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ISOPOD GUT MICROFLORA PARAMETERS AS ENDPOINTS IN TOXICITY STUDIES

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Abstract—Terrestrial isopods *Porcellio scaber* (Crustacea) were fed for five weeks on food contaminated by 250, 500, or 1,000 μg of Cd/g or for 10 d on diets with 50 or 250 μg Cd/g food. In both experiments, fecal production rate and colony forming units (CFUs) in the guts were determined. In addition, at the end of 10 d, each distinct colony morphotype obtained in gut samples was purified and characterized. Isolates were separated into 25 groups based on morphological and biochemical characteristics. These bacterial groups were used as units for calculating Shannon equitability indices (*J*) for each gut. The relative frequencies of the 25 bacterial units were determined in both cadmium groups (50 or 250 μg Cd/g food) and in the control. Cadmium-induced perturbations observed in the gut microbial communities were (1) increased number of morphologically distinct bacterial isolates in the group fed low-cadmium-dosed food (50 μg Cd/g) and reduced number of morphologically distinct bacterial isolates in the group fed high-cadmium-dosed food (250 μg Cd/g) compared with the control, (2) increased or decreased relative frequencies of almost all 25 bacterial units provoked by cadmium-contaminated food, (3) time-dependent increased numbers of gut CFUs in cadmium-fed animals (dose dependence was not observed), and (4) significant changes in community structure described by Shannon equitability indices at lower levels of food contamination (50 μg Cd/g) only. Gut microflora parameters are proposed as additional endpoints in the standardized single-species toxicity test with the terrestrial isopod *P. scaber* as a means of increasing the ecological relevance of the results.

Keywords—Culturable gut microflora Cadmium toxicity Toxicity endpoints Shannon–Wiener equitability index *Porcellio scaber*

INTRODUCTION

Metal contamination has been shown to affect the composition of microbial communities in terrestrial and aquatic environments due to a reduction in the numbers of particular members of the community, extinction of more sensitive species, and the appearance of metal-tolerant microorganisms [1–3].

Changes in bacterial communities were observed at similar or even lower metal concentrations in the substratum than those at functional levels (e.g., changes in N and C metabolism, respiration, and so on), indicating that microbial community analyses could be a sensitive means of detecting environmental perturbations [3]. Bacterial community structure would also be less affected by environmental factors like substratum moisture content, temperature, carbon availability, pH, and so on than would bacterial functions [4].

A well-known way of describing community structure is by diversity indices. A lowering of species diversity can show community perturbations, but an unchanged species diversity index does not indicate that the community structure remains unchanged [5]. Species diversity indices have rarely been applied to microbial communities because their use requires the specification of a large number of microorganisms, which is difficult to perform to the species level. This difficulty can be avoided by using higher taxonomic ranks [6] or different phenotypic characteristics for bacterial groupings [7,8]. The number of groups and the number of individuals within each group are then used to calculate the diversity indices.

The effects of metals on soil microorganisms measured in a variety of experiments are difficult to generalize or even compare. Data presented in different papers must be interpreted with caution because of variation in soil properties (clay and organic matter content), the wide range of concentrations applied (10 to a few thousand μg Cd/g dry wt soil), and the low standardization of experimental conditions [9–12].

Additional problems with these studies include how to define precisely the community boundaries and what control to use. In an attempt to minimize the effects of external abiotic factors on microflora, we directed our research to gut bacteria of terrestrial isopods after metal-induced stress. The advantage of gut microbiota studies lies in the lower susceptibility of the microbial community and their environment to external factors. In the experiments described here, it was possible to define the boundaries of the community and optionally to vary the period during which the community was subject to stress.

The microbial community in the intestinal tract of invertebrates has been studied extensively [13]. Unique among arthropods, the isopod gut is a simple tube lined with cuticle, which is renewed at molting [14]. Many investigations have shown that terrestrial isopods have an indigenous gut microflora composed of a diversity of microorganisms, some of them attached to cuticular spines [15]. Hassal et al. [16] reported a significant increase in the number of bacteria while food was passing through the gut of *Porcellio scaber*. No direct evidence of symbiotic bacteria in the isopod gut exists; instead, isopods maintain gut homeostasis by stimulating or inhibiting some bacterial groups. This presumably contributes to optimal food degradation [17].

