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Bioaccumulation and transformation of methylmercury and selenite using zebrafish (*Danio Rerio*) larvae as a model

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ABSTRACT

Bioaccumulation and possible transformation of methylmercury and selenite has been checked on a 72 h-cycle of bioaccumulation and depuration using larvae from zebrafish. The larvae were exposed to methylmercury and selenite at concentrations of 1% and 0.1% of their LC_{50} values. Quantitative extraction of methylmercury and selenite from exposed larvae was achieved by using ultrasonic probe-assisted extraction (USP), thus reducing extraction time and solvent consumption. Extracted species collected at different exposure times were characterized and quantified by liquid chromatography coupled to ICP-MS. Bioconcentration factors (BCFs) were estimated by two procedures: (i) as the ratio of the contaminant concentration in larvae and exposure media ($BCF_{48\,h}$) and (ii) fitting contaminant concentration in larvae to bioaccumulation models that describe uptake and depuration processes (BCF_k). The BCFs obtained for methylmercury were 5000 and 2333 for larvae exposed to 1 $\mu g\,L^{-1}$ and 10 $\mu g\,L^{-1}$, respectively; while for selenite the BCF was 74 for larvae exposed to 10 $\mu g\,L^{-1}$. The good correlation between the BCFs found and those previously reported in the literature shows the proposed method as a good and promising alternative to the OECD Bioconcentration Test 305. Actually, the use of zebrafish larvae reduces the bioaccumulation test time from forty two (OECD Bioconcentration Test 305) to three days. In addition, potential biotransformation of both methylmercury and selenite was evaluated by LC–ICP-MS. For this purpose, a method for species extraction in small size samples by using ultrasonic probe sonication was developed.

1. Introduction

REACH is the European Community Regulation (EC 1907/2006) [1] on chemicals and their safe usage, which main objective is to improve the protection of human health and environment by identification of the intrinsic properties of chemical substances. This regulation states that those chemicals whose production exceeds 10 tons per year and also those regarded as PBTs (Persistent, Bioaccumulable and Toxic) substances require a chemical safety report where information about its physical, chemical and health and safety data should be detailed. Besides chemical properties, studies about the ecotoxicity, mobility, persistence, bioaccumulation, and degradation of the contaminants are also required. REACH's Test Methods Regulation for bioaccumulation factor calculation [2] have established OECD Bioconcentration Test 305 [3] as the standard method, although other tests such as the ASTM E1022-94 from the American Society for Testing and Materials and OPPTS 850.1730 from US EPA are also considered as valid. The OECD Test Guideline describes a procedure for characterizing the bioconcentration factors of chemicals in fish based on the measurement of chemical content in both fish tissue and exposure solution at increasing exposure time until a steady response is reached (42 days). The long term study along with the high number of determinations required (at least 108 juvenile or adult fish specimens) results on a very expensive test (more than 100,000$ per compound studied) [4].

To overcome the mentioned drawbacks, REACH European legislation has proposed to replace animal testing wherever possible and to use animal-free approaches [5,6]. Among them zebrafish larvae has been considered as an excellent alternative model for toxicological assessment and bioaccumulation studies because it represents the complex dynamic, interactive and multi-organ events that occur *in vivo* in the context of a complete organism but with the additional benefit that is not considered as a laboratory animal according to the Directive 2010/63/EU. Other additional advantages are high reproductive capabilities (each female is capable of laying 200–300 eggs per week), a fast embryonic

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development and a genome similar to that of humans (over 80% similarity), thus facilitating extrapolation of the obtained results to humans. However, application of zebrafish larvae approach is not straightforward and requires first an adaptation of the protocol taking into account the following criteria [7]: (i) the substances and fish species used must be clearly specified in the protocol, (ii) test substance measurement should be performed in both fish tissue and exposure medium and (iii) BCFs values should always reflect steady state conditions.

Further, the determination of chemical concentration in larvae for BCF determination is still a challenge since it requires highly sensitive analytical techniques because only a small amount of substance might get accumulated due to the small volume of fish larvae.

Mercury is a well-known pollutant that can cause evolutionary changes due to their harmful effects on living organism. Mercury toxicity is highly dependent on its chemical form, being, organomercurial compounds more harmful than inorganic mercury. Due to its high lipophilicity [8], methylmercury accumulates throughout the food chain. Moreover, methylmercury can cross the blood–brain barrier causing damages in the brain and neurological disorders. Most of the knowledge on the toxic effects of methylmercury has come from catastrophic episodes of poisoning (Minamata and Niigata, Japan 1950s) [9,10].

Selenium is an essential trace element because it acts as a cofactor in several enzymes [11–13]. This element also has toxic properties, and there are evidences that can be responsible on reproductive failure in fish [13]. Selenium from both natural and anthropogenic sources enters surface waters primarily as the highly soluble Se(IV) and Se(VI) oxidation states. Organics selenides (Se(−II)), including selenoamino acids and selenoproteins, methyl selenides, and other Se-susbtituted analogs of organosulfurs compounds, are produced by biological reduction of selenite [14].

Selenium and mercury species determination in biological samples is not an easy task because of the low selenium and mercury concentration levels. Selenium and mercury speciation is commonly performed by HPLC or GC coupled to ICP-MS. Extraction of mercury and selenium species from a complex sample is recognized as one of the most crucial steps before their determination. A successful extraction procedure for speciation analysis requires high extraction efficiency while maintaining intact the original species distribution [15]. Ultrasound-assisted extraction has already been shown as a very promising technique for extraction of selenium and mercury species [16–18], however, very few have been reported about its application in zebrafish larvae. Selenium and mercury determination and speciation in zebrafish larvae is still a challenge because of its small size and high fat content.

