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GUT BACTERIAL COMMUNITY STRUCTURE (*PORCELLIO SCABER*, ISOPODA, CRUSTACEA) AS A MEASURE OF COMMUNITY LEVEL RESPONSE TO LONG-TERM AND SHORT-TERM METAL POLLUTION

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Abstract—Prokaryotes are of high importance in the assessment of environmental pollution effects. Due to fast responsiveness of bacterial communities to environmental physicochemical factors, it is difficult to compare results of bacterial community investigations on the temporal and spatial scale. To reduce the effects of variable physicochemical environmental conditions on bacterial microbiota when investigating the specific impact of contaminants on bacterial communities, we investigated the bacterial community in the gut of terrestrial isopods (*Porcellio scaber*, Isopoda, Crustacea) from clean and metal-polluted environments. Animals were collected from a chronically mercury-polluted site, a chronically multiple metal–(Cd, Pb, Zn) polluted site, and two reference sites. In addition, animals from an unpolluted site were laboratory exposed to 5 μg Hg/g food in order to compare the effect of acute and chronic Hg exposure. The bacterial gut microbiota was investigated by temporal temperature gradient gel electrophoresis (TTGE) and clone library construction based on polymerase chain reaction amplified 16S rRNA genes. The major bacterial representatives of the emptied gut microbiota in the animals from the chronically polluted environments seemed not affected when analyzed by TTGE. The detailed bacterial community structure investigated by 16S rRNA clone library construction, however, showed that the community from the Hg-polluted site also was affected severely (242.4 operational taxonomic units [OTU] in the polluted and 650.6 OTU in the unpolluted environment). When animals were acutely exposed to mercury, changes of bacterial community structures already were seen on TTGE profiles and no additional analysis was needed. We suggest the use of *P. scaber* gut bacterial community structure as a measure of effects caused by both long- and short-term exposure to pollution.

Keywords—Metals Gut *Porcellio scaber* 16S ribosomal RNA Bacteria

INTRODUCTION

Prokaryotes are a crucial component of the biosphere, catalyzing processes sustaining all life on Earth. They therefore are the engines of the biogeochemical cycles [1]. As a result, studies on ecosystem performance principally are closely linked to the determination of functioning of bacterial communities. Up to now, there are several reports of bacterial community analysis of polluted aquatic and terrestrial ecosystems [2–5], but there are no reports on changes of bacterial gut microbiota affected by contaminants. It also is well known that pollution of the environment with metals severely can change bacterial community structure [5–8]. However, the investigation of pollutants' effects on terrestrial microbiota is complicated. The sampling, as well as the isolation, of bacterial cells or their components from the terrestrial environment (e.g., soil) has several drawbacks, such as contamination of isolated microbial components with soil and the presence of polymerase chain reaction (PCR)–inhibitory substances [9]. On the other hand, the soil bacterial community can be influenced by several factors, such as wet-dry cycles, patchiness of pollution, temperature changes, animal and plant activity, and organic matter content, which are not related exclusively to pollution.

Because of the low culturability of environmental bacteria [7,10], analyses of the bacterial community structure only recently have become acceptable for scientists, that is, after molecular methods were developed. Consequently, today the use of microbial communities to ascertain the impact caused by anthropogenic stress in natural habitats is increasing [7]. The techniques in molecular biology permit characterization of bacterial community structures via 16S ribosomal RNA (16S rRNA) gene cloning and sequencing [11,12]. Denaturing gradient gel electrophoresis and temporal temperature gradient gel electrophoresis (TTGE) analyses of PCR partially amplified 16S rRNA genes provide information on the gross community structure [11,13]. The number of fragments in a denaturing gradient gel electrophoresis or TTGE profile yields a picture of the genetic structure of the community as a whole. Cloning of 16S rRNA genes shows a more detailed picture of genetic diversity. Although Wintzingerode et al. [9] and Ranjard et al. [14] reported several problems concerning 16S rRNA clone library survey, such as time-consuming analysis, presence of several 16S rRNA operons in bacteria, and PCR inhibition bias, this approach is one of the most comprehensive available today.

One of the main problems of the bacterial community investigations is fast responsiveness of bacteria to several environmental physicochemical factors. These environmental factors are rather noise-sensitive. This makes it difficult for the researchers to interpret the results of investigations of contaminations' effects on bacterial communities in the temporal and spatial scales.

According to problems concerning the soil and water sampling and changes of bacterial microbiota, we investigated the bacterial gut microbiota of soil invertebrates living in polluted and unpolluted environments. The microbiota of the animal

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gut, its interaction with animals, and physiology are highly complex, but it is expected to be less subjected to the variation affected by environmental abiotic conditions (moisture, temperature, pH, etc.). Moreover, the animal gut microbiota mostly is changed only in the range of the animal's homeostatic response. Also, the animal-bacterial interactions and the bacterial impact on the host organism are significant considering termites, other arthropods, and mammals, including humans [15,16].

As a model organism, we used the terrestrial isopod *Porcellio scaber* (Crustacea), in which we investigated bacterial gut community structure response to pollution. The microbiota of the *P. scaber* gut has been described in several recent publications [17–20], and it is strongly suggested that it consists of normal autochthonous and allochthonous microorganisms [21].

Porcellio scaber is detritivorous and involved in the fragmentation of plant material and the bacterial inoculation of partially digested food particles. This leads to a higher rate of detritus mineralization due to bacterial activity [21–28]. Therefore *P. scaber* is involved in soil formation and energy and matter cycling in terrestrial ecosystems. According to our knowledge, until now there were no reports of pollutants' effects on the bacterial community structure of invertebrate guts examined with molecular tools.

