Changes in culturable bacterial community of soil treated with high dosages of Cu or Cd

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ABSTRACT

Culturable bacteria numbers, kinetics of colony formation, community structure analyses in terms of ecological (r/K-strategists) and cytochemical approaches were used to assess the bacteria responses to soil treatment with high concentrations of Cu or Cd over time. The soil treatment resulted in high concentrations of water-soluble forms of the metals. Bacterial numbers significantly decreased in the metal-treated soil shortly after the soil contamination, their numbers however increased during incubation time. The short- or long-term contact of bacteria with the metals significantly decreased or increased the rate of cell proliferations, respectively. The populations represented by slow-growing K-strategists dominated the structures of bacterial communities with time. The results of fatty acid methyl ester analyses indicated that Gram-positive bacteria dominated shortly after soil contamination; however, changes in the community structures with stimulation of *Pseudomonas* in the Cd-treated soil occurred after prolonged exposure to the metal. This study shows that short- or long-term exposition to heavy metals may cause different quantitative and qualitative effects on culturable bacteria in soil.

Keywords: culturable soil bacteria; r/K-strategists; bacterial fatty acid analysis; bacterial colony formation; heavy metals

The toxic effects of heavy metals on soil microorganisms are well-recognized (Giller et al. 1998). Contrasting results were however presented, considering changes in bacterial counts, biochemical activities and community structures (Giller et al. 1998, Kozdrój and Van Elsas 2001a), which result from the differences in soil types, the level of contamination and time of exposure to bioavailable concentrations of heavy metals (Giller et al. 1998, Vig et al. 2003).

In pot experiments, established concentrations of heavy metals were used in treatments, and Cu or Cd were chosen for comparison as contrasting types of metals, a micronutrient and an element with no known biological function, respectively (Kozdrój 1995, Díaz-Raviña and Bååth 2001). Consequently, the impacts of these metals on soil microorganisms are supposed to be different. In some studies, Cu did not affect total bacterial numbers (Kozdrój 1995), while other studies showed significant increases in the numbers of Cu-tolerant populations and selection of specific species (Kunito et al. 1999). The toxicity of Cu highly depended on soil properties and time of incubation that affect the amount of available forms of Cu in the soil (Kunito et al. 1999, Rajapaksha et al. 2004). In terms of Cd, low concentrations were inhibitory to bacterial populations (up to 50 mg Cd/kg; Roane and Pepper 2000); however, similar contents (41 mg Cd/kg; Renella et al. 2004) or even environmentally unrealistic concentrations were not toxic at all (1000 mg Cd/kg; Fritze et al. 2000) or toxic only for 4 weeks (5000 mg Cd/kg; Kozdrój 1995).

Physiological changes may also occur in bacterial populations exposed to heavy metals in soil as indicated by the results of kinetics of colony formation (Hattori 1985). Culturable bacteria reacted to a soil treatment with Cu or Cd entering into the quiescent phase of low metabolic activity; however, some populations increased the rate of proliferation indicating that they entered into the vegetative phase (Kozdrój 1995, 2001). These changes in the physiological state of bacteria may have also been associated with changes in community structure. Under a long-term metal stress,

the genetic structure of soil microbial community changed toward reduction of its diversity (Giller et al. 1998, Kozdrój and Van Elsas 2001a). When the community structure was expressed in terms of r- and K-strategists, domination of slow-growing K-strategists was found in a soil polluted with Cu or Cd (Kozdrój 1995). Yet, some other authors reported that fast-growing r-strategists were stimulated in polluted soil (De Leij et al. 1993). Significant changes in microbial community structures were found using a cytochemical approach by extraction of cellular fatty acids (Kelly et al. 2003, Rajapaksha et al. 2004). A culturable fraction of soil bacteria did not change in soils contaminated with heavy metals (Kozdrój and Van Elsas 2001b), whereas Ellis et al. (2002) indicated that this fraction was sensitive to the effects of heavy metals. In addition, culturable bacteria revealed to be effective indicators of pollution in soil and reflected the perturbations seen in other components of the soil biota (Ellis et al. 2002).

Short-term experiments with pots were preferred to show the reaction of bacteria to heavy metal concentrations added to soil (Giller et al. 1998). Rajapaksha et al. (2004) reported that the reaction of bacterial community might change with time. In this study, our objective was to assess responses of the culturable fraction of soil bacteria to high dosages of Cu or Cd added to a compost soil during short- and long-term exposure to the metals.