It has been shown that rapid and complete degradation of organic materials is achieved only by concerted action of digestive enzymes of isopods and microorganisms within the gut and feces [16]. For this reason, disturbances of the gut microbial community by environmental contaminants might have severe consequences on decomposition processes.

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The aim of this study was to determine the suitability of some parameters of gut microflora to be used as additional endpoints in toxicity studies with *P. scaber*. For the sake of repeatability, simplicity, and ease of handling, only culturable gut bacteria were investigated. Also, those morphological and biochemical characteristics of isolates that could be rapidly and reliably determined were selected. The culturable gut microflora of control *P. scaber* and those fed with cadmium-dosed food was compared with data on feces production of the host animals.

MATERIALS AND METHODS

Five-week toxicity test for cadmium

The five-week toxicity test for cadmium was conducted with *P. scaber* (Lat.) (Isopoda, Crustacea) following the protocol proposed by Drobne and Hopkin [18]. Terrestrial isopods (*P. scaber*) were collected in the vicinity of the University Campus in Rozna dolina in Ljubljana.

During the experiment, six animals were held individually in 9-cm-diameter plastic Petri dishes at 19°C to 21°C. The animals were fed with hazelnut tree leaves (*Corylus avellana*) from the collecting site.

Cadmium chloride solutions in distilled water were applied to the lower leaf surfaces as small droplets and evenly spread over the surface with a paintbrush. The amount of solution applied was adjusted to give concentrations of 50, 250, 500, or 1,000 μg Cd/g dry weight of leaf. Control leaves were treated with distilled water.

The cadmium concentrations used were chosen on the basis of literature data and our previous tests on cadmium effects on food consumption rate of *P. scaber*. Donker and Bogert [19] report 20 μg Cd/g as the low observed effect concentration for feeding of *P. scaber*, but Khalil et al. [20] observed no effect on feeding of *P. scaber* even at 562 μg Cd/g in the food. Experimental conditions most similar to ours were those in experiments performed by Zidar [21], who, using the log-logistic model proposed by Haanstra et al. [22], proposed that EC10 (concentration causing 10% effect) for feeding was 64 μg Cd/g and that EC50 (concentration causing 50% effect) was 356 μg Cd/g food. On the basis of these literature data, we chose 50 μg Cd/g as close to the no-observed-effect concentration and 250, 500, and 1,000 μg Cd/g as concentrations that affected feeding of adult *P. scaber*. The cadmium concentration in the control and in a group of cadmium-dosed leaves was analyzed by flame atomic absorption spectrometry (Varian AA-5, Walnut Creek, CA, USA). Mean concentration of cadmium in control leaves was 0.1 ± 0.05 μg Cd/g. The levels of cadmium in cadmium-dosed leaves were within 5% of the expected values.

Feeding rate was determined as grams of feces produced per gram wet body weight in five weeks. Determination of feeding rate as amount of feces produced per body weight per day is misleading because production of feces at the beginning of the experiment is much higher than at the end of it. This must be taken into account when feces production of animals in experiments with different duration is compared.

10-d toxicity test for cadmium

The second cadmium test differed from the first in the concentrations tested and the duration of the test. Animals were exposed to cadmium for 10 d under the same conditions and experimental design as in the five-week test, but the concentrations were 50 and 250 μg Cd/g dry weight of leaf. Six animals were used in each experimental group. Feeding rate was expressed in grams of feces produced per gram wet weight of animal in 10 d.

Dissection of animals and counting and isolation of gut bacteria

At the end of the experiment, the animals were placed on sterile filter paper disks for 24 h to allow gut evacuation. The guts were then aseptically extracted by holding the body with tweezers, cutting off the head and last three segments of the pleon, and pulling out the gut. Only completely empty guts were handled further. Each gut was transferred into a glass tube containing 3 ml of physiological solution, one-third filled with glass beads. Guts were macerated with sterile tweezers by pressing them against the wall of the tube and than vortexing three times for 30 s together with the glass beads. The suspension was serially diluted up to 10^{-4} times in sterile physiological solution.

