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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Patrícia Alexandra Oliveira Pereira Kowalski, Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Filipa Santos Costa Pinto Ribeiro Lacerda, Professora Auxiliar da Escola de Medicina da Universidade do Minho.

Dedico esta dissertação aos meus pais,
o melhor da minha vida.

o júri

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palavras-chave

Mercúrio inorgânico; Metilmercúrio; Neurotoxicidade; Histopatologia; Morfometria; Peixes; Tecto óptico

resumo

O tecto óptico é uma área do cérebro que tem sido referida como particularmente vulnerável à exposição a iHg e MeHg em peixes. A integridade estrutural e funcional desta área é importante para a manutenção de diferentes comportamentos, sendo vital à sobrevivência dos peixes uma vez que está relacionada com a procura de alimento, fuga de predadores e reprodução. O atual estado de arte apresenta lacunas relativas aos efeitos morfológicos no tecto óptico desencadeados pela exposição de peixes a iHg e MeHg, nomeadamente: i) O iHg e o MeHg afetam de forma diferente as várias camadas do tecto óptico? ii) As formas de Hg afetam preferencialmente neurónios ou células da glia (ou ambos os tipos de células de forma idêntica)? iii) Será o tecto óptico capaz de recuperar da toxicidade desencadeada pelo iHg e MeHg em 28 dias de pós-exposição? iv) Qual a forma de Hg (iHg ou MeHg) mais tóxica para o tecto óptico? Com o objetivo de responder a estas questões, esta dissertação compreendeu duas experiências de exposição de sargos (*Diplodus sargus*) juvenis a $HgCl_2$ através da água (2 μg L^{-1}) e a MeHg (8.7 μg g^{-1}) através do alimento dos peixes, por períodos de 7 e 14 dias (E7 e E14, respetivamente) de exposição e 28 dias de pós-exposição (PE28). As análises morfométricas foram realizadas através de métodos estereológicos usados para delinear e quantificar as camadas do tecto óptico, assim como para a contagem de neurónios e células da glia. As análises histopatológicas foram realizadas por secção e por camada. O programa ImageJ foi usado para delinear e calcular a área analisada para posterior ajuste à área total de cada camada.

Os resultados demonstram que a exposição a iHg não conduziu à perda de neurónios durante o período de exposição (E7 e E14), enquanto uma diminuição de células da glia foi registada numa única camada do tecto óptico no período E14. Na experiência do MeHg, verificou-se a diminuição do número de neurónios e células da glia em diversas camadas do tecto óptico dos peixes durante o período de exposição. No período de pós-exposição (PE28), as duas formas de Hg induziram perda de neurónios, enquanto apenas a exposição a MeHg conduziu à perda de células da glia. A histopatologia sinalizou uma maior toxicidade do MeHg nas camadas do tecto óptico, particularmente no período de pós-exposição, sendo que não foram detetadas alterações significativas na exposição a iHg. Especificamente, foi possível demonstrar que ambas as formas de Hg têm como alvo preferencial os neurónios no tecto óptico dos peixes. Adicionalmente, os resultados não permitiram distinguir se alguma das camadas do tecto óptico é particularmente afetada pela exposição a cada uma das formas de Hg. Além disso, estes resultados apontam o iHg e o MeHg como neurotóxicos relevantes em peixes, sendo que a exposição a MeHg desencadeou uma maior toxicidade no tecto óptico em comparação com a exposição a iHg. Após 28 dias de pós-exposição, os efeitos neurotóxicos do iHg e do MeHg mantiveram-se proeminentes sugerindo um efeito prolongado destas duas formas de Hg. Assim, os efeitos neurotóxicos do iHg e MeHg no tecto óptico parecem ser dificilmente reversíveis, o que poderá comprometer o bem-estar e a sobrevivência do peixes. Ademais, a condição de saúde dos peixes deverá ser seguida ao longo de vários meses após a exposição às formas de Hg.

keywords

Inorganic mercury; Methylmercury; Neurotoxicity; Histopathology; Morphometry; Fish; Optic tectum

abstract

The optic tectum is a brain area that has been pointed out as particularly vulnerable in fish to inorganic mercury (iHg) and methylmercury (MeHg). The structural and functional integrity of this area is central for the maintenance of several behaviours including food search, predator escape and reproduction and is thus vital for fish survival. The current state of the art has knowledge gaps concerning the effects of iHg and MeHg on the optic tectum morphology, specifically in relation to these research questions: i) do iHg and MeHg differently affect specific layers of the optic tectum? ii) do iHg and MeHg target preferentially neurons or glial cells (or both indistinctly)? iii) is the optic tectum able to recover from iHg and MeHg exposure? iv) what is the most toxic form of Hg (iHg *vs*. MeHg) in the optic tectum? To answer these questions, two experiments exposing juvenile white seabream (*Diplodus sargus*) were performed under this dissertation, comprising both exposure (7 and 14 days; E7 and E14, respectively) and post-exposure (28 days; PE28) periods, namely: i) waterborne exposure to inorganic $HgCl_2$ (2 μg L^{-1}) and ii) dietary exposure to MeHg (8.7 μg g^{-1}). Morphometric assessments were performed using stereological methods where the layers of the optic tectum were outlined, while its area as well as the number of neurons and glial cells were quantified. The histopathological analyses were performed per section and per layer of the optic tectum. ImageJ was used to outline and calculate the area analysed for posterior adjustment of results to the total area of each layer.

Results showed that iHg exposure did not trigger the loss of neurons during the exposure periods (E7 and E14), while a decrease of glial cells was detected in a single layer of the optic tectum at E14. In the MeHg experiment, a decrease on the number of neurons and glial cells was found in several layers of the fish optic tectum during the exposure period. In the post-exposure timepoint (PE28), while both Hg forms triggered the loss of neurons, while only MeHg exposure led to a decrease on the number of glia cells. Histopathology pointed out a higher toxicity of MeHg in the optic tectum layers, particularly in the post-exposure period, while no significant alterations were found in fish previously exposed to iHg. Importantly, both forms of Hg target preferentially neurons rather than glial cells in the optic tectum. Additionally, it was difficult to perceive if Hg forms target specific layers of the optic tectum. Current findings point out iHg and MeHg as relevant neurotoxicants, with MeHg exposure leading to a higher neurotoxicity than iHg in the optic tectum of fish. After 28 days of post-exposure, neurotoxic effects of iHg and MeHg remained prominent, suggesting long-term effects of these Hg forms. Accordingly, the neurotoxic effects of iHg and MeHg in the fish optic tectum are hardly reversible over time, eventually compromising the fish well-being and survival. Moreover, the health condition of fish should be followed for several months after exposure to Hg forms.

Table of contents

List of figures

List of tables

1. Introduction

1.1. Mercury occurrence in aquatic systems

The aquatic environment has been severely impacted by metals. Mercury (Hg) is an environmentally important trace element and one of the most toxic metals due to its chemical and physical properties (WHO, 2003). According to the European Water Framework Directive (WFD), Hg is a priority hazardous substance in the European Union based on its toxicity, persistence and bioaccumulation (EU, 2003). Mercury is found in the aquatic environment due to different natural sources such as soil erosion, forest fires, active volcanoes and fossil fuels as coal and petroleum (Figure 1). In addition, anthropogenic emissions of Hg are increasing owing to human activities that can be grouped as primary when Hg emission is unintentional, such as mining, and extraction/burning of fossil fuels, or secondary when resulting from the intentional use of Hg, as in the industrial sector (*e.g.*, paper industries) (Pacyna et al., 2010). According to the United Nations Environment Programme (UNEP), until 2050 the emissions of Hg are expected to increase even more if no changes in pollution controls and other actions to reduce Hg emissions are implemented (UNEP, 2013). By that time, annual global Hg emissions can reach a maximum of 4860 tonnes while in 2006 the levels were 2480 tonnes (Streets et al., 2009). This prospect on Hg levels together with its hazardousness, propels more research and mitigation measures implementation on the environmental impacts of this trace element. In fact, the Minamata Convention on Mercury is a global treaty to protect human health and the environment from the adverse effects of Hg. The major goals of the Minamata Convention include the ban of new Hg mines and the closing of the existing ones, control measures on air emissions and land and water contamination (UNEP, 2019).

Mercury is naturally present in waters at very low levels, varying with the distance from contamination sources. It enters the aquatic systems via anthropogenic discharges, atmospheric deposition and natural processes (*e.g.*, river runoff) (Figure 1). Mercury in natural waters is present as methylmercury (MeHg) and inorganic Hg (iHg - Hg^{2+}) (Figure 2), either in dissolved or particulate forms, and as elemental Hg (Hg^0) that, together with dimethylmercury, occur as dissolved gases (Figure 1). Hg^0 is lost from the aquatic environment at normal temperatures, due to its relatively high volatility (Figure 1). Both iHg and MeHg have a high tendency to form complexes in water. For instance, more than 90% of Hg is complexed by organic matter in freshwaters.

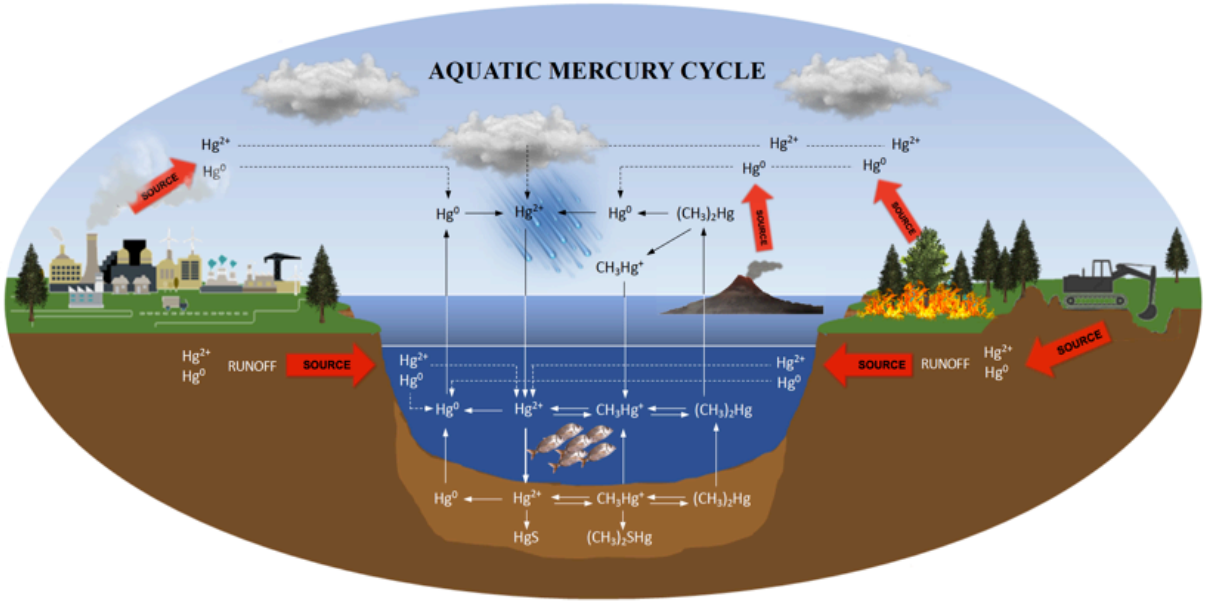


Figure 1 – Mercury cycle in aquatic systems. Natural (for example, volcanoes and forest fires) and anthropogenic (exemplified as hydroelectric, paper and mining industries) sources of Hg are indicated by the red arrows. (Adapted from Pereira et al., 2019).

Sediments constitute the main reservoir of Hg in aquatic systems (Ullrich et al., 2001) (Figure 1), being important sites for methylation. Hg^{2+} is transformed into MeHg through a process mediated by microorganisms, such as anaerobic sulphate-reducing bacteria, iron reducers, and methanogens (Authman et al., 2015). Interestingly, iHg is adsorbed into particles to a bigger extend than MeHg, even though the latter is more strongly absorbed by organisms than iHg (Pereira et al., 2019). Most of the time, MeHg is present in the sediment and ingested by zooplankton, thus entering the food chain and being then bioaccumulated (Chen et al., 2012). Sediments are considered a relevant source of iHg and MeHg to the water column, acting also as a sink for Hg forms (Cesário et al., 2016; Cesário et al., 2017). The release of Hg forms stored in the sediment to the water column represents a risk to the aquatic life (including fish). Accordingly, Wei et al. (2014) noted that benthic fish living closely to the lake-bed and feeding on benthic invertebrates displayed higher Hg levels than fish living in middle and upper areas of the water column. Both iHg and MeHg can accumulate in fish tissues, but generally the highest levels are recorded for MeHg (Lacoue-labarth et al., 2009).

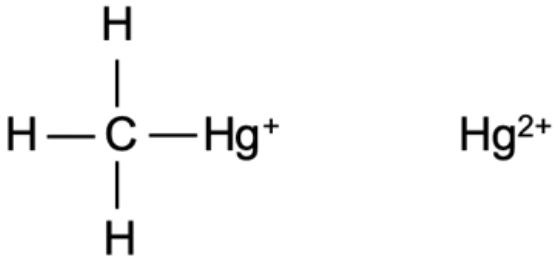


Figure 2 - Chemical form of methylmercury (on the left) and inorganic mercury (on the right).

1.2. Fish as bioindicators of mercury contamination

Fish species have been attracting substantial interest in studies evaluating biological responses to aquatic contaminants (Authman et al., 2015; Van der Oost et al., 2003). They are convenient indicators of ecosystem health since normally fish react with great sensitivity to changes in aquatic systems (Van der Oost et al., 2003). In ecotoxicological studies, there are several biomarkers routinely examined in fish (among physiological, histological and morphological alterations) that can provide useful information on a diverse range of steps during the risk assessment process such as exposure, effect and hazard assessment, risk characterization or classification, as well as on monitoring the environmental quality of aquatic ecosystems (Van der Oost et al., 2003). International monitoring protocols generally include measurements in fish species, which play a vital ecological role in aquatic food-webs due to their function as energy carriers from lower to higher trophic levels (Van der Oost et al., 2003). Additionally, fish are located at the top end of the aquatic food chain and thus they may bioaccumulate high levels of metals, such as Hg (Al-Yousuf et al., 2000). In fact, fish are vulnerable to Hg pollution related to their contaminated foods and when living in a polluted aquatic environment from where they cannot escape (Authman et al., 2015). Moreover, the accumulation of Hg in tissues reflects past exposures and can also elucidate about their current condition before Hg toxicity affects the ecological balance in the aquatic environment (Birungi et al., 2007). Understanding a toxicant uptake in fish, together with the extension of its effects (from biochemical changes to behavior alterations) is vital to perform a comprehensive environmental health assessment and study its ecological repercussions.

Fish have the ability to uptake and concentrate Hg directly from the water or indirectly from their diet (Authman et al., 2015). In the last decades, several studies have documented the accumulation of different forms of Hg in fish, such as iHg and MeHg, under realistic environmental and laboratory/experimental conditions (Liao et al., 2006; Maulvault et al.,

2016; Mieiro et al., 2010; Pereira et al., 2014; Korbas et al., 2012) as well as the toxicity of organic and iHg in fish organs such as the liver, gills and brain (Pereira et al., 2010; Pereira et al., 2014). Toxic effects of Hg can be due to different processes such as enzyme inhibition and modification of protein structure. Proteins (including enzymes) can be inactivated through the reaction of Hg with their functional groups (Pereira et al., 2019). Mercury binds to a variety of protein groups including sulfhydryl, phosphoryl, carboxyl, amide, and amine, either inactivating them or changing their structure. Besides that, part of Hg toxicity is related to its oxidative state and chemical form (organic vs. inorganic form).

The toxicity of Hg forms in fish has been investigated by screening the histopathological alterations in target organs, although most of the studies have been focused on kidney and liver. For instance, Sun et al. (2018) exposed *Pelteobagrus fulvidraco* to waterborne $HgCl_2$ and reported a decrease in lymphoid cells and an increase in melano-macrophage centers in the spleen, while the kidney displayed hepatocyte hypertrophy, decreased number of lymphoid cells and increased number of melano-macrophages (Sun et al., 2018). Identically, Ribeiro et al. (2002) studied the histopathological effects of waterborne iHg and dietary MeHg in the gills and liver of the arctic charr (*Salvelinus alpinus*). A severe disorganization of epithelial cells in gills after iHg exposure was recorded, while MeHg exposure triggered a severe necrosis and alterations of hepatocyte cytoplasmic organization. Wester and Canton (1992) and Mela et al. (2007) recorded large necrosis areas in the kidney after exposure to waterborne MeHgCl and dietary MeHg respectively, as well as an increased number of melano-macrophages centers, phagocytic areas and appearance of atypical cells. Accordingly, the liver showed leukocyte infiltration, an increased number of melano-macrophage centers, necrotic areas and lesions in Disse's space. Besides that, some histopathological studies have been studying the effects of Hg forms in fish. For instance, Berntssen et al. (2003) performed an examination of the Atlantic salmon (*Salmo salar*) brain after exposure to dietary iHg and MeHg. In this study were observed alterations such as necrosis, cell swelling and vacuolation in the brain.

Changes on fish brain morphology due to exposure to Hg forms have been more scarcely studied. Despite that, Pereira et al. (2016) recently studied the brain of *D. sargus* as a target organ of Hg forms both in field and laboratory studies. Pereira et al. (2016) and Puga et al. (2016) analyzed brain morphometry and swimming behavior, showing loss of cells (neurons and glial cells) in specific brain regions after 7 days of exposure to Hg forms, namely in the hypothalamus, optic tectum and cerebellum after iHg exposure (Pereira et al., 2016) as well as in the medial pallium and optic tectum after MeHg exposure (Puga et al., 2016). Both studies highlighted the optic tectum as the most vulnerable region to both Hg forms since the extent of cell loss was even greater at the end of the post-exposure period (28 days). In terms

of swimming behaviour, while MeHg showed no effects, iHg led to behavioural changes at the motor and mood/anxiety levels.

Effects of Hg in fish brain have been mostly studied by investigating physiological changes. For instance, Pereira et al. (2015) and Cardoso et al. (2017) searched for changes in brain oxidative stress endpoints in white seabreams (*D. sargus*), as well as Mieiro et al. (2011) in European sea bass (*Dicentrarchus labrax*). Results concerning *D. sargus* brain confirmed oxidative impact after iHg exposure, while MeHg seemed to trigger antioxidant protection, preventing damage in most of the exposure and post-exposure timepoints (Cardoso et al., 2017). *D. labrax* brain accumulated high levels of tHg also showing signs of increased susceptibility to oxidative stress due to alterations in antioxidant enzymes. The accumulation of Hg forms in the brain and their toxicity, namely at the histological/morphological level can seriously compromise fish fitness and survival (Mieiro et al., 2011).

1.3. Toxicokinetics of mercury in fish with a focus on the brain as a target organ

Toxicokinetics is a group of processes that comprises the absorption, distribution, biotransformation and excretion of toxicants throughout time (Gupta, 2016). Fish can accumulate Hg through different pathways such as directly from water, uptake of contaminated suspended particles and sediment or by the consumption of organisms of lower trophic level (Pereira, 2014). The absorption of Hg by fish includes its transfer to blood, mostly through the epithelial barrier of the gills, digestive organs and skin. Several studies have addressed Hg kinetics, though the majority are focused on the most explored tissues namely the liver, kidney, gills and muscle (Pereira et al., 2015). To understand Hg toxicokinetics, it is important to consider that the exposure routes, *i.e.*, waterborne or dietary, are closely related to each Hg form, since generally iHg is prevalent in the water while MeHg is mostly associated with a contaminated diet. Therefore, this duplicity between exposure route and Hg forms (waterborne iHg and dietary MeHg) will be presented in more detail below.

