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Abstract

This study aims at the assessment of mercury (Hg)-selenium (Se) antagonism in fish. For this purpose, rainbow trout fish (Oncorhynchus mykiss) were exposed to methylmercury (MeHg) under controlled conditions, in the presence or absence of selenomethionine (SeMet) using an in-house prepared diet (enriched with MeHg and SeMet at 0.2 μg/g and 5.0 μg/g, respectively). The total duration of the exposure study was 3 months. Fish was sampled and analysed for total Se (Se_T) and total mercury (Hg_T) content after 1, 2 and 3 months of exposure. Six feeding protocols were compared, depending on the exposure type: *(i)* no MeHg nor SeMet exposure (control group); *(ii)* exposure to SeMet solely; *(iii)* exposure to MeHg solely; *(iv)* exposure to both MeHg and SeMet; *(v)* exposure first to MeHg during 1 month and then to SeMet during 2 months and (vi), exposure to SeMet during 1 month and then to MeHg for 2 months. The levels of Se_T and Hg_T in the fish (control and supplemented with MeHg/SeMet) were measured by inductively coupled plasma-mass spectrometry (ICPMS).

Steadily (linear) bioaccumulation of MeHg in the fish muscle occurred when the fish were exposed individually to this species during the period. The bioaccumulation of MeHg is diminished when the fish are firstly exposed to SeMet and then to MeHg, hence indicating the MeHg detoxification due to SeMet supplementation.

Keywords: Mercury; selenium; antagonism; rainbow trout fish; dietary exposure; ICP-MS

1. Introduction

The high nutritional value of fish makes it an ideal component of a healthy and balanced diet for humans. However, some fish species accumulate substantial levels of trace toxic metals, such as mercury (Hg) hence hampering the consumption of such diet (Cabanero et al. 2004). Actually, the fish represents the main pathway (80-99%) of humans' exposure to Hg via methylmercury (MeHg) species most commonly found in fish and which is considerably more toxic than its inorganic precursor (Hg^{2+}) (WHO 2008; Eagles-Smith et al. 2018).

Humans' exposure to MeHg poses a risk for their health especially due to its neurotoxic effects, being considered a major public health concern by World Health Organization (WHO 2008). These effects arise from its high biological membranes permeability, potent bioaccumulation, stability and long-term elimination from tissues (Wang et al. 2003; EFSA 2012).

Fish is therefore a dietary product for which suitable measures should be taken to minimize the risks related to Hg exposure (McEneff et al. 2017). It is worth to note that fish is also an important contributor of selenium (Se) to humans, mostly as selenomethionine (SeMet), generally around 40–50% of the total Se diet (Misra et al. 2012). Nevertheless, Se has a narrow threshold between essentiality and toxicity and becomes toxic to fish at a slightly elevated level beyond the optimum intake (Misra et al. 2012; Ralston et al. 2016).

Although Hg interaction with sulphur-containing proteins/enzymes in fish is relatively well understood, the mechanism of its interaction with Se compounds such as selenoproteins is not completely known. Nevertheless, it appears that Se species can play a role in Hg detoxification hence mitigating the human exposure to MeHg contamination (Dang et al. 2011; Luque-Garcia et al. 2013). The current evidence suggests that the protective effect of Se against Hg may be related to the amount of Se available. It is generally accepted that the molar concentrations of Se and Hg in protected tissues need to exceed a 1 : 1 stoichiometry to be effective (Gochfeld et al. 2021).

Underlying the central paradigm that Hg exposure is less hazardous when tissue Se is in (molar) excess it is suggested that the Se protection occurs through Hg sequestration. Hg shows high

affinity for Se leading to HgSe precipitation, a species that seems to be metabolically inert (Raymond et al. 2020). In addition, it is suggested that the protective effect of Se depends on its availability for the selenoprotein synthesis (Manceau et al. 2021; Castriotta et al. 2020).

This study aims at the assessment of the dynamics of dietary MeHg in a fresh-water fish in the presence of SeMet, to understand the interactive effects of Se on Hg bioaccumulation as well as to elucidate the antagonistic relationship observed in other studies (Dang et al. 2011). For this purpose, an exposure study of dietary exposure to MeHg and SeMet of rainbow trout under various conditions such as exposure to MeHg and SeMet solely, simultaneously and sequentially, was carried out. The exposure study was carried out during a relatively long duration (3 months) to reflect the chronic exposure conditions (Ralston et al. 2009). The diet employed here prevented also acute, high exposure of fish with Hg and Se, which in the real world would happen only accidentally, as it was the case of MeHg poisoning in Minamata (Harada 1995). A novel method was also validated by using inductively coupled plasma-mass spectrometry (ICP-MS) for the simultaneous determination of trace and ultra-trace total Hg (Hg_T) and total Se (Se_T) in the muscle of the rainbow trout exposed to SeMet and MeHg.

2. Materials and Methods

2.1. Instrumentation

An ICP-MS iCAP Q (ThermoFisher Scientific, Courtaboeuf, France) equipped with a PFA standard nebulizer and a Teflon spray chamber was used for the determination of Hg_T and Se_T. Kinetic energy discrimination (KED) using helium (He) as a collision gas was employed throughout the study for interferences removal, namely the polyatomic interferences $^{40}Ar^{38}Ar^{+}$ and $^{38}Ar^{40}Ca^{+}$ on ^{78}Se isotope. The samples were digested using a closed microwave system (Multiwave 3000, Anton Paar, Courtaboeuf, France) equipped with a rotor for 80 mL quartz vessels.

98 ICP-MS measurement conditions were optimized daily by running short-term sensitivity and
99 stability tests using the KED mode. Data were processed using ThermoFisher Scientific Qtegra
100 software.
101 The optimum operating conditions of the iCAP-Q ICP-MS are summarized in Table 1.

Table 1. ICP-MS operating conditions for Se_T and Hg_T determination in fish.