The main aim of our work was to investigate the effects of metal pollution on the structure of the gut microbiota in chronically and acutely exposed *P. scaber* by the TTGE analysis. We also compared gut microbiota with soil and food samples collected in the closest vicinity of animal colonies. In addition, we investigated chronically exposed animal gut microbiota by clone library construction based on PCR partially amplified 16S rRNA eubacterial genes. Moreover, two additional aims were defined. The first goal was to determine the use of the analysis of animal gut bacterial community structure as a measure of the effects caused by long- or short-term pollution; the second goal was to assess the changes in the structure of the gut microbiota as a measure of pollution-induced community tolerance in chronically polluted environments.

In our research, we exposed two hypotheses. First, if gut bacterial microbiota is adapted to elevated metal concentrations in chronically exposed environments, then the gut microbiota of animals collected in polluted and unpolluted environments is expected to be similar when determined by TTGE, but not when clone library is compared. Second, contrary, acute changes of metal contamination significantly will affect animal bacterial gut microbiota when assessed by TTGE because it is not adapted to acute metal burden.

MATERIALS AND METHODS

Animals

Environmental samples. In 2003 and 2004, animals (*Porcellio scaber*, Latreille 1804) were collected in mercury (Idrija) and lead-, cadmium-, and zinc-polluted (Zerjav) areas as well as in two unpolluted areas (Radlek and Bovec) in Slovenia. At the sampling site in Idrija (more than 500 years of mercury mining), total Hg levels were up to 254 μg/g dry soil [29]. In Zerjav (more than 170 years of lead mining and smelting), up to 588 μg/g of Pb, 144 μg/g of Zn, and 5.27 μg/g of Cd were measured [30,31]. The emission of SO_2 into the air also was high due to the past Zerjav lead smelting activity [31]. From each location, a group of 10 animals was brought into the laboratory. Before dissection, their sex, weight, and molt stage were determined as described by Zidar et al. [32]. Another group of 10 animals from each location were food deprived for 24 h in order to empty guts before being dissected. Animals mostly are present in large colonies in Slovenia; therefore, soil and food (decaying leaves) were sampled from the same sites where the animals were collected. Food and soil samples were stored in cooled containers and brought into laboratory where they were frozen (−80°C) immediately until further processing. All samples were used for whole DNA isolation.

Laboratory animals. The animals for the acute Hg exposure were collected from the unpolluted area in Radlek. Decayed hazelnut tree (*Corylus avellana*) leaves and soil were collected on the same site to serve as food and substrate, respectively. The animals were kept in a terrarium for two weeks to molt and acclimatize. After two weeks, two groups of 15 animals were taken directly from the acclimatization culture. One of these groups was used for the isolation of DNA from the full guts; the other group was allowed to empty their guts (24 h of food deprivation) prior to DNA isolation (Fig. 1). The rest of the animals in the acclimatization culture were used in the laboratory mercury-exposure experiment that was performed after the 14-d acclimatization.

Laboratory experiment

Four groups of 15 animals from the acclimatization culture were used for the Hg exposure experiment that lasted 14 d. Two groups of 15 animals were exposed to a mercury ($HgCl_2$, Merck, Darmstadt, Germany, guaranteed reagent for analysis [99.5%]) concentration of 5 μg/g dry weight of hazelnut tree leaves; the other two groups were controls receiving leaves treated only with distilled water. Animals were kept individually. Every second day the amount of feces produced by each animal was determined, the food and the fecal pellets were removed, and freshly prepared food was given. Collected feces 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.

Animals were dissected as described before [33]. Guts were kept in phosphate buffer at pH 8 and frozen at −20°C until DNA isolation. The whole experimental setup is shown in Figure 1.

DNA isolation

In a preliminary study, we compared three different methods of DNA isolation: Enzymatic lysozyme lysis, freeze-thawing, and bead-beating procedure. With the bead-beating method, we isolated the highest amount of DNA and we used this method in all further experiments. The whole DNA isolation was performed on composite samples of 10 to 15 guts, 500 mg of soil, and 500 mg of food samples. The samples were put in phosphate buffer pH 8 in Eppendorf tubes. The samples of feces and leaves, as well as food environmental samples, were ground manually with a pestle. We added 350 μl sodium dodecyl sulphate lysis solution (10% sodium dodecyl sulphate, 100 mM NaCl, 500 mM Tris-[2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride] pH 8), 375 μl phenol:chloroform:isoamyl alcohol (25:24:1) and 375-mg glass beads (Sigma-Aldrich, St. Louis, MO, USA; 75–150 μm, 106 μm average radius). Samples were shaken at 26.6 Hz for 2 min (bead beating). After centrifugation at 12,000 *g* for 5 min, the water phase was transferred into a new Eppendorf tube. The

