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Long-term Hg pollution induced Hg tolerance in the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea)

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Isopods (Porcellio scaber) as well as their bacterial gut community from a mercury-polluted site are mercury tolerant.

Abstract

The aim of our work was to assess the pollution-induced community tolerance (PICT) of isopod gut microbiota and pollution-induced isopod population tolerance (PIPT). Animals collected from a chronically Hg polluted and an unpolluted location were exposed for 14 days to 10 μg Hg/g dry food under laboratory conditions. The lysosomal membrane stability, hepatopancreas epithelium thickness, feeding activity and animal bacterial gut microbiota composition were determined. The results confirm the hypothesis that the response to short-term Hg exposure differs for animals from the Hg polluted and the unpolluted field locations. The animals and their gut microbiota from the Hg polluted location were less affected by Hg in a short-term feeding experiment than those from the unpolluted environment. We discuss the pollution-induced population tolerance of isopods and their gut microbiota as a measure of effects of long-term environmental pollution. The ecological consequences of such phenomena are also discussed.

Keywords: Mercury; Hg tolerance; Lysosomal membrane stability; Gut bacterial community structure; TTGE; 16S rRNA

1. Introduction

Within the last decade, efforts have increasingly been dedicated to the use of biomarkers in environmental toxicity studies (Kammenga and Weeks, 2004). However, the presence of long-time stressors might establish a population or community with tolerant individuals, which might affect the response of the measured biomarkers and subsequently the risk interpretation. On the other side, the pollution-induced community tolerance (PICT) or population tolerance (PIPT) alone provide the tools for detection of effects of pollution at population or community levels (Blanck, 2002; Barata et al., 2002).

Several reports on the presence of tolerant populations of terrestrial invertebrates have been published, for example on earthworms (Oligochaeta) (earthworm populations – Spurgeon and Hopkin, 1999a; *Lumbricus rubellus* – Spurgeon and Hopkin, 1999b; Langdon et al., 1999; *Sparangophilus pearsei* – Vidal and Horne, 2003, *L. rubellus* and *Dendrodrilus rubidus* – Langdon et al., 2001), beetles (Coleoptera) (*Poecilus cupreus* – Kramarz, 1999; *Pterostichus oblongopunctatus* – Stone et al., 2001), springtails (Collembola) (*Onychiurus armatus*, *Isotoma notabilis* – Tranvik et al., 1993; *Folsomia fimetaria* – Scott-Fordsmand et al., 2000; *Orchesella cincta* – Sterenborg et al., 2003), roundworms (Nematoda) (*Caenorhabditis elegans* – Hsiu-Chuan Liao et al., 2002; nematode populations – Millward and Grant, 2000) and isopods (Isopoda)

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(*Porcellio scaber* – Donker et al., 1993, 1996, *P. scaber* and *Oniscus asellus* – Jones and Hopkin, 1996).

In general, tolerance of animals against metals is attributed to the duplication and higher transcription rate of metallothionein genes, metallothionein sequestering and metal–metallothionein adduct excretion, which was investigated on the cellular level (van Straalen and Roelofs, 2005). In addition, detoxifying mechanisms based on lysosomal proteins and their metal interaction are also reported (Hsiu-Chuan Liao et al., 2002). On the physiological level the difference between the tolerant and intolerant organisms can be expressed as the higher survival rate and better fitness of the tolerant animals when exposed to pollution.

While the importance of invertebrates-bacterial gut microbiota interaction is recognised in several publications on termites (Ohkuma and Kudo, 1998), collembolans (Thimm et al., 1998) and isopods (Zimmer, 2002; Kostanjšek et al., 2002), less is known about the contribution of the gut associated bacteria to the mitigation of metals consumed with food. In addition, it is known that bacteria can immobilise bioavailable metal ions which decrease metals toxicity. Therefore, one of the mechanisms of tolerance of animals to elevated environmental concentrations is metal detoxification by bacteria that are associated with those animals (Coughtrey et al., 1980; Wagner-Döbler et al., 2000; Stone et al., 2001). The community of soil dwelling animals and their autochthonous gut microbiota might cooperate in the mitigation of the toxic effects of metals on each member of this invertebrate–bacteria community (Coughtrey et al., 1980; Stone et al., 2001). The latter is expected to have an important role when considering the toxic effects of mercury.

Mercury is detoxified by mercury-resistant bacteria which are bearing the *mer* operon. The bacterial mercury reductase (MerA) reduces highly bioavailable Hg^{2+} into the volatile Hg^0 form, which evaporates from the bacterial cells (Barkay et al., 2003). The observed system, however, is becoming poorer with mercury present as Hg^{2+} as well as organic forms. Therefore, the bacterial community present in the gut is potentially very important when the environment is Hg polluted. Presumably, there are a number of factors which contribute to the Hg^{2+} tolerance, however at the present state of knowledge they are mainly unknown.

In this study, terrestrial isopods *P. scaber* were collected from two field locations. One location, Idrija, is severely Hg polluted, the other one, Radlek, is considered as unpolluted. In Slovenia, the Idrija region is highly polluted with mercury due to more than 500 years of mercury mining, resulting in high Hg environmental burden (Gnamuš and Horvat, 1999). The aim of our work was to assess the long-term pollution induced Hg tolerance in terrestrial isopod *P. scaber* (Isopoda, Crustacea) and its gut bacterial microbiota.

The tolerance was assessed in a two-week laboratory toxicity test, where animals from the polluted and unpolluted environments were fed with Hg-dosed food. We hypothesised that the Hg-tolerant animals would be less affected by the Hg exposure than the intolerant animals. We determined the lysosomal membrane stability (LMS) of digestive gland cells, hepatopancreatic epithelium thickness, feeding rate and gut bacterial community structure. We discuss the pollution-induced population tolerance of isopods and their gut microbiota as a measure of effects of the long-term environmental pollution. Also, the relevance of gut bacterial community structure coupled to organism level biomarkers is discussed in terms of providing environmentally relevant toxicity data in the controlled laboratory test.