Therefore, the aim of this work is to calculate the BCFs of methylmercury and selenite in zebrafish larvae, and checking the possible transformation of the species tested due to zebrafish larvae metabolism. The idea behind is to evaluate the potential of using zebrafish as an alternative to the high-time consuming and expensive models using adult fish. For this purpose, an analytical methodology based on the use of LC–ICP-MS and the application of several sample treatments have been developed for mercury and selenium species determination in zebrafish larvae.

2. Material and methods

2.1. Instrumentation

A Vibra cell VC×130 ultrasonic processor (CT, USA) equipped with a titanium 2-mm-diameter microtip and fitted with a high-frequency generator of 130 W at 20 kHz was used for leaching the analytes from larvae samples. Centrifugation was carried out in a centrifuge model type: Centrifuge 5415-R (Eppendorf, Germany). A quadrupole ICP-MS Thermo X-Series equipped with a meinhard nebulizer, a fusel torch, and impact bead quartz spray chamber cooled by a peltier system was used for selenium and mercury determination. The mass calibration of the ICP-MS instrument was tuned daily with a solution containing 1 $\mu g\,L^{-1}$ of Li, Co, Y, Ce, and Tl.

The liquid chromatographic system used for mercury and selenium speciation consisted of a PU-2089 LC pump (JASCO, Tokyo, Japan) fitted with a six-port injection valve (model 7725i; Rheodyne, Rohnert Park, CA, USA) with a 100 or 20-μL injection loop. The outlet of the column was directly connected to the nebulizer of an ICP-MS system using PEEK tubing (Ø = 0.13 mm). The optimal operation conditions and data acquisition parameters are summarized in Table 1.

2.2. Reagents and standards

All reagents used were of analytical grade. H_2O_2 (Panreac, Madrid, Spain) and HNO_3 (Merck, Damstadt, Germany) were used for acid digestion of samples. Non-specific protease type XIV (Sigma–Aldrich, Steinheim, Germany) and HCl (Merck) were used for enzymatic hydrolysis and acid leaching, respectively. The carrier solution for flow injection (FI) mercury determination contained KCl (Riedel-de Haën AG, Berlin, Germany), HCl and 2-mercaptoetanol (Merck). The carrier solution for selenium determination was 2% (v/v) HNO_3. Heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), formic acid, L-cysteine mono hydrochlorhydric from Sigma–Aldrich (Madrid, Spain) and methanol from Scharlau (Barcelona, Spain) were used in the chromatographic mobile phases.

All solutions and samples were prepared using high-purity water with a resistivity of 18.0 MΩ cm obtained from a Millipore (Bedford, MA, USA) ZMFQ 23004 Milli-Q water system. Inorganic selenium solution was obtained by dissolving sodium selenite (CAS no.: 10102-18-8, Merck) in deionized Milli-Q water. Stock solutions of 1000 mg L^{-1} were stored in the dark at 4 °C and working standard solutions were prepared daily by dilution. Methylmercury solution was obtained by dissolving methylmercury chloride (CAS no.: 115-09-3, Alfa Aesar, Karlsruhe, Germany) in methanol. This solution was stored in the dark at −18 °C.

2.3. Larvae contamination

Zebrafish larvae were supplied from ZF BioLabs (Madrid, Spain). Exposure solution was prepared in a way that had a similar composition as fresh river water. Briefly, 16 mL of concentrated solution (containing 2.9 g of $CaCl_2$, 17.2 g of NaCl, 0.76 g of KCl and 4.9 g of $MgSO_4$ per litre) were diluted to 1 L with distilled water. The final conditions of resulting exposure solution were: temperature 26 ± 2 °C, dissolved oxygen ≥60% and pH 6–8.5 (before and after renewal), values that fulfil the requirements of OECD guideline. To get the zebrafish larvae, it was necessary to develop the embryos to 72 h post fertilization (hpf), development stage that represents the moment when the embryos hatched. Zebrafish larvae remain classified as such until another 48 h later (120 hpf) when they are regarded as proper fish, but can be considered non-feeding other 24 h [19]. Bioaccumulation experiments were performed in three tanks, one as control (without the addition of the analyte) and two containing the target analytes at different concentration levels.

Bioaccumulation experiments were performed in two phases [3]: exposure (uptake) and post-exposure (depuration). For this,

Table 1
Operational parameter of the ICP-MS and HPLC–ICP-MS systems.

Plasma conditions	
Forward power	1250 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	0.73 L min^{-1}
Nebulizer gas flow rate	0.83 L min^{-1}
Nebulizer	Concentric
Spray chamber	Double-pass spray chamber
Measurement parameters (mercury)	
Acquisition mode	Time resolved analysis
Isotope monitored	^{202}Hg, ^{201}Hg and ^{200}Hg
Dwell time per point	100 ms
Replicates	3
Measurement parameters (selenium)	
Acquisition mode	Time resolved analysis
Isotope monitored	^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se (reaction cell with H_2)
Dwell time per point	100 ms
Replicates	3
HPLC conditions (1) (mercury speciation)	
Column	Symmetry shield RP 18 (150 × 3.9 mm, 5 μm)
Injection volume	100 μL
Column temperature	25 °C
Mobile phase	0.1% (v/v) formic acid, 0.1% (v/v) heptafluorobutyric acid, 2% (v/v) methanol, 10 mM L-cysteine
Elution	Isocratic
Flow rate	1 mL min^{-1}
HPLC conditions (2) (selenium speciation)	
Column	Luna C18(2)-HST (100 × 2 mm, 2.5 μm)
Injection volume	20 μL
Column temperature	25 °C
Mobile phase	0.1% (v/v) heptafluorobutyric acid, 0.05% (v/v) trifluoroacetic acid, 2% (v/v) MeOH
Elution	Isocratic
Flow rate	0.2 mL min^{-1}
HPLC conditions (3) (selenium speciation)	
Column	Synergi-Fusion (100 × 2 mm, 2.5 μm)
Injection volume	20 μL
Column temperature	25 °C
Mobile phase	0.1% (v/v) formic acid, 0.1% (v/v) heptafluorobutyric acid, 2% (v/v) methanol, 1 mM L-cysteine
Elution	Isocratic
Flow rate	0.2 mL min^{-1}

zebrafish larvae were first exposed during 48 h to the chemical (selenite or methylmercury) (exposure phase) and subsequently exposed during 24 h to the medium (depuration phase). About 20–25 larvae and several milliliters of the corresponding exposure solution were taken from the tanks after 2, 4, 6, 24, 28, 45, 48, 50, 69 and 72 h of exposure and the concentration of the target analyte were determined in both larvae and exposure media. The sampling times were selected according to our experimental results previously reported. Following OECD recommendation, the loading rate of larvae at the beginning of the experiments ranged between 0.1 and 1.0 g L^{-1} (wet weight) and the mortality of larvae was kept below 10% at the end of the test.

