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STRUCTURE OF MICROBIAL COMMUNITY IN SOILS CONTAMINATED WITH HEAVY METALS ASSESSED BY CULTURE AND FATTY ACID APPROACHES

Wydawnictwo Uniwersytetu Śląskiego
Katowice 2005

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Abbreviations

ARDRA	– amplified ribosomal DNA restriction analysis
CFE	– chloroform-fumigation extraction
CCA	– chromated copper arsenate
CD	– colony development index
cfu	– colony forming unit
CLPP	– community level physiological profiles
DG	– diglycerides
DGGE	– denaturing gradient gel electrophoresis
EP	– ecophysiological index
FAME	– methyl ester fatty acid
FAMEs	– methyl ester fatty acids
GC-MS	– gas chromatography/mass spectrometry
LPS	– lipopolysaccharide
LPS-OHFA	– lipopolysaccharide hydroxy fatty acids
MGE	– mobile genetic elements
MIDI	– Microbial ID, Inc.
PCR	– polymerase chain reaction
PHA	– poly-β-hydroxyalkanoic acid
PLFA	– phospholipid fatty acid
PLFAs	– phospholipid fatty acids
RISA	– ribosomal intergenic spacer analysis
RFLP	– restriction fragment length polymorphism
RQ	– respiratory quinones
SIR	– substrate-induced respiration
SLB	– signature lipid biomarkers
SLB/ENAP	– signature lipid biomarkers/environmental nucleic acid probes
SSU	– source substrate utilization
T-RFLP	– terminal restriction fragment length polymorphism
TG	– triglycerides
TGGE	– temperature gradient gel electrophoresis

1. Introduction

1.1. Heavy metals in soils

Heavy metals comprise a group of metallic elements, of density greater than 5 g/cm^3, with diverse physical, chemical and biological properties (Gadd, 1992). In terms of environmental pollution, heavy metals are generally regarded as harmful to living organisms, but actually this group includes essential nutrients such as Fe, Cu, Mn and Zn, as well as toxic Pb, Hg, Ag and Cd (Beveridge et al., 1997).

Soil heavy metals content is determined by the nature of the parent material and by the input of metal from anthropogenic sources. Common anthropogenic sources of soil heavy metals contamination are: metalliferous mining and smelting, industry, agriculture, municipal sewage sludge, waste disposal on agricultural land and fossil fuel combustion (Ross, 1994a; Kabata-Pendias and Pendias, 2001). Campbell et al. (1983) compared natural and anthropogenic quantities of heavy metals emitted to the atmosphere and found that around 15 times more cadmium, 100 times more lead, 13 times more copper, and 21 times more zinc were emitted by man's activities than by natural processes. Atmospheric deposition results in diffuse contamination, whereas a more concentrated contamination occurs where sewage sludge, metal-containing pesticides or fertilizers are used extensively (Chander and Brookes, 1993; Fliessbach et al., 1994; McGrath et al., 1995). Once incorporated into the soils, heavy metals remain for very long periods of time, up to several thousands of years (Kabata-Pendias and Pendias, 2002). Contamination with these metals is primarily restricted to the surface horizons and the extant of contamination may be 7–115-fold for Cu, 30-fold for Pb and 6-fold for Zn as compared with the subsurface level (Kabala and Singh, 2001). The heavy metals copper, zinc and cadmium are among the most common environmental contaminants resulting from anthropogenic activities and the weathering of natural mineral deposits.

Copper is present in the lithosphere at an average abundance of 70 mg kg^{-1} and its values reported for the earth's soil crust range from 24 to 55 mg kg^{-1}. The total content of Cu for soils of the world is approximately 30 mg kg^{-1}. Although Cu is one of the least mobile heavy metals in soils, this metal is abundant, as free and complexed ions, in solution of all types of soil and its concentration measured with various techniques varies from 3 to 135 μg l^{-1} (Bakker and Senft, 1995). The anthropogenic sources of Cu are related first of all to nonferric metal smelters, the use of fungicide sprays, fertilizers and utilization of municipal wastes. Contemporarily observed soil contamination has already brought Cu to extremely high concentrations in some sites of the world. In soils surrounding industrial sources of pollution Cu content reached the value of 3500 mg Cu kg^{-1} (Kabata-Pendias and Pendias, 2001). Zinc is distributed rather uniformly in the lithosphere and total Zn content of soils, depending on the composition of the parent rock materials, ranges from 40–120 mg kg^{-1}, with an average value of 80 mg kg^{-1}. The common range for total zinc concentrations in surface soils varies from 17–125 mg kg^{-1} (Kiekens, 1995). Soil contamination with Zn by metal industries and agricultural practices caused Zn accumulation in topsoils in certain areas over the world to reach extremely high values of up to 80 000 mg kg^{-1} (Kabata-Pendias and Pendias, 2001). Zn is considered to be readily soluble relatively to other heavy metals in soils. The Zn concentrations in soil solution range from 4–270 μg l^{-1}, depending on the soil and the techniques used for estimation of soluble ions (Kiekens, 1995). Cadmium occurs widely in nature in small amounts, with an average content of 0,2 mg kg^{-1} in the geosphere (Alloway, 1995). The concentration of Cd in soil solution varies significantly with soil properties and nature of management practices imposed on the system by farmers and other users (Alloway, 1995). In soils with no anthropogenic inputs of Cd, soil solution concentration ranged from 0,3–22,5 μg l^{-1} and in polluted soils, Cd concentration may exceed 10 μg l^{-1} in soil solution (Helmke, 1999).

The fate and behavior of heavy metals depend on the complex interactions that occur between metals and the soil. The distribution of heavy metals in soil profiles is continuously altered due to the natural turnover in the rock-soil-microorganisms-plants system. Heavy metals in soils exist in five major geochemical forms: exchangeable phase, bound to carbonate phase, bound to Fe-Mn oxides, bound to organic matter and residual metal phase (Asami et al., 1995; Kabala and Singh, 2001; Gupta et al., 1996). These associations determine their mobility and availability. Water-soluble and exchangeable forms are considered readily mobile and available for plants and microorganisms, whereas metals incorporated into crystalline lattices of clays appear relatively inactive. The other forms – precipitated as carbonate, Fe, Mn and Al oxides are considered to be relatively active or firmly bound,

depending upon the actual physico-chemical properties of soil (Shuman 1985; Gupta et al., 1996; Kabala and Singh, 2001). Organic complexation and chelation of metals in soil are one of the most important mechanisms that govern their bioavailability (Ross, 1994b). Studies on Cd speciation conducted by Krishnamurti and Naidu (2000) demonstrated that over 60% of Cd in the soil solution might be present in association with dissolved organic matter. Another important process in which heavy metals and other aqueous contaminant ions can be bound and sequestered is sorption on mineral surfaces (Davis and Batnagar, 1995; Brown et al., 1999). This process can dramatically reduce the mobility and bioavailability of contaminants in groundwater and, in the case of redox-sensitive elements, result in their transformation through reduction or oxidation reactions (Brown et al., 1999). Due to the fact that most major mechanisms of heavy metal toxicity are a consequence of their strong coordinating properties, a reduction in heavy metals bioavailability may reduce their toxicity and enhance microbial survival (Giller et al., 1998).

Mobility and the chemical speciation of heavy metals depend strongly on the physico-chemical soil properties, which can affect the long and short-term fate of metals. Soil properties that influence metal transformations and bioavailability include: particle size distribution and particle surface area, bulk density, temperature, aeration, redox status, pH, ion exchange capacity, quantity and quality of organic matter, type and amount of Fe, Mn and Al oxide and type and amount of clay (Ranjard et al., 1997; Naidu et al., 1997; You et al., 1999; Brown et al., 1999; Krishnamurti and Naidu, 2000). One of the most important factors controlling metal solubility and availability in soils is acidity. Many metals are relatively more mobile under acid, oxidizing conditions and are retained very strongly under alkaline and reducing conditions. Acidic conditions might increase metal availability, although H^+ may successfully compete with metal ions and reduce or prevent binding and transport into microbial cell. It has been shown that metal solubility increases with decreased pH. Experiments based on extracting soil solutions from a range of soil types maintained at different pH conditions have shown that Zn, Cd and Cu are much more soluble at pH range 4–5 than at pH range 5–7 (Ross, 1994b; Reddy et al., 1995). Giller et al. (1998) reported that decreased pH resulted in increased bioavailability of metals such as Zn and Cd in soil. Similarly, Khan and Scullion (2000) studying the effect of soil pH on microbial responses to metal contamination observed that metal inputs caused a marked increase in metal availability in the slightly acidic sandy loam and had little effect in the alkaline loam. Changes in organic matter and clay content can also significantly affect the microbial bioavailability of metals in soils (Bååth, 1989; Ranjard et al., 1997; Turpeinen et al., 2004). The important influence of organic matter

on heavy metal toxicity was confirmed by Moreno et al. (2001), who revealed that inhibitory effect of Cd on the ATP content, and the activity of both urease and dehydrogenase were significantly lower in soil with the highest organic carbon content.

Traditionally, the estimation of the environmental risk caused by heavy metals in soils and sediments is based on quantification of the total metals after digestion with strong acids followed by chemical analysis (Sheppard et al., 1992). However, the knowledge of the total content of heavy metals present in soil horizons provides limited information about their potential bioavailability to soil microorganisms and plants and does not predict the transport of toxic elements in the food chain. It has been shown that there is no correlation between the total concentration of heavy metals and microbial response (Leita et al., 1995; Almås et al., 2004).

Recently, many empirically-derived extraction procedures have been developed to simulate the availability of heavy metals such as Cd, Pb, Cr or Ni to plants. However, there are relatively few papers on their use to predict the effect of toxic elements on the activities of soil microorganisms in soils (Leita et al., 1995; Valsecchi et al., 1995; Insam et al., 1996; Chander et al., 2001). To predict the mobile fraction of heavy metals a sequential extraction procedure, using different extractants such as EDTA or acetic acid, is usually applied (Tessier et al., 1979; Quevauviller et al., 1997). This chemically mobile fraction has been interpreted as a bioavailable fraction but the correlation between the chemical mobility and bioavailability was not always observed (Turpeinen et al., 2004). Since chemical measurements alone do not provide complete information about the bioavailability of heavy metals the use of metal specific whole-cell biosensors seems to be a promising and suitable approach (Corbisier et al., 1996; Ivask et al., 2002). The determination of heavy-metal bioavailability by bacterial sensors is based on fusion between reporter genes and inducible promotor/operator sequences (Hansen and Sørensen, 2000). When the bioavailability of the tested metal reaches a threshold level, transcription derived from the inducible promotor/operator sequence will result in expression of the reporter system. Recently, various bacterial sensor strains specific for As, Zn, Cd, Cu, Cr, Pb and Ni have been developed and fully characterized in terms of specificity and detection limit (Tom-Petersen et al., 2001; Hansen and Sørensen, 2000; Tibazarwa et al., 2001). Reporter genes include those that encode bioluminescent proteins, such as luciferase (*luxAB*) and firefly luciferase (*lucFF*) or for β-galactosidase. The light produced is measured by luminometer, and the occurrence of β-galactosidase can be detected electrochemically or by using chemiluminescent substrates (Ramanathan et al., 1997; Corbisier et al., 1999; Bontidean et al., 2000).

1.2. Metal-microorganisms interactions

Metals play an integral role in the life processes of microorganisms. Some metals, such as calcium, cobalt, copper, iron, potassium, manganese, nickel and zinc, are essential, serve as micronutrients, and play a role in redox-oxidation processes. They stabilize molecules through electrostatic interaction, regulate osmotic pressure and form of charge and concentration gradients across membranes, which may be used to transport processes, and are components of various enzymes (Gadd, 1992; Bruins et al., 2000). For example, copper is present in cytochrome *c* oxidase, plastocyanin and some superoxide dismutases, whereas zinc is a component of the enzymes carbonic anhydrase, alcohol dehydrogenase, RNA and DNA polymerases and many DNA binding proteins (Beveridge et al., 1997). Many other metals, such as mercury, cadmium, lead and gold, have no biological role and are toxic to microorganisms. Toxicity of these metals occurs through the displacement of essential metals from their native bindings sites or through ligand interactions (Nies, 1999; Bruins et al., 2000). For example, Hg^{2+}, Cd^{2+} and Ag^{2+}, bind to SH groups resulting in inhibition of sensitive enzymes (Nies, 1999). At high concentrations, both essential and nonessential metals can block functional groups of biologically important molecules or transport systems for nutrient ions, damage cell membranes, alter enzyme specificity, disrupt cellular functions and damage the DNA structure (Bruins et al., 2000).

To play a physiological or toxic effect, metal ions have to enter the microbial cell. Many divalent metal cations (e.g. Fe^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}) are structurally very similar. Thus, to be able to differentiate between structurally similar metal ions, the microbial uptake systems have to be tightly regulated. Usually, microorganisms use two types of uptake systems for metal ions. The first one, non-specific, is fast and driven by the chemioosmotic gradient across the cytoplasmatic membrane. Since this mechanism is used by a variety of substrates, it is constitutively expressed (Nies, 1999). The second type of metal uptake system has high substrate specificity and uses ATP hydrolysis as the energy source. This system is slower and is only produced by the microbial cells at times of need, starvation or a special metabolic situation (Nies and Silver, 1995). High concentrations of nonessential metals may be transported into the cell by a constitutively expressed nonspecific system. This phenomenon is called "open gate" and it is one reason why metal ions are toxic to microorganisms (Nies, 1999).

As a consequence of the environmental exposure to heavy metals, microorganisms have evolved metal-ion homeostasis and genes encoding highly

specific mechanisms of resistance to heavy metals such as zinc, cadmium, copper, silver and mercury (Nies and Silver, 1995; Bruins et al., 2000). Metal-resistance strategies adopted by microorganisms include exclusion by permeability barrier, intra- and extra-cellular sequestration, enzymatic transformation and reduction in sensitivity of cellular target (Nies and Silver, 1995; Bruins, 2000; White et al., 1997b). Genetic resistance determinants are usually located on plasmids, only occasionally on transpozons, and frequently on the bacterial chromosome (Endo and Silver, 1995; Smit et al., 1998; Udo et al., 2000). The two most important types of resistance to heavy metals involve the energy dependent efflux of the metal ions from the cell and the reduction of metal to less toxic chemical form that can be excluded from the cells. Specific primary pumps have been identified for Cd(II), Zn(II), Cu(I), Ag(I), and As(III). Some of the efflux systems are ATPases and others are chemiosmotic cation/proton antiporters. The resistance mechanisms, based on ATPases, were described for cadmium and copper (Tsai et al., 1992; Silver, 1996). The cadmium resistance is encoded by the *cadA* operon, which consists of two genes *cadA* and *cadC*. The *cadC* gene encodes for the regulatory protein and the *cadA* gene for an energy-dependent pump, which is responsible for the efflux of Cd ions from the bacterial cells (Tsai et al., 1992). The best understood resistance system for copper is that of the Gram-positive pathogen *Enterococcus hirae*. This bacterium contains two genes, *copA* and *copB* that encode P-type ATPases responsible for copper uptake and export, respectively. These are regulated by two genes copY and copZ which are the first cistrons of cap operon and respond to both low and high copper concentrations (Odermatt and Solioz, 1995). The cadmium resistance ATPase of Gram-positive bacteria and the CopB copper efflux system of *E. hirae* are homologous to P-type ATPases of animals and plants (Nies and Silver, 1995). The three-component Czc (Cd, Zn and Co) chemiosmotic efflux pump of soil Gram-negative microorganisms consists of inner membrane (CzcA), outer membrane (CzcC) and membrane-spanning (CzcB) proteins which together transport cations from the cytoplasm across the periplasmic space to the outside of the cell (Silver and Phung, 1996). One or more of these resistance mechanisms allow microorganisms to survive under heavy metal stress.

