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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,