pollution monitoring. The huge distribution of blue sharks have the advantage of allowing scientists all over the world to use it as model organism, decreasing the need for different and sometimes less suitable candidates. This study was thus a starting point and an indication that by combining data from biochemical and molecular assays covering different biological pathways with chemical analysis of different types of contaminants in the sharks' tissues and ideally also population dynamics, a good set of tools can be developed to properly assess the health of the oceans.

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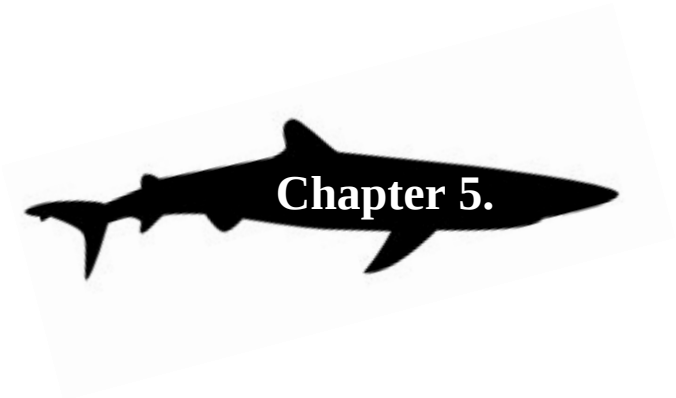

Chapter 5.

References

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References

Aires-da-Silva, A.M., Maunder, M.N., Gallucci, V.F., Kohler, N.E., Hoey, J.J., 2009. A spatially structured tagging model to estimate movement and fishing mortality rates for the blue shark (*Prionace glauca*) in the North Atlantic Ocean. Marine and Freshwater Research 60: 1029–1043.

Alpuche-Gual, L., Gold-Bouchot, G., 2008. Determination of esterase activity and characterization of cholinesterases in the reef fish *Haemulon plumieri*. Ecotoxicology and Environmental Safety. 71: 787–797.

Altarawneh, M., Dlugogorski, B.Z., Kennedy, E.M., Mackie, J.C., 2009. Mechanisms for formation, chlorination, dechlorination and destruction of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs). Progress in Energy and Combustion Science. 35: 245–274.

Al-Yousuf, M.H., El-Shahawi, M.S., Al-Ghais, S.M., 2000. Trace metals in liver, skin and muscle of Lethrinus lentjan fish species in relation to body length and sex. Science of the Total Environment. 256: 87–94.

Ambrosio, G., Tritto, I., 1999. Reperfusion injury: experimental evidence and clinical implications. American Heart Journal. 138(2):69–75.

Arufe, M.I., Arellano, J.M., García, L., Albendín, G., Sarasquete, C., 2007. Cholinesterase activity in gilthead seabream (Sparus aurata) larvae: Characterization and sensitivity to the organophosphate azinphosmethyl. Aquatic Toxicology. 84: 328–336

Baker, R.T.M., Martin, P., Davies, S.J., 1997. Ingestion of sub-lethalvlevels of iron sulphate by African catfish affects growth and tissuevlipid peroxidation. Aquatic Toxicology. 40: 51–61.

Ballantyne, J.S., 1997. Jaws, the inside story. The metabolism of elasmobranch fishes. Comparative Biochemestry and Physiology B-Biochemestry & Molecular Biology Biochem. Physiol. B 118: 703–742.

Barakat, A.O., Kim, M., Qian, Y., Wade, T.L., 2002. Organochlorine pesticides and PCB residues in sediments of Alexandria Harbour Egypt. Marine Pollution Bulletin. 44: 1426–1434.

Barrera-García, A., O'Hara, T., Galván-Magaña, F., Méndez-Rodríguez, L.C., Castellini, J.M., Zenteno-Savín, T., 2012. Oxidative stress indicators and trace elements in the blue shark (Prionace glauca) off the east coast of the Mexican Pacific Ocean. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology. 156: 59–66.

Barrera-García, A., O'Hara, T., Galván-Magaña, F., Méndez-Rodríguez, L.C., Castellini, J.M., Zenteno-Savín, T., 2013. Trace elements and oxidative stress indicators in the liver and kidney of the blue shark (Prionace glauca). Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology Part A. 165: 483–490.

Barton, B.A., Iwama, G., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases. 1: 3–26.

Baum, J.K., Worm, B., 2009. Cascading top-down effects of changing oceanic predator abundances. Journal of Animal Ecology. 78: 699–714.

Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.A., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdings, J., 1985. The Effects of Stress and Pollution on Marine Animals. Praeger, New York, USA.

Beauvais, S.L., Cole, K.J., Atchison, G.J., Coffey, M., 2002. Factors affecting brain cholinesterase activity in bluegill *Lepomis macrochirus*. Water Air and Soil Pollution. 135: 249–264.

