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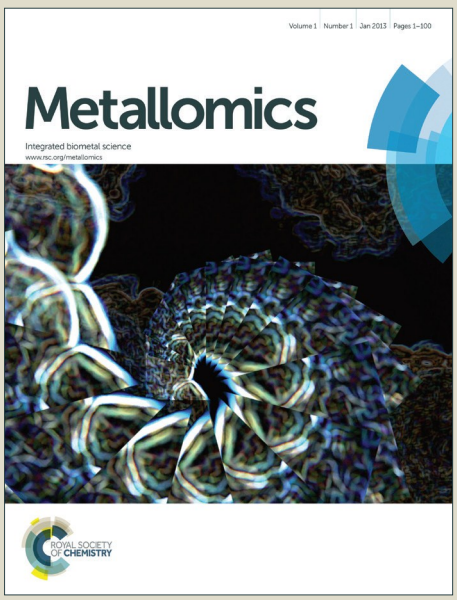

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A new page on the road book of inorganic mercury in fish body - tissue distribution and elimination following waterborne exposure and post-exposure periods

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Abstract

There are several aspects of inorganic mercury (iHg) toxicokinetics in fish that remain undeveloped despite its environmental ubiquity, bioaccumulation capacity and toxicity. Thus, this study presents the uptake, distribution and accumulation of iHg following water contamination by adopting a novel set of body compartments (gills, eye wall, lens, blood, liver, brain and bile) of the white seabream (*Diplodus sargus*) along 14 days of exposure. Realistic levels of iHg in water (2 μg L^{-1}) were adopted in order to engender reliable conclusions to fish health assessment. A depuration phase of 28 days was also considered with the purpose of clarifying iHg elimination. iHg was faster accumulated by gills (within 1 day) which also presented the highest accumulated levels among the target tissues/organs. Moreover, iHg increased gradually during the exposure time in all tissues/organs, except lens that showed relatively unaltered levels throughout the experiment. After 14 days of exposure, lower values of Hg were recorded in brain/eye wall comparatively with liver, probably related with the presence of blood-organ protection barriers which limit iHg influx. Even though, iHg reached brain earlier than eye wall (3 and 7 days, respectively) and higher accumulated levels were recorded in the former. A depuration period of 28 days did not allow the total elimination of iHg in any tissue/organ. Despite that, iHg was substantially eliminated in the gills, blood and liver through two temporal phases, while brain and eye wall were not able to eliminate iHg within this time-frame. Brain and eye wall are more “refractory” structures in what concerns iHg elimination and this could represent a risk for wild fish populations.

Keywords: Inorganic mercury; Accumulation dynamics; Tissue distribution; Elimination; Fish

1. INTRODUCTION

Mercury (Hg) compounds have triggered major concerns in terms of environmental and human health. This trace element is present in aquatic environment in organic (mainly methylmercury - MeHg) and inorganic forms (iHg), and both can be bioaccumulated by fish inducing toxic effects. There are several studies addressing Hg kinetics in fish body [e.g. 1; 2; 3; 4]. Most of them are focused in the widely explored tissues, such as liver, kidney, gills and muscle, while other potential target organs (e.g. brain and neurosensory structures) have been neglected. The lack of effective efforts on searching for Hg accumulation and pathways in fish brain and eyes is an intriguing aspect in ecotoxicology, when considering their crucial roles in fish fitness and survival. Both brain and eyes are protected by epithelial barriers (the blood-brain barrier [BBB] and the blood-retinal barrier [BRB]) that strictly regulate the selective transport of molecules from the bloodstream [5]. BBB and BRB are highly restrictive membranes but both can be crossed by essential elements like Mn and Fe [6]. In what concerns to Hg in fish, it is well established that it may also reach brain and eyes [5; 7]. However, the permeability of these barriers to Hg needs to be clarified in fish, particularly if it could be crossed bi-directionally, as well as the extent of Hg influx and efflux. The balance (or unbalance) between efflux and influx will lead to an inevitable accumulation, as reported for Fe in rodents brain [8]. Furthermore, fish lens has no direct blood supply but accumulates high levels of Hg both under field and laboratory exposures [5; 7]. A toxicokinetics trial that considers this eye component would elucidate about its high accumulation capacity. The presence of protective barriers in brain and eyes is a distinctive aspect from internal organs, such as liver, that would certainly have implications on Hg fate along time.

The evaluation of Hg toxicokinetics in post-exposure periods is still poorly documented. The presence of BBB and BRB would probably limit the elimination of Hg from the brain and eyes, respectively. This was reported for Fe in rodents' brain [8] but no information is available for fish brain or eyes regarding Hg. Again, both barriers would probably lead to distinct elimination patterns between barrier protected and non-protected organs (e.g. liver) but this hypothesis needs elucidation. Additionally, fish in their natural environment could easily move among areas with different contamination profiles, making critical the assessment of Hg fate in

their body after cessation of exposure. Thus, the follow up of Hg levels in fish key organs during a depuration period will also provide relevant indications for environmental health assessment.