Two dilutions (10^{-3} and 10^{-4}) with two replicates each were inoculated with 0.1 ml on Brain Heart Infusion agar plates (Difco, Grayson, GA, USA) and incubated for 8 d at 30°C. Each day, the CFUs were counted. Representatives of different morphological types of colonies, as judged by the colony shape, margins, pigmentation, size, and surface, were isolated in pure cultures. Bacterial isolates were grouped according to Gram staining, cell morphology, oxidative versus fermentative metabolism on oxidation–fermentation–glucose medium, colony morphology and pigmentation, motility, and the presence of catalase and oxidase [23–25]. The motility, catalase, and oxidase tests and oxidative versus fermentative metabolism were tested as described by Mac Faddin [23].

Diversity index

The Shannon–Wiener equitability index (J) was used to determine diversity of culturable gut bacteria in control and in cadmium-fed animals [26]. The index was calculated for each gut microbial community from the 10-d cadmium experiment. The Shannon–Wiener index H' is defined as $H' = -\Sigma\ (n_j/I)\ \log(n_j/I)$ [27], and equitability J is defined as $J = H'/H_{max}$ [27], where $H_{max} = \ln(S)$ and n_j/I is the proportion of the perturbed community belonging to the jth species, I is the total number of individuals in the community, and S is the total number of species.

Species here are bacterial units. The basis for the grouping was their morphological and biochemical characteristics, described in detail later in the text and presented in Table 1.

Statistical analyses

Data for feeding rate and number of colony forming units (CFUs) are presented as individual data and were not processed further. Shannon equitability indices are also presented as individual data. Nonparametric test (Kruskal–Wallis H test) was used to analyze whether the indices were significantly different from each other ($p < 0.05$) [28].

RESULTS

Effect of cadmium-dosed food on the feces production of P. scaber

The total amount of feces produced per gram wet body weight was taken as a measure of feeding activity during exposure to cadmium. In the five-week experiment, a trend of reduced feces production at a concentration of 250 μg Cd/g

Table 1. Characteristics of morphologically distinct bacterial isolates. On the basis of these characteristics, all morphologically distinct bacterial isolates were arranged into groups (= bacterial units). Number of morphologically distinct bacterial isolates in the guts of control *Porcellio scaber,* in the guts of animals fed differently cadmium-dosed food (50 and 250 μg Cd/g), and in the guts of animals from preliminary studies belonging to one of 26 bacterial units is shown[a]

Characteristics of isolates												No. morphologically distinct bacterial isolates in each bacterial unit			
Bacterial unit	Rods	Cocci	Gram stain	Oxidative metabolism[b]	Fermentative metabolism[b]	Oxidase	Catalase	Motility	Colony pigmentation	No further subcultured[c]		Control (five guts)	50 μg Cd/G (six guts)	250 μg Cd/g (four guts)	Preliminary study (nine guts)
1	+		−	+	+	−	+					6	5	0	6
2	+		−	+	+	+	+					1	1	1	1
3	+		−	−	+	+	+					0	0	2	
4	+		−	−	−	−	+					2	4	1	
5	+		−	−	−	+	+					1	8	6	6
6	+		−	+	−	−	+					1	0	0	
7	+		−	+	−	+	+					0	2	0	
8		+	+	+	+	−	+					1	3	0	2
9		+	+	+	+	+	+					0	1	0	
10		+	+	−	−	−	+					1	0	0	
11		+	+	−	−	+	+					0	2	0	0
12		+	+	+	−	−	+					0	0	1	
13	+		+	−	−	−	+		+			2	1		
14	+		+	+	−	−	+		+			0	0	1	4
15	+		+	+	+	−	+	+	y			0	3	0	
16	+		+	+	+	−	+	−	y			0	0	2	
17	+		+	−	+	−	+		+			1	0	0	
18	+		+	−	−	−	+		+			0	1	0	1
19	+		+	+	+	−	+	+	o			0	1	0	
20	+		+	+	+	−	+	−	o			1	1	0	
21	+		+			+	+		−			4	4	2	
22	+		+			−	+		−			11	8	5	14
23	+		+			−	−		−			2	1	0	
24	+		+			+	−		−			0	0	1	
25			v									2	3	6	ND[d]
											Sum	36	49	28	34
26										+		8	2	4	ND
										+	Sum	44	51	32	34

[a] + = positive reaction, − = negative reaction, v = variable, y = yellow, o = orange.
[b] Oxidation–fermentation test with glucose.
[c] Growth on the primary plates, but bacteria could not be further subcultured under established conditions.
[d] ND = not detected.

