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In Situ Analysis of Gut Residue of *Corophium volutator* (Pallas) by Electron Probe Microanalysis after Exposure to Barytes Spiked Sediments

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ABSTRACT: *Corophium volutator (Pallas) has been recommended by the Paris Commission as one of the standard species for testing chemicals used in offshore oil and gas exploration/ production. In the present study, gut contents of Corophium volutator have been analysed by electron probe microanalysis (EPMA) for $BaSO_4$ and other heavy metal impurities after exposure to spiked sediments following a standard ASTM sediment bioassay protocol. Results show that depuration of Corophium gut for 48 h in clean filtered seawater after exposure to sediments containing barytes, prior to whole body metal analysis, is required to reflect the true metal burdens.*

KEY WORDS: *Corophium volutator (Pallas), Electron probe microanalysis (EPMA), Barytes, Gut residue*

INTRODUCTION

An important aspect of estimating whole body metal burdens in the case of sediment dwelling marine organisms concerns the presence of contaminated sediments in their gut. Gut contents of marine organisms used in bioaccumulation studies not only increase the total body metal burdens but they are also a major source of significant increase in variability of metal concentration data [1-5]. Robinson *et al.* [6] confirmed the presence of particulate metals in the gut of *Mytilus edulis* as having a marked effect on the reported concentration of "whole body" Al, Cr, Fe and Ni concentrations in mussels. Rainbow [7] reported that the gut contents of deposit-feeding crustaceans often represent a significant component of the total body load because they ingest potentially metal-rich sediments. Levels of metals in the materials within the gut must therefore be accounted for if accurate estimates of tissue metal concentrations are to be made [8].

Corophium volutator (Pallas) is a selective deposit feeder. It has been reported to be a good indicator for heavy metal pollution [9-11]. It has several distinct features such as wide geographic distribution, ecological importance in estuarine system, high sensitivity to

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sediment contamination, broad salinity tolerance, high survival rate and ease of handling and maintaining in the laboratory, all of which suggest its use as a bioindicator to assess heavy metal pollution in sediments. Paris Commission (PARCOM) has chosen it as a standard species for testing chemicals used in offshore oil and gas exploration/production [12].

Approaches suggested to avoid the problem of contaminated sediments in *Corophium volutator* gut include removal of an animal's gut or elimination of contaminants (depuration of gut contents in clean seawater with or without clean sediments) Prior to whole body metal analysis. Physical removal of the gut of small animals like *Corophium volutator* is usually a tedious job. Therefore, depuration of gut contents in seawater is the preferred choice. Miramand *et al.* [13] found that 16 hours in running seawater was sufficient to eliminate the gut contents of the amphipod *Corophium volutator,* the bivalve *Scrobicularia plana* and the polychaete *Arenicola marina* following 14 days exposure to contaminated sediments. However, Icely and Nott [14] found that only 60% of *Corophium* had depurated their guts in filtered seawater after 48 h. Later, Icely and Nott [15] calculated that the average time for coarse material to pass through the gut of *Corophium* was 9 min. (rang 4 to 24 min.) so that contaminated fine material in the gut, which is usually retained longer, could be eliminated by allowing the animals to replace the finer gut contents with clean coarse sediment. Bat and Raffaelli [16] determined metal concentrations of Cu, Zn and Cd in *Corophium* tissues in individuals with gut contents, and with contents removed by three different protocols, and found significant differences in the concentrations of these metals remaining in those samples with and without gut contents. They recommended either depuration for 48 hours in constantly aerated clean seawater or complete removal of the gut. All of these studies were based on continued depuration until a "no-effect" level was reached, for total metal burdens. The aim of the present study was to investigate the presence of $BaSO_4$ and other trace heavy metal impurities remaining in *Corophium* gut after periods of depuration, following exposure to sediments spiked with barytes (the naturally occurring barium sulphate) in a 10-day static sediment bioassay protocol.