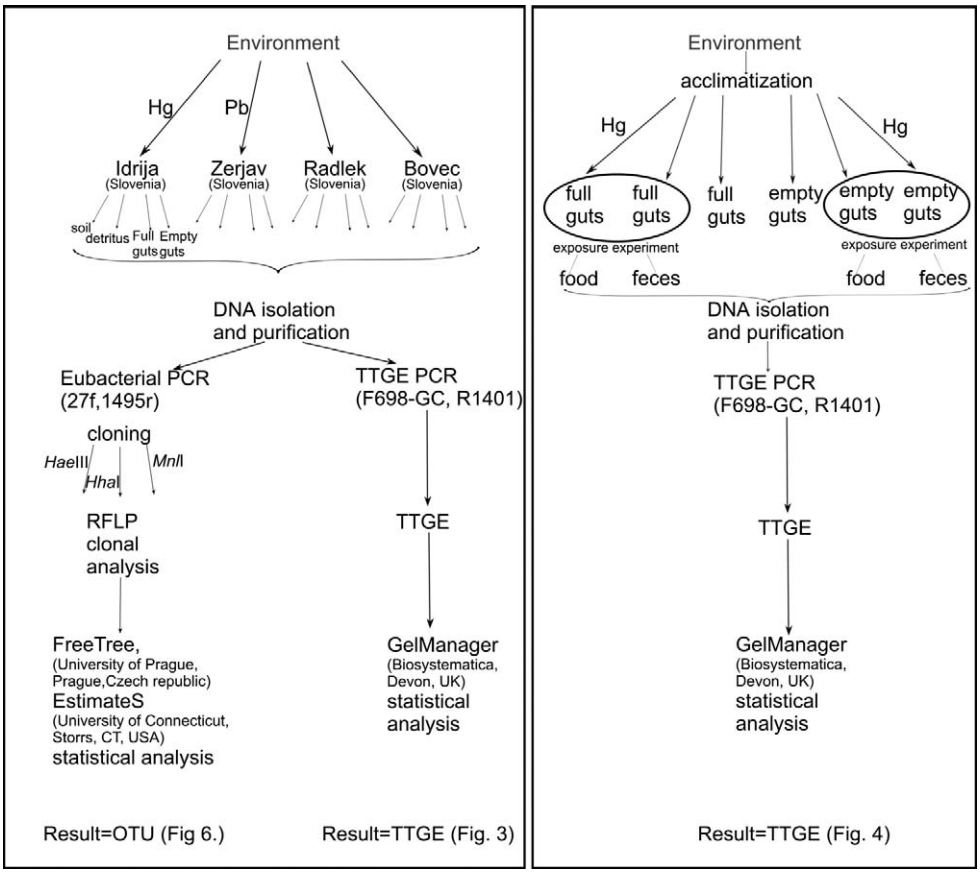

Fig. 1. Scheme of the experimental setup for the analysis of the bacterial community in the guts of chronically (left) and acutely (right) Hg-exposed isopods (*Porcellio scaber*). TTGE = temporal temperature gradient gel electrophoresis; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; OTU = operational taxonomic units.

phenol:chloroform:isoamyl alcohol was replaced and new sodium dodecyl sulphate lysis 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. [34], and put together to obtain one DNA sample. The DNA was cleaned through previously prepared sephadex G200 spun columns (Sigma-Aldrich, St. Louis, MO, USA) [35].

Polymerase chain reaction

We used two sorts of primers and conditions for partial amplification of 16S rRNA eubacterial genes. For TTGE analysis, we used U968-GC and L1401 primer pairs [36]. The amplification mixture and conditions were 1.5 μl of DNA sample amplified in a reaction mixture that consisted of 1 X reaction buffer (PerkinElmer, 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 (PerkinElmer, Warrington, UK). Amplification was performed on a GeneAmp 2400 thermal cycler (PerkinElmer, Norwalk, CT, 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, 1 min extension at 68°C, and a final extension step at 72°C for 7 min. Amplification products were separated by 1.5% agarose gel electrophoresis, and fragments of an expected length, approximately 700 base pairs, were cut from the gel and purified.

For the clone library construction, we amplified with PCR nearly the whole eubacterial 16S rRNA gene using 27f and 1495r primer pairs and conditions as described by Bianciotto et al. [37].

For both PCR amplifications, we used two separate PCRs for each DNA sample to minimize bias of the PCR amplification.

Temporal temperature gradient gel electrophoresis

We used the Dcode universal mutation detection system (Biorad, Paris, France) for sequence-specific separation of PCR products. Electrophoresis was performed through 1-mm thick and 16- × 16-cm polyacrylamide gels (8% 37.5:1 acrylamide: *N,N′* methylene bisacrylamide, 8 M urea, 125x TAE [Tris, acetic acid, ethylenediaminetetra-acetic acid buffer] buffer, 40 μl TEMED [*N,N,N′,N′*-Tetramethyl-1-,2-diaminomethane], and 400 μl 10% ammonium persulfate in 7 L of 125x TAE electrophoresis buffer). Electrophoresis was run 20 h at a constant voltage of 70 V in a temperature gradient from 54 to 68°C and ramp rate of 0.7°C/h. Gel was loaded with 200 μg of PCR-amplified DNA mixed with loading buffer (0.05% bromophenol blue, 0.05% xylene cyanolm, and 70% glycerol). Gels were stained by submersion into an ethidium bromide solution (1 mg/ml ethidium bromide in 125x TAE) for 20 min and destained for 5 min.

Clone library construction

We constructed two clone libraries from the 16S rRNA gene PCR products, which were amplified according to the eubacterial-specific binding of the 27f and 1495r primer pair (Fig.