Several advances were made elucidating bioaccumulation of Hg in fish, both from food and water sources [9; 10; 11; 12]. MeHg has been preferentially addressed in relation to iHg, probably based on the presumption of its higher toxicity related with the rapid uptake and distribution. However, it was stated that different forms of mercury share the same toxic chemical entity and that toxicity depends mainly on differential bioavailability [13]. It was also reported that iHg can display stronger acute effects on fish than organic forms [1]. iHg revealed to be more potent than MeHg in inhibiting glutamine synthetase activity in fish cortical astrocytes [14]. iHg compounds such as mercuric chloride can also act as a direct BBB toxicant increasing thus its permeability in rodents [15]. Moreover, iHg can occur as a product of MeHg demethylation in the intestine and in brain [16], pointing out the relevance of investigating the toxicokinetics of iHg forms. The importance of such knowledge is consubstantiate by the fact that the majority of Hg in natural waters occurs in inorganic forms, while MeHg often contributes to less than 5% of the total Hg in water [17].

The published data concerning the accumulation of iHg in key tissues/organs of fish following waterborne exposure are still insufficient to understand its toxicokinetics. Particularly, there is a lack of information on iHg disposition during a post-exposure period and elimination pathways. Fish neurosensory structures, such as the eyes, need to be considered in order to mitigate the lack of scientific knowledge on their role in iHg uptake and accumulation. Hence, this study presents the uptake, distribution and accumulation of iHg adopting a novel combination of tissues/organs (gills, eye wall, lens, blood, liver, brain) of the white seabream (*Diplodus sargus*) during 14 days of exposure. Afterward, a depuration period of 28 days was considered in order to evaluate the elimination of iHg in those tissues/organs, as well as bile's role in that process. Mercury enrichment factors were calculated to evaluate tissue/organ specific affinity for iHg. Moreover, the rate of Hg elimination was estimated in order to clarify the recovery of each tissue/organ. Fish were exposed to realistic waterborne Hg concentrations in order to produce reliable data to environmental health assessment.

2. Experimental

2.1. Experimental set-up and tissues/organs sampling

Juvenile white seabreams (*Diplodus sargus*) provided by an Aquaculture Research Station (IPMA - Olhão, Portugal), from the same cohort (weight: 146 ± 14 g; total length: 19 ± 1 cm), were used in the experiment. Fish were held in 300 L fibreglass tanks in an average initial density of 0.012 kg L^{-1}, under a 10:14 light:dark photoperiod. Seawater was renewed daily (around 80%) and fish were fed once a day with a commercial dry food [standard 3 mm from Sorgal (Portugal)], 1-2 hours before water renewal. Total Hg levels in food pellets were lower than 0.01 μg g^{-1}. In the sampling days, fish were not fed in the 12 hours preceding fish handling. Water temperature, salinity and pH were monitored daily throughout the experiment, varying as follows, respectively: 13.5 ± 0.3 ºC, 35 ± 2 and 7-8.

Prior to Hg exposure, fish were allowed to acclimatize to experimental conditions and routines for two weeks. Eight fish were sacrificed at the beginning of the experiment and used as the initial reference group (time zero; T0) (Fig. 1).

In exposure tanks, $HgCl_2$ (Sigma Aldrich) was added to the water in an aqueous solution in order to reach a final concentration of 2 μg L^{-1}. Mercury chloride was added on a daily basis after water renewal (i.e. daily water recontamination) during the exposure period. This iHg level was established considering previous studies in contaminated areas [7; 18] in order to mimic environmentally realistic conditions. Control fish were kept throughout the experiment in tanks filled with clean seawater. Fish wellbeing deserved a permanent attention, in accordance with national and international guidelines for the protection of animal welfare.

Fish were exposed to $HgCl_2$ for 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days. Thereafter, fish were transferred to clean water (post-exposure) and allowed to recover for 14 (PE14) and 28 days (PE28) (Fig. 1). In each sampling time, 8 fish were sampled per condition (n=8). The experiment had a total duration of 42 days. Immediately after collection, fish were anesthetized, weighed, measured, and sacrificed by cervical transection. Blood was collected with heparinised Pasteur pipettes from the cardinal vein, and gills, eyes, liver, brain and bile were removed. Gills were carefully washed with distilled water and filaments carefully separated. Eyes were dissected for isolation of lens and the remaining components hereafter collectively called “eye wall” to simplify, encompassing eye wall (retina, sclera, cornea, ciliar body, etc.),

chambers' content (vitreous and aqueous humours), and other small structures [7]. All biological samples were stored at -80 ºC until further processing for Hg determinations.

During the exposure period (at days 1, 3, 7 and 14), water samples were collected in triplicates from exposure and control tanks 24 hours after recontamination to quantify total Hg (tHg) levels, in order to prove that fish were subjected 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 concentration. Levels of tHg in the control tanks were below the detection limit throughout the experiment (0.1 ng L^{-1}). Identically, at days 28 and 42 (post-exposure period), both in control and in previously contaminated tanks, tHg was below the analytical detection limit.