1.3. Microbial influence on heavy metals mobility

Microorganisms play important roles in the environmental fate of heavy metals with a multiplicity of physico-chemical and biological mechanisms effecting the metal mobility and availability (White et al., 1997b; Ledin,

2000). The essential role of microorganisms in the fate of metals results from their ability to mediate mobilization or immobilization processes that influence the balance of metal species between soluble and insoluble phases. Microorganisms can mobilize metals through autotrophic and heterotrophic leaching, chelation by microbial metabolites and siderophores, methylation and by reduction and oxidation processes (Gadd, 2004). These processes can lead to the dissolution of insoluble metal compounds and minerals, including oxides, phosphates, sulfides and more complex ores, and the desorption of metal species from exchange sites on clay minerals and organic matter in soils, thereby increasing their bioavailability and potential toxicity (Gadd, 1993; White et al., 1997b; White et al., 1998; Ledin, 2000).

On the other hand, a number of processes mediated by microorganisms lead to immobilization of metals resulting in the reduction of the amount of free metal species in soil solution. Metals may be immobilized in soils by microbial accumulation and binding to metabolic products of microorganisms. Accumulation can occur either by metabolism-independent (passive) extracellular sorption on cell walls or membranes or by intracellular, metabolism-dependent, active transport into the cell by pathways existing for the uptake of metabolically necessary metals (Beveridge et al., 1997; Ledin, 2000; Daughney et al., 2002). Once inside cells, metal species may be bound, precipitated, localized within intracellular structures, or translocated to specific structures depending on the element concerned and the organism (White et al., 1997b; Gadd, 2000). Microbial metal accumulation received much attention last year due to the potential use of microorganisms for cleaning metal-polluted water. However, consideration should be paid to the role of bacterial accumulation for metal mobility in soils (Ledin, 2000). The interactions between microorganisms and metals present in the soil are considered to be the result of the competition for the metal between all components of the soil system (Kurek et al., 1996; Kurek and Majewska, 1998; Ledin et al., 1996). Ledin et al. (1999) compared the immobilization of metals by various soil components such as bacteria, fungi, peat, clay and aluminum oxide. They revealed that despite microorganisms being a minor fraction of the soil phase, they contributed considerably to the processes of heavy metals accumulation.

In addition, microorganisms produce a range of specific and nonspecific compounds. Nonspecific metal binding compounds range from simple organic acids and alcohols to macromolecules, such as polysaccharides, humic and fulvic acids (Gadd, 2000). Extracellular polymeric substances (EPS), a mixture of polysaccharides, mucopolysaccharides and proteins produced by different microorganisms also bind potentially toxic metals (White and Gadd, 2000). Some microorganisms in response to the presence of toxic metals produce specific, low molecular weight (6 000–10 000

Da proteins, termed metallothioneins (Howe et al., 1997)). Other metal-binding proteins, phytochelatins and related peptides, containing glutamic acid and cysteine have been found in several microorganisms (Rauser, 1995; Mejare and Bulow, 2001).

The immobilization of metals may be also based on a microbially induced process where metals are precipitated as insoluble sulfides indirectly by the metabolic activity of metal- and sulfate-reducing bacteria (White et al., 1997b). White and Gadd (2000) showed that sulfate-reducing bacteria exposed to Cd and Cu accumulated solid CdS and a mixture of Cu(I) and Cu(II) sulfides at the surface of bacterial biofilm.

In summary, microbiological processes can either solubilize metals or immobilize them, and thereby increase or reduce the bioavailability of metals. These biotransformations are fundamental components of biogeochemical cycles of metals and may also be exploited in bioremediation of metal contaminated areas (Gadd, 2004).

1.4. Microbial response to heavy metal contamination

Heavy metals at excessive concentrations can inhibit the growth and activity of bacterial cells (Gadd, 1992; Beveridge et al., 1997; Bruins et al., 2000). Since microorganisms play an essential role in the decomposition of soil organic matter, any reduction in their activity, abundance and diversity may affect the cycling of nutrients in the soil (Trevors, 1998; Giller et al., 1998). A large number of different bioassays have been used in the study of metal toxicity to soil microorganisms. The most common are measurements of global parameters such as microbial respiration (Valsecchi et al., 1995; Giller et al., 1998), organic matter turnover (Leita et al., 1995; Valsecchi et al., 1995), biomass (Kuperman and Carreiro, 1997; Chander et al., 1995; Kelly et al., 1999a) and more specific indicators based on specific microbial activities such as nitrogen fixation (Hirsch et al., 1993; Giller, 1998), nitrification (Hu et al., 2002), soil enzymes activity (Kandeler et al., 1996; Kelly et al., 1999a; Moreno et al., 2001; Majer et al., 2002) and enumeration and characterization of groups and communities of microorganisms (Dahlin et al., 1997; Stephen et al., 1999; Kozdrój and van Elsas, 2001a).

The results that have been reported on the toxic effects of heavy metals on soil microorganisms and their activities are contrasting. These are largely because of the differences in soil types and source of metal contamination (sewage sludge, soluble or insoluble metal salts), which would have a strong effect on the chemistry and bioavailability of the metals. There is little

reliable data on individual heavy metals, since most field studies on heavy metal-microorganism interactions are based on application of the sewage sludge containing multimetals and organic substances (Brookes, 1995; Chander et al., 2001; Kunito et al., 2001b).

1.4.1. Soil respiration

Measurement of microbial respiration (CO_2-C) and microbial biomass are the most commonly measured parameters in studies on soil microbial activity in heavy-metal contaminated sites. In most cases, heavy metal pollution has little effect on CO_2-C evolution at moderate and low levels of contamination (Giller et al., 1998; Post and Beeby, 1996). Only at very high metal concentrations is the evolution of CO_2 from soil significantly reduced (Doelman and Haanstra, 1984; Bååth 1989; Hattori 1992), probably due to the overall effect of the decrease in soil microbial biomass. In contrast, Chander and Brookes (1991) found an increase in CO_2 evolution in soils polluted with heavy metals. These observations were confirmed by Leita et al. (1995), who observed an increase in the soil respiration rate, expressed as CO_2-C evolved per hour, in soil contaminated with Zn and Pb. Another index for evaluating the toxic metal effects on soil microorganisms is the metabolic quotient (qCO_2), which is the respiratory CO_2 released per unit biomass. Similarly as in the case of the basal respiration rate, the results of the studies on qCO_2-response upon heavy metals contamination are contradictory. Some authors observed an increase of qCO_2 values along with increased levels of heavy metals (Bardgett and Saggar, 1994; Fliessbach et al., 1994; Dahlin et al., 1997). Enhanced qCO_2 and CO_2-C evolution might result from a low efficiency of substrate utilization for growth when microorganisms are under heavy metal stress (Flieissbach et al., 1994) and the greater demand for energy by microorganisms in order to cope with the toxicity of metal with a possible, consequent increase in the microbial turnover rate (Leita et al., 1995). On the other hand, a decrease of the qCO_2 in metal-contaminated soils was reported by Bååth (1989) and Shi et al. (2002a, b).

1.4.2. Microbial biomass

For the estimation of heavy-metal toxicity a measurement of microbial biomass has been commonly used. Brookes and McGrath (1984) have

provided evidence that heavy metals decrease the proportion of microbial biomass C in total soil organic matter and the ratio of soil microbial C to soil organic C has been proposed as a useful indicator of soil pollution by heavy metals. Metal exposure may result in the immediate death of cells due to disruption of essential functions, and to more gradual changes in population sizes due to changes in viability or ability of microorganisms to survive in metal-contaminated soils (Giller et al., 1998; Kandeler et al., 1996). It has been shown in many ecotoxicological studies that heavy metals added to soil in the sufficient high ratio affect the bacterial biomass (Aoyama et al., 1993; Leita et al., 1995; Speir et al., 1999; Kandeler et al., 1996). A decrease of bacterial biomass was observed in soils contaminated with heavy metals from different sources such as application of sewage sludge (McGrath et al., 1995), Cu and Zn in animal manures (Christie and Beattie, 1989), run-off from timber treatment plants (Yeates et al., 1994), past application of Cu-containing fungicides (Zelles et al., 1994) and metal-polluted army disposal (Kuperman and Carreiro, 1997). The reduction of microbial biomass occurs at a relatively modest, and sometimes even at a surprisingly low metal loading (Dar, 1997; Dahlin et al., 1997). Contrasting results were reported by Chander et al. (2001), who analyzed the influence of the soil contamination caused by sewage sludge and dump material from mining factory residues on microbial activity. They found that in long-term experiments, the contents of microbial biomass did not decrease with increasing heavy metal concentrations. Measurements of microbial biomass do not show the activity of the microorganisms. It is a measure of the "standing crop" of microorganisms in the soil and may be a useful indicator of changes in soil management or other environmental factors. However, different measurements must be made to examine whether the effects on specific microbially mediated processes in soil or on specific groups of organisms have occurred (McGrath et al., 1995).

1.4.3. Soil enzymes

Soil enzymes activities are attractive as indicators for monitoring the heavy metal impact on soils due to their important role in the soil environment. Metal ions, such as Zn, Cd, Ni, Pb or Cu may inactivate enzyme reactions by complexing the substrate, by combining with the protein-active groups of enzymes, or by reacting with the enzyme-substrate complex (Dick, 1997). Enzymes vary in their degree of inhibition by heavy metals. Among the soil enzymes tested so far, arylsulphatase appears to be the most sensitive to heavy metals, whereas urease and invertase are less affected by

metals (Yeates et al., 1994). Kandeler et al. (1996), studying activity of 13 enzymes involved in the cycling of C, N, P and S, found that enzyme activities decreased with increasing heavy metal pollution, and confirmed that the level of inhibition differed among the enzymes. Enzymes involved in C-cycling were least affected, whereas those related to the cycling of N, P and S showed a significant decrease in activity. In particular, arylsulfatase and phosphatase activities were strongly inhibited. Their activities decreased to a level of a few percent of their activities in the corresponding unpolluted control soil. In another study, where also a wide range of heavy metals have been tested, Hg, Ag, Cr and Cd generally caused the greatest inhibition of L-glutaminase, sulphatase, cellulase, L-asparaginase and β-glucosidase (Deng and Tabatai, 1995).

Dehydrogenase activity is also a very sensitive enzymatic microbial parameter (Majer et al., 2002). The inhibitory effect of heavy metals on dehydrogenase activity has been shown in laboratory (Doelman and Haanstra, 1984; Kucharski and Niklewska, 1992) and in field studies (Kelly and Tate, 1998a; Kelly et al., 2003). However, inconsistent responses of the individual enzymes were observed in soils from different locations, indicating that it is not possible to define a specific marker enzyme for metal contamination.

1.4.4. Microbial community structure

Toxic metals also affect communities of microorganisms in natural environments by reducing diversity and selecting metal-resistant strains from the populations (Bååth et al., 1998; Kelly et al., 1999a; Turpeinen et al., 2004). The trend towards a significant functional diversity loss becomes obvious even at low pollution levels (Doelman et al., 1994; Leita et al., 1995). Many studies have shown that metal contamination causes a shift within the soil microbial community from metal sensitive to less sensitive microorganisms (Maliszewska et al., 1985; Diaz-Raviňa and Bååth, 1996; Giller et al., 1998; Kelly et al., 1999a). The change in community structure could suggest that certain microbial species are no longer present in the heavy-metal contaminated soil, which could eventually lead to a loss of functions associated with these lost bacteria species. It has been shown that the presence of even small amounts of heavy metals might cause a substantial reduction in the total bacterial diversity and a change of the community structure, whereas other environmental conditions are responsible for only small differences in microbial community (Griffiths et al., 1997; Sandaa et al., 1999b; Bååth et al., 1998). Results obtained by Almås

et al. (2004) have shown that the long-term contamination with Cd and Zn forced a selection of the microbial community towards more tolerant species. Delorme et al. (2003) revealed that rhizobia isolated from uncontaminated soil differed both genetically and phenotypically when compared with those isolated from Zn- and Cd-contaminated soils. Moreover, isolates originating from the metal contaminated soil were more tolerant to both Cd and Zn when compared with those observed for isolates of control soil. In addition, Hirsch et al. (1993) reported that *Rhizobium leguminosarum* bv. *trifolii* exposed to heavy metals for a long time was unable to form effective symbiotic associations with white and red clover.

Changes in the microbial community structure and in the tolerance to metals appear to be very sensitive indicators of the biological availability of metals in soils. For instance, as an effect of heavy-metal contamination Frostegård et al. (1993b) reported gradual changes in the microbial community structure, based on changes in phospholipid fatty acid (PLFA) patterns. These changes were observed at metal concentrations below those affecting ATP synthesis and soil respiration. Bååth (1992) investigating metal induced community changes, developed a method based on the determination of the heavy-metal tolerance of whole bacterial communities. This approach involves short-time metal exposure of suspensions of extracted soil bacteria, during which the growth rate is determined by measuring the rate of [^{3}H] thymidyne or [^{14}C] leucine incorporation. This allows an approximate assessment of LC_{50} concentrations (i.e. the metal concentration which inhibits 50% of the activity), which is a measure of the overall metal tolerance of the community (Bååth, 1992). A greater LC_{50} value indicates increased metal tolerance, which is a strong indicator that the available heavy metals in the soil have reached sufficient concentrations to induce the changes in microbial community structure. Using this method, Díaz-Raviña and Bååth (1996) found that the community tolerance significantly increased at metal concentrations much lower than those necessary to alter microbial functions in the intact soil. Changes of microbial community structure after long-term exposure to heavy metals may result in reduced microbial activity. Doelman et al. (1994) observed that metal-contaminated soil contained more metal-resistant microorganisms, but these microorganisms had a restricted ability to degrade organic pollutants. Thus, heavy metal induced changes in community metal tolerance seem to be among the most sensitive indicators of biological effects caused by low heavy-metal concentrations.

