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Transformations of mercury in the terrestrial isopod *Porcellio scaber* (Crustacea)

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Abstract

The biological cycle of mercury in the terrestrial isopod *Porcellio scaber* was investigated. Testing the possibility of in vivo Hg^{2+} methylation was divided into two methodologically different parts. Firstly, concentrations of total mercury and MeHg in isopods *P. scaber* and their environment from a Hg-unpolluted area were measured by the use of validated methods (CV AAS, CV AFS). The data obtained show that the percentage of MeHg in leaves, soil and faeces was less than 1%. In contrast, the percentage of MeHg in gut and hepatopancreas was increased to 14 and 77%, respectively, indicating methylation of Hg^{2+} in the gut and its further accumulation in glands. To confirm this assumption, the second methodology was applied—a radiotracer technique with $^{203}Hg^{2+}$ of high specific activity. There are few radiotracer techniques for Hg-methylation assays; for our work we chose the method of Czuba et al. which includes alkaline leaching of Hg species, their extraction into dithizone–toluene, followed by specific separation of Hg dithizonates by thin-layer chromatography and gamma counting. All steps of the analytical protocol were checked and optimised by the use of aqueous solutions of $^{203}Hg^{2+}$ and $Me^{203}Hg^{+}$. The most important finding was that cleaning-up the extract through a florisil column is not appropriate, because the column retains different percentages of Hg^{2+} and $MeHg^{+}$ and consequently affects the accuracy of the final result. This optimised protocol was then applied to Hg transformation studies in the terrestrial isopod *P. scaber*. Leaching Hg species from *P. scaber* fed with $^{203}Hg^{2+}$ or $Me^{203}Hg^{+}$ dosed food was completely efficient only at elevated temperatures. Preliminary results of methylation/demethlytion studies are rather variable but they show that both processes ($Hg^{2+} \leftrightarrow MeHg^{+}$) take place in the isopod *P. scaber*. Additionally, an assessment of the mass balance of Hg in isopods *P. scaber* exposed to $^{203}Hg^{2+}$ indicates that volatile Hg species are also formed.

Keywords: Isopod; *Porcellio scaber*; Radiotracers; Mercury; Methylmercury; Methylation; Demethylation

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1. Introduction

The terrestrial isopod *Porcellio scaber* is a crustacean living on land. It colonizes upper soil layers and lives mostly under tree-leaves, decaying wood and bigger stones. *P. scaber* is an omnivore, but its most preferred food is decaying organic matter. Therefore, isopods are important for the decomposition and cycling of mineral matter in nature.

More than a decade ago, terrestrial isopods were introduced as biomonitoring organisms for metals in industrially polluted environments due to some suitable characteristics such as their convenient size, abundance, ease of handling in the laboratory and the ability to accumulate metals (Hopkin et al., 1986, 1993; Drobne, 1997). *P. scaber* is one of the most popular organisms in terrestrial ecotoxicology and ecophysiology. (Drobne and Hopkin, 1995; Drobne, 1997; Drobne et al., 2001). Still, there is nothing known about the accumulation, transformations and effects of Hg on terrestrial isopods, although they are abundant in mercury-contaminated environments such as, for example, the banks of river Idrijca in Slovenia.

Investigation of the biological cycle of mercury in *P. scaber* is of interest from two aspects; the animal could be an appropriate biomonitoring organism for terrestrial environments polluted with mercury, and secondly, there is a possibility that Hg^{2+} is methylated in the gut of *P. scaber.* Only quite recently, anaerobes in the gut of *P. scaber* were described (Kostanjšek et al., 2002) and sulphate-reducing bacteria, known to be principal methylators of Hg^{2+} in nature, were also found (Lapanje, 2000).

The first objective of our work was to measure total mercury (THg) and MeHg levels in *P. scaber* and its environment in a Hg-unpolluted area and to compare the percentages of MeHg in different compartments. We assume that Hg^{2+} is methylated in the gut and further accumulated in glands (hepatopancreas) or gut. Another approach to test this hypothesis is the use of tracers with which high sensitivity can be attained. According to the possibilities in our laboratory, we chose the radiotracer ^{203}Hg.

There are only a few analytical techniques available for $^{203}Hg^{2+}$ methylation studies (Furutani and Rudd, 1980; Czuba et al., 1981; Guimarães et al., 1995; Stordal and Gill, 1995; Gilmour and Riedel, 1995) and they differ in specificity of sample pretreatment and detection. In the present work, the method of Czuba et al. (1981) was chosen for two reasons: it separates $MeHg^+$ and Hg^{2+} efficiently, and provides information about the content of Hg^{2+} in the sample. The latter information can be quite helpful in transformation studies; beside formation and degradation of $MeHg^+$, there are also other processes that can take place, with reduction of Hg^{2+} to Hg^0 being the most critical one.

The second objective of this work was to test and optimise the analytical tracer technique for studies with isopod *P. scaber*. For accuracy of determination of the fraction of added $^{203}Hg^{2+}$ that was methylated to $Me^{203}Hg^+$ it is extremely important that the ratio of $MeHg^+/Hg^{2+}$ remains the same through all the steps of the analytical technique. The protocol was tested with aqueous solutions of $^{203}Hg^{2+}$ and $Me^{203}Hg^+$ of known concentrations and activities; attention was focused mainly on extraction efficiencies, cleaning-up the extracts and investigating possible interference due to other metals, especially copper, which is present in *P. scaber* in large amounts. Copper is present in haemocyanin in the blood of *P. scaber* and carries oxygen in the same way that hemoglobin does in mammals. Leaching of Hg species from isopods was tested on animals that were fed with $^{203}Hg^{2+}$ or $Me^{203}Hg$ dosed food following the protocol proposed by Drobne and Hopkin (1995).

By using the optimised analytical technique, a preliminary study on the methylation/demethylation potential in the digestive system of *P. scaber* was made.

An additional advantage of using a radiotracer is the ease of localisation of the radiotracer when physically following the metabolic path. Therefore, magnitudes of processes like metal (^{203}Hg) uptake, its retention and distribution in the animal and faecal excretion were assessed.