MATERIALS AND METHODS

Sediment bioassay methodology

The sediment bioassay methodology was based on that outlined by the American Society for Testing and Materials [17] and the US Environmental Protection Agency and the US Army Corps of Engineers (EPA/COE) [18] as developed by Swartz *et al.* [19]. *Corophium volutator* were collected from the nudflats of the Ythan estuary, Newburgh, Aberdeenshire, Scotland by sieving sediment through a 0.5 mm mesh. Large pieces of debris and any other macrofauna were discarded. Artificial seawater (32 ‰, 14 ± 1°C) used for the bioassay experiments was prepared from Seamix (Sea Salt supplied by Peacock Salt Ltd, Glasgow, UK). Seawater was put into a sediment-free tank and continually aerated. The amphipods were previously acclimatised to laboratory conditions for a period of 10 days. Equal numbers of adult (4-7 mm) male and female *Corophium* were used in the experiments.

Sediments were collected from an area of the Ythan estuary with a healthy population of *Corophium*. The sediment was washed through a 0.5 mm mesh into a tank to remove any *Corophium* and associated macrofauna, and then washed again through a 0.3 mm mesh to ensure a standardised sediment particle size for all experiments. Sediments were stirred and rinsed three times with filtered seawater, then allowed to stand for 24 hours in seawater. After that time the overlying water was poured off and the sediments were dried at 60°C for 72 hours in an oven. These sediments were used as controls in the bioassay experiment. The dried sediments were homogenised and mixed with different commercial barytes samples (B1-B10) to prepare mixtures containing 5% barytes by weight.

Temperature, salinity and pH were monitored throughout the experiment in all containers and in the holding tank used in the bioassay to ensure that all replicates and treatments were exposed to the same environmental parameters. All replicate containers were 9 cm in diameter and 8 cm deep. These contained 100 g of solids (approximately 2 cm deep) either of control sediment or of test sample (barytes/sediment mix, 5%, by weight). Some artificial seawater was added to each container and stirred thoroughly. Sediments were

allowed to settle and the supernatants were discarded. Then, fresh artificial seawater (300 mL) was added and each container was left to settle under constant aeration for 24 hours before any *Corophium* were added. Aeration was supplied through an air line connected to Pasteur pipettes. Gentle aeration, at a rate of approximately two or three bubbles per second was provided so as not to disturb the sediment surface. Oxygen concentrations (checked using a DO probe) were above 60% saturation in all containers and indicated acceptable conditions for 10-day sediment toxicity tests [17].

Corophium volutator samples for electron probe microanalysis

After the 10-day static sediment bioassay, living *Corophium* were removed from the containers and subjected to one of the following treatments:

(a) *Corophium* were rinsed with distilled water to remove any adhering sediment, blotted and transferred to the fixative. No time was allowed for these animals to clear their guts.

(b) *Corophium* were rinsed with distilled water to remove any adhering sediment and then placed in constantly aerated clean filtered seawater (32%) without any sediment for 24 hours in order to displace any treated sediment in their guts. They were then transferred to fixative for further processing.

(c) *Corophium* were rinsed with distilled water to remove any adhering sediment and then placed in constantly aerated clean filtered seawater (32%) without any sediment for 48 hours to clear their guts. They were then tranferred to fixative for further processing.

Corophium samples collected from the above three different protocols were studied for metal contents in their guts using electron probe microanalysis.

Fixation procedure

Corophium samples (categories a, b,c) were rinsed with distilled water and handled as follows:

(1) Primary fixation for 1-4 hour at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4).

(2) Wash for 2 hours at 4°C in 3 changes of 0.1 M buffer phosphate (pH 7.4).

(3) Wash for 2x5 minutes with distilled water.

(4) Dehydration in 100% ethanol for 15 minutes.

(5) Propylene oxide impregnation for 2x10 minutes.