2. Materials and methods

2.1. Animals

The animals (*P. scaber*, Latreille 1804) were collected in mercury polluted (Idrija) and unpolluted areas (Radlek) in Slovenia. At the sampling site in Idrija (more than 500 years of mercury mining) the total Hg levels were up to 263 μg/g of dry soil (SD = 8.528, $N = 4$) while in the unpolluted area they were 0.194 μg/g of dry soil (SD = 0.00532, $N = 3$). The animals were randomly collected from each location and their weight, sex and moulting stages were determined. Separately, from each location a group of 80 animals with similar average weight was brought into the laboratory for acclimatization prior to conducting the exposure experiment. The animals with similar weight were used to eliminate other possible differences resulting from weight dissimilarities. Two groups of 10 animals were immediately subjected to food deprivation for 20 h and used for DNA bacterial community analysis. From the same sites where the animals were collected also soil and decaying leaves were sampled and used as substrate and food source for the 14-day acclimatization period, respectively. After the acclimatization the acute mercury-exposure experiment was performed.

2.2. Laboratory experiment

After two weeks of acclimatization four groups of 15 animals (two groups from Idrija and two groups from Radlek) were exposed to the food dosed with 10 μg Hg ($HgCl_2$, Merck, Darmstadt, Germany, Guaranteed Reagent for analysis – 99.5%) of dry weight of hazelnut trees leaves. The acute exposure experiment lasted 14 days. Another four groups (two groups from each location) of 15 animals were control groups receiving leaves treated only with distilled water. Altogether 8 groups of 15 animals selected according to similar fresh weight (approximately 0.040 g) were handled in the 14-day experiment. During the experiment the animals were kept individually. Every second day the amount of faeces produced by each animal was determined, the food and the faecal pellets were removed and freshly prepared food was given. The collected faeces and food samples were frozen at −20 °C until DNA isolation. At the end of the experiment two groups of animals (one exposed and one control) were dissected immediately and the other two groups were allowed to empty their guts for 24 h. Before the dissection their sex, weight, and moult stage were determined as described by Zidar et al. (1998). The animals were dissected as described elsewhere (Drobne et al., 2002). The guts were kept in phosphate buffer at pH 8 and frozen at −20 °C until the DNA isolation.

2.3. Bacterial community response

2.3.1. DNA isolation

We used the bead-beating method in all further DNA isolation procedures. The whole DNA isolation was performed on composite samples of 10–15 guts, faeces collected over consecutive interval sampling in the laboratory experiment and food source (leaves), respectively. The samples were put in phosphate buffer pH 8 in Eppendorf tubes. The samples were manually ground with a pestle. We added 350 μl SDS lysis solution (10% SDS, 100 mM NaCl, 500 mM Tris pH 8), 375 μl phenol:chloroform:isoamyl alcohol (25:24:1) and 375 mg glass beads (Sigma–Aldrich, St. Louis, Missouri; 75–150 μm, 106 μm average radius). The samples were shaken at 26.6 Hz for 2 min (bead beating). After a centrifugation at 12000 *g* for 5 min, the water phase was transferred into a new Eppendorf tube. The phenol:chloroform:isoamyl alcohol was replaced and the new SDS lyses solution was added. The procedure

was repeated two more times. The subsamples were precipitated by adding 10 M ammonium acetate and 96% cold (−20 °C) ethanol, as described by Sambrook et al. (1989), and put together to obtain one DNA sample. DNA was cleaned through the previously prepared sephadex G200 spun columns (Miller et al., 1999).

2.3.2. PCR

For TTGE analysis we used the U968-GC and L1401 primer pairs (Nübel et al., 1996). The amplification mixture and conditions were as follows: 1.5 µl of the DNA sample was amplified in a reaction mixture that consisted of 1× reaction buffer (Perkin Elmer, Warrington, UK), 1.5 mM $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate, 9 µg of bovine serum albumin, 10 pmol of each primer and 2.5 U of Taq polymerase (Perkin Elmer, Warrington, UK). The amplification was performed on a GeneAmp 2400 thermal cycler (Perkin Elmer, Norwalk, Connecticut, USA) in a final volume of 25 µl. The amplification program consisted of initial denaturation at 93 °C for 3 min, followed by 35 cycles with 30 s of denaturation at 93 °C, 30 s annealing at 54 °C and 1 min extension at 68 °C, and a final extension step at 72 °C for 7 min. The amplification products were separated by 1.5% agarose gel electrophoresis, and the fragments of an expected length, approximately 700 bp, were cut from the gel and purified. We used two separate PCRs for each DNA sample to minimize bias of the PCR amplification.

2.3.3. TTGE

We used the Dcode universal mutation detection system (Biorad, Paris, France) for the sequence specific separation of PCR products. Electrophoresis was performed through 1 mm thick and 16 cm × 16 cm polyacrylamide gels (8% 37.5:1 acrylamide:*N*,*N*′ methylene bisacrylamide, 8 M urea, 1.25× TAE [Tris, acetic acid, ethylenediaminetetraacetic buffer] buffer, 40 µl TEMED [*N*,*N*,*N*′,*N*′-Tetramethyl-1-,2-diaminomethane] and 400 µl 10% ammonium persulfate in 7 L of 1.25× TAE electrophoresis buffer). The electrophoresis was run 20 h at a constant voltage of 70 V in a temperature gradient from 54 °C to 68 °C and ramp rate of 0.7 °C/h. The gel was loaded with 200 µg of PCR amplified DNA mixed with loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol and 70% glycerol). The gels were stained by submersion into an ethidium bromide solution (1 mg/ml ethidium bromide in 1.25× TAE) for 20 min and destained for 5 min.

2.4. LMS assessment

The lysosomal membrane stability (LMS) was assessed by the lysosomal latency (LL) assay and the neutral red retention (NRR) assay on *in toto* digestive gland tubes of *P. scaber*, as described in Nolde et al. (2006). The digestive glands (hepatopancreas) consist of four blind ending tubes, which were separated during isolation. Three of them were used for the LL assay, the last one was used for the NRR assay.

