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Whole-body autoradiography: An efficient technique to study copper accumulation and body distribution in small organisms

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ABSTRACT

Copepods have been widely used to evaluate toxicity of metals present in marine environments. However, a technical difficulty is to understand the possible routes of metal uptake and to identify in which tissues or organs metals are being accumulated. Traditional techniques are hard to be employed once each organ has to be analyzed separately. Autoradiography is an alternative technique to circumvent this limitation, since metal distribution in tissues can be visualized and quantified, even in small organisms like copepods. In the present study, accumulation and distribution of ^{64}Cu in the copepod *Calanus hyperboreus* was studied using autoradiography. Copepods were exposed for 2 h to copper (2.3 mg L^{-1}; 1.08 MBq ^{64}Cu mg^{-1} Cu) and then allowed to depurate for 2 h in clean seawater. Total ^{64}Cu was determined by gamma-spectrometry after a metal exposure and a depuration period. ^{64}Cu distribution was determined based on images generated by autoradiography. Metal accumulation was observed on all external surfaces of the copepods, being accumulated mostly on the ventral region, followed by dorsal, urossoma and internal regions. After depuration, radioactivity levels had a decrease in the sum of external body surface. Our results show that copper uptake by *C. hyperboreus* is fast and that a non-negligible proportion of the accumulated metal can reach internal tissues, which may lead to detrimental physiological effects. Moreover, whole-body autoradiography was demonstrated to be an efficient technique to study copper accumulation and body distribution in a very small organism such as the copepod *C. hyperboreus*.

1. Introduction

In crustaceans, tissue concentrations of trace metals show great interspecific variations, even in the absence of metal contamination associated with anthropogenic sources (Rainbow, 1988). Small crustaceans are typically permeable all over, whilst large ones may restrict permeability to selected regions, such as the gills (Rainbow, 1997a). In the former, a permeable cuticle is not considered to be a significant barrier to trace metal uptake. Under the cuticle, the epithelium cell membrane is the true barrier to the uptake of chemicals (including metals) into cells, protecting the cell biochemical pathways from the chemistry of the environment.

For many reasons, copepods have been widely used to evaluate toxicity of compounds present in marine environments. They are easily caught and cultured in laboratory (Barata and Baird, 2000). They play an essential role in the marine trophic chain. And finally, copepods are quite sensitive to trace metal contamination (Pinho and Bianchini, 2010).

In suspension feeders, like copepods, uptake from the dissolved phase and food ingestion can be equally important for metal accumulation. For copper, food has been shown to be the dominant source for uptake (Chang and Reeinfielder, 2000). Regarding bioavailability, it is important to understand all possible routes of metal uptake, and in which tissues or organs metals are being accumulated. While there is a number of studies reporting metal distribution and the amount of metal accumulated in the body of aquatic organisms, e.g. crabs and fish (Rainbow, 1985; Jeckel et al., 1996; Zimmermann et al., 2004; Martins et al., 2011), studies on metal quantification in small invertebrates using traditional techniques are technically difficult to be performed, once each organ has to be analyzed separately.

Autoradiography is an alternative to deal with the technical difficulty mentioned above, since fine-scale tissue distribution of metals can be visualized and quantified. As described by Ullberg et al. (1982), whole-body autoradiography is an imaging process that determines the *in situ* localization of radiolabelled xenobiotics in cryosections of a whole animal, which include representative

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samples of all major tissues. These sections are qualitatively and/or quantitatively evaluated for radioactive content after exposure to a phosphor imaging plate and analysis using a suitable software (Solon and Kraus, 2002). Therefore, the objective of the present study was to demonstrate the feasibility of this technique to determine metal accumulation and body distribution in small organisms. Using this technique, we studied the short-term accumulation and body distribution of ^{64}Cu in the copepod *Calanus hyperboreus*.