The absorption of waterborne iHg in fish occurs mainly at the gills, followed by distribution throughout different organs. In fact, the gills have been recognized as the main organ of trace elements uptake related to its direct exposure to the water medium and its wide surface of contact (Karan et al., 1998; Dalzell and Macfarlane, 1999). Dissolved Hg has the capacity to change the permeability of gills, increasing passive ionic effluxes (Lock et al., 1981). Specifically, Jagoe et al. (1996) showed that the gills of *Gambusia holbrooki* displayed

morphological modifications, such as thickening of primary lamellar epithelium after 14 days of exposure to Hg^{2+}.

The uptake of Hg by the gills occurs inadvertently by transport processes designed for essential cations (for instance uptake of Hg^{2+} instead of Ca^{2+}) (Klinck et al., 2005). Pereira et al. (2015) performed a toxicokinetic study of waterborne iHg in *D. sargus,* documenting its distribution and accumulation after 1, 3, 7 and 14 days of exposure, followed by a period of 14 and 28 days of post-exposure to address redistribution (Figure 3). Data pointed out the gills as the main organ of iHg uptake since accumulation was first noticed when compared with other organs (Figure 3).

The absorption of waterborne iHg can also occur through the skin or oral epithelia; however, its impact in adult fish is smaller due to increased skin thickness, low surface area and limited blood perfusion (Pereira et al., 2019). Moreover, due to the presence of mucus on the skin, the uptake of Hg is decreased, especially for iHg, as the mucus captures almost 80% of the total Hg (McKone et al., 1971). However, while Rouleau et al. (1999) also documented a strong binding of iHg to fish skin and oral mucosa, they proposed that water-exposed sensory cells on skin and oral epidermis, including mechanoreceptors of the lateral line system, cutaneous sensory cells and/or receptor cells of taste buds, served as a pathway for waterborne iHg to reach the brain. This axonal transport would begin across primary nerve pathways and ends at synaptic junctions with the second order neurons (Rouleau et al., 1999). The fish eyes were also claimed as a relevant uptake route for Hg forms related with its direct contact to the water (Pereira et al., 2014). However, Korbas et al. (2013) found that Hg can reach the eyes by the bloodstream and then pass the blood-retinal barrier (BRB) through which can easily pass due to its high affinity with the sulfhydryl groups of organic molecules (*e.g.*, cysteines) and this seems to be the preferential uptake route.

The main via of distribution of waterborne iHg is the blood, carrying it to different organs of fish. The study of Pereira et al. (2015) previously described in *D. sargus* confirmed this evidence. After 3 days of exposure to waterborne iHg, a significant accumulation of Hg was detected in the gills that are the uptake organ as described, followed by the blood, liver and brain. Once in the bloodstream, iHg can reach the liver and brain, explaining the common temporal pattern found in both organs during the exposure period (Figure 3). Even though accumulation of iHg is noticeable after 3 days of exposure in gills, blood, liver and brain, it is at 14 days of exposure when fish tissues seem to reach the highest Hg accumulation (8.1 ± 0.17, 1.8 ± 0.11, 2.5 ± 0.16, 1.4 ± 0.43 μg g^{-1}, at gills, blood, liver and brain, respectively) that are considered high for fish exposed to realistic iHg levels (2 μg L^{-1} of $HgCl_2$). The liver seems to accumulate higher iHg levels than the brain after 14 days of exposure, being the protection

afford by the blood–brain barrier (BBB) a possible reason to the lower accumulation of iHg in the brain. Differently, no physiological external barriers exist to protect the liver as the blood directly contacts hepatocytes through the large gaps of sinusoidal capillaries that exist in the hepatic lobules (Mieiro et al., 2010).

In the study of Pereira et al. (2015), the levels of Hg decreased in most of the surveyed organs in the post-exposure period, suggesting that iHg elimination took place. Indeed, after 28 days of post-exposure, a significant decrease of tHg levels in the gills, blood and liver was observed (Pereira et al., 2015). The elimination of iHg in the gills is probably the result of the high cellular turnover, while the liver can also excrete Hg^{2+} into the feces through biliary secretion (Pereira et al., 2015). By contrast, the Hg brain levels were not decreased after 28 days of post-exposure, pointing out the brain as a final target of iHg probably due to the fact the BBB can limit the release of iHg into the bloodstream and, thus, its elimination from the brain (Pereira et al., 2015). However, the accumulation of Hg in brain does not always imply toxicity which can be related to the chemical form in the cells and protective mechanisms (Korbas et al., 2010). iHg can be stored as a complex with selenium (HgSe) which is considered a non-toxic form. Nonetheless, it can cause a deficiency of essential Se-dependent enzymes (*e.g.*, glutathione peroxidase) which can be considered an indirect mechanism of iHg toxicity (Korbas et al., 2010).

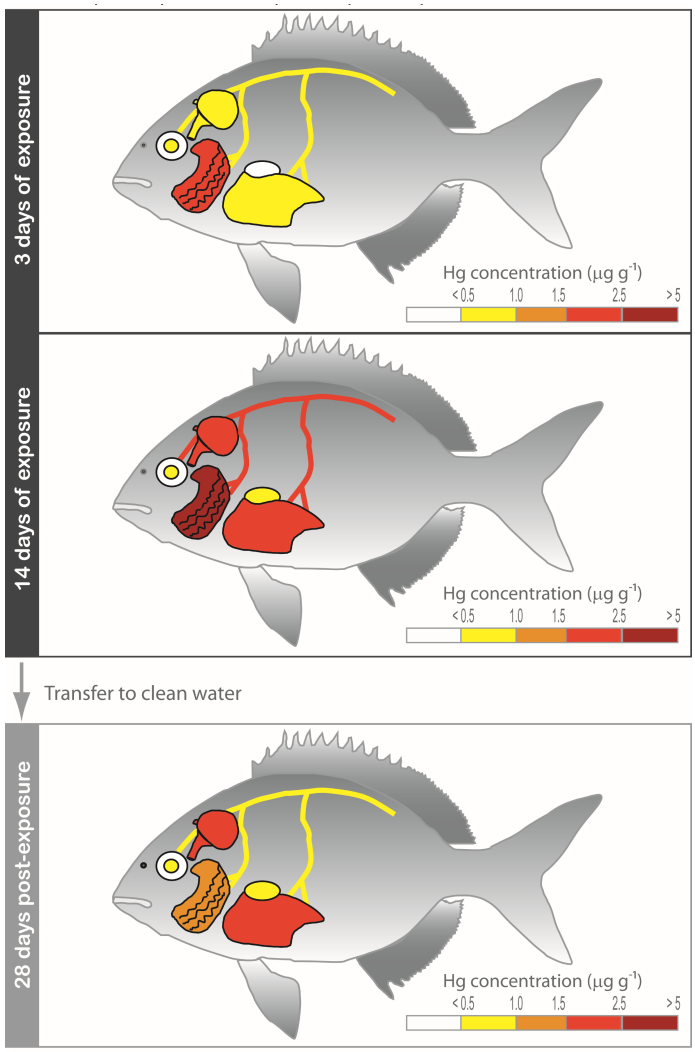


Figure 1 – Time-related accumulation/distribution of inorganic Hg upon exposure and post-exposure periods. Tissues/organs selected include gills, eye wall, lens, blood, liver, brain and bile. (Published in Pereira et al., 2015)

The uptake of MeHg occurs mostly through dietary exposure (Pereira et al., 2015). After ingestion of contaminated food, MeHg crosses the gut epithelia through an active process via the energy dependent L-type neutral amino acid transporters (Bradley et al., 2017). Once in the bloodstream, the liver is the first organ exposed to dietary MeHg, and, consequently, its ability to metabolize or eliminate it may dictate the level accumulated in other organs, such as the brain (Bradley et al., 2017). In fact, the toxicity of MeHg may decrease due to some hepatic defense mechanisms, such as sequestration by association to glutathione (GSH) and metallothioneins (Wei et al, 2014).

The bile and feces are the main pathways for excretion of dietary MeHg (Andréasson and Dave, 1995). MeHg and GSH form a complex that is secreted into the bile. MeHg binds with high affinity to the cysteine of GSH, forming a glutathione-MeHg complex that acts as an

endogenous ligand and contributes to MeHg efflux from the cells. After that, glutathione is hydrolyzed to its constituent amino acids, releasing the MeHg–cysteine complex (Clarkson and Magos, 2006). This last, can be reabsorbed in the gallbladder into the bloodstream or be secreted into the intestinal tract along with any unhydrolyzed glutathione complex. As it reaches the gastrointestinal tract, part of that is reabsorbed into the portal circulation as the cysteine complex and other part demethylated by intestinal microflora that is excreted in the feces in iHg form (Clarkson and Magos, 2006).

As mentioned above, there are several studies of Hg toxicokinetics in fish body, mainly focused in the widely explored tissues, such as liver, kidney and gills. On the contrary, the literature is scarce on studies focused on iHg and MeHg toxicokinetics including the brain. Despite that, there are studies reporting the accumulation of Hg forms in the brain of several species, pointing out the relevance of mitigating this knowledge gap. Pereira et al. (2014) evaluated total, organic and iHg levels in the brain of *Liza aurata* from a contaminated area at Aveiro lagoon (Portugal) and showed that it preferentially accumulated MeHg over iHg. Gentès et al. (2015) used zebrafish (*Danio rerio*) to compare the bioaccumulation of organic and iHg in the brain. Fish given a MeHg- or Hg^{2+}-enriched diet for 62 days (levels around 11 μg g^{-1} for both forms) displayed levels of organic Hg in brain 49 times higher than those of iHg, and higher than those found in the liver. By contrast, Bergés-Tiznado et al. (2015) evaluated Hg accumulation in different organs (liver, kidney and brain) of juvenile *Sphyrna lewini* and reported opposing results, as the brain registered the lowest Hg levels when compared to other organs. Incongruences between studies on Hg partitioning could be due to differences in metabolic rates among fish species (Pereira et al., 2019). Although there are not many studies reporting the distribution of Hg in different brain areas, more in-depth reports tried to map Hg distribution in fish brain (Korbas et al., 2012; Rouleau et al., 1999). Korbas et al. (2012) showed the optic nerve of zebrafish as one of the tissues with highest iHg and MeHg levels, while Rouleau et al. (1999), using the brown (*Salmo trutta*) and rainbow (*Oncorhynchus mykiss*) trout, showed that the optic nerve and tectum displayed the highest levels of iHg. Thus, the optic system seems to be especially prone to accumulate Hg forms, and the optic tectum a target area to study the effects of Hg forms accumulation in the brain.

The occurrence of Hg in the central nervous system (CNS) depends on its chemical speciation and points of entry (Pereira et al., 2015). The brain is highly protected from external toxicants by the BBB that controls the exchange of substances between the blood and the brain and has specialized transport proteins to allow access of nutrients (Pereira et al., 2015). In the body, MeHg exists as water-soluble complexes or attached to the sulfur atom of thiol ligands (Pereira et al., 2015). It enters the endothelial cells of the BBB as a complex with L-type large

neutral amino acid transported (Clarkson, 2002). Although few studies have been done about the uptake of iHg across BBB, it seems that thiol-conjugates of iHg present in blood can be actively transported across the membranes due to a molecular mimicry with endogenous molecules (Bridges et al., 2005).

1.4. Neurotoxicity of mercury in fish

The neurotoxic effects of Hg can be studied at various levels of biological organization, including biochemical analysis that can express initial signals of Hg exposure and precede higher levels analysis such as physiological and morphological parameters. When morphological parameters are affected, generally it indicates an irreversible damage or disease (Van der Oost et al., 2003). The behavioral effects of Hg forms have been also studied in fish as an apical manifestation of Hg neurotoxicity, which ultimately can have repercussions on fish performance and survival (Pereira et al., 2019). At this light, Table 1 summarizes laboratorial and field studies investigating the neurotoxicity of iHg and MeHg in fish at different levels of organization (from molecular to behavioral levels). Several fish species have been used to address the neurotoxicity of Hg forms, comprising both freshwater and seawater environments. Besides that, most of the studies used a multi-level approach combining morphology and behaviour (Pereira et al., 2016; Puga et al., 2016), histological and genetic (Cambier et al., 2012; Gentès et al., 2015) or molecular, physiological and histopathological analysis (Naïja et al., 2018). The most prominent studies comprising brain morphology and/or histopathology were summarized in Table 1 and are detailed in this section.

Table 1 - Summary of the studies reporting the neurotoxicity of inorganic and organic Hg forms in fish upon exposures in the laboratory (white) and under realistic field conditions (grey) (adapted from Pereira et al., 2015).

Main approach	Species	Developmental state	Habitat type	Hg species	iHg chemical form	oHg chemical form	iHg exposure route	oHg exposure route	Target organ	Main effects [1]	Reference
Morpho-structural changes	Zebrafish (*Danio rerio*)	Embryo	Freshwater	oHg	-	CH_3HgCl	-	Water	Brain neurons	Disrupted and degenerated nuclei, some neurons exhibiting different morphological patterns of cell death (necrosis, autophagy), swelling of mitochondria	Hassan et al. (2016)
Multi-approach	Zebrafish (*Danio rerio*)	Adult	Freshwater	oHg	-	CH_3HgCl	-	Diet	Brain	Preferential accumulation of MeHg in astrocytes triggering glutamate efflux, inhibition of glutamate reuptake and cysteine transport	Cambier et al. (2012)
	Zebrafish (*Danio rerio*)	Adult	Freshwater	oHg or iHg	unspecified	-	Diet	Diet	Multi-organ	MeHg levels being higher than iHg in zebrafish brain; induction of metallothionein production in the brain	Gentès et al. (2015)
	White seabream (*Diplodus sargus*)	Juvenile	Seawater	iHg	$HgCl_2$	-	Water	-	Brain	Decrease of the total number of cells (neurons and glia) in the optic tectum, cerebellum, and hypothalamus	Pereira et al. (2016)
	White seabream (*Diplodus sargus*)	Juvenile	Seawater	oHg	-	CH_3HgCl	-	Diet	Brain	Decrease of the number of brain cells (neurons and glia) in medial pallium and optic tectum	Puga et al. (2016)
	Peacock blennies (*Salaria pavo*)	Adult	Seawater	iHg	$HgCl_2$	-	Water	-	Brain	Several damages in the optic tectum and cerebellum including circulatory, regressive and progressive alterations; fish able to develop defense mechanisms	Naïja et al. (2018)
Morpho-structural changes	Golden grey mullet (*Liza aurata*)	Juvenile	Seawater	oHg	-	MeHg	-	Water	Brain	Larger optic tectum in summer; larger molecular layer of cerebellum with higher cell density in summer	Puga et al. (2018)

iHg – inorganic mercury; oHg – organic mercury; [1] – more details in the text

1.4.1. Biochemical alterations

Mercury-induced biochemical alterations have been recorded in fish CNS (the main target for Hg forms) with consequential inhibition of important enzymes for neurological function (Pereira et al., 2019). For instance, Verma et al. (1983) exposed *Notopterus notopterus* to waterborne $HgCl_2$ (17.6 - 88 μg L^{-1}) for 30 days and reported an inhibition of brain Na^+/K^+-ATPase. Identically, Reddy and co-workers (1988) showed a reduction of Ca^{2+}-ATPase activity in *Ictalurus punctatus* brain, which suggests that iHg leads to a disruption of calcium metabolism (essential for CNS).

MeHg intraperitoneal injection for 96 h on zebrafish altered the protein structure in the brain and induced oxidative stress that was proposed as a toxicity mechanism for this Hg form (Richter et al., 2011). Furthermore, zebrafish treated with dietary MeHg (mean value in the diet of 9.8 μg g^{-1} of total Hg levels) for 8 weeks showed modifications on brain proteome, indicating mitochondrial dysfunction, oxidative stress and disruption of calcium homeostasis (Rasinger et al., 2017). In addition, Gentès et al., (2015) reported an induction of metallothionein production in the brain of adult zebrafish exposed to dietary MeHg (11.58 μg g^{-1}) and iHg (11.92 μg g^{-1}).

As previously stated, oxidative stress has been associated with Hg neurotoxicity in fish. Considering iHg exposures, catfish *Heteropneustes fossilis* exposed to waterborne $HgCl_2$ (148 μg L^{-1} as Hg^{2+}) displayed a significant increase of lipid peroxidation and a depletion of total lipids (Bano and Hasan, 1989). In addition, dietary exposure to $HgCl_2$ (10 and 100 μg g^{-1} as iHg in feed dw) did not caused lipid peroxidation in the brain of juvenile Atlantic salmon *S. salar* (Berntssen et al., 2003). Also, organic Hg triggered changes on antioxidant enzymes (Berntssen et al., 2003). The brain of juvenile European sea bass *Dicentrarchus labrax* exposed to dietary MeHg (8.0 μg g^{-1} feed dw) for 28 days displayed alteration in the profile of antioxidant enzymes such as an increasing of catalase and an inhibition of superoxide dismutase activities (Maulvault et al., 2016). In addition, Berntssen et al. (2003) observed that a dietary exposure of *S. salar* to MeHg (4.3 and 8.6 μg g^{-1} as CH_3Hg^+) for 4 months triggered a general break-down of the redox defense system with the inhibition of superoxide dismutase and glutathione peroxidase in the brain.

More recently, Cardoso et al. (2015) compared the pro-oxidant potential of iHg and MeHg with similar daily exposure levels (~260 μg/day/kg bw). Exposure of juvenile white seabream (*Diplodus sargus*) to waterborne $HgCl_2$ (2 μg L^{-1} as Hg^{2+}) for 14 days resulted in oxidative damage (as protein oxidation) without activation of the antioxidant defense system in the brain. Interestingly, the dietary exposure to CH_3HgCl (8.7 μg g^{-1} as CH_3Hg^+ in feed dw),

for the same period, increased antioxidant protection, avoiding oxidative damage, and displaying a reduced neurotoxic effect compared to waterborne iHg. Importantly, this study challenges the belief that MeHg induces higher neurotoxic effects than iHg.

Mercury has a great affinity with sulfhydryl groups, forming covalent bonds with GSH and cysteine residues of proteins. MeHg binds directly to GSH acting as an endogenous ligand, contributing to MeHg efflux from the cells (Clarkson and Magos, 2006). MeHg promotes a decrease in intracellular GSH levels, which lead to cytotoxic effects (Choi et al., 1996). GSH has important protective effects against Hg related to its action that limits the amount of MeHg available for the interaction with sensitive macromolecules, and its ability as a reactive oxygen species (ROS) scavenger. Thus, the supply of GSH precursors to neurons via astrocytes and the maintenance of intracellular GSH concentrations are critical to protect cells against MeHg-induced neurotoxicity (Farina et al., 2011). Moreover, the inhibition of antioxidant enzymes in the CNS has been referred as a relevant mechanism involved in oxidative stress after Hg exposure (Roos et al., 2009). Research on oxidative stress as a chief mechanism of neurotoxicity has been mostly conducted with MeHg, since it has been developed in the human toxicology research context (humans are less prone to be exposed to waterborne iHg). Thus, little is known about oxidative stress induction in the brain after iHg exposure. iHg is likely to bound to thiol containing molecules such GSH that, presumably, can attach to Hg^{2+} resembling oxidized glutathione and being exported from liver cells by glutathione carriers (Clarkson and Magos, 2006).