Bejma, J., Ji, L.L., 1999. Aging and acute exercise enhance free radical generation in rat skeletal muscle. Journal of Applied Physiology. 87: 465–470.

Berntseen, M.H., Aatland, A., Handy, R.D., 2003. Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (Salmo salar) parr. Aquatic Toxicology 65: 55‑72.

Best, T.M., Fiebig, R., Corr, D.T., Brickson, S., Ji, L.L., 1999. Free radical activity, antioxidant enzyme, and glutathione changes with muscle stretch injury in rabbits. Journal of Applied Physiology. 87: 74–82.

Bird R.P., Draper H.H., 1984. Comparative studies on different methods of malonaldehyde determination. Methods in Enzymology. 105: 299–305.

Bloom, N.S., 1992. On the chemical form of mercury in edible fish and marine invertebrate tissue. Canadian Journal of Fisheries and Aquatic Sciences. 49: 1010–1017.

Boening, D.W., 2000. Ecological effects, transport, and fate of mercury: A General Review. Chemosphere. 40: 1335-1351.

Bonfil, R., 1994. Overview of world elasmobranch fisheries. FAO Fish. Tech. Pap. 341: 119.

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248–254.

Branco, V., Canário, J., Vale, C., Raimundo, J., Reis, C., 2004. Total and organic mercury concentrations in muscle tissue of the blue shark (Prionace glauca L.1758) from the Northeast Atlantic. Marine Pollution Bulletin. 49: 854–874.

Branco, V. Vale, V., Canário, J., dos Santos, M.N., 2007. Mercury and selenium in blue shark (Prionace glauca, L. 1758) and swordfish (Xiphias gladius, L. 1758) from two areas of the Atlantic Ocean. Environmental Pollution 150: 373-380

Brett, J.R., 1958. Implications and assessments of environmental stress. In: Larkin, P.A. (Ed.), Investigations of Fish-Power Problems, H.R. MacMillan Lectures in Fisheries. Univ. of British Columbia, Vancouver. 69–83.

Bresler, V., Bissinger, V., Abelson, A., Dizer, H., Sturm, A., Kratke, R., Fshelson, L., Hansen, P-D., 1999. Marine molluscs and fish as biomarkers of pollution stress in litoral regions of the Red Sea, Mediterranean Sea and North Sea. Helgoland Marine Research. 53: 219–243.

Bryan, G.W., 1976. Heavy metal contamination in the sea. In: Johnston, R. (Ed.). Marine pollution. Academic, London. 215-220.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology. 25: 201-268.

Burgeot, T., Bocquené, G., His, E., Vincent, F., Geffard, O., Beiras, R., Goraguer, H., Galgani, F., 2001. Monitoring of biological effects of pollutants: field application. In: Garigues Ph, Barth H, Walker CH, Narbonne JF, editors. Biomarkers in marine organisms: a practical approach. Amsterdam: Elsevier. 179–213.

Calabrese, E.J., 1991. Multiple Chemical Interactions. Lewis Publishers, Chelsea, MI, USA. 704.

Campana, S.E., Dorey, A., Fowler, M., Joyce, W., Wang, Z., Wright, D., Yashayaev, I., 2011. Migration pathways, behavioural thermoregulation and overwintering grounds of blue sharks in the Northwest Atlantic. PLoS One 6, e16854.

Canli, M., Atli, G., 2003. The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. Environmental Pollution. 121(1): 129-36.

Carson, H.S., 2013. The incidence of plastic ingestion by fishes: From the prey's perspective. Marine Pollution Bulletin. 74: 170–174.

Castro-González, M.I., Méndez-Armenta, M., 2008. Heavy metals: Implications associated to fish consumption. Environmental Toxicology & Pharmacology. 26: 263-271.

Chambers, J.E., Boone, J.S., Carr, R.L., Chambers, H.W., Strauss, D.L., 2002. Biomarkers as predictors in health and ecological risk assess- ment. Human and. Ecological Risk Assessment. 8 (1): 165–176.

Chen, C.-H., 2012. Activation and Detoxification Enzymes - Functions and Implications. Springer Science+Business Media, LLC, New York.

Chuiko, G.M., Zhelnin, Y.Y., Pod'gornaya, V.A., 1997. Seasonal fluctuations in brain acetylcholinesterase activity and soluble protein content in roach *Rutilus rutilus* L.: a freshwater fish from northwest Russia. Comparative Biochemestry and Physiology C-Pharmacology Toxicology & Endocrinology. 107: 251–257.

Chuiko, G.M., 2000. Comparative study of acetylcholinesterase and butyrylcholinesterase in brain and serum of several freshwater fish: specific activities and in vitro inhibition by DDVP, an organophosphorus pesticide. Comparative Biochemestry and Physiology C-Pharmacology Toxicology & Endocrinology. 127: 233–242.