(6) Propylene oxide: Resin (1:1 mixture) for 1-2 hours.

(7) Mixing the *Corophium* in the fresh resin overnight on a rotary mixer at 5 rpm.

(8) Embedding in moulds and polymerization in an oven at 60°C for 24 hours [20].

Resin blocks were then cut to expose the midgut region of *Corophium volutator.*

Electron probe microanalysis of Corophium gut

Equipment used for EPMA included: Cameca SX51 Electron Probe Microanalyser (Cameca, France) equipped with four wavelength dispersive spectrometers (using Lithium Fluoride (LiF), Pentaerythritol (PET), Thallium acid phthalate (TAP) & Pseudo-Crystal (PC1) analysing crystals). The instrument was operated using Cameca software in a Sun Microsystem environment. EMITECH K950 carbon coater (Emitech, England). Polisher Ecomet III Grinder (Buehler Ltd, Illinois, U.S.A.). Polishing plates and accesseries (Struers, Copenhagen, Denmark).

Sample preparation and measurements

Corophium samples of each category (a, b and c separately), previously embedded in little resin blocks, were set into a polymer resin (Epoxy resin) in a cylindrical mould (Epoform). Samples were left overnight to set and then heated in an oven at 60°C for 2 hours, ready to be polished. Polising took place in four stages:

(i) metallographic SiC grinding paper (GRIT P600).

(ii)metallographic SiC grinding paper (GRIT P1200).

(iii)6 μm diamond paste and paraffin oil.

(iv)0.3 μm alumina powder and paraffin oil.

The samples were washed thoroughly with isopropanol in an ultra-sonic bath after each stage. Finally, samples were coated with carbon (approx. thickness ~10-12 nm) using a carbon coator.

X-ray mapping of Ca, S, Ba and Pb in the gut of each of category Corophium samples

An accelerating voltage of 15 kV with a beam current of 20 nA was used (current was kept delibrately low so as to minimise damage to the animal tissue). The lines, Ba L_α, CaK_α, S K_α, and Pb L_α were measured.

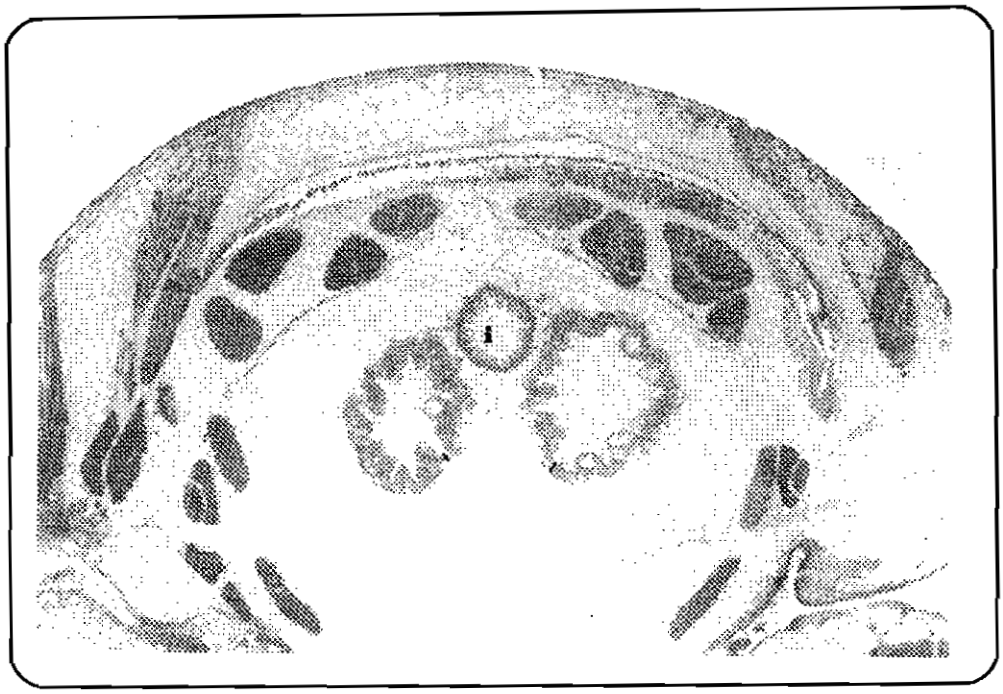

Fig. 1: Transverse section of Corophium midgut region; i(intestine), taken by electron microscopy