1.5. Methods of soil microbial community analyses

Despite considerable interest in the microbiology and biochemistry of soils, relatively little is known about the diversity and ecology of the microbial communities. Based on molecular studies it can be estimated that one gram of soil may harbor up to 10^9 microorganisms of possibly thousands of different species (Ward et al., 1990; Amann et al., 1995). Since less than 1% of the microorganisms observed under the microscope is cultivated and characterized, soils ecosystems are, to a large extent, uncharted. There is an increasing recognition of the essential functions fulfilled by soil microorganisms. They are responsible for biogeochemical cycling, decomposition of plant and animal wastes and pollutants in soil environments, and symbiotic relationships with higher organisms. Consequently, changes in the structure or function of microbial communities may have a major influence on ecosystem activities (Torsvik and Øvreås, 2002).

Microbial diversity describes complexity and variability at different levels of biological organization. This huge microbial diversity often makes it difficult to characterize the microbial community structure. A full description of community structure includes the number of species present (diversity), the number of individual cells of each species (evenness) as well as the physiological role of each species in the environment and its interactions with other species (Tiedje et al., 1999). A major obstacle to the evaluation and comparison of soil microbial communities has been the lack of effective methods for addressing community-level characteristics (Cavigelli et al., 1995). Because only a small percentage of soil microorganisms are culturable, traditional culture-based assays of microbial populations or estimates of microbial biomass and activity provide valuable, but very limited information on these complex soil communities. Since the knowledge of community structure becomes central to many ecological and environmental studies, a variety of techniques are currently used for analyzing various aspects of community structure. Strategies for community structure analysis may be divided into two general categories: those relying on laboratory cultivation and those based on the direct extraction and analysis of indicator molecules such as nucleic and fatty acids. Recently, several techniques useful for community-level characterization have been developed that do not require the isolation and subsequent culture of microorganisms. These methods include molecular analyses of RNA and DNA extracted directly from soil (Liu et al., 1997; Ritchie et al., 2000), methyl ester fatty acid (FAME) (Cavigelli et al., 1995; Kozdrój, 2000) and phospholipid fatty acid (PLFA) analyses (Zelles, 1997; Frostegård et al., 1993a, b, 1996; Kelly et al., 1999a, b). Another community-level approach often used in

ecological studies is profiling based on sole carbon source substrate utilization (SSU) also known as community level physiological profiles (CLPP) (Garland and Mills, 1991; Campbell et al., 1997).

1.5.1. Methods based on laboratory cultivation

Many commonly used methods of bacterial community analysis are based on the isolation of individual strains on a solid medium, followed by characterization of the isolated colonies (Ogram and Feng, 1997). This general strategy is appropriate for studies requiring identification or characterization of individual isolates or for genetic studies of a particular species. Cultivation methods always favour the growth of some community members and do not detect other microorganisms present in the samples. Some microorganisms may not be able to grow in pure culture without a very detailed knowledge of their physiological features and growth requirements, e.g. the need for specific growth factors, produced in mutualistic relationships (Liesack et al., 1997). Despite this limitation, standard microbiological culture-technique is the most popular method to determine the numbers of microorganisms in environmental samples and simultaneously allows the isolation of representative strains in pure culture. In addition, these techniques offer a sensitive approach for isolation and characterization of specific bacterial groups, if a suitable selective medium is available, that possess particular growth characteristics, such as the ability to degrade xenobiotics or resistance to antibiotics or/and to heavy metals (Ogram and Feng, 1997). Selective nutrient culture-based method may also provide additional information for the more technically challenging and time-consuming approaches based on the characterization of nucleic and fatty acids directly isolated from soil samples. The greatest limitation of this general approach is that relatively few species present in soils are readily cultivated, and many strains of interest may not be included in the analysis (Amann et al., 1995; Torsvik et al., 1996; Liesack et al., 1997). Another disadvantage of culture methods is that some taxa selected by this procedure may or may not be important contributors to the biodiversity of the active soil microbial community. Some of the bacteria might be in a dormant stage, becoming recognizable when they are provided with a suitable substrate for growth (Kandeler et al., 1996). Many of the problems connected with the culture of microorganisms are due to incorrect incubation temperature, medium pH, lack of a required nutrient, lower than required concentration of a carbon or energy source, or even excess of some medium components. The problem of the low recovery of the total population using viable count-

ing may be overcome in part by careful use of growth media, and by a choice of appropriate conditions (Tanner, 1997).

Traditional plate culture methods are still commonly used to assess the impact of pollutants on the number of readily culturable bacteria (Kozdrój, 1995; Konopka et al., 1999). Recently, Ellis et al. (2001) found a relationship between the number of colonies that grow on media and heavy metal concentrations in soils from the industrial sites and assumed that these plate counts were a useful indicator of the impact associated with the contamination. Readily culturable bacteria are probably the largest and most active portion of microbial communities and represent the ecologically relevant portion of the soil bacterial community (Bakken and Olsen, 1987) and so provide a useful, rapid assessment of biological responses to heavy-metal contamination. Ellis et al. (2003) concluded that recent advances in the design of solid media and culture methods for soil bacteria will make plate counting even more useful for assessing the impact of pollution.

De Leij et al. (1993) proposed a simple method based on bacterial colony development on agar plates for the characterization of bacterial communities in rhizosphere and non-rhizosphere soil. K- and r-strategy concept is derived from evolutionary ecology and based on the thesis that there are genetic differences in the ability of bacteria to exploit and survive in different environments. This implies that environmental conditions select organisms for r-strategists that can replicate rapidly under low density, nutrient-rich conditions and for K-strategists that are adapted to growth under nutrient-poor, crowded conditions. Generally, r-strategists are considered to dominate in unstable environments whereas K-strategists are characteristic of a stable environment (Pianka, 1970). The evolution of bacterial populations related with the content of soil nutrients was confirmed by Smit et al. (2001), who used the distribution of 16S rDNA sequences to search for a relationship between the abundance of microbial groups and soil nutritional status. Their results suggested that soil with a high content of readily available nutrients showed positive selection for α- and γ-*Proteobacteria*, this being indicative of r-selection, which is the selection for bacteria with potentially high growth rates. In low-nutrient soil or soil with a high content of recalcitrant substrates, the percentage of *Acidobacterium* increased, which indicated K-selection, which is the selection for bacteria with a lower growth potential but a higher capability to compete for substrates. The authors suggested that the ratio between the number of *Proteobacteria* and *Acidobacterium* indicated the nutritional status of soil.

It is impossible to identify r- and K-strategists *in situ* and the abundance of these bacterial groups is quantified by daily counting of agar plates inoculated with soil suspensions until no more colonies appear. De Leij et

al. (1993) and Sarathchandra et al. (1997) proved that the ratio between r- and K-strategists is a simple, reproducible, and highly sensitive indicator for the characterization of microbial communities in soil and on roots.

1.5.2. Methods based on DNA and RNA analysis

A number of molecular genetic techniques, such as total DNA isolation and characterization, G + C composition of DNA measured using thermal denaturation procedure, rRNA sequences, PCR amplification of rDNA, PCR amplification of functional genes, and *in situ* hybridization of rRNA oligonucleotide probes have been used successfully for the assessment of soil microbial communities (Akkermans et al., 1995; Hill et al., 2000; Kozdrój and van Elsas, 2001a).

The G + C analysis is based on the fact that prokaryotic DNA varies in G + C content from about 24% to 76% (Tiedje et al., 1999). Bacterial chromosomes have G + C contents that are characteristic of their taxonomic groups, and the relative proportion of G + C amounts in DNA extracted from bacterial community indicates the relative proportions of specific taxonomic group. Comparison of the distribution of G + C contents in the community DNA may be used to analyze shifts in microbial community structure in soils under heavy metal and organic pollutions stress. Griffiths et al. (1997) applied this method for the measurement of changes in microbial community structure in soil experimentally contaminated with Cd, Cu, Ni, Pb or Zn. After 34 months of incubation, according to %G + C distribution profiles obtained, three statistically distinct groups of the soil treatments (Pb and Ni), (Cd, Zn and control) and Cu, were described. They revealed that bacterial community in the soil contaminated with Cu significantly differed from communities in other soils, including control. In contrast, Sandaa et al. (1999b) did not observe pronounced differences in %G + C profiles obtained from bacteria of unpolluted, low and high heavy metal contaminated soils. The authors suggested that bacteria with a relatively high G + C content dominated in both unpolluted and heavy metal polluted soils.

The genetic complexity of microbial community may be assessed by reassociation of community DNA (Torsvik et al., 1990; Torsvik et al., 1996) and cross hybridization of different DNA samples (Griffiths et al., 1997; Clegg et al., 1998). In the DNA reassociation technique the DNA sequence complexity is determined by measuring the rate of reassociation of single-stranded DNA under defined conditions. The rate of reassociation is proportional to the square of the nucleotide concentration of homologous DNA strands. When microbial heterogeneity increases (i.e. there are a greater

number of unique genomes), the rate of reassociation of DNA isolated from a given community decreases (Stahl, 1997). Determination of the sequence complexity of DNA measured as reassociation rate has revealed that the size of the community genome in unperturbed organic soils is equivalent to 6 000–10 000 *Escherichia coli* genomes, but only to 350–1500 genomes in heavy-metal polluted soil (Torsvik et al., 1998).

Sandaa et al. (1999b) applied *in situ* hybridization technique and different probes to assess the diversity of larger phylogenetic groups of bacteria in metal-contaminated soil. They revealed a general decrease in the percentage of cells detected with probes for β-, γ-, δ-*Proteobacteria*, *Cytophaga-Flavobacterium* division, Gram-positive bacteria with high DNA G + C content, 23S rRNA division, and Gram-positive bacteria with low DNA G + C, 16S rRNA content division with increasing metal contamination. However, there was a pronounced increase in cells detected with probe ALF1b (α-*Proteobacteria*). This shift in bacterial community suggests that bacteria belonging to this subdivision might have selective advantages over other groups of bacteria in heavy metal contaminated soil.

1.5.3. Nucleic acid fingerprints of bacterial community

Many PCR-based community analysis methods, called "fingerprint" methods, are commonly used to assess differences in community composition between samples or treatments or to assess changes in microbial populations over time. Molecular fingerprinting includes methods of analysis that generate a pattern-based characterization of community structure, most commonly represented by a banding pattern of nucleic acid fragments resolved by gel electrophoresis (Stahl, 1997). Such techniques as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA) or restriction fragment length polymorphism (RFLP) yield complex community profiles that do not directly offer phylogenetic information but do allow analysis and comparisons of community composition. Differences in electrophoretic profiles between samples reflect differences in community composition and abundance of individual microbial populations in a community (Kent and Triplett, 2002). Direct information of specific microbial population identity can be obtained by further application of group/species probes from the gel (Stahl, 1997).

The denaturing/thermal gradient gel electrophoresis (DGGE/TGGE) methods of analysis are based on the analytical separation of DNA fragments

identical or nearly identical in length but different in sequence composition (Muyzer, 1999). The application of DGGE technique for comparison of the archean communities in heavy-metals polluted soils revealed qualitative differences in the structure of the archean community with increasing level of metal contamination (Sandaa et al., 1999a). The shift in the archean community structure was observed even in soil contaminated with heavy metals at the concentrations below the upper limit set by the European Commission. Kozdrój and van Elsas (2001b) used PCR-DGGE method to assess the microbial community structure in soil samples collected from highly industrialised areas contaminated with different levels of heavy metals. They found differences both in richness and the structure of dominating bacterial populations between the polluted and unpolluted soils. Torsvik et al. (1998) used PCR-DGGE analysis to assess the impact of methane on community structure of soil bacteria. They revealed that the methane induced profound changes in the community structure and diversity. DNA from the untreated soil had a genetical diversity index of 5700, whereas in methane -amended soil the value of this parameter was 270 indicating a significant reduction in diversity. The DGGE analysis of methane-amended soil gave a number of strong bands showing that the community contained some dominating bacteria. In another study TGGE method was applied to examine the bacterial community of a Zn-contaminated soil (Brim et al., 1999). Analysis of TGGE patterns indicated a higher diversity of bacteria than that demonstrated either by cultivation or by cloning and sequencing of 16S rRNA genes. In bacterial community patterns they observed the domination of *Arthrobacter*-like strains and disappearance of metal-resistant *Ralstonia eutropha*, previously dominant in this soil due to decreased content of heavy metal. Amplified ribosomal DNA restriction analysis (ARDRA) is a DNA fingerprinting method based on restriction enzyme digestion and agarose gel electrophoresis of PCR-amplified 16S rRNA genes using primers for conserved regions (Tiedje et al., 1999; Kozdrój and van Elsas, 2001a). Smit et al. (1997) used this method to study the impact of Cu contamination on soil microbial community. They analyzed ARDRA patterns of the whole microbial community and compared ARDRA patterns of cloned 16S rRNA gene sequences from soil and isolates obtained from soil by cultivation. The results obtained with this method revealed distinct differences in the bacterial community structure and a lower diversity in soil polluted with Cu when compared to unpolluted soil.

Another genetic fingerprinting method, based on the length polymorphism of the PCR amplified intergenic spacer between 16S rRNA and 23S rRNA, known as ribosomal intergenic spacer analysis (RISA) was used to study the impact of Hg(II) on the soil microbial community (Ranjard, 2000). Addition of Hg(II) to a silt loam caused an increase in abundance of two RISA

bands. These bands were excised, sequenced, and identified as originating from a *Clostridium*-like Gram-positive bacteria and *Ralstonia*-like β-*Proteobacteria*. Changes in RISA profiles were mainly due to the appearance of new bands and to an increase in relative intensity of bands previously obtained from uncontaminated soil.

A useful method for monitoring highly diverse soil bacterial community structure is the terminal restriction fragment length polymorphism (T-RFLP) technique. T-RFLP is an improvement of ARDRA, which measures only the terminal restriction of each 16S rRNA. Turpeinen et al. (2004) using this method indicated that exposure to high metal contamination or subsequent effects of this exposure permanently changed the microbial community structure.

1.5.4. Methods to assess community function in soil

As microbial ecology involves the study of both the structure and function of an ecosystem, assessment of microbial communities must consider not only the abundance and distribution of species but also the functional diversity and redundancy present in microbial community (Kent and Triplett, 2002). The Community Level Physiological Profiles (CLPP) approach, using the Biolog® system, are means of investigating the physiological diversity present in soils, since they reflect the catabolic potential of microbial communities to utilize a range of carbon substrates (Garland and Mills, 1991). The assay is based on measuring oxidative catabolism of 95 different substrates to generate patterns of potential sole C source utilization (Garland and Mills, 1991). Campbell et al. (1997) have developed the method to test C sources that are ecologically relevant to soil and it has been used to examine communities of grassland ecosystems Larkin, 2003) and pollutant stress (Bååth et al., 1998; Kelly et al., 2003). Knight et al. (1997) using this method have shown that Cu and Zn reduced the metabolic potential of the extracted soil microbial populations. In contrast, Bååth et al. (1998) did not find any significant differences between metabolic fingerprints of the microbial communities originating from Cu, Zn and Ni-contaminated soils and from uncontaminated soil. The use of the BIOLOG sole C-source test plates for testing selected part of soil communities, for example oligotrophic bacteria, has several limitations because the method primarily selects for a small proportion of the total community comprised mainly of fast growing bacteria (Kent and Triplett, 2002).

To gain a better insight into the potential of microbial activity, it is useful to study the functional diversity in combination with taxonomic diversity.