The nominal concentrations of the test substance were selected according to the requirements given by the OECD test 305, with the highest concentration being 1% of the LC_{50} value and second exposure concentration differing from the first by a factor of ten. LC_{50} values were selected according to the information appearing in the literature. For instance, METI-NITE Japan database has reported a LC_{50} value of 8.6 mg L^{-1} for selenite [20]. On the other hand, a LC_{50} value of 250 μg L^{-1} for methylmercury was obtained experimentally by ZF BioLabs (following the revised OECD protocol 403) [21]. Based on the above, nominal concentrations of 10 and 1 μg L^{-1} for selenite and methylmercury respectively, were chosen to carry out the bioconcentration experiments. Although a concentration of 10 μg L^{-1} of methylmercury does not fulfil recommendations given by OECD test (1% of the LC_{50}, 2.5 μg L^{-1}), however it was chosen since this species could not be quantified at lowest concentration which, as OECD 305 test establish, should be 10 times lower (0.2 μg L^{-1}).

2.4. Analytical procedure

2.4.1. Determination of total selenium and mercury concentration in exposure media by ICP-MS

Determination of methylmercury on exposure solutions was carried out by a flow injection (FI) system coupled to the ICP-MS using a carrier solution containing 0.1% (v/v) HCl, 0.1% (v/v) 2-mercaptoethanol and 0.15% (w/v) KCl in order to reduce mercury memory effects [22]. The same flow injection (FI) system coupled to the ICP-MS was employed for selenium determination but using 2% (v/v) HNO_3 as carrier solution instead [23]. The optimal operation conditions for ICP-MS determination are summarized in Table 1.

2.4.2. Determination of mercury and selenium species in zebrafish larvae by LC–ICP-MS

Prior to the determination of mercury and selenium species content in larvae, an extraction step was required. In case of mercury, two different extraction procedures (acid leaching and enzymatic hydrolysis), previously developed by our group [16] were applied with a few modifications. Briefly, in the acid leaching a pool of 20 larvae (12 mg) were sonicated with 150 μL of 7 mol L^{-1} HCl during 40 s at 40% ultrasonic amplitude and then diluted up to 800 μL with ultrapure water. For enzymatic hydrolysis, a pool of 20 larvae (12 mg) were sonicated with 7 mg of protease XIV during 40 s at 40% ultrasonic amplitude and then diluted up to 800 μL with ultrapure

Table 2
Selenium extraction yields from larva zebrafish compared with total extraction performed with MW-acid digestion.

Sample treatment		Recovery, $X \pm$ S.D.[a]
A	Ultrasonic probe 40 seg 40% ultrasound amplitude + water	49 ± 1
B	Ultrasonic probe 40 seg 40% ultrasound amplitude + protease XIV	47 ± 3
C	Ultrasonic probe 40 seg 40% ultrasound amplitude + double extraction + water	69 ± 1
D	Ultrasonic probe 80 seg 40% ultrasound amplitude + double extraction + water	77 ± 2

[a] Average value ± standard deviation ($n = 3$).

water. After extraction, 70 μL of methanol was added to precipitate the lipidic content of the samples and samples were filtered by 0.22 μm membrane filter before injection onto HPLC. Speciation of mercury species was carried out by reversed-phase chromatography coupled with ICP-MS using the conditions summarized in Table 1.

Selenium species extraction from larvae was performed by using different treatments based on the application of ultrasonic probe in an aqueous media (Table 2). The results obtained were compared with those provided after applying microwave acid digestion (total content of selenium). For that, a pool of 20 larvae (12 mg) were placed in a Teflon reactor and 200 μL and 100 μL of concentrated HNO_3 and H_2O_2 respectively were added and maintained for 4 h at 80 °C. Finally, samples were diluted to a total volume of 900 μL and total selenium concentration was determined following the conditions summarized in Table 1. Selenium speciation was carried out by using reversed-phase chromatography coupled with ICP-MS using two different chromatographic columns for a better confirmation of species identity (Table 1). Samples were filtered by 0.22 μm membrane filter before injection onto HPLC.

2.5. Quality assurance

Quality assurance steps included blanks, replicate analyses, certified reference material recoveries and calibrations. Linearity, drift check and spike recovery analyses were carried out using proper zebrafish larvae. Analyses were carried out by triplicate. Extraction procedures were optimized using the tuna fish tissue CRM-463 certified for methylmercury (obtained from Community Bureau of Reference, BCR (now renamed Standards, Measurements and Testing Programme)) [16] and a marine tissue reference material (Murst-ISS-A2, Antartic Krill, from Italian Istituto Superiore di Sanità), certified for total selenium.

3. Results and discussion

3.1. Quality assurance

The limits of detection for the complete method (MDLs) for mercury and selenium were 0.01 $\mu g\,g^{-1}$ and 0.03 $\mu g\,g^{-1}$ for larvae respectively, and 0.1 $\mu g\,L^{-1}$ and 0.2 $\mu g\,L^{-1}$ for exposure solution, respectively. Concentrations of all target compounds in blanks were below MDLs. A good reproducibility (4–7% for exposure solutions and 6–15% for larvae) was found for three replicates of each analysis. Calibrations showed good linearity ($R = 0.99$). Optimization using the certified materials show quantitative extraction for both methods used for methylmercury extraction: 99 ± 3% for acid leaching and 91 ± 4% for USP extraction. For total selenium the recovery achieved was 98 ± 4%.