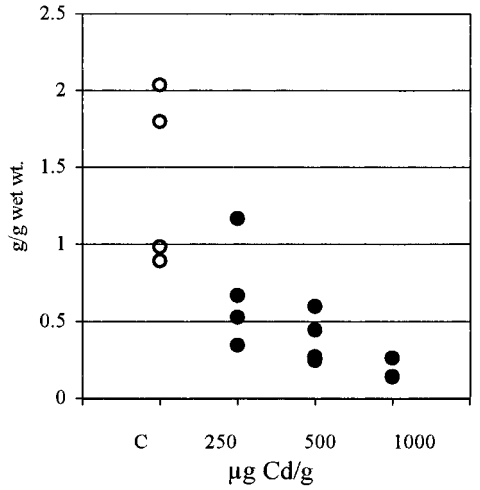

Fig. 1. Total amount of feces produced in five weeks by *Porcellio scaber*. Individual data are presented. *n* (control) = 4; *n* (250 Cd) = 4; *n* (500 Cd) = 4; *n* (1,000 Cd) = 3.

was observed (Fig. 1). However, feeding was evidently lower compared to controls when animals were fed with 500 or 1,000 µg Cd/g. Reduced feces production was observed also in the last week of exposure of control animals (results not presented). Reduction of feces production of control animals indicates a reduced state of health of animals or unfavorable conditions, such as isolation of an animal for too long, changes in food quality due to microbial activity, and so on. For that reason, the second experiment lasted only 10 d.

In the 10-d cadmium experiment, no clear differences were observed in feeding activity between controls and cadmium-fed animals (Fig. 2).

Effect of cadmium on the number of CFUs of gut bacteria of P. scaber

After five weeks of exposure to 250, 500, or 1,000 µg Cd/g food, an increase in the number of CFUs per gut was observed. No differences in the number of CFUs were observed among groups fed different cadmium concentrations in the food, indicating no dose dependence of the response during five weeks (Fig. 3). The numbers of CFUs in guts of animals from three

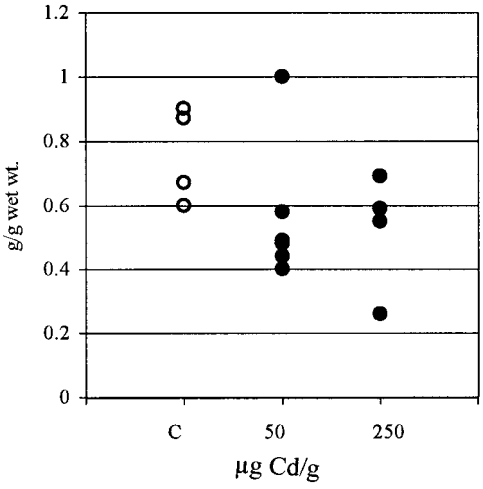

Fig. 2. Total amount of feces produced in five weeks by *Porcellio scaber*. Individual data are presented. *n* (control) = 5; *n* (50 Cd) = 6; *n* (250 Cd) = 4.

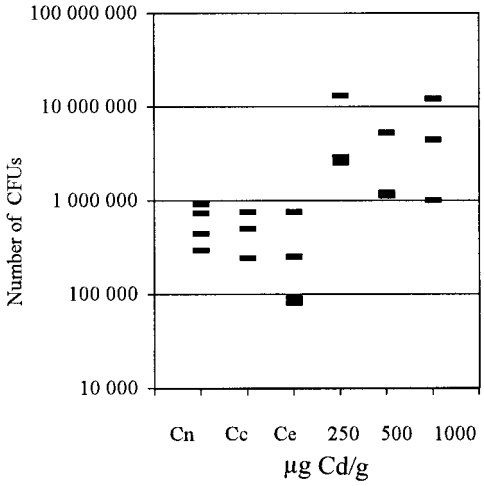

Fig. 3. Colony forming units (CFUs) per gut in five-week experiment. Bars show individual data. Cn: animals from nature, *n* = 4; Cc: animals from laboratory culture, *n* = 3; Ce: control in the experiment, *n* = 4; *n* (250 Cd) = 3; *n* (500 Cd) = 3; *n* (1,000 Cd) = 3.