2. Experimental

2.1. Reagents and materials

^{203}Hg as $^{203}HgCl_2$ in 1 M HCl solution with a specific activity of 3.6×10^{10} Bq/g Hg

(15.11.2000) (Isotope Products Laboratories, California, USA); ^{203}Hg as $^{203}HgCl_2$ in HCl solution with a specific activity of 7.1×10^{10} Bq/g Hg (01.07.1999) (Amersham Pharmacia Biotech); $(CH_3)_4Sn$ (for analysis, Merck); MeOH 98% (GR, dry, Merck); toluene (for residual pesticides, Carlo Erba); $Na_2S_2O_3\cdot5H_2O$ (p.a. Kemika Zagreb); $CuSO_4\cdot5H_2O$ (g.r. for analysis, Merck), NaCl (p.a. Merck); dithizone (p.a. Merck); 1 M NaOH (from NaOH, extra pure, pellets, Merck, and Milli-Q water), cleaned with toluene before use; 1 M HCl (from HCl, s.p. 30%, Merck, and Milli-Q); Florisil (60/80 mesh, Merck); Na_2SO_4 (s.p. Merck); Pasteur pipettes (145 mm in length, Brandt); NH_3 (s.p. 25%, Merck); POLYGRAM® SIL N-HR (0.2 mm silica gel N-HR, pre-coated sheets for thin-layer chromatography, Macherey-Nagel, Germany); hexane (nanograde, Promochem).

2.1.1. Synthesis of $Me^{203}Hg^+$

$Me^{203}Hg^+$ was synthesised directly from $^{203}Hg^{2+}$ solution as purchased from the supplier (Isotope Products Laboratories). We used $(CH_3)_4Sn$ in methanol solution as methylating agent according to a procedure described in Toribara (1985). The $Me^{203}Hg^+$ in toluene obtained was analysed for concentration by a GC-ECD detector and for γ-activity. The counting efficiency of the detector for our sample dimensions ($\approx$0.58 g of $Me^{203}Hg^+$ in toluene) was calculated using the Kayzero/Solcoi® program. The purity of the reaction product was checked using the method of Czuba et al. (1981).

For testing the analytical procedure and experiments with animals, we extracted $MeHg^+$ from toluene into aqueous solutions of either 1% cysteine in 20% citrate or 0.005 M Na_2CO_3.

2.1.2. Clean-up of toluene solution of dithizone

Twenty-five milliliter 1 M NaOH (previously purified with toluene) was added to 50-ml 0.1% dithizone in toluene in a separating funnel and shaken for 10 min in the dark. The organic phase was discarded. The orange coloured alkaline aqueous phase was neutralised with 1 M HCl. The dithizone obtained (dark violet colour) was extracted back into 50-ml of toluene. The dithizone in toluene solution should be freshly prepared.

2.2. Apparatus

Coaxial HPGe detector (Canberra), p-type.
Well-type HPGe detector (Ortec, USA).

2.3. Determination of THg and MeHg in P. scaber from a Hg-unpolluted area

Isopods *P. scaber* were collected in a Hg-unpolluted area (Laško, Slovenia) in October 1999. For analysis of MeHg and THg, two groups of 17 animals (male and female of an average weight of 60 ± 11 mg) were chosen. Due to the low expected concentrations of mercury and the low weight of organs, two composite samples of glands with an average fresh weight of approximately 60 mg, and two composite samples of guts with an average fresh weight of approximately 70 mg were prepared and analysed. THg was determined after wet digestion by CV AAS (Horvat et al., 1991). MeHg was determined by CV AFS after acid leaching, extraction of MeHg into CH_2Cl_2, re-extraction of MeHg from the organic solvent into Milli-Q water, followed by ethylation of Hg species and separation by gas chromatography (Bloom, 1989; Horvat et al., 1993a,b; Liang et al., 1994, 1996). For quality assurance, the reference materials IAEA-140, IAEA-142 and IAEA-356 were analysed together with samples.

2.4. Analytical procedure based on radiotracers

The procedure for determination of Hg methylation/demethylation in the isopod *P. scaber* is shown on Fig. 1.

The percentage of $^{203}Hg^{2+}$ present as $Me^{203}Hg^+$ was calculated using:

$$\mathrm{MeHg}(\%)=\frac{A_3}{\sum A}100\% \quad (1)$$

where A_3 is the activity of the third TLC strip which represents the activity of $Me^{203}Hg$ and $\sum A$ is the sum of activities of all four TLC strips. When measuring the percentage of ^{203}Hg present as $^{203}Hg^{2+}$, A_3 in Eq. (1) is replaced by A_2 which represents the activity of $^{203}Hg^{2+}$ (the second TLC strip). Measured activities on TLC strips were corrected for background and decay.