Recent studies have attempted to characterize the fraction of the microbial community that responds to nutrient availability by comparing fingerprints after incubation in individual BIOLOG wells (Smalla et al., 1998) or by isolating DNA from microbial community populations that responded to nutrient input by uptake and incorporation of the thymidyne nucleotide analog, bromodoxyuridine (Borneman, 1999).

1.5.5. Community structure analysis by fatty acid profiles

Another useful tool for identification of the microorganisms and characterization of microbial communities is signature lipid biomarker (SLB) analysis. In this approach microbial lipid analysis is based on the solvent extraction and separation of microbial lipids from cultured bacteria or directly from environmental samples, followed by quantitative analysis using gas chromatography and mass spectrometry (GC-MS). Several unique classes of lipids, including steroids, diglycerides (DG), triglycerides (TG), respiratory quinones (RQ), poly β-hydroxyalkanoate (PHA), phospholipid fatty acids (PLFA), lipo-amino acids, sphingolipids and lipopolysaccharide hydroxy fatty acids (LPS-OHFA) can be used as signature lipid biomarkers (White et al., 1998). The fatty acid analysis has been successfully applied to the characterization of microbial communities from agricultural soils, sediments, sites contaminated with heavy metals, aromatic compounds, alkaline dust, acid rain and from other diverse habitats (Bååth et al., 1992; Frostegård et al., 1993b; Ringelberg et al., 1997; Haack et al., 1994; White et al., 1998; Kelly et al., 1999a).

1.5.5.1. Fatty acids as biomarkers

Fatty acids are of great value in determining bacterial phylogeny, provide a useful set of features for characterizing strains and give important information about microbial communities present. One of the most important SLB classes, phospholipids have useful properties as biomarkers. PLFAs are essential membrane components and make up a relatively constant proportion of the bacterial biomass under natural conditions. They are degraded rapidly after cell death, are not found in storage lipids or in anthropogenic contaminants and have a high natural turnover rate (Tunlid and White, 1992; White and Ringelberg, 1997). Because different groups of microorganisms synthesise a variety of PLFA through various bio-

chemical pathways, the PLFA are effective taxonomic markers. Phospholipid fatty acid analysis can provide insight into the phylogenetic relationship between organisms similar to more specific phylogenetic methods based on the sequence homology of 16S rRNA (Kohring et al., 1994). Moreover, PLFA have been successfully extracted from environmental matrices such as soils and sediments providing a means for direct *in situ* measurements of bacterial and fungal community structure and biomass. The different subsets of the community may be characterized on the basis of various PLFA patterns. Changes in the community structure in response to any environmental stressor are monitored by the comparison of the relative abundance of certain PLFA that are characterized for a specific group of microorganisms. Specifically, the SLB approach allows measurement of three important attributes of microbial communities: viable biomass, microbial community structure and nutritional/physiological status (Morgan and Winstanley, 1997; White and Ringelberg, 1997; White at al., 1997a).

The total and specific amount of PLFAs has been used as an indicator of microbial biomass in environmental samples (Zelles et al., 1994; Frostegård and Bååth, 1996). The viable organisms have an intact membrane containing PLFAs. After cell death or cell lysis, cellular enzymes hydrolyse phospholipids within minutes releasing the polar head groups. The remaining lipid moiety, diglyceride, contains the same signature fatty acids as the phospholipids. An estimation of the total nonviable and the total viable biomass can be made by measuring the diglyceride fatty acids and phospholipid fatty acids, respectively (White and Ringelberg, 1997). Parker et al. (1982) used hydroxy fatty acids of the lipopolysaccharide (LPS) for estimating the biomass of Gram-negative bacteria in soil and sediments. The viable biomass determined by PLFAs is equivalent to estimations based on intercellular ATP, cell wall muramic acid and acridine orange direct counts (Balkwill et al., 1988). It means that the measurement of PLFAs provides an accurate estimation of the viable or potentially viable microbial biomass.

Information obtained from the lipid analysis provides insight into the community composition as well. The fatty acids extracted from sediments allow classification of distinct microbial groups: microeukaryotes (polyunsaturated fatty acids), aerobic prokaryotes (monounsaturated fatty acids), Gram-positive and other anaerobic bacteria (saturated and branched fatty acids in the range from C14 to C16), and sulfate-reducing bacteria including other anaerobic bacteria (Morgan and Winstanley, 1997). Branched fatty acids have been used as biomarkers for bacteria including anaerobic bacteria and the sulfate-reducing bacterium *Desulfovibrio*. Detection can be highly specific for a single strain. The fatty acid 10Me16:0 can be used as a signature for the presence of *Desulfovibrio* since it is not detected in other sulfate-reducing bacteria. Branched-chain fatty acids (iso and anteiso) are

characteristic for Gram-positive bacteria and the genera *Cytophaga* and *Flavobacterium* whereas cyclopropyl fatty acids are common in some Gram-negative strains and some anaerobic Gram-positive bacteria (Haack et al., 1994). Methyl branching on the tenth carbon atom in the acyl chain is specific for actinomycetes (Zelles, 1999a). Gram-negative bacteria contain unique hydroxyl fatty acids (LPS-OH) in the lipid portion of the lipopolysaccharides (LPS) in the outer membrane. In specific applications LPS-OH fatty acids were used as a general indicator of Gram-negative bacteria in environmental samples (White et al., 1977; Parker et al., 1982). The monounsaturated fatty acids are characteristic for, but not unique to, eubacteria, including mainly anaerobic Gram-negative microorganisms (Zelles, 1999a). The polyunsaturated fatty acids occur only in cyanobacteria and they are considered to be signature acids for eukaryotes. High levels of the unsaturated fatty acids with low level of the polyunsaturated fatty acids suggest bacterial dominance in soil sample. In addition, the differences in the relative proportion of branched and monounsaturated fatty acids have been used as a marker for the proportion of Gram-positive and Gram-negative bacteria in marine sediments (Morgan and Winstanley, 1997). There are also some fatty acids that have been considered as an indicator for soil fungi in the environment. Wells et al. (1996) detected long chain unsubstituted fatty acids (24:0–26:0) in hyphal forms of fungi. Also linoleic acid (18:2ω6,9) is a good indicator of the fungal biomass in soil (Frostegård and Bååth, 1996). Another fungal biomarker is 16:1ω5 typical for arbuscular mycorrhizal fungi (Kelly et al., 1999a). The potential application of the marker fatty acids of selected genus, species and microbial groups are presented in Table 1. Specific pattern of PLFAs can also indicate the physiological or nutritional status of bacteria. Lipids are one of the molecules that can adjust in accordance with various environmental disturbances (Sajbidor, 1997). Environmental factors such as temperature, pressure, pH, water activity, nutrients, toxicants, solvents, age of culture and enzyme action can cause a shift in the lipid composition of microorganisms (Denich et al., 2003; Ramos et al., 2001) in bacteria or triglyceride in the microeucaryota relative to the PLFAs (White and Ringelberg, 1997).

The physiological/nutritional status of bacteria can be estimated by measuring the amount of poly-β-hydroxyalkanoic acid (PHA). In unbalanced conditions, bacteria exposed to adequate carbon source but lacking some other essential nutrient, form PHA and they cannot grow and divide. When the essential components become available these bacteria catabolize PHA and form PLFAs as they grow and divide. Starvation and prolonged exposure to conditions inducing stationary growth phase lead to conversion of monoenoic PLFAs (16:1ω7c, 18:1ω7c) to cyclopropane PLFAs (cy17:0, cy19:0) (Guckert et al., 1986). The ratio of monoenoic PLFAs to cyclo-

Table 1

Marker fatty acids of selected species, genus and microbial groups

Species, genus or microbial group	Fatty acids	Reference
Desulfovibrio	i17:1ω7c, i15:1ω7c, i19:1ω7c	Edlund et al. (1985)
Desulfobacter	10Me16:0, cy18:0 (ω7,8)	Dowling et al. (1986)
Methanotrophs	16:1ω8c, 16:1ω5c	Nichols et al. (1987)
Pseudomonas	16:0 and 16:1 (equivalent proportions) 18:1ω7c/ω9t/ω12t	Haack et al. (1994)
Arthrobacter	a15:0 and 17:0 (high proportions)	Haack et al. (1994)
Gram-negative bacteria	OH FAs (e.g. 3OH 16:1, 3OH 18:1) monounsaturated FAs (16:1ω7c, 18:1ω7t), cyclopropane FAs (e.g. cy 17:0, cy 19:0)	Cavigelli et al. (1995) Zelles (1999a)
Gram-positive bacteria	iso- and anteiso FAs (e.g. i15:0, a15:0, i17:0, a17:0)	Pennanen et al. (1998b) Zelles (1999a, b)
Actinomycetales	10Me FAs (e.g. 10Me16:0, 10Me17:0)	Frostegård et al. (1993b)
Fungi	18:2ω6,9, 18:3ω3, 18:3ω6 18:1ω9c, 20:4 16:1ω5c	Frostegård et al. (1993b) Lindahl et al. (1997) Olsson (1997)
Cytophaga – Flavobacterium	16:1ω5c	Frostegård et al. (1993b) Kelly et al. (1999b)

propane PLFAs varies from organism to organism and environment to environment but usually falls within the range of 0.05 in the expotential phase to 2.5 or greater in the stationary phase. An increase in cyclopropyl PLFAs formation has also been associated with increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies (White and Ringelberg, 1997). Exposure of bacteria to organic compounds (alcohols, phenol toxicants, short-chain carboxylic acid) leads to an increase in the proportion of *trans* PLFA compared to the *cis* homologues (Heipieper et al., 1995). In addition, *trans/cis* ratios of greater than 0.1 indicate nutrient starvation in bacterial isolates. This value is usually 0.05 or less in healthy and non-stressed populations. Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity. Environments with high potential terminal electron acceptors induce formation of benzoquinones in bacteria whereas microorganisms respiring on organic substrates form naphthoquinones.

1.5.5.2. FAME and PLFA analyses in microbial ecology studies

The FAME and PLFA analyses can be used to detect changes in soil microbial diversity in response to different soil perturbations. The MIDI-FAME (Microbial ID, Inc.) method was designed to extract fatty acids from pure cultures of bacterial isolates for identification purposes, but it has been also applied for characterization of culturable fractions of soil microbial communities. The recognition of fatty acid patterns allows identification of bacteria to the species or subspecies level and establish taxonomic relationships between the species (Zelles 1999b; Sasser, 1990; Buyer, 2002). Brim et al. (1999) used FAME analysis for the identification of dominant strains of the bacterial community in zinc-polluted soil. They revealed that the majority of the isolates were identified as *Arthrobacter* spp. (18 out of 23). Most of the isolates were zinc tolerant but only seven could be considered as zinc-resistant. The FAME method was also used for whole community profile determination. These profiles may be valuable as a marker of the degree to which communities are similar or different. Thompson et al. (1995) used assessment of community diversity by FAME content of bacterial isolates to reveal that a genetically modified *Pseudomonas fluorescens* had less impact on the bacterial community than the wild-type in a clear separation of two different communities. In other studies Cavigelli et al. (1995) applied principal-components analysis of MIDI-FAME profiles to identify similarities and differences among soil microbial communities. They observed many differences between FAME profiles obtained from direct soil samples and from isolates cultured on solid media, indicating that profiles of fatty acids extracted from isolates revealed a portion of the microbial community that was not culturable on the solid medium. Also Haack et al. (1994) used this method to compare microbial communities that differed in taxonomic status. They found relative similarities and differences of tested microbial communities. Few studies have focussed on the application of the whole soil fatty acid methyl ester profiles as an indicator of changes of microbial community affected by physico-chemical factors such as temperature, pH, matrix water potential, and the presence of toxic compounds. For example, Petersen and Klug (1994) observed a major shift in the fatty acid profiles obtained from soil incubated at near freezing temperatures and at 25°C. Kozdrój (2000) used FAME analysis to estimate microbial community structure of industrial wastes such as coal-mine spoil, non-ferrous metallurgical slag and coal fly-ash. He found that fungi dominated in metallurgical slag, since a high content of fungal marker fatty acid, 18:2ω6,9, was detected in this waste. In contrast, representatives of the *Cytophaga-Flavobacterium* group, for which 16:1ω5c was used as the marker, dominated in the coal fly-ash.

The ester-linked fatty acids in the phospholipids (PLFA) are currently one of the most sensitive and the most useful chemical measures of microbial community structure in the environment and have been used for the characterization of microbial community structure in sediments and soils (Frostegård et al., 1993a, b; Rajendran et al., 1994; Bååth et al., 1998). Rajendran et al. (1994) analysed the PLFAs to determine regional differences in microbial groups in sediments from Osaka bay. These workers demonstrated the predominance of prokaryotes in the sediment by the abundance of three major groups of C10 to C19 PLFA (saturated, branched and monounsaturated), which comprised 84% to 97% of the total PLFAs. By examining the PLFAs, they distinguished two characteristics of the distribution pattern: the predominance of anaerobic bacteria and Gram-positive prokaryotes characterized by the high proportion of branched PLFAs and the predominance of aerobic prokaryotes and eukaryotes as evidenced by the large amounts of monounsaturated PLFAs.

Many studies concerning the changes of PLFA profiles in bacterial communities affected by soil pollution have been studied extensively under field and laboratory conditions (Bååth et al., 1992; Frostegård et al., 1993b; Kelly et al., 1999b; Turpeinen et al., 2004). In studies on the influence of Zn pollution on soil microorganisms Frostegård et al. (1996) observed that the differences in PLFA pattern, due to the Zn pollution, increased gradually over time during long-term incubation. They attributed these changes in PLFA patterns to either the gradual development of microbial community, which became increasingly tolerant to Zn, or to the successive breakdown of PLFAs of those cells and subsequent proliferation of the organisms feeding on them. Phospholipid fatty acids were also analysed in forest humus and in an arable soil experimentally polluted with Cd, Cu, Ni, Pb and Zn at different concentrations. In both soil types, gradual changes in the PLFA patterns for the different levels of metal contamination were observed (Frostegård et al., 1993b). Soil bacterial biomass, pH tolerance and PLFA pattern were studied by Bååth et al. (1992) in a forest area polluted with alkaline dust. The largest proportional increase was found for the fatty acid 10Me18:0, which indicated an increase in the number of actinomycetes in the polluted sites. The levels of the fatty acids i14:0, 16:1ω5, cy17:0, 18:1ω7 and 19:1 also increased in the polluted sites while those of fatty acids 15:0, i15:0, 10Me16:0, 16:1ω7t, 18:1ω9 and 19:0 decreased when compared with unpolluted sites.