3.2. Exposure of larvae to methylmercury

3.2.1. Determination of methylmercury in exposure media and zebrafish larvae

According to the conditions given by the OECD test 305, a proper estimation of the bioaccumulation factor requires that chemical concentration should not fluctuate more that 20% of the mean of the measured values during the uptake. To achieve this, methylmercury concentration in the exposure solution was maintained constant along the uptake phase (6.7 ± 0.8 $\mu g\,L^{-1}$ and 0.3 ± 0.1 $\mu g\,L^{-1}$ for the highest and lowest concentration, respectively, Fig. 1). Variation of experimental data obtained for the lowest concentration is higher than the limit established by OECD 305 requirements (32% of variation versus an allowed limit of 20%). It is important to highlight that the strict control of such low concentration (lower than 0.5 ppb) on an exposure media containing living organisms is difficult to achieve. This statement has been confirmed from experimental data on bioconcentration and bioaccumulation studies published [20]. Due to the lack of experimental data of concentration of the exposure media along the uptake period, our experimental results were very useful for further calculations. Methylmercury content in the exposure solution during depuration was either not detected or always below detection limits.

As it is shown in Fig. 2 no significant differences were observed between the methylmercury accumulation curves obtained at the two levels of methylmercury exposure, 10 and 1 $\mu g\,L^{-1}$. The concentration of methylmercury increased during the uptake phase reaching a maximum value at 48–50 h of exposure. However, methylmercury was not significantly eliminated from larvae during the depuration step which could be justified for its high affinity to fatty tissues and the sulfhydryl groups of proteins. This slow

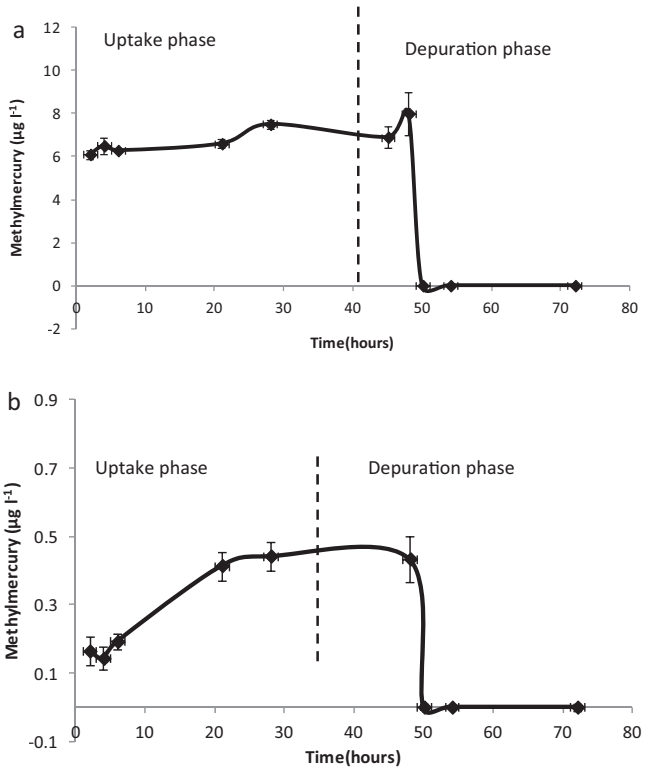

Fig. 1. Methylmercury concentration ($\mu g\,L^{-1}$) in the culture medium. (a) Nominal content of 10 $\mu g\,L^{-1}$ and (b) nominal content of 1 $\mu g\,L^{-1}$.

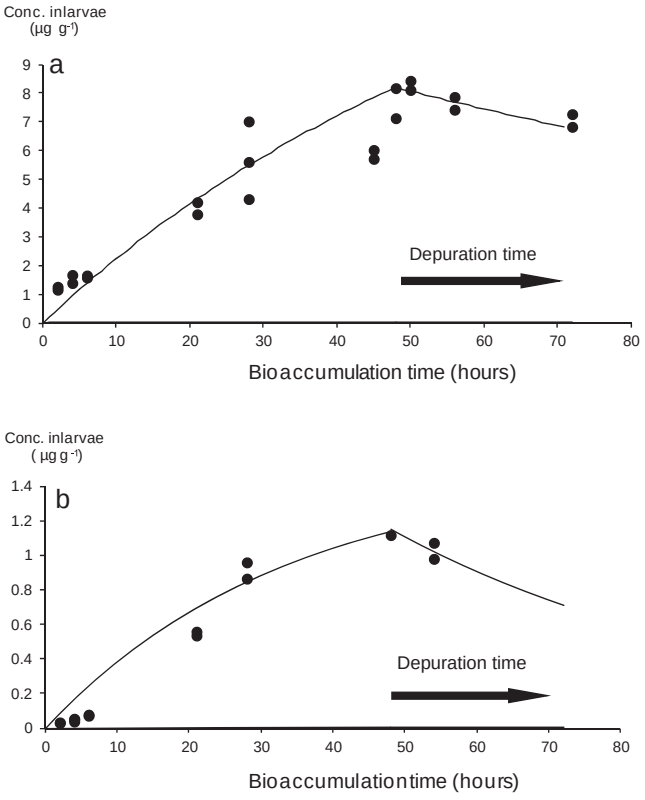

Fig. 2. Accumulation of methylmercury ($mg\,g^{-1}$) in larvae exposed to (a) 10 $\mu g\,L^{-1}$ and (b) 1 $\mu g\,L^{-1}$ of methylmercury. Solid circles (●) represent the experimental points and lines (—) the expected values based on the model calculations.

excretion of methylmercury has been also observed by other authors [8,24–26].