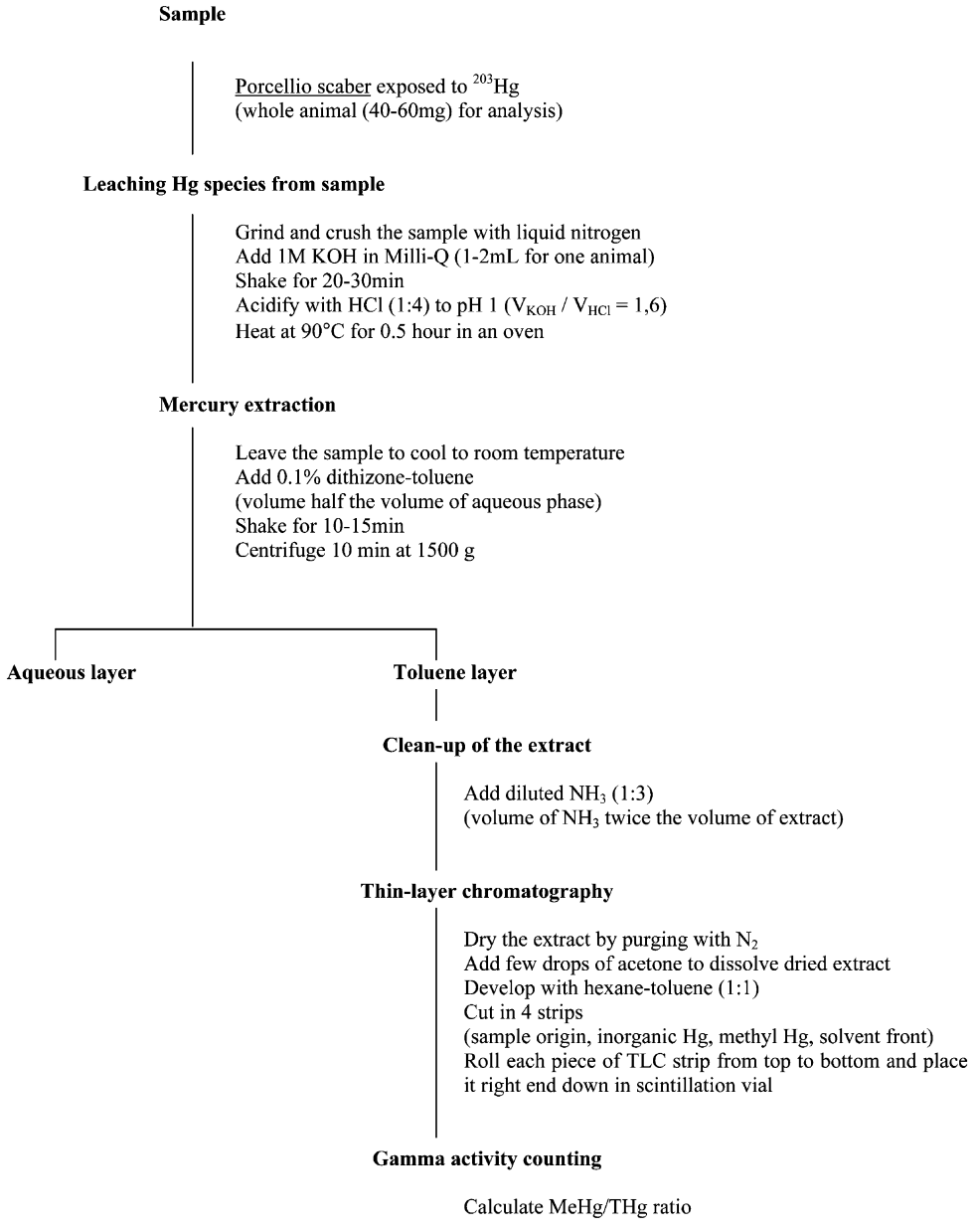

Fig. 1. Analytical protocol for ^{203}Hg methylation/demethylation in *P. scaber*.

2.5. Optimisation of the analytical procedure

2.5.1. Extraction efficiency

The extraction efficiency for Hg species from aqueous solution into 0.1% dithizone–toluene (Dz–Tl) was tested at different pH values (0, 1, 2, 3, 4, 5, 6, 7, 10) and different volume ratios of Dz–Tl to aqueous phase $R = V_{org}/V_{aq}$ (5, 2, 1, 0.5, 0.1). Solutions of different pH values were prepared by mixing Milli-Q water with HCl or NaOH. Appropriate volumes of Dz–Tl were added to aqueous solutions of ^{203}Hg, shaken for 10 min,

Table 1
Experimental design for testing florisil column

Ratio $V_{extract}/m_{column}$	$V_{extract}$ (ml)	Florisil (g) Na_2SO_4 (g)
2:1	2	0.5 0.5
4:1	2	0.25 0.25
8:1	2	0.125 0.125
8:1	1	0.063 0.063
12:1	2	0.083 0.083
16:1	2	0.063 0.063

centrifuged for 10 min at $1500\times g$ and finally the organic and aqueous phases were separated. The activities of small aliquots of the organic and aqueous phases were measured on the well-type HPGe detector. The efficiency of extraction was determined from the measured activities.

2.5.2. Clean-up of extracts

Clean-up of the extract initially consisted of two steps; clean-up through a florisil column and washing the excess of dithizone with alkaline aqueous solution.

To check the florisil column, different volumes of $^{203}Hg^{2+}$ or $Me^{203}Hg^{+}$ extracts were applied on different columns. Florisil columns were prepared by filling Pasteur pipettes with florisil and then with Na_2SO_4 in the ratio 1:1 (w/w) as described in Table 1. Before column preparation, florisil was activated by heating at 130 °C overnight in an oven, and Na_2SO_4 was dried at 500 °C for 2 h. Both materials were then stored in a desiccator.

The excess of dithizone was washed with alkaline aqueous solution. Different volumes of diluted solutions of NH_3 (1:99, 1:9, 1:3, v/v) and NaOH (0.1, 2 and 5 M) were tested to find the most efficient washing solution and the best volume ratio $R=V_{org}/V_{aq}$.

2.6. Application of the optimised analytical procedure to the isopod P. scaber

Several groups of animals were exposed to $^{203}Hg^{2+}$ or $Me^{203}Hg^{+}$ of different concentrations and activities as shown in Table 2. The mercury tracers were spotted on hazelnut leaves (*Corylus avellana*) at the surficial concentrations shown in Table 2. The prepared leaves were used as food for the isopods. Groups of exposed animals were left in separate plastic Petri dishes for 10 or 17 days. Faeces were not separated from the system. The inner surface of the plastic Petri dishes was sprayed with water every second day to assure a sufficiently high humidity. After exposure, whole animals were taken for analysis. From each group, we analysed 1, 2 or 3 animals together. Before analysis, isopods were put in the freezer for a few minutes to stun them and then they were crushed and ground with the addition of liquid nitrogen. The procedure continued with the addition of 1 M KOH in Milli-Q and shaking on a reciprocal action shaker for 20–30 min. Samples were acidified with HCl (1:4) to pH 1 and extracted with 0.1% Dz–Tl. The mixture was shaken for 10 min, centrifuged for 10 min at $1500\times g$ and the phases were separated. The isolation efficiency was determined by comparing the activities of aqueous phases before extraction and the activities of the extracts obtained. The volumes of reagents used in the sample pre-treatment are shown in Table 2.

The extracts obtained were washed with NH_3 (1:3) using volumes twice as great as the volumes of extracts and the procedure was then continued as shown in Fig. 1.

2.7. Estimation of Hg mass balance in P. scaber fed with $^{203}Hg^{2+}$ dosed food

To assess the magnitude of food consumption, Hg^{2+} uptake, retention of mercury in the animal, accumulation in glands and excretion of Hg by faeces, three groups of isopods were fed with three different mass concentrations of $^{203}Hg^{2+}$ dosed food.

Animals were fed with hazelnut leaves (*Corylus avellana*) from a Hg-unpolluted area with added $^{203}Hg^{2+}$. The preparation of the leaves and the

Table 2
Different groups of isopod *P. scaber* exposed to ^{203}Hg species spotted on leaves

Sample	Solution	Activity and concentration of solution	Volume (ml)[a]	Exposure to ^{203}Hg (days)	No. of animals for analysis	Volume 1 M KOH (ml)	Volume HCl (1:4) (ml)	Volume 0.1% Dz–Tl (ml)
1	$MeHg^+$ in cysteine	1250 Bq/ml	0.3	10	1	2	1.25	1.5
2		28 ng/ml	0.3		3	6	3.75	5
3	$MeHg^+$ in Na_2CO_3	910 Bq/ml	0.2	17	1	1.23	0.77	1
4		20 ng/ml	0.2		1	1.23	0.77	1
5			0.2		2	2.46	1.54	2
6			0.5		2	2.46	1.54	2
7	Hg^{2+} in diluted HCl	2650 Bq/ml	0.2	17	1	1.23	0.77	1
8		38 ng/ml	0.2		1	1.23	0.77	1
9			0.2		1	1.23	0.77	1
10			0.2		3	3.69	2.31	3
11		5300 Bq/ml	0.2	17	1	1.23	0.77	1
12		76 ng/ml	0.2		2	2.46	1.54	2
13			0.2		3	3.69	2.31	3
14		13250 Bq/ml	0.2	17	1	1.23	0.77	1
15		190 ng/ml	0.2		1	1.23	0.77	1
16			0.2		2	2.46	1.54	2
17			0.2		3	3.69	2.31	3

[a] Volume of corresponding solution applied on 100 mg leaf.

application of tracer to leaves was done by following the procedure described in Drobne and Hopkin (1995). Three concentration levels were chosen according to concentrations of Hg in soil; background level (0.3 ng/mg), slightly elevated mercury in soil (3 ng/mg) and very contaminated soil (300 ng/mg).