Chuiko, G.M., Podgornaya, V.A., Zhelnin, Y.Y., 2003. Acetylcholinesterase and butyrylcholinesterase activities in brain and plasma of freshwater teleost: cross-species and cross-family differences. Comparative Biochemestry and Physiology B-Biochemistry & Molecular Biology. 135: 55–61.

Compagno, L.J.V., 1999. Systematics and body form. In: Hamlett, W.C. (Ed.), Sharks, Skates and Rays: The Biology of Elasmobranch Fishes. John Hopkins University Press, Baltimore. 1–42.

Costanza, R., Folke, C., 1997. Valuing ecosystem services with efficiency, fairness and sustainability as goals. In: Daily, G. (Ed.), Nature's Services: Societal Dependence on Natural Ecosystems. Island Press, Washington, DC, pp. 49–70.

Dautremepuits C, Paris-Palacios S, Betoulle S, Vernet G., 2004. Modulation in hepatic and head kidney parameters of carp (Cyprinus carpio L.) induced by copper and chitosan. Comparative Biochemestry and Physiology. 137: 325–333.

De Bragança, C. (1904). Ichthyologia. II - Esqualos obtidos nas costas de Portugal durante as campanhas de 1896 a 1903. Resultados das Investigações scientificas feitas a bordo do yacht "Amélia" e sob a direcção de D. Carlos de Bragança. Imprensa Nacional, Lisboa, 115 pp., 2 gravuras a cores.

De Carvalho, G.G.A., Degaspari, I.A.M., Branco, V., Canário, J., de Amorim, A.F., Kennedy, V.H.K., Ferreira, J.R., 2014. Assessment of Total and Organic Mercury Levels in Blue Sharks (Prionace glauca) from the South and Southeastern Brazilian Coast. Biological Trace Elements Research. 159: 128–134.

Depledge, M.H., Fossi, M.C., 1994. The role of biomarkers in environmental assessment (2). Ecotoxicology. 3: 161-172.

Derraik, J.G.B., 2002. The pollution of the marine environment by plastic debris: a review. Marine Pollution Bulletin. 44: 842–852.

Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. Environmental and Toxicological Chemestry. 8: 1103-1123.

Di Giulio, R.T., Meyer, J.N., 2008. Reactive oxygen species and oxidative stress. In: Di Giulio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. CRC Press, Boca Raton, FL. 273–326.

Duffy, J.E., 2002. Biodiversity and ecosystem function: the consumer connection. Oikos. 99: 201–219.

Egaas, E., Falls, J.G., Svendsen, N.O., Ramstad, H., Shkaare, J.U. and Dauterman, W.C. 1995. Strain- and sex-specific differences in the glutathione S-transferase class pi in the mouse examined by gradient elution of the glutathione-affinity matrix and reverse-phase high performance liquid chromatography. Biochimica et Biophysica Acta. 1243: 256- 264.

Elia, A.C., Galarini, R., Dörr, A.J.M., Taticchi, M.I., 2006. Bioaccumulation of heavy metals, organochlorine pesticides, and detoxication biochemical indexes in tissues of Ictalurus melas of lake Trasimeno. Bulletin of Environmental Contamination and Toxicology. 76: 132–139.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric of acetylcholinesterase determination. Biochemical Pharmacology. 7: 88–95.

Escobar-Sánchez, O., Galván-Magaña, F., Rosíles-Martínez, R., 2011. Biomagnification of mercury and selenium in blue shark Prionace glauca from the Pacific Ocean off Mexico. Biological Trace Elements. Research. 144: 550–559.

Eto, M., 1974. Organophosphorus Pesticides. Organic and Biological Chemistry. CRC Press.

Farombi, E., Adelowo, O., Ajimoko, Y., 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African cat fish (Clarias gariepinus) from Nigeria Ogun River. International Journal of Environmental Research and Public Health. 4: 158–165.

Fernandes, C., Fontaínhas-Fernandes, A., Peixoto, F., Salgado, M.A., 2007. Bioaccumulation of heavy metals in Liza saliens from the Esmoriz–Paramos coastal lagoon, Portugal. Ecotoxicology and Environmental Safety. 66: 426–431.

Filho, D.W., 1996. Fish antioxidant defenses - a comparative approach. Braz. Journal of Medical and Biological Research. 29: 1735-1742.

Fischer, L.J., Seegal, R.F., Ganey,P.E., Pessah, I.N., Kodavanti', P.R.S., 1998. Symposium Overview: Toxicity of Non-Coplanar PCBs12. Toxicological Sciences. 41: 49-61.

Flammarion, P., Noury, P., Garric, J., 2002. The measurement of cholinesterase activities as a biomarker in chub *Leuciscus cephalus*: the fish length should not be ignored. Environmental Pollution. 120: 325–330.

