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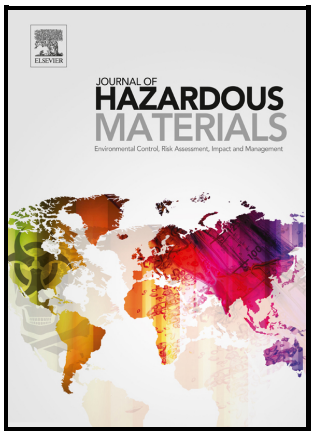


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# Exploring mercury detoxification in fish: the role of selenium from tuna byproduct diets for sustainable aquaculture.

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## Abstract

Exposure to mercury (Hg) through fish consumption poses significant environmental and public health risks, given its status as one of the top ten hazardous chemicals. Aquaculture is expanding, driving a surge in demand for sustainable aquafeeds. Tuna byproducts, which are rich in protein, offer potential for aquafeed production, yet their use is challenged by the high content of heavy metals, particularly Hg. However, these byproducts also contain elevated levels of selenium (Se), which may counteract Hg adverse effects. This study examines the fate of dietary Hg and Se in an aquaculture model fish. Biomolecular speciation analyses through hyphenated analytical approaches were conducted on the water-soluble protein fraction of key organs of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to various combinations of Hg and Se species, including diets containing tuna byproducts, over a six-month period. The findings shed light on the dynamics of Hg and Se compounds in fish revealing potential Hg detoxification mechanisms through complexation with Hg-biomolecules, such as cysteine, glutathione, and metallothionein. Furthermore, the trophic transfer of selenoneine is demonstrated, revealing novel opportunities for sustainable aquafeed production. Understanding the

interactions between Hg and Se in aquaculture systems is crucial for optimizing feed formulations and mitigating environmental risks. This research contributes to the broader goal of advancing sustainable practices in aquaculture while addressing food security challenges.

**Environmental implication:**

Exposure to mercury through fish consumption poses significant environmental and public health risks, as mercury ranks among the top ten hazardous chemicals. However, the metabolic pathways of mercury in biota remain incompletely understood. This study offers groundbreaking insights into the molecular-level fate of mercury in fish, revealing potential detoxification mechanisms involving its binding to low molecular weight compounds. Understanding the interaction between mercury and selenium presented in this research is crucial for optimizing feed formulations. Furthermore, it highlights the potential of utilizing tuna byproducts to foster sustainable aquaculture practices, addressing pressing environmental and food security challenges.

**Keywords:** aquafeed, speciation, selenoneine, methylmercury, metallothionein.

## 1. Introduction

Fish and seafood consumption is recognized as the main pathway of human exposure to mercury (Hg), one of the top ten chemicals of major public health[1]. Current regulatory criteria primarily focus on methylmercury (MeHg) content[2,3], despite recent studies proposing new and more precise risk criteria based not only on MeHg levels, which are known to be neurotoxic, but also on selenium (Se) concentration. Selenium is an essential element for living organisms, recognized for its antioxidant role[4-8]. Moreover, recent research suggests that Se may play a crucial role in mitigating Hg toxicity, prompting proposals for its inclusion in regulatory frameworks[4,9-11].

The fate of Hg and Se in fish, and in biota in general, remains largely elusive. Despite the efforts of the large scientific community studying Hg in biota and recent advancements in the field, the metabolic pathways remain incompletely elucidated[12,13]. Understanding their fate in fish tissues is crucial for assessing their bioavailability, among others. While studies often focus on quantifying MeHg in muscle tissue, it is essential to also investigate which Hg molecular forms are occurring, especially when Hg(II) or MeHg binds to specific biomolecules. Similarly, the simultaneous characterization of the chemical forms

of Se is mandatory to unravel metabolic pathways and potential interactions between both elements[4,9,10,14,15]. The necessity for speciation studies becomes imperative to better comprehend the dynamics of these elements in fish tissues and potential health repercussions. Speciation analyses of both Hg and Se represent significant analytical challenges due, among others, to the instability of the target compounds, their low abundance, and the complex biological matrices. Typically, hyphenated techniques involve a preliminary step of compound separation by liquid chromatography, followed by specific element detection and structural characterization using mass spectrometry. Other approaches, such as X-ray absorption spectroscopy[16], have also been successfully employed.

Aquaculture management confronts unprecedented challenges due to the escalating global demand, climate change, and intensified competition for natural resources. The urgent need for sustainable aquafeeds, as alternatives to traditional fish meals sourced from fishing, is indisputable[3]. In this context, the utilization of fish byproducts has emerged as a compelling solution, with tuna byproducts garnering particular interest. Given that the tuna canning industry produces waste levels of up to 65% of the original material, these byproducts offer a promising and environmentally friendly resource for fish meal production. However, the elevated levels of heavy metals, for instance Hg, in these byproducts remain of concern. Heavy metals can induce harmful effects such as disruption in hormone secretion affecting animal and human reproduction[17]. Recent promising studies on tilapia and trout aquaculture have revealed that diets based on tuna byproducts containing concentrations close to the non-observed effect level (NOEL) of 0.5 µg Hg $g^{-1}$ for salmonids[18] do not result in Hg levels in flesh exceeding regulatory limits[15,19]. Investigations into Hg bioaccumulation in juvenile trout further suggest the potential role of basal Se in tuna in regulating Hg levels in fish tissues[15]. To gain a deeper understanding of the interaction between Hg and Se, chemical speciation studies and analysis of various organs are essential. However, until now, the studies of the dietary Se effect on Hg bioaccumulation and/or toxicity are mainly limited to the quantification of total Hg and/or MeHg in the fish flesh[20-25].

The main aim of this work is to obtain new insights at the molecular level into the dietary fate of Hg and Se in fish. Utilizing rainbow trout (*Oncorhynchus mykiss*) as an aquaculture fish model, biomolecular speciation was conducted across blood, kidney, brain, liver, and muscle tissues of animals exposed to a variety of Hg and Se species,

including those naturally present as in tuna byproducts or added under controlled conditions. This approach explores for the first time the resulting Hg and Se metabolites of farmed fish fed with tuna byproducts. This comprehensive study contributes to the ongoing efforts to optimize aquafeed formulations, mitigate environmental and health risks, and promote sustainable practices in aquaculture while addressing food security challenges.