The leaves were air-dried, cut into smaller pieces and all pieces of one leaf were stored in a separate plastic Petri dish until the experiment. Each animal was put on a plastic grid which was placed in the middle of a 10 cm high plastic tube together with a few pieces of a ^{203}Hg labelled leaf. Only a few small pieces were added at a time, in order to prevent coprophagy (eating their own faeces). Faeces were collected in glass Petri dishes, which were placed under each plastic tube. The glass cover at the top of each tube was sprayed with Milli-Q water to maintain high humidity. Tubes with animals were put in glass boxes, covered with PVC foil and dark paper. The PVC foil was also sprayed with water (each few days). The temperature during the experiment was 20 ± 2 °C.

The weight of leaves was determined before and after the experiment. At the end of exposure, the faeces of each animal were collected, weighted and counted for their γ-activity in a well-type detector. Several times during the experiment, animals were counted for their γ-activity to follow their uptake and retention dynamics of ^{203}Hg. The term retention is used to describe the amount of Hg species that are already absorbed from the gut and also Hg species present in the gut content. All data for γ-activity were normalised for background, decay and sample geometric counting efficiency. All parameters (date, number and weight of animals, their gender, amount of food consumption, weight of faeces, moult frequency) were carefully recorded.

In some animals, especially in those exposed to 300 ngHg/mg leaf, the distribution of ^{203}Hg was investigated. Before dissection, animals were counted for their γ-activity. After dissection, glands and gut of each animal were also counted for γ-activity to determine the distribution of ^{203}Hg within the digestive system.

Table 3
Concentrations of THg and MeHg in *P. scaber* and its environment, collected in a Hg-unpolluted area

Sample	THg (ng/g) (CV AAS)	MeHg (ng/g) (CV AFS)	% MeHg[a]
Glands	9.9±1.4 (5)	7.33±1.04 (7)	77
Gut	30±3 (4)	4.16±0.48 (9)	14
Faeces	45±5 (6)	0.37±0.11 (4)	0.8
Tree-leaves	52±7 (4)	0.20±0.07 (3)	0.4
Soil	146±37 (5)	0.70±0.08 (6)	0.5

Number of determinations for two independent pooled samples are in parentheses.
[a] Average value.

The number of animals in each group was different (from 5 to 15) and the times of exposure (5–35 days) were also different. After exposure to $^{203}Hg^{2+}$, some animals were dissected, and some of them were fed with non-radioactive leaves for some time (1–19 days). To compare food consumption, those animals from each group were chosen which were exposed to $^{203}Hg^{2+}$ dosed food for the same time period. The first group of animals was exposed to $^{203}Hg^{2+}$ dosed food of 0.3 ngHg/mg leaf concentration for 16 days (group A), the next group was fed with leaves of 3 ngHg/mg leaf concentration for 16 or 35 days (subgroups B16 and B35) and the last group of animals was exposed to $^{203}Hg^{2+}$ dosed food of 300 ngHg/mg leaf concentration for 7 days (group C). For better comparability of food consumption, a control group K was included as well; this was a group of animals fed with leaves from a Hg-unpolluted area with no ^{203}Hg added.

3. Results and discussion

3.1. Determination of THg and MeHg in P. scaber from a Hg-unpolluted area

Concentrations of MeHg and THg in *P. scaber* and its environment, collected from a Hg-unpolluted area, are summarised in Table 3. The concentrations of THg are low and are in the range from 10 to 200 ng/g. These concentrations represent the natural background. The lowest THg concentrations ($\approx$10 ng/g) were found in glands of *P. scaber*. THg concentrations in the gut of *P. scaber* are approximately 3 times higher. In contrast, the concentrations of MeHg in guts and glands are about one order of magnitude higher than MeHg concentrations in soil, leaves and faeces. MeHg concentrations in glands are approximately 7 ng/g, which is about twice as much as in guts.

Comparison of the percentages of THg as MeHg shows that Hg^{2+} is most probably methylated in the gut of *P. scaber* to $MeHg^{+}$, and further accumulated in glands. The percentage of MeHg in gut and glands is 14 and 77%, respectively, whereas the percentage of MeHg in faeces, soil and leaves is less than 1%.

3.2. Optimisation of the analytical procedure

First results of measuring the amount of methylmercury $Me^{203}Hg^{+}$ in glands and guts of the isopod *P. scaber* using the original analytical procedure showed that a small fraction (0–3%) of $^{203}Hg^{2+}$ incorporated in vivo is transformed to $Me^{203}Hg^{+}$ (data not shown). However, the results were highly variable (mainly due to analytical uncertainties and/or individual variability of animals) and with high associated uncertainties. In order to reduce the analytical uncertainties, the next step was validation of the analytical protocol. Following Eq. (1), it is clear that the ratio of MeHg/THg must remain the same throughout all the steps of the analytical technique, even though recoveries of individual steps are not quantitative.

3.2.1. Extraction efficiency

The best extraction efficiency was found at pH 1 for all volume ratios. We chose a volume ratio $R=0.5$ (volume of extract half the volume of leachate), because small extracts are preferred for further drying by purging with nitrogen.