MATERIAL AND METHODS

Soil contamination. A soil enriched with compost made for plant cultivation (69.9% sand, 10.5% clay, 7% silt, 0.79 g/cm^3 bulk density, 8.7 meq/100 g cation exchange capacity, 12.6% organic matter, 0.36% total N and pH_{H2O} 6.5) was used in this study. The soil was air-dried to about 15% (v/w) moisture content, sieved (2 mm), placed in plastic containers (300 g) and wetted with distilled water to about 35% (w/w) moisture content, corresponding to about 50% (w/w) of water holding capacity. The soil portions were equilibrated at room temperature for one week prior to the treatment. The samples were treated with single dosages of 1.5 or 2.5 mg Cu/g soil, 0.5 or 1.5 mg Cd/g soil or were left untreated. There were five treatments with three replicates giving 15 pots in the experiment. The metals were added as chlorides; the salts were dissolved in 15 ml redistilled water and added dropwise to the soil, which was then mixed thoroughly with a sterile spatula. The control soil received the same volume of redistilled water followed by mixing. All pots were kept at 22°C in darkness.

Water-soluble metal concentrations and pH values were measured after soil contamination on day 1, weeks 4, 8, 12, 16 and 20. To measure the metal concentrations, soil samples (5 g) were suspended in 50 ml of redistilled water and shaken for 2 h at 180 rev/min at room temperature. After filtration, the metal concentrations were determined by atomic absorption spectrometry (Unicam 939/959). The pH values of aqueous soil extracts (1:2.5, w/v) were measured with a glass electrode by Jenway pH-meter at 20°C.

Plating analyses. After 0, 4, 8, 12, 16 and 20 weeks of exposure the total numbers of culturable bacteria were determined by the dilution plate method. Duplicate samples (5 g) collected from each container were shaken (30 min, 180 rev/min) in Erlenmeyer flasks containing 45 ml of 0.1% sterile sodium pyrophosphate (pH 7.0). Then, serial tenfold dilutions of soil suspensions were plated onto 0.1-strength tryptic soy agar (TSA, Difco) followed by incubation for 10 days at 27°C.

The colony formation of soil bacteria was estimated by the FOR model (Hattori 1985). Simultaneously, the structure of the culturable community was determined by counting bacterial colonies appearing on the 0.1-strength TSA in terms of r (rapid colony formation within 24 h) and K (colony formation after 24–48 h) strategists (De Leij et al. 1993). For this purpose, plates were incubated at 27°C for 10 days and enumerated on a daily basis for five times (days 1 to 5) and, in addition, on day 10. Several classes of culturable bacteria were thus defined per plate. To characterise the community composition, the eco-physiological (EP) index proposed by De Leij et al. (1993) was calculated. The more even was the distribution of bacterial classes observed within the community, the higher was the EP-index obtained.

Bacterial community structure by FAME analysis. After 4 and 16 weeks, the community structures of culturable soil bacteria were determined by the fatty acid methyl ester (FAME) analyses. All colonies growing on an agar plate from the dilution taken for bacterial counts, were scraped off into a reaction tube. The biomass was saponified and methylated following the procedure given for the Microbial Identification System. Saponification of lipids was conducted in a sodium hydroxide-methanol solution at 100°C for 30 min, liberating the fatty acids from cellular lipids, followed by acid methanolysis in HCl-methanol at 80°C for 10 min.

Table 1. Concentrations of water-soluble Cu or Cd (mg/g soil) in soil treated with different concentrations of the metals (as chlorides)

Weeks	Cu-treated soil (mg/g soil)			Cd-treated soil (mg/g soil)		
	control	1.5	2.5	control	0.5	1.5
0*	0.0121^{a}	0.0934^{b}	0.1287^{c}	0.0028^{a}	0.0146^{b}	0.1527^{c}
4	0.0120^{a}	0.0276^{a}	0.0723^{c}	0.0027^{a}	0.0172^{b}	0.1278^{c}
8	0.0123^{a}	0.0206^{a}	0.0698^{c}	0.0026^{a}	0.0164^{b}	0.1243^{c}
12	0.0122^{a}	0.0167^{a}	0.0676^{c}	0.0028^{a}	0.0213^{b}	0.1053^{c}
16	0.0120^{a}	0.0174^{a}	0.0476^{c}	0.0027^{a}	0.0178^{b}	0.1079^{c}
20	0.0119^{a}	0.0220^{a}	0.0345^{c}	0.0029^{a}	0.0176^{b}	0.1177^{c}

*concentrations measured one day after soil contamination
Different letters indicate significant differences between means at $P < 0.05$ level, using the Tukey's honestly significant difference (HSD) test

The fatty acid methyl esters were extracted into hexane-methyl tertiary butyl ether and washed with aqueous NaOH.