## 2. Materials and methods

### 2.1. Feeding trial and sample collection

The 6-month feeding trial, involving 1800 all-female diploid rainbow trout juveniles (initial and final mean body weights: 26 ± 1 and 410 ± 29 g, respectively) was conducted at the French National Research Institute for Agriculture Food and Environment (INRAE) as described elsewhere[15,26]. There were 50 fish per replicate tank. All experimental procedures complied with the European Directive 010/63/EU for the protection of animals used for scientific purposes, and the French Decree no. 2013-118 for animal experimentation (project agreement number: APAFIS#27846-2020102812241350v2). The experimental iso-nitrogenous (50% crude protein) and iso-lipidic (21% total lipid) feeds consisted of 6 plant-based diets and 6 tuna meal-based diets, with different dietary Se and Hg species (including selenomethionine, sodium selenite and methylmercury(II) chloride) and concentrations (ranging from 0.25 to 11.2 µgSe $g^{-1}$ and 0 to 2.6 µgHg $g^{-1}$, respectively)[15]. Each diet was assessed in triplicate. Liver, kidney, muscle, brain, and blood samples from rainbow trout juveniles, euthanized with an overdose of benzocaine, were collected from three individuals per dietary condition after overnight starvation for 16 h at 2, 7, 21, 84, and 168 days of the feeding trial. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

### 2.4. Speciation by SEC-ICP-MS, HILIC-ICP-MS and HILIC-ESI-MS

The water-soluble fraction was extracted from fresh samples (approximately 0.3 g) by ultrasonication (30 s at 21 % at 100 W) in 1 mL of 100 mM ammonium acetate (pH 7.4), followed by centrifugation, as described elsewhere[27]. The resulting water-soluble fraction was analysed using SEC-ICP-MS under conditions compiled in Table S1.

In parallel, to identify low-molecular-weight Hg and Se species, an aliquot of the water-soluble fraction was diluted with acetonitrile (1:2 v/v). The mixture was centrifuged at 14000 g for 10 min, and the obtained supernatant was directly analyzed by hydrophilic

interaction liquid chromatography (HILIC) coupled to ICP-MS and electrospray ionization ESI-MS (gradient detailed in Table S1)[14,28]. Derivatized samples were also prepared, with iodoacetamide and tris(2-carboxyethyl) phosphine (TCEP)[29].

ESI-MS was operated in the positive ion mode under the following optimum settings: an ion spray voltage of 2.80 kV; a capillary temperature of 285°C; a source heater temperature of 120 °C; a nitrogen sheath gas flow of 15, an auxiliary gas flow of 5, and a sweep gas of 1 (arbitrary units); and an S-lens RF level of 95%. Mass spectrometer calibration was performed with a mixture of caffeine (m/z 195.08765), Met-Arg-Phe-Ala (MRFA) (m/z 524.26499) and Ultramark polymer (m/z 1221.99063) dissolved in 50 % acetonitrile and 0.1 % formic acid solution. Mass spectral data were processed with Xcalibur 2.1 software (Thermo Fisher). In full scan mode, a m/z range of 120−1800 was scanned for the detection of Hg and Se biocompounds at a resolution of 100 000 (m/Δm, FWHM at m/z 400). The Hg and Se isotopic patterns were searched using Thermo MetWorks 1.2.1 software.

***Reagents and standards***

All solutions were prepared using Milli-Q (ultrapure) water (18.2 MΩ cm, Millipore Bedford, MA) and all analytical reagent grade chemicals were purchased from Sigma Aldrich (Saint Quentin, Fallavier, France).

***Instrumentation***

Liquid chromatographic separations were conducted with an Agilent 1100 liquid chromatography (Agilent, Wilmington, DE) equipped with an autosampler and a binary HPLC pump. An Agilent inductively coupled plasma mass spectrometer (ICP-MS) 7500ce (Yokogawa Analytical Systems, Tokyo, Japan) was used for detection of Hg, Se, and other elements (Table S1) after liquid chromatography separation. The ICPMS instrument was daily tuned to fully optimize and validate the entire mass range of the instrument. Two size exclusion chromatographic (SEC) columns were employed and coupled to ICP-MS: Superdex 200 and Superdex peptide HR 10/30 (10×300 mm, 13 µm, GE Healthcare, Uppsala, Sweden). The hydrophilic interaction chromatography (HILIC) separation system with TSK gel amide 80 column (250×1 mm, 5 µm, Tosoh Biosciences, Stuttgart, Germany) was coupled to ICP-MS or an electrospray hybrid linear trap quadrupole Orbitrap Velos mass spectrometer (ESI LTQ Orbitrap MS) from Thermo Fisher Scientific (Bremen, Germany) using a heated electrospray ionization source (H

ESI II) (Thermo Fisher Scientific). An ultrasonic probe—Vibracell (USP) 75115 (Bioblock Scientific, Illkirch) instrument with a 3 mm diameter and a nominal power of 500 W was used for water-soluble fractions extraction. A centrifugation system using a MiniSpin plus model centrifuge (Eppendorf, Hamburg, Germany) at 14 100 g was used.

## 3. Results and discussion

### 3.1. Screening of Se and Hg in the water-soluble fraction of trout tissues

Investigating the water-soluble fraction of blood, liver, kidney, brain and muscle tissues from rainbow trout exposed to various aquafeeds (plant-based or tuna byproducts-based, enriched or not with Hg and Se species) revealed interesting insights through SEC-ICP-MS. Figures S1 and S2 provide the chromatographic profiles for Se and Hg, respectively, across these organs. The singularities observed during the screening of both elements are presented and discussed hereafter.

#### *3.1.1. Se species screening in the water-soluble fraction*

Selenium was distributed over a wide range of molecular weight in trout tissues showing specificities according to the investigated tissue and dietary exposure (Figure S1). Notable differences were observed in blood according to the exposure to tuna byproducts and plant-based diets (Figure 1). It is important to note that the basal Se naturally present in the tuna byproducts contributes to 8 µgSe $g^{-1}$ of food, which represents 80 % of the final Se concentration in the tuna byproducts based-diets enriched with selenomethionine (SeMet) or Se(IV)[26]. The profile of Se-biomolecules showed no significant differences between the blood of the control group exposed to tuna byproducts and the blood when aquafeed was enriched with Se species (Figure 1a). These similarities align perfectly with the consistent Se content values across the various dietary treatments using tuna-based aquafeeds[15]. These findings strongly suggest that the Se in blood is predominantly influenced by the basal Se content in such byproducts.