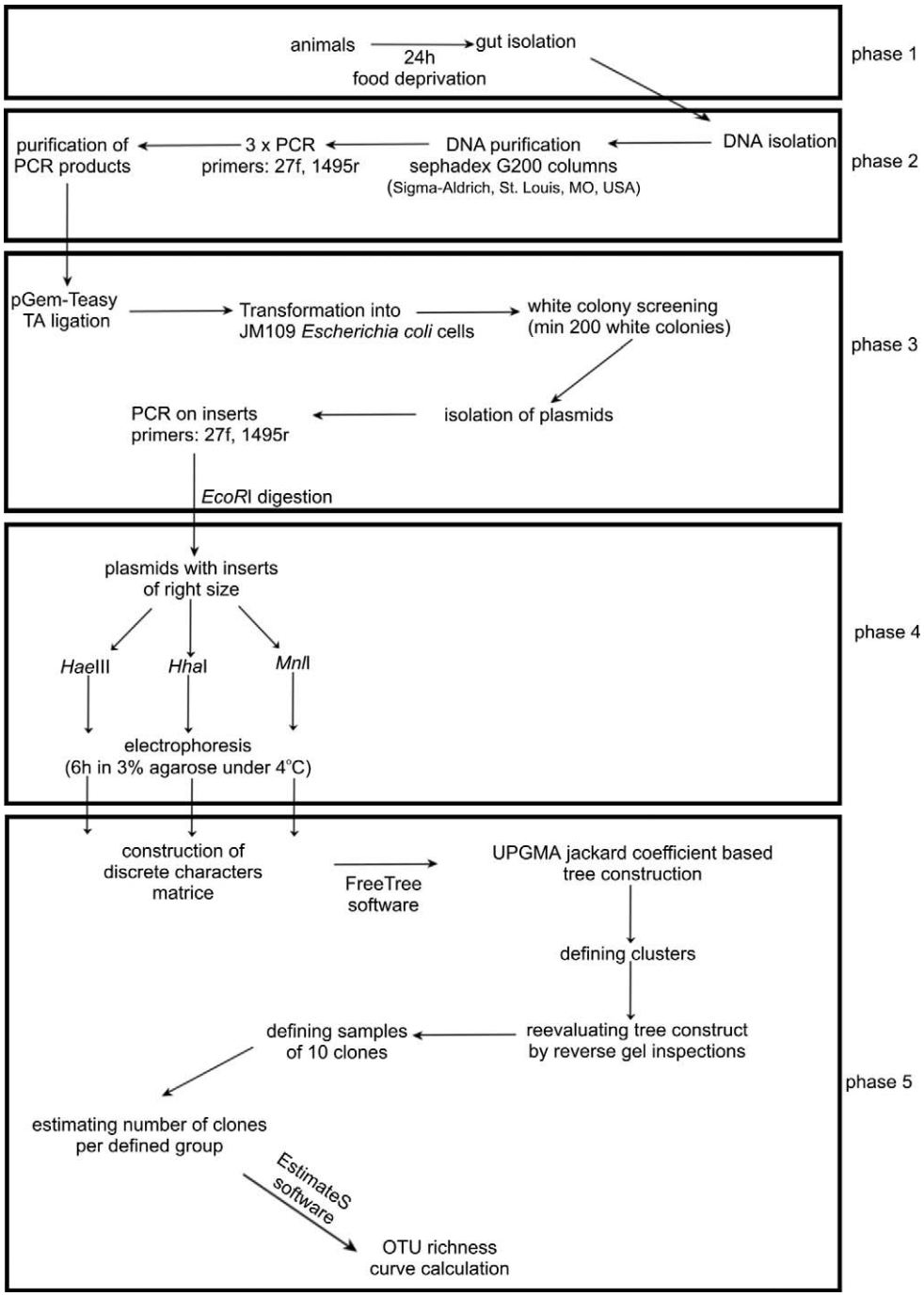

Fig. 2. Scheme of the detailed analysis of the isopod gut bacterial community by clone library construction and its analysis. PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; OTU = operational taxonomic units; UPGMA = unweighted pair group method with arithmetic mean.

2). For the PCR amplification, we used DNA samples isolated from the 10 empty guts of animals collected from the Hg-polluted (Idrija) and one unpolluted site (Radlek). Products of PCR amplifications with primer pair 27f and 1495r were cloned into the pGEM-T easy plasmid and transformed into *Escherichia coli* JM109 cells (Promega, Madison, WI, USA), as described in the Promega manual. Approximately 200 clones were obtained. Plasmids with inserts were isolated, resuspended in 50 μl sterile distilled water, and diluted 100 times. The inserts were PCR reamplified with the same primer pairs (27f and 1495r). On the PCR-amplified products, restriction fragment length polymorphism analysis was performed. The PCR products were digested using the restriction enzymes *Hae*III, *Mnl*I, and *Hha*I (New England Biolabs, Beverly, MA, USA and Fermentas, ON, Canada), each separately. Products were applied to a 3% agarose gel, separated by electrophoresis at 70 V for 6 h at 4°C, stained with ethydium bromide, and visualized. Sizes of the bands were determined by comparison of their positions with those of the bands of the 100 base pair marker (Invitrogen, Carlsbad, CA, USA) on the agarose gels.

Statistical analysis

TTGE analysis. The TTGE data were analyzed using the GelManager 1.5 software package (Biosystematica, Devon,

UK). We used unweighted pair group method with arithmetic mean clustering for tree construction based on the matrix of the Pearson product moment coefficient of the similarities of the patterns.