1.4.2. Histomorphological alterations

Histopathological analysis is a useful tool to indirectly assess the degree of aquatic contamination by Hg, especially for sub-lethal and chronic effects (Bernet et al., 1999; Mishra and Devi, 2014; Pereira et al., 2018). Furthermore, the classification of histopathological lesions allows determining which organs have been damaged and to what extent (Bernet et al., 1999).

Berntssen et al. (2003) performed a histopathological study of the Atlantic salmon (*S. salar*) brain after 120 days of dietary exposure to MeHg (4.3 and 8.6 μg g^{-1} dw) and $HgCl_2$ (10 and 100 μg g^{-1} dw). MeHg exposure induced vacuolation and necrosis in the medulla oblongata, ventral regions of the optic tectum and cerebrum, with effects being more severe in fish exposed to the higher dose. Nevertheless, the severity of effects was not consistent with brain accumulation levels that were higher after exposure to the lower dose of MeHg (accumulation

levels in the brain of 1.16 μg g^{-1}) in comparison to the higher exposure level (accumulation levels in the brain of 0.68 μg g^{-1}). The authors proposed that the discrepancy between exposure dose and histopathological effects was due to the occurrence of brain edema that would apparently decrease Hg tissue concentration when expressed on a wet weight basis. On the other hand, $HgCl_2$ induced much less effects than those recorded for MeHg. After 120 days, $HgCl_2$ (7.4 μg g^{-1}) triggered an increase of cellularity of astrocytes in optic tectum and throughout the medulla oblongata. The necrosis extent after $HgCl_2$ exposure was moderate when compared with the severe necrosis caused by MeHg (at identical exposure levels of 8.6 μg g^{-1}).

Naïja et al. (2018) investigated the histopathological effects of waterborne $HgCl_2$ (66 μg L^{-1}) in peacock blennies (*S. pavo*) brain, specifically in the optic tectum. This brain area was screened by its different layers, namely from the innermost to outer layers as following: *stratum periventriculare* (C1), *stratum album centrale* (C2), *stratum griseum centrale* (C3), *stratum fibrosum et griseum superficiale* (C4), *stratum opticum* and *stratum marginale* (C5/6). This study revealed in the first day of the experiment blood vessel abnormalities and hypertrophy of C1, congestion, necrosis and atrophy of C3, fusion of layer C4 and C5 and vacuolated areas in C6. After 4 days of exposure to $HgCl_2$, it was observed an abundance of neurons as well as clumping, spongiosis and fusion of C5 and C6 as well as increased vacuolization. On the 10^{th} day of exposure, they reported severe clumping with congestion and dilatation of blood vessels and fusion of C2 with C3 and of C4 with C5/C6. Finally, on the last day of exposure a fusion of layers from C2 to C6 was reported as well as severe vacuolization of all layers. Congestion, necrosis, clumping, blood vessel abnormalities, atrophy and dilatation was also noted, but to a less extent than for previous timepoint. Interestingly, these effects were not accompanied by a significant accumulation of Hg in the brain with the authors claiming that the brain of peacock blennies was able to eliminate iHg. Additionally, other studies reported alterations in the neuronal cells with some nuclei appearing to be disrupted (presence of clumped or condensed chromatin) during MeHg exposure (Hassan et al., 2016; Cambier et al., 2012).

There are only a few studies that employed stereological methods to evaluate the effects of Hg in fish brain morphometry. Pereira et al. (2016) showed that iHg significantly decreases the total number of cells (neurons and glia cells) in the optic tectum, cerebellum and hypothalamus of *Diplodus sargus* after 7 days of exposure. In the same research group, Puga et al. (2016) investigated the effect of MeHg in different brain areas of *D. sargus*, showing a decrease in the total number of brain cells (neurons and glia cells) in medial pallium and optic tectum after 7 days of exposure while the hypothalamic volume was increased.

As mentioned above, there are only a few studies on histopathological alterations in fish brain exposed to Hg forms (which normally consist in exposure to dietary MeHg or iHg separately). Differently, the morphology of rodent brain has been extensively examined following Hg exposure revealing adverse effects in cell populations (Sager et al., 1984; Roegge et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). These studies showed adverse effects on cell populations within the hippocampus and cerebellum exposed to MeHg. For instance, Aragão et al. (2018) exposed rats to $HgCl_2$ (0.375 mg/kg/day) by gavage for 45 days and demonstrated that iHg induced deleterious effects in the CNS, resulting in cognitive impairments and hippocampal damage, namely a decrease in the number of astrocytes and neurons, after long-term exposure to low doses.

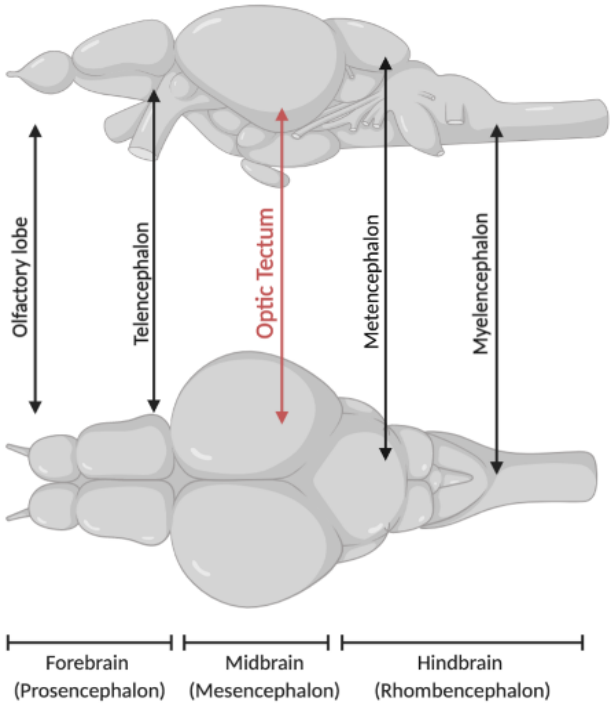


Figure 2 – Representation of fish brain anatomy with the identification of main areas. Red arrow indicates the optic tectum that was the brain region addressed in the current study (This figure was elaborated using https://biorender.com/).

The brain is a primary target organ for Hg forms accumulation (Mishra and Devi, 2014) with the optic tectum displaying a higher vulnerability to both iHg and MeHg accumulation (Rouleau et al., 1999; Korbas et al., 2012). The optic tectum is a midbrain area (Figure 4) that processes information and controls behaviour in all fish species (Northmore, 2011). It is also considered the primary visual center in the brain since the majority of the optic nerve fibers,

the axons of retinal ganglion cells, terminate in the optic tectum (Northmore, 2011). This region comprises 6 layers (Figure 5), specifically from the innermost to outermost: the *stratum periventriculare* (L1), *stratum album centrale* (L2), *stratum griseum centrale* (L3), *stratum fibrosum et griseum superficiale* (L4), *stratum opticum* and *marginale* (L5/6).

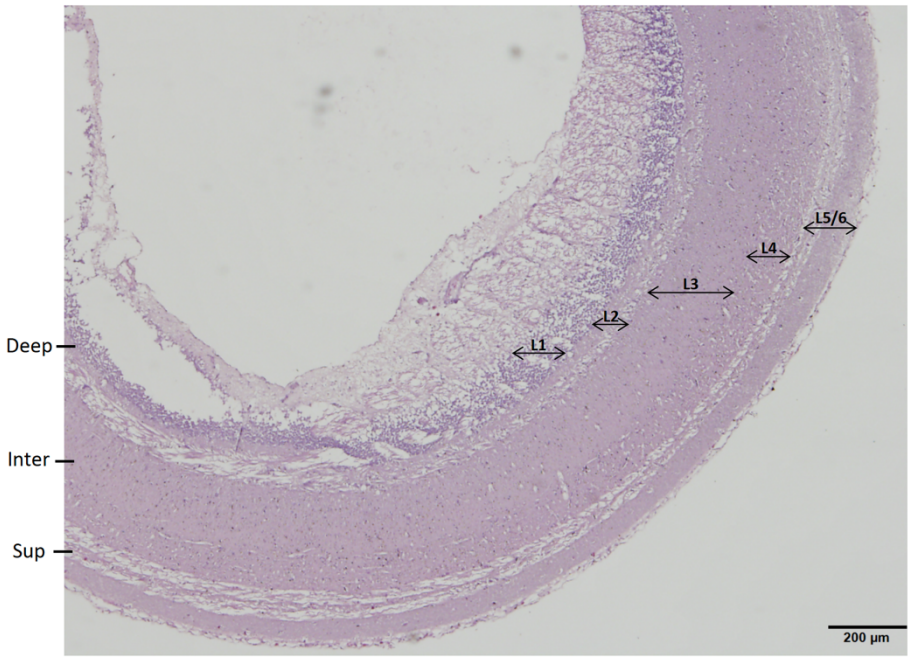


Figure 3 – Layers of *Diplodus sargus* optic tectum. *Stratum periventriculare* (L1), *stratum album centrale* (L2), *stratum griseum centrale* (L3), *stratum fibrosum et griseum superficiale* (L4), *stratum opticum and marginale* (L5/6). (Deep – deep layers; Inter – intermediate layers; Sup – superior layers).

Anatomically, some of the layers contain many fibers (axons) (L2, L5/6) or neuronal cell bodies, fibers and blood vessels (L3 and L4), while the innermost layer (L1) comprises densely packed neuronal cell bodies (Northmore, 2011) and the tectal proliferation zone (Alunni et al., 2010). Studies performed in medaka and zebrafish showed that, although the new generated cells can be found in all layers of the optic tectum, the majority stays within L1 (Ganz and Brand, 2016). In fish brain, the optic tectum receives information from the optic nerves and its large size is related to the importance of the vision in this animal group (Naïja et al., 2018). Importantly, the optic tectum is interconnected with different brain areas that vary by layer. Most fibers existing in L2 originate in the torus semicircularis, an auditory center which contains directionally selective neurons that respond to underwater vibrating in a particular direction (Northmore, 2011). L3 fibers are connected with the telencephalon as well

as with the nucleus isthmi, a cell mass below the torus semicircularis which functions to sustain hunting behavior by providing state-dependent feedback facilitation to the optic tectum (Northmore, 2011). Moreover, optic fibers from retina to L4 and to other layers are the major neural connections of the optic tectum. Layer L5 receives inputs from the telencephalon and the outermost layer (L6) receives inputs from the torus longitudinalis, which consists in a group of many small and medium neurons located in a longitudinal eminence attached to the optic tectum, involved in visual information processing (Northmore, 2011). A partial or localized lesion of one tectal lobe induces a loss of responsiveness to visual stimuli (Northmore, 2011). In addition, damage to the optic tectum also results in losses beyond blindness (Northmore, 2011). Fish start to bump into objects and impair their ability to navigate the environment using non-visual senses, since the source of spatial information underwater is believed to be represented in the optic tectum too (Northmore, 2011). The integrity of the optic tectum is central for the maintenance of many behaviors vital for fish survival, such as food search, predator escape or reproduction (Northmore, 2011).

1.4.3. Neuronal cell types and deleterious effects of mercury

Similar to mammals, the CNS of fish presents two major cellular components, namely: neurons and glial cells (Figure 6). Numerous studies confirmed the similarity of neurons and glia between fish and mammals (Bullock, 2004; Genten et al., 2009; Lyons and Talbot, 2015); however, it is still unknown if neurogenesis and gliogenesis are conserved in fish (Lyons and Talbot, 2015). Neurons, considered the basic unit of the brain, are responsible for processing and responding to inputs (Sahota et al., 2019). These cells are characterized by a wide variety of shapes (*e.g.*, multipolar neuron in Figure 6) and sizes (*e.g.*, small and large neurons) and can be classified by the neurotransmitters they release (*e.g.*, cholinergic, GABAergic, glutamatergic) (Garman, 2011). Usually, neurons present multiple dendrites arising from their cell bodies, which receive incoming signals, and a single axon that is specialized for transport, conduction of depolarization and synaptic transmission. Moreover, axons contain a large number of neurofilaments and microtubules that are especially important in the maintenance of cell integrity and axonal transport (Garman, 2011).

CNS glia of fish is composed by macroglia (including astroglia, oligodendrocytes) and microglia (Figure 6) (Lyons and Talbot, 2015). Glial cells perform a diverse range of functions including guidance cues for neuronal proliferation, electrical differentiation of neurons, as well

as the maintenance of neuronal homeostasis and synaptic plasticity and repair (Aschner et al., 1999).

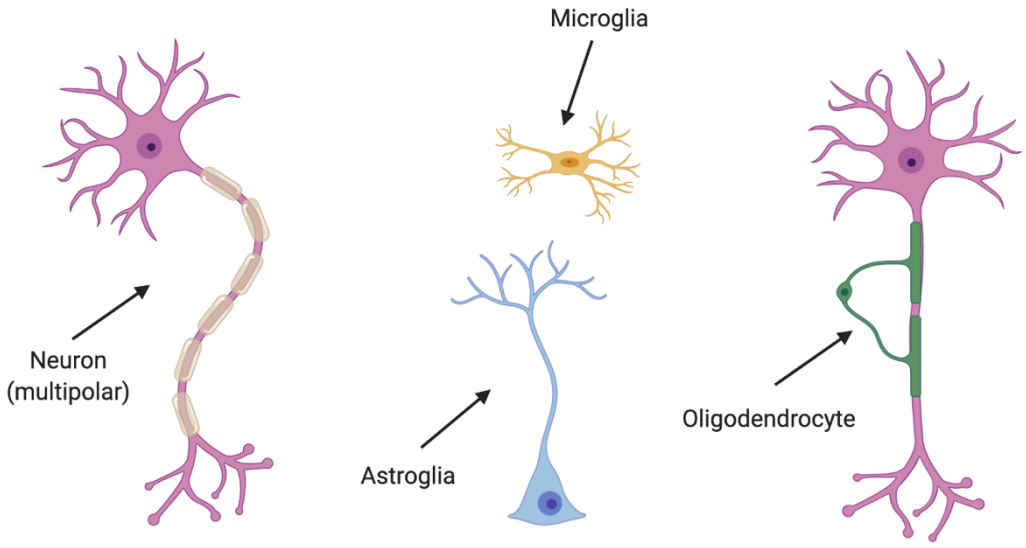


Figure 4 – Schematic representation of central nervous system cells (this figure was elaborated using https://biorender.com/).

Astroglia (also known as radial glia or radial astrocytes) are the most numerous glial cells within the fish CNS and have the particular feature of expressing glial fibrillary acidic protein (GFAP), which is used to identify astroglia in immunochemistry assays (Butt, 2009). Several studies consider astroglia as a homologue of mammalian astrocytes (Grupp et al., 2010; Lyons and Talbot, 2015); however, astroglia in fish exhibit morphological characteristics of mammalian radial glia with radial extensions (Figure 6). In teleost fish, astroglia maintains proliferative capacity and, at the same time has many functions in common with mammalian astrocytes (Jurisch-Yaksi et al., 2020).

Moreover, astroglia is involved in cell-cell communication and in monitoring the extracellular matrix of the CNS, as well as recycling electrolytes released by neurons during transmission of a nerve impulse and also metabolize and recycle many neurotransmitters such as glutamate and GABA (Cambier et al., 2012). Furthermore, astroglia also process neuronal metabolic waste and metabolize certain toxins (Cambier et al., 2012). In fact, after an insult, radial astrocytes can respond to damage within the CNS and become larger (hypertrophy) or proliferate in response to a toxic stimulus (Cambier et al., 2012; Aschner et al., 2007). Moreover, these cells can respond to neuronal activity by intracellular calcium increase, influencing neurons by several mechanisms such as transmitter release or modulation of

extracellular potassium concentration (Mu et al., 2019). Astrocytes can also modulate circuit function and behavior, as well as integrate neuronal signals over time (Mu et al., 2019; Deemyad et al., 2018).

Fish oligodendrocytes are highly specialized glial cells that are responsible for the formation and maintenance of the myelin sheaths of the CNS (Lyons and Talbot, 2015; Garman, 2011). Myelin sheaths are extremely large extensions of the oligodendrocyte cell membrane that are enfolded around the axon (Sahota et al., 2019). Being analogous to Schwann cells in the peripheral nervous system (PNS), oligodendrocytes are fundamental for myelin formation and wrap around axons to ensure proper and fast transmission of the nerve impulses (Kuhn et al., 2019; Butt, 2009). While myelinating oligodendrocytes are abundant within CNS white matter, non-myelinating oligodendrocytes (also called satellite oligodendrocytes) are found in CNS gray matter. These non-myelinating oligodendrocytes are not involved in myelination and are close to neuronal cell bodies or to blood vessels (Butt, 2009).

As the name suggests, microglia are the smallest glial cells. Considered the primary immune effector within CNS (Sahota et al., 2019) and, similarly to monocytes and macrophages, they secrete cytokines in response to inflammatory stimulus, function as antigen-presenting cells and maintain homeostasis by phagocytosis and remove cell waste after an injury (Sahota et al., 2019). Interestingly, even though microglia cells normally do not contact with each other, a subpopulation called satellite microglia is known to be in frequently contact with healthy neurons of CNS (Aschner et al., 1999).

Teixeira et al. (2018) observed a decrease of neurons and astrocytes in the motor cortex of adult rats after $HgCl_2$ exposure due to apoptosis. Interestingly, studies in fish and in mammals show a preferential accumulation of MeHg by astrocytes but not of iHg (Cambier et al., 2012; Aschner et al., 2007; Allen et al., 2002). MeHg in astrocytes triggers glutamate efflux, inhibition of glutamate reuptake and cysteine transport, leading to depletion of the cellular pools of glutamate and glutathione, and promoting the generation of ROS (Aschner et al., 2007). Tong et al. (2016), after evaluating the effects of iHg and MeHg in neural cells *in vitro* of different species (green turtle, mouse and human), concluded that MeHg is much more toxic than iHg *in vitro*. Human cell culture of neurons and astrocytes exposed to MeHg chloride (MeHgCl) and mercury chloride ($HgCl_2$) showed the major effects for the organic form of Hg in cell viability and proteomic analysis (Lohren et al., 2015). On contrary, the same study showed that iHg induces a more pronounced cytotoxicity than MeHgCl, after a long-term exposure.

In general, neurons seem to be more susceptible to Hg-induced cytotoxicity as compared to astrocytes (Lohren et al., 2015); however, data are contradictory. After MeHg exposure, Ni et al. (2012) showed lower levels of intracellular MeHg in neurons, while glial

cells showed a higher accumulation of MeHg. Even though studies *in vitro* and *in vivo* documented a greater accumulation of MeHg in astrocytes, Cambier et al. (2012) showed higher mortality of neurons. Additionally, in an experiment involving rat brain cell culture, neurons were shown to be the first cells irreversibly affected by MeHg (Monnet-Tschudi et al., 1996). In relation to iHg exposure, some studies reported an increased susceptibility of astrocytes to iHg toxicity when compared to neurons in mammals (Aragão et al., 2018; Charleston et al., 1994), while others referred to neurons as more sensitive than astrocytes (Teixeira et al., 2018). Interestingly, long-term exposure to iHg induced a more pronounced cytotoxicity compared to organic Hg (Lohren et al., 2015). In summary, more studies are necessary to understand which brain cells are more vulnerable to each Hg form in fish species.