The comparison of Se-biomolecule profiles in the blood of animals exposed to tuna byproducts (Figure 1a) or plant-based diets (Figure 1b) revealed significant differences in the Se fraction eluting around 30 min. This prominent fraction in the blood of animals fed with tuna byproducts is either absent or present at low intensity when animals were fed with Se-fortified plant based-diets. It is noteworthy that this Se-fraction was consistently found in all trout exposed to tuna byproducts-based diets, including the

control group, suggesting its origin from the tuna byproducts used in aquafeed formulation (discussed in Section 3.2.1).

The Se water-soluble fraction in the liver, the organ exhibiting the highest Se bioaccumulation, is predominantly composed of a fraction eluting around 28 min, corresponding to low molecular weight (LMW) species (Figure S1b). Despite the co-supplementation of Se(IV) with MeHg in both types of diets leading to the highest hepatic Se levels compared to other diet conditions[15], the chromatographic profiles remain similar across the different dietary treatments. The specific increase in hepatic Se is exclusively observed when animals were exposed to Se(IV) and MeHg simultaneously (370 µg Se $g^{-1}$). However, this increase is not reflected in the profile of the soluble extractable Se fraction, strongly suggesting the formation of insoluble Se compounds. Previous studies have already reported the formation of insoluble Se nanoparticles (NPs) combined with Hg (tiemannite), considered the end product of Hg detoxification, as well as with other elements such as Cd and As[13,16,30,31]. While the mechanisms underlying the bioformation of Se NPs remain incompletely understood, existing literature consistently supports the notion that an excess of Se compared to Hg plays a crucial role in the formation of detoxifying NPs. It is interesting to notice that the Se:Hg hepatic molar ratio was around 100 when Se(IV) and MeHg were simultaneously supplemented in both tuna byproducts- and plant-based diets[15]. These values exceed more than twice those obtained with SeMet and MeHg simultaneous dietary exposure[15].

In the kidney of animals exposed to both plant and tuna-based diets, Se is primarily associated with a fraction eluting at around 28 min, corresponding to LMW compounds (Figure S1c). Interestingly, a lack of specificity in Se bioaccumulation was observed in the kidney of animals exposed to Se(IV) and SeMet-enriched diets. Animals fed with tuna byproducts-based diets showed approximately 30 % higher total Se levels in the kidney compared to those fed with plant-based diets[15]. However, dietary supplementation with Se(IV) or SeMet did not induce differences in bioaccumulation of total Se levels compared to the control tuna based aquafeed[15]. These findings suggest that the basal Se content in tuna byproducts and/or the specific role of the kidney play a key role in the regulation of Se accumulation, regardless of the dietary Se species.

Muscle and brain tissues exhibit the lowest Se intensity in the chromatographic profiles, in comparison with the rest of the tissues (Figure S1), in total agreement with the Se bioaccumulation resulting in these organs[15]. The distribution of water-soluble Se species

in these tissues was similar for tuna-based diets with and without supplementation, while for animals fed with plant-based diets two additional Se fractions were observed when supplemented with Se(IV) or SeMet.

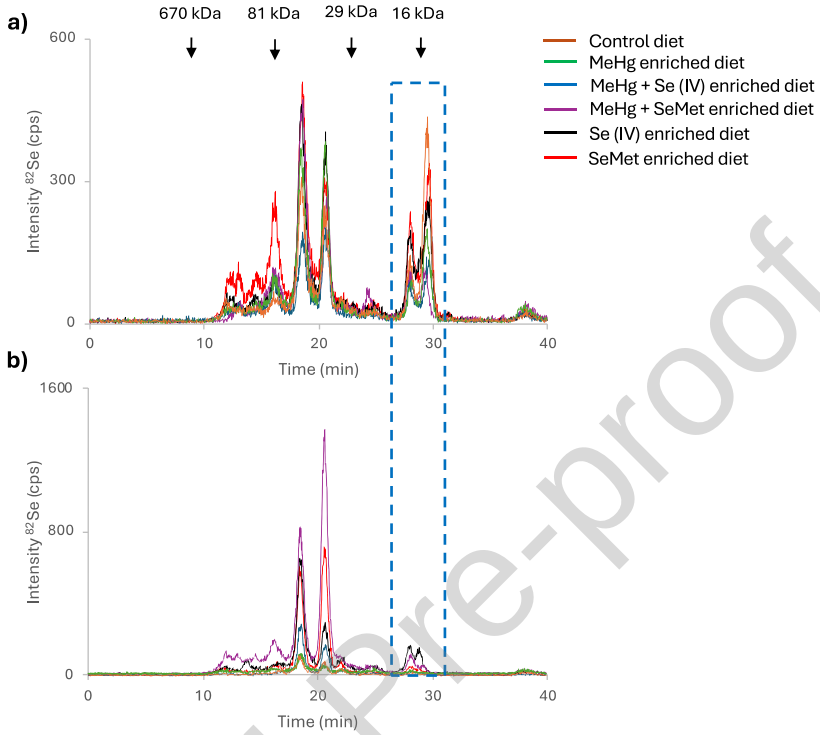


**Figure 1.** Typical $SEC_{200}$-ICP-MS chromatograms corresponding to the water-soluble fraction analyses of Se in blood of animals fed with a) tuna byproducts-based diet and b) plant-based diet after 168 days of exposure.