Clustering of the Pearson product moment coefficients was used for comparison of the bacterial communities. Relative positions as well as intensities of the bands, when compared to other bands on the TTGE gels, were used in the calculations. The Pearson moment coefficients at values above 0.8 were taken as the borderline for delineation of major clusters. The TTGE profiles showed that the majority of the most distinctive bands in TTGE profiles are present at the same positions at coefficient 0.8. We described as the most similar TTGE profiles those at Pearson moment coefficients larger than 0.95. Coefficients between borderlines were used for descriptions of the similarities and dissimilarities among subclusters. It has to be stressed that each band represents a population of similar and not necessarily identical nucleotide sequences of partially amplified 16S rRNA genes.

Restriction fragment length polymorphism analysis of the clone libraries. Each band in the agarose gels was assigned with a discrete number according to its position on the gel. The GelProAnalyzer software (Media Cybernetics, Silver Spring, MD, USA) determined the sizes and positions of the bands. According to the presence or absence of the bands at the defined positions, matrices of discrete (0/1) characters were constructed and put into the FreeTree program [38]. For the calculation of similarities between different patterns, we used Jackard similarity index and unweighted pair group method with arithmetic mean tree construction method. Each cluster in the tree was assigned a different operational taxonomic unit (OTU) number and checked for the right clustering by gel inspection. Each restriction fragment length polymorphism result of 20 subsamples per 10 clones of the clone library was put into the EstimateS 6b1a software ([39]; http://viceroy.eeb.uconn.edu/EstimateS7Pages/EstS7UsersGuide/EstimateS7UsersGuide.htm#AppendixC) and Chao2 accumulation curves with corresponding standard errors were calculated.

RESULTS

Environmental samples

Environmental samples did not show clear effects of the pollution on bacterial gut microbiota nor to the soil or food samples. However, TTGE analysis showed a major difference in eubacterial 16S rRNA community profiles between detritus (food) and soil samples on one hand and full and empty isopod guts on the other (Fig. 3).

The soil and food eubacterial TTGE profiles were clustered with Pearson product moment coefficient of 0.83 (Fig. 3). In this cluster, the TTGE profiles of bacterial community present in soil and food samples from the reference sites Radlek and Bovec differed from each other. Also, TTGE profiles of the bacterial community of two samples of soil and food from the Hg (Idrija) and Pb-, Cd-, Zn- (Zerjav) polluted sites were different from each other. The TTGE profiles of the bacterial community present in the soil from the Hg-polluted site Idrija were grouped with the Radlek samples, but TTGE profile of bacterial microbiota in the detritus (food) collected in the mercury-contaminated site was grouped with Bovec samples (Fig. 3). The TTGE bacterial community profiles of food and soil samples collected from the two reference sites and the Pb-, Cd-, Zn-polluted site (Zerjav) were similar to each other, which is shown as clustering into one group with Pearson product moment coefficients of 0.92. The TTGE bacterial community profiles of the two samples from Zerjav were separated into the subcluster from the other soil and detritus samples in this cluster.

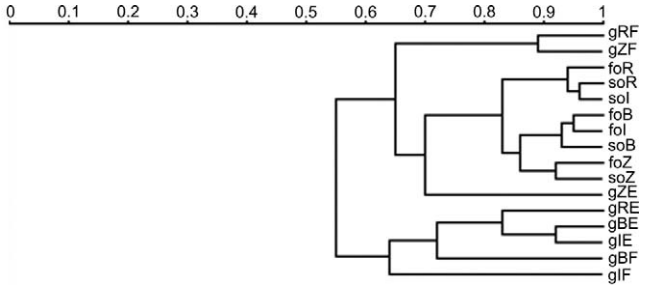

Fig. 3. Dendrogram based on Pearson product moment coefficients calculated from temporal temperature gradient gel electrophoresis (TTGE) profiles of 16S rDNA fragments from the predominant bacterial populations from *Porcellio scaber* guts (g), soil (so) collected at the animal collecting sites, and detritus (fo) as potential food source. All samples are collected in polluted and unpolluted areas in Slovenia. E = empty guts; F = full guts; I = Idrija (mercury-polluted area); Z = Zerjav (lead-polluted area); R = Radlek (unpolluted area I); B = Bovec (unpolluted area II).

Two clusters of TTGE eubacterial profiles of full and empty guts of animals from the four locations were observed (Fig. 3). The majority of full and empty gut TTGE eubacterial profiles were grouped in one cluster. Differences existed in TTGE eubacterial profiles between full and empty guts of animals from all four locations. Empty gut TTGE eubacterial profiles of animals from two reference locations and the Hg-polluted location were grouped into one subcluster. The TTGE eubacterial 16S rRNA profiles of empty guts collected from a lead-polluted environment (Zerjav) differed from the other samples.

Laboratory experiment

Measured feces production according to the number of fecal pellets per day of the laboratory-exposed animals did not show any statistical differences between Hg-exposed and nonexposed animals, and no other signs of toxic effects of Hg exposure were seen (data not shown). Furthermore, we were interested in changes in the bacterial community structure between laboratory mercury–exposed and nonexposed animals.

In general, the Pearson product moment coefficients were lower in field samples of animals and feces (0.55) than in laboratory samples (0.66). We explain this by the fact that animals for laboratory experiments came from the same population. Therefore direct comparison between TTGE gels of field and laboratory samples is not possible. In our study we compared field samples among each other and laboratory samples among each other.