2.2. Analytical procedures

Total dissolved mercury was determined following U.S.EPA method 1631 [19]. Briefly, water samples were preserved by the addition of 0.5% BrCl until analyses (less than one week after collection). The samples were then analyzed by cold-vapour atomic fluorescence spectrometry (CV-AFS) with a PSA model Merlin 10.023 equipped with a detector PSA model 10.003 using $SnCl_2$ reduction. BCR-579 reference material was used to control the accuracy of the procedure and the obtained values were consistent with the certified ones.

Gills, eye wall, lens, blood, liver, brain and bile samples were firstly lyophilised and homogenised. Samples were then analysed for tHg by atomic absorption spectrometry (AAS) with thermal decomposition followed by gold amalgamation, using a mercury analyser (AMA) LECO 254 [20]. Certified reference materials (DORM-3, DOLT-4) were used to ensure the accuracy of the procedures and the obtained values were consistent with the certified ones.

In the current work, tHg levels in biological samples allowed interpretations on iHg toxicokinetics based on the assumptions that fish were exposed to iHg and that no methylation was so far reported to occur in fish.

2.3. Data analysis

Statistical software (Statistica 6.0) was used for statistical analyses. All data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to meet

statistical demands. One-way analysis of variance (ANOVA) was performed to compare tHg levels in control and exposed fish for each experimental time. The comparison was performed for the six analysed tissues/organs (gills, eye wall, lens, blood, liver, brain) and the bile. One-way ANOVA was also used to compare experimental times for tHg levels accumulated in control and exposed fish. The Tukey test was applied for post-hoc comparison. Differences between means were considered significant when $p<0.05$.

The quotient of tHg levels (mean values) in exposed and control fish were calculated for each experimental time and for all the analysed biological matrices. That quotient corresponds to the Hg enrichment factor.

The Spearman analysis was used to test the significance of correlations between all the analysed biological matrices for Hg levels. The significance of correlations between Hg levels (in the exposed fish) and time (in days) during the exposure period was also tested by the Spearman analysis. Correlations were considered significant for $p<0.05$.

A crude estimation of the rate of tHg elimination per day (k) was made for data obtained in the post-exposure period, as following: $([Hg]_{day14} - [Hg]_{day28\ or\ 42})$/ number of days (14 or 28 days). The elimination rate (k) was expressed as µg of Hg per g of tissue per day.

3. RESULTS

No fish mortality was observed during the experiment. Though feeding was not strictly monitored, no alterations were perceptible during and after treatment on fish feeding behaviour.

3.1. Mercury levels in fish tissues/organs and bile

Figure 2 presents the variation of total Hg (tHg) in gills, eye wall, lens, blood, liver, brain and bile of white seabream exposed to inorganic Hg (iHg) (2 µg L^{-1}), as well as in control fish. In gills, tHg levels differed significantly between control and exposed fish after the first day of exposure. Hence, tHg levels in gills increased gradually and reached a maximum at E14, which presented significantly higher values than those recorded at days 1, 3 and 7. Moreover, tHg levels at E7 were significantly higher than values at E1. In the post-exposure period (PE14 and PE28), tHg levels in gills decreased significantly in relation to E14 but remained above values in control.

A similar variation pattern was found for blood, liver and brain in the exposure and post-exposure periods, being characterized by significant differences between control and exposed fish at conditions E3, E7, E14, PE14 and PE28 (Fig. 2). As observed for gills, tHg levels in blood, liver and brain reached a maximum at day 14. Concentrations of tHg in blood and liver decreased significantly in the post-exposure period (PE14 and PE28) but remained always above the control values. Contrarily, tHg levels in the brain were identical in conditions E14, PE14 and PE28. Similarly, tHg levels in eye wall did not decrease significantly in the post-exposure period in relation to the last day of exposure (E14). During the exposure period, significant differences were found between tHg levels in eye wall of control and exposed fish at E7 and E14. Maximum levels of tHg in eye wall were recorded at E14, as described for the remaining tissues. No statistical differences were found between tHg levels in control and exposed fish for lens, with the sole exception of E14. Moreover, tHg levels in lens of exposed fish did not vary significantly over the experiment. Regarding the bile, tHg levels differed significantly between control and exposed fish at E14, PE14 and PE28 while values did not decrease significantly in the post-exposure period in relation to E14.

With the exception of lens, tHg levels in the post-exposure period never reached the levels found in control fish (Fig. 2). In general, no temporal variations were found for tHg levels in control fish.

The highest tHg levels accumulated throughout the experiment in exposed fish were observed in gills (8.1 μg g^{-1}), followed by liver (2.5 μg g^{-1}), blood (1.8 μg g^{-1}) and brain (1.5 μg g^{-1}), and then by lens (1.0 μg g^{-1}), bile (0.76 μg g^{-1}) and eye wall (0.34 μg g^{-1}) (Fig. 2).