2. Material and methods

2.1. Copepod collection and acclimation

Calanus hyperboreus were collected in May 2006 in the Saint Lawrence estuary (48°40′000N lat. −068°35′000W long; Quebec, Canada). Copepod collection was performed using a 1 m × 3 m conical net (333 μm mesh size) towed vertically at 0.5 m s^{-1}. Approximately 200 adult females were selected and transported to the Aquaculture Station of the "Institut de Sciences de la Mer" (Pointe-au-Père, Quebec, Canada) and acclimated to laboratory conditions for 2–4 weeks. Copepods were kept in 10 L plastic buckets containing water at salinity 27. Water was not aerated, but completely renewed every 4 d. Copepods were fed every 96 h with an algae mixture (*Isochrysis galbana*, *Pavlova lutheri* and *Nannochloropsis* sp.). Temperature was kept at ~10 °C by keeping the plastic buckets in an aquarium with running seawater. Natural photoperiod was used. For both acclimation and toxicity tests, filtered seawater (0.7 μm mesh GF/F filter) from the Saint Lawrence estuary was employed.

2.2. Isotope origin and preparation of the radioactive solution

^{64}Cu (half-life = 12.7 h) was obtained from the McMaster University nuclear reactor (Hamilton, ON, Canada) by neutron irradiation of a copper wire weighing 63.8 mg. Activity of the wire at reference time was 848 MBq. In the following day, the copper wire was dissolved in 0.6 mL of HNO_3 and the total volume was then brought to 10 mL with deionised water (final Cu concentration = 6.38 mg mL^{-1}). At the beginning of the experiment ($t = 0$), the decay-corrected activity of the solution was 6.88 MBq ^{64}Cu mL^{-1} and the specific activity was 1.08 MBq ^{64}Cu mg^{-1} Cu. All radioactivity data obtained were decay-corrected to $t = 0$, as follows:

$$\text{Activity}_{\text{at } t} = \text{Activity}_{\text{at } t=0} \exp(-(t - t_0) * 0.0546\ \text{h}^{-1}) \quad (1)$$

where t is time (h) and 0.0546 h^{-1} is the decay rate constant for ^{64}Cu obtained as follows:

$$-(\ln 0.5)/\text{half-life}\ ^{64}\text{Cu} = 0.693/12.7\ \text{h} \quad (2)$$

2.3. Copper exposure

Nominal metal concentration added to the water was a compromise between copper toxicity to *C. hyperboreus* (2-h LC_{10}: lethal concentration to 10% of individuals exposed to copper for 2 h) and technical requirements for a successful whole-body autoradiography (WBARG). It is clear that the copper concentration employed in the present study is not environmentally relevant.

However, this concentration was used to assure a significant copper accumulation and body distribution after a short period of exposure (2 h) without causing copepod mortality. The ^{64}Cu half-life is of only12.7 h. Therefore, a successful WBARG using ^{64}Cu can only be performed following a very short-time exposure. Based on previous results obtained, copepods were acutely exposed (2 h) to radioactive copper as $^{64}CuNO_3$ at a final concentration of 2.71 MBq ^{64}Cu L^{-1}, which corresponded to a metal concentration in the experimental media of 2.33 ± 0.05 and 2.35 ± 0.05 mg Cu L^{-1} for groups 1 and 2, respectively.

^{64}Cu exposure was performed in duplicate (groups 1 and 2) in glass jars containing 200 mL of filtered seawater at 10 °C. Temperature was kept constant by placing the jars in an aquarium with running seawater. In each jar, 20 copepods were placed in a plastic container, opened at one end and closed at the other end with a 300 μm mesh Nytex net. By using these plastic containers, it was possible to handle the copepods during the experiment without actually manipulating them.