### *3.1.2. Hg species screening in the water-soluble fraction*

Notable differences in the Hg profile were observed across the analyzed organs (Figure S2). Although total Hg concentrations were significantly higher in organs of animals exposed to plant-based diets compared to those exposed to tuna byproducts-based dietary treatments[15], similar Hg profiles were observed under both feeding conditions. A major Hg fraction, eluting at 20 min, was consistently found in blood of both groups of animals. Remarkably, this main fraction was detected in the blood of animals exposed to tuna byproducts-diets without dietary MeHg supplementation, suggesting its association with the basal dietary Hg content present in the tuna byproducts used for aquafeed preparation (Figure S2). This specific Hg fraction increased with dietary MeHg supplementation, most likely corresponding to the binding of MeHg to hemoglobin. Although the identification of this complex was not performed in the current study, the specific increase

of this fraction with dietary MeHg exposure along with its retention time matching the previously identified MeHg-binding cysteine residues on the hemoglobin β chain in mammals using the same chromatographic mechanism[28], strongly supports our hypothesis. The Hg binding by hemoglobin has been associated to the transport of MeHg in animals[28]. However, unambiguous identification of this metabolite requires specific structural analyses, which face several challenges[28], including species integrity preservation, preconcentration, and unambiguous characterization through HPLC-ESI-MS/MS.

The mercury profiles in liver corresponding to animals exposed to both type of aquafeeds (plant- and tuna-based) were quite similar (Figure S2b). Both groups of animals exhibit two main Hg fractions, eluting at around 25 and 28 min, respectively. Meanwhile in kidney, after 168 days of exposure, Hg appears to be associated to three main fractions eluting at around 25, 28 and 30 min (Figure S2c). In this organ specifically, the distribution of Hg fractions varied with diet exposure time (Figure 2). After 21 days of exposure to both diet groups, Hg is principally associated with the fraction eluting at 20 min. However, at the end of the trial (168 days), the mentioned fraction is not observed on the animals fed with tuna byproducts-based diets, in which Hg seems to be associated to fractions appearing in the LMW range. As observed in blood, this fraction eluting at 20 min is exclusively present when MeHg is supplemented to the diet, regardless of the co-exposure with Se. The observed trend suggests that dietary MeHg reaches the kidney transported by the hemoglobin (corresponding to the fraction eluting at 20 min in Figure 2a) and is kinetically transferred to LMW compounds during storage and/or detoxification (peaks appearing at 28 and 30 min in Figure 2c) and discussed in Section 3.2.2. Interestingly, regarding the kidney of animals exposed to plant-based diets, the Hg distribution shows a similar tendency. However, after 168 days of dietary exposure, a small proportion of Hg-containing peak eluting at 20 min (hypothesized as hemoglobin) is still present (Figure 2d).

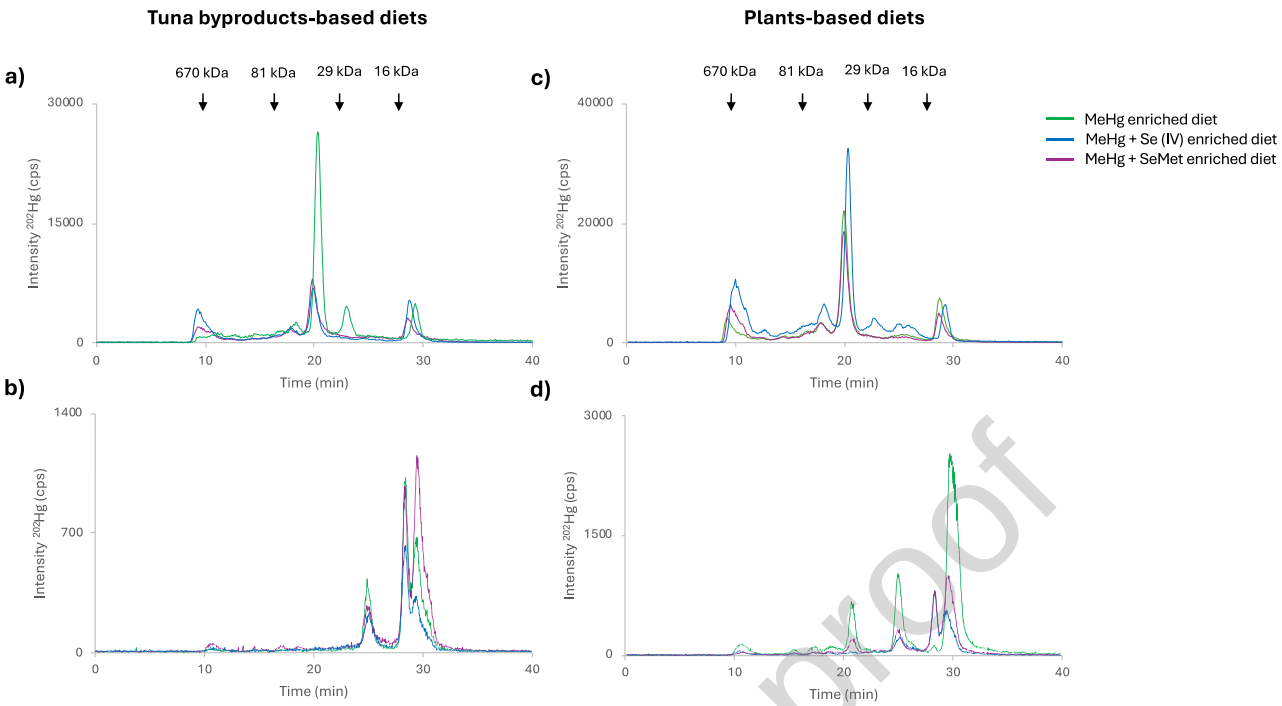


**Figure 2.** Typical $SEC_{200}$-ICP-MS ($^{202}Hg$) corresponding to the water-soluble fraction of kidney of juvenile trout fed with tuna byproducts (left panels) and plant based aquafeeds (right panels) for 21 (a, c) and 168 days (b, d).

Regarding muscle and brain tissues (Figure S2d and S2e, respectively), Hg was distributed in a wider range of molecular weight in comparison to blood, liver and kidney (Figure S2a-c), exhibiting around five different Hg-fractions. It is worth noting that the Hg naturally present in the tuna byproduct-based diet representing the control study results in two Hg-containing fractions in muscle (Figure S2d), where the major fraction elutes at 28 min. Dietary MeHg supplementation leads to a dissimilar Hg distribution, inducing the presence of two abundant Hg-containing fractions eluting at 20 and 25 min, respectively (Figure S2e, left panel). The distribution of water-soluble Hg species was similar in muscle of animals exposed to tuna and plant-based diets supplemented with MeHg, despite the differences previously found in their total Hg content, being 30 % higher in the muscle of fish fed with plant-based diets[15].