In laboratory samples, two large clusters and two separated branches were observed. The separated branches represent bacterial communities in the full guts of the animals from the control animals, the Hg-exposed animals, and the laboratory-acclimatized animals. Two large clusters of similar bacterial communities represent where one is composed of empty guts from control nonexposed animals and from acclimatization culture, and another cluster is composed of fecal samples of all animals and full and empty guts from Hg-exposed animals.

The TTGE eubacterial profiles of empty guts of *P. scaber* exposed to the 5 μg/g Hg were significantly different from those of control and laboratory-acclimatized animals (Fig. 4).

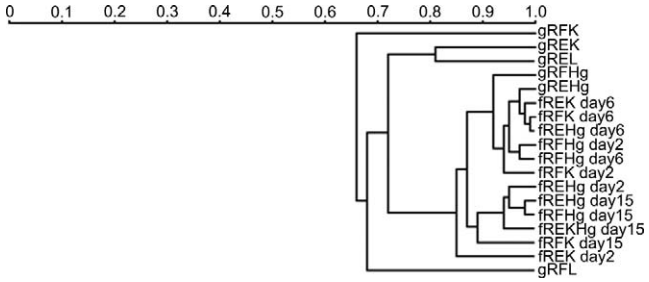

Fig. 4. Dendrogram based on Pearson product moment coefficients calculated from temporal temperature gradient gel electrophoresis (TTGE) profiles of 16S ribosomal RNA fragments from the predominant bacterial populations from *Porcellio scaber* guts (g) collected from the unpolluted area (Radlek, Slovenia [R]) and their feces (f) 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 d; K = guts from the control groups of animals from the exposure experiment.

Very similar TTGE profiles were observed between empty guts of laboratory-acclimatized animals and a control group in the Hg-exposure experiment that clustered together according to the Pearson product moment coefficients. Full guts of both control groups differed from each other, although they shared several major bands in the TTGE profiles (Fig. 5).

Eubacterial TTGE profiles of feces showed no major differences between control and Hg-exposed animals. Analysis by the GelManager software, however, did separate the group of TTGE profiles of the last sampling interval of the 14-d experiment. In this group, the TTGE eubacterial profiles of

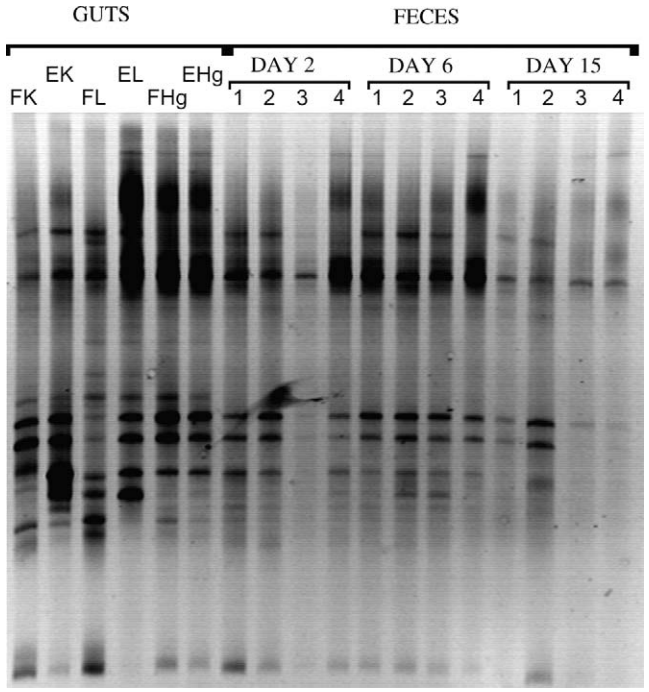

Fig. 5. Example of the temporal temperature gradient gel electrophoresis (TTGE) profiles of the 16S ribosomal RNA polymerase chain reaction (PCR) amplified genes of the gut and fecal samples from the laboratory experiment with *Porcellio scaber* (F = full guts; E = empty guts; L = animals from acclimatization culture; K = control groups of animals from laboratory experiment; Hg = mercury-exposed [5 μg Hg/g dry food] animals). Numbers 1 and 2 indicate TTGE bacterial community profiles of fecal samples of control animals; numbers 3 and 4 are profiles of fecal samples of Hg-exposed animals.

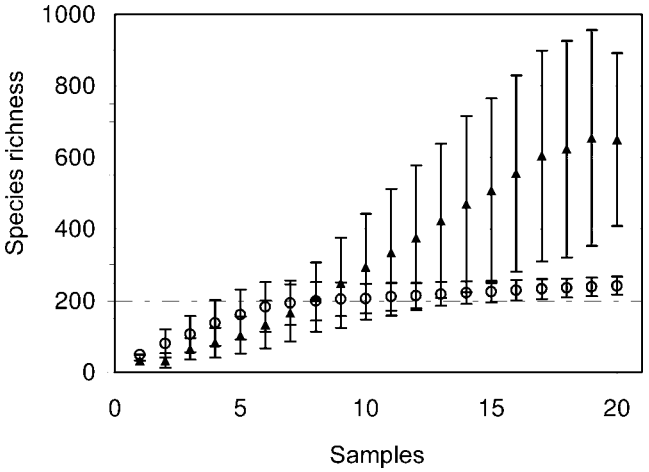

Fig. 6. Cumulative bacterial operational taxonomic units (OTU) richness curves for the bacterial microbiota in the *Porcellio scaber* guts calculated on the basis of Chao2 estimate parameter. Error bars represent the Chao2 standard deviation for each subsample. Filled triangles represent bacterial OTU richness from animals collected in the reference (Radlek, Slovenia) area; circles show values for animals from the Hg-polluted region (Idrija, Slovenia).

feces from Hg-exposed animals grouped closer together than those of the two control groups. The TTGE profiles of feces from other sampling intervals showed one separated cluster, regardless of whether the feces samples were produced by control animals or Hg-exposed animals (Fig. 5).

