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In vivo bioconcentration of a metal mixture by *Danio rerio* eleutheroembryos

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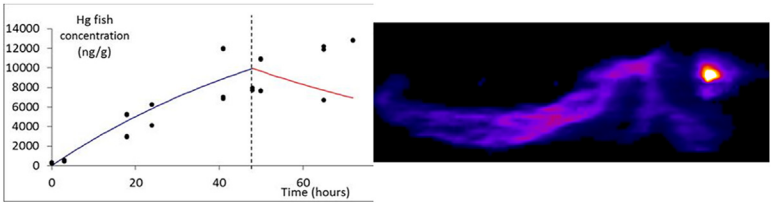
HIGHLIGHTS

- These data are among the very few published data on bioconcentration of metals mixture, and also by monitoring the exposure media concentration to comply with OECD35 requirements.
- LA-ICP/MS experiments are totally complementary to the BCF's data obtained.
- The combination of both set of experiments is unique within the environmental data published up to date.

GRAPHICAL ABSTRACT



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ABSTRACT

Exposure to heavy metals has represented one of the most serious health risks of environmental pollution over the last 50 years. Most of the bioconcentration studies that have been carried out to date explored only individual contaminants, unlike the real situations that occur in the environment. In this work, zebrafish eleutheroembryos were exposed to a mixture of $CH_3Hg(II)$, iAs(III), Ag(I) and Cd(II), and new BCFs were calculated and compared with those calculated from single metal exposures. In both cases, experimental conditions meet the OECD Test 305 conditions established for aquatic systems. In addition, spatial imaging obtained by laser ablation coupled to inductively plasma mass spectrometry (LA-ICP/MS), has been directly performed in these samples providing complementary information. The new BCF's have revealed some differences compared to single metal exposures when eleutheroembryos were exposed to the metal mixture, especially for iAs(III) and Cd(II). LA-ICP/MS images are in good agreement with the BFC's found, representing an interesting approach to get spatial distribution of metals that reinforces the toxicokinetic information.

1. Introduction

From the time of the industrial revolution anthropogenic inputs to the environment have created serious risks to human health associated with exposure to heavy metals, producing negative effects on fauna and flora and causing severe damage to human health either via the food chain or through direct uptake (Abboud and Wilkinson, 2013; Bharti, 2012; Su et al., 2014). The adverse effects of single metals on organisms have been well documented

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and water quality criteria have mainly been defined by performing single metal toxicity tests (Jaishankar et al., 2014; Tchounwou et al., 2012). However, aquatic organisms inhabiting metal-contaminated natural waters are often exposed to metal mixtures. This point has been recognized by the U.S. Environmental Protection Agency (USEPA) as being a key gap in metal risk assessments (Fairbrother et al., 2007). Interpretation of bioaccumulation or toxicity results in the case of metal mixtures is complex because there can be chemical interactions with media components, physiological processes leading to metal biotransformations, and competition at the toxicity site(s).

Over the past few years, the biotic ligand model (BLM) has been proposed as a tool to quantitatively model the manner in which metals "*bind*" to biological systems and how water composition (salts, organic matter, etc.) affects the transformation and biological availability of metals in aquatic systems (Paquin et al., 2002; Slaveykova and Wilkinson, 2005). Although the BLM model is broadly accepted, empirical data in the last few years has demonstrated that several mixtures do not behave as predicted by the process of competitive inhibition of metal binding to the organism described by the BLM. Synergetic and partial inhibition effects may appear, so new models including non-competitive, anti-competitive, and mixed inhibition forms must be developed (Chen et al., 2010; Komjarova and Blust, 2009).

It is recognized that mixtures should sometimes be modeled using a single uptake site and sometimes using multiple independent uptake sites, depending on the identity of the mixed compounds (Balistrieri and Mebane, 2014; Norwood et al., 2003). In a single uptake site model, each chemical contributes to toxicity in a proportional way and the same effect can be exactly produced by replacing one chemical with another (Altenburger et al., 2000). In this way, the *single uptake site model* when applied to a mixture will always predict results leading to unchanged or decreased bioaccumulation and consequently decreased toxicological effects (Balistrieri and Mebane, 2014; Norwood et al., 2003; Vijver et al., 2011). If a *more than one binding site model* is used, several other possibilities can occur and accumulation is dependent on the nature of the tested compounds. Thus, passive diffusion across the lipid bilayer of biological membranes following Fick's Law is the main process for uptake of neutral organic substances (McKim, 1994). On the other hand, as lipophilicity of metals is rather low, their accumulation in organisms can follow different pathways such as specific channels in the cell membrane, active transport, endocytosis, biotransformation to less toxic species, etc. (Faust, 1992; Tsopelas et al., 2005). For a model involving independent uptake sites, increased accumulation of a metal in the presence of other metals or partial inhibition is possible.