Fatty acid extracts were analysed by the gas--liquid chromatography (Hewlett-Packard 6890, USA) using capillary column Ultra 2-HP (cross--linked 5% phenyl-methyl silicone; 25 m, 0.22 mm ID; film thickness 0.33 μm) and hydrogen as the carrier gas. FAME compounds were detected by a flame ionisation detector (FID) and identified using the MIDI Microbial Identification System software (Sherlock TSBA40 method and TSBA40 library; MIDI Inc., Newark, DE, USA).

Statistics. Statistical analyses were performed using two-way ANOVA (Statistica 6.0, PL) and Tukey's honestly significant difference (HSD) test. Values were considered to be significantly different at the 95% confidence level.

RESULTS AND DISCUSSION

Metal concentrations and bacterial numbers

The concentrations of water-soluble Cu and Cd in the soil treatments are presented in Table 1. Addition of the metals to the soil resulted in pH decrease by 0.4 to 0.5 for the lower treatments and by 0.7 to 0.8 for the higher treatments of Cd and Cu, respectively, compared with the control soil (pH 6.5). The decreased pH was observed during the whole experiment (data not shown).

Culturable bacteria counts significantly decreased ($P < 0.05$) shortly after the soil treatment with the metals compared with the control; however, the significant increase ($P < 0.05$) in the numbers was found on week 4 (Table 2). Differences in the CFU counts between the treatments of respective

Table 2. Effect of soil treatment with different concentrations of Cu or Cd on numbers (log CFU/g dry soil) of culturable bacteria

Weeks	Metal-treated soil (mg/g soil)				
	control	1.5 Cu	2.5 Cu	0.5 Cd	1.5 Cd
0*	7.88^{a}	6.94^{b}	6.42^{c}	6.83^{b}	6.58^{b}
4	7.96^{a}	9.05^{c}	8.59^{d}	8.87^{d}	8.92^{d}
8	8.93^{c}	9.08^{c}	8.77^{d}	8.81^{cd}	8.91^{cd}
12	8.92^{ce}	8.95^{ce}	8.79^{de}	8.87^{cd}	7.96^{f}
16	8.10^{a}	7.60^{f}	7.71^{f}	7.81^{af}	7.97^{af}
20	7.86^{a}	7.53^{f}	7.41^{f}	7.90^{af}	7.63^{af}

*numbers determined one day after soil contamination
Means within rows and columns followed by different letter are significantly different at $P < 0.05$ level, using the Tukey's honestly significant difference (HSD) test

Table 3. Changes in the kinetics of colony formation by culturable bacteria in soil treated with different concentrations of Cu or Cd

Weeks	Parameter	Metal-treated soil (mg/g soil)				
		control	1.5 Cu	2.5 Cu	0.5 Cd	1.5 Cd
4	N_{inf}	75.16^{a}	72.72^{a}	20.61^{b}	70.17^{ac}	25.04^{b}
	λ	0.95^{a}	0.38^{b}	0.45^{b}	0.46^{b}	0.42^{b}
8	N_{inf}	84.23^{a}	100.49^{a}	24.54^{b}	57.22^{ac}	28.29b
	λ	0.43^{a}	0.37^{ab}	0.84^{a}	1.13^{c}	1.12^{c}
12	N_{inf}	80.79a	86.91^{a}	55.83^{a}	62.55^{ac}	38.16^{b}
	λ	0.60^{a}	0.45^{ab}	0.41^{a}	1.84^{d}	0.85^{ac}
16	N_{inf}	99.10^{a}	81.25^{a}	23.49^{b}	44.84^{bc}	28.74^{bc}
	λ	0.56^{a}	1.10^{c}	0.24^{a}	0.80^{a}	0.93^{a}
20	N_{inf}	77.25^{a}	63.57^{a}	62.00a	50.76^{c}	21.11^{bc}
	λ	0.59^{a}	0.83^{a}	0.67^{a}	0.46^{a}	0.75^{a}