Potential biotransformation of methylmercury in larvae during accumulation was evaluated by speciation analysis. Acid leaching and enzymatic hydrolysis were applied for mercury species extraction before their identification and quantification by reversed-phase chromatography coupled with ICP-MS. The chromatograms obtained using both sample treatments (Fig. 3) showed only a single peak which was identified by retention time and spiking experiments as methylmercury, concluding that zebrafish larvae do not biotransform methylmercury during the uptake process.

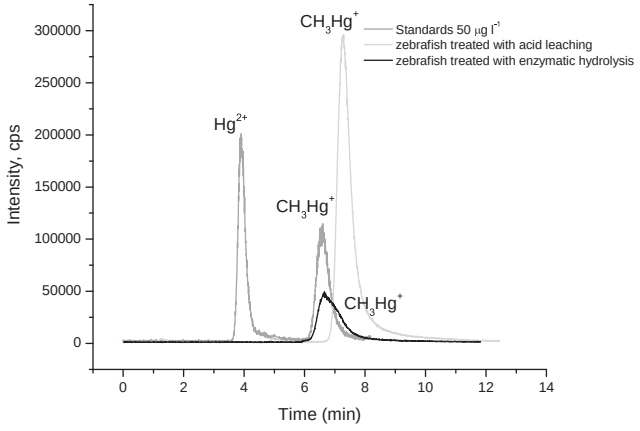

Fig. 3. Chromatographic profile obtained by reverse-phase LC–ICP-MS corresponding to 50 $\mu g\,L^{-1}$ of Hg^{2+} and $MeHg^{+}$, zebrafish treated with acid leaching and zebrafish treated with enzymatic hydrolysis. Chromatographic column: Symmetry Shield RP 18.

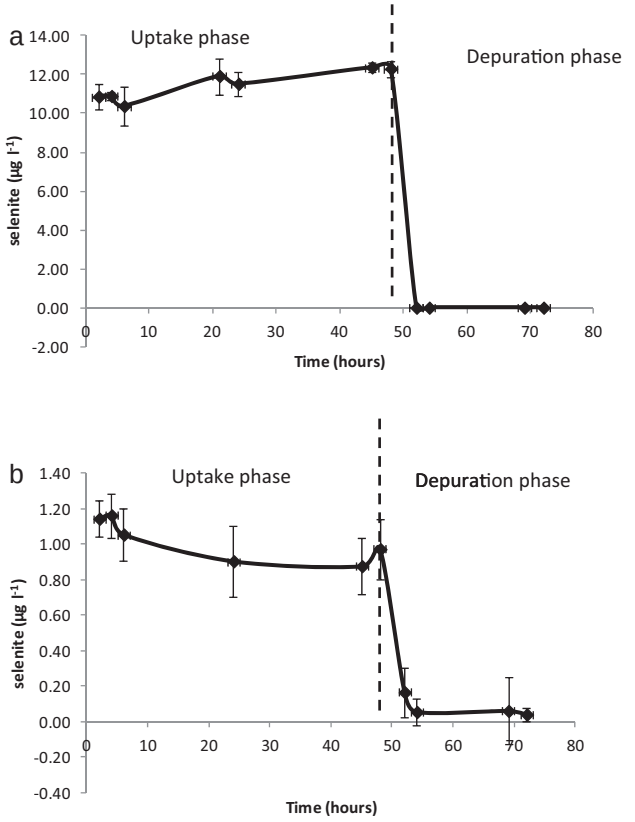

Fig. 4. Selenium concentration ($\mu g\,L^{-1}$) in the culture medium. (a) Nominal content of 10 $\mu g\,L^{-1}$ and (b) nominal content of 1 $\mu g\,L^{-1}$.

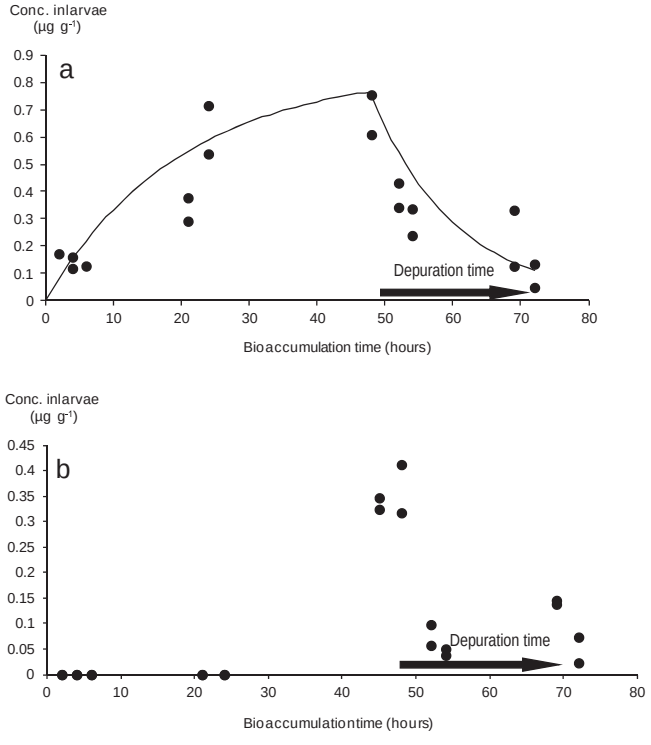

Fig. 5. Accumulation of selenite ($mg\,g^{-1}$) in larvae exposed to (a) 10 $\mu g\,L^{-1}$ and (b) 1 $\mu g\,L^{-1}$ of selenite. Solid circles (●) represent the experimental points and lines (—) the expected values based on the model calculations.

Table 3
BCFs and k_1 and k_2 values for larvae exposed to different concentrations of methylmercury and selenite.