The Hg concentration in brain of animals fed with tuna byproducts diets without and with MeHg enrichment was quite different with more than 10-fold concentration increases with MeHg enrichment but Hg chromatographic profiles exhibiting a similar Hg distribution (Figure S2e). Animals exposed to MeHg with plant diet with and without Se supplementation exhibited similar profiles to those fed with tuna byproducts. These

resemblances on the Hg profile in brain, may stem from specific processes of Hg complexation to limit the toxicity within this critical organ of the nervous system.

### 3.2. Exploring mercury and selenium biomolecules in fish tissues: insights and challenges

The screening of the Se and Hg containing biomolecules previously detailed revealed the presence of a large variety of these compounds in the fish tissues analyzed. The identification of such biomolecules could greatly contribute to the understanding of the fate and interaction of Hg and Se species in biota, comprising the Hg detoxification mechanisms by Se. The unambiguous characterization of Hg-containing biomolecules represents a great analytical challenge due to complex instability and low concentration. Until now, there is a scarce number of speciation studies revealing the identity of Hg compounds[16,28,32,33], with a focus being principally on MeHg quantification. The hyphenation of HILIC with ICP-MS and ESI-MS/MS has been successfully used for the characterization of Hg and Se low molecular weight compounds[14,29,34]. This approach was employed in the current work, revealing the identity of several Hg and Se species, as detailed below.

#### *3.2.1. Trophic transfer of selenoneine in tuna byproducts-based diet fed animals: evidence from HILIC-ESI-MS/MS analysis*

Concerning Se speciation, this study focuses on the characterization of the fraction exclusively found in organs of animals fed with tuna byproducts, as highlighted in the screening of Se in blood (Figure 1). Further analysis of the water-soluble extract on an additional column (Superdex peptide), offering improved mass resolution (operational separation range of 0.1 to 7 kDa), also unveiled the presence of a Se fraction at 27 minutes, exclusively in the blood of animals fed with tuna-based diets, as well as in some other organs within this group of animals (Figure S3). To ascertain the identity of the detected Se-containing fraction, an additional hybrid approach was employed by coupling HILIC with ICP-MS and ESI-MS[14]. Two Se-containing peaks (Figure S4) that elute around 23 and 24 min, exclusively in tissues of animals fed with tuna byproducts-based meal, were found. At these retention times, spectra obtained by HILIC-ESI-MS revealed a Se isotopic pattern at m/z 278.03995 and at m/z 292.05549, corresponding to selenoneine and methyl-selenoneine, respectively. Selenoneine and methyl-selenoneine were unambiguously identified in blood, liver and kidney tissue of rainbow trout fed by tuna

by-products (Figure S4). To confirm that methyl-selenoneine was originally present in rainbow fish samples and that it was not an artifact due to sample preparation, some samples were reduced with TCEP and derivatized with iodoacetamide. In both cases, methyl-selenoneine was also detected.

The limited Se content in muscle and brain hampers the unequivocal identification of selenoneine by HILIC-ESI-MS/MS in these organs. Nevertheless, the potential presence of selenoneine in these two organs is strongly supported by analyses conducted with two size exclusion chromatography columns with different operational molecular weight separation range (Figure S1 and S3) and the unambiguous identification in the rest of tissues investigated. Selenoneine typically elutes at around 27 min when using SEC peptide[14] column and this Se fraction was observed in the brain and muscle of animals exposed to tuna byproducts-based diets (Figure S3). In a recent study, selenoneine was identified as a key Se species in the brain of top predator marine seabirds[14]. However, due to the limited available mass of trout brain samples, the unambiguous characterization of this Se compound in brain was not achieved in the current study. Further research is needed to identify this compound in fish brains, as this aspect has not been explored before.

The fact that selenoneine was exclusively found in animals fed with tuna byproducts-based diets definitively supports the proposed trophic transfer of this Se species. A kinetic rise of this specific Se-fraction is evidenced in blood (Figure 3). The Se concentration increases with exposure time is much less pronounced in the Se-fractions associated to high molecular weight compounds (between 10-22 min) than for selenoneine, suggesting their regulation and essential implication in metabolism. The Se compounds eluting between 10 and 22 min are probably selenoproteins, recognized for playing essential metabolic roles, like selenoprotein P, glutathione peroxidase among others[14,35,36]. The recent comparison of selenoneine levels in chicks and adult seabirds[14] provides preliminary insights into such trophic transfer, but the present fish study unequivocally demonstrates it.

Selenoneine has been reported as the primary Se species in tuna blood and other organs[37,38]. It has been predominantly identified in marine organisms such as beluga mattaaq (skin)[39], mackerel (blood)[40], sardine (muscle)[41], giant petrel (blood, muscle, brain, kidney and liver)[14] and dolphin (liver)[28]. The current study reports the presence of selenoneine, a potent antioxidant compound, in farmed fish through dietary transfer. This

finding establishes a solid foundation for enriching aquaculture fish species with this beneficial Se compound.

Despite the fate and role(s) of selenoneine in biota not being fully elucidated[41], several studies demonstrate the antioxidant and cytoprotectant functions of this compound in a variety of living organisms[42,43]. The methylated form, Se-methylselenoneine, a metabolite of selenoneine also found in fish organs exposed to tuna byproducts, has been previously reported in human blood and urine[29,43]. Isotopically tracing Se in orally ingested selenoneine ($^{76}$Se) by mice revealed the methylation of this compound in the liver and/or kidney before urinary excretion[29,41].

Numerous studies evoke a potential role of selenoneine in Hg detoxification[14,39,42,44]. Despite the mechanisms being unknown, it has been associated to the demethylation of MeHg through the formation of tiemannite (HgSe)[14,16]. This hypothesis is also supported by previous research, where a diminishment in MeHg bioaccumulation and toxicity on zebrafish embryos was attributed to the presence of selenoneine[44]. In the investigated fish set, dietary MeHg exposure induced a transient reduction in growth performance in rainbow trout that were fed on a plant-based diet along with a transient inflammation characterized by elevated plasma levels of IL-1β and TNF-α[26]. The MeHg intake also induced a long-term oxidative stress with increased mRNA levels of antioxidant enzymes in the brain and liver and a reduction in body mineral retention. These detrimental effects were not observed in fish fed a tuna-based diet.