Radioactive copper was added to the jars 1 h before the beginning of the experiment. The plastic containers with the copepods were then placed into the glass jars and exposed to copper for 2 h. After the exposure period, the plastic container with the copepods was lifted from the experimental medium, washed for few seconds in a copper-concentrated solution (10 mg L^{-1}) to displace the loosely bound ^{64}Cu, and then successively dipped into three jars containing filtered seawater to rinse out the copper solution. After blotting to remove the excess of water from the Nytex net, the plastic container was placed into a plastic Petri dish and the radioactivity from the sample was measured for 1 min with a 76 mm diameter NaI(Tl) gamma detector (Canberra), using the 511-keV gamma peak of positron annihilation. A planar standard made from a paper filter cut to the size of the Nytex net was spiked four times with 5 μL of the ^{64}Cu solution in a centered square pattern, for a total of 137.8 kBq. This standard was used to calculate the efficiency of the detector for that particular geometry and to check its stability during the experiment (Fig. 1). A sample (2 mL) from the exposure medium was collected in duplicate and the radioactivity measured with a Wizard 1480 gamma counter (3.3% absolute efficiency for 511 keV annihilation gamma ray of ^{64}Cu for a 2 mL sample in a 12 × 75 mm test tube geometry).

At the end of the exposure period, radioactivity was measured in each copepod (μCi g^{-1} ww). Then, half of the Cu-exposed copepods was blotted dry, weighed, and prepared for WBARG. The other half was transferred to clean filtered seawater and allowed to depurate for 2 h. At the end of the depuration period, radioactivity was measured in each copepod and finally copepods from groups 1 and 2 ($n = 10$ for each group) were blotted dry, weighed, and prepared for WBARG.

2.4. Whole-body autoradiography

A drop of a methylene blue solution was first added onto the copepods, allowing for a better visualization of the organisms during sectioning. Each group of copepods was embedded in a layer of carboxymethylcellulose gel and subsequently frozen in a slurry of

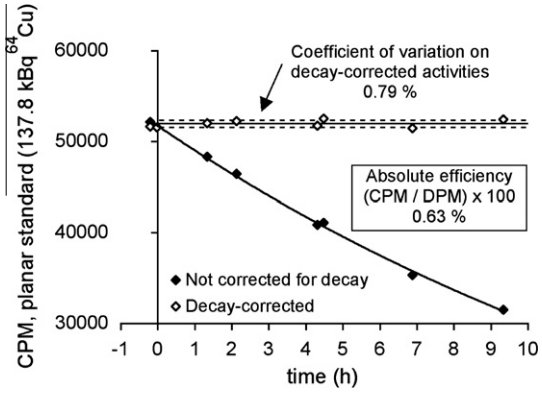

Fig. 1. ^{64}Cu radioactivity in exposure water (CPM) as a function of the exposure time. Insert shows average ^{64}Cu concentrations for the whole exposure period.

ethanol and dry ice. Then, a second layer of carboxymethylcellulose gel was added onto the first layer and frozen, forming a block.

Blocks were sectioned at −20 °C on a Leica CM3600 cryomicrotome to a thickness of 100 μm. Sections obtained were exposed on a phosphor screen for 1 h, at −20 °C. The screens were then scanned with a Cyclone Phosphor Imager, and the radioactivity distribution in the sections visualized and quantified using the software Optiquant. Radioactivity was measured in sections for which five regions of interest could be seen: ventral and dorsal surfaces, urossoma, internal environment, the sum of the external regions and the sum of all regions (which corresponded to the whole-body). They were used only those blocks with copepods in a good position for sections. Sixteen tissue sections from 16 different copepods and 13 tissue sections from 13 different copepods were selected from the replicates of each accumulation and depuration group, respectively. In the case with more than one tissue section from a same copepod, was used those with the highest total accumulated radioactivity (DLU mm^{-2}). Therefore, was possible to do a standardization, using the most similar section area in copepods from both groups (accumulation and depuration). Results obtained were corrected for decay and expressed as digital light units per mm^2 of the section surface (DLU mm^{-2}). Percentage of ^{64}Cu radioactivity in each tissue relative to the whole body was also calculated.

