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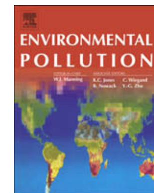

Impact of hypoxia on hemolymph contamination by uranium in an aquatic animal, the freshwater clam *Corbicula fluminea*

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Uranium contamination enhanced by hypoxia can deeply impair circulatory hemolymph flow in aquatic animals.

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ABSTRACT

Multi-stress situations are a major question and low-oxygenated waters (hypoxia) are a growing problem. Importantly, hypoxia stimulates the ventilatory flow rate in aquatic animals and this increases gill exposure to contaminants. Surprisingly, in the freshwater clam *Corbicula fluminea*, this is associated with increased bioaccumulation of uranium in gills but not in deep tissues. We searched for an explanation by analyzing hemolymph *U*-transport in *Corbicula* exposed to 0.36 μM dissolved uranium at various O_2-levels for 10 days. In hypoxia, one observed an increased *U* concentration in the arterial hemolymph flowing from gills to tissues but this was not associated with an increased *U* concentration in the venous hemolymph nor in the other tissues. We conclude that the cardiac flow rate must have decreased to explain this absence of over-accumulation. In addition to its already known deleterious effects, uranium can thus deeply impair cardiac flow rate in exposed aquatic animals during multi-stress exposures.

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1. Introduction

Uranium is naturally present in numerous hydrosystems and is potentially dangerous for both ecosystems and human health. The World Health Organization (Paris, France) guidelines for uranium concentration give a maximum value of 8.4 nmol L^{-1} in drinking water (WHO, 1998). However, this concentration may be further increased by anthropogenic contributions ranging from mining activities to management of nuclear fuel waste and military land use. Uranium behavior in natural ecosystems has been extensively studied (Ragnarsdottir and Charlet, 2000; Colle et al., 2001). Total uranium concentrations in natural waters range widely from below 0.05 nmol L^{-1} to 8.5 μmol L^{-1} (WHO, 2001). Importantly, few studies of uranium's biological effects in aquatic systems are available. Existing ecotoxicological data for freshwater organisms are still limited and until recently mostly came from acute exposure toxicity tests (Colle et al., 2001; Ribera et al., 1996).

Heavy metal bioaccumulation processes in aquatic animals are now well documented (Deforest et al., 2007; Goodyear and McNeill, 1999; Wang and Fisher, 1999). However, the understanding of the mechanisms involved remains a question still debated. This is especially true for metals like uranium whose fate in the environment remains an open question. Most of the time one relies on chemical arguments to explain the bioavailability of the dissolved metal to the biological membrane, and many theories show how this drives the uptake and internalization of the contaminant into the organism (Morel, 1983; Campbell, 1995; Paquin et al., 2002). The recently described physiological mechanism, in which the ventilatory activity of the organism is a potent contributor to the uptake rate of trace metals, offers a deeper understanding of the metal bioaccumulation process. Specifically, the limiting role of ventilation was demonstrated in bivalves (Tran et al., 2001, 2002, 2005), crustaceans (Pierron et al., 2007a) and fish (Lloyd and Herbert, 1962; Pierron et al., 2007b). Campbell (2004) suggested that it could at least partly explain the scattered differences in metal accumulation and toxicity, at constant contamination pressure, reported in the literature.

However, the physiological adaptations of water-breathers to varying oxygen levels are quite complex (Massabuau, 2001). This is obviously the case for the physiological mechanisms underlying metal transfer from gill to internal tissues. This study was done in

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an invertebrate, the freshwater clam *Corbicula fluminea*, to get more insight into the hemolymph and circulatory component during uranium contamination by the direct exposure route, i.e. water. In addition, we are also presenting new information about mollusk bivalve valve activity, which is the very first physiological limiting step in bivalve contamination processes. Valve activity recording is currently receiving a renewed interest in different bivalve types (Tran et al., 2003, 2004, 2007; Liao et al., 2005, 2007; Riisgard et al., 2006; Wilson et al., 2005; Robson et al., 2007). This technique is clearly of great interest in terms of both bivalve ecology and water biomonitoring. However, its interpretation still requires a solid documentation to test and estimate its potential under various types of environmental changes. In this work, we used the water oxygenation level to experimentally manipulate, at constant water uranium concentration, the accumulation of metal in the hemolymph of *C. fluminea*. In *C. fluminea* it was shown that the kinetics of uranium uptake and bioaccumulation are enhanced under hypoxia in the gills but not in deeper tissues such as the adductor muscle, the mantle and the visceral mass (Tran et al., 2005). We previously speculated that the gills could act as a primary filter limiting uranium export to the internal organs. We will see that this report offers an alternative explanation.