2.4.1. LL assay

For the lysosomal membrane stability (LMS) assessment, the cytochemical procedure based on evaluation of the activity of the lysosomal enzyme *N*-acetyl-β-hexosaminidase was performed on *in toto* digestive gland tubes of *P. scaber*. A series of gland tubes was incubated in a labilizing buffer (0.1 M Na-citrate buffer at pH 4.5) at 37 °C for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180 and 200 min, respectively, in order to artificially labilize the membrane. After the acid labilization the gland tubes were incubated in 50 ml 0.1 M Na-citrate buffer containing 3.5 mg of a low viscosity peptide (for membrane stabilization) and 20 mg of substrate (naphthol AS-BI *N*-acetyl-β-glucosaminide), previously dissolved in 2.5 ml 2-methoxyethanol for 20 min at 37 °C. Then the gland tubes were rinsed with physiological solution for *P. scaber* (Hagedorn and Ziegler, 2002) and incubated for 10 min at room temperature in 0.1 M Na-phosphate buffer (pH 7.4) containing the diazonium coupler Fast Violet B Salt (Sigma–Aldrich Chemie, Steinheim, Germany) (1 mg/ml). After a 5-min rinse in tap water, the gland tubes were fixed for 10 min at 4 °C in calcium formol and mounted in Kaiser glycerine gelatine (Merck KGaA, Darmstadt, Germany).

Two parameters, the maximum amount of stained lysosomes and the staining of the cytoplasm, were assessed microscopically (Axioskop 2 MOT, Carl Zeiss, Goettingen, Germany) in each series of gland tubes by two independent observers. The LMS was assessed as the time of incubation of the gland tube in the labilizing buffer when the maximal amount of stained lysosomes or the staining of the cytoplasm occurs.

2.4.2. NRR assay

The neutral red stock solution was made following the procedure described in Ringwood et al. (2005): 4 mg of the neutral red powder (The British Drug Houses, London, England) was dissolved in 1 ml of dimethyl sulphoxide (DMSO) (Sigma, Sigma–Aldrich Chemie, Steinheim, Germany). The working solution was prepared by diluting 10 µl of the stock solution with 9.9 ml of the physiological solution for *P. scaber*.

For each NRR assay, one *in toto* digestive gland tube was placed on a slide and 60 µl of the working solution of the neutral red (NR) dye was added to the tissue (Nolde et al., 2006). The time when the NR dye was added to the gland tubes was noted. The slides were left for 20 min in a light protected humidity chamber that consisted of a glass Petri dish ($r = 9.5$ cm) with a lid. To maintain high humidity, the lid was sprayed with distilled water and the base of the dish was covered with moistened filter paper. After that the slides were covered with the cover slip and inspected under a microscope (Axioskop 2 MOT, Carl Zeiss) every 30 min. At every inspection, the fraction of the gland tube was assessed, which became coloured pink to red because of the leakage of the NR dye from the lysosomes into the cytoplasm.

2.5. Average epithelial thickness

One digestive gland tube from each animal was fixed in Carnoy B fixative (ethanol:chloroform:acetic acid; 6:3:1) for 2 h at room temperature. The fixed digestive gland specimens were dehydrated in ethanol, cleared in xylene and embedded in Paraplast Plus (Sigma). Eight-micrometer sections (Reichert-Jung 2040 rotatory microtome, Reichert-Jung, Vienna, Austria) of the entire digestive gland tube were cut and stained with eosin (Drobne and Drobne, 2005). The sections were examined with light microscope (Carl Zeiss Axioskop 2 MOT, Carl Zeiss, Jena, Germany). For the determination of the average epithelial thickness 12 sections equally apart were chosen from the middle part of the gland tube (2/3 of the whole tube length). The average epithelial thickness on a section was determined using computer program for image analyses KS 400 (Carl Zeiss Vision, Carl Zeiss Jena GmbH, Oberkochen, Germany) and manual contouring of the inner and outer epithelial surface (Drobne and Drobne, 2005).

2.6. Statistical analysis

2.6.1. LMS, NRR and average epithelial thickness

For statistical analysis of the data, the Windows statistical software (SPSS 12.01, SPSS, Chicago, IL, USA) was used. The differences in the LMS among the pairs of exposed and unexposed groups of animals were tested with the Mann–Whitney test, at the significance level $p < 0.05$. *t*-test and the Mann–Whitney test were used for the statistical analysis of the hepatopancreatic epithelia thickness at the significance levels $p < 0.01$ and < 0.05.

2.6.2. TTGE analysis

The TTGE data were analysed using the GelManager 1.5 software package (Biosystematica, Devon, UK). We used the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering for the tree construction based on the matrix of the Pearson product moment coefficient of the similarities of the TTGE patterns.

3. Results

3.1. Field data

3.1.1. Weight of field animals

The animals ($N = 88$) collected in Radlek had 0.044 g average fresh weight (SD = 0.011). Animal group consisted of' 26

females and 62 males. Eleven animals were in various moulting stages (13.6%) which were previously described by Zidar et al. (1998). The animals collected in Idrija ($N = 64$) had 0.029 g of average fresh weight (SD = 0.008) and included 27 males and 37 females. Six of these animals (9.4%) were in one of the moulting stages. Even though the animals were collected in the same period, according to the *t*-test ($p < 0.01$) the animals from Idrija were statistically lighter than the animals from Radlek.

3.2. Laboratory experiment

3.2.1. Feeding rate

The animals from the clean site had a higher initial feeding activity (measured as number of faecal pellets per day) than the animals from the polluted site (*t*-test, $p < 0.01$) (Fig. 1A, B).

For all animals, the feeding rate decreased with time (two-way ANOVA, $p < 0.01$). The reduced feeding activity is indicated by the linear regression line ($R^2 = 0.95$) calculated on the basis of the average production of faeces of the mercury exposed and control groups as a function of time. The decreased feeding rate may be due to the unfavourable conditions in the laboratory, which were apparently stressful for all animals.

In the animals from the clean site, the decrease in feeding rate was much faster when fed with Hg-dosed food (coefficient of linear function is −8.89) compared to clean food (coefficient of linear function is −6.46; two-way ANOVA, $p < 0.01$); such difference was not found for animals from the polluted site (coefficient of linear function of control animals −2.67 and Hg-fed animals −1.54; two-way ANOVA, $p > 0.05$). This may be an indication for tolerance in the animals from the polluted site.

3.3. Lysosomal stability determined by lysosomal staining and neutral red retention

According to the arbitrarily defined borders naming the animals as *normal*, *concerned* and *stressed* (Nolde et al., 2006), the staining of the lysosomes in the lysosomal latency (LL) experiment showed that the animals collected in Radlek, exposed to mercury, had a response which was classified close to the line of demarcation between the *concerned* and *normal* lysosomal membrane stability (Fig. 2). The discolouration of the lysosomes and staining of the cytosol showed that the animals from Radlek are placed in the area arbitrarily defined as *concerned* when exposed to the mercury in the food exposure experiment (Fig. 2). In the NRR assay a drop from the normal state to the concerned state was observed after 80 min of observation, when the animals were fed with mercury-dosed food (Fig. 3). The NRR assay also showed that the response to the elevated Hg concentrations in the food occurred as faster colourization of cytosol when compared to the lysosomal response of the control group of animals (Fig. 4).