different controls are presented also. These are the control animals from the experiment, animals brought from the field, and animals cultivated under laboratory conditions. No significant differences were observed in the number of CFUs between the controls.

In the 10-d cadmium experiment, no differences were observed in the number of CFUs in the guts of control and cadmium-fed animals (Fig. 4).

Description of gut bacterial isolates of control and cadmium-fed animals for 10 d

From primary plates, 127 macroscopically distinct bacterial isolates were subcultured. Isolates were arranged into 25 groups based on their morphological and biochemical characteristics (Table 1). These groups were subsequently termed ''units.'' The classification performed here enabled distinct discrimination among them. Isolates that could not be further subcultured under established laboratory conditions were taken as a noncoherent Unit 26 of bacteria and not included in calculating diversity.

In animals fed 250 µg Cd/g, the total number of morpho-

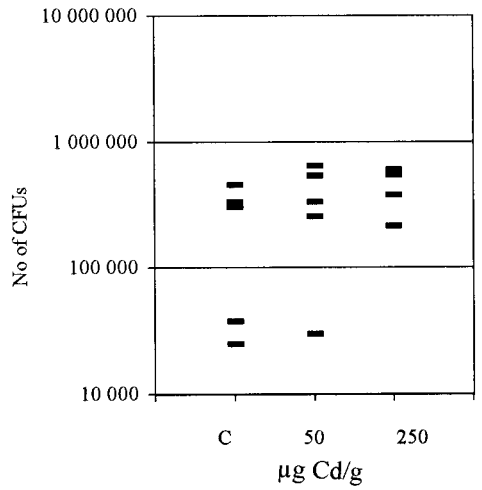

Fig. 4. Colony forming units (CFUs) per gut in 10-d experiment. Bars show individual data. *n* (control) = 5; *n* (50 Cd) = 6; *n* (250 Cd) = 4.

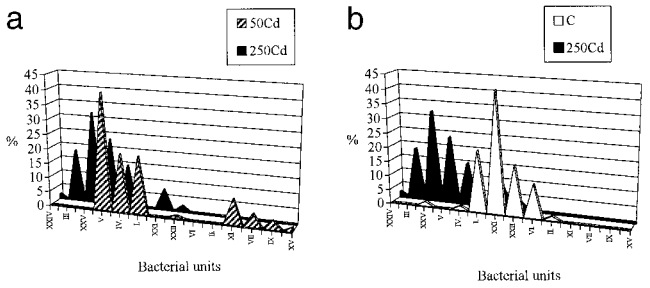

Fig. 5. (**a**) Relative frequencies of bacterial units in control and in the cadmium-fed group (250 μg Cd/g). (**b**) Relative frequencies of bacterial groups in guts of both cadmium exposed groups. Bacterial units that have relative frequencies in control and both cadmium groups lower than 1% are not presented in the plot.

logically distinct bacterial isolates was almost one-third lower than that in controls (Table 1). This reduction was especially evident in Unit 1 and Unit 22. In animals fed 50 μg Cd/g, the total number of distinct bacterial isolates was 15% higher than in the control.

For all three experimental groups of animals, relative frequencies of bacterial units were calculated also (Fig. 5a and b). The sum of CFUs in guts of each of three groups of animals (control and animals fed 50 and 250 μg Cd/g) was taken as 100%. Then relative frequencies for the 25 bacterial units were determined in each group.

It appeared that in the group fed 50 μg Cd/g, some bacterial units with relatively high frequency (Units 6, 9, 11, and 15) were not found either in controls or in animals fed 250 μg Cd/g (Fig. 5a). In the group fed 250 μg Cd/g, relative frequencies of bacterial units are clearly different compared to control guts (Fig. 5b), but not that much different from guts of animals fed 50 μg Cd/g (Fig. 5a).