Values were expressed as mean ± one standard deviation (SD) of the mean. As homogeneity of variances assumption was not met, differences in copper accumulation (expressed as DLU mm^{-2} and as % of whole body) in each body region (ventral, dorsal, internal, and urossoma), and differences in copper accumulation (expressed as DLU mm^{-2}) between accumulation and depuration groups (in each body region; in internal and sum of external regions) were assessed by one-way non-parametric analysis of variance (Kruskal–Wallis ANOVA). Significance level adopted was 95% ($\alpha = 0.05$).

3. Results

No copepod mortality was observed during the experiment. Once corrected for decay, the radioactivity measured in the planar standard varied by only 0.8% ($n = 8$). Radioactivity in water varied by less than 4% during the exposure period (Fig. 1) and was then assumed to be constant.

3.1. Uptake and elimination of ^{64}Cu

At the end of the exposure and depuration periods, whole-body ^{64}Cu accumulation was 72.9 ± 20.8 and 34.3 ± 12.1 μCi g^{-1} ww (mean ± SD) using gamma counter.

3.2. Whole-body autoradiography

For anatomical reference, a light microscopy picture of *C. hyperboreus* is shown in Fig. 2.

For both exposure and depuration periods, radioactivity labeling was found on all external surfaces, as well as in the internal environment of the copepods. However, labeling was much lower in the internal environment than on external surfaces (Fig. 3). After the depuration period, a decrease of radioactivity labeling on the sum of external surfaces (ventral, dorsal and urossoma regions) was observed, being individually significant on ventral region (Fig. 3). It was also noted that specific regions showed higher levels of ^{64}Cu after the exposure period. They include the mouth area (mandible, maxilla, and maxillipeds), which is part of the ventral region, and the genital segment, which is located in the urossoma region (Fig. 3).

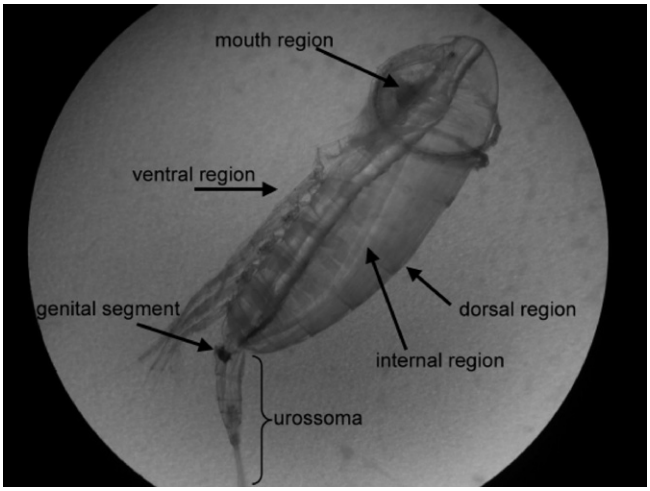

Fig. 2. Light microscopy picture of *Calanus hyperboreus* showing the pre-defined regions analyzed in the autoradiograms (ventral, dorsal, internal and urossoma regions), mouth region, and genital segment.

Quantitative analysis of the autoradiograms showed that accumulation of radiolabelled copper in the internal region of copepods was around fourfold lower than that observed in the other three regions analyzed (ventral, dorsal and urossoma regions) after exposure period. A similar finding (around threefold lower) was observed after depuration period (Fig. 4).

Most of copper accumulation was found to occur in the ventral region (47.8 ± 5.7%), followed by the dorsal region (23.1 ± 6.0%), urossoma (12.6 ± 4.6%) and internal region (9.3 ± 2.2%) (mean ± SD; Fig. 4). After the depuration period, proportions of radioactivity in the ventral, dorsal, urossoma and internal regions did not change respect to those observed after the exposure period (41.6 ± 7.4, 25.3 ± 4.8, 13.0 ± 6.9, and 12.1 ± 3.1%, respectively; mean ± SD; Fig. 5).