Plasma power	1550 W
Reflected power	<5 W
Plasma gas flow (Ar)	14.0 L/min
Auxiliary gas flow (Ar)	0.8 L/min
Nebuliser gas flow (Ar)	1.0 ± 0.1 L/min (depending on daily optimisation)
KED gas flow (He)	5.0 mL/min
Spray chamber type & temperature	Quartz, concentric, 2.7 °C
Nebuliser type	PFA-ST Microflow
Monitored isotopes	^{78}Se, ^{202}Hg, ^{89}Y, ^{185}Re
Dwell time	0.1 s
Measurements number	5

102

103 ***2.2. Chemicals and reagents***

104 All solutions were prepared using analytical reagent grade chemicals and ultrapure water (18.2 MΩ
105 cm^{-1}, Millipore Milli-Q™, Merck Millipore, Saint Quentin in Yvelines, France). Suprapur HNO_3
106 (67% m/m) was purchased from VWR (Fontenay-sous-Bois, France). The standard stock solutions
107 at 1000 mg/L of Hg, Se, Au, Re and Y were purchased from SCP Science (Courtaboeuf, France).
108 The intermediate solution of Se and Hg at 1.0 mg/L (available for one month when stored at 4-5 °C)
109 was used for the daily preparation of the calibration solutions.
110 The tuning solution for the daily optimization of the ICP-MS consisted of Ba, Bi, Ce, Co, In, Li and
111 U at 1.0 µg/L in HNO_3 2% (v/v) and HCl 0.5% (v/v) (Tune B iCAP Q/RQ) and it was purchased
112 from Thermo Fischer Scientific (Courtaboeuf, France).
113 The certified reference materials (CRMs) used in this study were ERM®-BB-422 (fish muscle) and
114 ERM®-CE101 (trout muscle) from the European Joint Research Centre (JRC), DORM-4 (fish
115 protein) from the National Research Council Canada and 7402-a (codfish tissue) from the National
116 Metrology Institute of Japan and they were purchased from LGC Standards (Molsheim, France).

117 Ultrapure grade (99.9995%) argon and helium employed in ICP-MS were supplied by Linde
118 (Montereau, France).
119 High purity seleno-DL-methionine (≥99%) and methylmercury chloride (802 µg/mL ± 2%) were
120 obtained, from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and LGC Standards, respectively.
121 The fish feed used for the dietary exposure study was purchased from Nouvelles Vague, Wimereux,
122 France. Commercial dried pellets (T-PRIMA - 5 mm and semi-floating) were obtained from Le
123 Gouessant Aquaculture, Lamballe-Armor, France. The different enriched diets were prepared by
124 Nouvelle Vague company using an in-house developed process (based on vacuum technology)
125 allowing the enriching compounds (contained in 1% of an aqueous phase of an oil emulsion) to
126 achieve the pellets' core. The aim was to avoid the dispersion of MeHg and/or SeMet into the tank
127 water during fish feeding. For confirming that, Se_T and Hg_T levels were measured by ICP-MS in the
128 water solution in contact with the pellets. Once the optimum conditions were achieved, appropriate
129 amounts of control and supplemented (semi-floating) diet were produced, as follows:
130 *(i)* 5 kg of non-supplemented diet (pellets), coated with the emulsion solely (without MeHg or
131 SeMet);
132 *(ii)* 10 kg of diet supplemented with MeHg at a final concentration of 0.2 mg/kg;
133 *(iii)* 10 kg of diet supplemented with SeMet at 5.0 mg/kg;
134 *(iv)* 5 kg of diet supplemented with both MeHg (0.2 mg/kg) and SeMet (5.0 mg/kg).
135 The levels of Hg_T and Se_T in the fish diet (control and enriched with SeMet and MeHg,
136 respectively) were measured by ICP-MS using an accredited method (by COFRAC, the French
137 accreditation body), as reported by Chevallier et al. 2015; each diet was analysed in three
138 independent replicates to assess the average levels of MeHg and/or SeMet but also their
139 homogeneity. Hg_T levels measured in the control and MeHg-spiked diets were 0.052 ± 0.002
140 (RSD=3.6%) and 0.24 ± 0.01 mg/kg (RSD=4.1%), respectively, whereas SeT were 0.95 ± 0.02
141 mg/kg (RSD=1.7%) and 5.41 ± 0.36 mg/kg (RSD=6.6%) in the control and the spiked diets
142 enriched individually with MeHg and SeMet. SeT and HgT were 6.08 ± 0.02 (RSD=8.8%) and 0.26

± 0.02 (RSD=6.3%), respectively, in the diet enriched with both MeHg and SeMet. The relative standard deviation values (reported into the brackets) are < 10% in all cases, hence confirming the satisfactory homogeneity of the diets used in this study.

2.3. Fish

In this study, 95 specific pathogen-free rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) including a mixture of males and females from the same genetic group were used. They were handled at the protected and monitoring facilities of the Plouzané (France) laboratory of ANSES. The fish were 18 months old, with a weight of 266 ± 20 g. They were fed once a day by using the in-house prepared diet (dry pellets) at 1.5% of their body weight.

2.4. Experimental exposure of fish to MeHg and SeMet species

2.4.1. Authorization to experiment and ethical aspects

This exposure experiment was carried out in accordance with the European guidelines and recommendation on animal experimentation and welfare (European Union Directive 2010/63). The ANSES (Plouzané) laboratory is authorized to carry out experiments on fish in its facilities in compliance with the Administrative Order No. E29-212-03 issued by the Prefecture of the Finistère (France). This animal experimental procedure was also approved by the ethics committee on animal experimentation ANSES/ENVA/UPC No. 16 and was authorized by the French Ministry of Research, under the number APAFIS# 2019071109391903.

2.4.2. Experimental design

The experimental system consisted of six cylindrical 400 L resin tanks. In order to minimize the tank effects, the same fresh river water flow at a flow of 0.5 m^3/h was added to each tank to maintain optimal water conditions for the fish throughout the experiment, such as: river water temperature ≅ 12 ± 2 °C, oxygen saturation >80% of full air saturation, pH ≅ 8 and water free of

nitrate and nitrite. Additionally, all the tanks were maintained in a natural light/dark cycle (10 h/14 h in winter, approximately) in a room with air volume changes every hour.

The fish were randomly distributed into the six tanks. After one week of acclimation, six groups were defined according to the daily diet supplemented with MeHg and/or SeMet for a duration of three months (from January 2020 to April 2020), as follows: control (G0), exposed to SeMet (G1), exposed to MeHg (G2), exposed simultaneously to SeMet and MeHg (G3), exposed initially to SeMet (one month) and then to MeHg (two months) (G4), and exposed initially to MeHg (one month) and then to SeMet (two months) (G5). The experimental scheme of the exposure study is provided in Fig. 1.

The dietary exposure to MeHg of the trout used in this study was chosen in agreement with the EC Regulation No. 1881/2006, which defines the maximum limit of Hg in fishery products, namely in the rainbow trout, at 0.5 mg/kg (wet weight, ww). The exposure level used in the study was set at 40% below the EC maximum limit, to avoid mortality, taking into account the duration and the slow rate of Hg elimination from fish. The dietary exposure concentration for Se was fixed at an intermediary level in agreement with previous results from the literature (Misra et al. 2012; Sele et al. 2018; McEneff et al. 2017).