3.2.2. Clean-up of extracts

The retention of mercury species on the florisil column is shown in Fig. 2. The fraction of Hg species retained changes with the volume of the Hg extract and column size; the bigger is the volume of the extract in comparison to the mass of the column, the smaller is the fraction of Hg retained. In each case, Hg^{2+} is much more strongly

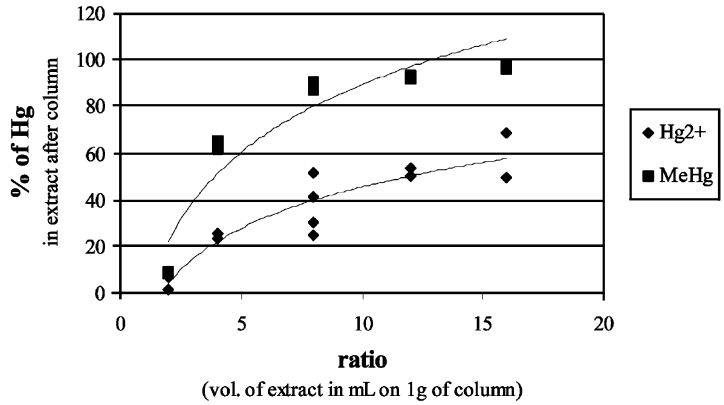

Fig. 2. Retention of ^{203}Hg species on florisil column.

retained on the column than $MeHg^+$. Therefore, for accurate determination of $MeHg^+/Hg^{2+}$ ratios, clean-up through a florisil column is not appropriate. Clean-up through a florisil column does not affect the appearance of the thin-layer chromatogram from the isopod *P. scaber*.

The results of consecutive washing of the excess of dithizone with different diluted alkaline solutions are presented in Table 4. After washing with NH_3 (1:99), Hg remains completely in the organic phase, but the efficiency of washing is poor and the extracts are still slightly green in colour. Washing with NH_3 (1:9) solution is appropriate for $MeHg^+$-dithizonate extracts but less for Hg^{2+}-dithizonates, where only 90% of the original activity was found after washing. We also measured lower activities in the Hg^{2+}-dithizonate extract after washing with diluted solutions of NaOH. The

Table 4
Percentage of Hg in organic phase after consecutive washing the excess of dithizone with different diluted alkaline solutions

Solution	Ratio $V_{extract}/V_{aq}$	% of Hg in organic phase after washing					
		NH_3 1:99	NH_3 1:9	NH_3 1:3	NaOH 0.1 M	NaOH 2 M	NaOH 5 M
$^{203}Hg^{2+}$	2:1	100	87	100	92	–	–
		104	90	104	95		
		100	91	103	84		
		102	91	104	82		
$^{203}Hg^{2+}$	2:1	98	87	105	–	–	–
		100	90	106			
		97	91	109			
		101	91	105			
$^{203}Hg^{2+}$	1:1	–	–	–	–	78	78
						79	79
$^{203}Hg^{2+}$	1:5	92	89	91	–	48	–
		98	90	98		8	
$Me^{203}Hg^+$	1:2	95	96	96	–	101	–
		101	103	105		93	
$Me^{203}Hg^+$	1:3	109	104	104	–	105	–

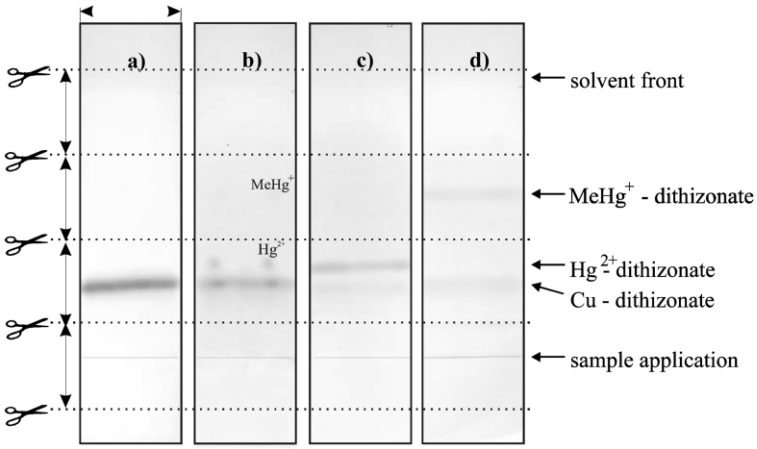

Fig. 3. Thin-layer chromatograms. (a) Cu^{2+}-dithizonate prepared from Cu^{2+} aqueous standard solution. (b) Isopod *P. scaber* together with non-radioactive Hg standards applied in two spots along the sample application line. (c) Non-radioactive Hg^{2+}-dithizonate. (d) Non-radioactive $MeHg^{+}$-dithizonate.

best results for $MeHg^{+}$- and Hg^{2+}-extracts were obtained by using diluted NH_3 (1:3). For further experiments we chose the latter solution and a volume of washing solution twice as great as the volume of the extract.

3.2.3. Interferences

Possible interferences due to the coextraction of metals other than Hg were checked by the comparison of thin-layer chromatograms of dithizonates extruded from the isopod *P. scaber* and of prepared aqueous solutions of different metals that can also be present in the isopods (Zn, Cd, Co, Pb, Cu). The next step was to measure the extraction efficiency for ^{203}Hg in the presence of other metals and chelating agents (10% EDTA). At pH 1 the only metal which is coextracted with Hg is Cu, as is also well indicated by the violet colour of the washed extract and an intensive violet line on the thin-layer chromatogram (Fig. 3). Copper is present in isopods at approximately up to 250 μg/g of dry body mass (Hopkin et al., 1993) but it does not affect the extraction of Hg-species at this level.

Fig. 3 shows thin-layer chromatograms of Cu^{2+}-dithizonate, of the dithizonate extract from *P. scaber* and non-radioactive Hg^{2+}- and $MeHg^{+}$-dithizonates. Non-radioactive Hg dithizonates are used to indicate the position of ^{203}Hg dithizonates. When applying a real sample on the TLC sheet, non-radioactive standards of Hg-dithizonates must be added as well. We added the non-radioactive standards just in two spots along the application line. The TLC chromatograms (a) and (b) on Fig. 3 confirm the presence of Cu in the isopod.

3.3. Application of the optimised analytical procedure to the isopod P. scaber

The results of the isolation of Hg species from isopods *P. scaber* fed with $^{203}Hg^{2+}$ or $Me^{203}Hg^{+}$ are presented in Fig. 4. The first separation of Hg species performed by leaching with 1 M KOH in Milli-Q and extraction into 0.1% Dz–Tl was not completely successful; isolation was complete only for the group of animals exposed to $Me^{203}Hg^{+}$, whereas the efficiency for the animals exposed to $^{203}Hg^{2+}$ was only $(70.1 \pm 7.5)\%$. It seems that part of the $^{203}Hg^{2+}$ is more strongly bound in the samples. The extraction efficiency of Hg species was checked with aqueous solutions of $^{203}Hg^{2+}$ and $Me^{203}Hg^{+}$ of known activities as shown in Table 5. We also tried to separate the mercury that remained in the samples after the first leaching: aqueous solutions of animals, already acidified to pH 1, were heated in an oven at 90 °C for half an