4. Discussion

Knowledge regarding metal toxicity in aquatic animals was initially originated from studies performed on relatively large organisms, like fish and crabs (Grosell et al., 2002). Assisting the interpretation of these experiments a number of studies reporting metal distribution and the amount of metal accumulated in the bodies of the studied organisms have been performed (Rainbow, 1985; Jeckel et al., 1996; Zimmermann et al., 2004; Martins et al., 2011). Studies on metals bioaccumulation in mussels were also performed worldwide, as for example the studies done in the scope of the "Global Mussel Watch Program", which has been developed since the 1970s. Data generated from these studies, are generally not sensitive to metal exposure. Presently, the use of an "Artificial Mussel", a cylinder containing a polymer-ligand, has been suggested to correct interferences of biological factors on metal accumulation (Gonzalez-Rey et al., in press). However, as a non-living device, other surface problems and limitations occur, such as the detrimental effect of biofouling on metal accumulation (Gonzalez-Rey et al., in press) and the impossibility to relate accumulation with physiological effects.

The number of studies using small animals, such as copepods, has been increased. In fact, marine copepods have been employed in toxicity tests for regulatory purposes in some countries (ISO, 1999). These small crustaceans are very sensitive to metal toxicity (Hook and Fisher, 2001) and are being used in toxicological studies either in laboratory conditions (Pinho and Bianchini, 2010) or field conditions (Bianchi et al., 2003).

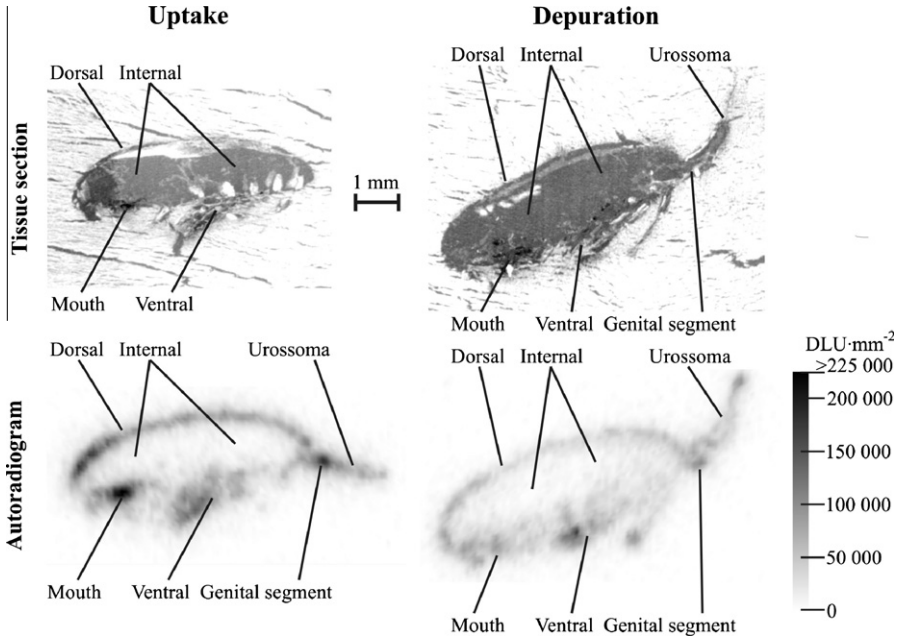

Fig. 3. Autoradiograms and corresponding tissue sections of *Calanus hyperboreus* after 2 h of exposure to 2.71 MBq ^{64}Cu L^{-1}, and after a 2 h depuration period. Darkest areas in autoradiograms correspond to the highest levels of radioactivity.