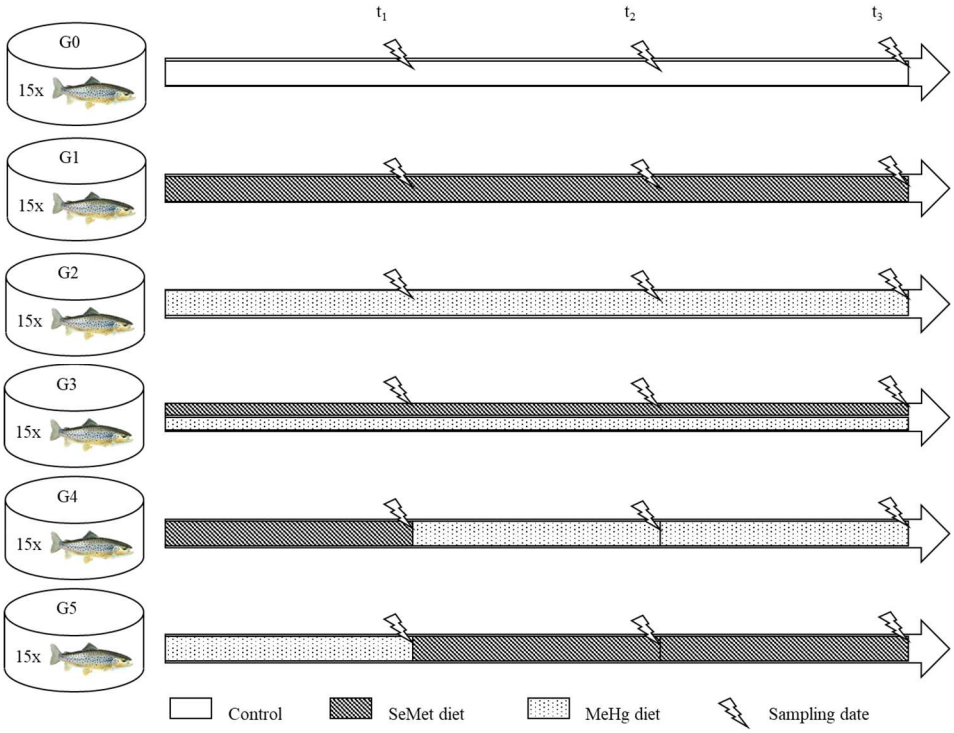


Fig. 1. Schematic representation of the *in vivo* exposure of rainbow trout to MeHg and SeMet.

2.5. Samples and sampling date

Five control fish were randomly sampled in the fish farming of ANSES just before the beginning of the trial (t_0). Amongst the fish exposed to MeHg and SeMet, five individuals of the corresponding exposure tank were sampled after one (t_1), two (t_2) and three (t_3) months of exposure. The fish were sacrificed by cutting their spinal cord; they were then measured and weighed. The fish muscles were sampled using a standardised cut. Scalpel blades were used to cut the entire fillet from the operculum to the tail, along the vertebral axis. The samples were kept on ice during sampling and transportation to the laboratory and then stored at −20 °C until analysis. The data related to the fish growth, including the global growth during the whole exposure study (t_3-t_0) are reported in Table 2.

Table. 2 Fish weight (g ww, mean ± SD[a], n = 5) for each condition at the different sampling dates.

Exposure group	**Fish weight (g)**				**Global growth [a] (g)**
	t_0	**t_1**	**t_2**	**t_3**	
G0: Control	256.0 ± 22.7	279.6 ± 19.6	499.2 ± 47.6	542.4 ± 35.3	286.4
G1: SeMet		334.4 ± 53.3	590.8 ± 140.1	636.4 ± 135.1	380.4
G2: MeHg		392.0 ± 37.1	541.6 ± 23.8	692.4 ± 197.8	436.4
G3: SeMet and MeHg		395.6 ± 44.0	512.8 ± 107.5	725.6 ± 72.2	469.6
G4: SeMet (one month) then MeHg (2 months)		361.2 ± 58.5	492.0 ± 136.6	640.0 ± 92.8	384
G5: MeHg (one month) then SeMet (2 months)		376.8 ± 38.7	510.8 ± 64.0	667.2 ± 72.1	411.2

[a] standard deviation

[b] weight at t_3 – weight at t_0

As can be seen from the Table 2, similar growth was observed for all exposure conditions, hence no corrections related to the growth process were necessary.

2.6. Analytical procedures

2.6.1. Sample preparation

The frozen fillets were thawed and then grounded and homogenized, prior to analysis. Approximately 0.5 g of samples were accurately weighed into a 80 mL quartz vessel and then thoroughly mixed and digested with a mixture of 3 mL of concentrated HNO_3 and 3 mL of ultrapure water using a closed microwave system; for this purpose, the blend was exposed to

206 microwave power of 800 W for 10 min, then increasing the power up to 1000 W and the sample
207 kept in this condition during 10 min. Then the digests were quantitatively transferred into
208 polypropylene tubes (50 mL) and then diluted to 50 mL with ultrapure water after the addition of
209 the internal standards (Y for Se and Re for Hg at 2.0 µg/L.
210
211 *2.6.2. Determination of total Hg and Se by ICP-MS and the internal quality control assurance*
212 The quantification of Se_T and Hg_T in the fish digests was carried out by ICP-MS using the external
213 calibration in the range 1.0 to 20 µg/L. Several internal quality controls (IQCs) were set up to
214 ensure the reliability of the results (Chevallier et al. 2015; Ghosn et al. 2020; Saraiva et al. 2021);
215 the data were valid only when all the acceptance criteria were satisfied.
216 The method trueness was assessed by analysing certified reference materials (CRM) in parallel with
217 each batch of samples. Each analysis run included also one digestion blank (to monitor possible
218 contaminations due to sampling preparation), one spiked sample (to assess the matrix effects), and a
219 mid-range standard solution containing Hg and Se (at 5.0 µg/L) that was analysed every ten
220 samples and also at the end of the sequence, to monitor instrumental drift; the data were validated
221 only when the deviation of the concentration of this control standard was ≤20% compared to the
222 theoretical value.
223 The variation of the IS (Y and Re) was also monitored to assess the instrumental drift and matrix
224 effects. In most cases, the recovery of IS was between 80% and 120%.
225 Finally, a solution of gold (Au) standard at 10 mg/L prepared in 6% HNO_3 (v/v) was used
226 throughout as a washing solution in between the analyses to reduce the memory effects related to
227 Hg analysis. Additionally, the elimination of the memory effects was assured by employing the
228 «Intelligent Rinse» mode of the ICP-MS. Briefly, this consists of the rinsing of the system after
229 each measurement as long as the analytes signal has not fallen below a certain threshold in terms of
230 intensity: in our case, this was set at 20 cps for ^{82}Se and at 800 cps for ^{202}Hg.
231
232 ***2.7. Data and Statistical analysis***