Franke, C., Studinger, G., Berger, G., Bohling, S., Bruckmann, U., Cohors-Fresenborg, D., Johncke, U., 1994. The assessment of bioaccumulation. Chemosphere. 29: 1501-1514.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organopho- sphorus insecticide exposure and effects. Environmental Toxicology and Chemestry. 20: 37-45.

Galgani, F., Bocquené, G., Cadiou, Y., 1992. Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. Mar. Ecol. Prog. Ser. 91: 77–82.

Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamates exposure in the bivalve mollusk Mytilus edulis using combined esterase activities as biomarkers. Aquat. Toxicol. 61: 169–180.

Garcia, L.,M., Castro, B., Ribeiro, R., Guilhermino, L., 2000. Characterization of cholinesterase from guppy *Poecilia reticulata* muscle and its in vitro inhibition by environmental contaminants. Biomarkers. 5 (4): 274-284

Gochfeld, M., 2003. Cases of mercury exposure, bioavailability, and absorption. Ecotoxicology and Environmental Safety. 56(1): 174–179.

Gomes, I.D.L., Lemos, M.F.L., Soares, A.M.V.M., Barata, C., Faria, M., 2014. The use of cholinesterase as potential biomarker: In vitro characterization in the polychaete *Capitella teleta*. Marine Pollution Bulletin. 85: 179–85.

González-Mille, D.J., Ilizaliturri-Hernández, C.A., Espinosa-Reyes, G., Costilla-Salazar, R., Díaz-Barriga, F., Ize-Lema, I., Mejía-Saavedra, J., 2010. Exposure to persistent organic pollutants (POPs) and DNA damage as an indicator of environmental stress in fish of different feeding habits of Coatzacoalcos, Veracruz, Mexico. Ecotoxicology. 19: 1238–1248.

Gorbi, S., Pellegrini, D., Tedesco, S., Regoli, F., 2004. Antioxidant efficiency and detoxification enzymes in spotted dogfish Scyliorhinus canicula. Marine Environmental Research 58: 293–297.

Gramatica, P., Papa, E., 2007. Screening and Ranking of POPs for Global Half-Life: QSAR Approaches for Prioritization Based on Molecular Structure. Environmental Science and Technology. 41(8): 2833–2839.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere. 32: 727.

Guzzi, G., La Porta, C.A., 2008. Molecular mechanisms triggered by mercury. Toxicology. 244(1):1–12

Halliwell, B., Gutteridge, J.M.C., 2001. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, UK. 936

Heath, A.G., 1995. Water pollution and fish physiology. Lewis Publishers Boca Raton, New York, London, Tokyo. p.359.

Howcroft, C.F., Gravato, C., Amorim, M.J.B., Novais, S.C., Soares, A.M.V.M., Guilhermino, L., 2011. Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham. Ecotoxicology. 20 (1): 119–130.

Hueter, R.E., Fong, W.G., Henderson, G., French, M.F., Manire, C.A., 1995. Methylmercury concentration in shark muscle by species, size and distribution of sharks in Florida coastal waters. Water, Air and Soil Pollution. 80: 893–899.

Imlay, J.A., 2003. Pathways of oxidative damage. Annu. Rev. Microbiol., 57: 395‑418.

IOC/UNESCO, IMO, FAO, UNDP. 2011. A Blueprint for Ocean and Coastal Sustainability. Paris: IOC/UNESCO.

Jacoby, W.B., Ziegler, D.M., 1990. The enzymes of detoxication. Journal of Biological Chemistry. 265: 20715–20718.

Ji, L.L., 1999. Antioxidants and oxidative stress in exercise. Proceedings of the Society for Experimental Biology and Medicine. 222: 283–292.

Jones, K.C., de Voogt, P., 1999. Persistent organic pollutants (POPs): state of the science. Environmental Pollution. 100: 209-221

Kalay, M., Ay, O., & Canli, M., 1999. Heavy metal concentrations in fish tissues from the Northeast Mediterranean Sea. Bulletin of Environmental Contamination & Toxicology. 63: 673-681.

Kirby, M.F., Morris, S., Hurst, M., Kirby, S.J., Neall, P., Tylor, T., Fagg, A., 2000. The use of cholinesterase activity in flounder (Platichthys flesus) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Marine Polluttion Bulletin 40(9): 780–791.

Koenig, S., 2012. Bioaccumulation of persistent organic pollutants (POPs) and biomarkers of pollution in Mediterranean deep-sea organisms. Universitat de Barcelona.

Korhonen, M., Verta, M., Lehtoranta, J., Kiviranta, H., Vartiainen, T., 2001. Concentrations of polychlorinated dibenzo-p-dioxins and furans in fish downstream from a Ky-5 manufacturing. Chemosphere 43: 587–593.