In contrast, the Hg exposed animals from the Hg polluted area had stable lysosomes in the LL assay (Fig. 2). On the

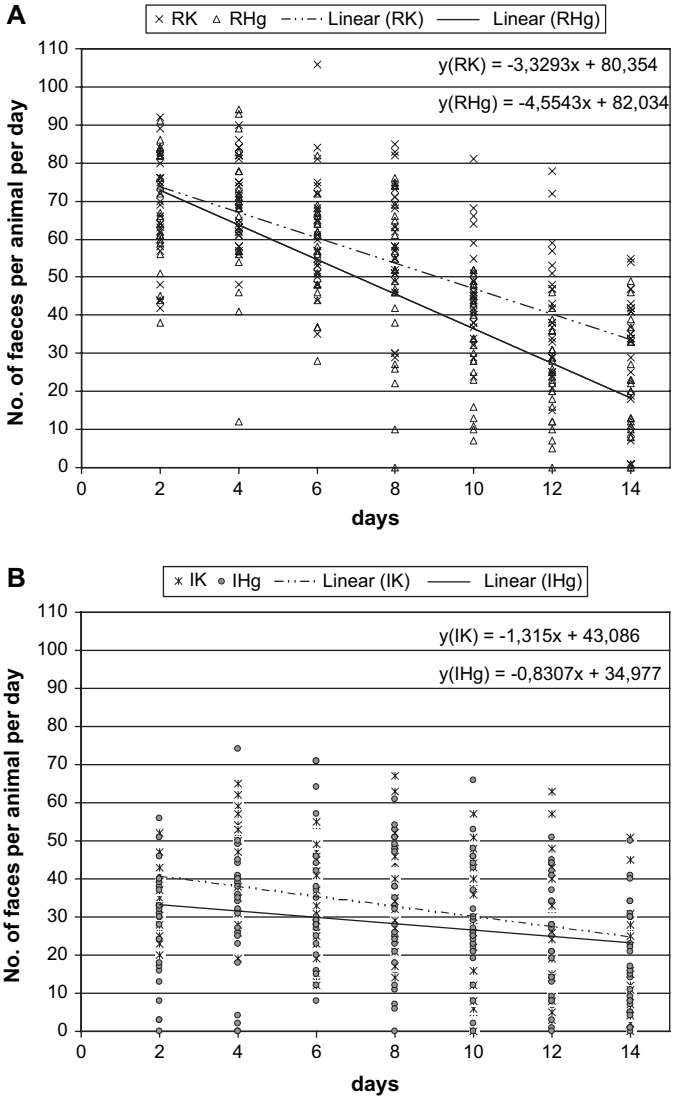

Fig. 1. Feeding rate of *Porcellio scaber* from non-polluted (Radlek) and Hg-polluted (Idrija) sites, exposed for 14 days to clean or 10 μg Hg/g dry weight polluted food. Feeding rate is expressed as faecal pellet production per animal per day. Dashed lines represent linear regression of control groups, solid lines of the Hg-exposed groups. (A) Animals from the non-polluted Radlek area (RK = control, RHg = exposed). (B) Animals from the Hg-polluted Idrija area (IK = control, IHg = exposed).

basis of lysosomal colouration and discolouration observations in the LL assay showed that the animals from Idrija were sorted into the normal state of the arbitrarily defined area. However, the lysosomal discolouration showed that the animals were in the state close to the borderline between the concerned and normal conditions (Fig. 2). In the NRR assay, the lysosomes mostly maintained neutral red even at the last inspection after 140 min of observations. This result places the animals into the normal state (Fig. 3). The lysosomal response in the NRR assay was highly diverse especially after 40 min of incubation in neutral red (Fig. 4). Accordingly, there were no statistically significant differences between the control and mercury exposed groups of animals in the LL as well as in the NRR assay.

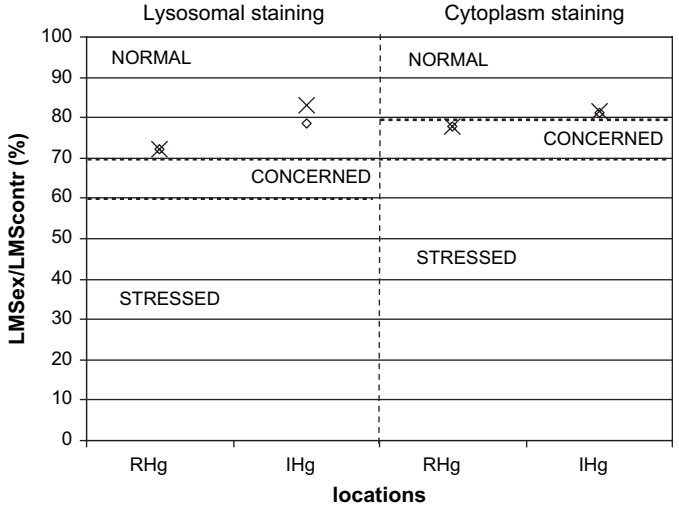

Fig. 2. Comparison of lysosomal membrane stability ratios of exposed and control groups of animals after 14 days study with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). Horizontal dashed lines represent the borders of normal (upper dashed lines), concerned (between upper and lower dashed lines) and stressed response of animals (lower dashed lines) (R – animals from Radlek, I – animals from Idrija, Hg – $HgCl_2$ exposed groups of animals). ◇ – average, × – m.