1.5. Dissertation motivation, outline and objectives

Findings from a couple of studies regarding Hg toxicity in fish pointed out the optic tectum of the brain as particularly vulnerable to iHg and MeHg exposures. For instance, Puga et al. (2016) and Pereira et al. (2016) studied the neurotoxicity of iHg (2 μg L^{-1}) and MeHg (8.7 μg g^{-1}) in *Diplodus sargus*, respectively, through a regional morphometric analysis of the brain. Both studies detected a decrease in the total number of cells in the optic tectum after 7 days of exposure to waterborne iHg and dietary MeHg (Pereira et al., 2016; Puga et al., 2016). Interestingly, both studies agreed on the hypothesis of a preferential accumulation of iHg and MeHg in the optic tectum and on a lack of recovery after exposure to both Hg forms that explain the higher vulnerability of this brain area. Yet, some knowledge gaps remain concerning the morphological effects of iHg and MeHg in the optic tectum, specifically what is the most toxic form of Hg (iHg vs. MeHg) and what are the type of cells (neurons or glial cells) more susceptible to each Hg form. Thus, this dissertation compares, for the first time, the toxicity of iHg and MeHg in the optic tectum of fish (*Diplodus sargus*) through an elaborate quantification of neurons and glia cells, associated to the histopathological analysis. This evaluation was performed for each layer of the optic tectum, considering that a regional vulnerability could be related to the different composition of the layers. For that purpose, two experiments were performed, both comprising exposure and post-exposure periods, namely: i) waterborne exposure to $HgCl_2$ (2 μg L^{-1}); and ii) dietary exposure to MeHg (8.7 μg g^{-1}). Realistic levels of Hg in water and in food were tested in order to produce reliable data for environmental health assessment.

Fish brain was surveyed after 7 and 14 days of Hg exposure, as well as following a post-exposure period of 28 days, for a comparative analysis of Hg forms (iHg or MeHg) and exposure routes neurotoxicity (waterborne iHg or dietary MeHg) through the clarification of morphological effects in the optic tectum. Hence, the specific aims of this dissertation were the following:

1. To evaluate stereologically the number of neurons and glial cells, in the optic tectum layers of *D. sargus* after exposure to waterborne iHg and dietary MeHg, and relate the observed effects with the toxicokinetics of each Hg form;
2. To clarify the histopathological effects of iHg and MeHg in the optic tectum layers of *D. sargus*;
3. To evaluate the extent of the optic tectum recovery after exposure to iHg and MeHg.

By achieving these aims, this dissertation will provide answer to the research questions:

a. Do iHg and MeHg differently affect the specific layers of the optic tectum of *D. sargus*?
b. Do iHg and MeHg target preferentially neurons or glial cells (or both indistinctly)?
c. Is the optic tectum able recover from iHg and MeHg toxicity within 28 days?
d. What is the most toxic form of Hg (iHg vs. MeHg) in the optic tectum of *D. sargus*?

2. Material and Methods

2.1. Experimental set-up and sampling

Juvenile white seabreams (*Diplodus sargus*), provided by the Aquaculture Research Station (IPMA – Olhão, Portugal), were used to perform two experiments with the same design. The white seabream was selected as a model organism in the present dissertation since it is abundant in estuarine systems where Hg contamination can occur (Pereira et al., 2019). Moreover, *D. sargus* was previously selected as a model organism to explore the iHg and MeHg toxicokinetics (Pereira et al., 2015) and its neurotoxicity by assessing brain morphometric changes and behavioural alterations (Pereira et al., 2016; Puga et al., 2016).

Fish wellbeing deserved permanent attention during both experiments, in accordance with national and international guidelines for the protection of animal welfare. All fish were allowed to acclimatize to experimental conditions and routines for two weeks prior to Hg exposure. Water temperature, salinity and pH were monitored daily throughout both experiments, varying as follows, respectively: 13.5 ± 0.3 ºC, 35 ± 2 and 7-8.

Fish condition was assessed along both experiments through the Fulton's condition factor (K), according to the expression:

$$K = \frac{W \times 100}{L^3}$$

where W = weight (g) and L = total length (cm).

The iHg experiment concerned the exposure of fish to $HgCl_2$ [inorganic Hg – iHg] via water contamination, while in the MeHg experiment contaminated feed was used to expose fish (Figure 7). Fish were held in 300 L fiberglass tanks in an average density of 0.062 kg L^{-1} in the iHg experiment (initial weight: 19 ± 0.5 g; initial total length: 146 ± 14 cm) and 0.056 kg L^{-1} in the MeHg experiment (initial weight: 18 ± 0.5 g; initial total length: 119 ± 8 cm) under a 10 h light:14 h dark photoperiod. In both experiments, seawater was renewed daily (around 80%) and fish were fed once a day, namely 1-2 hours before water renewal. In all sampling days, fish were not fed in the 12 hours preceding handling that started around 09:00 am.

In the iHg experiment, fish were fed with a commercial feed (standard 3 mm pellets; Sorgal, Portugal) with total Hg residues lower than 0.01 μg g^{-1}. In exposure tanks, $HgCl_2$ (Sigma Aldrich) was added to the water in an aqueous solution (Milli-Q water acidified with 1% of HNO_3 supra-pure) to reach a final concentration of 2 μg L^{-1}. $HgCl_2$ was added daily after water renewal (*i.e.*, daily water recontamination) during the exposure period that lasted 14 days. This divalent Hg level was established considering previous studies in contaminated areas (Horvat et al., 2003; Li et al., 2009) to mimic environmentally realistic conditions. Control fish were kept in tanks filled with clean seawater throughout the experiment.

Contaminated feed (3 mm pellets), produced by SPAROS Company (Olhão, Portugal), was used to expose fish to MeHg. A solution of MeHg chloride (Sigma-Aldrich; prepared in ethanol) was added during the process of pellets production, with a homogenous distribution of toxicant throughout the batch. MeHg levels in contaminated pellets (8.7±0.5 μg g^{-1} dry weight) used to expose fish to this Hg form presented concentrations similar to those previously detected in natural food of *D. sargus* (*e.g.*, *Nereis diversicolor*) (Pereira et al. unpublished data). Control fish were fed with uncontaminated pellets, with the same size and nutritional formulation, which were produced following an identical methodology but without addition of MeHg solution (MeHg residues lower than 0.01 μg g^{-1}).

To compare the neurotoxic potential of both Hg forms, comparable daily exposure levels were estimated for both experiments, which were translated in the following values: 265 and 261 μg/day/kg of body weight, respectively for iHg and MeHg. Daily exposure values were estimated considering the amount of food ingested and the corresponding mass of metal vehiculated for MeHg experiment, while for the iHg experiment the difference between the initial nominal concentration and the concentration measured before recontamination (maximum value corresponding to 18% of the initial concentration) was considered to represent a rough measure of the Hg that was taken up by fish, assuming negligible losses by volatilization and adsorption by tank surface (plausible due to daily recontamination) in line with previous findings (Ribeiro et al., 2002).

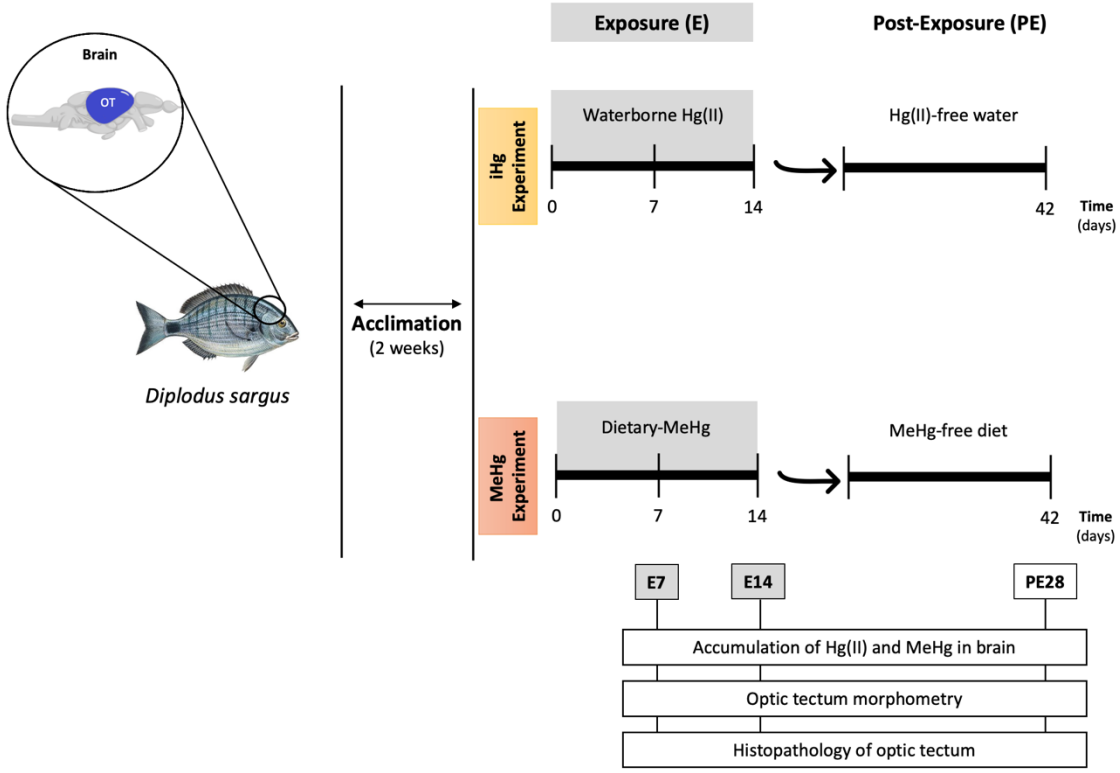


Figure 5 – Design of the experiments with white seabream (*D. sargus*) comprising iHg (2 µg L^{-1}) or MeHg (8.7 µg g^{-1}) exposure. Before Hg exposure, fish had a period of acclimation of 2 weeks. Fish were then exposed for 7 and 14 days (conditions E7 and E14, respectively). Thereafter, fish were transferred to clean water/uncontaminated feed and allowed to recover for 28 days (condition PE28). In parallel, control groups were also considered following an identical design as the exposed groups. (OT – Optic tectum)

Both experiments followed the same experimental set-up. Fish were exposed to divalent Hg or MeHg for either 7 (E7) or 14 (E14) days. Thereafter, fish were either transferred to clean water (post-exposure period of the iHg experiment) or fed with uncontaminated pellets (post-exposure of the MeHg experiment) and allowed to recover for 28 days (PE28) (Figure 7). The experiment had a total duration of 42 days. At each sampling time (E7, E14 and PE28), in both experiments, fish were sacrificed for different analyses, as following: eight fish for Hg determinations and another five for morphometric and histopathological examination (Figure 7), although in some groups four fish were used for morphometric analysis. When the region of interest was damaged related to the histological and/or sectioning procedures, the sample was not included in data processing (the total fish number was: n_{iHg}= 39 and $n_{iHg\text{-}Control}$=37; n_{MeHg}= 38 and $n_{MeHg\text{-}Control}$=38).

During the exposure period (on days 7 and 14) of the iHg experiment, water samples were collected in triplicates from exposure and control tanks, 24 hours after recontamination, to quantify total Hg (tHg) levels in order to demonstrate that fish were actually exposed to the toxicant. Values of tHg in the exposure tanks varied between 0.05 and 0.36 µg L^{-1}, which would

probably correspond to the minimum exposure concentrations. Levels of tHg in the control tanks were below the detection limit throughout the experiment (< 0.1 ng L^{-1}). Identically, after 42 days (28 days of post-exposure), both in control and in previously contaminated tanks, tHg was below the analytical detection limit.

2.2. Brain sampling and histological procedures

During sampling, fish were anesthetized with 0.2 mg L^{-1} tricaine methanesulfonate (MS-222, buffered with $NaHCO_3$), weighed, measured, and sacrificed by cervical transection. Fish were then properly bled from the cardinal vein, using heparinized Pasteur pipettes, the whole brain was excised, and one set of samples stored at -80 ºC until further processing for Hg determinations. For morphometric and histopathologic evaluations, another set of brain samples was preserved by immersion in a solution of 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for a minimum of 72 hours, with no rinses.

For morphometric analysis and histopathology examination, each brain was firstly sliced. For that purpose, the portion corresponding to the optic tectum was dehydrated using increasing concentrations of ethanol, diaphanized with xylol and embedded in paraffin (6774006, Thermo Scientific, USA). The paraffin block containing the optic tectum region was then serially cut in 4 μm coronal sections in a microtome (Mícron HM325; Leica Microsystems, Nussloch, Germany), sections were mounted in microscope Menzel-Gläser Superfrost plus slides (J1800AMNZ, Thermo Scientific, USA) and stained with hematoxylin Haris (Merck, Darmstadt, Germany) and eosin Y alcoholic (Thermo Scientific, Cheshire, UK). Finally, slides were dehydrated using increasing concentrations of ethanol to remove all traces of water, rinsed in two baths of xylene and a thin layer Entellan mounting medium (Merck) was applied, followed by glass cover slipping (Menzel-Gläser).

2.3. Mercury analysis in the brain

Whole brain samples from the iHg experiment were firstly lyophilized, homogenized and then analyzed for tHg concentration by atomic absorption spectrometry (AAS) with thermal decomposition followed by gold amalgamation in a Hg analyzer (AMA) LECO 254 according to the protocol by Costley et al. (2000). Certified reference materials (Fish protein - DORM-3, Dogfish Liver Tissue - DOLT-4) from the Canadian National Research Council were used to ensure the accuracy of the procedure and obtained values were consistent with the

certified ones. In the current work, tHg levels in the whole brain allowed interpretations on iHg toxicokinetics based on the assumption that fish were exposed to iHg and that no methylation was so far reported to occur in fish.

All samples of MeHg experiment were processed and analyzed in the Center for Analytical Research on the Environment (CARE) at Acadia University (Nova Scotia, Canada). Dried and homogenized brain samples were weighed using a Sartorius ultra-balance and approximately 10 mg of sample was transferred to a 2 mL polypropylene vial for Hg speciation analysis. MeHg was quantified in whole brain samples using alkaline digestion, ethylation, and purge and trap gas chromatography – atomic fluorescence spectrometry in accordance with the protocols from Liang et al. (1994) and Edmonds et al. (2012). Quality control measures included the use of alkaline digest method blanks, sample replicates, analytical replicates, and analysis of two certified standard reference materials [dogfish liver tissue (DOLT-4) and dorsal fish muscle (DORM-3); from the Canadian National Research Council]. The certified reference materials DOLT-4 and DORM-3 showed excellent recoveries. This technique has been used in previously published works (Liang et al., 1994; Edmonds et al., 2012).

Data on Hg accumulation levels presented in this dissertation were previously published by the supervision team (Cardoso et al., 2017). These data are presented here to support interpretations on the toxicity of iHg and MeHg in the optic tectum of *D. sargus*, namely at the morphological level.

2.4. Morphometric analysis of the optic tectum

All morphometric measurements were performed under an Olympus Stereology Microscope (Upright BX51, Germany) and the Visiopharm software (Version 2.12.3, Visiopharm, Hovedstaden, Denmark) was used to outline and quantify the area of the optic tectum layers and to count the number of neurons and glial cells.

To the best of our knowledge, no brain atlas is available for *D. sargus.* Hence, the identification of the optic tectum layers was performed by comparison with the brain atlas of gilthead seabream *Sparus aurata* (Muñoz-Cueto, 2001), which also belongs to the *Sparidae* family. In brief and as shown in Figure 8, the optic tectum was outlined (final magnification of 25x) in six layers namely the *stratum periventriculare* (L1), *stratum album centrale* (L2), *stratum griseum centrale* (L3), *stratum fibrosum et griseum superficiale* (L4), *stratum opticum/marginale* (L5/6).

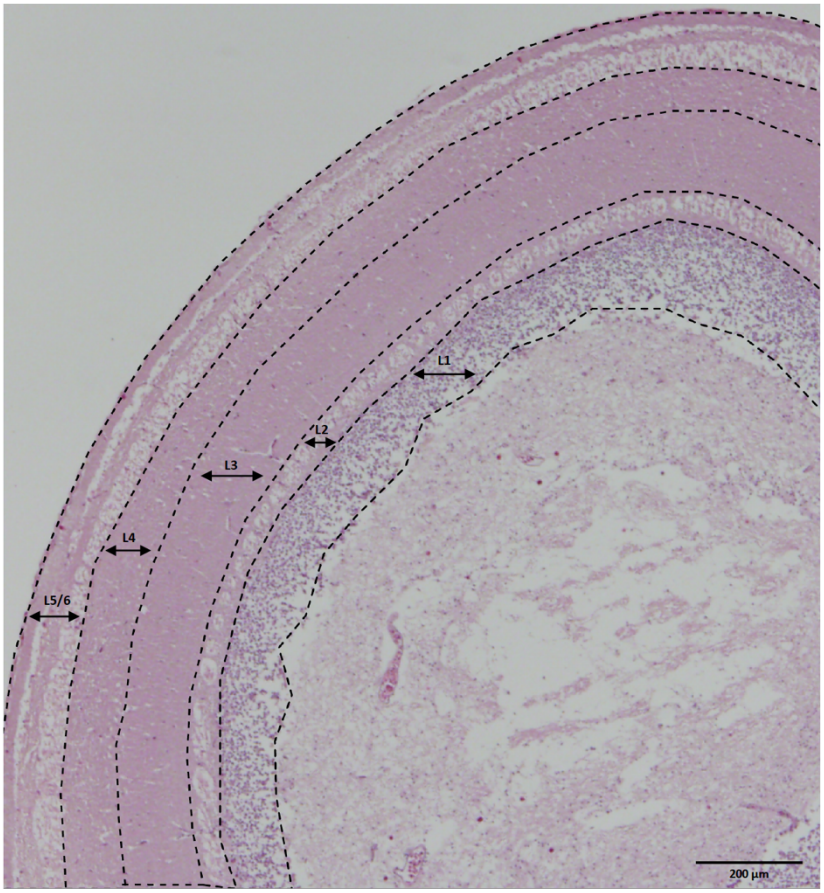


Figure 6 – Illustration of layer delimitation in *D. sargus* optic tectum. [(L1 - *stratum periventriculare*; L2 - *stratum album centrale;* L3- *stratum griseum centrale*; L4- *stratum fibrosum et griseum superficiale*; L5/6 - *stratum opticum and stratum marginale* (magnification 25x)].

The quantification of the number of neurons and glial cells was performed, at a magnification of 1000x, per section and per layer with the mean value per animal being considered in the statistical analysis. The researcher was blind to the fish treatment to eliminate any bias. An example of *D. sargus* neuron (black arrowheads) and glial cell (black arrows) is depicted in Figure 9. Neurons were considered to present larger cell bodies in which was possible to see the axons (Kaufman et al., 2012; Butt et al., 2019; Garman, 2011), while glial cells were considered as small round cells close to neurons with little observable cytoplasm and relatively small or indistinct nucleus (Butt et al., 2019; Garman, 2011).

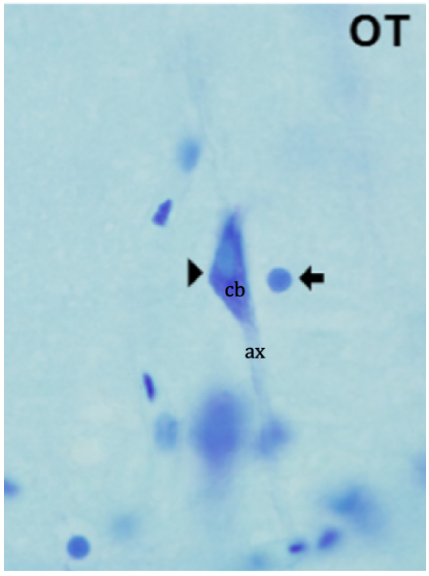


Figure 7 – Photomicrography showing a neuron (black arrowhead) and a glial cell (black arrows) in the optic tectum of *D. sargus* (Adapted from Puga et al., 2016). OT – optic tectum; cb – cell body; ax – axon.