3.2. Enrichment factors of mercury in fish tissues/organs

The enrichment factors of tHg at each sampling time varied between the analysed tissues as well as over time (Fig. 3). Gills exhibited the highest enrichment factors during the exposure period (1-14 days) (6-49), followed by blood (1-16), liver (1-15), brain (1-3) and eye wall (1-2). In general, enrichment factors increased along the exposure period (reaching maximum values at day 14), being followed by a decrease in the post-exposure phase. Gills, blood and liver showed an accentuated decrease of enrichment factors between E14 and PE14, as following described, respectively: from 49 to 16; from 16 to 6; from 15 to 11. Enrichment

factors of gills and blood continued to drop down between PE14 and PE42 (from 16 to 9 and from 6 to 3, respectively), while values in liver remained identical in both post-exposure times. A different temporal pattern was recorded for brain and eye wall in comparison with the previous biological matrices since enrichment factors were the same in E14, PE14 and PE28 conditions (always 3 for brain and 2 for eye wall). No substantial time-related changes were observed for lens during both experimental periods, being enrichment factors always around 1.

3.3. Relationships between mercury levels and exposure time

In the exposed fish, tHg levels in all biological matrices (except lens) increased linearly with exposure time (Fig. 4). The slope of the relationship between tHg and time in gills (0.535) was around three- and five-fold higher than those of the liver (0.174) and blood (0.117), respectively. The slopes of the relations of tHg in brain (0.073), bile (0.033) and eye wall (0.015) vs. time were one order of magnitude lower than the previous ones. Contrarily, no significant correlation was found between tHg levels in lens and exposure time.

3.4. Relationships between the analysed biological matrices for mercury levels

Figure 5 presents the significant correlations found between the analysed biological matrices for tHg levels after a Spearman analysis (non-significant correlations are omitted). Data of the exposure period were separated from those obtained in the post-exposure sampling days.

In the exposure period, tHg levels recorded in gills were significantly correlated with values found in blood, liver and brain, while liver was highly correlated with blood and brain. Additionally, tHg levels in blood were significantly correlated with those in brain.

In the post-exposure phase, tHg levels were not significantly correlated among the analysed tissues. Interestingly, a sole exception was found for brain vs. blood with tHg levels in both tissues being negatively correlated.

3.5. Rate of mercury tissue elimination

At PE14, the rate of iHg elimination (k) in gills (0.40) was 20-, 10- and 6-fold higher than those of brain (0.02), liver (0.04) and blood (0.07), respectively (Table 1). The k values

estimated for eye wall and lens were one order of magnitude below the previous ones (0.004, 0.008, respectively). In general, the elimination of tHg slowed down in the following period (15-28 days of recovery) in all the biological matrices. Particularly, brain and lens presented a negligible rate of iHg elimination in that period.

4. DISCUSSION

4.1. Mercury uptake, distribution and accumulation in key tissues

Current results revealed that gills accumulated iHg faster (within 1 day) than the remaining tissues when fish are exposed to realistic levels of this Hg counterpart via water. The high responsiveness of gills was also corroborated by the maximum values of Hg enrichment factors during the exposure period. This is not surprising since it is well established that gills are the primary route of waterborne iHg entrance in fish [e.g. 1; 3]. The preponderance of gills as an uptake surface could explain the significant correlations obtained for tHg levels in gills and liver in the exposure period, as well the strong association of gills with blood and brain. In fact, gills are in direct contact with water and suspended particles, being thus a relevant interface with metal ions [21; 22], including iHg [3]. Several authors claimed that iHg is less accumulated than MeHg due to its lower lipophilicity [e.g. 23]. However, current results suggest that iHg could also be rapidly taken up by gills (i.e. significant differences from control within 1 day of exposure). It is still unclear whether iHg absorption by gills is through physiologically regulated transport or by passive diffusion. It was previously suggested that iHg uptake involves a number of mechanisms, both active and passive, and that iHg binds strongly to the gills (e.g. to SH groups) [24]. Additionally, accumulation of non-essential waterborne metals by gills of freshwater fish is generally thought to occur when metals (like Hg) are taken up inadvertently by transport processes designed for essential cations (e.g. Cd^{2+} uptake instead of Ca^{2+}) [24]. The Hg entry into gills might probably be also facilitated by the physiological gradient enhancement due to the counter-current principle between the flows of water and blood, outside and inside the gill structures. Present data on *D. sargus* confirmed that iHg taken up by gills enters the bloodstream as indicated by the strong relationship found between tHg levels in gills and in blood, which is in line with previous studies [12].

Fish eyes are also in straight contact with the surrounding medium but the significant increase of accumulated Hg in eye wall was only noticed after 7 days of exposure. The temporal delay found between gills and eye wall for iHg load increase could be attributed to the distinct tissues nature and physiology. Despite the direct contact of fish eye with water, this organ seems to be, in some extent, impervious to the dissolved iHg, being thus physiologically protected. The epidermal mucus secretions covering fish eyes can be a first line of defence against metals [25]. Several mucus constituents of fish skin such as the sialic acid and other glycoprotein components may bind and immobilize iHg [1] preventing its direct uptake by eyes. iHg can also induce mucous secretion in gills of various species of fish as a defensive mechanism [25] but data in seabream suggest that its chemical composition is probably different from eyes' mucus and does not entrap efficiently iHg at gills surface. Additionally, current data suggest that water is not the main vehicle of iHg to fish eyes, pointing to the occurrence of an alternative pathway for iHg to reach eye wall. iHg can be distributed through the blood to eye wall and this seems to be the preferential uptake route. Such distribution was previously observed for MeHg in zebrafish [5] and invoked to explain the iHg accumulation in wild fish [7].