Generally, Hg bioaccumulation in individuals fed with a tuna byproduct-based diet was lower compared to those fed by plant-based diets, despite the identical dietary MeHg exposure levels in both MeHg-spiked aquafeeds[15]. Several hypotheses have been proposed to explain this trend. One suggests a lower Hg digestibility from tuna byproducts, while another posits an enhancement of Hg excretion by fish as a detoxification mechanism[15,26]. The latter is supported by the notable difference in Hg fecal content between the two dietary groups[26]. Fish exposed to tuna byproduct-based diets exhibit significantly higher levels (four times higher) compared to those fed plant-based diets[26]. Naturally present seleno-compounds in tuna byproducts, accounting for the 80 % of total dietary Se, such as selenoneine[37,44], may play a crucial role in regulating Hg in rainbow trout, potentially masking the effects of supplemented Se species, even if other components of tuna-based diet could be implicated. Further investigation through

toxicological studies is essential to better comprehend this phenomenon and the potential role of selenoneine.

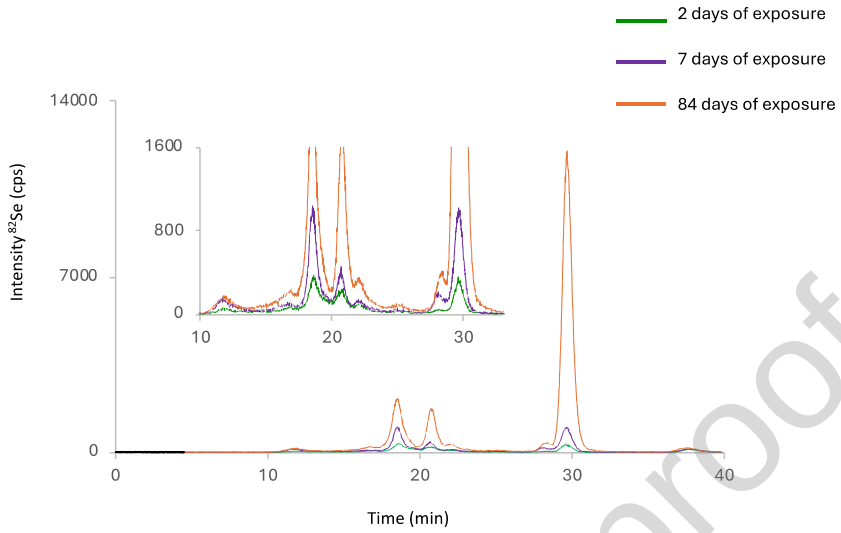


**Figure 3.** $SEC_{200}$-ICP-MS chromatograms ($^{82}Se$) corresponding to the water-soluble fraction of blood of trout after 2, 7 and 84 days of dietary exposure to tuna byproducts-based diet without Se supplementation. The inset shows the corresponding zoomed-in view.

### *3.2.2. Low molecular weight Hg compounds*

#### *Potential occurrence of Hg-metallothioneins (MTs)*

The screening of Hg-containing biomolecules (Figure S2) revealed that, except in blood and muscle, most of the investigated organs primarily contain low molecular weight compounds (eluting after 25 min). In the liver, a target organ for Hg bioaccumulation in juvenile trout, further analysis with a SEC peptide column that offers an enhanced resolution for low molecular weight molecules (Figure 4, Figure S5b) unveiled variations in Hg species distribution among various dietary treatments. Although similar Hg hepatic bioaccumulation was observed in animals exposed to plant-based diets supplemented with MeHg with or without Se species (8 µg Se $g^{-1}$), significant disparities were noted in their biomolecular distribution (Figure 4a).

Interestingly, the fraction eluting at 13.8 min using the SEC peptide column was not observed in the hepatic organ of animals fed a plant-based diet and co-exposed to both MeHg and SeMet (Figure 4). This observation contrasts with the results of exposure to MeHg alone and the co-exposure of MeHg and Se(IV). The co-exposure of Se(IV) or SeMet with MeHg, reduces the intensity of this Hg fraction in animals exposed to supplemented tuna-based diets (Figure 4a). The divergences between Hg distribution

according to the un-supplemented based aquafeeds could be due to the presence of selenoneine, exclusively found in the tuna-based ones. The unambiguous identification of this Hg fraction can provide valuable insights into the dynamics and interactions of Hg and Se within this crucial organ. Animals exposed to supplemented tuna-based diets display a different trend. In this case, the Hg fraction at 13.8 min was observed under all treatments supplemented with MeHg (2.5 μg Hg $g^{-1}$) and it was not present in the control group (exposed to Hg levels at 0.2 μg Hg $g^{-1}$). Notably, this Hg fraction co-elutes with elements typically associated with metallothioneins (MTs), such as Zn, Cu, and Cd (Figure S6 left panel). The retention time (13.8 min) is also consistent with MTs molecular weight fraction (6-7 kDa). Analysis by HILIC-ICP-MS consistently reveals patterns where the major Hg fraction co-elutes alongside these metals (Figure S6 right panel). Metallothioneins, characterized by their high sulfur content, primarily as thiolate functional groups (~30% of cysteines), are well-known for their capacity to chelate a wide range of metals[27,45]. The co-elution of Hg with these elements is not a definitive marker for identifying such fractions, but strongly indicates the potential presence of Hg-bound MTs. While the exact biological functions of MTs are still not fully understood, it is hypothesized that they play pivotal roles sequestering and maintaining the balance of essential elements such as Zn and Cu[46], as well as in the detoxification mechanisms targeting Cd and Hg[27,33,47]. The mentioned Hg-fraction was detected in liver, kidney and brain of rainbow trout exposed to all tuna aquafeeds and to MeHg-plant based diets. The binding of Hg by MTs has been reported in dolphin liver[33] and wild blue mussels[48]. However, to the best of our knowledge, there is no structural identification of MTs binding Hg in fish. Kinetic tracking of this fraction in the liver revealed an increase over time of this specific fraction (retention time 13.8 min) when animals were dietary exposed to MeHg (Figure S7). To confirm the co-elution and presence of Hg, Cu, Zn and Cd MT complexes additional characterization of the major Hg-containing fraction was performed by the HILIC-ESI-MS analysis of the liver extract. Several molecules from 3 to 7 kDa were observed around the retention time of the major peak of Hg observed by HILIC-ICP-MS. Among these molecules, a few of them had an isotopic pattern much broader than other molecules, which is typical of the presence of several metallic ions in MTs[33]. As shown in Figure S6, copper is the predominant metal ion present at this retention time among the metal able to bind MTs (Cu, Zn, Cd, Hg). Therefore, it was expected to find holo-MTs forms containing almost only Cu. Some proteins ranging from 3.2 to 3.6 kDa were fragmented and were found to be fragments of the β chain of trout MTs (Table S2