Chemical analysis for heavy metals in Corophium tissues

At the end of 10 days, surviving *Corophium* were transferred to "clean" filtered artificial seawater (32%) for 48 hours to depurate sediment present in the gut. Following the depuration stage. *Corophium* were rinsed in distilled water to remove any excess salt, then dried at 60°C for 24 hours. *Corophium* collected from the control and different barytes treatments were weighed and digested. To *Corophium* whole body sample (0.02 g), 3 mL of ARISTAR grade HNO_3 and 2 mL of H_2O_2 were added in a PTFE vessel. The sample was left to stand for 10 minutes with occasional shaking and then heated in a microwave oven using an optimised microwave heating programme (10 minutes at 50% power). After cooling to room temperature, the PTFE vessel was opened and the volume was made up to 20 mL with distilled water. Samples were stored in clean polyethylene containers for metal analysis. The accuracy of the HNO_3 / H_2O_2 microwave digestion procedure used for *Corophium* tissue was checked by determining the heavy metal concentrations in Dogfish liver certified reference material (DOLT-2). Cu and Zn were measured by flame AAS, Hg by CV-AAS, while Ba, Mn and Pb were determined by ICP-MS using rhodium as an internal standard. Results agreed with the certified values within ±10% relative or better. Concentrations of heavy metals in *Corophium* tissues were expressed as $\mu g\ g^{-1}$ dry weight [21].

RESULTS AND DISCUSSION

Removal of solids from the gut

The morphology and fine structure of the alimentary canal in *Corophium volutator* have been described in detail by Icely and Nott [22]. A transverse section of midgut region of a normal *Corophium volutator* obtained by electron microscopy is shown in Fig. 1. In order to find the presence of mineral residues (i.e. $BaSO_4$ and other heavy metal impurities) in the gut of *Corophium volutator, in situ* analysis of gut contents was carried out by EPMA.

X-ray images for Ba , Ca , Pb and S in the midgut of *Corophium* specimens were collected following the different protocols (a, b & c). The white areas in the X-ray images correspond to the presence of the relevant element. Fig. 2 shows the presence of Ba , S and Ca when

Corohium were not allowed to clean their guts after 10 days exposure to barytes spiked sediments. The Pb image was actually noise which can probably be attributed to extremely low levels of Pb in the barytes spiked sediment. Fig. 3 shows the presence of traces of Ba, S and Ca remaining in the gut of *Corophium* depurated in constantly aerated clean seawater for 24 hours. However, no traces of Ba , Ca , S and Pb were observed in *Corophium* guts after depuration of 48 h in constantly aerated clean seawater.

The results confirm the presence of $BaSO_4$ as well as of sediment particulate matter in *Corophium* gut when the amphipod was not allowed to clean its gut contents after exposure to barytes spiked sediments. Even a depuration time of 24 h to clean the gut contents might lead to an overestimation of whole body tissue metal concentrations. It can be concluded that 48 h of depuration in clean constantly aerated seawater are required to clear the gut mineral residues to reflect true metal burdens in *Corophium volutator* after exposure to barytes spiked sediments. This finding agrees well with the recommendations made by Bat and Raffaelli [16] who suggested a depuration time of 48 h for *Corophium* to clean its gut contents in clean aerated seawater before carrying out