Currently, laboratory studies are performed to provide information on the progress of bioremediation of an oil spill and aromatic hydrocarbon contaminated sites. MacNaughton et al. (1999) reported that the community structure of all oiled samples shifted away from that of the control samples with time. The major difference in the PLFA profiles was that the microbial

communities in the oiled samples contained significantly more monoenoic PLFAs, specifically 16:1ω7c, 18:1ω7c, 16:1ω7t and 18:1ω7t, indicative of Gram-negative bacteria, than those in the control samples. Ringelberg et al. (1997) observed significant negative correlation between three-ring polycyclic aromatic hydrocarbons (PAH) and specific PLFA. For example, significant correlation of i17:0 with the loss of fluorene, phenanthrene and anthracene, is common to species of *Arthrobacter*, *Streptomyces* and *Rhodococcus*. Kelly et al. (2003) used PLFA analysis to assess the impacts of heavy metal contamination and subsequent remediation on soil microbial communities from sites surrounding a zinc smelter. They reported differences in the PLFA profiles for soil with different levels of metal contamination and decrease in indicator PLFAs for fungi, Gram-positive bacteria and actinomycetes in highly polluted soils. They also found that PLFA profiles for remediated sites showed increases in indicator PLFAs for fungi, Gram-positive bacteria and actinomycetes, suggesting a recovery of several microbial populations resulting from remediation. Signature lipid biomarker analysis combined with environmental nucleic acid probes (SLB/ENAP) was used to establish the impact of the jet fuel on microbial community structure (White et al., 1998). They found that the viable biomass increased as was indicated by the increase in PLFA and DNA. Principal-component analysis showed that the PLFAs that contributed to the hydrocarbon-influenced increase were those typical of Gram-negative heterotrophs (16:1ω7c, 18:1ω7c) and actinomycetes (10Me 18:0). The toxic stress reaction was observed in Gram-negative bacteria as evidenced by the increase in the *trans/cis* PLFA ratio. SLB/ENAP analysis can provide a comprehensive and powerful integrating tool for the determination of changes in microbial community that can be correlated to natural and accelerated bioremediation.

2. Aim of study

The fate and behavior of heavy metals in the environment is of great importance with respect to cycling of nutrients and assessment of environmental quality. Microorganisms are the first biota that undergoes direct and indirect effects of heavy metals. When present in high concentrations, they are well known to be toxic and influence microorganisms by adversely affecting their growth, biochemical activity, diversity and their community structure.

Our current understanding of microbial community structure is limited since the methods used to study microorganisms in natural environments have mostly been based on cultivation and isolation techniques. Recent advances in fingerprinting methods using signature biomarkers such as lipids provide qualitative and quantitative measures of microbial community composition in undisturbed and polluted soils.

The aim of this study was to determine microbial community structure in soil contaminated with different concentrations of selected heavy metals using both culture and culture-irrespective approaches. To determine the culturable fraction of the microbial community, whole-cell fatty acid methyl ester (FAME) profiling and r- and K-strategists approach were used. The total microbial community structure was assessed by phospholipid fatty acid (PLFA) analysis. The characteristics of the microbial community structure were supported by estimation of bacterial and fungal biomass in the soil. There are a variety of methods currently used for this purpose, however the FAME analysis of the culturable fraction of soil microorganisms and PLFA was selected in this study. While the FAME analysis still relies on the growth of bacteria, PLFA was used to determine major differences in the structure of the entire soil community irrespective of culturing.

3. Material and methods

3.1. Soil

The studies were performed using a sandy-loam soil with the following characteristic: 80% sand, 12% clay, 8% silt, specific gravity 0.79 g/cm^3, organic C 10.6%, total N 0.36%, cation exchange capacity (CEC) 8.7 me 100 g^{-1} and pH(H_2O) 6.9. In the laboratory, the soil was air-dried at room temperature, sieved (2 mm mesh) and soil samples (500 g) were placed in plastic containers. The soil samples were adjusted to 50% of its water-holding capacity and equilibrated for 7 days at 22°C, prior to use.

3.2. Soil contamination

The soil portions were contaminated with two concentrations of Cu (1000 or 2000 μg g^{-1} dry soil), Zn (1000 or 2000 μg g^{-1} dry soil) or Cd (500 or 1000 μg g^{-1} dry soil). High doses of metals used in this study correspond to the amount of these metals in heavily contaminated soils over the world. All metals were added as chlorides. The solutions of $CuCl_2$, $ZnCl_2$ and $CdCl_2$ were dissolved in 50 cm^3 of redistilled water and added dropwise to soil, followed by mixing thoroughly with a sterile spatula. The untreated soil portion was used as a control. For each metal treatment and control two soils were prepared. All soil samples were incubated at room temperature. The moisture content of soil was maintained throughout the study by periodic addition of distilled water, establishing 45% to 55% of the soil maximum water-holding capacity.

3.3. Chemical analysis

Bioavailable Cu, Zn and Cd were measured on 15, 60 and 90 days after soil contamination. Triplicate soil samples (5 g) were suspended in 50 cm^3 of distilled water and shaken for 1h at 180 rev min^{-1} at room temperature. After shaking, the water phase was filtered through a membrane filter (Whatman 0.45 μm pore size). A 1 cm^3 aliquot sample was mixed with 1 cm^3 of 0.1 mM Hg (II) in 100 mM HCl and 2 cm^3 of 1 M KCl, and immediately analysed by anodic stripping voltametry (deposition time 60 s, deposition potential 1.35 V, scan speed 600 mV s^{-1}, current range 200–500 mA), using a microtrace analyzer (AMAK, Poland).

The pH values of the aqueous soil extracts (1:2.5 w/v) were measured in triplicate with a glass electrode by a Jenway pH-meter at 20°C.

3.4. Microbial analyses

3.4.1. Bacterial numbers

After 15, 60 and 90 days of exposure the total numbers of heterotrophic culturable bacteria were determined by a soil-dilution plate method. Duplicate soil samples (10 g) were shaken for 30 min (180 rev min^{-1}) in Erlenmayer flask containing 90 cm^3 of sterile 0.85% NaCl. Serial 10-fold dilutions of soil suspensions were plated in triplicate on 0.1-strength Tryptic soy agar (TSA). To inhibit the growth of fungi cycloheximide (100 μg ml^{-1}) was added to the medium. The inoculated plates were incubated at 28°C for 5 days, prior to colony counting.

3.4.2. Bacterial community structure: r- and K-strategists

To determine the community structure of culturable fraction of soil bacteria, the method proposed by De Leij et al. (1993) was used. On 15, 60 and 90 days after soil contamination, serial 10-fold dilutions of soil samples in 0.85% NaCl were plated in triplicate on 0.1-strength TSA. Plates were incubated at 28°C for 10 days. Colonies were enumerated on a daily basis for seven consecutive days and on day 10. This way, eight classes of culturable bacteria were generated per plate, i.e. colonies that were visible

after 1, 2, 3, 4, 5, 6, 7 and 10 days of inoculation. The plates containing between 20 to 200 colonies were selected for enumeration. The number of bacteria in each class was expressed as a proportion (%) of the total number of culturable bacteria. The different distribution of the classes presented the distribution of r- and K-strategists in each soil sample. In this study, r-strategists were defined as fast growing bacteria that formed more than 50% of total visible colonies at 28°C on 0.1-strength TSA within 24 to 48h, whereas K-strategists were characterised as slow growing bacteria that produced colonies later (Kozdrój, 1999).

3.4.3. Ecophysiological (EP) and colony development (CD) indices

To characterise the community composition in the form of a single value the eco-physiological (EP) index (De Leij et al., 1993) and colony development (CD) index (Sarathchandra et al., 1997) were calculated. The EP index (a modification of the Shannon diversity index) was calculated according to the following equation:

$$EP = - \Sigma (p_i \times \log p_i)$$

where p_i represents each of the seven classes defined as a proportion of the total population in the soil sample. The more even the distribution of the classes, the higher the EP index.

The colony development (CD) index was calculated using the equation:

$$CD = (N_1/1 + N_2/2 = N_3/3 + \ldots + N_{10}/10) \times 100$$

where N_1, N_2, N_3 ..., N_{10} represent the proportion (i.e. bacterial colonies appearing on each counting day expressed as a proportion of the total number of colonies appearing over the 10 day period) of bacterial colonies appearing on days 1, 2, 3, ..., 10. The more even distribution of the classes is, the lower the CD index will be.

3.4.4. Determination of microbial biomass

Bacterial biomass in soil samples was calculated by the sum of the fatty acids considered to be of bacterial origin. These fatty acids included branched fatty acids i15:0, a15:0, i16:0, i17:0, a15:0, a17:0, odd-numbered straight

chain fatty acids (15:0 and 17:0), three types of unsaturated fatty acids (16:1ω9, 16:1ω7, 18:1ω7) and cyclopropyl fatty acids cy17:0 and cy19:0 (Frostegård and Bååth, 1996). Several other PLFA were detected, which could also be considered of bacterial origin. However, to estimate the bacterial biomass, PLFA completely identified by GC-MS and present in all soil samples were used.

3.4.5. PLFA analysis of microbial community

Phospholipid fatty acids (PLFAs) were assayed for uncontaminated and Cu, Zn or Cd-contaminated soils on each sampling day. Two PLFA profiles were produced for each soil by taking two soil subsamples from each soil. Phospholipid fatty acids were extracted using a procedure proposed by Frostegård et al. (1991), which is a modification of the one-phase method (Bligh and Dyer, 1959). Soil samples (1–2 g dry weight) were agitated in 18.3 cm^3 of a single-phase mixture of chloroform, methanol and citric buffer in a ratio 1:0.8:1 (v/v/v). After 3 hours extraction at room temperature the samples were centrifuged at 2500 rev min^{-1} for 15 min and the supernatants were transferred to other tubes. The pellets were washed with 5 cm^3 of the extraction mixture. Combined supernatants were split into two phases by adding 6.2 cm^3 of chloroform and 6.2 cm^3 of citric buffer. After shaking for 2 min, the samples were left to separate into phases at 4°C overnight. A portion (1–2 cm^3) of the lipid-containing lower phase was collected into new test tube and dried under N_2 at 30°C in a water bath. Lipid material was stored at -20°C until further analysis.

Phospholipids were separated from neutral and glycolipids fractions using solid phase extraction columns containing 500 mg of silicic acid. The columns were conditioned with 2 cm^3 of $CHCl_3$. Lipids were transferred to the columns in 1 cm^3 of $CHCl_3$. Glycolipids, neutral lipids and polar lipids were fractionated with 5 cm^3 chloroform, 10 cm^3 acetone and 5 cm^3 methanol, respectively. Polar lipids (phospholipids) were dried under N_2 at 30°C in a water bath. The phospholipids were subjected to mild alkaline methylation, which yielded fatty acid methyl esters (FAME) (Findlay, 1996). A 1 cm^3 volume of methanol-toluene (1:1 vol/vol) and 1 cm^3 of 0.2 M methanolic KOH were added to tubes containing dried phospholipids. The FAMEs were extracted with 2 cm^3 of hexane, 2 cm^3 of H_2O and 0.3 cm^3 of 1.0 M acetic acid, and then, hexane fractions were collected. The remaining solutions were washed once with 2 cm^3 of hexane and combined hexane fractions were dried under a stream of N_2 at room temperature and stored at -20°C prior to gas chromatography (GC) analysis. Before GC analysis, samples were dissolved

in chloroform and nonadecanoate (19:0) was added as an internal standard. The FAMEs were analyzed on Hewlet Packard 438A gas chromatograph equipped with Ultra 1 (crosslinked methyl siloxane) capillary column (12m; 0.2 mm i.d.; film thickness 0.33 μm) and a flame ionisation detector (FID). Hydrogen was served as the carrier gas. Data were integrated and printed on Shimadzu CR3A Chromatopac integrator.

The identification of particular PLFAs was carried out by GC equipped with a mass spectrometry detector (GC-MS), comparing their retention times and mass spectra to those of standard fatty acids. The fatty acids were quantified by correlating the peak areas to the peak area of the internal standard 19:0. Fatty acids with chain length exceeding 20 carbons, which are generally more characteristic of eukaryotic organisms than prokaryotes (Cavigelli et al., 1995), were not included in analyses in order to avoid interferences from animal and plant cells.

Fatty acids were designated by the number of carbon atoms, number of double bonds followed by the position of the double bond from the methyl end of the molecule. Configurations *cis* and *trans* are indicated by *c* and *t*, respectively. The prefixes *i* and *a* indicate *iso* and *anteiso* branching, 10Me indices a methyl group on the 10th carbon atom from the carboxyl end of the molecule, and *cy* refers to cyclopropane fatty acids.

3.4.6. MIDI-FAME analysis of the culturable bacterial community

The analysis of whole-cell derived FAMEs obtained from the culturable soil bacteria was used as a possible method to characterise the community structure, based on the average fatty acid composition of bacterial cells. For the analysis, diluted soil suspensions in 0.85% NaCl were plated on 0.1-strength TSA. After a 4-day incubation period at 28°C, all bacterial colonies (100–120 colonies per plate) were transferred in duplicate from plates to reaction tubes. FAMEs were extracted and purified according to the MIDI procedure (Sasser, 1990). Briefly, to each tube 3 cm^3 of a saponification reagent (45 g NaOH; 150 cm^3 methanol; 150 cm^3 water) was added, then vortexed and heated at 100°C in a water bath for 30 min to liberate fatty acids from cellular lipids. Once cooled, 2 cm^3 of a methylation reagent (325 cm^3 6.0 M HCL; 275 cm^3 methanol) was added to each tube. The tubes were vortexed again and incubated for 10 min at 80°C in a water bath to form methyl esters of the fatty acids. The FAMEs were extracted from the aqueous phase by adding 1.25 cm^3 of hexane/methyl *tert*-butyl eter (1:1 v/w) reagent, and the tubes were rotated end-over-end for 10 min. Next, after removing the aqueous phase, the organic phase was washed with 3 cm^3 of

1.2% NaOH and the tubes were again rotated for 5 min. Finally, the organic phase containing fatty acid methyl esters was transferred to a gas chromatography vial.

The extracted fatty acids were analysed by gas-liquid chromatograph (Hewlett-Packard 6890), using an HP Ultra 2 capillary column (5% cross-linked phenyl-methyl silicone; 25 m x 0.2 mm i.d.; film thickness 0.33 μm) and hydrogen as a carrier gas. The fatty acid methyl esters were detected by a flame ionisation detector (FID) and identified using the Microbial Identification Software (Sherlock aerobe method and TSBA library version 3.90) developed by MIDI (Microbial ID Inc., Newark, Delaware, USA). FAME standard mixtures obtained with the manufacturer were used according to the instructions to adjust to calibrate the system.

All fatty acids were divided by structural classes, including saturated straight chain, monounsaturated, polyunsaturated, branched and hydroxy fatty acids. These classes and selected individual fatty acids were used as indicators for particular microbial groups (Cavigelli et al., 1995; Zelles et al., 1999a). Only fatty acids that accounted for at least 0.50% of the total fatty acid content over all observations from any given sampling data were included in the analysis. This approach eliminated fatty acids that were only sporadically detected or unreliably quantified that might influence the results.

Statistics

The results of this study were expressed as the means based on duplicate or triplicate measurements. To show significant differences, comparisons of means were carried out by one-way ANOVA. For analysis of FAME and PLFA data, a joining method of the cluster analysis module (CA) by using the unweighted pair group with mathematical averages (UPGMA) was applied. This way, the dendrograms showing clustering trends among analysed samples were generated. All statistical calculations were performed using Statistica 5.0 PL.