The TTGE profiles of food samples from Hg-exposed or control groups showed no differences during the 14-d exposure period (data not shown). Additional investigations, like restriction fragment length polymorphism analysis, were not performed because the TTGE 16S rRNA profiles already showed a clear distinction between normal autochthonous bacterial communities in the empty guts from the mercury-exposed and nonexposed animals. This was not the case when animals from chronically polluted environments were investigated. Therefore, in the latter, a more detailed bacterial community analysis was needed.

OTU richness estimation

Comparison of the TTGE eubacterial 16S rRNA community profiles of the emptied gut microbiota did not show major differences between food-deprived *P. scaber* from the polluted Idrija environment and animals collected from the reference site Radlek. Because of that, a more detailed analysis of the emptied gut bacterial community structure was conducted. Investigation by 16S rRNA clone library construction showed a lower bacterial OTU in the emptied guts of animals collected from the Hg-polluted environment compared to the reference site Radlek. According to our results, the minimal clone number in the clone library should be more than 150 to observe differences in species richness between two samples (Fig. 6).

The cumulative gut bacterial community structure of *P. scaber* from the unpolluted area (Radlek) showed a sigmoid curve with a higher OTU richness (650.6 OTU) than in animals from the Hg-polluted area (242.4 OTU). The cumulative gut bacterial community structure of animals collected from the Hg-polluted site (Idrija) showed a logarithmic shape where the steep beginning of the curve changes to a gentle rise already after analyzing 90 clones (Fig. 6). The curve describing the gut bacterial community structure of animals collected from

the reference site has a sigmoid shape. The maximum species richness in samples from Radlek was reached after analyzing 150 to 200 clones.

DISCUSSION

Our results showed that TTGE could not distinguish among differences of microbiota from soil, food, or the terrestrial isopod *P. scaber* guts from pristine versus chronically contaminated locations. The OT units showed differences between the chronic Hg-contaminated and one of the noncontaminated sites, when comparing the pooled sample of 10 animals. When the acute exposure experiment was performed on animals from pristine environment, TTGE apparently showed differences in normal microbiota communities present in the gut of isopods, but not in those from fecal samples.

In our study, culture-independent methods were employed to assess long- and short-term effects of elevated concentrations of metals on the microbial community in isopod guts. Earlier, conventional microbiological methods were used to study the isopod gut microbiota as endpoint in laboratory toxicity studies [33]. Results of the culture-independent methods based on 16S rRNA genes provide a more comprehensive view on bacterial community changes [10].

The bacterial microbiota present in the gut of animals is defined as indigenous and consists of autochthonous (real symbionts), normal, and pathogenic bacteria [40]. Several assumptions have been made about the isopods digestive system as a suitable environment for symbiotic as well as pathogenic bacteria [20,21]. However, it was suggested that, in arthropod-bacteria interactions, representatives of normal bacterial microbiota might be even more important than symbiotic ones [41,42]. In our work, we interpret the gut bacterial community in empty guts as normal autochthonous, although in full guts the normal allochthonous microbiota is prevailing.

When TTGE profiling was conducted on the gut microbial community of animals from a chronically polluted environment, the major representatives of the normal autochthonous microbiota were not affected (Fig. 3: gRE [empty guts of animals from Radlek], gBE [empty guts of animals from Bovec], and gIE [empty guts of animals from Idrija] branches). However, when the species richness was investigated further by clone library construction, bacterial species richness was lower in the gut of *P. scaber* collected from the chronic mercury-polluted area (Idrija) than from the reference site Radlek (Fig. 6). Further, after acute Hg exposure, TTGE profiling indicated significant alterations in the gut bacterial community (Fig. 4: REKg, RELg vs. REHg branches, where REKg, REL, and REHg represent empty guts of animals collected from Radlek and used in the laboratory-exposure experiment as a control group of animals, acclimatized group of animals, and mercury-exposed group of animals, respectively). The disappearance of major representative members and/or a reduced relative presence were detected at much lower concentrations of Hg (5 μg/g Hg) than those measured in Idrija by Gnamuš and Horvat [29] (Fig. 5). We speculated that, in animals from a chronically polluted environment (500 years of Hg pollution), TTGE profiles were similar to those in animals from an unpolluted environment, probably due to adaptation of major bacterial representatives mostly by acquisition of *mer* genes [43] or other genes that help them detoxificate elevated metal concentrations (data not shown). As expected, acute laboratory Hg exposure of the isopods also affected major representatives of the gut bacterial community (Fig. 5: REL, REK lines compared to REHg). On the basis of our results, we conclude that TTGE profiling method is sensitive enough to detect changes in acutely stressed bacterial communities.

The similarity of the TTGE patterns of the emptied gut microbiota from different populations collected from geographically separated locations (polluted and unpolluted) indirectly leads us to think about the presence of the normal autochtonous microbiota in the *P. scaber* gut.