Labrada-Martagón, V., Rodríguez, P.A.T., Méndez-Rodríguez, L.C., Zenteno-Savín, T., 2011. Oxidative stress indicators and chemical contaminants in east Pacific green turtles (Chelonia mydas) inhabiting two foraging coastal lagoons in the Baja California peninsula. Comparative Biochemestry and Physiology C-Pharmacology Toxicology & Endocrinology. 154: 65–75.

Lackner, R., 1995. "Oxidative stress" in fish by environmental pollutants. In: Braunbeck, T., Hinton, D., Streit, B. (Eds.), Fish Ecotoxicology. Birkhauser, Berlin. 203–224.

Lalli, C.M., Parsons, T.R., 1993. Biological Oceanography. Elsevier Science, Oxford. 301

Lauterburg, B.H., Smith, C.V., Hughes, H., Mitchell, J.R., 1983. Determinants of hepatic glutathione turnover: toxicological sig- nificance. In: Lamble, J.W. (Ed.), Drug Metabolism and Distribution. Elsevier Biomedical Press, Amsterdam, Netherlands. 166-180.

Lemos, M.F.L., Soares, A.M.V.M., Correia, A.C., Esteves, A.C., 2010. Proteins in ecotoxicology - how, why and why not? Proteomics 10: 873–87.

Lenártová, V., Holovská, K., Pedrajas, J.R., Martínez-Lara, E., Peinado, J., López-Barea, J., Rosival, I., Košúth, P., 1997. Antioxidant and detoxifying fish enzymes as biomarkers of river pollution. Biomarkers. 2(4):247-52.

Lionetto, M.G., Caricato, R., Giordano, M.E., Schettino, T., 2004. Biomarker application for the study of chemical contamination risk on marine organisms in the Taranto marine coastal area. Chemical Ecology. 20: 333–343.

Liska, D.J., 1998. The detoxification enzyme systems. Alternative Medicine Review 3, 187–98.

Livingstone D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull. 42: 656–66.

Lopes, P.A, Pinheiro T, Santos, M.C., Mathias, M.L., Pereira, M.J.C., Viegas-Crespo, A.M., 2001. Response of antioxidant enzymes in freshwater fish populations (Leuciscus alburnoides complex) to inorganic pollutants exposure. Science of Total Environment. 280: 153–163.

López-Cruz, R.I., Zenteno-Savín, T., Galván-Magaña, F., 2010. Superoxide production, oxidative damage and enzymatic antioxidant defenses in shark skeletal muscle. Comparative Biochemistry and Physiology. A 156: 50–56.

Lucas, D.T., Szweda, L.I., 1998. Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. Proceedings of the National Academy of Sciences of the United States of America. 95: 510-514.

Lund, S.A., Fulton, M. H., Key, P.B., 2000. The sensitivity of grass shrimp, *Palaemonetes pugio*, embryos to organophosphate pesticide induced acetylcholinesterase inhibition. Aquatic Toxicology. 482-3: 127-134.

Mack, A., Robitzki, A., 2000. The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-50 butyrylcholinesterase-DNA study. Progress in Neurobiology. 60: 607–628.

Matthiessen, P., Law, R.J., 2002. "Contaminants and their effects on estuarine and coastal organisms in the United Kingdom in the late twentieth century", Environmental Pollution, 120: 739–757.

Mart, L., Rfitzel, H., Klahre, P., Sipos, L., Platzek, U., Valenta, P., Niimberg, H.W., 1982. Science of Total Environment. 26:1.

Maz-Courrau, A., López-Vera, C., Galván-Magana, F., Escobar-Sánchez, O., Rosíles-Martínez, R., Sanjuán-Munoz, A., 2012. Bioaccumulation and Biomagnification of Total Mercury in Four Exploited Shark Species in the Baja California Peninsula, Mexico. Bulletin of Environmental Contamination and Toxicology. 88: 129–134.

McCarthy, J.F., Shugart, L.R., 1990. Biological markers of environmental contamination. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, FL, USA, 3-16.

McCarthy, J.F., Halbrook, R.S., Shugart, L.R., 1991. Conceptual strategy for design, implementation, and validation of a biomarkerbased biomonitoring capability. Publication no. 3072, ORNL/TM-11783. Environmental Sciences Division, Oak Ridge National Laboratory, Tennessee, USA.

Monteiro, M., Quintaneiro, C., Morgado, F., Soares, A.M.V.M., Guilhermino, L., 2005. Characterization of the cholinesterases present in head tissues of the estuarine fish *Pomatoschistus microps*: application to biomonitoring. Ecotoxicology and Environmental Safety. 62: 341–347.

Myers, R.A., Baum, J.K., Shepherd, T., Powers, S.P., Peterson, C.H., 2007. Cascading effects of the loss of apex predatory sharks from a coastal ocean. Science, 315: 1846–1850.

