Chemosphere 111 (2014) 412–417

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Different uranium distribution patterns in cytosolic protein pool of zebrafish gills after chronic and acute waterborne exposures

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HIGHLIGHTS

- Screening of U distribution among proteins in gill cytosols of U exposed zebrafish.
- Significant U bioaccumulation in gills after 3 d of realistic chronic exposure.
- Different U-protein distribution patterns in cytosols as function of exposure level.
- Higher amount of U in the cytosolic fraction in case of high exposure level.
- Potential effect of U on Zn cytosolic burden.

ARTICLE INFO

Article history:
Received 17 July 2013
Received in revised form 17 March 2014
Accepted 22 March 2014
Available online 16 May 2014

Handling Editor: Klaus Kümmerer

Keywords:
Zebrafish
Gills
Uranium speciation
SEC–ICP-SFMS
Cytosolic distribution

ABSTRACT

The toxicity of uranium (U) to aquatic organisms depends notably on its compartmentalization in organs, tissues, cells as well as on its distribution among biomolecules. In order to contribute to the understanding of U accumulation and associated toxicity mechanisms in case of waterborne exposure, this study focused on U fate in the gills epithelia, uptake pathway, of the fish model *Danio rerio* (zebrafish). U distribution among cytosolic biomolecules was investigated after no addition (0 μg L^{-1} (c_0) for 3 and 30 d), chronic (20 μg L^{-1} (c_{20}) for 30 d) and acute (20 μg L^{-1} (c_{20}) and 250 μg L^{-1} (c_{250}) for 3 d) exposures to depleted U. Cytosolic U accounted for an average of 24–32% of gills burden for c_{20} and c_{250}, respectively. Size Exclusion Chromatography (SEC) coupled with Inductively Coupled Plasma-Sector Field Mass Spectrometry (ICP-SFMS) allowed identification of ecotoxicologically relevant U-containing fractions among cytosolic biomolecules as a function of exposure conditions. In c_0 and c_{20} samples, most U (*ca.* 80%) was found in the Low Molecular Weight fraction (LMW, <18 kDa), often considered as a detoxifying fraction. In c_{250} exposed fish, U was equally distributed between LMW (40%) and High Molecular Weight (HMW, 150–670 kDa; 40%) fractions, the latter including sensitive metalloproteins. Uranium-biomolecules were co-eluted with endogenous essential metal (Fe, Cu and Zn) species, however, no major influence on their cytosolic concentration and distribution pattern among cytosolic proteins was found.

1. Introduction

Various approaches have been developed to assess metal toxicity in aquatic living organisms over many years (Campbell et al., 2004). The Critical Body Residue (CBR), which proposes to link the toxicity of a metal to its bioaccumulation within organisms, suggests that organisms are able to distribute the metal burden among metabolically available (*e.g.* cytosol, organelles) and detoxified (*e.g.* metal-rich granules) cell compartments (Vijver et al., 2004; Adams et al., 2011). Thus, the determination of metal compartmentalization at the organ and subcellular levels is necessary (i) to better understand the kinetics of metal distribution into a biological cell or tissue and (ii) to better define the relationship between metal bioaccumulation and its biological effect. Within this compartmentalization, the cytosol of cells is of significant importance in the identification of toxic metal fractions as this compartment is known to have an important role in the toxicokinetics and toxicodynamics of metals (Amiard et al., 2006; Perceval et al., 2006; Geffard et al., 2010). It contains several proteins dedicated to detoxification, the latter occurring mainly *via* an association between proteins or other organic molecules and

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http://dx.doi.org/10.1016/j.chemosphere.2014.03.110
0045-6535/

metals. Potential transporters should then be studied to explain metal distribution and understand their fate and toxicity in organs (Szpunar, 2004).

Among the non-essential elements, uranium (U) is naturally occurring and exhibits both radio- and chemo-toxicity. It is found in freshwater ecosystems worldwide at concentrations ranging from 0.01 μg L^{-1} to 12.4 mg L^{-1} (Salonen, 1994; WHO, 2011). Its speciation in aquatic organisms remains poorly described (Bresson et al., 2011) despite significant knowledge on coordination chemistry and affinity for proteins (Ansoborlo et al., 2006; Michon et al., 2010). Previous studies showed that U was mainly bound to acidic proteins in exposed rat kidney (Frelon et al., 2009) and that hard Lewis cation (*i.e.* Ca^{2+}, Zn^{2+}) binding proteins as well as phosphorylated proteins were likely to complex uranyl (Dedieu et al., 2009; Pardoux et al., 2012). One major issue of this complexation is the possible perturbation of essential metal metabolic pathways as reported for Fe in rats (Donnadieu-Claraz et al., 2007).

The zebrafish is a model organism for aquatic vertebrates that has been extensively studied to assess the biological effects of different contaminants (Hill et al., 2005). Studies on zebrafish exposed to U are increasingly described (Goulet et al., 2011), however, mechanisms of toxicity are not well understood. Gill epithelia are the entry pathway following waterborne exposure to U, and the corresponding cytosolic fraction which contains soluble biomolecules are prone to undergo intra-cellular exchanges as well as metal detoxification.

Therefore, the objective of this study was to investigate the compartmentalization of U in zebrafish gills epithelia following different waterborne exposure conditions chosen to be representative of previous ecotoxicological studies. U compartmentalization was then studied at both the subcellular and the cytosolic protein levels to identify the ecotoxicologically relevant U fraction in this organ. Finally, the influence of this distribution on endogenous essential metals was also studied at both organization levels.