λ – parameter indicating the rate of colony formation; N_{inf} – expected number of colonies at the finite time of incubation

For each parameter, different letters indicate significant differences between means within corresponding row or column at $P < 0.05$ level, using the Tukey's honestly significant difference (HSD) test

metal were observed for Cu on 0, 4 and 8, and for Cd on 0, 12 and 20 weeks of soil incubation. The prolonged incubation of soils resulted in the significant decrease ($P < 0.05$) in the CFU counts on week 16 and 20; still, these numbers (except the control) were higher compared with those on week 0 (Table 2).

Colony formation rate

The values of parameters that characterize the kinetics of bacterial growth are presented in Table 3. For the higher metal treatments, the first parameter N_{inf} showed lower values than the control. However, N_{inf} value of the bacterial population in the soil treated with 2.5 mg Cu/g soil did not differ significantly ($P < 0.05$) from that of the control on week 20. Generally, N_{inf} values did not change significantly with time in all treatments and uncontaminated soil (Table 3). As for the second parameter, a decrease in λ values of bacteria in Cu- and Cd-treated soils compared with that of the control was observed on week 4. In contrast, increased values of λ were observed for the soil treated with 1.5 mg Cu/g soil on weeks 16 and 20 as well as for the soil amended with both dosages of Cd on weeks 8, 12 and 16 (Table 3).

Community structure by plate count data

Bacterial colonies, growing on 0.1-strength TSA, were generally represented by slow-growing organisms when they were isolated from contaminated soil. The culturable bacteria belonging to fast-growing organisms were found in the similar proportions in both Cd-treatments and untreated control on week 4 (Figure 1); by contrast, bacterial populations forming colonies after 7 days of incubation dominated in both Cu-treatments. Similarly, the same bacterial class dominated in the soil contaminated with both concentrations of Cu over time. On weeks 16 and 20, the bacteria appearing after 10 days of incubation dominated in the soil treated with either 1.5 or 2.5 mg Cu/g soil, respectively. For both Cd-treatments, the dominance of slow-growing colonies revealed over time, but the patterns obtained were different (Figure 1). Also, a shift towards the dominance of slow-growing bacteria was found in the untreated control after 8, 12 and 16 weeks of soil incubation.

The calculation of EP indices showed that soil contamination with the metal dosages decreased the EP values for all treatments as compared with that of the uncontaminated control on week 4 (Table 4). In addition, the significant ($P < 0.05$) decrease was ascertained for the soil polluted with 2.5 mg