Nakano, H., Seki, M.P., 2003. Synopsis of biological data on the blue shark, Prionace glauca Linnaeus. Bulletin of Fisheries Research. Agency 6: 18–55.

Newman, M.C., 2008. What exactly are you inferring? A closer look at hypothesis testing. Environmental Toxicology and Chemistry. 27: 1013‑1019.

NRC: Committee on Biological Markers of the National Research Council, 1987. Biological markers in environmental health research. Environmental Health Perspectives. 74: 3-9.

Ohkawa, H., Ohishi, N, Yagi K., 1979. Assay for lipid peroxides in animal-tissues by thiobarbituric acid reaction. Analitical Biochemistry. 95: 351–358.

Oliveira, M.M., Filho, M.V.S., Bastos, V.L.F.C., Fernandes, F.C., Bastos, J.C., 2007. Brain acetylcholinesterase as a marine pesticide biomarker using Brazilian fishes. Marine Environmental Research. 63: 303–312.

Payne, J.F., Mathieu, A., Melvin,W., Fancey, L.L., 1996. Acetylcholinesterase, and old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Marine Pollution Bulletin. 32: 225–231.

Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish Wallago attu (Bl. & Schn.). The Science of the Total Environment. 309: 105–115

Péan, S., Daouk, T., Vignet, C., Lyphout, L., Leguay, D., Loizeau, V., Bégout, M.L., Cousin, X., 2013. Long-term dietary-exposure to non-coplanar PCBs induces behavioral disruptions in adult zebrafish and their offspring. Neurotoxicology and Teratology. 39: 45–56.

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly *Sericostoma vittatum*: Biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicology and Environmental Safety. 104: 263-268.

Pezzementi, L., Sanders, M., Jenkins, T., Holliman, D., Toutant, J.P., Bradley, R.J., 1991. Structure and function of cholinesterase from amphioxus. In: Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology. Massoulié, J., Bacou, F., Barnard, E.A., Chatonnet, A., Doctor, B.P., Quinn, D.M. (Eds.), American Chemical Society, Washington, D.C. 24–31.

Pohanka, M., 2011. Cholinesterases, a target of Pharmacology and Toxicology. Biomed Biomedical papers of the Medical Faculty of the University Palacký Olomouc Czech Republic. 155(3): 219–230.

Porcella, D., 1994. Mercury in the environment: biogeochemistry. In: Watras, C.J., Huckabee, J.W. (Eds.), Mercury Pollution: Integration and Synthesis. CRC Press, Boca Raton, FL. 3–19.

Powers, D.A., 1989. Fish as model systems. Science 246: 352-358.

Richardson, B.J., Mak, E., De Luca-Abbott, Sh.B., Martin, M. and McClellan, K., 2008. Antioxidant responses to polycyclic aromatic hydrocarbons and organochlorine pesticides in green-lipped mussels (Perna viridis): do mussels "integrate" biomarker responses? Marine Pollution Bulletin. 57: 503–514.

Rodríguez-Fuentes, G., Mélina, M., Soto, Luna-Ramírez, K., 2013. Characterization of cholinesterases in thedamselfish Sergeant major *Abudefduf saxatilis*. Ecotoxicology and Environmental Safety. 96: 99–102.

Ross, P.S., De Swart, R.L., Reijnders, P.J.H., Van Loveren, H., Vos, J.G., Osterhaus, A.D.M.E., 1995. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbour seals fed herring from the Baltic Sea. Environmental Health Perspectives. 103: 162-167.

Safe, S.H., 1994. Polychlorinated biphenyls (PCBs)-environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Review Toxicology. 24: 87-149.

Santos, M.N., Garcia, A., Pereira, J.G., 2002. Historical review of the by-catch from the Portuguese surface long-line swordfish fishery: observations on the blue shark (Prionace glauca) and short-fin mako (Isurus oxyrinchus). ICCAT, Collective Volumes of Scientific Papers. 54 (4): 1333–1340

Schlenk, D., Sapozhnikova, Y. Irwin, M.A., Xie, L., Hwang, W., Reddy, S., Brownawell, B.J., Armstrong, J., Kelly, M., Montagne, D.E., Kolodziej, E.P., Sedlak, D., Snyder, S., 2005. In vivo Bioassay Guided Fractionation of Marine Sediment Extracts from the Southern California Bight for Estrogenic Activity. Environmental Toxicology and Chemistry. 24: 2820-2826

Schlichting, I., Berendzen, J., Chu, K., Stock, A.M., Maves, S.A., Benson, D.E., Sweet, R.M., Ringe, D., Petsko, G.A., Sligar, S.G., 2000. The catalytic pathway of cytochrome p450cam at atomic resolution. *Science.* 287(5458): 1615-1622.