On the contrary, the high variability and no correlation with metal contamination of bacterial microbiota TTGE patterns of the full guts of animals collected from different locations (polluted and unpolluted) showed that the prevailing bacterial microbiota in the full guts is introduced with food particles (Fig. 3). Also, the TTGE profiles of the 16S rRNA eubacterial genes from food as well as from soil were different from the full gut. The environmental soil or food samples are exposed to differential environmental conditions, which affect the bacterial community structure [3,9,44,45].

The emptied gut microbiota consequently is less variable and not directly affected by changing environmental conditions. The normal autochthonous isopod gut microbiota in emptied guts corresponded with the environmental metal concentrations. For example, in Idrija, mercury is present constantly at elevated concentrations (less variable environment) and the presence of major representatives of the gut bacterial community was not different from that in animals collected in unpolluted environments (Fig. 3: gIE [Idrija], gRE [Radlek], gBE [Bovec] compared to gZE [Zerjav]). However, the detailed analysis showed that the operational taxonomic units are less numerous in animals from the chronically polluted environment (Fig. 6). Additionally, in Zerjav (Fig. 3: gZE), several metals are present and their concentrations fluctuated with time, making the environment highly variable. We speculate that the highly variable environment, as well as the presence of several different metals in elevated concentrations, affected the gut microbiota to such an extent that it was detected by the TTGE method (Fig. 3: gZE branch). The environmental metal concentrations were not significantly different during the isopods' life span. Animals also did not move out of the contaminated sites to the clean areas because of patchy distribution of *P. scaber* colonies and large areas of polluted sites (Žerjav more than 304 km^2; Idrija more than 293 km^2). The average life span of the *P. scaber* is up to five years [46], which is too short to experience different environmental metal concentrations. However, the normal bacterial microbiota of *P. scaber* is not built up with the proctodeal trophallaxis as observed in termites [47] and consequently the normal autochthonous microbiota is not transferred from one generation to the next. Accordingly, it is expected that the normal microbiota of the *P. scaber* is obtained from the consumed food, and the bacterial strains are selected in accordance with the gut internal environmental conditions. Therefore, different bacterial strains with the same functional characteristics belonging even to different bacterial species might fulfill the isopods' requests for the functional normal microbiota. Moreover, if the environmental metal concentrations change at the larger timescales, the future generations may consume bacterial communities different from previous generations. Thus, the normal autochthonous microbiota is expected to be different. The bacterial analyses of empty guts are always more reliable than that of full guts, because bacterial microbiota present in the food also is analyzed in the full gut.

The TTGE bacterial microbiota profiling is a rather rough

estimate of the whole bacterial community [14]. The bands observed in the TTGE gels demonstrate only the presence of the major representatives of the bacterial community. The TTGE method itself is relatively simple and is one of the most rapid profiling methods that may show major changes of the microbial community [48]. Additionally, the observed TTGE bands presenting major representatives of the bacterial community might be extracted for further determination of the DNA origin. The emptied gut microbiota also was compared on the basis of 16S rRNA gene by clone library construction. The clone library construction provides a more detailed analysis than in TTGE profiling [49]. By TTGE methodology we could observe only major representatives mostly, whereas by 16S rRNA clone libraries we also could detect minor-underrepresented groups of bacteria. Therefore, higher differences in bacterial OTU richness were observed when we used clone libraries (comparing Figs. 3 and 6). Contrary, the results of TTGE separation did not reveal such differences in emptied gut bacterial microbiota because it was observed by clone library analysis. In the animals from a stable environment (chronic pollution or clean environment), the major representatives (observed by TTGE) of the normal autochthonous bacterial microbiota were preserved (Fig. 3: gRE, gBE, gIE). This is not the case when the animals acutely were exposed to elevated Hg concentrations (Figs. 4 and 5). As a proof of changes of the community, several authors reported the disappearance of different major groups of bacterial representatives upon acute elevated mercury exposure in, e.g., soil [3,50]. However, after long-term mercury exposure, the bacterial community in the soil was restored, which has been proven by rapid profiling methodology, e.g., denaturing gradient gel electrophoresis or TTGE [4,7].

The rarefaction curves of 16S rRNA gene clone libraries of bacterial gut microbiota became well separated after the 15th sample (see Fig. 6). That means that one needs to make the restriction analysis on more than 150 clones to detect the differences between OTU richness in bacterial emptied gut microbiota between animals from chronically polluted and clean environments (Fig. 6).

Changes in the microbial community in the environment might affect the isopods' performance. For example, isopods can detect food by the smell of the microbial components [51–54]. Food choice in a polluted environment could affect the uptake of bacteria and subsequently the distribution of normal allochthonous and autochthonous gut bacteria. Moreover, changes in the animal–microbial interaction might have even more widespread effects. Because isopods are important decomposers of organic matter in the environment, any changes in the animal–bacterial interactions potentially can affect the flow of energy and matter in the ecosystem. Besides that, metal-tolerant populations of *P. scaber* can be present in metal-contaminated areas [55]. The occurrence of metal-tolerant animal populations in polluted environments provides a closer look into the mechanism of metal effects. This is one of the special issues in ecotoxicological studies, because pollution-induced population tolerance is becoming an important parameter for determining effects of environmental pollution from past anthropogenic activities [56].

CONCLUSION

The results of our study showed that the bacterial gut microbiota of isopods might be used in toxicological studies of chronically and acutely polluted environments when completed with additional parameters. The methods used in the present paper (TTGE profiling and clone library) do not enable direct assessment of pollution-induced microbial community tolerance in metal-polluted terrestrial environments, but can serve as a first step before the molecular microbial functional approach is applied.

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