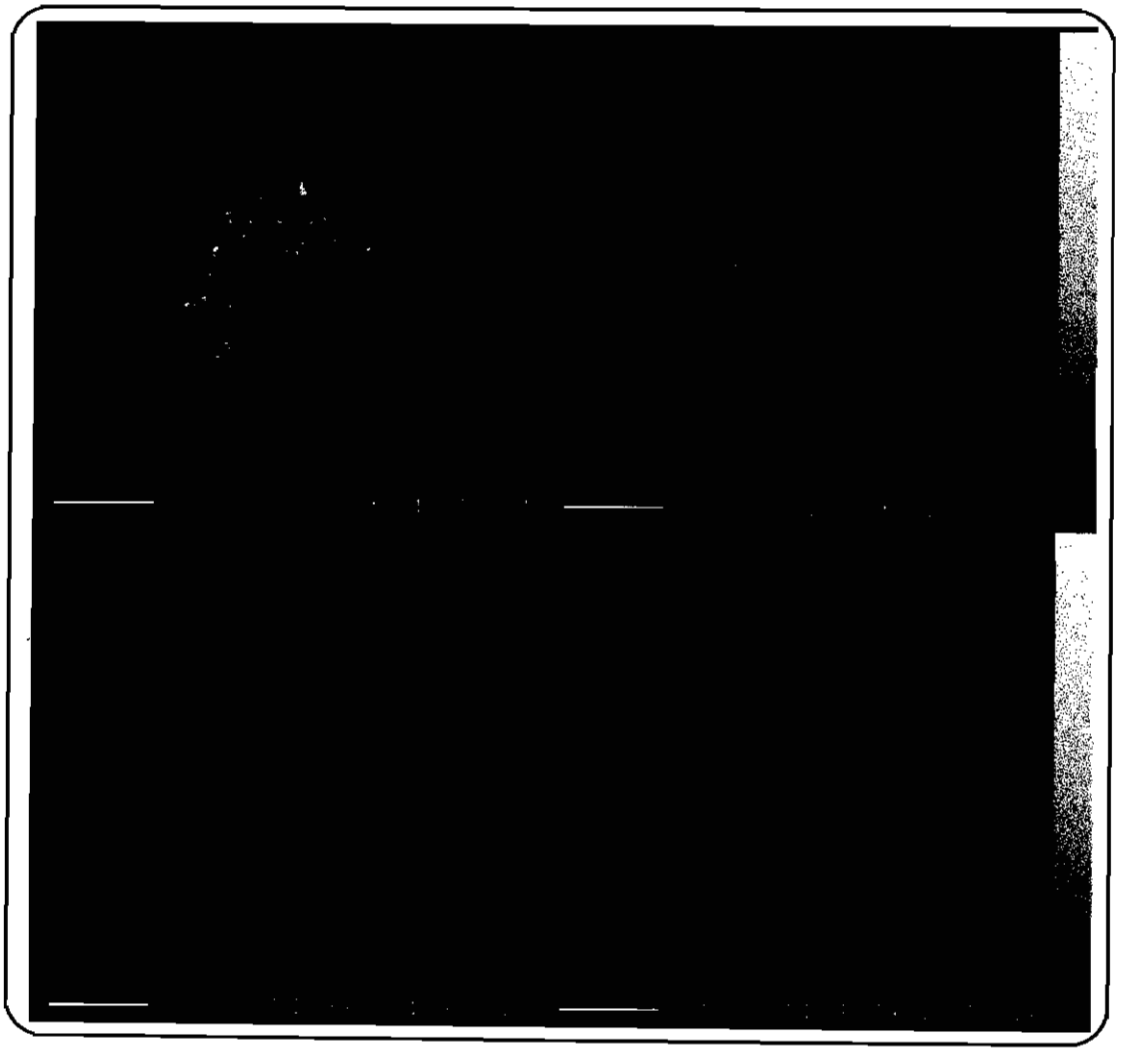

Fig. 2: X-ray images of Corophium gut for Ba , Ca , S and Pb after 10 days exposure to barytes spiked sediment (amphipod was not allowed to depurate the gut contents).