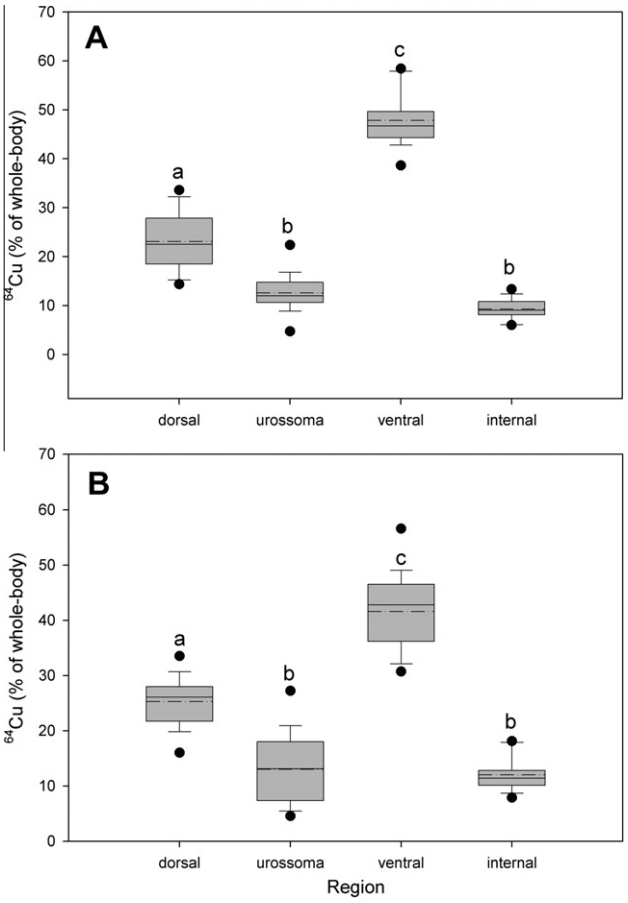

Fig. 4. Radiolabelled copper concentrations expressed as DLU mm^{-2} in *Calanus hyperboreus* after 2 h of exposure to 2.71 MBq ^{64}Cu L^{-1} (A), and after a 2 h depuration period (B). Box represents median ± 95% IC, solid line represents mean, dash-dot line represents median, and * represents the 5th and 95th percentiles of outliers. Different letters indicate significantly different means ($P < 0.05$).

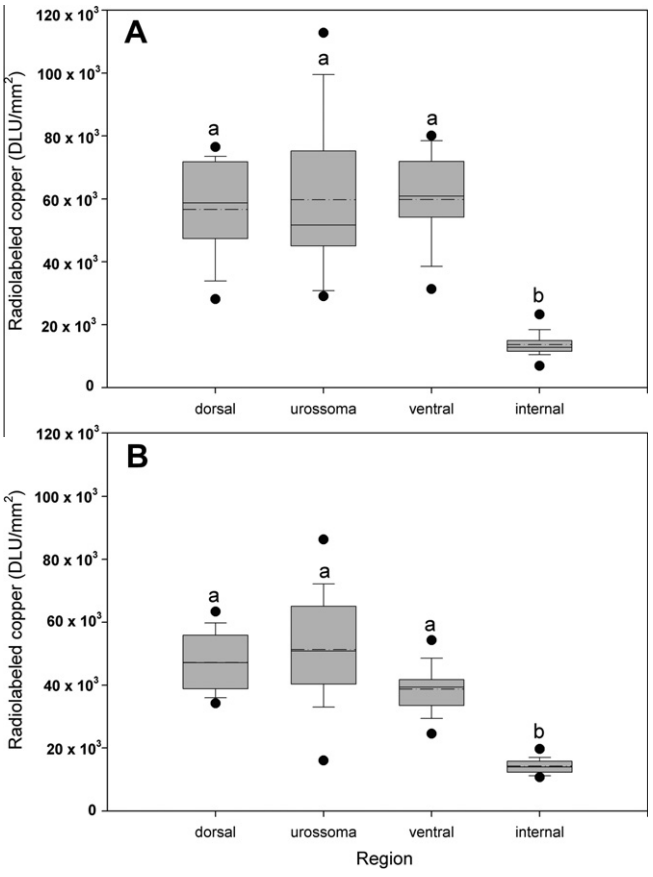

Fig. 5. Radiolabelled copper accumulation expressed as % the of whole-body accumulation in *Calanus hyperboreus* after 2 h of exposure to 2.71 MBq ^{64}Cu L^{-1} (A), and after a 2 h depuration period. Box represents median ± 95% IC, solid line represents mean, dash-dot line represents median, and * represents the 5th and 95th percentiles of outliers. Different letters indicate significantly different means ($P < 0.05$).