2. Materials and methods

2.1. General conditions

Experiments were done with 70 freshwater clams, *C. fluminea* collected in Saint-Seurin sur l'Isle (Gironde, France), between February and March 2003. During the holding period, they were kept in aerated tap water (tank volume 200 L). Half the volume of water was changed once a week. The animals were fed daily with a suspension of unicellular algae (*Scenedesmus subspicatus*, total density of 1–2×10^5 cells mL^{-1} in the tank immediately after addition). These conditions were maintained for at least 3 weeks to settle animal behavior. Before experiment, bivalves were allowed to acclimate for 7 days (t_{-7} to t_0) to the experimental set-up composed of two tanks (volume 20 L, supplied at a renewal rate of 10 L day^{-1}) whose bottoms were covered with quartz sand. These tanks were isolated from laboratory vibrations by anti-vibrating benches to minimize external disturbances that could interfere with the natural behavior of *C. fluminea* (Tran et al., 2003).

2.2. Water quality parameters

The experiments were performed at 15.0 ± 0.5 °C. The pH was regulated at 7.00 ± 0.05, controlled by a pH-CO_2 stat. Two O_2 partial pressures (PO_2) were selected: 21 kPa for normoxic conditions (air-equilibrated water; corresponding to an O_2 concentration of 10 mg L^{-1}) and 4 kPa for hypoxic water (20% of air saturation, 2 mg L^{-1}). Hypoxic water was obtained by bubbling an N_2/air gas mixture via mass flow controllers (Tylan General, model FC-260) driven by a laboratory-constructed programmable control unit. The gas mixtures were bubbled directly at the bottom and the middle of the water column to improve algal mixing and gas–water equilibration, and to control the respiratory status of the bivalves. During experiments, the ionic composition (mmol L^{-1}) of water, controlled by adding synthetic water using a pump under flow-through conditions, was as follows: Ca^{2+}, 0.37 ± 0.02; Na^+, 0.52 ± 0.01; K^+, 0.35 ± 0.01; Mg^{2+}, 0.21 ± 0.01; Cl^-, 0.29 ± 0.01; NO_3^-, 0.08 ± 0.01; SO_4^{2-}, 0.05 ± 0.01; HCO_3^-, 1.55 ± 0.01. Cations were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometer (Perkin Optima 4300 DV), and anions by ion chromatography (Dionex DX 120, detection limit 10 µmol L^{-1}). During the experiments, *C. fluminea* were continuously fed with *S. subspicatus*. The algal concentration was measured with a Coulter counter and held constant at $0.85 \pm 0.15 \times 10^5$ algae mL^{-1} for the two PO_2 conditions, with and without uranium addition, the objective being to avoid the stress induced by starvation of the bivalves during the experiments and to moderately stimulate their ventilatory activity for feeding purposes (Tran et al., 2002).

2.3. Uranium exposure conditions

Treatments corresponding to *U* exposure conditions were performed with a constant total uranium water concentration fixed at 0.36 µmol L^{-1}. Uranium was added from a stock solution of uranyl nitrate [$UO_2(NO_3)_2 \cdot 6H_2O$; Sigma–Aldrich; 10 mmol L^{-1} as *U* concentration]. The uranium concentration was held constant throughout the experiment by the flow-through system. During the experiments, the 20-L glass tanks were supplied at a renewal rate of 10 L day^{-1}. Concentration of dissolved uranium (water filtered through 0.45 µm Whatman polycarbonate–propylene filters) and total uranium (dissolved and algal-bound forms) were analyzed daily. All water samples were acidified by addition of 2% HNO_3 and kept in darkness at 4 °C prior to analysis. The total uranium content was determined by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin–Elmer Optima 4300 DV, detection limit: 13.9 nmol L^{-1}). *U* concentration from t_0 to t_{10} (no *U* added) in both experimental tanks was below detection limits and from t_{10} to t_{20}, the mean values measured in 0.45 µm filtered water (algae removed) were 0.369 ± 0.005 µmol L^{-1} at $PO_2 = 21$ kPa and 0.357 ± 0.006 µmol L^{-1} at 4 kPa. They were 0.373 ± 0.004 µmol L^{-1} and 0.362 ± 0.006 µmol L^{-1}, respectively, in unfiltered water (algae included). Note that the *U* fraction on algae (absorbed and adsorbed) was only 1.1% of the total uranium concentration at $PO_2 = 21$ kPa and 1.4% at $PO_2 = 4$ kPa of total water contamination.