4. Results

4.1. Bioavailable metal concentrations and soil pH

The water-soluble fractions of Cu, Zn and Cd in the soil samples constituted only a small proportion of the total metals added (Table 2). After 15 days of incubation, the concentrations of water-soluble Cu and Zn in soils contaminated with the lower doses of these metals were 43.5 and 47.3 μg g^{-1}, respectively, representing 4.4 and 4.7% of the total metals added. In soils contaminated with 2000 μg g^{1} of Cu or Zn the water-soluble fraction represented 3.9% and 2.6% of the total metals, respectively. Compared to Cu and Zn, Cd was characterized by greater water extractability. In the soil contaminated with Cd at the dose of 1000 μg g^{-1}, the water-soluble fraction of Cd reached the level of 69.0 μg g^{-1}, representing 6.9% of the total metal

Table 2

Concentrations of water-soluble Cu, Zn, or Cd in soils contaminated with heavy metals (μg g^{-1})

Treatment		Incubation time (days)		
		15	60	90
Control	Cu	3.1 ± 0.2	2.9 ± 0.2	3.1 ± 0.1
	Zn	2.7 ± 0.2	2.6 ± 0.07	2.6 ± 0.1
	Cd	1.5 ± 0.09	1.4 ± 0.07	1.4 ± 0.07
Cu 1000 μg g^{-1}		43.5 ± 3.5	35.0 ± 4.2	14.2 ± 2.1
Cu 2000 μg g^{-1}		77.5 ± 9.4	38.0 ± 3.2	18.5 ± 2.1
Zn 1000 μg g^{-1}		47.3 ± 3,0	32.0 ± 3.6	14.3 ± 2.1
Zn 2000 μg g^{-1}		52.3 ± 2.5	36.6 ± 2.5	16.3 ± 1.5
Cd 500 μg g^{-1}		32.6 ± 1.5	18.3 ± 1.5	16.6 ± 1.6
Cd 1000 μg g^{-1}		69.0 ± 5.6	48.3 ± 3.1	24.7 ± 3.2

amount. The water-soluble concentrations of Cu, Zn and Cd decreased progressively over incubation time. On day 90 after soil contamination, the soluble fractions of Cu and Zn ranged from 0.8 to 1.4% of the total metal treatments. The water-soluble fraction of Cd in soil contaminated with the higher dose of this metal reached the value of 24.7 μg g^{-1}, representing 2.5% of the total metal content.

After 60 and 90 days of incubation, water-soluble concentrations of Cu and Zn were not significantly different in soil contaminated with either lower or higher doses of these metals. In contrast, significant differences between treatments were observed in soil polluted with Cd (Table 2).

Soil contamination with Cu, Zn and Cd decreased pH in each treatment (Table 3). The highest decrease of pH was observed on day 15 in soil contaminated with 2000 μg Cu g^{-1}. In this case, soil pH was significantly ($p < 0.05$) lower (5.91) when compared to control soil (7.21). The significant decrease ($p < 0.05$) of soil pH was also found in soils contaminated with the higher doses of Zn and Cd. Over time, the pH in the control soil slightly decreased from 7.01 on day 15, to 6.86 on day 90. In contrast, in metal-treated soil samples a slight increase in pH was observed (Table 3).

Table 3

Effect of additions of Cu, Zn or Cd on soil pH (H_2O)

Treatment	Incubation time (days)		
	15	60	90
Control	7.01 ± 0.03^{a}	6.98 ± 0.02^{a}	6.86 ± 0.07^{a}
Cu 1000 μg g^{-1}	6.11 ± 0.02^{b}	6.20 ± 0.03^{b}	6.24 ± 0.08^{b}
Cu 2000 μg g^{-1}	5.91 ± 0.03^{c}	5.98 ± 0.03^{b}	6.02 ± 0.07^{c}
Zn 1000 μg g^{-1}	6.30 ± 0.02^{d}	6.49 ± 0.05^{c}	$6.55 \pm 0.06^{d,e}$
Zn 2000 μg g^{-1}	6.18 ± 0.03^{b}	6.36 ± 0.08^{c}	$6.41 \pm 0.06^{b,e}$
Cd 500 μg g^{-1}	6.52 ± 0.02^{d}	6.47 ± 0.04^{c}	$6.51 \pm 0.07^{d,e}$
Cd 1000 μg g^{-1}	6.16 ± 0.01^{b}	6.04 ± 0.05^{b}	6.22 ± 0.06

Means within a column followed by different letters differ significantly at $p < 0.05$.

4.2. Bacterial numbers

The Cu, Zn and Cd contamination of soil significantly decreased viable counts of the total heterotrophic bacteria. However, this effect lasted only a few weeks, when stress caused by the metals and decreased pH killed sensitive strains. On day 15 in higher contaminated soils bacterial counts for

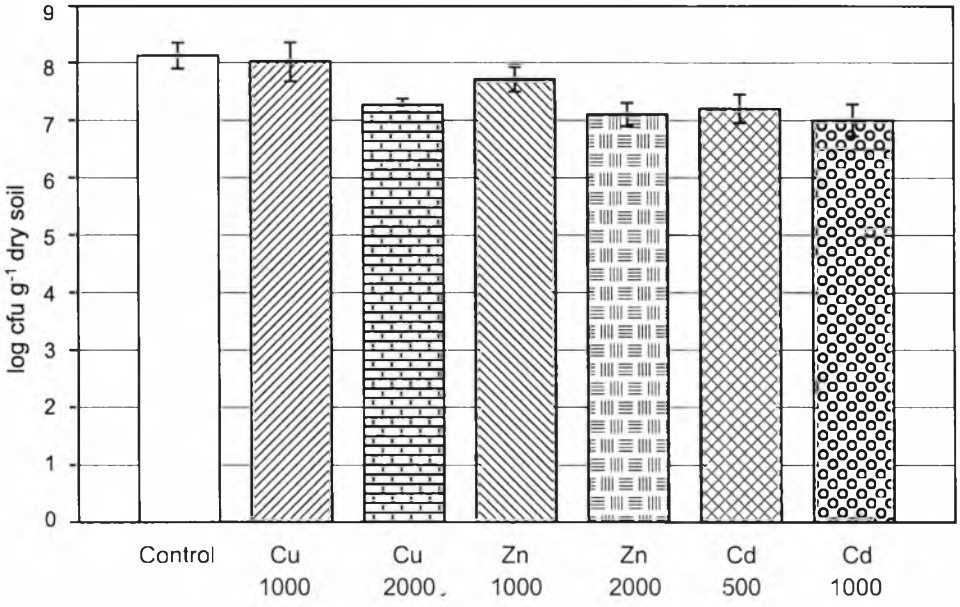

Fig. 1. Numbers of total heterotrophic bacteria in soils contaminated with Cu, Zn or Cd (in μg g^{-1}) 15 days after the addition of metals (mean ± standard deviation)

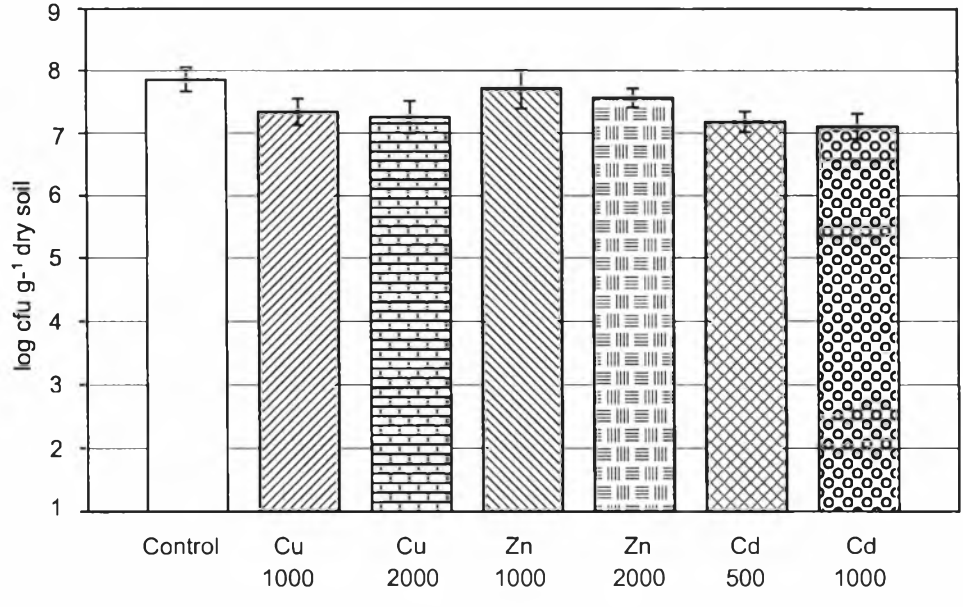

Fig. 2. Numbers of total heterotrophic bacteria in soils contaminated with Cu, Zn or Cd (in μg g^{-1}) 60 days after the addition of metals (mean ± standard deviation)

Cu, Zn and Cd-polluted soils decreased 86, 90 and 92%, respectively, when compared to the control sample (Fig. 1). The number of heterotrophic bacteria also changed significantly ($p < 0.05$) in the soil polluted with the lower dose of Cd. In Cd-polluted soil the bacterial counts were one log unit lower than in the control.

By day 90, the cfu counts in metal-treated soils increased and bacterial numbers were not significantly different than those in the control (Fig. 2 and 3).

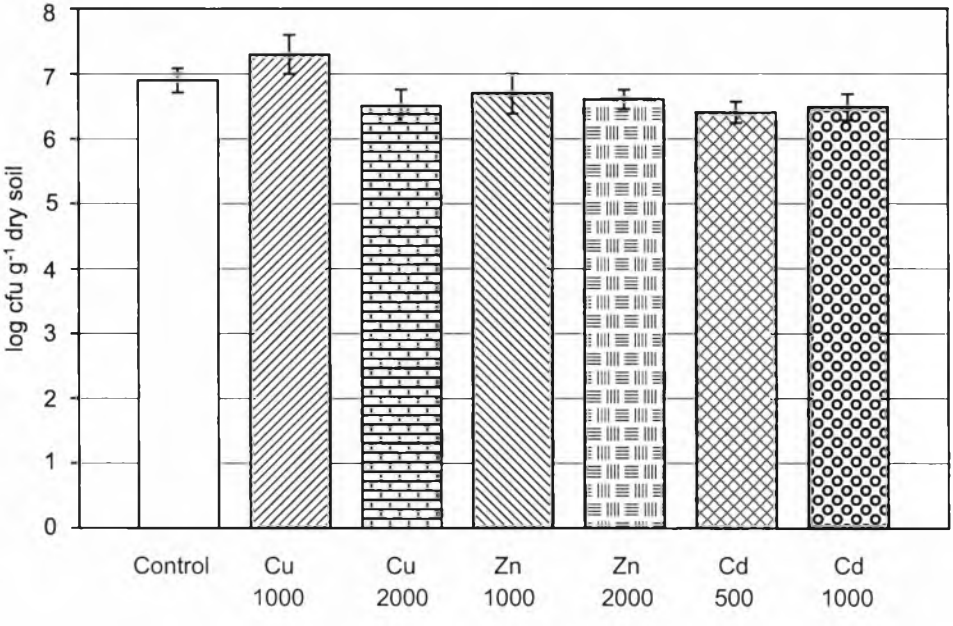

Fig. 3. Numbers of total heterotrophic bacteria in soil contaminated with Cu, Zn or Cd (in μg g^{-1}) 90 days after the addition of metals (mean ± standard deviation)

4.3. Bacterial community structure determined by r- and K-strategists

In unpolluted soil, the community structure of culturable bacteria differed from those in metal-treated soils. Differences in the development of r- and K-strategists during the experiments were observed both between the metals level of contamination and the sampling time. Differences between the evolution of the bacterial populations in the contaminated and uncontaminated soils samples were observed at each sampling day. Gen-

erally, a shift towards slow-growing bacteria was found in the contaminated soils over time.

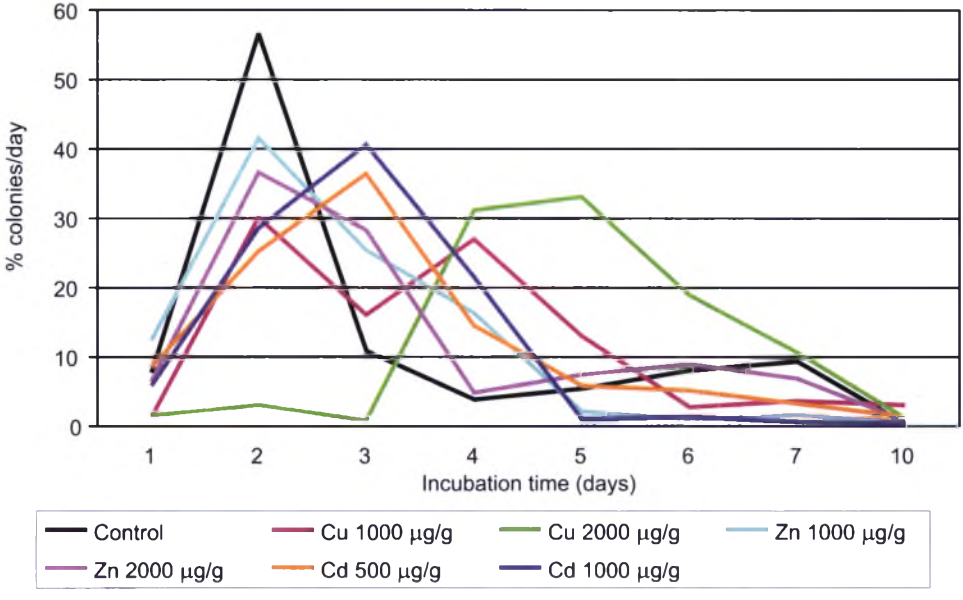

Fig. 4. Community structure of culturable fraction of bacteria in soils contaminated with Cu, Zn or Cd 15 days after the addition of metals

On day 15, in the soil contaminated with Zn (1000 μg g^{-1}) and in the control soil the bacteria that formed colonies within 24–48 h dominated. They constituted 41 and 56% of the total bacteria isolated from Zn-treated and control soil samples, respectively, whereas in the soil polluted with the higher dose of Zn and lower dose of Cu this fraction of bacteria represented about 30% of the total counts (Fig. 4). In the soil contaminated with 2000 μg Cu g^{-1} soil, visible colonies appeared mainly on day 4 and 5 of incubation. The domination of K-strategists was also observed in the soil contaminated with Cd. In these soil samples, the bacterial communities comprised a greater percentage of slower growing isolates (i.e. 60 and 70% for the soil contaminated with 500 or 1000 μg Cd g^{-1} soil, respectively) than that in the control (35%). In Cd-treated soils, the bacteria that produced visible colonies after 3 days dominated. They constituted from 36 to 40% of the total culturable bacteria.