2.5. Histopathological examination of the optic tectum

Slides used for the histopathological analysis were photographed in an Olympus Microscope (Upright BX61, Germany) at magnifications of 100x and 400x. For each fish, one photograph at magnification of 100x and four photographs at magnification of 400x were taken. Images were transferred to the ImageJ software (Version 1.53a, National Institutes of Health, USA) where the optic tectum areas were outlined (as in section 2.4) to quantify the area sampled. The screening of abnormalities was performed per section and per layer, adjusted to the total area of the layer and the mean value per fish was considered in further analyses. The researcher was blind to the fish treatment to eliminate any bias. Control and exposed fish were compared to detect and quantify the occurrence of Hg-induced abnormalities. Three reaction patterns were identified in the optic tectum according to Bernet et al. (1999), namely: circulatory disturbances, progressive changes and regressive changes. The circulatory disturbances result from a pathological condition of the circulatory system and tissues including congestion, blood vessel abnormalities and spongiosis (Table 2). Additionally, progressive changes lead to an increase or altered activity of cells and include dilatation and hypertrophy (Bernet et al., 1999) (Table 2). Regressive changes are processes that can culminate in a functional reduction or loss of an organ. This reaction pattern comprises the

following alterations: architectural and structural changes, vacuolation, necrosis, atrophy, fusion of layers and the detachment of layers (Table 2).

Table 2– Histopathological alterations identified in the optic tectum of *D. sargus*. The alterations are divided in three reaction patterns as circulatory disturbances, regressive and progressive changes. All alterations observed have an importance factor associated, as previously established by Bernet et al. (1999).

Reaction pattern	Alteration	Description	Importance factor
Circulatory disturbances	Congestion	Excess of blood within blood vessels;	1
	Blood vessel abnormality	Altered wall structure, perivascular edema or/and inflammatory cells;	1
Progressive changes	Spongiosis	Edema (abnormal accumulation of fluid);	1
	Dilatation	Dilatation of the blood vessels;	2
	Hypertrophy	Enlargement of cell volume or tissue without increase in cell number;	1
	Architectural and structural alterations	Changes in tissue structure as well as in shape;	1
	Vacuolation	Increase in the number of vacuoles in the cytoplasm;	1
Regressive changes	Necrosis	Morphological state of a cell which appears after loss of cell functions;	3
	Atrophy	Reduction in the volume of cells and/or a decreasing amount of intracellular substances;	2
	Fusion of layers	Impossibility of distinguish layer limit;	1
	Detachment of layers	Separation of the layers;	1

The optic tectum of control fish showed a normal architecture and morphology (Figure 10, first left column), while histological alterations surveyed in the optic tectum layers of fish exposed to iHg and MeHg are presented in Figure 10.

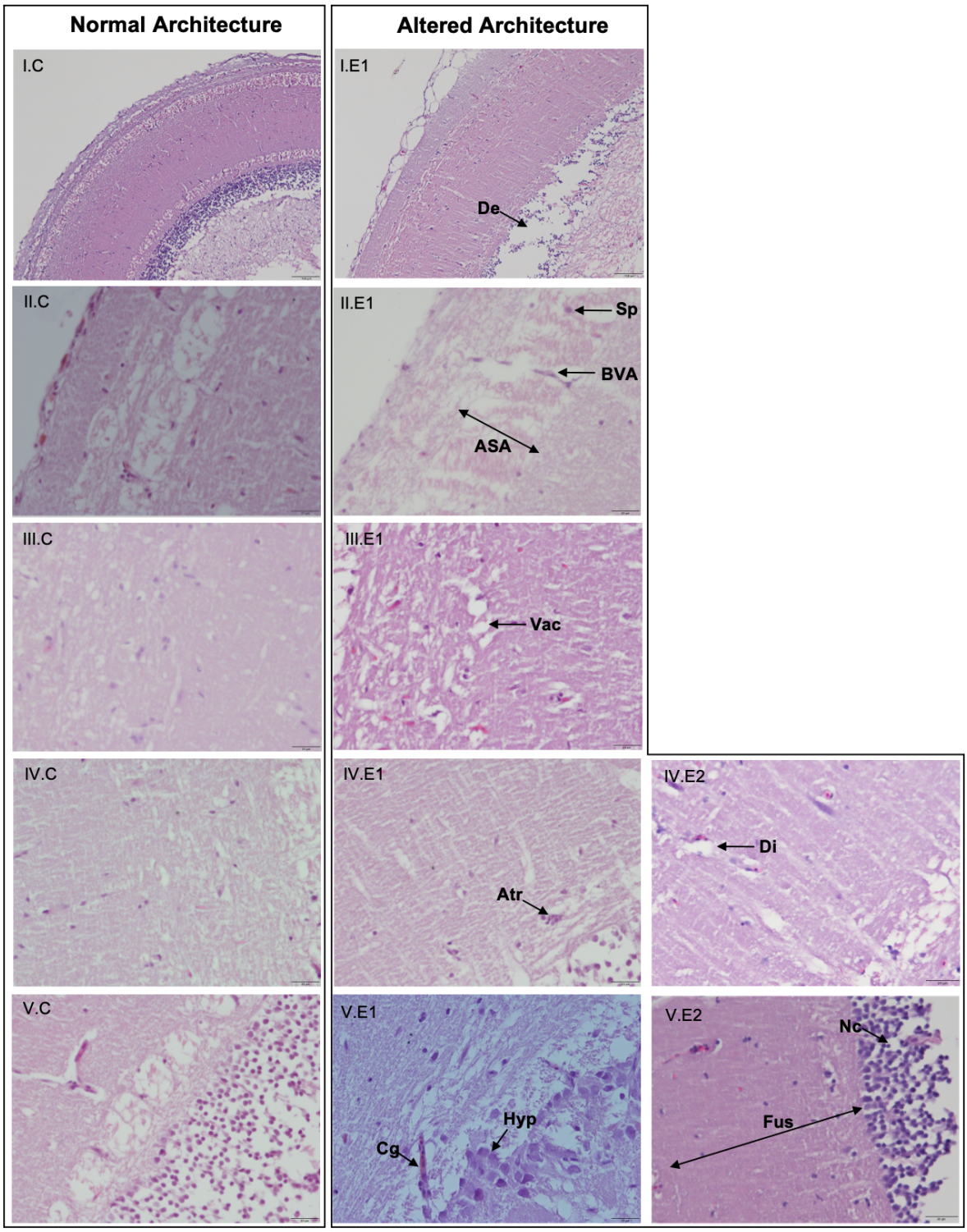


Figure 8– Optic tectum sections of *D. sargus* demonstrating the normal and altered features identified in the histopathological analysis. Normal architecture (I.C, II.C, III.C, IV.C and V.C) and altered architecture caused by iHg (II.E1, III.E1, IV.E1 and V.E1) or MeHg exposure (I.E1, IV.E2 and V.E2). Exposure to both Hg forms displayed an impact on the layers of the optic tectum including detachment of layers (De), spongiosis (Sp), blood vessel abnormality (BVA), architectural and structural alterations (ASA), vacuolation (Vac), dilatation (Di), atrophy (Atr), congestion (Cg), hypertrophy (Hyp), fusion of layers (Fus) and necrosis (Nc).

The organ index (I_{Lx}), representing the damage degree of the optic tectum layer and was calculated according to the following formula (Bernet et al., 1999):

$$I_{Lx} = \sum_{rp} \sum_{alt} (a_{Cxalt} \times W_{Cxalt})$$

where:

- Lx is the optic tectum layer;
- rp = reaction pattern;
- alt = alteration;
- a = score value;
- w = importance factor.

Table 2 shows all identified alterations with the respective importance factor, as previously defined by Bernet et al. (1999). An importance factor (w) (Table 2) was attributed to each pathological alteration identified in the optic tectum layers associated to its potential of affecting the organ function in accordance with Bernet et al. (1999) and Naïja et al. (2018), namely: i) importance factor 1 reflects minimal pathological importance, meaning that the lesion is reversible after the exposure to the toxicant ends; ii) importance factor 2 reflects moderate pathological importance, meaning reversible lesions if stressor is neutralized; iii) importance factor 3 reflects marked pathological importance where the lesion is irreversible even after exposure ends.

A score range (a) corresponding to the degree and the extent of the alterations was adopted from Bernet et al. (1999) and Naïja et al. (2018), namely: 0 attributed to an unchanged profile, 2 to mild occurrence, 4 for moderate occurrence and 6 was attributed to severe occurrence.

The index was calculated by the sum of the multiplied importance factors (w) and score values (a) of all alterations (Table 2) recorded in each layer of the optic tectum separately, for a given fish. Throughout the calculation of this histopathological index, it is possible to compare the degree of damage in the optic tectum layers of fish exposed to iHg and MeHg with the respective controls. The classification of the I_{Lx} used to determine the degree of damage inflicted by the Hg forms is represented in Table 3, following the works of Naïja et al. (2018), Zimmerli et al. (2007) and Agbohessi et al. (2015a,b).

Table 3– I_{Lx} classification system used to categorize the severity of iHg and MeHg effects in the optic tectum layers of *D. sargus* (Zimmerli et al., 2007; Agbohessi et al. 2015a,b).

Class	Severity of response
1 (index ≤10)	Normal/healthy structure
2 (10<index≤20)	Slight modifications of normal tissue architecture and morphology
3 (20<index≤30)	Moderate modifications of normal tissue architecture and morphology
4 (30<index≤40)	Pronounced modifications of normal tissue architecture and morphology
5 (index>40)	Severe alterations of normal tissue architecture and morphology

2.6. Data analysis

All the statistical analyses were performed using the GraphPad software (GraphPad Prism version 8.4.2 for macOS, GraphPad Software, San Diego, California). The normal distribution of all variables was assessed by the Shapiro-Wilk test. On normalized data, an unpaired Student's t-test with a significance level of 0.05 (two-tailed) was employed to assess the differences between control and exposed fish at each sampling time for both experiments. Otherwise, a nonparametric Mann-Whitney U-test (two-sided, unpaired) was used. Additionally, the effect of time on the accumulation of Hg species within experimental groups (exposed or control) was tested using a one-way ANOVA. Statistical differences between control and exposed fish for the histopathological alterations were assessed using Chi-test. Statistically significant differences between means were considered when $p < 0.05$. Data are presented as mean ± standard error of the mean.

3. Results

No fish mortality was observed during both experiments and, although feeding was not strictly monitored, no observable alterations were perceptible during and after treatments on fish feeding behaviour. Fish condition was assessed during the experiments through the use of the Fulton's condition factor (K). Table 4 shows the K values for control and exposed fish to iHg or MeHg, where no significant differences were found within each sample time-point for both experiments.

Table 4 – Condition factor (K) of *D. sargus* at 7 (E7) and 14 (E14) days of exposure and 28 days of post-exposure (PE28) concerning iHg and MeHg experiments. Data presented as mean ± standard deviation (n=8).

Experimental time	iHg experiment		MeHg experiment	
	Control	**Exposed**	**Control**	**Exposed**
E7	2.1±0.3	2.1±0.3	2.1±0.2	2.2±0.1
E14	2.3±0.2	2.5±0.2	2.3±0.1	2.2±0.1
PE28	2.2±0.2	2.1±0.3	2.3±0.2	2.2±0.1

3.1. iHg and MeHg accumulation in the brain

The levels of tHg in the whole brain of white seabream exposed to inorganic Hg (iHg) (2 μg L^{-1}) and the respective control groups are presented in Figure 11. Total Hg levels in the brain differed between control and exposed groups after 7 (E7) and 14 (E14) days of exposure, as well as at 28 days of post-exposure (PE28). After 7 and 14 days of exposure, exposed fish showed an increase of tHg concentration (E7: *t*-test, $p < 0.001$; E14: *t*-test, $p < 0.001$) when compared with controls. In the post-exposure period (PE28), the accumulated levels of tHg remained higher in exposed fish (*t*-test, $p < 0.001$). Additionally, temporal variations were noticed in exposed fish between the 7th and 14th day of exposure with the increase of tHg levels (one way ANOVA, Bonferroni *post hoc* test, $p < 0.001$) as well as between the 14th of exposure and 28th days of post-exposure (one way ANOVA, Bonferroni *post hoc* test, $p < 0.001$). Finally, no temporal variations were recorded for tHg concentration in the brain of control fish (one way ANOVA, Bonferroni *post hoc* test, $p > 0.05$).

In the other experiment, the levels of MeHg were assessed in the whole brain of white seabream exposed to dietary MeHg and in its respective controls (Figure 11). The concentration of MeHg was higher in the brain of E7 exposed fish (*t*-test, $p < 0.001$) and reached a maximum at E14 (*t*-test, $p < 0.001$). The levels of MeHg remained higher in exposed fish at 28

days of post-exposure (PE28) (*t*-test, $p < 0.001$) relatively to its control, although not as high as at E14 (*t*-test, $p < 0.001$). Temporal variations showed a significative increase of MeHg levels between the 7th and 14th days of exposure (one way ANOVA, Bonferroni *post hoc* test, $p < 0.001$), while the opposite occurred between E14 and PE28 (one way ANOVA, Bonferroni *post hoc* test, $p < 0.001$). Albeit the decrease of MeHg levels at PE28, the values did not reach those of control fish. In general, no temporal variations were recorded for MeHg levels in the brain of control fish (one way ANOVA, Bonferroni *post hoc* test, $p > 0.05$).

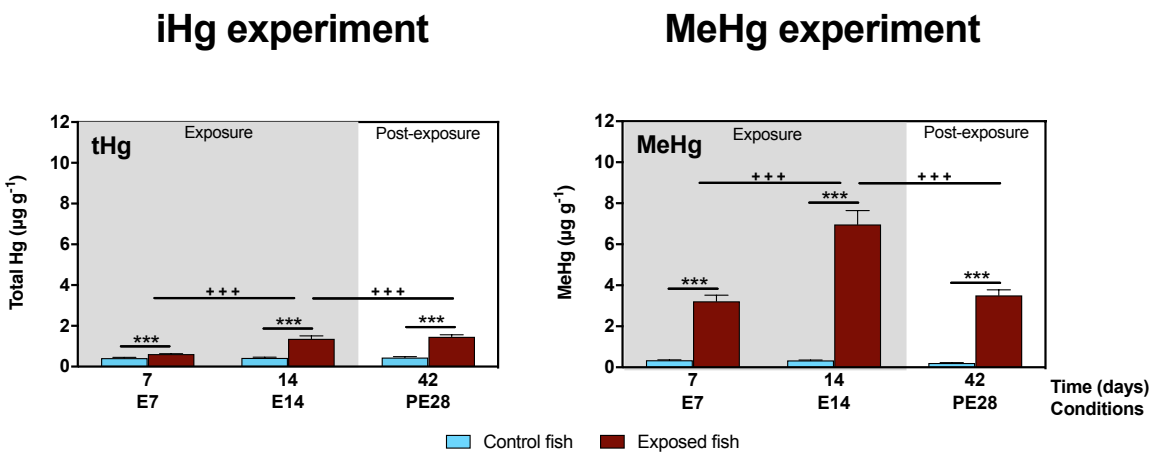


Figure 9 - Time variation of total Hg levels (tHg) (left graph) and methylmercury (MeHg) (right graph) in the whole brain of *D. sargus* after 7 (E7) and 14 (E14) days of exposure to iHg or MeHg (grey areas), respectively, and after 28 days (PE28) of post-exposure (light areas), respectively. Data presented as mean ± standard deviation (n=8). Asterisks represent significant differences in relation to the control group for each experimental time, assessed using a t-test: **p*<0.05, ***p*<0.01 and ****p*<0.001; +++ represents significant differences between exposed groups, assessed using one-way ANOVA. These data on Hg accumulation levels were previously published by the supervision team (Cardoso et al., 2017).

3.2. Morphometric alterations in the optic tectum

3.2.1. Number of neurons

The number of neurons in the optic tectum of fish exposed to iHg was not altered at E7 (L1: *t*-test, $p > 0.05$; L2: *t*-test, $p > 0.05$; L3: *t*-test, $p > 0.05$; L4: *t*-test, $p > 0.05$; L5/6: *t*-test, $p > 0.05$) and E14 (L1: *t*-test, $p > 0.05$; L2: *t*-test, $p > 0.05$; L3: *t*-test, $p > 0.05$; L4: *t*-test, $p > 0.05$; L5/6: *t*-test, $p > 0.05$). By contrast, the number of neurons decreased significantly in layers L3 (*t*-test, $p = 0.007$), L4 (*t*-test, $p = 0.008$) and L5/6 (*t*-test, $p = 0.04$) at 28 days of post-exposure to iHg (Figure 12).

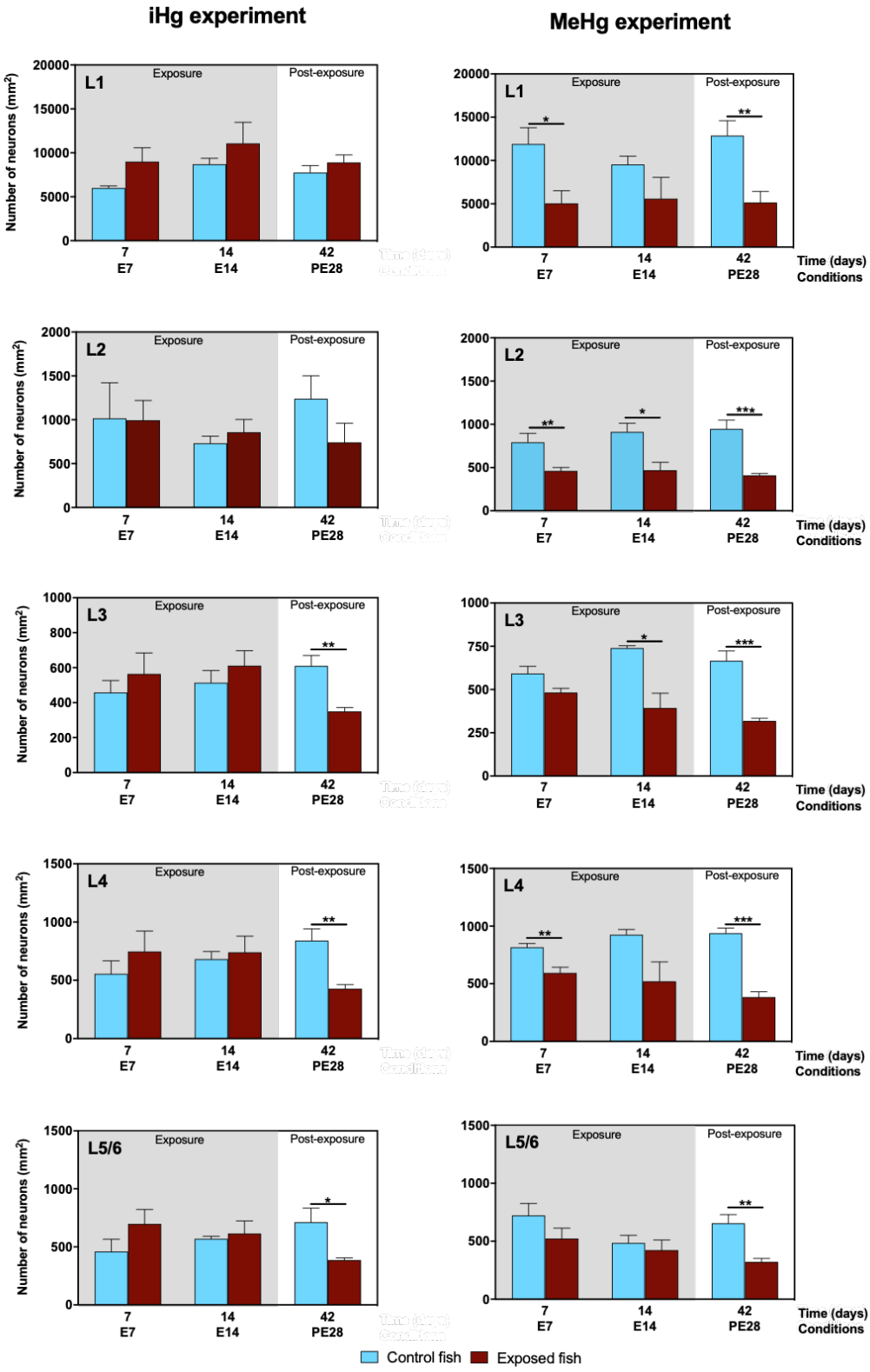


Figure 10 – Number of neurons in each layer (L1 to L5/6) of the optic tectum of *D. sargus* after 7 (E7) and 14 (E14) days of exposure to iHg or MeHg (grey area) and after 28 days of post-exposure (PE28; light area). Data presented as mean ± standard deviation (* represents significant differences in relation to the control group for each experimental time: *p<0.05, **p<0.01 and ***p<0.001; L1 - *stratum periventriculare*; L2 - *stratum album centrale;* L3 - *stratum griseum centrale*; L4 - *stratum fibrosum et griseum superficiale*; L5/6 - *stratum opticum and stratum marginale*).