2.4. Experimental procedure

Following the 7-day acclimation period, two groups of animals were exposed for 10 days (t_0–t_{10}) to two water PO_2-levels (21 and 4 kPa) without uranium. Then, uranium was added and held at 0.36 µmol L^{-1} for the following 10 days (t_{10}–t_{20}). Note that t_{10} is a reference point and that animals were sampled before *U* addition. Analysis was conducted on 70 individuals (mean body fresh weight 2.31 ± 0.16 g fw) 20 of which were equipped with impedance electrodes (see below).

2.5. Valve movement activity

To evaluate the effect of the hypoxic stress alone and its interaction with uranium contamination in *C. fluminea,* we continuously, from t_0 to t_{20}, recorded the valve activity with a valvometer by impedance. This experiment was performed on two groups of 10 *C. fluminea* exhibiting a weight 2.30 ± 0.16 g fw (mean ± SE), where fw corresponds to the fresh weight of the animals without the shell. Briefly, animals were equipped (at t_{-7}) with lightweight (20 mg) impedance electrodes glued to both shells. These allow the animals to be free to move around and to position themselves in the sand with minimal experimental constraints. The measurement principle is based on the application of Ohm's Law, $U = R \times I$. The apparatus measures a current that varies according to the distance between the electrodes. For more details, the technique is described in Tran et al. (2003). The daily valve-opening duration was determined for each animal. As there were no significant trends during the 10-day experimental period, a mean value per animal was calculated for each 10-day exposure period. Then, a mean value was calculated with the 10 animals studied for each condition. Each mean opening value was then compared to the paired hemolymph analysis performed in the same animal.

2.6. Arterial and venous hemolymph and gill sampling

For each PO_2 condition, groups of 10 animals were sampled at t_{10}, t_{11}, t_{14} and t_{20}. At t_{20}, the groups equipped with electrodes were sampled, to determine the relationship with valve-opening duration. On each sampled animal, arterial hemolymph was taken from the heart ventricle, and venous hemolymph from the lacunae of the posterior adductor muscle. For hemolymph sampling (method developed by Tran et al., 2000), animals were prepared 7 days prior to the experiment. A groove was drilled into the shell above the heart to weaken the shell. For the sampling, each bivalve was gently taken from water to not disturb the others, and instantaneously, its shell was broken with a knife. Under a binocular microscope and using a 100 µL glass capillary tube, the heart ventricle was punctured for arterial sampling (mean ± SE of volume sampled: 0.16 ± 0.01 mL), then the posterior adductor muscle was punctured with a 1 mL syringe for venous sampling (mean ± SE of volume sampled: 0.61 ± 0.03 mL). Total hemolymph sampling – arterial and venous – was completed in less than 2 min after removal from water. This sampling technique was critically assessed in Tran et al. (2000) for bivalves. Then, the gills were dissected, blotted dry with tissue paper and weighed. The mean gill tissue wet weight was 0.140 ± 0.005 g.

2.7. Measure of uranium accumulation in gills and hemolymph

Each sample was mineralized in a polypropylene tube: 3 h with HNO_3 (65%, Merck) at 95 °C, then 1 h with H_2O_2 (30%, Merck) at 120 °C. Digested samples were then diluted with ultra-pure water (USF, Elga, Vivendi) up to a volume of 20 mL for gills and 1 mL for hemolymph. Uranium concentration in gill tissue was analyzed by ICP-OES (Perkin Optima 4300 DV, detection limit: 13.9 nmol L^{-1}) and in hemolymph by ICP-MS (Perkin, detection limit: 0.03 nmol L^{-1}).

2.8. Statistical analysis

Results are expressed as mean ± standard error. Treatment differences were determined using one-way analysis of variance (ANOVA) after checking assumptions (normality and homoscedasticity of the error term). Where assumptions were not met, the non-parametric Kruskall–Wallis test was used. If the null hypothesis was rejected, the Tukey test was applied to detect significant differences between conditions. For all statistical results, a probability of $p < 0.05$ was considered significant. Statistical analyses were performed using the Sigma Stat software for Windows, Version 3.1 (SYSTAT, Richmond, CA, USA).

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