The bioaccumulation factor (BF, %) of Hg from the diet to the muscle of the rainbow trout fed with MeHg supplemented diet (G2) was assessed. The same study was carried out for Se, i. e., calculation of the BF of Se from the diet to the fish muscle (%) in the fish group fed with SeMet (G1), using the equation provided below.

$$BF\ (\%) = ((C_{muscle} \times BM)_{\text{three months}} - (C_{muscle} \times BM)_{\text{control group}})/(C_{diet} \times FI)) \times 100$$

where:

BM, biomass muscle corresponding to 66% of the weight of the whole fish (mg);

C_{muscle}, Se or Hg mass fraction in muscle (mg/kg);

C_{diet}, Se or Hg mass fraction in diet (mg/kg);

FI, feed intake, corresponding to the amount of the diet consumed by the fish (1.5% body weight) (mg).

The statistical data treatment was carried out using Statgraphics Centurion® software. All data sets were first tested for normality (Shapiro–Wilk test). To detect significant differences among the different dietary exposed groups, Hg_T and Se_T levels in muscle were analysed using one-way analysis of variance (ANOVA) followed by the application of Tukey–Kramer HSD test with a $p < 0.05$. For all analyses, 95% confidence interval was applied with a level of $p < 0.05$ considered to be significant. All data related to the fish analysis are reported as mean ± standard deviation (n = 5).

3. Results and discussion

3.1 Method validation

The method employed in this study for the simultaneous quantification of Hg_T and Se_T in fish samples is based on a previously published study dealing with multi-elemental determination in food matrices (Chevallier et al. 2015). The sample preparation consisted of microwave (acidic) assisted digestion as described briefly in the section 2.5.1. The validation of the method was carried out using the accuracy profile approach based on the French standard NF V03-110: 2010 (NF V03-110 2010). Briefly, the accuracy profile combines the systematic (trueness) and the random error

(repeatability and/or intermediate precision) for a series of analytes' levels in various matrices within a range of concentrations called the validity domain (Ghosn et al. 2020; Mermet et al. 2012). To construct the accuracy profile, the accuracy was assessed for each analyte (Se_T and Hg_T) at 3 different levels corresponding to three CRMs, as follows: ERM®-CE101 (trout muscle), NRC DORM-4 (fish proteins) and the NMIJ CRM 7402-a (codfish tissue), comprising, respectively, a low level, an intermediate level and a high level of the target elements. A series of five measurement were carried out in duplicate on (five) different days over a timespan of three months. For each validation series, an (external) calibration was carried out also in duplicate at seven levels (0, 1.0, 2.0, 5.0, 7.5, 10 and 20 µg/L).

In this study, the probability β was set at 80% for Se_T and at 70% for Hg_T, which means that the risk of expected results falling outside these limits is <20% and <30%, respectively. The acceptance limits (λ) were set to ± 25% for Se_T and to ± 35% for Hg_T. The accuracy profiles obtained for Se_T and Hg_T determination in fishery products by ICP-MS are provided in Figs. 2 and 3.

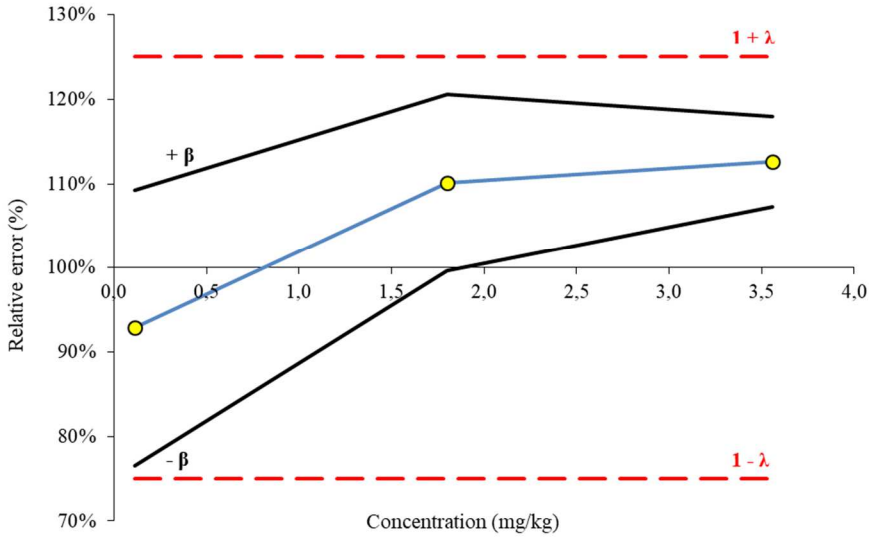


Fig. 2. Accuracy profile for Se_T determination in fish by ICP-MS following (closed) microwave acid digestion (β = 80%; λ = 25%). Upper acceptance limit: 1+λ; lower acceptance limit: 1-λ; upper acceptance limit: +β and lower acceptance limit: -β.

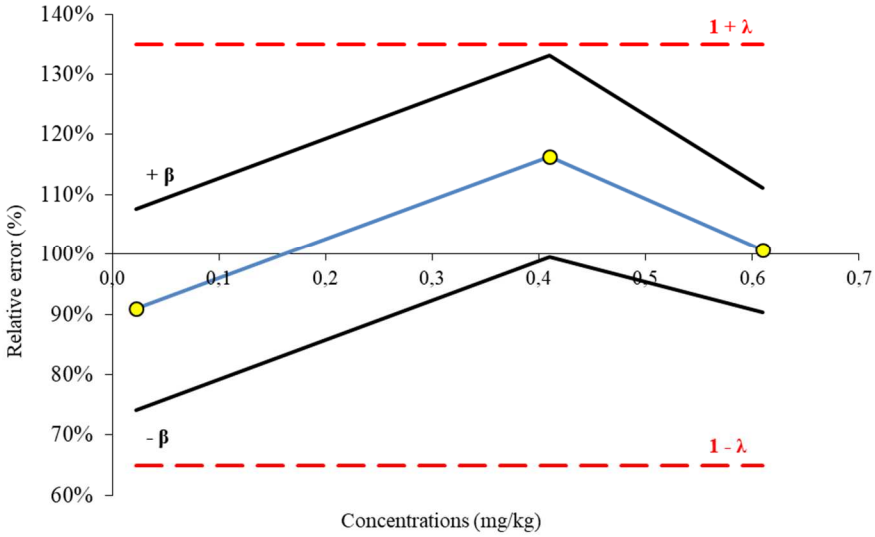


Fig. 3. Accuracy profile for Hg_T determination in fish by ICP-MS following (closed) microwave acid digestion (β = 70%; λ = 35%). Upper acceptance limit: 1+λ; lower acceptance limit: 1-λ; upper acceptance limit: +β and lower acceptance limit: -β.