Diversity of culturable gut bacteria of control and cadmium-fed animals

Shannon–Wiener equitability indices (*J*) showed that the diversity of culturable gut bacteria of animals fed with 50 μg Cd/g is statistically significantly lower than those of animals fed 250 μg Cd/g or those of control animals (Kruskal–Wallis test, $p < 0.05$; Fig. 6). Diversity of culturable gut bacteria is evidently changed only at lower cadmium concentrations in the food. At higher cadmium concentrations in the food, the diversity of culturable gut bacteria is similar to that in control guts.

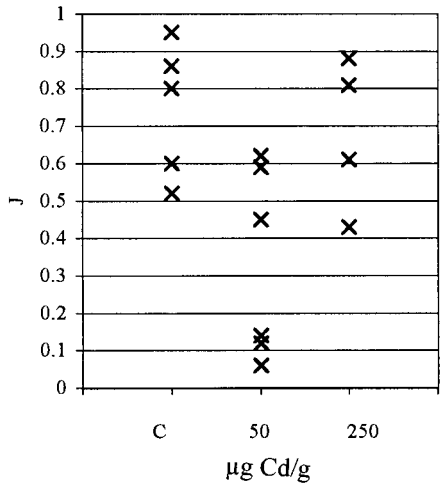

Fig. 6. Individual values of the Shannon–Wiener equitability indices (*J*) for culturable gut microflora in each experimental animal. *n* (control) = 5; *n* (50 Cd) = 6; *n* (250 Cd) = 4.

DISCUSSION

The effect of cadmium on bacterial species extinction is indicated by the reduced number of distinct bacterial isolates in the group fed with 250 μg Cd/g compared with controls and by lower relative frequencies of some bacterial units compared with the control. However, the effect of Cd on bacterial species extinction could not be generalized since even 50 μg Cd/g increased the number of morphotypes in comparison to the control.

Expansion of opportunistic and/or tolerant species is indicated by higher relative frequencies of some other bacterial units as well as by increased number of gut CFUs in cadmium-fed animals.

The lower Shannon equitability index could be a result of selective stress to the community that caused changes in community structure [27]. Significant reduction of gut bacterial diversity was observed when animals were fed 50 μg Cd/g. With increasing Cd concentration, however, diversity was similar to that of the controls. Because data on the frequency of bacterial groups showed that cadmium contamination had markedly changed the bacterial community, one could speculate that, until a new community was established, bacterial diversity was low. Later, bacterial diversity was restored. This is only a speculation because the experiment had no temporal aspect. Still, diversity indices can be used as an early warning sign of community changes but not as a measure of the degree of community perturbation. Other ecotoxicological studies lead to such conclusions as well [10].

The primary aim of our study was to couple gut microbial toxicity data with toxicity data on host organisms to increase the relevance of the test results. We found that parameters such as number of morphologically distinct bacterial isolates, number of CFUs in the guts, classification of culturable gut isolates into bacterial units based on biochemical and morphological characteristics, data on relative frequencies of these units, and data on Shannon equitability indices enable simple, fast and repeatable quantitative comparison of gut microflora between isopods dosed with different levels of metals.

Coupling toxicity data from the host with the toxicity data of its gut microflora increases the relevance of test results as more endpoints are observed and as both structural (changes in gut microflora community) and functional (changes in feces production of the host) endpoints are analyzed. The other requirements (sensitivity, robustness, reproducibility, and reliability) for a good toxicity test remain satisfactorily fulfilled [29].

Although differences in gut bacterial community between control and cadmium-fed animals were evident, nothing can be concluded about the changed structure or function of the entire gut bacterial community subjected to cadmium-induced stress and the ecological consequences that this would have. That is, culturable bacteria represent less than 0.1% of the soil bacterial community [30]. For establishing a direct ecological cause-and-effect relationship, more knowledge of the structure and functioning of the gut microflora and their relation to the external environment is needed. Still, we find that gut microflora toxicity studies are a promising way to get ecologically

relevant data on the effects of chemicals in terrestrial environments [16].

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