As for large organisms, the quantification of the whole-body accumulation in copepods is the first step in the interpretation of results from laboratory and field studies (Barka et al., 2001; Fang et al., 2006). The second step is to describe where the metal is accumulated into the body. However, studies on metal quantification and body distribution in small invertebrates using traditional techniques are technically difficult, once each organ has to be analyzed separately. For instance, the most detailed study reported for copepods showed a differentially copper accumulation in external and internal tissues, without providing more detailed information on how much copper was accumulated in the distinct organs and surfaces of the copepod (Hook and Fisher, 2001).

Autoradiography is an alternative to deal with this technical difficulty in small organisms, since fine-scale tissue distribution of metals can be visualized and quantified. Therefore, the present study is novel as it demonstrates how this technique can be applied in tiny animals such as copepods. Moreover, relating autoradiography data to basic information on copper toxicity is a further step for the understanding of metal toxicity in small organisms.

Data from the present study on whole-body ^{64}Cu accumulation after exposure and depuration treatments indicated the occurrence of two distinct pools of Cu in the copepod *C. hyperboreus*. It is important to note that a primarily permeable cuticle covers the surface of crustaceans, and small crustaceans are permeable all over. This cuticle acts as a site for passive adsorption of dissolved trace metals (Rainbow, 1988). Under the cuticle, there are many proteins that are key to the passage of trace metals across the membranes (Rainbow, 1997a).

The general two-compartment model for water uptake is represented in other studies (Ruzic, 1972), and many variants exist. However, the mathematical equations describing these models contain too many parameters, which cannot be evaluated properly with the set of data from the present study, and this limitation must be kept in mind. Nevertheless, it is noteworthy that the values of copper accumulation calculated from the uptake and depuration phases agree reasonably well, allowing a useful insight of the process involved in Cu uptake and elimination in *C. hyperboreus*.

In general, aquatic organisms have a fast compartment characterized by higher uptake and elimination rate constants, and a slow compartment. Data gained from the autoradiograms showed that external surfaces accumulated more radioactive Cu than the internal region. They also showed a higher decrease of radioactivity in the external surface than in the internal region after the depuration period. This finding indicates that the external surfaces likely belong to the fast compartment, while the internal region belongs to the slow one. The higher ^{64}Cu accumulation onto the external surfaces at the end of the uptake period could be explained by the short duration of our experiment and the much faster uptake kinetics of this compartment.

After the depuration period, nearly 50% of the radioactivity remained in the copepods (72.9 ± 20.8 and 34.3 ± 12.1 $\mu Ci\ g^{-1}$ ww for exposure and depuration periods, respectively). In a study on silver accumulation and depuration in *Daphnia magna*, it was reported that the metal was not eliminated even after 5 h of depuration in clean water (Bianchini et al., 2005), showing that elimination of accumulated metals is not a simple and direct process. The low elimination rate of Cu from copepods may result from its high affinity for binding proteins. Copper is considered a borderline metal that exhibits chemical reactivity with sulfur, nitrogen, and oxygen-bearing functional groups (Nieboer and Richardson, 1980). The low turnover of copper in copepods may also indicate a significant biological requirement of this essential metal, which supports reports for other marine invertebrates that this metal is regulated (Langston and Spence, 1995).

Generally, Cu is required by crustaceans as a component of enzymes and hemocyanin. However, as copepods may not have hemocyanin, Cu requirements are largely related to enzymatic activity, which reflects the life-history processes such as egg production and growth (Kahle and Zauke, 2003).