As can be seen, β values are comprised within the acceptability limits for the three levels for which the method validation was carried out; this corresponds to a validity domain comprised between 0.11 and 3.56 mg/kg for Se_T and 0.022 and 0.61 mg/kg for Hg_T (ww in all cases).

The bias ranged from 1.0% to 16% for Hg_T and between 7.0% and 13% for Se_T.

The repeatability (expressed as the coefficient of variation, CV_r), varied from 3.3% (highest level) to 6.0% (lowest level) for Se_T and between 1.4% to 6.5% (highest level) for Hg_T determination. The intermediate reproducibility (CV_R) was comprised between 8.6% (highest level) and 14.4% (lowest level) for Hg_T and between 3.3% and 11.0% for Se_T determination.

The limits of detection (LOD) and quantification (LOQ) were determined by the analysis of 21 analytical blanks. The LOQ was calculated as 10 × standard deviation of the blank's levels (n = 21) and the LOD as 3/10×LOQ. LOQ of 0.050 mg/kg for Se_T and 0.019 mg/kg for Hg_T determination, respectively, were obtained, while the LOD were 0.015 mg/kg for Se_T and 0.0057 mg/kg for Hg_T. The method accuracy at the LOQ levels was also assessed in this study. For this purpose, ten solutions spiked with Se_T and Hg_T at the LOQs levels were analysed and in all the cases the method accuracy at such low levels was confirmed.

298 ***3.2. Exposure study***

299 In this study, physiological status parameters, as the weight and the length of the fish before the
300 exposure study and at the moment of the sampling, were determined. The muscle biomass was
301 estimated based on the weight of the whole fish, assuming that 66% of its weight consists of muscle
302 (Sele et al. 2018). As it was already mentioned, the feed intake was set at 1.5% of the body weight.
303 Using this feed intake, no mortality at all was encountered during this exposure study.

304 It is worth to note that the choice of Se and/or Hg species to be used for the fish exposure studies is
305 still a matter of debate (Ralston et al. 2009). Several studies used inorganic Hg and Se forms, which
306 may not accurately reflect the natural conditions (Ralston et al. 2007).

307 In our study, MeHg and SeMet species were chosen for the exposure study to reflect the real-life
308 conditions. Due the fact that rainbow trout is a carnivore fish, and the fish bioaccumulate Hg in the
309 form of MeHg (Dang et al. 2011; Wang et al. 2019), whereas the predominant Se form in marine
310 animals is present as selenoproteins, including SeMet (Pedrero et al. 2009; Dang et al. 2011; Misra
311 et al. 2012), we chose to expose the fish to SeMet and MeHg during our exposure study. Therefore,
312 enriching the (in house) diet used for the exposure study with these species mimics the chemical
313 forms present in the natural prey, thus, the natural fish diet (Dang et al 2011; Misra et al. 2012).

314 In this study, Se_T and Hg_T levels solely were determined; indeed, speciation analysis of MeHg and
315 SeMet (and other Se species) in the fish muscle could have provided more comprehensive data
316 regarding the distribution of Hg and Se species in fish upon exposure. Nevertheless, it is known that
317 the predominant species in fish muscle are SeMet and MeHg and when the fish diet is supplemented
318 with organic species (e. g. MeHg and SeMet), Se_T and Hg_T are highly correlated with the levels of
319 SeMet and MeHg, respectively (Sele, et al. 2018; Dang et al 2011; Misra et al. 2012).

320 The results in terms of Hg_T and Se_T levels in diets, in muscle and the BFs of Hg and Se from the
321 diet to the muscle of the Rainbow trout are reported in the Table 3.

Table 3. Hg_T and Se_T levels in diets (mg/kg ww, mean ± SD, n = 3) and muscle tissue (mg/kg ww, mean ± SD, n = 5), as well as the bioaccumulation factors (BF, %) from the diet in the muscle tissue of rainbow trout.

Sample	Mass fraction (mg/kg ww)		BF (%)	
	Hg	Se	Hg	Se
Diet	0.241 ± 0.049	5.41 ± 1.62		
Muscle at t_0	0.027 ± 0.002	0.14 ± 0.013	368	99
Muscle at t_3	0.140 ± 0.008	0.91 ± 0.117		

As can be seen from Table 3, excellent bioaccumulation of MeHg (368%) and SeMet (≅ 100%) took place from the in-house made diets to the muscle of the rainbow trout exposed to these species through the diets. This confirms, in the time tested in our study, that for fish, MeHg and SeMet are highly bioavailable species of Hg and Se, respectively.

It is also worth to note that the steady state between absorption and elimination of the SeMet was achieved. For Hg, the bioaccumulation of MeHg from the diet to the muscle after 3 months of exposure is still predominant (>100%), hence explaining the bioavailability of this species (Gochfeld et al. 2021). The high bioaccumulation of MeHg in the muscle could also result from the low elimination rate of this element (Larsen et al. 1995).

Hg_T levels in the fish muscle tissue after exposure in different conditions are reported in Table 4.

Table 4. Hg_T levels in rainbow trout muscle tissue (mean ± SD, n = 5) exposed to SeMet and MeHg via an in-house prepared diet, in various conditions during one (t_1), two (t_2) and three (t_3) months, respectively (also compared to the controlled conditions, t_0).

Exposure group	Hg_T mass fraction (mg/kg ww)				RSD (%)[a]		
	t_0	t_1	t_2	t_3	t_1	t_2	t_3
G0: Control	0.027 ± 0.002	0.029 ± 0.002	0.031 ± 0.002	0.034 ± 0.003	6.9	6.5	8.8
G1: SeMet		0.033 ± 0.003	0.031 ± 0.002	0.030 ± 0.003	9.1	6.5	10.0
G2: MeHg		0.074 ± 0.006	0.107 ± 0.008	0.140 ± 0.008	8.1	7.5	8.1
G3: SeMet and MeHg		0.064 ± 0.005	0.127 ± 0.013	0.135 ± 0.006	7.8	10.2	5.7
G4: SeMet (one month) then MeHg (2 months)		0.030 ± 0.003	0.073 ± 0.004	0.103 ± 0.008	10.0	5.5	7.8
G5: MeHg (one month) then SeMet (2 months)		0.064 ± 0.002	0.068 ± 0.004	0.056 ± 0.001	3.1	5.9	1.8

[a] relative standard deviation (%) for the analysis of 5 fish individually.