On the other hand, a significant decrease on the number of neurons in L1 and L4 was observed after 7 days of exposure to dietary MeHg (E7) (*t*-test, $p = 0.02$; *t*-test, $p = 0.005$, respectively), as well as in the post-exposure time (PE28) (*t*-test, $p = 0.007$; *t*-test, $p < 0.001$, respectively). Moreover, layer L2 showed a decrease in the number of neurons in all time points (E7: *U*-test, $p = 0.008$; E14: *t*-test, $p = 0.02$; PE28: *t*-test, $p < 0.001$).

Even though no changes were found at E7 in L3 (*U*-test, $p > 0.05$), the number of neurons decreased significantly at E14 (*U*-test, $p = 0.03$) and 28 days after exposure to MeHg (*t*-test, $p < 0.001$). Lastly, layer L5/6 displayed a decrease in the number of neurons in the post-exposure time point (*t*-test, $p = 0.003$), while no changes were recorded at E7 and E14 (E7: *t*-test, $p > 0.05$; E14: *t*-test, $p > 0.05$).

No temporal changes were found for the number of neurons in each layer of the optic tectum of control and exposed fish.

3.2.2. Number of glial cells

The number of glial cells was not changed significantly after waterborne exposure to iHg. An exception was found in L1 layer at the post-exposure time of 28 days, when a significant increase in the number of glial cells was recorded (*t*-test; $p = 0.01$). Differently, a decrease of glial cells in the L5/6 layer of exposed fish at E14 (*t*-test, $p = 0.03$) was observed (Figure 13).

Concerning the dietary exposure to MeHg, while the number of glial cells was not altered in layers L1 (E7: *t*-test, $p > 0.05$; E14: *t*-test, $p > 0.05$; PE28: *t*-test, $p > 0.05$) and L5/6 (E7: *t*-test; $p > 0.05$; E14: *t*-test, $p > 0.05$; PE28: *t*-test, $p > 0.05$) through the experiment, significant differences between exposed and control fish were recorded in all the other layers. A decrease in the number of glial cells was found in the L2 layer of the optic tectum of fish after 7 and 14 days of exposure to MeHg (E7: *t*-test, $p = 0.02$; E14: *t*-test, $p = 0.03$). A similar pattern was recorded for L3 and L4 with a decrease on the cells number at E14 (L3: *t*-test; $p < 0.001$; L4: *t*-test, $p = 0.009$) and PE28 (L3: *t*-test, $p = 0.02$; L4: *t*-test, $p = 0.03$).

Temporal variations on the number of glial cells occurred occasionally, namely at: L1 of control fish of the iHg experiment where E14 was higher than PE28 (one way ANOVA, Bonferroni *post hoc* test, $p = 0.0045$); L3 of exposed fish of the MeHg experiment where E7 was higher than E14 (one way ANOVA, Bonferroni *post hoc* test, $p = 0.04$).

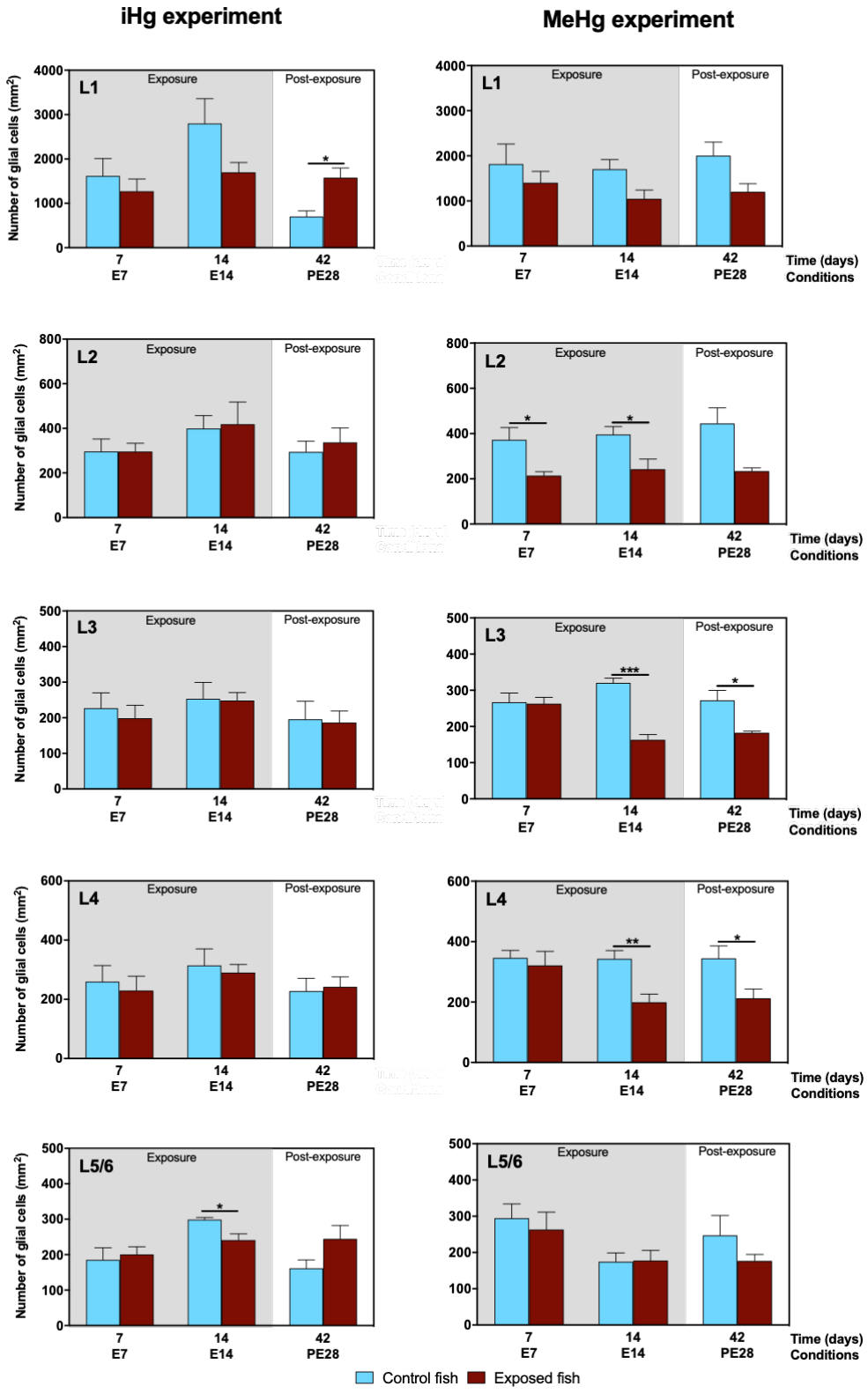


Figure 11 – Number of glial cells in each layer (L1 to L5/6) of the optic tectum of *D. sargus* after 7 (E7) and 14 (E14) days of exposure to iHg or MeHg (grey area) and after 28 days of post-exposure (PE28; light area). Data presented as mean ± standard deviation (* represents significant differences in relation to the control group for each experimental time: *p<0.05, **p<0.01 and ***p<0.001; L1 - *stratum periventriculare*; L2 - *stratum album centrale*; L3 - *stratum griseum centrale*; L4 - *stratum fibrosum et griseum superficiale*; L5/6 - *stratum opticum and stratum marginale*).

3.3. Histopathological alterations in the optic tectum

3.3.1. Semi-quantitative assessment

Control fish presented a normal architecture of the optic tectum in both iHg and MeHg experiments, which was consistent with a normal juvenile teleost fish with clearly defined and distinct layers of this brain region (Tables 5 and 6). Despite that, a few alterations were observed in control samples, mostly congestion, spongiosis, necrose and dilatation.

Differently, iHg exposure triggered histological alterations in the fish optic tectum in exposure and post-exposure periods (Table 5). Although statistically significant results were scarce when comparing control and exposed fish at each exposure time, related to an elevated dispersion of the data, notorious tendencies were found as described below. After 7 days of waterborne iHg exposure, spongiosis and necrosis were detected in all of the five surveyed layers of the optic tectum, with a significant difference being found at L5/6 for necrosis (X^2, p = 0.037). Moreover, this layer also presented a higher incidence of blood vessel abnormality, dilatation, architectural and structural alterations (X^2, p = 0.09), and detachment, while L4 was additionally impaired with hypertrophy, architectural and structural alterations and fusion of layers. All of the mentioned alterations were absent in control fish at this experimental time. Also at E7, L1, L2 and L3 were only occasionally altered by impairments other than spongiosis and necrose.

Moreover, at E14, spongiosis and necrosis occurred at all layers of exposed fish, but with higher incidence at L3, L4 and L5/6 since at these layers those alterations were not detected in control fish (X^2, p = 0.09). Some alterations should be highlighted at L4, namely dilatation and vacuolation, while L3 of exposed fish presented a significantly higher vacuolation than control ones. Still, in the post-exposure period (PE28), the optic tectum of pre-exposed fish was altered, namely with spongiosis (higher at L5/6), architectural and structural alterations, vacuolation and necrose.

The dietary exposure of fish to MeHg elicited significant histological changes in the optic tectum after 7 days of exposure, with necrosis and spongiosis being the most preponderant alterations occurring at every surveyed layer (Table 6). Despite that, significant differences from control fish were only recorded for necrosis at L3 (X^2, p = 0.037) and L4 (X^2, p = 0.037). Besides that, congestion and architectural/structural alterations were observed at almost every layer of exposed fish and fusion of layers in L2 and L3. Other alterations such as

blood vessel abnormalities, vacuolation and hypertrophy were detected occasionally in some layers of the optic tectum with no significant differences from control fish.

An identical pattern was observed at E14 and PE28, with significant differences for spongiosis in L4 at E14 (X^2, $p = 0.037$) as well as in L2 at PE28 (X^2, $p = 0.037$). Besides that, it was noticed vacuolation occurring in L3 and L5/6 layers at E14, and detachment in layers 1, 2 and 5/6 at PE28.

Table 5 – Estimated number of changes for each surveyed histopathological alteration in the optic tectum layers of *D. sargus* exposed to waterborne iHg (red area) and the respective controls (blue area). Data are presented as mean ± standard deviation (* represents significant differences in relation to the control group for each experimental time, namely: *$p < 0.05$; Δ represents conditions with higher odds ratio corresponding to a p value <0.1); E7 – 7 days exposure; E14 – 14 days exposure; PE28 – 28 days of post-exposure; L1 – *stratum periventriculare*; L2 – *stratum album centrale*; L3 – *stratum griseum centrale*; L4 – *stratum fibrosum et griseum superficiale*; L5/6 – *stratum opticum and stratum marginale*; Cg – congestion; BVA – blood vessel abnormalities; Sp – spongiosis; Di – dilatation; Hyp – hypertrophy; ASA – architectural and structural alterations; Vac – vacuolation; Nc – necrosis; Atr – atrophy; Fus – fusion; Det – detachment).

	Reaction patterns		Circulatory disturbances			Progressive changes		Regressive changes					
	Alterations		**Cg**	**BVA**	**Sp**	**Di**	**Hyp**	**ASA**	**Vac**	**Nc**	**Atr**	**Fus**	**Det**
E7	**Control**	**L1**	0	0	6±12	0	0	0	0	34±47	0	0	0
		L2	0	0	0	0	0	0	0	5±11	0	0	0
		L3	0	0	0	0	0	0	0	0	0	0	0
		L4	0	0	0	0	0	0	15±31	8±15	0	0	0
		L5/6	17±20	0	16±19	0	0	0	0	0	0	0	0
	Exposed	**L1**	0	0	255±219	0	10±19	10±19	0	413±190	13±26	0	13±26
		L2	4±8	0	5±10	0	0	14±6	0	22±13	0	0	0
		L3	0	0	34±28	23±6	10±20	0	0	68±17	0	4±7	0
		L4	0	0	97±74	0	33±66	32±18	42±33	86±46	0	16±33	0
		L5/6	45±90	30±42	157±119	8±16	0	**55±31Δ**	0	**93±85***	0	0	9±18
E14	**Control**	**L1**	0	0	135±65	0	0	0	0	97±30	0	0	0
		L2	0	0	28±25	0	0	0	0	13±2	0	0	0
		L3	0	0	0	7±15	0	0	0	0	0	0	0
		L4	0	0	0	0	0	0	0	0	0	0	0
		L5/6	6±11	0	0	0	0	0	0	0	0	0	0
	Exposed	**L1**	0	0	181±98	0	0	0	0	213±101	0	0	**59±57Δ**
		L2	7±14	0	23±6	0	0	7±13	0	31±17	10±20	0	0
		L3	0	0	24±8	9±11	10±19	59±70	**27±17Δ**	25±8	35±70	0	0
		L4	0	0	7±14	41±16	4±8	0	20±39	40±17	0	0	0
		L5/6	42±17	0	25±4	0	0	25±4	0	**29±8Δ**	22±44	0	0
PE28	**Control**	**L1**	0	0	115±99	0	0	0	0	60±39	0	0	8±17
		L2	0	0	8±15	0	0	0	0	5±11	0	0	0
		L3	4±8	0	4±8	4±8	0	0	0	8±16	0	0	0
		L4	0	0	9±17	9±17	0	0	0	0	0	0	0
		L5/6	15±19	0	0	0	0	0	0	0	0	0	10±19
	Exposed	**L1**	0	0	131±134	0	0	0	6±12	79±64	13±15	0	56±32
		L2	0	0	0	0	0	3±7	0	22±6	18±36	5±9	0
		L3	9±17	0	12±11	6±11	0	7±14	18±6	4±7	0	4±7	0
		L4	4±9	0	0	4±9	0	6±12	9±18	27±35	0	0	0
		L5/6	6±12	0	**16±11Δ**	0	0	7±15	0	**19±11Δ**	0	0	0

Table 6 – Estimated number of changes for each surveyed histopathological alteration in the optic tectum layers of *D. sargus* exposed to dietary MeHg (red area) and the respective controls (blue area). Data are presented as mean ± standard deviation (* represents significant differences in relation to the control group for each experimental time, namely: *p <0.05; ᐞ represents conditions with higher odds ratio corresponding to a p value <0.1); E7 – 7 days exposure; E14 – 14 days exposure; PE28 – 28 days of post-exposure; L1 - *stratum periventriculare*; L2 - *stratum album centrale*; L3 - *stratum griseum centrale*; L4 - *stratum fibrosum et griseum superficiale*; L5/6 - *stratum opticum and stratum marginale*; Cg - congestion; BVA – blood vessel abnormalities; Sp – spongiosis; Di – dilatation; Hyp – hypertrophy; ASA – architectural and structural alterations; Vac – vacuolation; Nc – necrosis; Atr – atrophy; Fus – fusion; Det – detachment).

Reaction patterns			Circulatory disturbances			Progressive changes		Regressive changes					
Alterations			**Cg**	**BVA**	**Sp**	**Di**	**Hyp**	**ASA**	**Vac**	**Nc**	**Atr**	**Fus**	**Det**
E7	Control	L1	0	0	43±65	0	0	0	0	32±38	0	0	0
		L2	0	0	0	0	0	7±13	0	8±16	0	0	0
		L3	10±14	0	7±15	6±12	0	6±13	9±12	0	0	0	0
		L4	0	0	16±33	0	0	0	0	6±12	0	0	0
		L5/6	5±9	0	35±42	0	0	0	0	14±27	0	0	0
	Exposed	L1	4±7	0	146±75	0	0	0	0	202±31	4±7	0	11±15
		L2	0	0	30±60	0	0	27±12	0	**29±10**	0	4±9	0
		L3	13±16	0	39±20	6±13	0	40±12	36±73	**172±228ᐞ**	0	12±24	0
		L4	35±16	0	67±49	0	8±16	34±18	0	**47±32ᐞ**	0	0	0
		L5/6	**47±26ᐞ**	20±32	82±85	0	0	6±12	0	25±50	0	0	0
E14	Control	L1	0	0	23±19	0	0	0	0	37±21	0	0	0
		L2	0	0	22±35	0	0	0	0	10±12	0	0	0
		L3	0	0	3±6	8±15	0	0	0	21±7	0	0	0
		L4	0	0	0	0	0	0	0	0	0	0	0
		L5/6	18±36	0	9±18	5±9	0	0	0	0	0	0	0
	Exposed	L1	0	0	141±87	0	0	0	0	263±161	0	0	**34±5ᐞ**
		L2	0	0	4±9	0	0	21±8	0	39±34	0	0	0
		L3	0	0	78±18	91±182	0	91±182	9±18	187±127	29±57	0	0
		L4	9±17	0	**86±36***	0	0	0	0	**92±36***	0	9±17	0
		L5/6	7±14	0	**64±40ᐞ**	7±14	0	0	4±7	**62±19ᐞ**	0	10±19	0
PE28	Control	L1	0	0	19±29	0	0	0	0	14±19	0	0	2±4
		L2	3±5	0	0	0	0	0	0	0	0	0	0
		L3	0	0	17±10	0	0	0	4±8	0	0	0	0
		L4	0	0	9±11	5±10	0	0	0	5±11	0	0	0
		L5/6	0	8±16	8±15	0	0	0	0	8±15	0	0	0
	Exposed	L1	0	0	257±143	0	0	0	0	331±189	0	0	54±28
		L2	8±16	0	**37±19***	0	0	0	0	**30±16ᐞ**	0	38±14	4±8
		L3	25±12	0	42±19	23±10	0	11±22	0	**65±33***	0	27±8	0
		L4	0	0	7±14	0	0	77±66	62±21	**44±24ᐞ**	0	0	0
		L5/6	**54±45ᐞ**	9±18	54±21	9±18	0	41±8	9±18	54±15	0	0	9±18

3.3.2. Histopathological index

After 7 days of fish exposure to iHg, a significant increase of the histopathological index was recorded in optic tectum layers, namely: L1 (*t*-test, $p = 0.03$), L3 (*t*-test, $p = 0.03$) and L5/6 (*t*-test, $p = 0.002$). On the contrary, no alterations were found in L2 and L4 (Figure 14). Among the three categories of surveyed alterations (circulatory, progressive and regressive), the latter contributed the most for the total estimated histopathological index. At E14, the index only increased significantly in L3 (*U*-test, $p = 0.03$), while no differences were found in the optic tectum layers of fish in the post-exposure period (PE28).