Bio-imaging analytical techniques are an emerging field in the life sciences. Laser ablation together with inductively coupled plasma mass spectrometry (LA-ICP/MS) is a versatile method for obtaining images from biological systems. This technique was initially established for elemental detection in inorganic samples, mainly of a geological nature (Russo et al., 2002) but was applied later to analyze metalloproteins after separation by gel electrophoresis (Becker et al., 2008). From these first applications to biological matrices, LA-ICP/MS has been increasingly used for elemental mapping in biological samples such as animal tissues (Becker et al., 2010), plants (Wu et al., 2009), organ sections, etc. (Bonta et al., 2014; Gholap et al., 2010). An advantage over other imaging techniques is the ability of LA-ICP/MS to provide isotopic information and multielemental detection as well as the sensitivity offered in comparison with other imaging techniques such as XRF, TEM, etc. By contrast, this technique needs specific sample preparation and the acquisition procedure is divided into several steps: first, the biological sample is fixed and embedded in a matrix (usually paraffin); second, the sample is metallized with a thin film (usually gold), and finally, the surface is irradiated with a pulsed laser to produce an aerosol containing the elements of interest. The sample is positioned on a motorized plate which moves with a specific spatial resolution under the pulsed laser beam to obtain ionic intensities corresponding to the selected m/z, and data is then be represented as a matrix that can be transposed to an image using specific software (Gut et al., 2015).

In this work we quantified the amount of different metals accumulated by zebrafish eleutheroembryos exposed to a mixture of CH_3Hg(II), iAs(III), Ag(I) and Cd (II) and evaluated the bioconcentration factors (BCF) of each metal in the mixture by comparing with the BCFs previously obtained in individual exposures. The exposure procedure has already been published (Zarco-Fernández et al., 2016) and complies with the main requirements of OECD Test 305. An analytical methodology based on LA-ICP/MS was developed and employed to evaluate metal distributions inside the zebrafish eleutheroembryos after exposure with the aim of supporting the bioconcentration data.

2. Materials and methods

2.1. Instrumentation

An MSP microwave oven (CEM MSP 1000, Matheus, NC) was employed to digest eleutheroembryos for the determination of total metals and ERM-CE278k was used as the reference material for validation. An inductively coupled plasma mass spectrometer (ICP/MS) HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan) equipped with a Conikal nebulizer, Fassel torch and double pass Scott-type spray chamber cooled by a Peltier system was employed to determine total cadmium, mercury, arsenic and silver content. The ions were detected at m/z corresponding to ^{111}Cd, ^{114}Cd, ^{200}Hg, ^{202}Hg, ^{75}As, ^{109}Ag and ^{89}Y, ^{115}In, ^{209}Bi were selected as internal standards for data collection. The digests were introduced into the ICP/MS via a Flow Injection Analysis (FIA) system, employing a sample loop of 300 μL.

Laser ablation coupled to ICP/MS for spatial distribution of metals was performed using a KGW-Yb crystal infrared femtosecond laser (ALFAMET, Nexeya Sa, Amplitude Systèmes, France) coupled to an ICP/MS (Perkin Elmer Sciex ELAN DRC2). The ablated material, approximately $2 \times 2\ mm^2$, was transported using He as a carrier gas to the ICP/MS. ^{111}Cd, ^{202}Hg, ^{75}As, ^{109}Ag, ^{197}Au, isotopes were measured. Taking into account the ablation cell washout time, the laser beam size and the XY stage speed the spatial resolution was evaluated to be in the range of 20 μm. ICP/MS data acquisition was set up to obtain square pixels of 20 μm. Data obtained were exported for further data processing using a VBA macro and images were processed with Image J (PAMAL, Plataforme d'Analyze des Métaux traces par Ablation Laser), for imaging processing (Sarrat et al., 2011).

2.2. Reagents

Analytical grade chemicals were used for all experiments. Standard solutions of cadmium (1000 mg L^{-1} Cd, Sigma Aldrich), mercury (1000 mg L^{-1} Hg, Merck, Darmstadt, Germany), silver (1000 mg L^{-1} Ag, Sigma Aldrich), arsenic (10150 mg L^{-1} As, Sigma Aldrich), yttrium (1000 mg L^{-1} Y, Sigma Aldrich), bismuth (1000 mg L^{-1} Bi, Spectrosol, England) and indium (1000 mg L^{-1} In, CPI international, USA) were employed. Working solutions were prepared daily by diluting the appropriate amount using Milli-Q Element ultrapure water (Millipore, Ohio, USA). Sub-boiled nitric acid (60% HNO_3, Scharlau, Barcelona, Spain), hydrogen peroxide