Excellent precision (RSD ≤10%, see Table 4) was obtained in terms of MeHg bioaccumulation by the five fish individuals exposed to this contaminant in various conditions. The precision in SeMet accumulation is lower, especially when the fish were exposed to SeMet individually, when the RSD was ≅ 15% (see Table 5).

As can be seen from Table 4, Hg_T level in the control group (G0) and in the fish supplemented with SeMet solely (G1) remained constant throughout the study (also compatible with the control group). This suggests that vestigial Hg in the diet did not contribute to any Hg bioaccumulation in the muscle and hence the results obtained in this study are related solely to the enriched diet (with SeMet and MeHg). On the other hand, Hg_T levels in the muscle of the exposed to MeHg solely (G2) increased 2.7 (t_1), 4 (t_2) and 5 (t_3) fold, respectively, compared to the control group (G0), which shows a quasi linear bioaccumulation ($R^2 \cong 0.99$) during the whole period of the exposure. Similarly, the levels of Se_T in the fish (muscle tissue) after exposure in the same conditions (G0-G5) are reported in Table 5.

Table 5. Se_T levels in muscle tissue (mean ± SD, n = 5) of rainbow trout exposed to SeMet and MeHg via an in-house prepared diet, in various conditions during one (t_1), two (t_2) and three (t_3) months, respectively (also compared to the controlled conditions, t_0).

Exposure group	**Se_T mass fraction (mg/kg ww)**				**RSD (%)[a]**		
	t_0	**t_1**	**t_2**	**t_3**	**t_1**	**t_2**	**t_3**
G0: Control	0.141 ± 0.013	0.151 ± 0.012	0.152 ± 0.018	0.158 ± 0.013	7.9	11.8	8.2
G1: SeMet		0.523 ± 0.072	0.867 ± 0.132	0.910 ± 0.117	13.8	15.2	12.9
G2: MeHg		0.162 ± 0.007	0.166 ± 0.007	0.168 ± 0.014	4.3	4.2	8.3
G3: SeMet and MeHg		0.537 ± 0.040	0.921 ± 0.034	1.115 ± 0.088	7.4	3.7	7.9
G4: SeMet (one month) then MeHg (2 months)		0.526 ± 0.029	0.529 ± 0.016	0.436 ± 0.015	5.5	3.0	3.4
G5: MeHg (one month) then SeMet (2 months)		0.149 ± 0.004	0.557 ± 0.023	0.783 ± 0.079	2.7	4.1	10.1

[a] relative standard deviation (%) for the analysis of 5 fish individually.

The levels of Se_T in the control group (G0) did not differ significantly during the whole experiment; the relative standard deviation of the values corresponding to t_0, t_1, t_2 and t_3 was ≅ 5% and $p = 0.33$. This indicates also that vestigial Se in the diet did not contribute to any Se bioaccumulation from diet to fish when fish were not supplemented with the Se rich diet. However, unlike the G0, where Se_T levels maintained constant in the absence of Se supplementation, in fish with the same Se exposure conditions, but with MeHg dietary exposure (G2), a slight increase in Se_T levels was observed ($p = 0.004$).

When the fish were supplemented with SeMet enriched diet (G1), the Se_T content in the muscle was 3.7 (t_1), 6 (t_2) and 6.5 (t_3) higher compared to the control group. It is interesting to note that the increase of the Se_T level up to 2 months was linear ($R^2 = 0.99$) while at t_2 a saturation occurred (no statistical difference between Se_T levels at t_2 and t_3). Thus, following 90 days of dietary SeMet exposure, Se_T muscle burden increased significantly, but the values reached were still lower than the toxic effect threshold of Se in fish (1.0 µg/g ww).

It is worth to note that the Se antagonistic effects on Hg can be seen on two different sides. On one side, one can consider the reduced MeHg bioaccumulation by fish in the presence of (relatively) high levels of Se species (SeMet in our study). On the other side, the detoxification of fish (related to its MeHg content) when it is exposed to SeMet can also be considered. In our study we took into consideration both sides, by studying the simultaneous co-exposure of fish to MeHg and SeMet (G3) but also sequentially (G4 and G5, respectively).

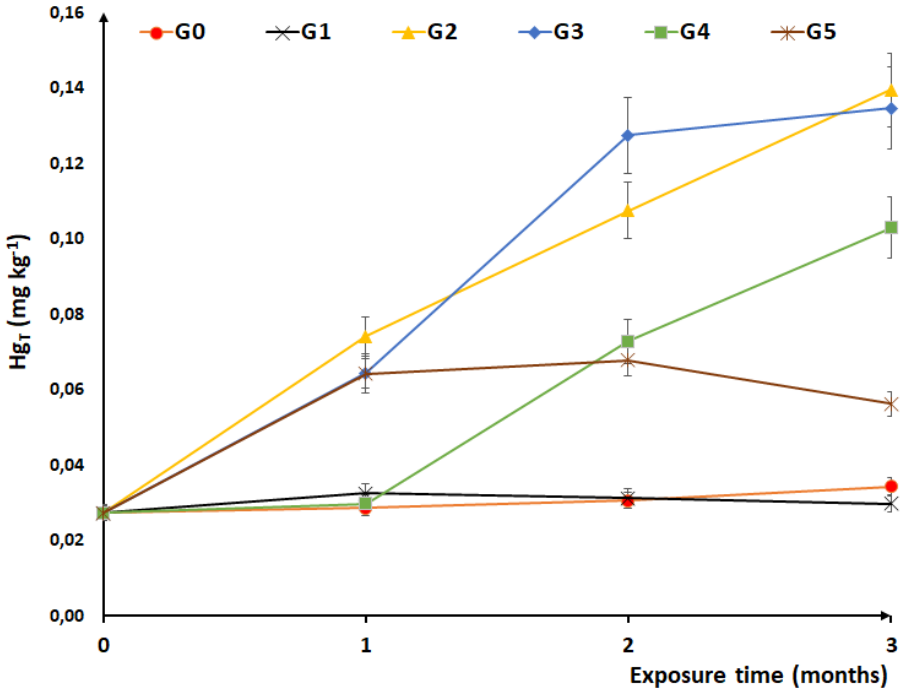
When the fish were exposed to SeMet and MeHg simultaneously (G3), significant MeHg uptake occurred only up to t_2 (see also Fig. 4a), while Se_T increased steadily for the whole exposure study ($R^2 \cong 0.98$). Additionally, a slightly higher bioaccumulation of SeMet was noticed when the fish was supplemented with MeHg and SeMet in the same time than when it was supplemented alone (G1). When the fish were supplemented with MeHg and SeMet simultaneously (G3), MeHg level is similar ($p = 0.31$) to that obtained by supplementation with MeHg solely (G2, t_3), while at short exposure time (t_1), a lower accumulation of MeHg (-14%) was observed (see Table 4).