	Uptake phase				Depuration phase
	k_1 (L kg^{-1} h^{-1})	k_2 (h^{-1})	BCF$_k$	BCF$_{48\,h}$	k_2 (h^{-1})
Conc. MeHg (μg L^{-1})					
10	35	0.015	2333	1119	0.008
1	150	0.03	5000	3748	0.02
Conc. Se(IV) (μg L^{-1})					
10	3.7	0.05	74	67	0.08
1	–	–	–	354	–

3.2.2. Calculation of bioconcentration factors

Bioconcentration factor (BCF), the most employed parameter to asses the mobility of a contaminant from surrounding media to living organisms [27], is defined by of the OECD test 305 as the ratio of a chemical concentration of the studied substance in an organism to the concentration in water once the equilibrium is reached [28]. The kinetics of the bioconcentration process can be described by a first order two-compartment (water and aquatic organism) model [29,30]. So, if the chemical concentration in water (C_w) is kept constant during the exposure period, the variation of a persistent chemical concentration on fish could be described by Eq. (1).

$$C_f = \frac{k_1}{k_2} \cdot C_w(1 - e^{-k_2 t}) \quad (1)$$

where k_1 is the uptake rate constant (L kg^{-1} h^{-1}), k_2 is the elimination or depuration rate constant (h^{-1}), C_w is concentration of exposure media (expressed in mg L^{-1}) and C_f is the chemical concentration in fish (expressed in mg kg^{-1} wet weight). When the equilibrium is reached (steady-state), Eq. (1) may be simplified as follows:

$$\frac{C_f}{C_w} = \mathrm{BCF}_k = \frac{k_1}{k_2} \quad (2)$$

The elimination or depuration of chemicals from aquatic and terrestrial organism often follows a first order kinetics that can be described by Eq. (3).

$$C_f = C_{f,o} \cdot e^{-k_2 t} \quad (3)$$

where $C_{f,o}$ is the concentration at the start of the depuration period and as in Eq. (1), k_2 is the elimination or depuration rate constant (h^{-1}).

So, BCFs values for methylmercury were calculated by using two procedures (Table 3): (1) ratio between the concentration of methylmercury in larvae and exposure media at the maximum time of uptake phase (48 h) (BCF$_{48\,h}$) and (2) by applying Eq. (1) (BCF$_k$). For the later method, methylmercury concentration in larvae was displayed versus uptake time and data were fitted to a non-linear regression curve using the software OriginPro v.8.5 (from OriginLab, Northampton, MA, USA). Data obtained in the depuration phase were fitted to Eq. (3). Thus k_1 and k_2 values were obtained and finally the BCF$_k$ was deduced from Equation (2).

Different values of BCF$_k$ and BCF$_{48\,h}$ were obtained for methylmercury, but not for selenium (Table 3) due to the fact that a steady-state during the uptake phase was not reached. Organometallic compounds with high octanol/water partition coefficient (K_{ow}) values could require longer exposure time than the 48 h used to reach a steady state. BCF$_k$ values, calculated under these conditions using the adjustment to the kinetic model, are considered quite accurate and will be used forward to compare our experimental data with those found in the literature. The BCF$_k$ values experimentally obtained in the present study are in good agreement with those BCFs compiled in the ECOTOX database [31], which are within the range of 1113–5900 for a 1 μg L^{-1} exposure and with other data obtained (3344 for a concentration of 1 μg L^{-1} and within the range of 595–3000 for a concentration of 10 μg L^{-1}) from experiments with medaka fishes (*Oryzias latines*) [24].

Bioconcentration factor has been often related with K_{ow} because of the link between K_{ow} and cell membrane permeability [29]. In general, the bioconcentration factors of chemicals increases as K_{ow} values increase. Table 4 lists the regression values obtained for BCF estimation from K_{ow} values [30,32,33]. By applying these values, a log BCF within the range 0.37–1.01 was obtained for methylmercury. This value is, far away from the experimental data ($\log K_{ow} = 1.71$) given by other authors [34] and also from the extrapolated values (3.04–3.69) obtained in this study. Some authors have questioned whether use of the BCF model is appropriate for describing the relationship between bioaccumulation and

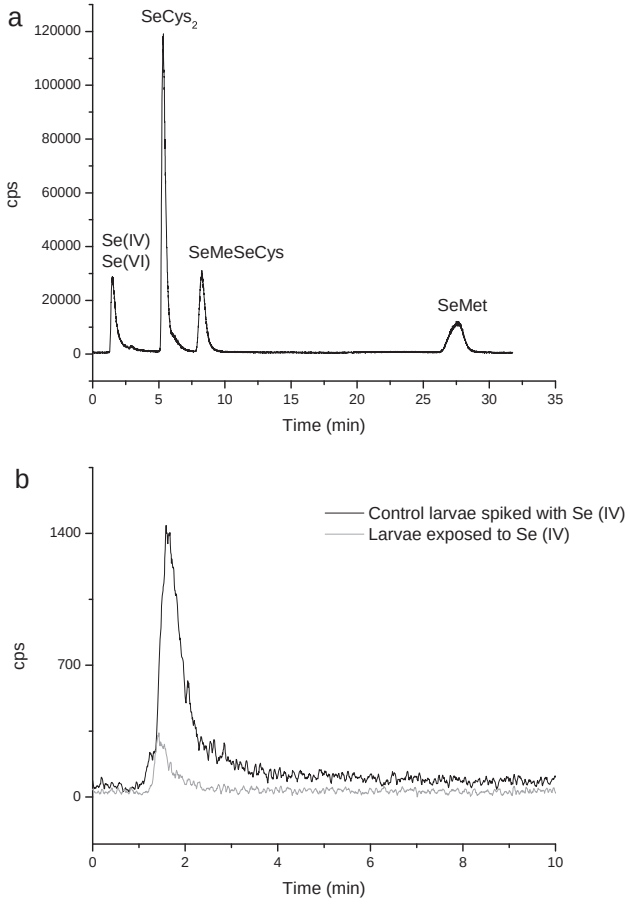

Fig. 6. Chromatographic profile obtained by reverse-phase LC–ICP-MS corresponding to (a) 50 μg L^{-1} of selenium mixture standards and (b) selenium extracted from the zebrafish larvae. Chromatographic column: Luna C18(2)-HST.