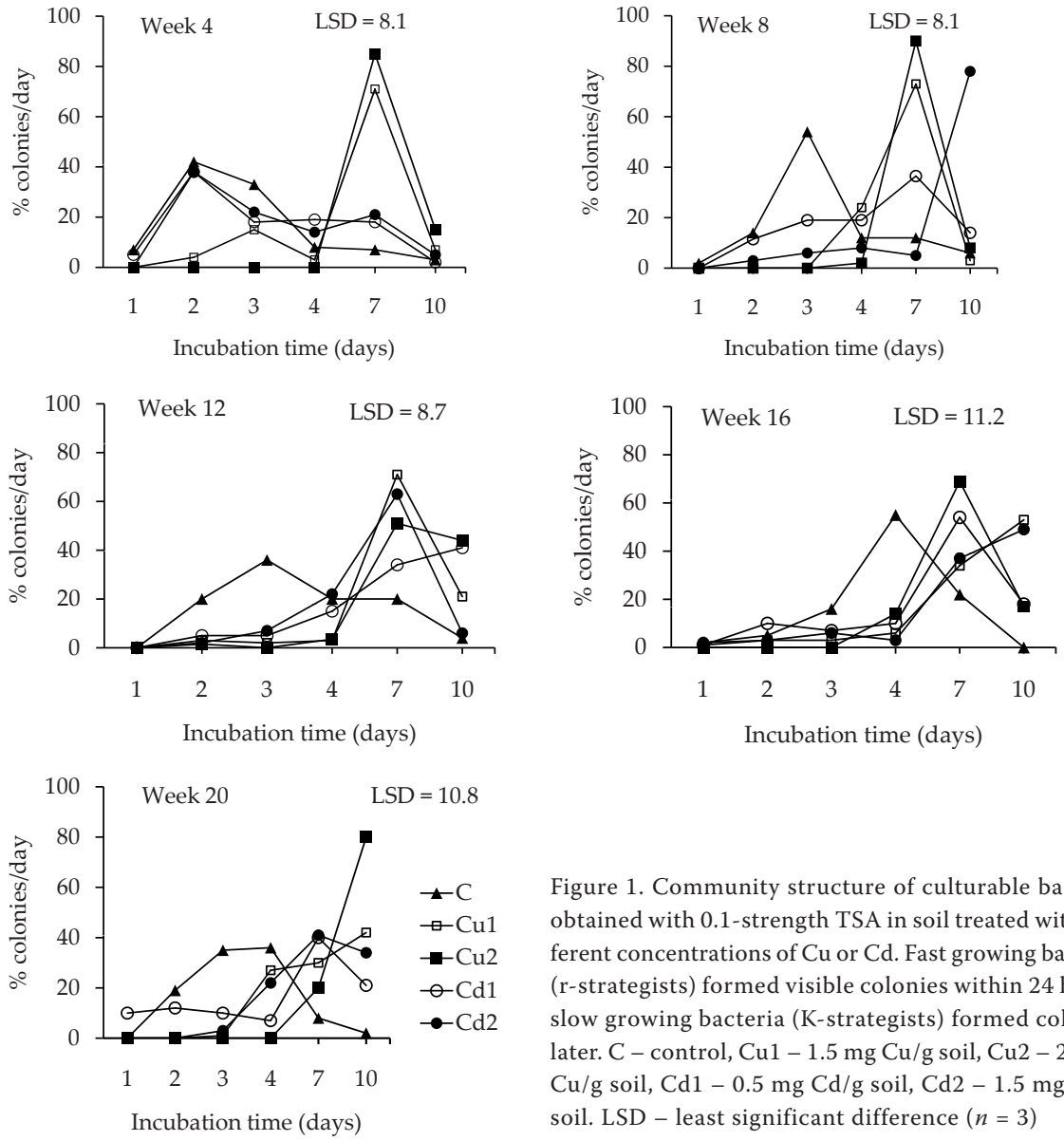

Figure 1. Community structure of culturable bacteria obtained with 0.1-strength TSA in soil treated with different concentrations of Cu or Cd. Fast growing bacteria (r-strategists) formed visible colonies within 24 h, and slow growing bacteria (K-strategists) formed colonies later. C – control, Cu1 – 1.5 mg Cu/g soil, Cu2 – 2.5 mg Cu/g soil, Cd1 – 0.5 mg Cd/g soil, Cd2 – 1.5 mg Cd/g soil. LSD – least significant difference (n = 3)

Cu/g soil on weeks 8 and 20, as well as for both concentrations of Cu on week 12. For Cd-treated soil, EP-indices also decreased on weeks 12 and 20. Generally, EP values did not differ significantly regarding the effect of time, except that found for 2.5 mg Cu/g soil. Moreover, there were no significant ($P < 0.05$) differences between the EP indices of the bacterial populations when effects of two dosages of each metal were compared (Table 4).

Community structure by FAME analysis

In total, 26 different FAMEs with chain lengths from C12 to C18 were identified in the communities of culturable bacteria obtained in this study. On average, 17 to 24 FAMEs were detected on week 4, and 11 to 17 on week 16. Among these FAMEs, 18 were common in the profiles obtained at both sampling times (data not shown). Taking into account all FAMEs identified, seven classes comprising single or multiple FAMEs were distinguished (Figure 2). The branched-chain (iso/anteiso) fatty acids containing 15 or 17 carbon atoms (i/a C15/17) were found in the highest proportions in the FAME profiles. However, significantly higher concentrations of this class were determined in the bacterial profiles obtained from the metal-treated soil portions on week 4 (Figure 2). In contrast, lower levels of i/a C15/17 were found in the profiles obtained from the soil samples contaminated with both concentrations of Cd on week 16, as compared with the untreated control and Cu-treated soils.