The bioaccumulation of SeMet and MeHg does not present the same behaviour in fish exposed to both species in comparison to the ones exposed individually to SeMet and MeHg. An increase in the Hg_T and/or Se_T levels from t_0 to t_3 is observed when they are present in the diet, in all tested conditions, hence confirming that these elements are bio-accumulated along the 3 months of the experiment.

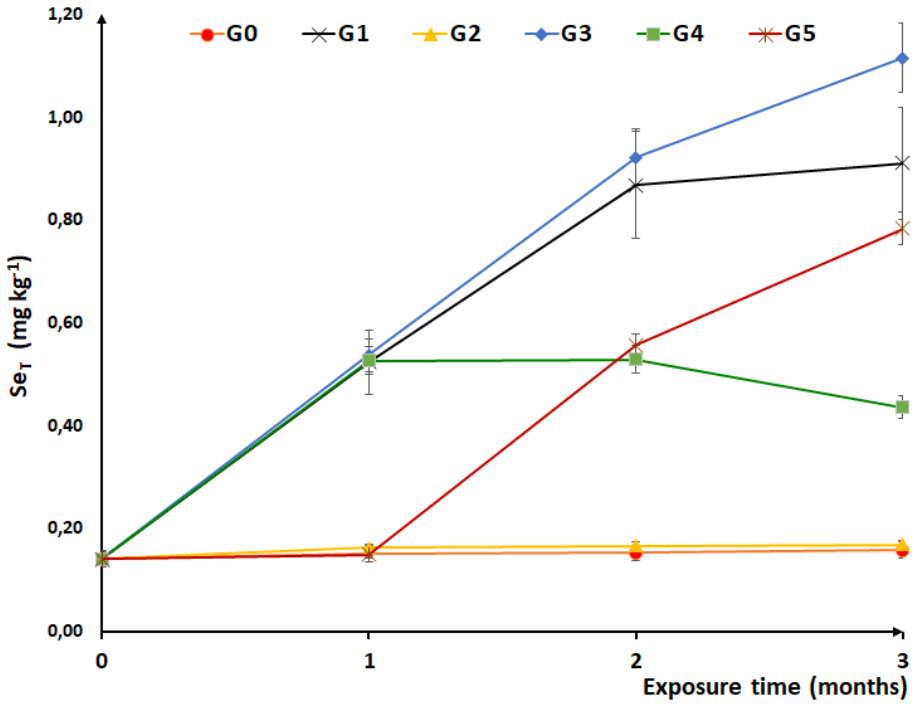
It is interesting to note that in the case of G3, a saturation of the MeHg bioaccumulation appeared at t_2 (see Fig. 4a). ANOVA was performed to compare the Hg_T levels at t_2 and t_3, which confirmed that they were not statistically different ($p = 0.30$).

To assess the Hg-Se antagonism in fish, a group of rainbow trout was preliminarily exposed to SeMet during one month and then to MeHg during the following two months (G4). As can be seen from Table 4 and Fig. 4a, at t_3, the fish bioaccumulates less MeHg when exposed firstly to SeMet (1 month, G4) compared to the exposure to MeHg solely (G2). However, this is not necessarily due to the inhibitory effect of SeMet but most likely due to the lower duration of exposure to MeHg (two months only). Actually, MeHg bioaccumulation at t_3 in G4 (two months exposure) is comparable ($p = 0.41$) with that of t_2 in G2 (2 months exposure in both cases).

As can also be seen from Table 5 and Fig. 4b (G4), SeMet level remains constant ($p = 0.87$) one month after ending the supplementation (see t_1 and t_2) but it declines (≅ -18%) two months after the supplementation is stopped (see t_3).



(a)



(b)

Fig. 4a-b. Variation of Hg_T (a) and Se_T (b) levels in the muscle of rainbow trout during different scenarios of exposure to SeMet and MeHg in a dynamic system via an in-house prepared diet. G0: control group, G1: group exposed to SeMet enriched diet, G2: group exposed to MeHg enriched diet, G3: group exposed simultaneously to SeMet and MeHg; G4: group exposed initially to SeMet (one month) and then to MeHg (two months) and G5: group exposed initially to MeHg (one month) and then to SeMet (two months).

To assess the detoxification role of SeMet towards MeHg in fish, a group of rainbow trout was initially exposed to MeHg (1 month) and then to SeMet via the corresponding enriched in-house prepared diets (G5). When comparing the bioaccumulation of MeHg observed in the first month of exposure in this group (G5, t_1), to the fish also exposed to MeHg for the same period but with simultaneous exposure to SeMet, (G3, t_1), it was observed that MeHg was bioaccumulated similarly (p = 0.95). That means that the presence of SeMet did not influence the bioaccumulation of MeHg. Additionally, it was observed that MeHg level remained constant (p = 0.14) one month after the substitution of MeHg with SeMet (G5, t_2). However, a slight decrease (≅ -18%) in MeHg level was noticed after two months of fish exposure to SeMet and no MeHg (G5, t_3). We can relate this observation to a detoxification effect of the fish in terms of MeHg as a result of its supplementation with SeMet enriched diet, which is also in agreement with other studies (Amlund et al. 2015; Bjerregaard et al. 2011). On the other hand, we could associate the observed decrease in MeHg concentration to a natural detoxification due to the fact that MeHg exposure was stopped. However,

this hypothesis it is not likely because when comparing the MeHg bioaccumulation behaviour observed in the control group (non MeHg enriched diet), the levels of MeHg in the fish remained constant throughout the exposure experiment suggesting that no natural detoxification occurs in this case.

However, as observed by several authors (Ralston et al. 2009; Huang et al. 2013; Khan et al. 2009), the overall net protective effect of Se over Hg toxicity will depend on the relative concentrations of Hg and Se, justifying the importance of evaluating also the Se/Hg molar ratio. Se and Hg molar ratios in the diets and the fish muscle are shown in Table 6.