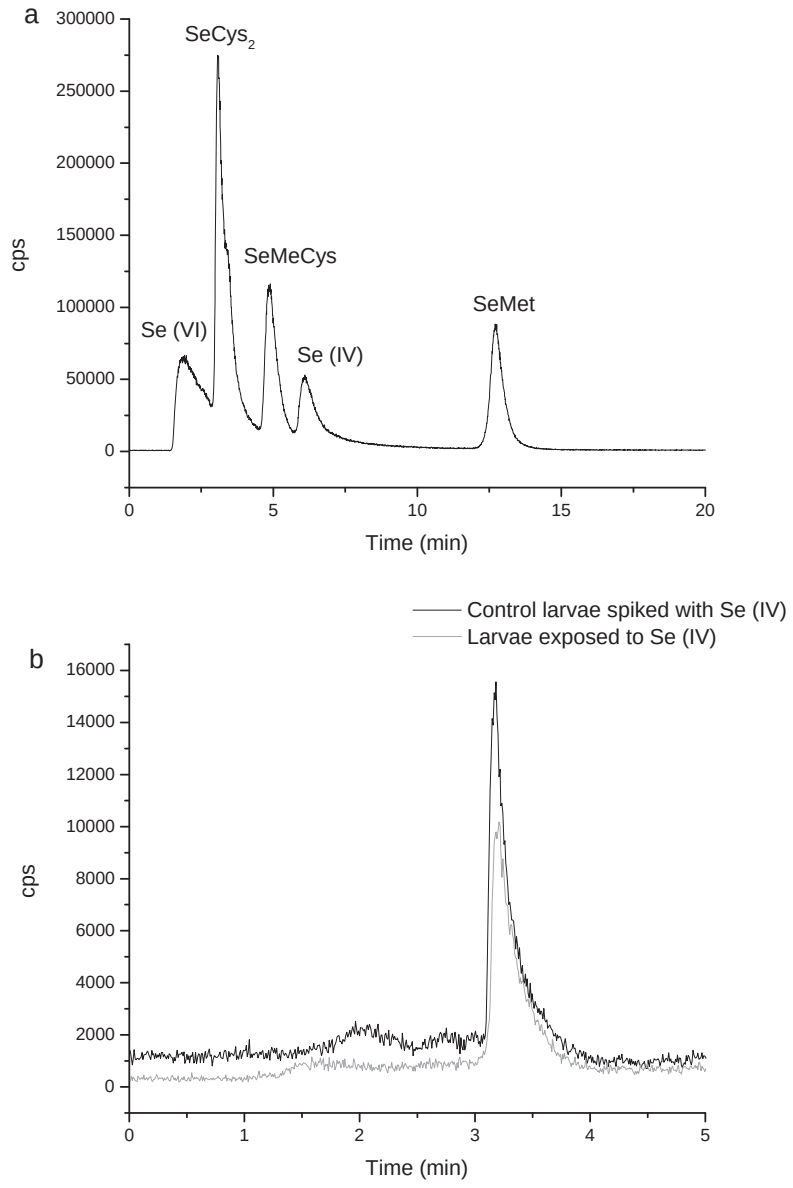

Fig. 7. Chromatographic profile obtained by reverse-phase LC–ICP-MS corresponding to (a) 50 $\mu g\,L^{-1}$ of selenium mixture standards and (b) selenium extracted from the zebrafish larvae. Column: Synergi-Fusion.

the potential effects of inorganic substances, as metals [27,35]. The reason of that could be attributed to the low lipophilicity of metals [11,34] compared to neutral organic substances, and therefore presenting other accumulation mechanisms in biota than those predicted by Fick's law [36]. Metals present complex internal dynamics of uptake (specific channels in the cell membrane, active transport, or endocytosis [37]) and storage (as detoxified forms, such as inorganic granules or bound to metallothionein-like proteins, active elimination) [35].

The high bioaccumulation capability of methylmercury has been explained by its ability to associate to sulfhydryl groups of proteins [8,24–26,38]. The differences in the values of the depuration constant (k_2) obtained after adjustment of data to the Eqs. (1) and (3) (Table 3) is another issue to be considered. These differences could be attributed to either the lack of experimental data to obtain a proper fitting or to the fact that the uptake of methylmercury could be affecting somehow, the depuration of methylmercury.

3.3. Exposure of larvae to selenite

3.3.1. Determination of total selenium and selenium species in exposure media and zebrafish larvae

As it is shown in Fig. 4, the concentration of selenium, added as Se(IV), in the exposure solutions (see Section 2) remains constant along the uptake phase ($11.4 \pm 0.7\ \mu g\,L^{-1}$ and $1.03 \pm 0.19\ \mu g\,L^{-1}$ for the highest and lowest concentration, respectively). No selenium was detected during the depuration phase, neither on the control group (always below detection limits), thus fulfilling the restrictions of the OECD 305 test. Selenium concentration found in larvae exposed to $1\ \mu g\,L^{-1}$ was rather low and only detectable after 45 h exposure (Fig. 5).