Indeed, blood is the main vehicle of mercury (re)distribution in fish body (similarly to other xenobiotics). Despite eye wall, the blood can also transport substantial amounts of iHg to liver and brain, explaining the common temporal pattern found between the two organs in the exposure period. However, liver accumulated higher iHg levels than brain at E14 (mean values of 2.5 μg g^{-1} and 1.4 μg g^{-1}, respectively) and showed greater enrichment factors. These differences could be attributed to the fact that iHg only reached the brain after crossing the blood-brain barrier (BBB), while 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. Moreover, the liver has a well-recognized detoxification function, being a preferential site of metals accumulation in fish [26; 27; 28] including in response to iHg water exposure [29]. iHg in fish liver cells was reported to be mainly located in lysosomes and nuclei [30]. Mercury levels in blood were highly correlated with values in brain and liver but the slope was almost two-fold higher in liver *vs*. blood (1.44) than in brain vs. blood (0.61), indicating a higher transference of iHg in the first case. Such difference supports the previous hypothesis

that higher iHg levels in the liver can result from the absence of protective external barriers in opposition to what occurs for the brain.

As previously stated for the brain, also fish eye is protected by an epithelial barrier (blood-retinal barrier- BRB). Current exposures of seabream revealed that iHg could cross both BBB and BRB within a few days of exposure and under realist contamination levels of iHg in water. In fact, BBB and BRB strictly regulate the transport of molecules from the bloodstream to the cells of brain and retina, but Hg chloride can penetrate both barriers by membrane carrier systems [5]. Interestingly, current data suggest that the BBB is more permeable to iHg than BRB. The temporal comparison of tHg levels in both organs allows concluding that iHg reached the brain more rapidly (within 3 days of exposure) than the eye wall (7 days of exposure). The greater susceptibility of brain to iHg exposure is also pointed by the higher accumulated levels (0.42-1.4 μg g^{-1}) relatively to eye wall (0.17-0.34 μg g^{-1}) and also by the enhanced enrichment factors along the exposure period (1.1-3.2 for brain and 0.82-2.4 for eye wall). The higher permeability of BBB in comparison with BRB to iHg is also supported by the fact that significant correlations were found between tHg levels for brain *vs*. blood, while no associations were obtained for eye wall *vs*. blood. Fish eye wall comprises probably pseudo-isolated components of the eye, which provides to such structures a singular iHg toxicokinetics, as suggested by the absence of significant correlations between tHg levels in eye wall and the other biological matrices. Tissue-specificities concerning tHg load in fish were also found under field exposure, with values ranging from 0.11 to 0.61 μg g^{-1} in brain and 0.05 to 0.30 μg g^{-1} in eye wall [7]. Since mercury accumulation by eye tissues has only recently been revealed [5; 7], more research is still needed to clarify the higher permeability of BBB to iHg in comparison with BRB. Thiols and MTs cysteine-rich intracellular proteins are important ligands for iHg in central nervous system (CNS) [31] and this could be related with the distinct accumulation capacity of brain and eye wall.

Hg(II) could also reach the brain by axonal transport as previously stated [9], but this pathway is probably less important than iHg transport through the BBB. The mechanism by which iHg could reach the brain is still a controversial issue. iHg appearance in the brain was previously attributed to organic Hg uptake and subsequent demethylation. The transport of iHg to the brain via the blood after demethylation in liver (where it is well established) is another

widely accepted hypothesis. Current data contributed to demystify this controversy revealing that iHg could reach the brain after waterborne exposure and via bloodstream. iHg would probably pass the BBB by diffusion on the contrary of previous assumptions of Rouleau et al. (1999) [9] that described BBB as impervious to iHg. Mercuric chloride can also act as a direct BBB toxicant, affecting its structure and thus increasing its permeability [15].

Lens did not reflect iHg exposure by water within 14 days of exposure. In fact, tHg levels in fish lens remained relatively unchanged over the exposure time and similar levels were recurrently found between the control and exposed fish. These results are in line with our most recent data for iHg accumulation in lens of wild fish [7]. Apparently, lens is unable to reflect different environmental availabilities related with seasonal changes, as well as within the time-frame currently considered for seabream. The absence of significant tHg increases in lens of exposed fish could be related with the chemical form of this trace element. Lens showed to accumulate preferential MeHg (more than 96% of total Hg) than iHg under field exposure, presumably due to its high protein nature [7]. Korbas and co-authors (2013) [5] investigated the uptake and accumulation of MeHg in zebrafish larvae and found the highest levels in the secondary lens fibers underlying the lens epithelium. Lens is the site of particularly high protein production (named as crystallins) and deposition [32]. MeHg reaches lens from the aqueous humours since it has no direct blood supply. Thus, it is plausible that under iHg exposures, this chemical form does not cross from the surrounding aqueous humours to the lens due to a very low chemical affinity.