Schindler, D.E., Essington, T.E., Kitchell, J.F., Boggs, C., Hilborn, R., 2002. Sharks and tunas: fisheries impacts on predators with contrasting life histories. Ecological Applications. 12: 735–748.

Semenza, G., 2000. HIF-1 and human disease: one highly involved factor. Genes Development. 14: 1983‑1991.

Serrano, R., Fernández, M., Rabanal, R., Hernández, M., Gonzales, M.J., 2000. Congener-specific determination of polychlorinated biphenyls I shark and grouper livers from the Northwest African Atlantic Ocean. Archives of Environmental Contamination and Toxicology. 38: 217–224.

Shanshan, Z., Lu, T., Qiaozhi, T., Xiaoting, G., Bin, X., Weiping, L., 2013. Residues, sources and tissue distributions of organochlorine pesticides in dog sharks *Mustelus griseus* from Zhoushan Fishing Ground, China Marine Pollution Bulletin. 73: 374–380.

Silver, A., 1974. The biology of cholinesterases. In: Neuberger, A., Tatum, E.L. Eds., Frontiers of Biology, vol. 36.

Skomal, G.B., Mandelman, J.W., 2012. The physiological response to anthropogenic stressors in marine elasmobranch fishes: A review with a focus on the secondary response. Comparative Biochemistry and Physiology. 162: 146–155.

Solé M., García de la Parra, L.M., Alejandre-Grimaldo, S., Sardá, F., 2006. Esterase activities and lipid peroxidation levels in offshore commercial species of the NW Mediterranean Sea. Marine Pollution Bulletin. 52: 1708–1716.

Solé M, Lobera G, Aljinovic B, Ríos J, García de la Parra LM, Maynou F, Cartes, J.E., 2008. Cholinesterases activities and lipid peroxidation levels in muscle from shelf and slope dwelling fish from the NW Mediterranean: its potential use in pollution monitoring. Science of Total Environment. 402: 306–317.

Sparling, D.W., Fellers, G., 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. Environmental Pollution. 147 3: 535–539.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Forlin, L., Fowler, B.A., Sanders, B.M., van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett, R.J., Kimerly, R.A., Mehrle, P.M., Jr, Bergman, H.L. (Eds.), Biomarkers: Biochemical, Physiological and Histological markers of Anthropogenic Stress. Lewis Publishers, Chelsea, MI, USA. 235-335.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenase: current perspective on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic toxicology; Molecular, Biochemical and Cellular Perspectives. Lewis Publishers, CRC press, Boca Raton, pp. 87-206.

Steinberg, D., 1997. Low density lipoprotein oxidation and its pathological significance. Journal of Biological Chemistry. 272: 20963–20966.

Stevens, J.D., 1976. First results of shark tagging in the north-east Atlantic, 1972–1975. J. Mar. Biol. Assoc. U. K. 56: 929–937.

Stevens, J.D., Bonfil, R., Dulvy, N.K., Walker, P.A., 2000. The effects of fishing on sharks, rays, and chimaeras (chondrichthyans), and the implications for marine ecosystems. ICES Journal of Marine Sciences. 57, 476‑494.

Stevens, J., 2009. Prionace glauca. The IUCN Red List of Threatened Species. Version 2014.2. www.iucnredlist.org. 17 September 2014.

Stevens, J.D., Bradford, R.W., West, G.J., 2010. Satellite tagging of blue sharks (Prionace glauca) and other pelagic sharks off eastern Australia: depth behaviour, temperature experience and movements. Marine Biology. 157: 575–591

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M., Lafaurie, M., 1998. Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean sea. Environmental Pollution. 99:339–45.

Stohs, S. J., Bagchi, D., 1995. Oxidative Mechanisms in the Toxicity of Metal ions. Free Radical Biology & Medicine. 18(2): 321-336

Storelli, M.M., Marcotrigiano, G.O., 2001. Persistent organochlorine residues and toxic evaluation of polychlorinated biphenyls in sharks from the Mediterranean Sea (Italy). Marine Pollution Bulletin. 12: 1323–1329.

Storelli, M.M., Storelli, A., Marcotrigiano, G.O., 2005. Concentration and hazard assessment of polychlorinated biphenyls and organochlorine pesticides in shark liver from the Mediterranean Sea. Marine Pollution Bulletin. 50: 850–858.

Storelli, M.M., Marcotrigiano, G.O., 2006. Occurrence and accumulation of organochlorine contaminants in swordfish from Mediterranean Sea: a case study. Chemosphere 62: 375–380.

Storelli, M.M., Barone, G., Piscitelli, G., Storelli, A., Marcotrigiano, G.O., 2007. Tissuerelated polychlorinated biphenyls accumulation in Mediterranean cetaceans: Assessment of toxicological status. Bulletin of Environmental Contamination and Toxicology. 78: 206–210.