Selenium speciation in the zebrafish larvae was performed by LC–ICP-MS after selenium species extraction by using ultrasonic probe sonication. Table 2 shows selenium extraction efficiency obtained by applying different sample treatments. As it is shown, the maximum recovery (77%) was obtained with ultrasonic probe assisted-double aqueous extraction (sample treatment D) by

Table 4
Regression values for estimating the BCF values from $\log K_{ow}$ using a linear relation $\log BCF = a + b \log K_{ow}$.

a	b	n	r^2	Life stage	Reference
−0.46 ± 0.46	0.86 ± 0.09	11	0.91	Larvae	[30]
−0.23 ± 0.05	0.60 ± 0.01	2393	0.52	Adult fishes	[31]
0.06 ± 0.11[a]	0.0006 ± 0.05[a]	84	0.00	Adult fishes	[31]
−1.336	1	71	0.95	Adult fishes	[32]

[a] Only when $K_{ow} < 1$.

applying a sonication time of 80 s and 40% of ultrasonic amplitude. The efficiency of the extraction was not improved by further increasing the sonication time and the number of extractions. Therefore sample treatment D was selected for further speciation of selenium species since it provides the highest extraction yield while keeping species integrity [39–41]. It is important to point out that the use of enzymatic hydrolysis does not improve the efficiency of selenium extraction, which could indicate that selenium in zebrafish larvae is not bound or associate to peptide and proteins.

Fig. 6a and b shows the chromatographic profiles from Se standards solutions and zebrafish larvae exposed to Se(IV), respectively. The single peak that appears in the chromatograms of zebrafish larvae was identified by spiking experiments as Se(IV) (Fig. 6b). However, Se(VI) and Se(IV) co-elute from this column, being impossible to distinguish between these two species. To overcome this problem, different chromatographic conditions were employed using a reversed phase column with polar embedded groups (Synergi-Fusion) and a different mobile phase (Table 1. HPLC conditions (3)). By using these conditions, Se(VI) and Se(IV) were successfully separated (Fig. 7a). The chromatographic extracts of larvae exposed to Se(IV) exhibit only one peak at the same retention time that those from no exposed larvae spiked with Se(IV) (Fig. 7b). Thus, it can be concluded that Se(IV) is the unique selenium species found on exposed larvae, and therefore biotransformation of selenite did not occur.

3.3.2. Calculation of bioconcentration factors

BCF_k and $BCF_{48\,h}$ values were calculated (Table 3) following the two procedures previous applied for methylmercury. BCF_k values were only calculated for larvae exposed to 10 $\mu g\,L^{-1}$, because those exposed to 1 $\mu g\,L^{-1}$ accumulated selenium only after 45 h, which means that there were not enough experimental data for a good fitting to a non-linear regression. Values of BCF_k and $BCF_{48\,h}$ from larvae exposed to 10 $\mu g\,L^{-1}$ of Se(IV) were similar, showing that 48 h was enough time to reach the steady-state. BCFs values calculated in this experiment (60–74 for 10 $\mu g\,L^{-1}$) are in good agreement with others published using bluegill fish as a model, which reported a bioconcentration factor of 56 at 10 $\mu g\,L^{-1}$ [14] and also with those compiled on the ECOTOX database (bioconcentration factors ranging from 35 to 1850 [31]). BCFs values from METI-NITE Japan database [20], obtained according to the OECD 305 Guidelines but not published (BCF <8.1–10 for an exposure concentration of 10 $\mu g\,L^{-1}$ and lower than 85 for 1 $\mu g\,L^{-1}$) are slightly lower but still within the range.

Due to the lack of experimental K_{ow} values for selenite, BCF values were calculated by using a value of $\log K_{ow} = -6.13$ provided by the estimation software KOWWIN™ powered by EPI Suite™ of U.S. Environmental Protection Agency [5]. By using this data (see Table 4 for $K_{ow} < 1$), a value of BCF of 1.13 was obtained, which is markedly lower than those previously mentioned (60–74 for 10 $\mu g\,L^{-1}$). As methylmercury, no correlation between octanol–water partition model and BCF was obtained for selenite, and even a reverse relationship between BCFs and concentration of exposure is attained. These results strongly suggest what it was previously mentioned, that the bioaccumulation model is not suitable to be applied to inorganic compounds.

In summary, BCFs calculated in the present work by using zebrafish larvae are in good agreement with others published previously using adult fish; however a linear relation between BCFs and octanol-to-water partition was not achieved. Despite that, none of these issues diminishes the importance of bioaccumulation as a key parameter in assessing the environmental hazard associated to metals [33]. Based on the above, other metal-specific bioaccumulation models such as the biotic ligand model [42] and the free-ion activity model [43] might be used. Moreover, additional aspects such as relation between bioaccumulation and the covalent index [44,45] and the fish metabolism [46] should be explored. Some of these further studies are currently being carried out by our group.

4. Conclusions

BCFs calculated in the present work by using zebrafish larvae in a 48 h accumulation plus 24 h of depuration coupled to adjustment of experimental data to a first order kinetic accumulation model, are in good agreement with others published previously using adult fish which it makes a good and promising alternative to the OECD Bioconcentration Test 305. For this study a simple method to extract both selenium and methylmercury species from small and complex samples such as fish larvae, has been proposed. Optimized procedures for the extraction have allowed quantification of mercury and selenium species in both type of samples (water and larvae) as well as the determination of the bioaccumulation factors of these species. The extraction and cleanup methodology of larvae, a very small (less than 0.010 g of wet weight the pools of 20 larvae) and complex (lipid content around 15%) samples, presented here represents a relevant analytical advance in terms of rapidity and effectiveness for analyte leaching, low solvent consumption and low hazardous residues production, detection limits achieved, etc. Analytical procedures developed for speciation of mercury and selenium have shown that the biotransformation of species of the metals tested during the accumulation-depuration steps do not occur.

Although the use of BCFs for metals as a hazard criterion is complicated, and further studies should go in this direction, developing analytical tools that allow distinguishing between accumulated metals through biological mechanisms than those from only sequestering and storing is an important issue. The good agreement between the BCFs values found in this work and those reported in the literature clearly demonstrate that the proposed method using larvae exposed to both species is adequate. Zebrafish larvae has been shown as a good alternative to the OECD 305 bioconcentration test, which requires many adult fishes, implying a high cost, as well as complex, time-consuming experiments.

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