and Figure S8). To find partially degraded MTs is coherent with protein recycling that can occur in liver and with the fact that the amino acids between the two MTs domains are probably the more exposed to enzymatic cell lysis when MTs are binding metals. Two intact proteins around 6.7 kDa were detected. According to the composition of metallothionein β domain and to the exact masses of the two intact MTs, their expected amino acids composition and metal composition was confirmed as shown in Table S2 and Figure S9. They correspond to metallothionein A (P68503 · MTA_ONCMY) and metallothionein B (P68501 · MTB_ONCMY) from the rainbow trout, *Oncorhynchus mykiss*, in their N-terminal acetylated forms and that are binding 10 Cu(I) ions each. As demonstrated in previous studies[49], β domain is expected to be metalated first by 6 Cu(I) ions and then the complete MTs is found to be more stable with 10 Cu(I), which is totally consistent with the experimental observation in trout MTs. Even if the adduct of Hg ions is not detectable because of its low abundance compared to Cu, the coelution of Hg in this fraction with Cu, Zn, Cd and MTs in both chromatographic settings are clearly due to its binding to trout MTs.

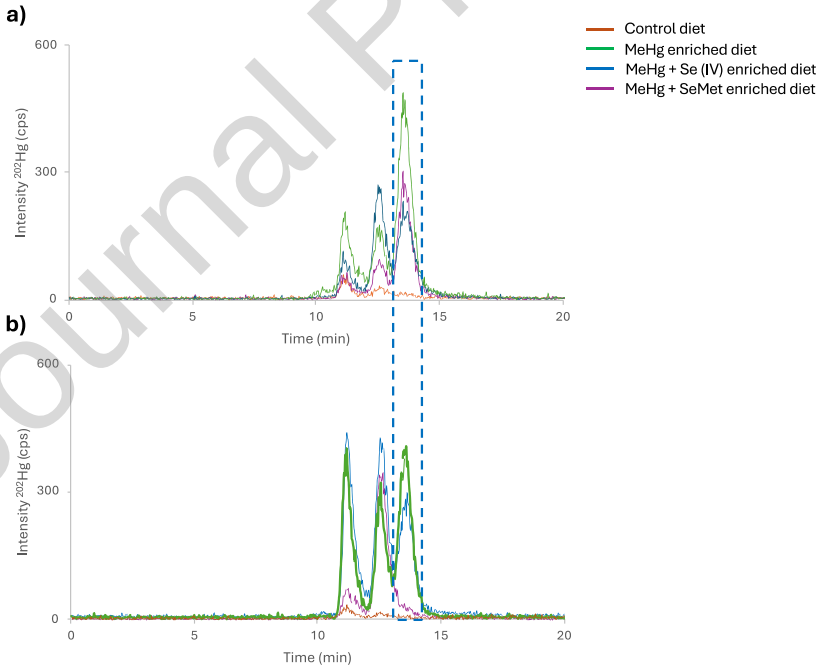


**Figure 4.** Typical SEC peptide column-ICP-MS chromatograms of $^{202}$Hg corresponding to the water-soluble fraction of the liver of juvenile trout exposed during 168 days to different dietary conditions a) tuna byproducts-based diets and b) plant-based diets.

***MeHg binding cysteine and glutathione***

In the low molecular weight fraction, alongside the previously described fraction eluting at 26 min using the HILIC-ICP-MS, two other Hg-containing fractions were observed, eluting at 22.4 and 23 min (Figure S10a). The analyses by HILIC-ESI-MS reveal that these two Hg species (Figure S10 b and c) correspond to MeHg-glutathione (GSH) (m/z=524.08 $C_{11}H_{20}HgN_3O_6S^+$) and MeHg-cysteine (Cys) (m/z=338.13 $C_4H_9HgNO_2S^+$).

The GSH-MeHg complex was identified in all tissues of trout fed by both tuna and plant-based diets, irrespective of co-exposure to Se species. Mercury bioaccumulation in fish has been strongly associated with oxidative stress, although the underlying mechanisms remain understood[50-52]. GSH is recognized for its role in eliminating reactive oxygen species[53-55] and has been utilized as a biomarker of Hg exposure. The correlation between GSH and MeHg concentrations, along with the coeluting retention times of MeHg-GSH and MeHg standards, is often interpreted as indicative of their interaction[56]. Few studies report the unambiguous identification of this complex, as seen in HepG2 cells[56] and recently in phytoplankton[34].

Rat model experiments demonstrate MeHg's biliary excretion from the liver, mediated by GSH[57]. More recently, the MeHg-GSH complex was unambiguously identified as an excretion product in HepG2 cells, understood as a MeHg detoxification process[56]. The antioxidant GSH system is an important target in mediating Hg toxicity[58], and different studies have shown a reduction of GSH in the living organisms exposed to this element[59,60]. Herein the identification of this complex for the first time in tissues of (freshwater) fish suggests a potential key role in Hg metabolism, warranting further investigation.

MeHg was also found bound to Cys, forming the MeHg-Cys complex, in several tissues of trout depending on the administered diet. In blood, kidney, and brain tissues, MeHg-Cys was detected in all samples analyzed, regardless of the supplementation condition of MeHg and/or Se in both tuna byproduct- and plant-based diets. Regarding muscle tissue, MeHg-Cys was identified in all samples fed with tuna byproduct-based diets, regardless of supplementation with MeHg and/or Se, while in plant-based diets, it was only detected in those supplemented with MeHg. Finally, in the liver, MeHg-Cys was found in all conditions of MeHg and/or Se supplementation for both tuna byproduct- and plant-based diets, except for the supplementation of MeHg along with SeMet in plant-based diets.