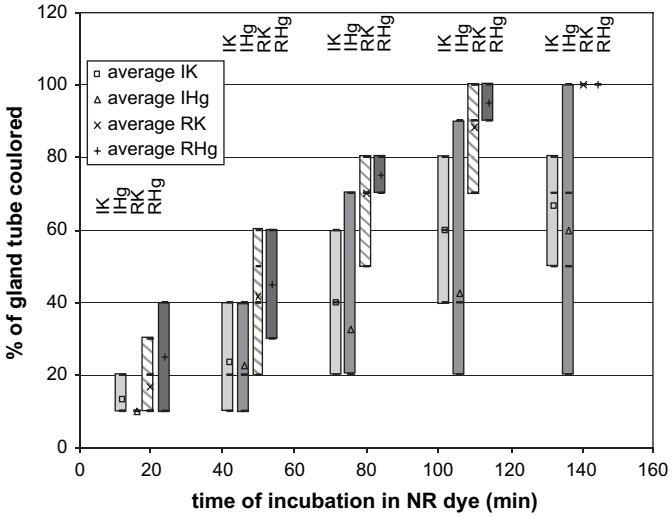

Fig. 4. Neutral red lysosomal retention time after 14 days exposure experiment with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). The *y*-axis represents the percentage of the coloured hepatopancreatic tubes (R – animals from Radlek, I – animals from Idrija, K – control groups of animals, Hg – $HgCl_2$ exposed groups of animals). Bars represent the range of the observed results (Idrija control goup – □, Idrija $HgCl_2$ exposed group – ■, Radlek control group – ▨, Radlek $HgCl_2$ exposed group – ▩).

When comparing the lysosomal staining and cytoplasm staining in the LL assay the Hg exposed animals from the unpolluted area had less stable lysosomal membranes than those from Idrija after mercury exposure (Fig. 2). In addition, the control group of animals from Idrija had higher lysosomal membrane stability in both assays than those from Radlek after the feeding experiment. The NRR assay showed that the variance of cytoplasm staining was higher in the group of animals from Idrija when compared to the group of animals from Radlek. This difference in dispersion of response was most obvious after 80 min of observation when comparing the groups of Hg exposed animals from each location (Fig. 4).

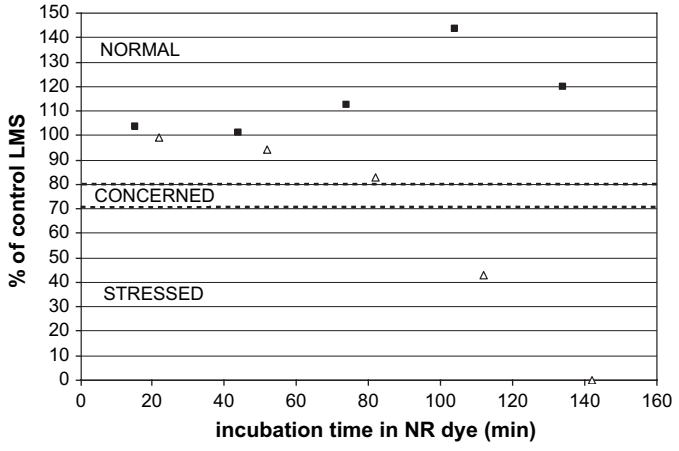

Fig. 3. Neutral red lysosomal retention time after 14 days experiment with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). The *x*-axis represents consecutive observations in the first 20 min and consecutive every additional 30 min until the last observation at 140 min. The *y*-axis represents the percentage of the coloured hepatopancreatic tubes. △ – animals from Radlek (unpolluted environment) after 14 days Hg exposure, ■ – animals from Idrija after 14 days Hg exposure.

3.4. Hepatopancreas epithelium thickness response

Right after laboratory acclimatization, the animals from Radlek had a thinner epithelium than those analysed directly after collection from the field (*t*-test, $p < 0.05$, Table 1, Fig. 5). In the laboratory feeding experiment, the mercury exposed animals from Radlek had a thinner hepatopancreas epithelium compared to the average values of control groups (Fig. 5, Table 1). However, this difference was not statistically significant. In contrast, the Hg exposed animals from the laboratory experiment had a significantly thinner hepatopancreas epithelium when compared to the animals directly taken from the environment – Radlek ($p < 0.05$, Table 1).

After acclimatization, the animals from Idrija also had a thinner epithelium. However, both the mercury exposed group and the control group of animals from the chronically polluted environment had thicker hepatopancreatic epithelia than before the laboratory exposure experiment (Fig. 5).

The epithelium thickness of *P. scaber* hepatopancreatic glands was similar in both groups of animals collected in Idrija or Radlek when animals were inspected immediately after sampling (Fig. 5). In addition, after 14 days of acclimatization, in both groups of animals a reduced hepatopancreatic epithelium thickness was observed. However, the difference was not statistically significant between the animals from the polluted and pristine environments. When the animals were subjected to 14-day acute mercury exposure, the animals from

Table 1
Statistical differences between groups of animals in the 14 days $HgCl_2$ food exposure experiment – Student's *t*-test

	R1	R14	RFK	REK	RFHg	REHg	I1	I14	IFK	IEK	IFHg	IEHg
R1												
R14	*											
RFK	*											
REK	*											
RFHg	*	*										
REHg	*											
I1		*	*	*	*	*						
I14	*						*					
IFK	*		**		*	**	*					
IEK	**		**		*		*					
IFHg	*						*					
IEHg	**		*	**	*	*	*					

Analysis was performed after 14 days exposure study with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). R – animals from Radlek, I – animals from Idrija, R1 and I1 – animals directly from the environment, R14 and I14 – animals after 14-day acclimatization, F – animals with full guts, E – animals after food deprivation, K – control group, Hg – mercury exposed group, * $p < 0.05$, ** $p < 0.1$.

Idrija on average had a thicker hepatopancreatic epithelium ($p < 0.05$) than in the Radlek animals fed with Hg (Fig. 5, Table 1).

However, differences of average epithelial thickness in the Hg-exposed groups of animals from Radlek as well as from Idrija during the exposure experiment were not observed ($p > 0.5$) even though the average values decreased when compared to the control groups from Radlek or Idrija.

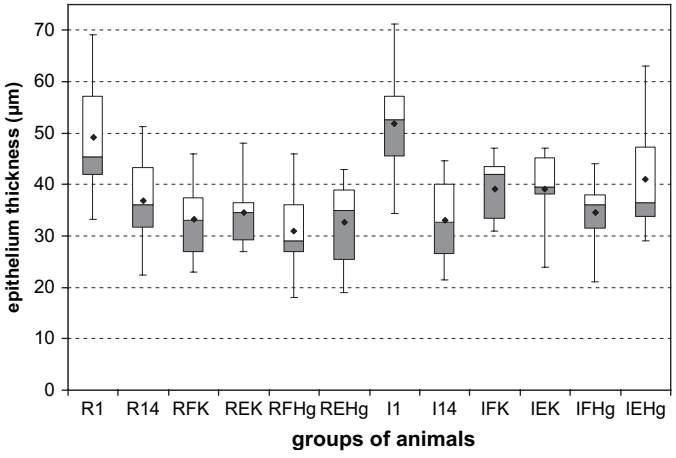

Fig. 5. Analysis of the hepatopancreatic epithelial thickness after 14 days exposure study with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). Box and Whisker plots represents minimum, 1st quartile, median, 3rd quartile and maximum values of the hepatopancreas epithelia thickness. In the graph average values are added (◆ – average). R – animals from Radlek, I – animals from Idrija, R1 and I1 – animals directly from the environment, R14 and I14 – animals after 14-day acclimatization, F – animals with full guts, E – animals after food deprivation, K – control group, Hg – mercury exposed group.