Fig. 3: X-ray images of Corophium gut for Ba, Ca and S after 24 h of depuration of gut contents in clean aerated seawater (amphipod was exposed to barytes spiked sediment for 10 days).

whole body tissue metal analysis.

Uptake of metals by Corophium volutator

Sediment bioassays carried out with *Corophium volutator* have shown significant accumulation of barium, lead and manganese in tissue providing evidence of the bioavailability of these metals from commercial barytes used as weighting agent in drilling fluids in oil and gas industry [21]. Fig. 4 shows the accumulation of heavy metals in *Corophium volutator* from different barytes samples after a 10-day static sediment bioassay. Results on accumulation of barium and other heavy metals by *Corophium volutator* exposed to barytes spiked sediments is a subject of a detailed report which is under prepration.

Bioaccumulation of barium, lead and manganese also confirms the ability of *Corophium volutator* to immobilise and to detoxify these metals. It has been suggested that toxic metals can either be detoxified and distributed evenly throughout body tissues, or can be accumulated in granular form and stored in specific tissues [14,23]. Findings of this study suggest that, at least for *Corophium*, the heavy metals barium and lead are accumulated and distributed throughout the body tissue, and are not

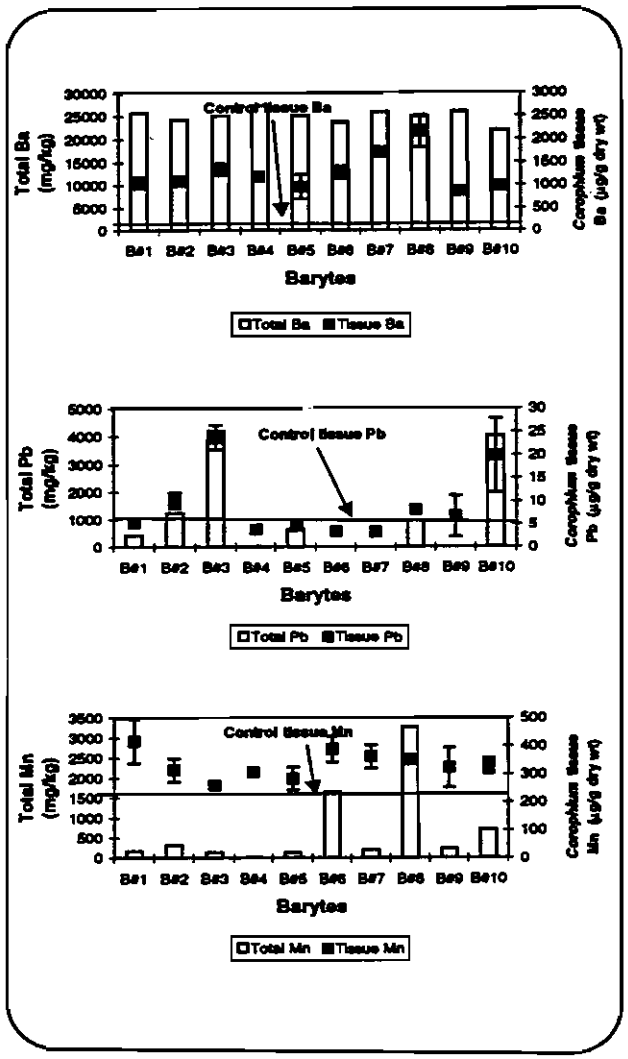

Fig. 4: Accumulation of heavy metals in Corophium volutator from different barytes after 10-day static sediment bioassay (Corophium depurated the gut contents for 48 h in clean aerated seawater prior to chemical analysis; error bars- SEM).

stored as particulates. Further research is needed to reveal the exact nature of metal detoxification mechanisms in *Corophium* exposed to heavy metal bearing barytes.

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