MeHg-Cys may act as a substrate for Hg transport to key internal tissues[58,61-63]. This complex is known to mimic methionine, allowing among others, MeHg to cross the blood-brain cell membranes[62]. MeHg has been reported to bind with Cys residues in proteins as thioredoxin reductase[64] and hemoglobin[28]. In fish, most of the studies on Hg speciation have been exclusively conducted on muscle tissue. X-ray absorption analyses have revealed that MeHg in fish muscle is primarily bound to Cys containing compounds[65,66], corresponding to animals from environments with varying levels of Hg. HPLC-ESI-MS studies on dogfish muscle highlights the presence of both ‘‘free’’ MeHg-Cys and ‘‘bound’’ MeHg-Cys residues after enzymatic hydrolysis[63].

The screening for Hg in the water-soluble fraction unveils a significant trend across various organs of animals exposed to both types of diets. It clearly indicates that Hg shows a strong association to LMW biomolecules (< 10 kDa, Figure S2). Moreover, the discussed kinetic tracking in kidney unveils a significant transfer of Hg from HMW proteins to the LMW metabolites. These findings strongly suggest a potential MeHg detoxification mechanism in the investigated fish model, emphasizing the need for further comprehensive analyses.

It should be noted that the Hg biomolecular distribution observed in this study is linked to specific dietary sources. Further research on animals exposed to different conditions could be valuable to investigate the fate of Hg from other sources, such as emissions and discharges from offshore oil, gas and gas production which may pose a threat to aquatic environments due to potential leakage and discharges relating to the production processes. More recently several publications have highlighted the environmental impact from decommissioning activities for offshore platforms which a potential release of mercury from contaminated equipment and pipelines[67-69].

One of the main perspectives of the current work is the isotopic characterization of Hg[12,70-72] and Se to obtain complementary and dynamic information about such mechanisms.

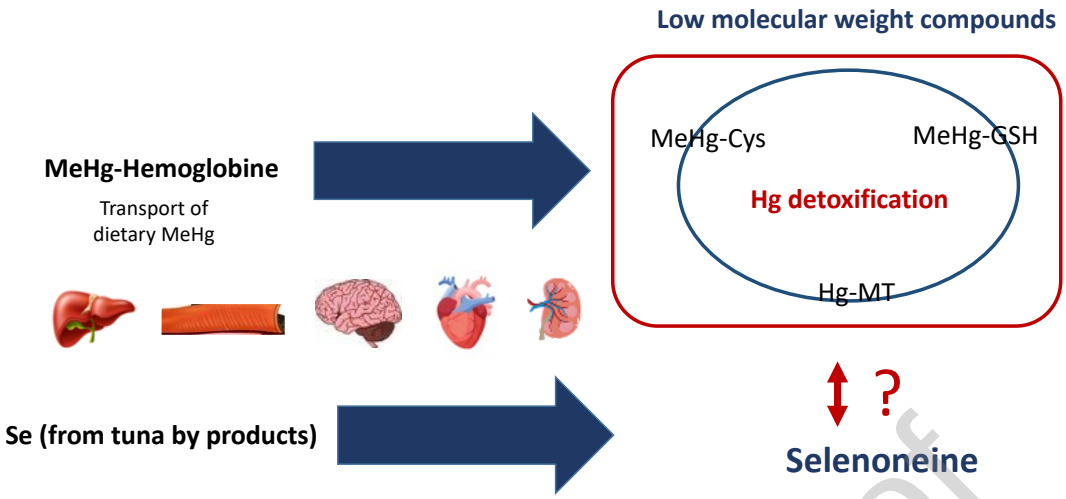


**Figure 5.** Diagrammatic representation of concluding results.

## 4. Conclusions:

In summary, this comprehensive study provides new insights into Hg dynamics in fish, with global implications for understanding the environmental impact of Hg in biota. The findings suggest a detoxification mechanism through the binding of Hg to low molecular weight compounds such as cysteine, glutathione, and metallothionein. Additionally, the research highlights the trophic transfer of selenoneine, a key antioxidant Se species, observed for the first time in freshwater fish. These findings underscore the importance of further exploring Hg-Se interactions in fish to optimize feed formulations and mitigate environmental risks. This research advances the potential use of tuna byproducts in sustainable aquafeed formulations, promoting environmentally friendly aquaculture practices and addressing food security challenges.

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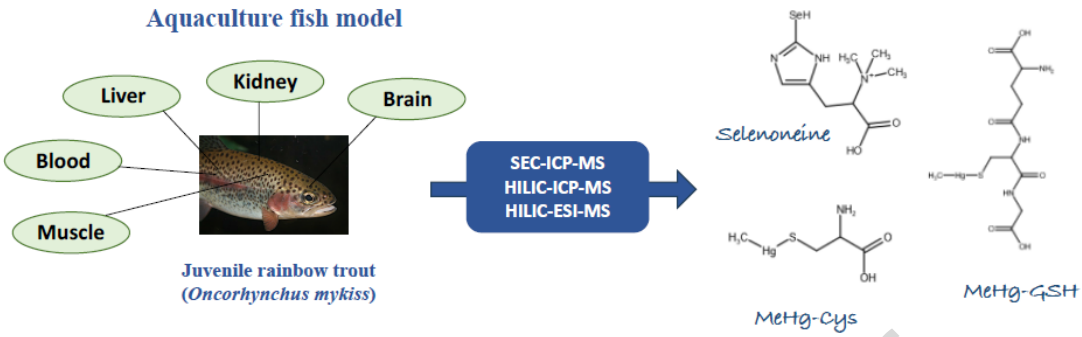
Graphical abstract



**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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

**Highlights:**

- New insights on the fate of dietary Hg exposure in fish.
- Potential MeHg detoxification mechanism in fish model unveiled.
- First time identification of MeHg-GSH in fish
- Aquafeed sustainability enhanced by tuna byproducts.
- First evidence of selenoneine trophic transfer in freshwater fish.