The dietary exposure of fish to MeHg elicited an increase of the histopathological index in L1 (*t*-test, $p = 0.03$), L2 (*t*-test, $p = 0.045$) and L4 (*t*-test, $p = 0.01$) in the optic tectum of fish after 7 days of exposure (Figure 14), while no changes were recorded at L3 and L5/6. After 14 days of exposure to MeHg, layers L4 (*U*-test, $p = 0.03$) and L5/6 (*t*-test, $p = 0.004$) showed an enhancement of the index, while at PE28 changes were found significantly at L1 (*t*-test, $p = 0.03$) and L3 (*U*-test, $p = 0.03$). The regressive changes were the most abundant, contributing mostly to the estimated index at every layer and experimental condition (Figure 14).

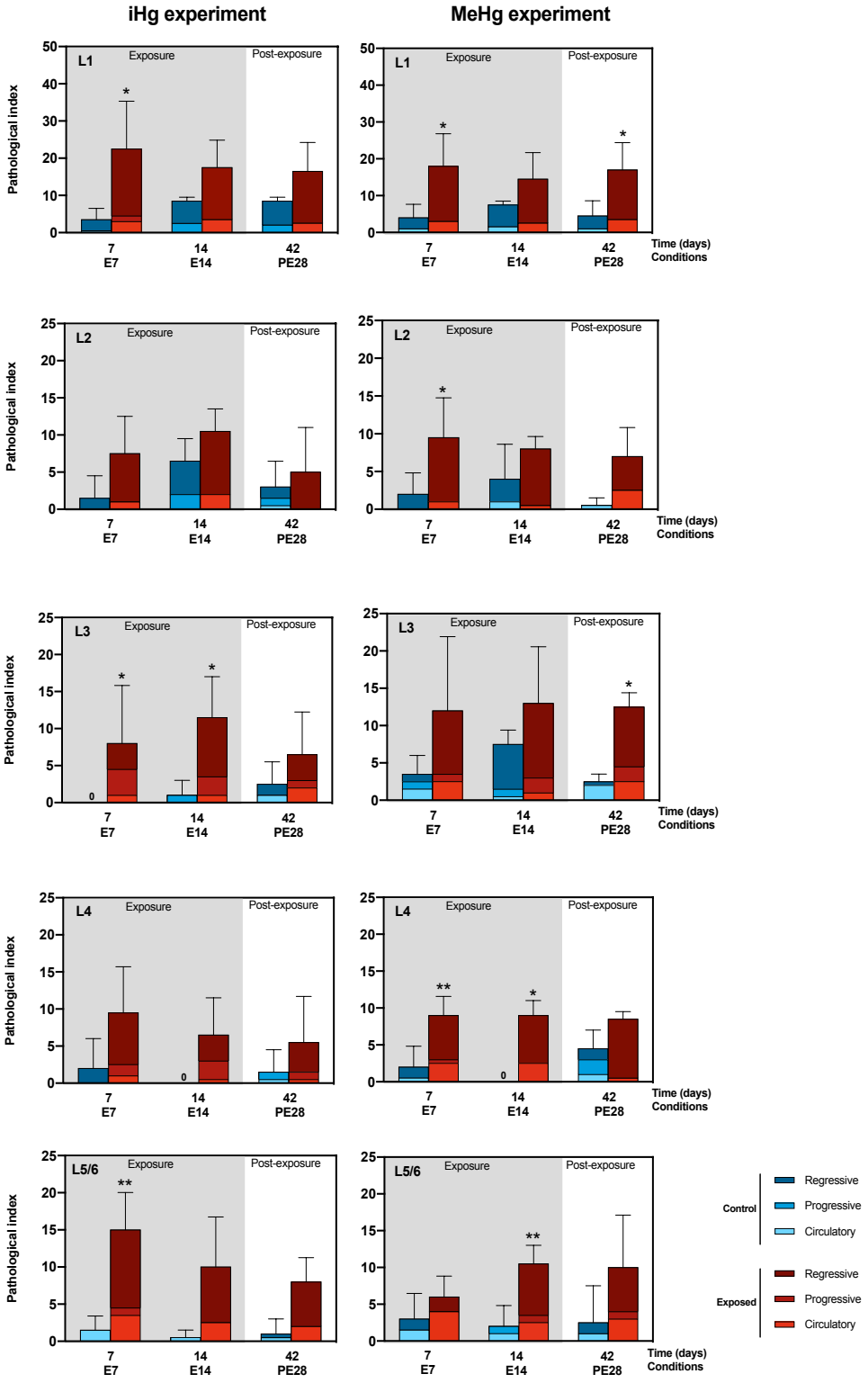


Figure 12 – Histopathological condition index (circulatory, progressive, regressive and total) by layer (L1 to L5/6) of the optic tectum of *D. sargus* exposed to iHg via water or MeHg via food after 7 (E7) and 14 (E14) days of exposure (grey area) and after 28 days of post-exposure (PE28; light area). Data presented as mean ± standard deviation (* represents significant differences in relation to the control group for each experimental time: *p<0.05 and **p<0.01; L1 - *stratum periventriculare*; L2 - *stratum album centrale*; L3 - *stratum griseum centrale*; L4 - *stratum fibrosum et griseum superficiale*; L5/6 - *stratum opticum and stratum marginale*).

4. Discussion

The comparable daily exposure levels achieved for waterborne iHg (265 µg $HgCl_2$/day/kg of body weight) and dietary MeHg (261 µg MeHg/day/kg of body weight) allows parallel interpretations on the accumulation dynamics and toxicity of both Hg forms. In the current study, although a heterogeneous distribution of iHg and MeHg in the brain is expected based on evidence presented for other species, iHg and MeHg levels were determined in the whole brain of *D. sargus* mainly due to analytical restrictions. For instance, Korbas et al. (2012) performed a screening of both iHg and MeHg distribution in zebrafish larvae and found a preferential accumulation of both Hg forms in the pineal gland. Moreover, in a study with brown (*Salmo trutta*) and rainbow (*Oncorhynchus mykiss*) trout, Rouleau et al. (1999) documented a preferential accumulation of iHg in specific areas of the brain including the optic tectum. Similarly, a heterogeneous distribution of iHg and MeHg with a preferential deposition in the optic tectum is expected to occur here in seabream's brain, which could trigger more adverse effects in this specific area. An optic tectum impairment may compromise fish fitness, reproduction and survival, considering that this brain region is the primary visual centre where sensory inputs (via *e.g.*, vision, audition) are processed (Northmore, 2011). The optic tectum of teleost fish is a highly developed neural processor, which is crucial for sensory discrimination and rapid decisions (Northmore, 2011).

As published in Cardoso et al. (2017), levels of MeHg in the brain of *D. sargus* were higher than those of iHg in exposure and post-exposure periods. These results are in line with previous levels in gills, carcass, head and viscera of *Oreochromis niloticus* that pointed out a higher propensity of the brain to accumulate MeHg over time in comparison to iHg (Wang et al., 2010). During the exposure period (E7 and E14), MeHg levels were 5 times higher than iHg, which is in agreement with Gentès et al. (2015) data that mentioned MeHg levels being higher than iHg in zebrafish brain. Different areas of the brain could have distinct affinities for Hg accumulation. In this context, Korbas et al. (2012) found a higher accumulation of iHg and MeHg in the pineal gland of zebrafish, while Rouleau et al. (1999) documented a preferential accumulation of iHg in the optic tectum. The mapping of Hg accumulation in the fish optic tectum layers was not done so far, but a differential accumulation per optic tectum layers could be speculated based on the different layer's composition. While L1 is mainly composed by a dense neuronal cell bodies, L3 and L4 are formed by a high number of myelin fibers and L5/6 is formed by unmyelinated fibers. The mapping of Hg forms by X-ray Fluorescence Imaging (XFI) was initially considered in this dissertation in order to support the interpretation of the

surveyed effects in the optic tectum. This would be done on the scope of a collaboration of the research group. This aim was postponed after delays related to the pandemic event.

iHg toxicokinetic in the brain of exposed fish was characterized by a significant increase of accumulation levels just after 7 days of exposure, which continued to increase in the next 7 days and remained stable in the post-exposure period that lasted 28 days. MeHg accumulation levels also increased significantly in the first 7 days of exposure, doubling between E7 and E14. Interestingly, the levels of MeHg after 28 days of depuration decreased to half while iHg accumulation seem to remain very stable over time. These results for iHg could be related to the formation of a stable complex with selenium (HgSe) that is very difficult to be eliminated (Korbas et al., 2010; Korbas et al., 2012). The complexation of Hg with Se can interfere with cellular components that depend on this essential element (*e.g.*, Se-dependent enzymes). Actually, Cardoso et al. (2015) investigated the effects of iHg exposure on antioxidant enzymes of the *D. sargus* brain and documented a consistently inhibition of glutathione peroxidase. Since the fish condition factor remained stable over time in both experiments, variations on the accumulation levels of Hg forms in the brain and associated toxic effects cannot be attributed to any compromise of fish fitness during the experiments.

4.1. Impairments on the optic tectum morphometry in relation to iHg and MeHg accumulation

No significant changes in the number of neurons in the optic tectum of seabream were found during the exposure period to iHg, namely after 7 (E7) and 14 (E14) days of exposure, despite the noteworthy increase of its levels in the brain. iHg effects in the optic tectum were only detected after 28 days of post-exposure (PE28), as pointed out by the significant decrease in the number of neurons in optic tectum layers L3, L4 and L5/6 (Table 7). Accumulation levels in the whole brain at PE28 (mean accumulation level of 1.47 μg g^{-1}) were similar to those recorded at E14 (mean accumulation level of 1.36 μg g^{-1}), suggesting a delay of iHg toxicity. Naïja et al. (2018) reported neurotoxic effects of $HgCl_2$ in the peacock blenny *Salaria pavo* when Hg accumulation levels were undetectable, claiming that this discrepancy could be due to delayed effects of iHg. This Hg form was previously related with the loss of brain cells in fish. Pereira et al. (2016) surveyed several brain areas (lateral pallium, medial pallium, hypothalamus, optic tectum, cerebellum) of *D. sargus* for changes in the number of cells (neurons and glial accessed indistinctly) upon iHg exposure. This Hg form elicited a decrease in the number of brain cells in the hypothalamus, cerebellum and optic tectum.

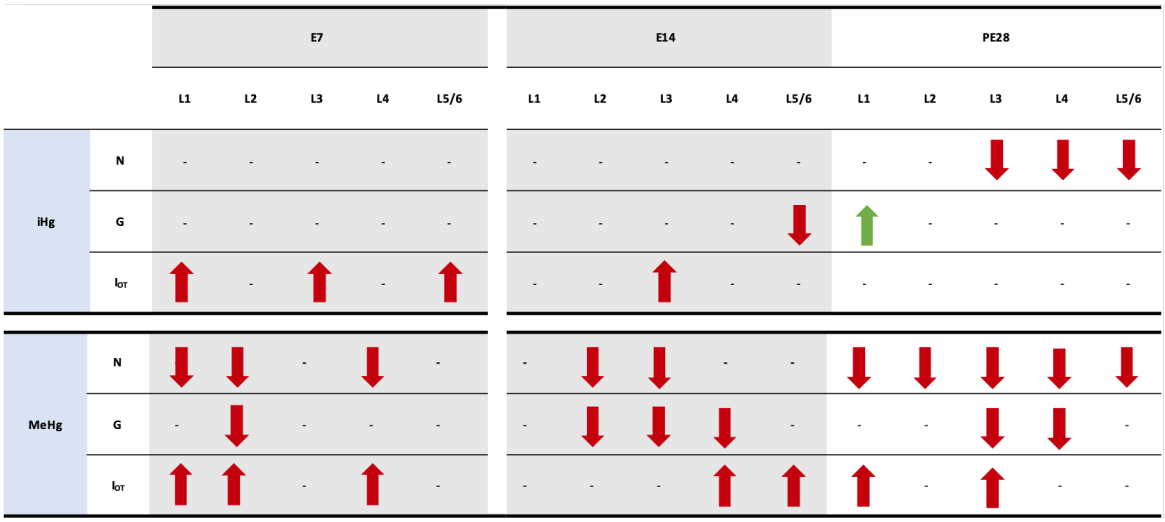
This latter region was particularly affected by iHg, since the decrease in the number of cells was observed at E7, E14 and PE28. These previous findings were a motivation for this dissertation research topic, since the neurotoxicity of specific Hg forms in fish is poorly understood and the available information is sometimes contradictory. Current data on the number of neurons in the optic tectum layers after fish exposure to iHg are in accordance with Pereira et al. (2015) findings for this region at PE28. The discrepancy between current data and that described in the previous article of the team (Pereira et al., 2015) where effects at E7 and E14 were reported could be related to differences on the methodological approach. While herein iHg toxicity was surveyed specifically in neurons and glial cells (assessed separately), in the previous work changes on the number of both types of cells was estimated (with no distinction between neurons and glial cells). At the light of the whole data together, more prevalent changes on the number of glial cells could be speculated on the previous work, in accordance with current findings.

The mechanisms of iHg neurotoxicity in fish remain elusive, but evidence in mammals suggest apoptosis as a key process that could lead to the loss of brain cells (Toimela and Tähti, 2004). Apoptosis is a highly conserved cell death process involved in tissue remodeling and degeneration in cells (Ceccatelli et al., 2010). Besides apoptosis, iHg can exert toxicity in neurons by leading to oxidative stress (Cardoso et al., 2015). Despite the activation of the antioxidant defense system, Cardoso et al. (2017) found an increase of lipid peroxidation and protein oxidation in the brain of fish exposed to iHg, pointing out oxidative stress as a possible mechanism of neurotoxicity. This process together with glutamate and calcium dyshomeostasis are claimed as important interplaying phenomena that mediate Hg toxicity, which can culminate in cell death (Farina et al., 2011). Moreover, the loss of neurons in the optic tectum of *D. sargus* can be also related with an inhibition of cell proliferation, as previously observed in the cerebellum and hippocampus of rats exposed to iHg (Burke et al., 2006).

Interestingly, iHg toxicity in neurons occurred only at the post-exposure period (PE28). At this time, accumulation levels remained similar to those detected at the end of the exposure period (E14). This finding suggests that the fish neurons were able to maintain the homeostasis for at least 14 days upon daily iHg exposure. Later than that period, the homeostatic mechanisms that were preventing iHg toxicity in neurons had probably decreased on efficiency, leading to a loss of neurons. An activation of antioxidant enzymes could have afforded protection to neurons against iHg toxicity during the exposure period, since oxidative stress is a chief event on Hg neurotoxicity (Roos et al., 2009). This hypothesis was not confirmed by a previous study of the research team, which comprised the exposure of fish to

iHg and the measurement of antioxidants and damage indicators in the brain. Results revealed an activation of antioxidant enzymes in the brain upon iHg exposure, which have not prevented the occurrence of damage on proteins (Cardoso et al., 2107). The proper maintenance of calcium homeostasis during the exposure period of *D. sargus* to iHg can be speculated, considered that a disruption of this process has been related to the loss of neuronal cells and neurotoxicity (Farina et al., 2011). Besides that, iHg can be complexed with Se leading to the formation of HgSe that is a non-toxic form. However, over time this complexation can cause a deficiency of essential Se-dependent enzymes, such as glutathione peroxidase. The loss of neurons at PE28 is in line with Pereira et al., (2016) that recorded a decrease of cells (neurons and glia cells) in the optic tectum of *D. sargus* after 28 days of post-exposure to iHg.

Table 7 – Synopsis of results obtained for morphometric and histopathological analysis after exposure to waterborne iHg and dietary MeHg. Decrease of neurons (N) and glia cells (G) are signalized by ↓ and the increase of these cells is signalized by ↑ while the increase of histopathological index (I_{LX})is signalized by ↑.



As stated, current data are in line with previous findings for the whole optic tectum of *D. sargus* in the post-exposure period to waterborne iHg. Besides that, results on the number of neurons clearly demonstrate that the outer layers of the optic tectum (L3, L4, L5/6) are the most vulnerable to iHg toxicity (Table 7). These layers are highly vascularized (Northmore, 2011) and as iHg enters the brain via bloodstream it can be distributed and deposited more effectively in the most irrigated layers, which can explain our findings in layers L3, L4 and L5/6. iHg effects were almost inexistent during the exposure period (E7 and E14), occurring mainly in the post-exposure time (PE28), suggesting a low plausibility of the previous hypothesis. Moreover, the number of myelinated fibers is high in L3 and L4 (Northmore, 2011). Myelin sheath can be destabilized due to the high affinity of iHg for proteins, which could

hypothetically lead to a neuronal loss, as previously claimed by Vieira et al. (2017). Interestingly, the layers affected by the loss of neurons receive inputs of visual information (L4) and are related to fish behaviour (L3 and L5/6). Thus, the late loss of neurons probably associated with iHg deposition in these layers can hypothetically alter the performance of *D. sargus* in searching for food as well as when running away from the predators (Northmore, 2011). In agreement, Pereira et al. (2016) recorded alterations of *D. sargus* behaviour when fish were exposed to iHg, pointing out that this Hg form alters motor behavior and change the fish mood, potentially increasing its anxiety status.

Consistently with findings in the optic tectum neurons during the exposure period, adverse effects of iHg in glial cells were only occasional. A lower number of glial cells was found in L5/6 layer at E14, pointing out to a low toxicity of iHg to glial cells. Despite Hg forms accumulate preferentially in glial cells (mainly astrocytes and microglia) (Cambier et al., 2012; Aschner et al., 2007; Allen et al., 2002), these cells are not the principal neuronal cells affected by Hg (Charleston et al., 1994). Accordingly, Lohren et al. (2015) documented a major susceptibility of Hg to induce cytotoxicity in human neurons rather than in astrocytes. Interestingly, even though in mammals the proportion of glial cells is higher than neurons (Giaume et al., 2007), the contrary seems to occur in fish. Actually, Hassan et al (2016) refer neurons as the majority of brain cells in zebrafish embryos. Also, Teixeira et al. (2018) documented, in rats, a higher toxicity of iHg neurons than glia cells. Moreover, a higher number of glial cells was recorded at PE28 in optic tectum L1 layer of exposed *D. sargus*, suggesting the occurrence of cell proliferation. This finding consubstantiates the lower toxicity of iHg to *D. sargus* glial cells. Brain inflammatory processes, including the occurrence of glial cell proliferation and hypertrophy, have also been reported in the cerebral cortex of mammals after MeHgCl exposure (Monnet-Tschudi et al., 2006).