Table 6. Se and Hg molar ratio in the different types of diet as well as in the muscle tissue of rainbow trout exposed to MeHg and SeMet in different conditions.

Diet	n_{Se} / n_{Hg}[a]	**Exposure group**	n_{Se} / n_{Hg}[a]			
			t_0	t_1	t_2	t_3
Control	44	**G0**: Control	13	13	12	12
MeHg enriched	9	**G1**: SeMet		40	71	77
SeMet enriched	274	**G2**: MeHg		6	4	3
MeHg + SeMet enriched	55	**G3**: SeMet and MeHg		21	18	21
		G4: SeMet (one month) then MeHg (2 months)		45	18	11
		G5: MeHg (one month) then SeMet (2 months)		6	21	36

[a] n: number of moles

The Se/Hg molar ratio is always considerably >1, suggesting that Se is always in excess relative to Hg, both in the fish muscle and the diet (including in the diet control fish and diet, respectively). Therefore, for fish, n_{Se} / n_{Hg} >1 assures both the normal Se metabolism and the MeHg detoxification (Ralston et al. 2009).

As expected, the lowest Se/Hg molar ratios are observed in G2 (exposure to MeHg solely), while the highest Se/Hg molar ratios are measured in the fish from G1 (exposure to SeMet solely). Deserves mention that some authors reported that the benefit of Se supplementation in prevention of MeHg accumulation is dependent on the chronology of the exposure treatments, being only observable when Hg and Se are provided simultaneously (Naganuma et al. 1983). Lower mortality rate was observed in fish pre-treated with SeO_2 before $HgCl_2$ administration in comparison to those exposed to $HgCl_2$ treatment only (Cuvin et al. 1988). Nagamuna et al. (1983) reported that a

437 delayed administration of Na_2SeO_3 had no protective effect in mice, while Agarwal et al. (2007)
438 observed even a greater accumulation of Hg in mice undergoing a post-treatment with Na_2SeO_3
439 (Naganuma et al. 1983; 387 Agarwal et al. 2007). To respond to this controversy, as it was already
440 presented above, we carried out an exposure experiment by supplementing the fish firstly with
441 MeHg (one month) and then with SeMet during two months (G5). Our results show that when the
442 supplementation with SeMet starts one month after the exposure to MeHg, the Se/Hg molar ratio
443 (t_2) is comparable with that at t_2 in case of simultaneous exposure (G3). The data also show that
444 bioaccumulation of equimolar Se and Hg levels in the fish muscle does not occur in any of the
445 conditions of our study (using diet with or without supplementation with SeMet and/or MeHg
446 species). Finally, no correlation can be established between the molar ratio in the diet and the fish
447 muscle in any of the exposure condition.
448
449 **Conclusions**
450 This study focused on the dietary exposure of a river water fish species (rainbow trout) to MeHg
451 and/or SeMet via an in-house prepared diet supplemented with these Hg and Se species. The study
452 was carried out in dynamic conditions by using various scenarios, relying on exposure to MeHg and
453 SeMet individually, simultaneously but also sequentially. For the simultaneous determination of the
454 levels of total Hg and Se in fish muscle, a novel method based on ICP-MS was validated using the
455 accuracy profile. Excellent precision and accuracy were obtained, hence making it fully applicable
456 to the trace and ultra-trace determination of total Hg and Se in fish undergoing exposure to SeMet
457 and MeHg.
458 Our results show a quasilinear bioaccumulation of MeHg in the fish muscle during three months
459 when they are exposed individually to this species, while saturation occurs for SeMet after two
460 months of exposure. Also, a threshold in total Hg level is reached when the fish are exposed
461 simultaneously to MeHg and SeMet. It is also interesting to note a decrease of MeHg burden was
462 observed when the fish were exposed to SeMet after being first exposed to MeHg, hence indicating

463 the MeHg detoxification due to SeMet supplementation. This in-vivo exposure study confirms the
464 previous reports regarding the possible antagonistic effect of Se and Hg in fish.
465 The study must be a pursuit with the speciation analysis of Hg (MeHg and its inorganic precursor,
466 Hg^{2+}) and Se (SeMet and other species such as inorganic Se(IV)/Se(VI) and selenocysteine/SeCys)
467 to decipher the mechanism of the in-vivo interaction between Se and Hg in fish and the protective
468 effect of Se towards Hg contamination. It could also be extended to carrying out the exposure
469 during a longer duration (>3 months) using other fish species and also using Hg^{2+} and other Se
470 species (e.g. SeCys, Se(IV), Se(VI)) to deeply understand the Hg-Se antagonism in fish.
471
472 **Acknowledgements**
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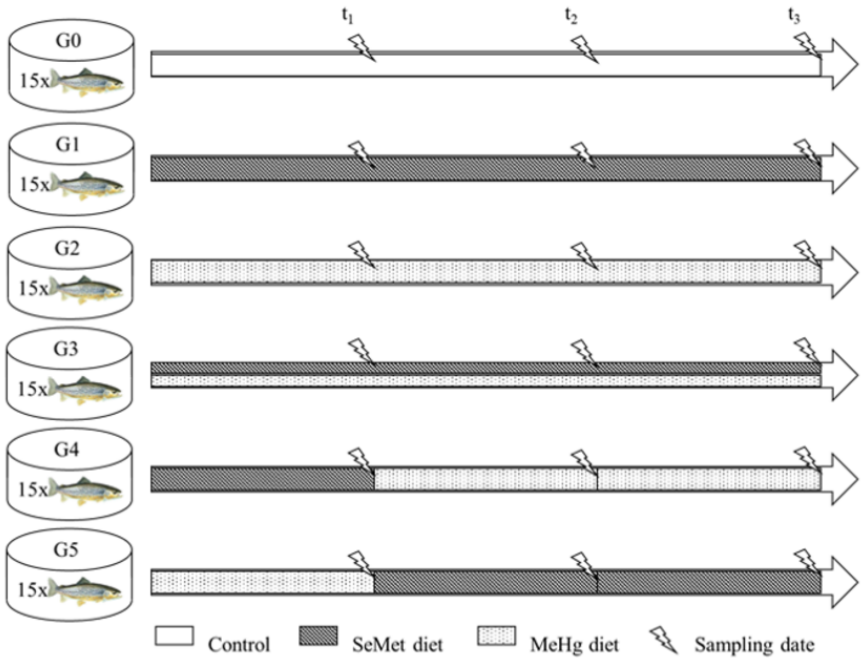
Exposure study



Se and Hg Quantification

Antagonistic effect of Se and Hg in fish