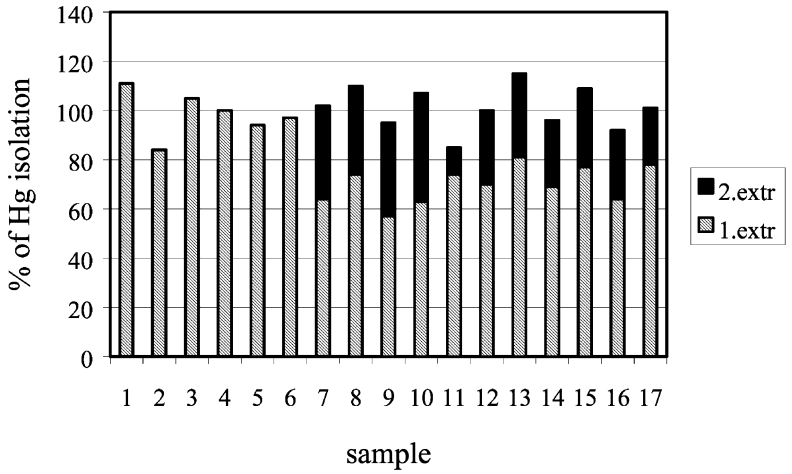

Fig. 4. Isolation of Hg species from *P. scaber* previously exposed to $^{203}Hg^{2+}$ or $Me^{203}Hg^{+}$. Animals 1–6 were fed with $Me^{203}Hg^{+}$ dosed food. Animals 7–17 were fed with $^{203}Hg^{2+}$ dosed food.

hour. After heating, the samples were shaken and left to cool to room temperature. Immediately after cooling, Hg was extracted into 0.1% Dz–Tl. (Addition of Dz–Tl at high temperature destroys dithizone). The data obtained after the second extraction show that an additional (30 ± 9)% of mercury was isolated from the samples and thus separation of Hg was complete.

The extracts obtained were washed with diluted NH_3 (1:3) and all Hg (on average 103.7%) remained in the organic phase. The procedure was then continued as shown in Fig. 1 and the final results are presented in Fig. 5. Fig. 5 consists of two parts; the first (Fig. 5a) shows the percentage of ^{203}Hg present as $^{203}Hg^{2+}$ in animals fed with $Me^{203}Hg^{+}$ dosed food. Fig. 5b shows the percentage of ^{203}Hg present as $Me^{203}Hg^{+}$ in animals fed with $^{203}Hg^{2+}$ dosed food. These are preliminary results from methylation/demethylation studies of mercury in the isopod *P. scaber* and they are

Table 5
Checking extraction efficiency with aqueous solutions of $^{203}Hg^{2+}$ and $Me^{203}Hg^{+}$ of known activities

Hg species	Standard[a]	0.5 ml aliquot of aqueous phase		0.5 ml aliquot of extract		Recovery (%)
		Activity (Bq)	R.S.D. (%)	Activity (Bq)	R.S.D. (%)	
$^{203}Hg^{2+}$	Std 1a	4.10	1.3			
	Std 1b			4.02	1.3	98
	Std 2a	4.62	1.4			
	Std 2b			4.69	1.2	105
$Me^{203}Hg^{+}$	Std 3a	2.45	1.9			
	Std 3b			2.51	1.9	105
	Std 4a	0.27	5.2			
	Std 4b			0.27	6.1	105

[a] Standards a and b were aqueous solutions of ^{203}Hg prepared in the same way, but the volumes of standards a were half the volumes of standards b. Standards b were then extracted into half the volume of 0.1 % Dz–Tl.

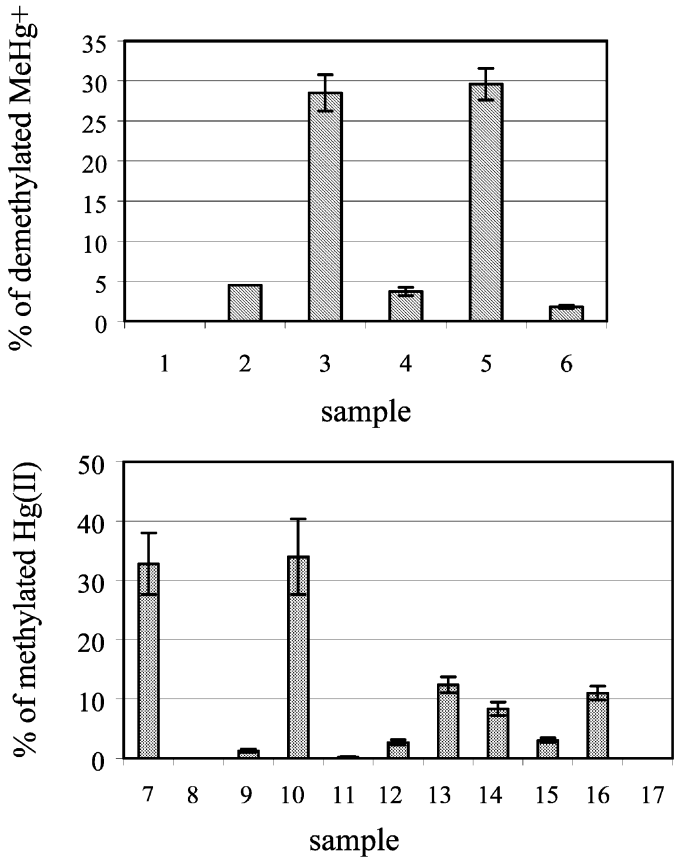

Fig. 5. Hg transformations in *P. scaber*. (a) Fraction of ^{203}Hg present as $^{203}Hg^{2+}$ in animals fed with $Me^{203}Hg^{+}$ dosed food. (b) Fraction of ^{203}Hg present as $Me^{203}Hg^{+}$ in animals fed with $^{203}Hg^{2+}$ dosed food.

highly variable. It should be noted, however, that it was not our intention to measure precisely the fractions of transformed Hg species and elucidate possible factors influencing the processes (Hg concentration etc.) but rather just to see whether these transformations occur in *P. scaber* or not.

3.4. Estimation of Hg mass balance in P. scaber fed with $^{203}Hg^{2+}$ dosed food

3.4.1. Food consumption

Fig. 6 shows the amount of consumed food as a function of Hg concentration on the leaves. The total amount of food consumed in milligrams was normalised to the time unit (day) and to the fresh weight of an animal (mg). There was no difference in the food consumption between control group K and groups A, B16 and B35. A significant reduction of feeding rate was recorded only in the group of animals fed with leaves of 300 ngHg/mg concentration (group C). It is known that a reduced feeding rate in *P. scaber* is an indication of the adverse effect of chemicals added to the food.