2. Materials and methods

2.1. Experimental workflow

Details on chemicals and reagents used in this study are given in Supplemental Document 1. Adult male zebrafish (fresh weight 400 ± 50 mg) were separated into three groups (2.75 fish L^{-1}) and exposed to waterborne U: 0 μg L^{-1} (control, c_0) for 3 and 30 d; 20 μg L^{-1} (c_{20}) for 3 and 30 d; 250 μg L^{-1} (c_{250}) for 3 d. No mortality was observed throughout the experiment. The pH (set at 6.5 ± 1.0), temperature (set at 26 ± 1 °C), major cation concentrations, total ($[U]_{total}$) and dissolved ($[U]_{dissolved}$) concentrations of U in water were monitored daily. On days 3 (3 d) and 30 (30 d), gills were collected after fish exsanguination. The epithelium was dissected, discarding the branchial arch, quick-frozen in liquid nitrogen and stored at −80 °C prior to sample preparation and analysis. 10 fish per exposure condition were randomly selected for bioaccumulation assessment and 5 gills were collected and prepared as described by Barillet et al. (2010) for U microlocalization by TEM–EDX imaging. Details about fish exposure can be found in Supplemental Document 1.

Fresh gill epithelium (6–12 mg) stored at −80 °C was thawed and homogenized in 600 μL ice cold 100 mM ammonium acetate buffer "B" (pH 7.4) using a 4 mL Potter–Elvehjem grinder (Dutscher, France; 2 × 1.5 min grinding, 30 s pause). Cytosol from gill epithelium was then obtained after centrifugation (Himac CS 120GX, Hitachi, Japan) of the homogenate at 100 000*g* for 1 h at 4 °C. Three aliquots of homogenate, cytosol and pellet were kept for further protein, U, Fe, Cu and Zn quantification and fractionation by SEC. Further details can be found in Supplemental Document 1.

2.2. Fractionation of uranium- and metal–biomolecule complexes by SEC coupled to ICP-SFMS detection

Freshly prepared cytosol were directly analyzed using a size exclusion chromatography (SEC) column (Superdex 200 10/300 GL, 600–10 kDa, 300 × 10, GE Healthcare, Sweden) connected to an Agilent 1200 series HPLC system (BinPump G1312A, Germany). The mobile phase was buffer "B" delivered at 0.7 mL min^{-1} and the injection volume was 100 μL. Calibration of the SEC column was performed using protein standards (thyroglobulin 670 kDa, ferritin 474 kDa, transferrin 80 kDa, Mn-SOD 40 kDa, myoglobin 16 kDa, Cd-MT2 6.8 kDa and cobalamin 1.3 kDa). Detection was achieved on-line by both a MWD (Multiple Wavelength Detector Agilent G1365B, Germany) recording the absorbance at 280 nm (aromatic aminoacids) for proteins and an ICP-SFMS (Element XR, ThermoFisher, Germany; Scott chamber, 1 mL min^{-1} conikal nebulizer and 2 mm i.d. injector) for highly sensitive elemental detection (^{235}U, ^{238}U, ^{64}Zn, ^{63}Cu, ^{54}Fe). To ensure reproducible and comparable results, the ICP-SFMS was tuned daily and sensitivity tests were performed by analyzing different standard solutions and running flow injection analysis (Supplemental Document 1).

To ensure the lowest ^{238}U background (≈100 cps), the column was systematically cleaned using the following procedure: (1) the column was first flushed with a mixture "M" of 200 mM NH_4HCO_3 and 2 mM EDTA for at least 2 h at 0.2 mL min^{-1} and conditioned with "B" for 2 h at 0.5 mL min^{-1}; (2) between each cytosol injection a wash run consisting of five 200 μL injections of "M".

Protein recovery from biological samples was assessed by injecting different cytosols with and without column and comparing the UV signal areas at 225 nm.

The U recovery was assessed by comparing U amount in the whole eluate (*ca.* 42 mL) and the corresponding amount in the 100 μL of cytosol injected.

2.3. Data processing and statistical analysis

Chromatographic and ICP-MS data processing and statistical analysis were carried out using Microsoft Excel 2003 (v11.8302.8221 SP3) with Grimmersoft Statbox (v7.1.9). Unless otherwise stated, statistically significant results are given at $p < 0.05$.

3. Results

Throughout the manuscript "c_x–yd" corresponds to the exposure condition "x μg L^{-1} – y days".

3.1. Uranium monitoring in exposure media

Irrespective of exposure condition and duration, the pH was maintained at 6.3 ± 0.2 and so within the set point of 6.5 ± 1.0.

The mean total U concentrations in water were 0.034, 0.12, 20.8, 18.6 and 265 μg L^{-1} for c_0-3d, c_0-30d, c_{20}-3d, c_{20}-30d and c_{250}-3d, respectively and were close to the nominal concentrations. The $[U]_{dissolved}/[U]_{total}$ ratio was similar for c_{20}-3d and c_{250}-3d but decreased from 69% to 52% for c_{20}-30d. Detailed data on exposure conditions are presented in Supplemental Table S1.

3.2. Uranium compartmentalization in zebrafish gills

The mean U concentrations in gill epithelia were 5.5, 4.3, 413, 553 and 5379 pg mg^{-1} wet wt. for c_0-3d, c_0-30d, c_{20}-3d, c_{20}-30d