During the second sampling time, the domination of fast growing bacteria was also found only in the control soil. For this soil, 63% of colonies appeared during two first days of incubation (Fig. 5). In contrast, higher numbers of visible colonies were formed after two days of incubation for bacteria isolated from soil contaminated with heavy metals. In the soil treated

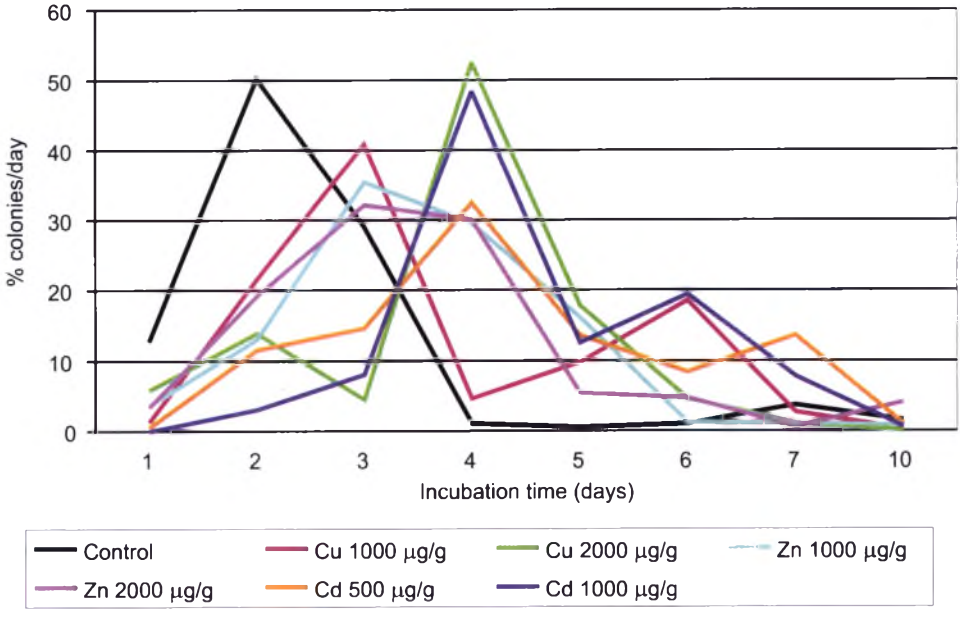

Fig. 5. Community structure of culturable fraction of bacteria in soils contaminated with Cu, Zn or Cd 60 days after the addition of metals

with Cu, Zn or Cd the structures of bacterial communities were not significantly affected by the level of contamination. However, in the soil treated with the lower dose of Cu the bacteria that formed colonies after 4 days of incubation (52% of the total bacterial community) dominated, whereas in the higher polluted soil there were the bacteria that produced colonies after 3 days of incubation (40% of the total bacterial number) which dominated. The bacterial communities in Cd-polluted soils were dominated by bacteria that formed visible colonies after 3 days; they represented from 38 (the lower dose) to 48% (the higher dose) of the total bacterial community. In turn, bacteria forming colonies after 3 and 4 days of incubation dominated in Zn-polluted soils.

Similarly, the bacterial colonies isolated from metal-treated soils were generally represented by slow-growing microorganisms on day 90 (Fig. 6). In the unpolluted soil, the bacterial communities were dominated by fast growing bacteria that produced colonies within 0–48 h (about 50% of the total bacterial communities). In contrast, only 20% of the bacterial community were able to produce colonies during that time for the soils treated with Cu (2000 mg Cu g^{-1}) or Zn. Similarly to Cu- (lower dose) and Zn-treated soils, the populations that formed visible colonies after 3 days dominated in both Cd-polluted soils. They constituted about 40, 55 and 45% of the bacterial communities in soil polluted with Cu, Zn and Cd, respectively. In contrast, the

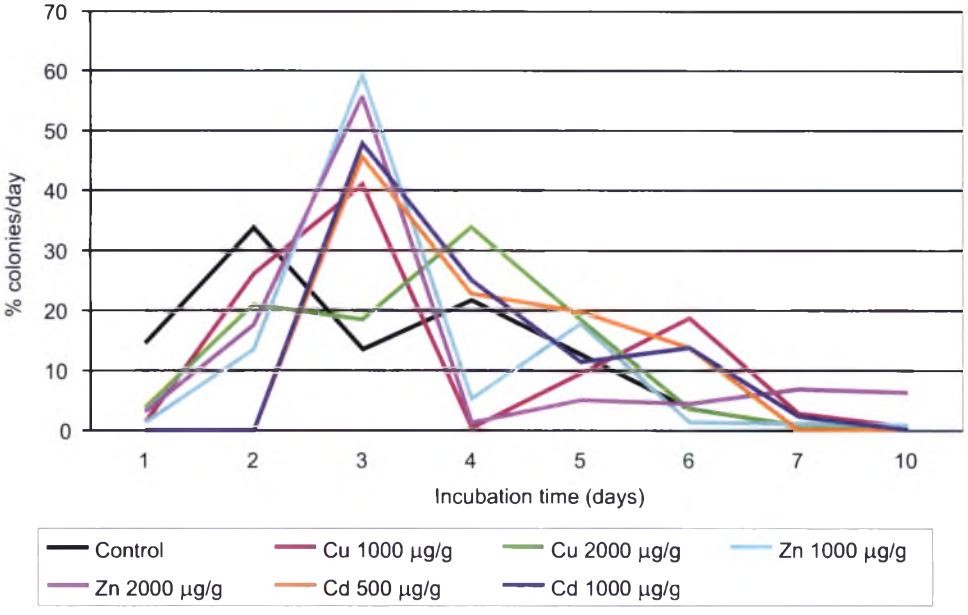

Fig. 6. Community structure of culturable fraction of bacteria in soils contaminated with Cu, Zn or Cd 90 days after the addition of metals

bacteria forming colonies after 4 days dominated in soil polluted with the higher concentration of Cu.

4.4. Ecophysiological (EP) and colony development (CD) indices

The effect of soil contamination with heavy metals on ecophysiological (EP) and colony development (CD) indices of the bacterial communities growing on 0.1-strength TSA was found. This effect was especially seen after 60 and 90 days of the soil exposure to the metals (Table 4 and 5). Generally, EP and CD indices of the bacterial populations isolated from the contaminated soils were lower than those from the control.

Considering the EP-index, bacterial diversity appeared to be higher in the bacterial community of the uncontaminated soil when compared to soil samples contaminated with the metals. On 15 day the value of EP-index decreased from 0.702 in the control soil to 0.653, 0.670 and 0.600 in the soil polluted with the higher levels of Cu, Zn or Cd, respectively; however, the differences were statistically significant, as compared to the control, only for soil contaminated with 1000 µg Cd g^{-1} (Table 4). The differences among EP indices became more noticeable with time. After 60 days, significant dif-

Table 4

Values of eco-physiological (EP) index of culturable heterotrophic bacteria isolated from soil contaminated with different concentrations of heavy metals

Treatment	Incubation time (days)		
	15	60	90
Control	0.702 ± 0.021^{a}	0.699 ± 0.022^{a}	0.707 ± 0.049^{a}
Cu 1000 μg g^{-1}	0.701 ± 0.046^{a}	$0.679 \pm 0.033^{a,c}$	$0.635 \pm 0.046^{a,c}$
Cu 2000 μg g^{-1}	$0.653 \pm 0.034^{a,b}$	$0.612 \pm 0.036^{a,c,d}$	$0.605 \pm 0.039^{a,b,c}$
Zn 1000 μg g^{-1}	$0.690 \pm 0.023^{a,b}$	$0.624 \pm 0.064^{b,d}$	$0.518 \pm 0.021^{b,d}$
Zn 2000 μg g^{-1}	$0.670 \pm 0.030^{a,b}$	$0.574 \pm 0.045^{b,c,d}$	$0.550 \pm 0.031^{b,c}$
Cd 500 μg g^{-1}	$0.665 \pm 0.048^{a,b}$	$0.552 \pm 0.018^{b,c,d}$	$0.539 \pm 0.020^{b,c}$
Cd 1000 μg g^{-1}	0.600 ± 0.035^{b}	$0.547 \pm 0.030^{b,c,d}$	$0.553 \pm 0.018^{b,c}$

Means within a column followed by different letters differ significantly at $p < 0.05$.

Table 5

Values of colony development (CD) indices of culturable heterotrophic bacteria isolated from soil contaminated with different concentrations of heavy metals

Treatment	Incubation time (days)		
	15	60	90
Control	51.6 ± 2.01^{a}	45.8 ± 2.2^{a}	43.1 ± 1.6^{a}
Cu 1000 μg g^{-1}	$42.6 \pm 3.2^{b,c}$	43.2 ± 2.4^{a}	$39.9 \pm 0.8^{a,c}$
Cu 2000 μg g^{-1}	37.4 ± 1.8^{c}	31.2 ± 1.2^{b}	33.8 ± 2.6^{b}
Zn 1000 μg g^{-1}	$45.3 \pm 2.3^{a,b}$	$35.5 \pm 1.5^{b,c}$	$38.2 \pm 2.1^{a,b}$
Zn 2000 μg g^{-1}	$40.3 \pm 0.8^{b,c}$	$40.8 \pm 3.3^{a,c}$	$35.6 \pm 2.4^{b,c}$
Cd 500 μg g^{-1}	$42.7 \pm 1.2^{b,c}$	30.8 ± 1.5^{b}	28.3 ± 1.66^{d}
Cd 1000 μg g^{-1}	$40.6 \pm 3.1^{b,c}$	35.1 ± 0.6^{b}	$26.8 \pm 0,97^{d}$

Means within a column followed by different letters differ significantly at $p < 0.05$.

ferences ($p < 0.05$) were observed between EP indices of the bacterial populations from the control soil and soil treated with both doses of Cd and Zn. During this sampling time the value of EP-index decreased from 0.699 in the control soil to 0.552 and 0.547 in the soils contaminated with the lower and higher concentrations of Cd, respectively. In Zn-contaminated (1000 μg g^{-1}) soil the EP-index reached the value of 0.574. After 60 days of incubation in the soil samples contaminated with Cu the EP-indices were also lower than in the control soil, but the differences were not significant. The similar trend was observed during the last sampling time. The values of the EP-in-

dices were significantly higher ($p < 0.05$) in unpolluted soil than those in the soil polluted with Zn or Cd. The highest decrease of EP index was found in the soil contaminated with Zn (1000 μg g^{-1}); the EP-index was 0.518 in this soil, while in the control soil EP showed 0.707.

The culturable bacteria in the uncontaminated soil showed higher CD values than those of the soils contaminated with the metals (Table 5). This finding was associated with the dominance of fast-growing bacteria in these soils. On day 15, CD values of the bacterial communities were significantly lower ($p < 0.05$) in the soils contaminated with the higher dose of Zn and both doses of Cu and Cd than those found in the control soil. In the soils polluted with the higher doses of metals CD showed the values of 37.4, 40.3 and 40.6 in the soil treated with Cu, Zn or Cd, respectively, while in the control soil the CD value was 51.6. Similar trends were observed during the second and third sampling times. After 90 days of incubation, CD values of the bacterial communities were significantly lower ($p < 0.05$) in the soil treated with higher doses of Cu or Zn, and both doses of Cd. The highest decrease of CD index was found in the soils contaminated with Cd. CD-index was 28.3 and 26.8 for bacterial communities isolated from the soils treated with the lower and higher doses of Cd, respectively, while in the control soil the CD showed the value of 43.1. In contrast to the EP-index, significant differences among CD values of the bacterial communities originating from the metal-polluted soils were observed during the second and third sampling times (Table 5).

4.5. Bacterial and fungal biomass

In soil contaminated with heavy metals bacterial biomass was estimated as amounts of PLFAs that are considered to be of bacterial origin. The amounts of bacterial PLFAs extracted from the soil samples were variable and ranged from 4.66 μmol g^{-1} to 13.2 μmol g^{-1} (Fig. 7–9).

The metal-treatment had a significant negative effect on microbial biomass that was noticeable on each sampling day (Fig. 7). On day 15, the microbial biomass significantly decreased in contaminated soils regardless of the treatment level. Even at the lower doses of the metals, the contaminated soils differed from the control. In all metal-polluted soils, significant decreases ($p < 0,05$) in the bacterial biomass in comparison with the uncontaminated soils were observed. The highest decreases were found in the soils contaminated with both doses of Cd and the higher dose of Cu or Zn. In the Cd-treated soils, the microbial biomass was about 55% (for 500 μg Cd g^{-1}) and 65% (for 1000 μg Cd g^{-1}) lower than in the control samples.

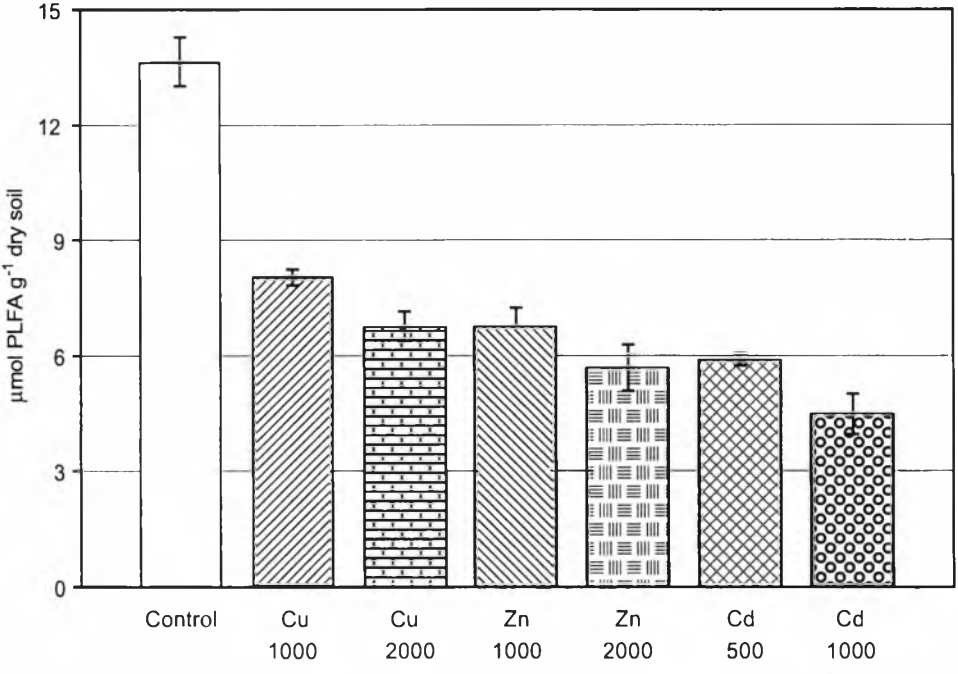

Fig. 7. Bacterial biomass in soils contaminated with Cu, Zn or Cd (in µg g^{-1}) 15 days after the addition of metals (mean ± standard deviation)

For the Zn-contaminated soils, the bacterial biomass constituted 52% (for 1000 µg Zn g^{-1}) and 45% (for 2000 µg Zn g^{-1}) of the bacterial biomass estimated for the control soil. The total amounts of microbe-derived PLFAs appeared to be dependent on the doses of the metals. The bacterial biomass in the soil contaminated with Cu at the dose of 1000 µg g^{-1} was significantly higher than that in the soil treated with 2000 µg g^{-1} of Cu, as well as in the soils polluted with other metals.