The maximum levels of Hg accumulation were recorded after 14 days of exposure for all the target tissues/organs (except lens). This lead to a maximum of enrichment factors at E14. The mean levels of tHg reached by gills, blood, liver and brain at E14 (8.1±0.17, 1.8±0.11, 2.5±0.16, 1.4±0.43 μg g^{-1}, respectively) were high considering that fish were exposed to realistic waterborne iHg levels (2 μg L^{-1} $HgCl_2$). Those tissues exhibited concentrations higher than those found in another estuarine species (*Liza aurata*) from a severe contaminated area by Hg (mean values around 0.10 μg g^{-1}in gills, 0.05 μg g^{-1} in blood, 1.0 μg g^{-1} in liver and 0.20 μg g^{-1} in brain) [26]. Under field exposure, fish were subject both to inorganic and organic Hg counterparts, as well as to different absorption pathways, i.e. contaminated water and food.

Thus, the higher levels of tHg currently found in seabream tissues/organs point out the relevance both of iHg chemical form and water as vehicle for Hg entrance into fish body.

Mercury is highly reactive with sulphydryl groups of proteins, forming covalent bonds with reduced glutathione (GSH) and cystein residues of proteins. GSH is the primary antioxidant and conjugating agent, being the first line of defence against Hg. GSH was previously determined in gills, liver and brain of *Liza aurata* from Aveiro lagoon [33; 34]. A significant decrease of GSH was recorded in liver and brain of fish from the most contaminated area suggesting the release of GSH-Hg conjugates, while no spatial changes were found for gills.

4.2. Fish recovery after cessation of waterborne iHg exposure

The post-exposure periods of 14 and 28 days allowed a significant decrease of tHg accumulation in gills, followed by blood and liver, indicating that such tissues eliminate iHg within a few days in the absence of the compound in the water. The tissue elimination rates estimated for seabream blood and liver are within the reported efflux-rate constant for the first depuration phase in whole sweetlips [0.07 d^{-1}] [35]. Values of k estimated for gills were higher than those of blood and liver, as well as higher than those previously presented for whole body of sweetlips [35]. Elimination of iHg is probably significantly promoted by the high cellular turnover of the gills.

The liver could also excrete iHg into the faeces, as a result of biliary secretion [36]. However, tHg levels in liver and bile did not vary concomitantly over exposure and post-exposure periods. Concentrations in the bile only increased significantly in the last exposure time (E14) and in both recovery times (PE14 and PE28). This temporal lag points to the involvement of other hepatic defence mechanisms (at E3 and E7) such as MTs and glutathione. The exhaustion of these detoxification strategies probably lead to the significant excretion of iHg through the bile.

Despite the significant reduction of accumulated tHg levels in gills, blood and liver during the post-exposure period, values did not reach baseline levels (i.e. recorded in control fish). Thus, more than 28 days are probably required for complete iHg elimination in those tissues, which is in line with previous estimations of iHg half-life ($t_{1/2}$) in whole tilapia [18.0 days] [3] and whole sweetlips [25.4 days] [35] after waterborne exposure.

Mercury load in eye wall and brain did not decrease in the post-exposure period diverging from the temporal pattern previously described for gills, blood and liver. In fact, no significant differences were found for tHg levels recorded in eye wall and brain at E14 *versus* PE14 and E14 *versus* PE28, suggesting negligible iHg elimination within this time-frame. A similar finding was previously reported for zebra-seabream brain exposed to MeHg in water [4] and zebrafish (eyes and brain) subjected to several Hg counterparts [5]. Mercury levels in seabream brain were negatively correlated with those in blood, indicating that iHg was not removed from the brain. This is a quite relevant finding regarding iHg kinetics in brain that indicates low elimination ability within 28 days of recovery. Current data pointed also that eye wall and brain are final targets of iHg, as previously suggested for tilapia that accumulated significant levels of Hg(II) in the head at the end of 30 days of depuration [3]. This is probably due to the high affinity of iHg to cellular and molecular components of both tissues. Moreover, iHg that reaches both eye wall and brain could only be removed via the blood, implying its passage through BBB and BRB, respectively. It seems that both barriers can also limit the release of iHg to the bloodstream and thus its elimination. Efflux of mercury from the brain has received very little attention and only regarding the MeHg form, while no data exist for eye wall. The efflux of MeHg through brain capillary endothelial cells of BBB was already proved to occur in association with glutathione [37], as reported in other cell systems [23]. Despite iHg could cross BBB bi-directionally, iHg influx and efflux from brain is probably unbalanced, leading inevitably to its accumulation in brain over time, as previously described for Fe [8]. It could also be hypothesized an iHg redistribution between eye wall and lens leading to a decrease of accumulated levels in eye wall, as previously proposed for MeHg in a field study [7]. However, current data did not support this hypothesis since tHg levels in lens did not vary significantly over depuration time and iHg enrichment factors were around 1.0 for PE14 and PE28.

The slow release of iHg from the eye wall and brain upon cessation of exposure is an important aspect considering their main physiological roles. Regarding the fish eyes, there are several implications on organism's health and survival that could be expected due to the iHg presence. Blindness of fish was reported after iHg exposure [38]. Visual deficits were also observed [39], as well as disorganized retinas, abnormal pigment distribution, and invasive blood sinuses in eyes of medaka embryos exposed to MeHg [40].