3.4.2. Uptake and retention in the animal and excretion of ^{203}Hg by faeces

A comparison of the processes of uptake and retention of mercury in the animal and excretion of ^{203}Hg by faeces in three different groups of *P. scaber* is shown in Table 6. Table 6 also shows the weights of the animals and the duration of exposure for each isopod. The activity in consumed food was calculated from the amount of leaves consumed and the specific activity of

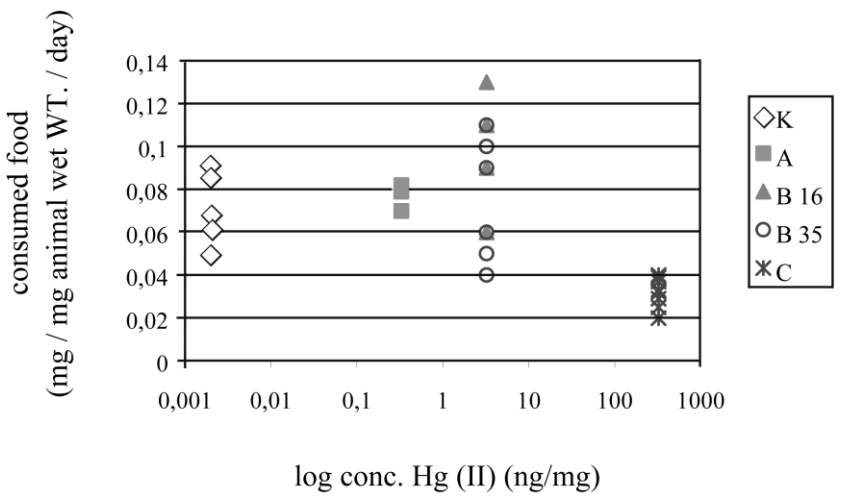

Fig. 6. Amount of food consumed as a function of Hg^{2+} concentration in the food. K—control group. A—group of animals fed with $^{203}Hg^{2+}$ dosed food of 0.3 ngHg/mg leaf concentration for 16 days. B—group of animals fed with $^{203}Hg^{2+}$ dosed food of 3 ngHg/mg leaf concentration for 16 days (sugroup B16) or 35 days (subgroup B35). C—group of animals fed with $^{203}Hg^{2+}$ dosed food of 300 ngHg/mg leaf concentration for 7 days.

$^{203}Hg^{2+}$ on the leaves. Data on the activities in animals refer to the last day of the exposure. The results from Table 6 are also presented in Fig. 7.

The data obtained show that the majority of Hg consumed is excreted from the animals (30–60%); only a few percent ($\approx$1–4%) are retained. The percentage of retained Hg changes with the amount of ^{203}Hg consumed, reaching a minimum in group B. When examining the dynamics of uptake and retention of ^{203}Hg during exposure, a small peak in uptake/retention activity was indicated in the first week of exposure to $^{203}Hg^{2+}$ (data not shown). Later, activities were lower and relatively constant. In further studies, it would be of interest to follow the dynamics in more detail at the beginning of the experiment.

An assessment of the mass balance indicates that the larger fraction of ^{203}Hg is 'lost' from the experiment; the sum of the activities in an animal and its faeces is much less than the activity in the food consumed. The loss of Hg is particularly high in the group of animals exposed to the highest Hg concentrations in the food (Table 3). This observation suggests that volatile Hg species are formed, most probably Hg^0. This could be either the result of an abiotic process due to the high humidity, or the result of biological processes. Reduction of Hg^{2+} to Hg^0 can occur either on the leaves, or in the gut of the isopod, or in its faeces, due to the presence of micro-organisms which possess genetic determinants for mercury resistance called mer-operon (Summers and Silver, 1978; Barkay, 1992; Silver, 1996). Expression of the operon is inducible and dependent on the presence of Hg^{2+}; for example Barkay (1992) reported that inducible concentrations of Hg^{2+} are in the range 0.2–20 μg/ml. This evidence may explain the observation in our study that the highest losses of Hg were found in the animals fed with the highest concentration of Hg in food. Another possibility could be formation of volatile $Me_2{}^{203}Hg$ and therefore further studies should include measurement and speciation of volatile Hg compounds.

3.4.3. Distribution of ^{203}Hg in P. scaber

The distribution of Hg retained in whole animals, their glands and guts was investigated in animals fed with leaves of 300 ngHg/mg concentration and the results are shown in Fig. 8.

These results show that the majority of Hg retained is distributed between glands and gut. Hg in glands represents (34$\pm$7)% of the total retained

Table 6
Uptake, retention and excretion of ^{203}Hg in *P. scaber*

Group[a]		Weight of animal (mg)	Duration of exposure (day)	Weight of eaten food (mg)	Activity in consumed food (Bq)	Activity in animal (Bq)	Percent of ^{203}Hg in animal (%)	Activity in faeces (Bq)	Percent of excreted ^{203}Hg by faeces (%)
A	A1	46	16	57.2	21.4	0.6	2.8	15.5	70.1
0.33 ng/mg	A2	46	16	54.8	20.5	0.6	3.1	13.4	65.4
0.37 Bq/mg	A3	42	16	52.9	19.8	0.8	4.2	10.0	50.5
	A4	36	16	47.5	17.8	0.6	3.2	8.5	47.8
	A5	32	16	43.1	16.1	0.2	1.4	10.7	66.5
$X \pm$ S.D.							3.0 ± 0.1		60 ± 11
B	B1	55	35	78.8	344	2.4	0.7	272	79.2
3.23 ng/mg	B2	29	16	43.7	191	4.3	2.3	118	61.9
4.4 Bq/mg	B3	26	16	54.7	239	4.4	1.9	186	78.0
	B4	44	16	45.3	198	3.9	2.0	116	58.7
	B5	35	33	89.1	389	3.2	0.8	282	72.6
	B6	32	35	96.3	420	1.0	0.2	45	10.7
	B7	40	35	86.1	375	6.7	1.8	232	61.8
	B8	55	35	96.5	421	4.8	1.2	222	52.8
	B9	55	28	96.3	420	1.1	0.3	94	22.4
	B10	35	22	50.1	218	4.1	1.9	69	31.6
	B11	29	16	70.3	307	6.2	2.0	237	77.3
	B12	31	35	93.7	409	3.6	0.9	300	73.4
	B13	45	35	92.7	404	3.0	0.8	131	32.4
	B14	28	35	92.8	405	4.4	1.1	166	41.0
	B15	24	35	85.8	374	2.8	0.8	166	44.4
$X \pm$ S.D.							1.2 ± 0.7		53 ± 22
C	C1	53	5	9.30	4790	253	5.3	1510	31.6
323ng/mg	C2	54	5	10.2	5260	199	3.8	2020	38.3
433Bq/mg	C3	80	7	13.9	7160	210	2.9	2140	29.9
	C4	60	7	12.4	6390	254	4.0	1040	16.3
	C5	63	7	12.5	6440	294	4.6	1750	27.2
	C6	76	7	13.8	7110	452	6.4	3740	52.6
	C7	64	7	12.9	6650	119	1.8	1140	17.1
	C8	58	7	13.5	6960	209	3.0	1440	20.6
$X \pm$ S.D.							4.0 ± 1.4		29 ± 12