The second class, odd-numbered fatty acids, was also found in higher proportions among the cultur-

Table 4. Effect of soil treatment with different concentrations of Cu or Cd on eco-physiological indices (EP) of culturable bacteria

Weeks	Metal-treated soil (mg/g soil)				
	control	1.5 Cu	2.5 Cu	0.5 Cd	1.5 Cd
4	0.638^{a}	0.548^{a}	0.572^{a}	0.470^{bc}	0.513^{bc}
8	0.552^{a}	0.446^{ac}	0.360^{bc}	0.559^{ac}	0.551^{ac}
12	0.655^{a}	0.354^{bc}	0.424^{bc}	0.500^{bc}	0.462^{bc}
16	0.496^{a}	0.472^{ac}	0.376^{ac}	0.478^{ac}	0.489^{ac}
20	0.601^{a}	0.437^{ac}	0.208^{d}	0.418^{bc}	0.438^{bc}

Means within rows and columns followed by different letter are significantly different at $P < 0.05$ level, using the Tukey's honestly significant difference (HSD) test

able bacteria from the metal-treated soils compared with that of the control on week 4 (Figure 2). The third class, other i/a (i.e. i14:0 and i16:0), also showed higher contents in the profiles of bacterial communities obtained from the contaminated soils on week 4. Further incubation of soils resulted in an increase of other i/a concentrations in the control and soil treated with the higher concentration of Cu. In contrast to the described classes, the even-numbered FAMEs established significantly larger proportion in the bacterial profile of the untreated control compared with those of the metal-contaminated soils on week 4 (Figure 2). However, both concentrations of Cd increased the proportions of even-numbered FAMEs in the obtained profiles on week 16. In contrast, soil contamination with the lower concentration of Cu resulted in the increased proportion of the fatty acids on week 16 (Figure 2).

The untreated control and soil contaminated with 0.5 mg Cd/g soil were the habitats in which cy17:0 was only detected in the bacterial communities at both sampling times. Methylated fatty acids (i.e. 10Me) were solely identified in the community obtained from the soil contaminated with 0.5 mg Cd/g soil on week 4. The last FAME class represented by 18:1ω9c/ω12t/ω7c was found in a higher proportion in the control than metal-treated soil samples on week 4. However, significantly higher proportions of the FAME were observed in the Cd-treatments after 16 weeks (Figure 2).

Microorganisms react to the presence of heavy metals in soil depending on concentrations of soluble forms of the metals (Giller et al. 1998, Vig et al. 2003). In laboratory studies, concentrations of soluble heavy metals have decreased in contaminated soils with time (Kozdrój 1995). For cadmium, Vig et al. (2003) observed an exponential decrease in the metal concentration in freshly contaminated soil from 3 mg/kg to less than 0.0006 mg/l measured in the soil solution within 50 days of ageing. In this study, the significant decrease in the metal concentrations was only found for the lower dosage of Cu, and a reducing trend was observed for the higher dosages of both metals. This discrepancy in the results may be associated with different properties (e.g. organic matter content) of the soil used in the studies. Prolonged contact of heavy metal with soil results in a reduction of the metal bioavailability due to its decreased desorbability over time and/or formation of insoluble complexes (Vig et al. 2003). However, the concentrations of soluble metals, especially those of Cd, were even higher than the permitted levels accepted by EU for total concentrations of metals in soil. In addition to the soluble metals, the impact of decreased pH of the contaminated soils (especially those higher polluted) cannot be excluded.

The increase in the numbers of culturable bacterial in the metal-treated soil found on week 4 was probably associated with growth of metal-tolerant populations. These bacteria survived and proliferated due to nutrients released from sensitive populations killed by the metals (Kozdrój 1995, 2001). Angle et al. (1993) proposed that metal-tolerant bacteria are present at low population level in uncontaminated soil, and short-term increase in CFU is associated with multiplication of the bacteria (Kunito et al. 1999). However, the increase in CFU, observed after prolonged exposure to heavy metals in soil, may indicate acquired nature of the tolerance by different bacterial populations (Kunito et al. 1999). In contrast, the numbers of culturable bacteria decreased in all metal-treatments with time in this study. This fact suggests that the structure of bacterial community may