Neurodegeneration was previously associated with iHg presence in the brain [31] and iHg was also responsible for the inhibition of crucial molecular mechanisms, such as the thioredoxin system [4]. Oxidative stress in fish brain was attributed to accumulation of inorganic Hg and organic Hg species under field exposure [41], other neurotoxic effects such as the reduction of neural monoamine oxidase activity and astrocyte proliferation were also attributed to iHg [e.g. 42], as well as behavioural impairments [43].

The time retention of iHg in the fish eye wall and brain and/or the delayed efflux is toxicologically relevant and should be taken into account when studying the health risk of wildlife exposed to iHg. Furthermore, current findings can also be considered informative, on an extrapolation basis, to predict the risk of human exposure to iHg.

4.3. Contributions to the design of strategies for environmental health assessment using fish

Understanding the iHg toxicokinetics is of utmost importance in the choice of the fishes' tissue/organs that could better reflect waterborne field contamination. Under this context, short- and long-term exposures of fish to iHg need to be considered. According to current data of seabream, gills can be proposed as the most adequate tissue/organ to reflect short-term exposures to realistic levels of iHg. This conclusion is provided by two evidences: (i) gills of exposed fish accumulated significantly higher levels of iHg within 1 day of exposure in comparison with control specimens; (ii) gills exhibited accumulation enrichment factors higher than 1 during the entire exposure period (14 days). Regarding long-term exposure of fish to iHg, a more "refractory" tissue/organ seems to be appropriately to reflect faithfully water contamination. Brain and eye wall were the most "refractory" tissues/organs in relation with iHg exposure based on their slow elimination capacity. In fact, no significant elimination of iHg was detected in brain and eye wall within 28 days of fish depuration. The iHg stability in those tissues/organs is particularly important when fish are considered in the assessment of aquatic contamination due to its mobility. Such outcomes need to be considered in order to minimize the occurrence of false positive or negative results in environmental risk assessment.

5. CONCLUSIONS

These main findings were provided by the exposure of fish to realistic levels of waterborne iHg (within 14 days) followed by a depuration period of 28 days:

- Gills accumulated iHg faster than eye wall, blood, liver and brain, reaching also the highest accumulation levels after exposure. Contrarily, lens was not able to accumulate iHg within this exposure time-frame;
- The physiological protection provided by BRB and BBB seems to be related to the lower iHg accumulation in eye wall and brain, respectively, though the BBB showed to be more permeable to iHg;
- 28 days of depuration were not enough to ensure the total elimination of iHg from any of the tissues, though biliary excretion showed to be involved in iHg elimination during post-exposure. Moreover, eye wall and brain were unable to carry out a significant elimination of iHg;
- The slow elimination of iHg in eye wall and brain could represent a risk for wild populations of fish. These body compartments seem to be particularly informative of iHg water contamination under long-term exposures, while gills could faithfully reflect short-term exposures.

Ethical statement

This study was conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities.

Conflicts of interest

There are no conflicts of interest in this work.

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6. REFERENCES

1. C. A. Oliveira Ribeiro, J. R. D. Guimarães and W. C. Pfeiffer, *Ecotoxicol. Environ. Saf.*, 1996, **34**, 109.
2. C. A. Oliveira Ribeiro, E. Pelletier, W. C. Pfeiffer and C. Rouleau, *Environ. Res. Section A*, 2000, **83**, 286.
3. R. Wang, M.-H. Wong and W.-X. Wang, *Environ. Pollut.,* 2010, **158**, 2694.
4. V. Branco, J. Canário, A. Holmgren and C. Carvalho, *Toxicol. Appl. Pharmacol.*, 2011, **51**, 95.
5. M. Korbas, B. Lai, S. Vogt, S.-C. Gleber, C. Karunakaran, I. J. Pickering, P. H. Krone and G. N. George, *ACS Chemical Biology*, 2013, **8**, 2256.
6. R. A. Yokel, *Neuromol. Med.*, 2009, **11**, 297.
7. P. Pereira, J. Raimundo, O. Araújo, J. Canário, A. Almeida and M. Pacheco, *Science of the Total Environment*, 2014, **494–495**, 290.
8. J.-H. Chen, N. Singh, H. Taya and T. Walczyk, *Metallomics*, 2014, **6**, 1417.
9. C. Rouleau, K. Borg-Neczak, J. Gottofrey and H. Tjälve, *Environ. Sci. Technol.*, 1999, **33**, 3384.
10. R. P. Mason, J.-M. Laporte and S. Andres, *Arch. Environ. Contam. Toxicol.,* 2000, **38**, 283;
11. C. A. Oliveira Ribeiro, C. Rouleau, E. Pelletier, C. Audet and H. Tjalve, *Environ. Sci. Technol.*, 1999, **33**, 902.
12. J. J. Leaner and R. P. Mason, *Environmental Toxicology and Chemistry*, 2004, **23**, 2138.
13. S. De Flora, C. Bennicelli and M. Bagnasco, *Mutat. Res.*, 1994, **317**, 57.
14. J. W. Allen, L. A. Mutkus and M. Aschner, *Brain Res.*, 2001, **891**, 148.