Whole-body autoradiograms revealed two sites of higher copper accumulation, namely around the mouth and genital segment. In the case of the mouth, the high metal accumulation could be a result from the higher water circulation around this area, which would lead to a higher interaction between the contaminated water and animal surface. In fact, it is shown that different invertebrates accumulate trace metals at different concentrations in their tissues and organs (Rainbow, 2002). Indeed, oral intake of water is known to take place in crustaceans (Fox, 1952). In *D. magna*, the digestive tract was shown to be the most important compartment for silver accumulation (Bianchini et al., 2005). It is evident that the metal needs to pass through the mouth to reach the digestive tract. Similar finding was reported for *Ceriodaphnia dubia*, when metal exposure via water and food resulted in the highest metal (Cd) accumulation in the midgut (Munger et al., 1999). Copepod mouthparts have a very important function in the uptake of food particles. It has been shown that copepods generate water currents at the ventral side of their body by means of the mouthparts, for improved detection of food particles and directed transport of the particles to the stomach (Michels and Schanck-Schiel, 2005). If copepods have the capacity to detect food particles (physically and/or chemically) one speculation could be that these animals have also a capacity to identify necessary micronutrients as copper and other important ions. To date, a specialized site (like gills) for ions exchange and water uptake has not been described in copepods.

An explanation for the higher copper accumulation in the genital segment and the mouth in the copepod *C. hyperboreus* could be the presence of a protein and/or another substance in these sites that would show a higher affinity for copper binding. Trace metals have the potential to bind to any molecule with a certain affinity. Since trace metals typically have a higher affinity for sulfur and nitrogen, and the fact that proteins are made up with amino acids, many of which contain sulfur and/or nitrogen. Therefore, there is no shortage of potential binding sites for trace metals (Rainbow, 1997b). Autoradiograms from *D. magna* also showed conspicuous accumulations of radioactive silver on the compound eyes (Bianchini et al., 2005). A suggested explanation was a high affinity of proteins in the compound eyes for Ag, particularly crystalline lens proteins. The genital structures of female calanoid copepods act in the storage of seminal products, fertilization and laying of oocytes, formation of the egg-sac and release of egg-shell material (Cuoc et al., 1997). An explanation to the high copper accumulation in this region can be related to the composition of the substances present in this region to facilitate the reproduction, as for example, the fixation of spermatophores from males into the female genital structures, and that could be an attractive for copper binding. However, this speculation needs to be carefully interpreted, since there is no evidence to support it.

In a study with the copepod *Acartia* sp. exposed to metals via either food or water, it was observed a differential distribution relative to the via of exposure. When uptake from food occurred, copper accumulation was mainly found in internal tissues, and a small amount was detected on the exoskeleton. However, when copper uptake occurred from the dissolved phase (water), the accumulation pattern was opposed, with a higher copper accumulation on the exoskeleton and a smaller amount being accumulated in internal tissues (Hook and Fisher, 2001). It is worthy noting that in both cases, metals accumulated in the exoskeleton was tightly bound and not easily mobilized to result in a higher internal metal accumulation. This finding is in agreement with our results where metal was not easily mobilized to reach the internal tissue.

5. Conclusion

Findings reported in the present study indicate that copper is quickly bound to the surfaces of the copepod *Calanus hyperboreus*, but only a small metal fraction is accumulated in the internal organs. Whole-body autoradiograms revealed two sites with higher copper accumulation, namely around the mouth and the genital areas. A first explanation is that these sites have a highest copper affinity for copper binding in the copepod *C. hyperboreus*. The high metal accumulation could also be a consequence of the high water flux around this body region. Thus, whole-body autoradiography was proved to be an efficient technique to study copper accumulation and body distribution in a very small organism, the copepod *C. hyperboreus*, results obtained with this technique were more informative than those generated using traditional techniques.

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