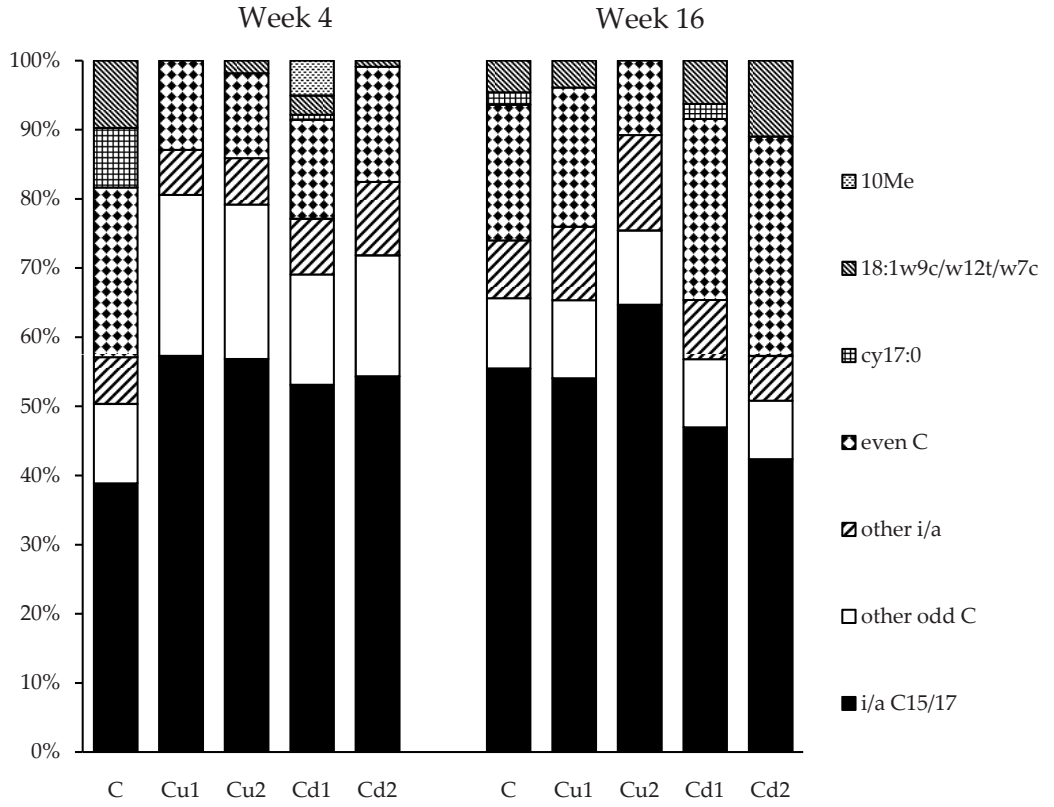

Figure 2. Proportions of major fatty acids in FAME profiles of culturable bacteria in soil treated with different concentrations of Cu or Cd. The odd-numbered fatty acids comprised chains with 13, 15 or 17 carbons. Other iso fatty acids comprised i14:0 and i16:0. The even-numbered fatty acids had chains with 12, 14, 16 and 18 carbon atoms. C – control, Cu1 – 1.5 mg Cu/g soil, Cu2 – 2.5 mg Cu/g soil, Cd1 – 0.5 mg Cd/g soil, Cd2 – 1.5 mg Cd/g soil

have been changed towards domination of a few populations and disappearing of several sensitive bacterial species with prolonged exposure to higher concentrations of Cu or Cd in the soil (Kozdrój and Van Elsas 2001b).

Surviving bacterial populations adapted to the contaminated habitats probably by changing their physiology as indicated by higher values of λ while N_{inf} did not change with time (Hattori 1985). The short-term contact of the bacteria with Cu or Cd concentrations may induce a defence mechanism based on the decline of the rate of cell proliferations (low λ) and a shift towards the quiescent phase. However, when the bacteria are exposed to the metal concentrations for a longer period of time they may increase the rate of proliferation and shift towards the vegetative phase. As a result, the bacterial cells increasing frequency of proliferation decrease their exposure time to the metals; thus they cannot take up toxic concentrations of the metals. Hattori (1985) reported that bacteria increase λ values in polluted environments, while some previous studies indicated that soil contamination with heavy metals results in the domination of bacterial populations being into the quiescent phase (Kozdrój 1995, 2001). However, these results were obtained using short-term experiments.