15. W. Zheng, M. Aschner and J. F. Ghersi-Egea, *Toxicol. Appl. Pharmacol.*, 2003, **192**, 1.
16. M. Korbas, P. H. Krone, I. J. Pickering and G. N. George, *Journal Biological Inorganic Chemistry*, 2010, **15**, 1137.
17. C. J. Watras, *Science of the Total Environment*, 1998, **219**, 183-208.
18. P. Pereira, J. Raimundo, J. Canário, A. Almeida and M. Pacheco, *Mar. Pollut. Bull.*, 2013, **77**, 375.
19. U. S. EPA. Method 1630: Mercury in Water by Oxidation, Purge and Trap, and Cold Vapor Atomic Fluorescence Spectrometry, 2002, Washington, United States, Environmental Protection Agency.
20. C. T. Costley, K. F. Mossop, J. R. Dean, L. M. Garden, J. Marshall and J. Carrol, *Analytica Chimica Acta*, 2000, **405**, 179.
21. C. Fernandes, A. Fontaínhas-Fernandes, F. Peixoto and M. A. Salgado, *Ecotoxicol. Environ. Saf.* 2007, **66**, 426.
22. P. Pereira, H. de Pablo, C. Vale and M. Pacheco, *Mar. Environ. Res.,* 2010, **69**, 53.
23. G. P. Guzzi and C. A. M. Porta, *Toxicology*, 2008, **244**, 1.
24. J. Klinck, M. Dunbar, S. Brown, J. Nichols, A. Winter, C. Hughes and R. C. Playle, *Aquat. Toxicol.*, 2005, **72**, 161.
25. W. E. Coello and M. A. Q. Khan, *Arch. Environ. Contain. Toxicol*., 1996, **30**, 319.
26. C. L. Mieiro, M. Pacheco, M. E. Pereira and A. C. Duarte, *Journal of Environmental Monitoring,* 2009, **11**, 1004.
27. P. Pereira, H. de Pablo, M. Pacheco and C. Vale, *Ecotoxicol. Environ. Saf.*, 2010, **73**, 805.
28. J. Raimundo, C. Vale, M. Caetano, B. Anes, E. Giacomello and G. Menezes, *Deep-Sea Research Part II*, 2013, **98**, 137.
29. A. C. Elia, R. Galarini, M. I. Taticchi, A. J. M. Dörr and L. Mantilacci, *Ecotoxicol. Environ. Saf.*, 2003, **55,** 162.
30. E. Baatrup, M. G. Nielsen and G. Danscher, *Ecotoxicol. Environ. Saf.*, 1986, **12**, 267.
31. M. Farina, D. S. Avila, J. B. T. Rocha and M. Aschner, *Neurochem. Int.*, 2013, **62**, 575.
32. M. Korbas, P. H. Krone, I. J. Pickering and G. N. George, *Journal Biological Inorganic Chemistry*, 2010, **15**, 1137.

33. C. L. Mieiro, I. Ahmad, M. E. Pereira, A. C. Duarte and M. Pacheco, *Ecotoxicology*, 2010, **19**, 1034.

34. C. L. Mieiro, A. C. Duarte, M. E. Pereira and M. Pacheco, *Ecotoxicol. Environ. Saf.*, 2011, **74**, 2225.

35. W.-X. Wang and R. S. K., Wong, *Mar. Ecol. Prog. Ser.,* 2003, **261**, 257.

36. R. K. Zalups, *Journal of Toxicology and Environmental Health, Part A*, 1998, **53**, 615.

37. L. E. Kerper, E. M. Mokrzan, T. W. Clarkson and N. Ballatori, *Toxicol. Appl. Pharmacol.*, 1996, **141**, 526.

38. A. K. Panigrahi and B. N. Misra, *Arch. Toxicol.*, 1980, **44**, 269.

39. C. W. Hawryshyn, W. C. Mackay and T. H. Nilsson, *Canadian Journal of Zoology*, 1982, **60**, 3127.

40. N. A. Dial, *Teratology*, 1978, 17, 83.

41. C. L. Mieiro, M. E. Pereira, A. C. Duarte and M. Pacheco, *Aquat. Toxicol.*, 2011, **103**, 233.

42. M.H.G. Berntessen, A. Aatland and R. D. Handy, *Aquat. Toxicol.*, 2003, **65**, 55.

43. L.R. Vieira, C. Gravato, A.M.V.M. Soares, F. Morgado and L. Guilhermino, *Chemosphere*, 2009, **76**, 1416.

Table 1 – Estimated rate (k) of inorganic mercury (iHg) elimination for each tissue/organ during the post-exposure period (PE).

	Rate of iHg elimination (k) (µg Hg/g/day)					
Condition	**Gills**	**Eye wall**	**Lens**	**Blood**	**Liver**	**Brain**
PE14	0.40	0.004	0.008	0.07	0.04	0.02
PE28	0.07	0.002	-0.01	0.02	0.001	-0.02