Storelli, M.M., Casalino, E., Barone, G., Marcotrigiano, G.O., 2008. Persistent organic pollutants (PCBs and DDTs) in small size specimens of bluefin tuna (Thunnus thynnus) from the Mediterranean Sea (Ionian Sea). Environmental International. 34: 509–513.

Storelli, M.M.; Barone, G.; Storelli, A.; Marcotrigiano, G.O., 2011. Levels and congener profiles of PCBs and PCDD/Fs in blue shark (Prionace glauca) liver from the South-Eastern Mediterranean Sea (Italy). Chemosphere. 82: 37–42.

Strid, A., Jörundsdóttir, H., Päpke, O., Svavarsson, J., Bergman, Å., 2007. Dioxinsand PCBs in Greenland shark (Somniosus microcephalus) from the North-East Atlantic. Marine Pollution Bulletin. 54: 1514–1522.

Sturm, A., da Silva Assis, H.C., Hansen, P.D., 1999a. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Marine Environmental Research. 47: 389–398.

Sturm, A., Wogram, J., Hansen, P.D., Liess, M., 1999b. Potential use of cholinesterase in monitoring low levels of organophosphates in small streams: natural variability in three-spined stickleback *Gasterosteus aculeatus* and relation to pollution. Environmental Toxicology and Chemistry. 18: 194–220.

Sturm, A., Wogram, J., Segner, H., Liess, M., 2000. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback (*Gasterosteus aculeatus*): application in biomonitoring. Environmental Toxicology and Chemistry. 19: 1607-1615.

Suter, G.W., II, 1993. Ecological Risk Assessment. Lewis Publishers, Boca Raton, FL, USA. 538.

Thomas, P., Wofford, H.W., 1993. Effects of cadmium and Arochlor 1254 on lipid peroxidation, glutathione peroxidase acitivity, and selected antioxidants in Atlantic croaker tissues. Aquatic Toxicology. 27: 159–178.

Tillitt, D.E., Ankley, G.T., Giesy, J.P., Ludwig, J.P., Kurita-Matsuba, H., Weseloh, D.V., Ross, P.S., Bishop, C.A., Sileo, L., Stromborg, K.L., Larson, J., Kubiak, T.J., 1992. Polychlorinated biphenyl residues and egg mortality in double-crested cormorants from Great Lakes. Environmental Toxicology and Chemistry. 11: 1281-1288.

Tortelli, V., Colares, E.P., Robaldo, R.B., Nery, L.E.M., Pinho, G.L.L., Bianchini, A., Monserrat, J.M., 2006. Importance of cholinesterase kinetic parameters in environmental monitoring using estuarine fish. Chemosphere. 65: 560–566.

Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R. et al., 2002. Oxidative stress in the mussel Mytella guyanensis from polluted mangroves on Santa Catarina Island, Brazil. Marine Pollution Bulletin. 44: 923–932.

Toutant, J.P., 1989. Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. Progress in Neurobiology. 32: 423–446.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. International Journal of Biochemistry. Cell Biol. 39: 44–84.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology. 13(2): 57–149.

Van Gestel, C.A.M., Van Brummelen, T.C., 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology 5: 217-225.

Van Leeuwen, C.J., Hermens, J.L.M., 1995. Risk Assessment of Chemicals: an Introduction. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Vélez-Alavez, M., Labrada-Martagón, V., Méndez-Rodriguez, L.C., Galván-Magaña, F., Zenteno-Savín, T. 2013. Oxidative stress indicators and trace element concentrations in tissues of mako shark (Isurus oxyrinchus). Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology. 165: 508–514.

Wang, W.X., 2002. Interactions of trace metals and different marine food chains. Marine Ecology Progress Series. 243: 295–309.

Whitehead, A., Anderson, S.L., Ramirez, A., Wilson, B.W., 2005. Cholinesterases in Aquatic Biomonitoring: Assay Optimization and Species-Specific Characterization for a California Native Fish. Ecotoxicology. 14: 597–606.

WHO International Programme on Chemical Safety (IPCS), 1993. Biomarkers and risk assessment: concepts and principles. Environmental Health Criteria 155, World Health Organization, Geneva.

Wilhelm, D., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). Aquaculture. 203: 149–158.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquatic Toxicology. 19: 137-161.

Winzer, K., 2001. Oxidative stress in the marine environment - prognostic tools for toxic injury in fish liver cells. Academic Thesis, University of Amsterdam.

Worthington, D.J., Rosemeyer, M.A., 1974. Human glutathione reductase: purification of the crystalline enzyme from erythrocytes. European Journal of Biochemistry. 48: 167-177.

Wu, W.Z., Schramm, K.W., Xu, Y., Kettrup, A., 2001. Accumulation and partition of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) in the muscle and liver of fish. Chemosphere 43: 633–641.