Data on animal and faeces activities are normalised for background, decay and geometric counting efficiency.
[a] Groups of animals (A, B, C) exposed to $^{203}Hg^{2+}$ of defined concentrations and activities. Concentrations and activities refer to Hg on leaves (ngHg/mg of leaf, Bq/mg of leaf).

Hg in isopods C1–C5. The percentage of Hg in their guts is much more variable, also depending on whether the gut was full or empty. The sums of the activities in glands and guts of isopods C2 and C3 were much smaller than the activities of the live animals measured just prior to dissection but so far, we cannot explain these results. Animals C6–C8 were left for another 5 days with non-contaminated food; the activities of the animals were therefore lower and the percentage of Hg accumulated in glands increased to approximately (55 ± 19)% of retained Hg.

4. Conclusions

The main objective of the present work was to optimise an analytical method in order to follow Hg^{2+} methylation and $Me^{203}Hg^{+}$ demethylation

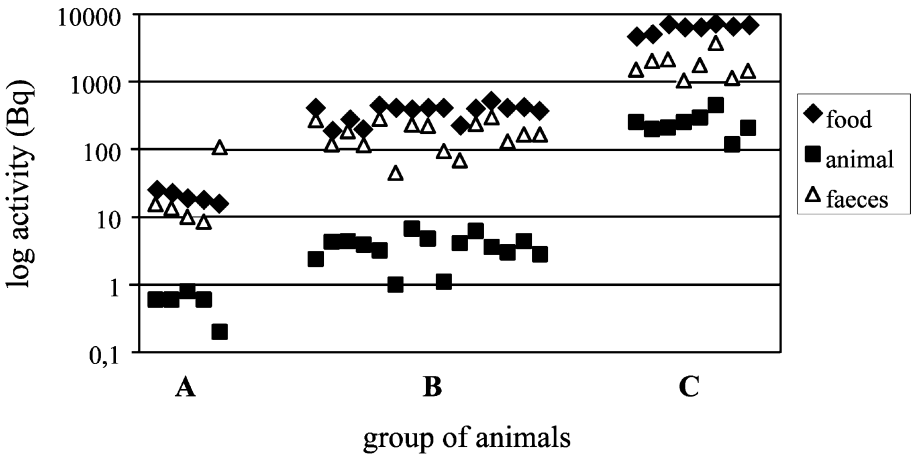

Fig. 7. Comparison of ^{203}Hg uptake and retention in animal and excretion of ^{203}Hg by faeces in three groups of *P. scaber* exposed to $^{203}Hg^{2+}$.

in the isopod *P. scaber*. Special attention was focused on separation of Hg species from the sample. Individual analytical steps were tested with $^{203}Hg^{2+}$ and $Me^{203}Hg^{+}$ aqueous tracer solutions and with animals previously exposed to one of the two ^{203}Hg species. One of the most important observations is that a florisil column retains different fractions of Hg^{2+} and $MeHg^{+}$, and is therefore not appropriate for accurate determination of the percentage of transformed Hg species. Cold leaching of Hg species with 1 M KOH from the sample is not completely efficient and part of the Hg^{2+} remains in the sample, but can be released by leaching at an elevated temperature. Copper, which is originally present in *P. scaber* in large amounts, does not interfere with the extraction and separation of Hg species.

Preliminary results of the Hg methylation/demethylation study are very variable, probably due to different concentrations and activities of Hg in the food of the exposed animals and to individual variability between animals. In the future,

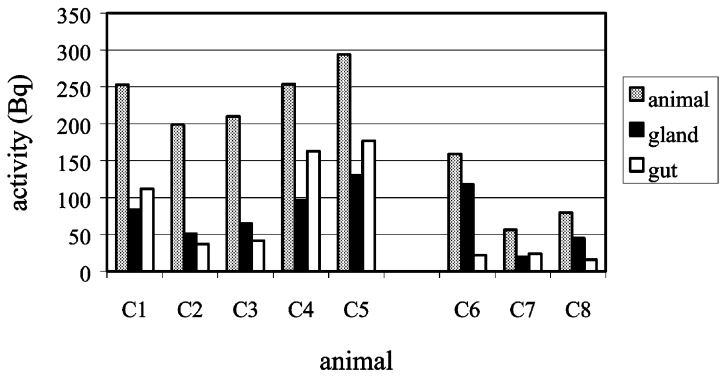

Fig. 8. Distribution of ^{203}Hg between glands and gut in *P. scaber*. Animals C1–C8 were exposed to $^{203}Hg^{2+}$ for 7 days. Animals C1–C5 analysed immediately after exposure (C2, C3 had empty gut). Animals C6–C8 left for another 5 days, fed with non-contaminated food.

larger groups of animals should be investigated to avoid individual variability. Additionally, some improvements of counting conditions should be made in order to reduce the counting time and improve sensitivity of detection. However, the data obtained show that in the isopod *P. scaber* formation and degradation of MeHg takes place. Demethylation studies can be made now using either $Me^{203}Hg^{+}$ or $^{14}CH_3Hg^{+}$ (Oremland et al., 1991; Marvin-Dipasquale et al., 2000; Hines et al., 2000).

An assessment of mercury uptake, retention and excretion in *P. scaber* were also performed in three groups of isopods exposed to different concentrations of $^{203}Hg^{2+}$. Based on the results of this experiment, a mass balance was estimated. One of the important findings was that part of Hg was 'lost' from the system, most probably due to the formation of volatile Hg species. The percent of 'lost' Hg is dependent on the Hg concentration in food, which could probably be related to the induction of mer-operon, also responsible for reduction of Hg^{2+} to Hg^0. In future studies, all possible transformation mechanisms of mercury (i.e. methylation, demethylation, volatilization) should be included.

In further work it would be also of interest to compare these data on Hg concentrations and speciation in *P. scaber* from a background area with Hg concentrations in *P. scaber* sampled in Idrija, an environment highly polluted with mercury.

Acknowledgments

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