The agar plating method for the characterization of a bacterial community on the basis of r/K concept can give an overall picture of r/K distribution of organisms and can be considered an ecological fingerprint of bacterial communities (De Leij et al. 1993). In this study, the structures of bacterial communities were dominated by slow-growing populations of K-strategists with time. Similar results were obtained in the previous study (Kozdrój 1995). K-strategists are organisms well adapted to habitats polluted with metals because they seem to be more tolerant to Cu or Cd (De Leij et al. 1993, Kozdrój 1995). In contrast with the previous study (Kozdrój 1995), changes in diversity of the culturable bacterial communities, expressed by decreased values of EP-indices, were found in the contaminated soils, especially those treated with Cu. This observation indicates that one or a few metal-tolerant populations could dominate

in the bacterial communities. Decreased microbial diversity in soil polluted with heavy metals was reported in previous studies (Kozdrój and Van Elsas 2001a, b).

To show perturbations at the community structure level of the culturable bacteria, an additional analysis was performed by the MIDI-FAME profiling. This method supported the results of the bacterial community structure analysis by plate counts (r/K-strategists, EP-index), indicating that the structure was less complex in the contaminated soil portions. The culturable fraction of bacteria was represented by high biomass of Gram-positives (e.g. *Bacillus*, *Arthrobacter*) as indicated by the contents of branched FAMEs (especially iso/anteiso 15:0/17:0). Culturable communities of bacteria in soil were often dominated by Gram-positive species (Kozdrój and Van Elsas 2001a, Rajapaksha et al. 2004). These bacteria appear to be less tolerant to heavy metal stress compared with Gram-negative species (Kelly et al. 2003). However, *Bacillus*, *Corynebacterium* or other *Firmicutes* dominated in some metal contaminated soils (Rajapaksha et al. 2004). In this study, the soil contamination with both concentrations of Cu or Cd enhanced the growth of Gram-positive bacteria on week 4; this group was however negatively affected by both concentrations of Cd on week 16. This would suggest that the development of Gram-positive bacteria in the soil might depend on the metal type (i.e. lower tolerance to Cd) and time (i.e. shift in the population structure).

Odd-numbered fatty acids are markers of the *Cytophaga/Flavobacterium* group (Olsson and Persson 1999), whose contribution to the composition of the soil culturable bacteria could not be excluded in this study. Nevertheless, since another marker of the C*ytophaga/Flavobacterium* group, 16:1ω5c (Kozdrój and Van Elsas 2001a), was not found in any soil sample, the marker FAME presumably originated from the group of Gram-positive bacteria. The species containing these FAMEs may have proliferated in contaminated soil on week 4; however, Cd may have been toxic to some of them as indicated by contents of the FAMEs on week 16. A search of the MIDI-TSBA40 library has revealed that other i/a fatty acids (i.e. i14:0 and i16:0) are present in the FAME profiles of *Arthrobacter*, *Bacillus*, *Micrococcus*, *Paenibacillus*, *Cellulomonas* and some *Actinomycetes*. The data of these FAMEs found on weeks 4 and 16 suggest that the bacteria mentioned might have been those Gram-positives that dominated in the contaminated soil and were impacted by Cd with time.

In contrast, the proportion of Gram-negative bacteria, specifically *Pseudomonas*, increased within the culturable community of the control compared with those of the contaminated soils on week 4, as indicated by contents of even-numbered, cyclopropane and 18:1ω9c/ω12t/ω7t fatty acids (Olsson and Persson 1999). Gram-negative bacteria were often found in metal polluted soils (Kozdrój and Van Elsas 2000, Rajapaksha et al. 2004). The metal-tolerant Gram-negative bacteria, identified in these soils, belong to *Pseudomonas*, *Alcaligenes*, *Ralstonia*, *Burkholderia*, *Comamonas*, *Variovorax*, *Methylobacterium*, and *Flavobacterium*. In this study, the increased contents of even-numbered FAMEs in the Cd-contaminated soils on week 16 appear to be associated with the proliferation of Cd-tolerant Gram-negative species and/or spreading of the Cd-tolerance by a plasmid among the bacteria (Lawlor et al. 1999). The bacteria especially selected by Cd might be *Pseudomonas*, as indicated by contents of 18:1ω9c/ω12t/ω7t (Olsson and Persson 1999). This finding confirms other results suggesting that *Pseudomonas* tend to dominate in contaminated soils (Abaye et al. 2005, Piotrowska-Seget et al. 2005). However, Cu negatively affected the bacterial growth, which was still noticeable on week 16, especially for the higher concentration.

The nature of the time-depended differences (e.g. spreading of plasmid-carried Cd-tolerance and/or changes in species diversity) between the effects of Cu or Cd on the community structure of culturable bacteria in contaminated soil seems to be worth further studies.

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