3.5. Bacterial community response

When the autochthonous bacterial microbiota (emptied gut bacterial microbiota) of animals immediately after collection from polluted and unpolluted sites was investigated with the TTGE 16S rRNA profiling, the gut microbiota was similar to each other – the Pearson moment coefficient was 0.83 (Fig. 6). However, after the 2-week laboratory feeding experiment, differences between the gut bacterial community of animals from polluted and unpolluted environments became evident when we inspected the TTGE 16S rRNA profiles (comparing Fig. 6 and Fig. 8 – the Pearson moment coefficient of separate groups from Radlek and Idrija was below 0.53) (Fig. 7a).

When comparing the 16S rRNA TTGE profiles of faecal samples over consecutive samplings in the food exposure experiment, bacterial communities in faecal samples of mercury exposed and control groups were more similar in animals from Radlek in each consecutive sampling interval than in animals from Idrija (Fig 7a). When comparing the TTGE 16S rRNA profiles of full guts there were no evident differences or observable patterns between the exposed and control groups of animals from Radlek or Idrija (Fig 7b). After the 14-day laboratory exposure experiment, the community structure of the autochthonous microbiota showed bigger differences between the Hg exposed and control groups of animals collected in Radlek (Pearson moment coefficient was 0.81) than in animals collected in Idrija (Pearson moment coefficient was 0.91) (Fig. 7b, c).

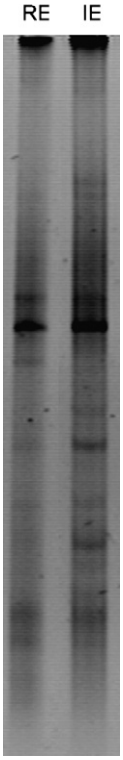

Fig. 6. Comparison of TTGE profiles of empty guts of the animals collected in Radlek (RE) and the animals collected in Idrija (IE) before the mercury-exposure experiment.

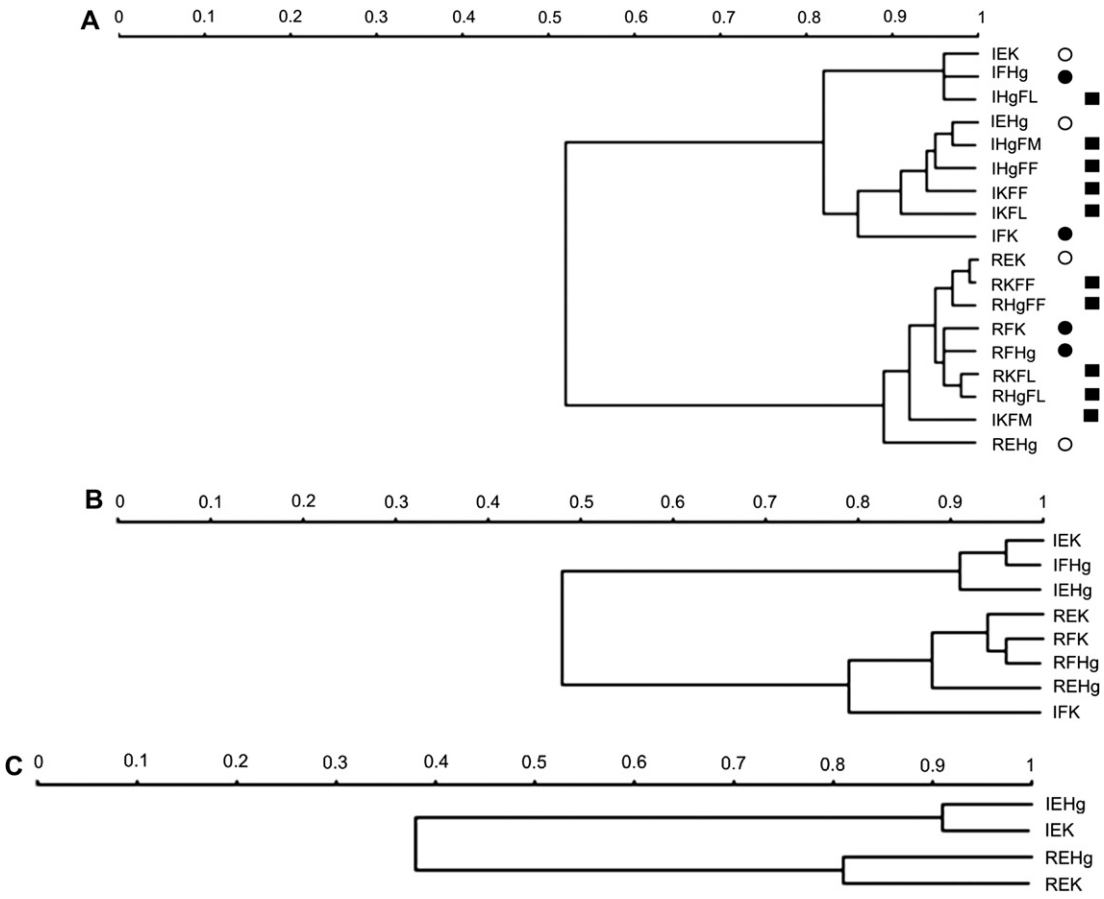

Fig. 7. Changes of the gut bacterial community structure after 14 days exposure study with *Porcellio scaber*, using 10 μg Hg/g dry food, and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). Dendrogram based on the Pearson product moment coefficients calculated from TTGE profiles of 16S rRNA fragments from the predominant bacterial populations from *Porcellio scaber* guts. (A) Comparison between empty and full guts, and faecal bacterial community (circle – ○ empty guts and ● full guts) collected from the unpolluted area (Radlek – R), and their faeces (■) from the laboratory experiment. E – empty gut, F – full gut, Hg – mercury exposed, L – guts of the animals brought from the environment and acclimatized for 14 days, K – guts from the control groups of animals from the exposure experiment. (B) Comparison between empty and full guts bacterial community (I – Idrija, R – Radlek, E – emptied guts, F – full guts, K – control, Hg – $HgCl_2$). (C) Comparison between bacterial communities of empty guts of animals from different locations (I – Idrija, R – Radlek, K – control, Hg – $HgCl_2$).