A different picture was found in the optic tectum after dietary exposure of fish to MeHg. This Hg form caused loss of neurons in the optic tectum after 7 days of exposure with L1, L2 and L4 layers presenting a significant decrease in the number of neurons (Table 7). These findings are in line with a previous study of the dissertation supervision team on the effects of MeHg in the morphometry of fish brain (Puga et al., 2016), where several brain regions were surveyed for the loss of cells (neurons plus glial cells indistinctly) and a lower number of cells was recorded in the medial pallium and optic tectum after 7 days of exposure. As previously hypothesized for iHg exposure, MeHg could also have triggered neuronal toxicity leading to neurons loss by enhancing apoptosis, autophagy, and oxidative stress and/or by inhibiting neurons proliferation. All these phenomena have been previously reported in MeHg neurotoxicity studies. For instance, Berntssen et al. (2003) studied the lesions in different brain

areas of *Salmo salar* including optic tectum, medulla oblongata, and cerebellum, after a four-month dietary MeHg exposure (5 and 10 mg Hg kg^{-1} dw). Optic tectum showed diffuse necrosis that resulted in the loss of pyramidal and Purkinje cells (Berntssen et al., 2003). Hassan et al. (2016) documented morphological patterns of cell death in neurons of zebrafish exposed to MeHg, while Monnet-Tschudi et al. (1996) reported that MeHgCl can specifically induce neuronal apoptosis in rats. Aside from apoptosis, it was also documented that autophagy was triggered in human neural stem cells (Chang et al., 2013) and in rat primary astrocytes (Yuntao et al., 2014) after MeHg exposure.

The levels of MeHg in *D. sargus* whole brain increased two-fold from E7 to E14, which was accompanied by a decrease in the number of neurons in L2 and L3 layers of the optic tectum. Similar mechanisms of MeHg toxicity were hypothesized to explain the decrease on the number of neurons at E7 could be operating at E14. By the contrast, the accumulated levels of MeHg decreased significantly from the end of the exposure period (mean accumulation level of 6.97 μg g^{-1}) to the post-exposure time (mean accumulation level of 3.51 μg g^{-1}), although this decrease was not accompanied by an attenuation of MeHg neurotoxic effects. In fact, a significant decrease on the number of neurons was observed in all the optic tectum layers at PE28, suggesting a more severe toxicity of MeHg at this time point. Puga et al. (2016) results correlate with our data as the optic tectum of *D. sargus* also displayed a decrease in the number of cells (neurons and glia cells) after 28 days of depuration, although accumulation levels in the brain had decreased from the end of the exposure period to this time point.

MeHg exposure elicited a decrease on the number of glial cells at both exposure periods, specifically in L2 at E7 and in L2, L3 and L4 at E14. These changes are in line with those recorded in neurons for layers L2 at E7 and L2 and L3 at E14, suggesting MeHg exposure caused loss of both cell types. The effects of MeHg on glial cells are also observed after exposure, since a lower number of cells was found in L3 and L4 at PE28. Curiously, L5/6 was the only layer that did not recorded a decrease of neurons or glia cells during the exposure period (both at E7 and E14). This result could be related to the composition of this layer which has a high number of unmyelinated fibers (Northmore, 2011). As previously mentioned, myelinated fibers may be related to a higher vulnerability of the layers to Hg forms.

Overall, current data suggest a higher toxicity of MeHg to both neurons and glial cells of the optic tectum when compared to iHg (Table 7). Thus, while iHg induced decreases in the number of neurons (although more accentuated at PE28) and almost no changes on the glial cell numbers, MeHg exposure triggered a significant decrease on the number of both neurons and glial cells in several layers of the optic tectum (with particular severity at PE28 also).

4.1.1. Comparative vulnerability of neurons and glial cells to Hg forms

Based on the current results, iHg seems to target preferentially neurons rather than the glial cells. In fact, only occasionally iHg exposure triggered a decrease on the number of glial cells (L5/6 at E14), suggesting a lower toxicity of this Hg form to these brain cells. The toxicity mechanisms of iHg to fish neuronal cells remains elusive, while it is well established that this Hg form is not evenly distributed in mammals' brain but it is preferentially deposited in certain cell types. Charleston et al. (1994) estimated the number of neurons, astrocytes, reactive glia, oligodendrocytes, endothelia, and pericytes in the cortex of the calcarine sulcus of adult female *Macaca fascicularis* following a long-term subclinical exposure to iHg. Monkeys were administered with iHg (as $HgCl_2$; 200 μg Hg kg^{-1} body wt/day) by constant rate intravenous infusion for 3 months. An increase of 165% in the number of reactive glia was observed in the exposed group, suggesting that iHg may be responsible for the increase of these cells. This finding pointed out the reactive glial cells (most likely a form of microglia) as the most sensitive cell type (in terms of changes in its population) to iHg exposure (Charleston et al., 1994). The meaning of a large increase of activated microglia was not known at the time, but it was hypothesized that astrocytes and reactive glia were probably sequestering Hg to protect neurons and other cells of the central nervous system. These findings were opposed to those of the current dissertation. Herein, iHg seems to target preferentially neurons rather than the glial cells, as previously stated. Accordingly, diverse studies have been considering neurons to be more sensitive to Hg forms than glia cells, as occurred in *D. sargus* optic tectum. Teixeira et al. (2018) reported a higher propensity of rat neurons to death than glia after iHg exposure. Moreover, after exposing human neurons and astrocytes to iHg, Lohren et al. (2015) concluded a major susceptibility of neurons to iHg cytotoxicity than astrocytes. While the most prominent changes observed in *D. sargus* optic tectum were the decrease of neurons numbers, a significant increase of glial cells was also found in L1 of the optic tectum in the post-exposure time. As mentioned before, this increase of glial cells suggests cell proliferation pointing out a higher plasticity of glial cells upon iHg exposure. The interpretation of current results on the glial cells is difficult to be done, since the assessed cells correspond to multiple glial cell types such as astroglia, oligodendrocytes and microglia. For this reason, even though these cells are all glia cells, it is not possible to compare between them since they have so many distinct functions that would be possible each cell type having different responses to Hg exposure.

Similarly, MeHg toxicity seemed to be higher to neurons than glial cells of the optic tectum of *D. sargus*, namely at E7 and PE28, following the pattern recorded for iHg. In fact, after 7 days of exposure to MeHg layers L1, L2 and L4 exhibited a significant lower number of

neurons, while a decrease of glial cells number was only recorded in L2. Moreover, MeHg decreased neurons number in all layers at PE28, while changes on glial cells density was only recorded in L3 and L4. The study of Charleston et al. (1994) also evaluated changes on the number of neuronal cells in monkeys after a long-term exposure to MeHg, reporting a significant increase of reactive glia in exposed monkeys, as described for iHg. This vulnerability of neurons when compared to glia cells is corroborated with previous studies. Ni et al. (2012) also refers astrocytes and microglia as the cells with higher MeHg levels, while neurons show significantly lower MeHg concentration. Besides that, other cells such as oligodendrocytes and endothelial cells were rarely observed with MeHg (Ni et al., 2012). Lohren and colleagues (2015) recorded a higher propensity of human neurons to die after MeHg exposure than human astrocytes. Moreover, Cambier et al. (2012) reported that even though the highest MeHg accumulation was detected in astrocytes of rats, cell death occurs mainly in neurons after MeHg exposure. Moreover, Monnet-Tschudi et al. (1996) refers that even though MeHg target glial cells, neurons are the first cells to be irreversibly affected by this Hg form. MeHg cause damage in the other cell types, however with low severity compared to neurons (Monnet-Tschudi et al., 1996).

4.2. iHg- and MeHg-induced histopathological alterations in the optic tectum

Exposure of *D. sargus* to waterborne iHg induced significant histopathological alterations in the optic tectum just after 7 days of exposure. The most notorious alterations were spongiosis and necrose that were detected in all of the five surveyed layers of the optic tectum of exposed fish (although significant statistical results were scarce). Spongiosis is an intracellular edema (abnormal accumulation of fluid) that signals an almost inevitably cell death (Naïja et al., 2018). Despite these alterations, the morphometric analysis did not record a decrease in the number of neurons or glia cells in the exposure periods (E7 and E14). This may be related to the loss of optic tectum volume or the occurrence of cell proliferation, which would replace the loss of cells. Previous studies also found spongiosis as the main histopathological alteration in the brain of peacock blennies after exposure to iHg (Naïja et al., 2018).

Additionally, necrosis is a morphological state of the cell that appears after the loss of cell function (Bernet et al., 1999). This lesion is irreversible and can lead to a partial or total loss of the organ function (Bernet et al., 1999). Our results are in line with findings of other studies, such as Berntssen et al. (2003), which recorded necrosis in the Atlantic salmon brain

after dietary exposure to iHg. Besides that, layer L5/6 also presented other significant alterations such as blood vessel abnormality, detachment of layers and architectural and structural alterations. Structural disturbances such as detachment of layers and architectural/structural alterations can imply alterations on the behaviour of fish, leading to an altered motor control, as suggested by Berntessen et al. (2003) for the Atlantic salmon upon iHg exposure. In addition, BBB is formed by endothelial cells surrounding capillaries so the occurrence of blood vessel abnormalities can possibly affect the efficiency of this barrier (Risk, 1992). Although there are not many studies investigating the neurotoxicity of iHg in fish by addressing brain histopathology, Naïja et al. (2018) documented the presence of blood vessel abnormalities, necrosis and spongiosis in the optic tectum of peacock blennies. Our results are also in agreement with those of Ranjan et al. (2015) which showed that iHg can induce spongiosis and necrosis in neuronal cells of the rat's cerebellum. Similar pathologies were currently recorded in the optic tectum of exposed *D. sargus* after 14 days of exposure, although the occurrence of vacuolation at L3, as well as dilatation at L4 are notable. The occurrence of vacuolization can affect the good functioning of mitochondria as well as disrupt motor coordination of organisms (Naïja et al., 2018). Naïja and colleagues (2018) also reported vacuolation, hypertrophy and dilatation of blood vessels in fish optic tectum, in line with our data. In the post-exposure period (PE28), the optic tectum continued altered by several pathologies, with necrosis being once again the most prominent lesion.

After 7 days of dietary exposure, MeHg elicited significant histopathological changes in the optic tectum being spongiosis and necrosis the most predominant alterations occurring in all surveyed layers. As stated above, these alterations denote cell death (necrosis) or can imply cell death (spongiosis) and further loss of organ function which, in case of damage in the optic tectum, can lead to a loss of responsiveness to visual stimuli (Northmore, 2011). Accordingly, Berntssen et al. (2003) documented cell swelling in the brain of Atlantic salmon exposed to MeHg, while Fujimura et al. (2009) documented necrosis in mice exposed to MeHg. Moreover, congestion and architectural/structural alterations were also observed in almost every layer of the optic tectum. The congestion of blood vessels can result in haemorrhage due to the increase blood pressure in consequence of toxicants exposure (Javed et al., 2015), while alterations on the structure of optic tectum can lead to modifications on fish behaviour as previously claimed for iHg and in accordance with Northmore (2011). A similar pattern was observed at E14 and PE28, with a higher incidence of spongiosis in every layer of optic tectum in these time points. At PE28, vacuolation was also elevated at L4, eventually with negative implications on mitochondria functioning and then on motor coordination of fish, as proposed

by Naïja et al. (2018). Also, Berntssen et al. (2003) recorded severe vacuolization in brain of fish exposed to dietary MeHg.

The histopathological index that computes all the recorded alterations to provide an overview picture of the iHg toxicity in optic tectum layers revealed significant alterations at layers L1, L3 and L5/6 after 7 days of exposure (Figure 14). According to this index, the prevalence of histopathological effects seemed to attenuate along the experiment as at E14 significant changes were only recorded in L3 while at PE28 no changes were found. These results agree with Naïja et al. (2018) that showed a higher histopathological index after 4 days of exposure to iHg than later after 10 and 15 days. In general, layer L1 showed a higher index than the rest of the layers, reaching a value within class 3, denotating moderate modifications of optic tectum layer structure and morphology. Differently, the index of the other layers is within class 2, pointing out slight modifications only. The particular susceptibility of L1 layer can be related with its higher cell density in comparison with the other surveyed layers. The regressive alterations contributed mostly to the estimated total histopathological index at all time points of the iHg experiment. The implications of regressive alterations on the organ function are the functional reduction or loss of the organ. Despite moderate modifications (Class 3) of the optic tectum architecture and morphology, *D. sargus* exposed to waterborne iHg appears to activate defense mechanisms to recover from the damage while this do not seem to occur in fish exposed to dietary MeHg (Naïja et al., 2018). Interestingly, during the exposure to iHg (E7 and E14) histopathological alterations were more frequent than morphometric alterations while in the post-exposure (PE28) the reverse occurred. These results denote the complexity of the toxicologic processes involved and points out to the need of an integrated approach at the cellular and supracellular levels to effectively understand the neurotoxicity of iHg in fish.

Histopathological alterations in the optic tectum of fish exposed to MeHg revealed changes at L1, L2 and L4 in the histopathological index after 7 days of exposure. Regressive alterations are the changes that influence the histopathological index the most; however, in layer L5/6 circulatory changes seem to be the most dominant. While the implications of regressive alterations were previously mentioned, circulatory changes are related to inflammatory processes in result of a pathological condition of blood and tissue fluid flow. After 14 days of MeHg exposure, significant alterations of the histopathological index were detected at L4 and L5/6, while at PE28 differences were found at L1 and L3. These findings point out to a higher prevalence of alterations in the optic tectum of fish exposed to MeHg than to iHg since for the latter effects were attenuated over time.

Interestingly, in both iHg and MeHg exposure L1 was the most affected layer presenting a higher I_{Lx} than all the other layers (Figure 14). The histopathological index reached Class 3 while the great majority of the time points and layers the index fall within Class 2 showing slight modifications of the optic tectum morphology and architecture. In addition, these results do not correlate with morphometric analysis where intermediate layers were the most affected by the loss of neurons and glia cells.

4.3. Contributions to the knowledge of Hg neurotoxicity in fish

Still, there are knowledge gaps on the mechanisms of iHg and MeHg neurotoxicity in fish, and occasionally some of the existing findings point out to divergent conclusions. A main aspect is related to the Hg form that is more neurotoxic to fish. Most of the studies indicate MeHg as the main toxic form in fish brain, related to the higher liposolubility that facilitates its mobility across cells, including those of the BBB. Besides that, most of the studies performed in fish under this topic had investigated the neurotoxicity of MeHg rather than iHg. This is probably because the human neurotoxicology research on Hg had paved the way to the studies implemented in fish. Despite that, it is now known that both Hg forms can trigger a wide range of neurotoxicological effects in fish, namely from brain biochemical and physiological changes to morphological alterations and behavioral impairments (Table 1). Under this context, this dissertation investigated the effects for the first time to the of iHg and MeHg in *D. sargus* optic tectum, which was selected based on previous findings that revealed this region as particularly vulnerable to Hg exposure (Pereira et al., 2016; Puga et al., 2016). Current findings on the optic tectum morphometry and histopathological alterations supports a higher toxicity of MeHg than iHg (Table 7).

In general, iHg had not triggered the loss of neurons or glial cells in the optic tectum during the exposure period. On the contrary, a decrease in the number of neurons and glial cells was found in the optic tectum of fish exposed to MeHg at E7 and E14. Additionally, in the post-exposure period of 28 days, the effects of MeHg were more pronounced than those prompted by iHg. Results of the histopathology index corroborate this tendency, pointing out a higher toxicity of MeHg in the optic tectum layers, particularly in the post-exposure period when no significant alteration was found for iHg. Beyond that, the different profiles of neurotoxicity found for iHg and MeHg could be related to distinct patterns on toxicokinetics and toxicodynamics of both Hg forms and, subsequently lead to different effects in the temporal patterns.

Besides that, iHg and MeHg seemed to target preferentially neurons than glial cells of the optic tectum. For instance, a decrease of the glial cells number occurred occasionally only after iHg exposure (L5/6), while a decrease on the number of neurons was found at L3, L4 and L5/6 at PE28. Moreover, MeHg exposure was the basis of a decrease on the number of neurons in all layers at PE28, while for glial cells this tendency only occurred at L3 and L4. Based on these findings, it is recommended an evaluation of the number of neurons in the optic tectum of fish exposed to Hg forms, as this endpoint seems to be more responsive and informative about the toxicity of iHg and MeHg (particularly in short-term exposures). This recommendation is particularly relevant considering that a morphometric analysis of the optic tectum layers by stereological methods is a time-consuming approach.

The morphometric analyses and histopathology evaluation was performed per optic tectum layer (L1-L5/6), considering that the toxicity of Hg forms could vary between layers due to their different composition and distribution in the organ. Despite that, it was difficult to identify the most vulnerable layers to iHg and MeHg exposure, even if the current data suggest that intermediate layers were the most affected by Hg forms in the morphometric analysis. In this analysis, L3 appear to be most affected by waterborne iHg, while layers L2, L3 and L4 seem to be the most affected by dietary MeHg. This does not correlate with the histopathological index results where L1 was the most affected layer showing moderate modifications of the optic tectum morphology.

Despite the lower toxicity of iHg in the optic tectum of *D. sargus* in comparison to MeHg, current results pinpointed the relevance of considering, in future field and laboratory studies, iHg as a potential neurotoxicant in fish. For instance, when considering the iHg-induced histopathological alterations, the occurrence of necrosis in all optic tectum layers right after 7 days of exposure was observed. This cellular pathology may imply the partial or total loss of the organ function, highlighting a high toxicity of iHg in such a short time of exposure. This finding supports the previous recommendation of including iHg in future studies regarding the neurotoxicity of Hg in fish.

5. Conclusions and Future Research

According to the present results, it is possible to conclude that:

1. It was difficult to perceive if different Hg forms target specific layers of the optic tectum. However, intermediate layers seem to be more prone to the loss of cells with L3, L4 and L5/6 being the most affected after MeHg exposure and L3 and L5/6 after iHg exposure. However, these results do not correlate with the histopathological index where L1 was the layer revealing higher damage.
2. It was evident that Hg forms were particularly detrimental to neurons than glial cells. Even though iHg triggered significantly fewer losses of neurons and glial cells than MeHg, it is possible to conclude that both Hg forms increased neuronal death.
3. Neurotoxic effects of iHg and MeHg were recorded even after a post-exposure period of 28 days. This finding suggests long-term effects of Hg forms in fish, pointing out the need to follow the recovery of a Hg-contaminated system for several months after the inactivation of sources, particularly in what concerns the fish health.
4. Both iHg and MeHg are relevant neurotoxicants to fish optic tectum. MeHg demonstrated a higher neurotoxicity than iHg in the optic tectum of *D. sargus.* The morphometric analysis of the optic tectum pointed out a higher loss of neuronal cells upon MeHg exposure (particularly of neurons), an effect further supported by the histopathological analyses which also suggested a higher toxicity of MeHg to the optic tectum since the alterations were observed during the experimental time.

This dissertation contributed to advance knowledge on the Hg neurotoxicity in fish, namely by uncovering the optic tectum vulnerability to dietary MeHg and waterborne iHg. Despite that, the observed effects haven't followed the toxicokinetics of Hg forms based on accumulation levels detected in the whole brain. A mapping of iHg and MeHg would be more informative as it could pinpoint the optic tectum as a preferential area of iHg and MeHg accumulation, while it would elucidate if Hg deposition change with the layer. This was an initial aim of the dissertation planned to be achieved in collaboration with the Canadian Light Centre, which was postponed due to the pandemic situation. Moreover, further studies on this research line should comprise immunohistochemical analysis to reinforce our results. Specifically, different markers could be used to identify the different neuronal cells targeted by Hg forms, namely GFAP (marker of astrocytes) and Iba1 (marker of microglia).

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