After the exposure, in the TTGE 16S rRNA profiles there were some shared bands between the groups of Hg exposed and control groups of animals from Idrija and also some other shared bands in the TTGE 16S rRNA profiles of animals from Radlek. The shared bands indicate a possible similarity in the bacterial community. Therefore, despite clear differences in bacterial communities determined by the Pearson coefficient, part of the bacterial community remained unaffected (Fig. 8).

4. Discussion

The results of this investigation showed that the animals and their gut microbiota from the clean or Hg polluted environments responded differently to mercury-dosed food in the laboratory experiment. We detected a less affected feeding rate and more stable lysosomal membranes of hepatopancreatic cells after acute Hg exposure for the animals from the Hg polluted environment. These animals had, in addition, a less affected epithelium after acute Hg exposure than those from the pristine location. Moreover, the animal autochthonous microbiota was less affected in the animals from the chronically mercury-polluted environment than in those from the pristine environment when fed with mercury. We attribute all these differences in the response to the Hg-dosed food to tolerance of animals and of their gut microbiota, when *P. scaber* was collected from the chronically Hg polluted location. However, some precautions of the interpretation of the results should be taken into account, because the experiment was not conducted in such a way that several different concentrations of Hg were applied and therefore no dose dependant response could be measured. Therefore, only the differences in dose responses between populations of animals from pristine and chronically polluted areas when exposed to several Hg-dosed food should show obvious PIPT and PICT responses of animals and their gut bacterial community. However, the aim of this study was comparison of the responses between animals and their gut microbiota from chronically polluted and pristine environments when exposed to the elevated Hg concentrations in the laboratory experiments. These observations should point out the responses which are expected to be result of PIPT of animals and PICT of animal gut bacterial community.

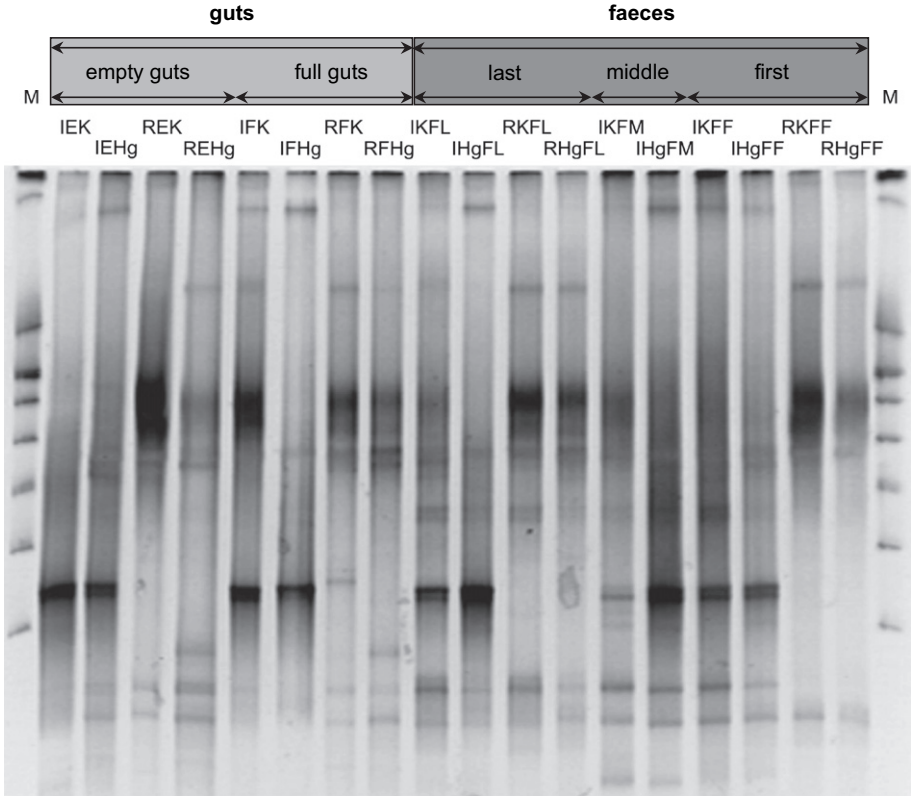

Fig. 8. TTGE profiles of the 16S rRNA PCR amplified genes of the gut and faecal samples from the 14 days laboratory exposure experiment with *Porcellio scaber*, using 10 μg Hg/g dry food and with animals from a non-polluted (Radlek) and a Hg-polluted site (Idrija). (I – animals from Idrija (mercury-polluted site), R – animals from Radlek (unpolluted site), K – control group of animals, Hg – $HgCl_2$ exposed group of animals, F – full guts (gut samples) or faeces (faecal samples), E – empty guts, L – sampling of faecal pellets at the end of experiment, M – sampling of faecal pellets in the middle of experiment and F – sampling of pellets at the beginning of the experiment). M at the left and right sides of the gel stands for marker λ/*Pst*I.

The animals directly taken from the chronically mercury-polluted area had a significantly lower body weight than the animals from the unpolluted environment. In addition to the pollution, we attribute the differences in their average fresh body weight to the differences in environmental parameters. The reason for the lower weight of the animals might be in the lower life expectancy and shift of the reproduction period to the younger animals (Donker et al., 1993; Jones and Hopkin, 1996). Additionally, in the laboratory Hg exposure experiment, the population of animals from Idrija had a lower feeding rate than the animals from the unpolluted environment (Fig. 1A, B). One can speculate that if animals consume less food, the lower average weight of these animals is expected in their natural environment also. The consequence of the lower food uptake of animals from the polluted location results in higher mortality when animals are subjected to additional stress like food deprivation (Stone et al., 2001; Tranvik et al., 1993).

After the Hg feeding experiment also the lysosomal membrane stability of the hepatopancreatic cells and the epithelium thickness of hepatopancreas were assessed. At the cellular level, lysosomal stability is most frequently applied as a cytotoxicity biomarker (Moore, 1990; Moore et al., 2004; Nolde et al., 2006). In our experiment, NRR and lysosomal latency showed that the animals collected in the polluted environment were less susceptible to the acute effects of mercury (Figs. 2 and 3). Our results support the data presented by Nolde et al. (2006) obtained also on the isopods from Idrija. The higher diversity of the NRR response of the animals from Idrija is also in congruence with observations of other researchers, where the larger variance in observed responses was found in populations of *Daphnia magna* (Barata et al., 1999), *Artemia* sp. (brine shrimp) (Sarabia et al., 2002) and *L. rubellus* (earthworms) (Langdon et al., 1999, 2001) from the contaminated environment. These large variances in responses are explained as one of the indications of the PIPT.

The epithelium thickness is a reliable measure of the state of organisms, applied also on isopods (Odendaal and Reinecke, 2003; Znidarsic et al., 2003; Drobne and Drobne, 2005). In our experiments we expected epithelial thinning to occur after feeding with the Hg-dosed food. However, at the end of the 14-day feeding period, in the animals from the Hg polluted locations the epithelium was evidently thicker than at the beginning of the exposure. This was not the case in the animals from the pristine location. With these data we proved the differences also in the histological response between the animals from the Hg polluted and unpolluted locations. But to induce histopathological changes as a result of feeding with Hg, most probably longer exposure time or higher doses are needed (Znidarsic et al., 2003).

When investigating toxic effects of mercury it is important to have in mind that the bioavailable concentrations of metals, which enter the cells, are highly dependant on the microbiota present in the gut of isopods. Accordingly, the microbiota might play a protective role against the elevated concentrations of mercury by Hg^{2+} reduction (Barkay et al., 2003). It is not known how much of the mercury is actually present near-by epithelial cells and furthermore transported into the cells. Accordingly, the differences of the observed biomarkers between the animals from the polluted and unpolluted environments could be due to the tolerance of the animals or due to the decreased bioavailable Hg fraction resulting from bacterial activity. Because of the fact that mercury toxicity might be lowered due to the microbial community activities and that invertebrates are closely associated with their gut microbiota, the changes of the bacterial gut community structure during the 14-day experiment were investigated.

The animals from Radlek had a similar autochthonous gut bacterial community to those from Idrija at the time of sampling (Fig. 6). However, after the laboratory Hg exposure experiment, the gut bacterial microbiota was significantly different among experimental groups. We explained these results with two phenomena: (i) more intensive horizontal gene transfer of resistant genes in the gut of animals from polluted sites and/or (ii) effects of the stress on the animals and their immune system affecting the animal–bacteria interaction.

Bacteria living in the contaminated environment develop mechanisms of resistance by the horizontal gene transfer (Smets and Barkay, 2005; Licht et al., 1999). Very intensive horizontal gene transfers were described to occur in guts of invertebrate species, such as springtails (Colembola – Hoffmann et al., 1998), earthworms (Daane et al., 1996), butterflies (Lepidoptera) and beetle (Coleoptera) larvae (Thomas et al., 2000). The horizontal transfer of resistant genes is also expected to occur in isopods. Therefore, on the one hand the intensive horizontal gene transfer preserves bacterial community species composition, and on the other hand, in the case of mercury it increases the potential for mercury detoxification.

Because isopods are soil dwelling organisms and their food source is poor in nutrients they are expected to be dependant on microorganisms associated with the gut (Lapanje, 2005; Kostanjšek et al., 2004; Wang et al., 2004; Zimmer, 2002). Therefore, slight changes in the animal fitness might have effects on the community. As is seen from our results the bacterial community present in the gut of the isopods during experiment is changed compared to the initial state (Figs. 7a, 8). This could be also attributed to the laboratory-induced stress which was observed as reduced faecal production in the control group of animals. The effects on the animal gut microbiota of the non-specific stress could be due to a reduced fitness of the animals. The lower fitness has high consequences on the immune system. The immune system of the animals and associated gut microbiota are normally in balance (Loker et al., 2004; Guarner and Malagelada, 2003). Moreover, the microbiota present on the epithelium have mostly the host protective function (Loker et al., 2004; Engel et al., 2002), which was observed in common cuttle (*Sepia officinalis*) (Grigioni et al., 2000; Barbieri et al., 2001), aphids (*Acyrthosiphon pisum*) (Scarborough et al., 2005), shrimp embryos (*Palaemon macrodactylus*) (Gil-Turnes et al., 1989) and American lobster (*Homarus americanus*) (Gil-Turnes and Fenical, 1992). The results of the comparison of autochthonous gut microflora of animals from the Hg-polluted site and gut microflora of animals from the unpolluted location after the laboratory experiment could be partially interpreted as the consequence of higher fitness of the animals from Idrija, or by adaptation of the gut bacterial community to the elevated mercury pollution in the animals collected in the Hg polluted environment.

The presence of bacteria that are capable of reducing Hg^{2+} is expected, because Nolde et al. (2005) reported the reduction of Hg in the gut of *P. scaber*. Additionally, the Hg resistant bacteria were isolated from the *P. scaber* gut and they were in higher numbers in animals from the mercury-polluted environments (Lapanje et al., in preparation, data not shown). As a result, it is expected that the bacterial gut community could adapt to the metal pollution. Accordingly, the gut bacterial community structure analysis should be coupled to organism level biomarkers. For that reason, the analysis of the bacterial community structure must be considered in terms of providing good quality and environmentally relevant toxicity data in controlled laboratory exposure experiments.

The pollution-induced tolerance can be considered as a valuable marker and good predictor for future changes of the biota in the contaminated environment (Grant, 2002). Additionally, ecologically more relevant data are obtained if animals and their gut microbiota are studied. This is of high importance in metal ecotoxicity studies, because gut microbiota play a role in metal speciation (Nolde et al., 2005).

Our results showed that a combined approach of the determination of PIPT of *P. scaber* population and PICT of its gut microbiota could be a valuable tool for assessing the effects of pollution in the environment by comparing populations from different locations in a standardised laboratory test protocol. Lysosomal stability, feeding activity and gut bacterial community structure proved to be particularly suitable in such studies. Our study also indicates that if acute toxicity is studied the past pollutant exposures of the population must be considered.

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