Significant differences ($p < 0.05$) between bacterial biomass in the lower and higher polluted soils were also observed in Zn- and Cd-contaminated samples. After 60 and 90 days of incubation, the similar trend was observed and the bacterial biomass in the metal-contaminated soils was significantly lower ($p < 0.05$) than in the control soil (Fig. 8). On these days, the highest decreases in bacterial biomass were found in the soils contaminated with a higher dose of Zn or Cd, in which the biomass was 30% lower than that in the control soil. No significant differences were observed in the amount of bacterial biomass between the metal-polluted soils (Fig. 8). On day 90, the decrease in the bacterial biomass observed was lower as compared to the first sampling days. However, a significant decrease in the bacterial biomass was still noticeable in the Zn and Cd-contaminated soil (Fig. 9).

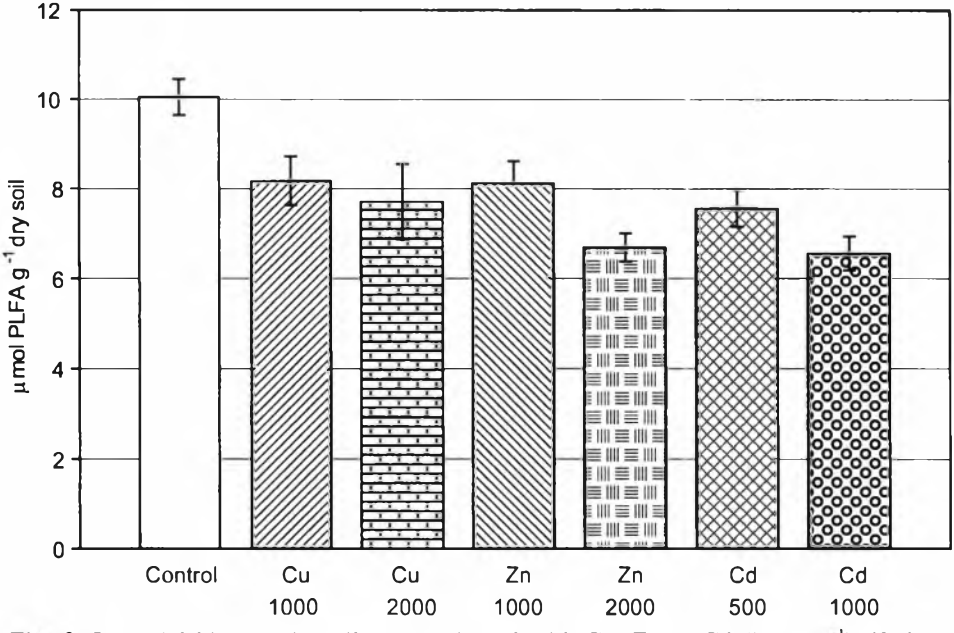

Fig. 8. Bacterial biomass in soils contaminated with Cu, Zn or Cd (in µg g^{-1}) 60 days after the addition of metals (mean ± standard deviation)

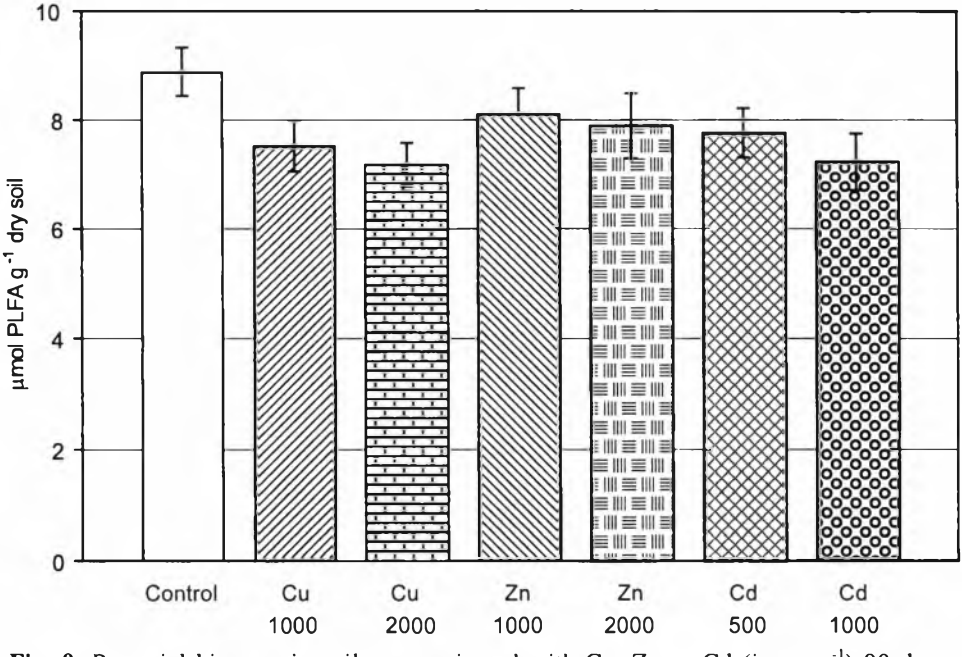

Fig. 9. Bacterial biomass in soils contaminated with Cu, Zn or Cd (in µg g^{-1}) 90 days after the addition of metals (mean ± standard deviation)

The response of fungal biomass to metal input, measured as the amount of an indicator fatty acid 18:2ω6,9, was different in the Cu-contaminated soil than that compared to the soil treated with either Zn or Cd. Only Cu had a strong negative effect on the fungal biomass. In the soil contaminated with Cu a decrease of fungal biomass was found, especially at the higher dose on day 15. For this concentration level the fungal biomass estimated was 30% lower than in the control soil (Fig. 10).

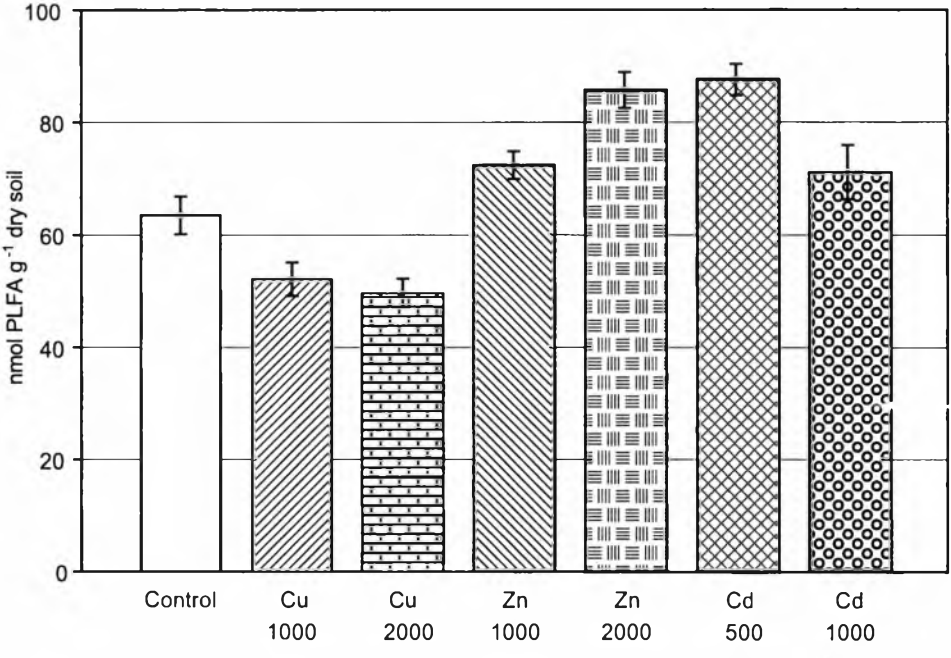

Fig. 10. Fungal biomass in soils contaminated with Cu, Zn or Cd (in μg g^{-1}) 15 days after metals addition (mean ± standard deviation)

The similar biomass reduction was observed over time. The fungal biomass in the soil treated with the higher dose of Cu was 40% and 30% lower as compared to the control on day 60 and 90, respectively (Fig. 11 and 12). On the last sampling time, the decrease of fungal biomass in the lower Cu-treated soil was less pronounced and there was no significant difference of the fungal biomass in that soil in comparison with the control (Fig. 12). In contrast, Zn and Cd inputs resulted in a progressive increase in the biomass of fungi over time. On day 15, a significant increase ($p < 0.05$) in fungal biomass was detected in the soil treated with Zn at the dose of 2000 μg g^{-1} and in that treated with the lower dose of Cd (Fig. 10). During next sampling times, significant increases in the fungal biomass were found in all samples contaminated with Zn or Cd. In the Zn-polluted soil (1000 μg g^{-1}) the fungal biomass was even twofold higher than that in the control soil (Fig. 11 and 12).

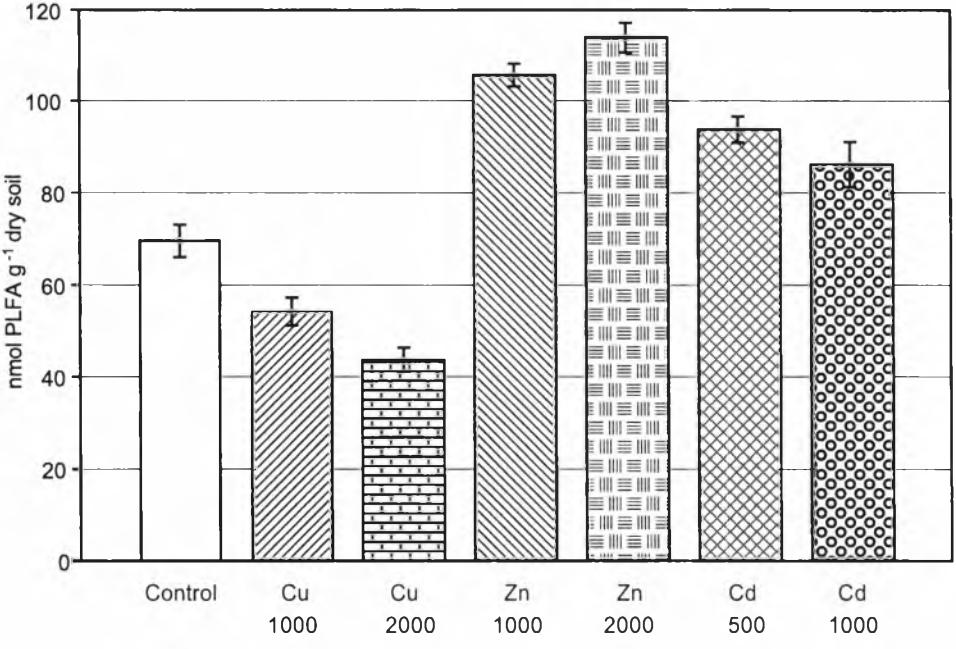

Fig. 11. Fungal biomass in soils contaminated with Cu, Zn or Cd (in μg g^{-1}) 60 days after metals addition (mean ± standard deviation)

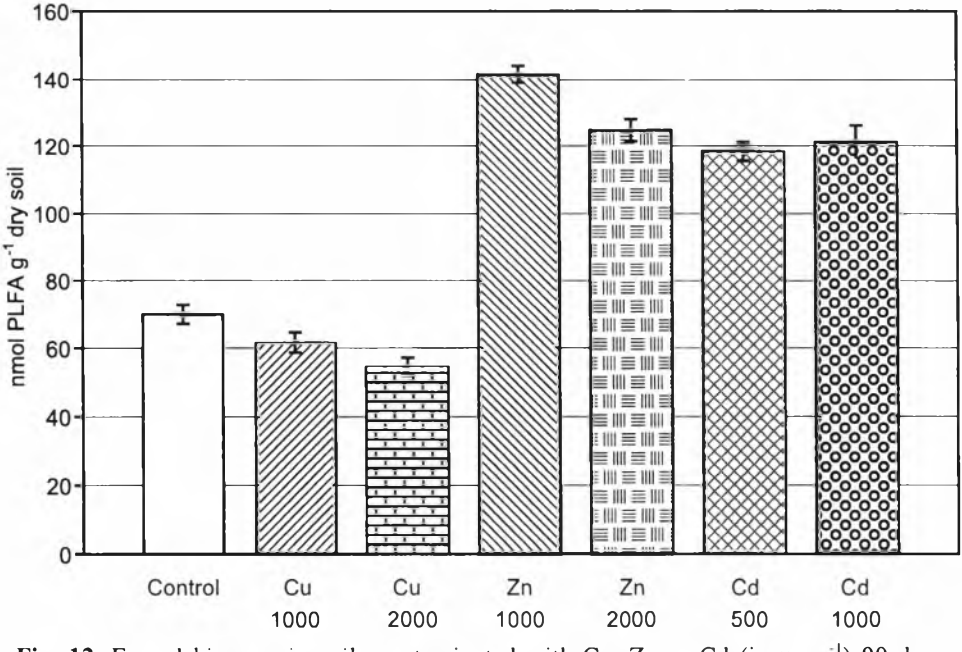

Fig. 12. Fungal biomass in soils contaminated with Cu, Zn or Cd (in μg g^{-1}) 90 days after metals addition (mean ± standard deviation)

4.6. PLFA analysis

In total, 26 different PLFAs with a chain length up to C_{20} were detected and identified by GC-MS (Tables 6–8). Changes in PLFA patterns were found irrespective of which metal was added to the soil. The analyses of phospholipid fatty acid patterns revealed distinct differences between the PLFA profiles obtained from uncontaminated and metal-contaminated soils. The changes in PLFA composition due to the metal contamination were illustrated by the changes in the amount of individual PLFAs at each sampling time (Tables 6–8). For the control soils, the proportions (%) of the isolated PLFAs were given. To show the effects of different heavy metals on bac-

Table 6

Distribution of PLFAs in soil contaminated with Cu, Zn or Cd 15 days after the addition of metals

PLFA	Proportion of PLFA (%) in control soil	Ratio with given metal					
		Cu ($\mu g\ g^{-1}$)		Zn ($\mu g\ g^{-1}$)		Cd ($\mu g\ g^{-1}$)	
		1000	2000	1000	2000	500	1000
i14:0	1.20	0.93	0.86	0.89	0.64	0.90	1.01
14:0	1.72	0.81	0.80	0.69	0.90	0.96	0.90
i15:0	5.11	0.75	1.56	1.07	1.15	1.11	0.55
a15:0	4.08	0.88	1.37	0.83	1.15	1.03	0.82
15:0	1.80	0.85	1.04	0.90	0.91	0.65	0.70
i16:1	2.47	0.57	0.87	1.04	0.95	0.53	0.57
i16:0	1.95	1.11	0.65	1.01	1.06	0.79	1.11
16:1ω9c	1.06	0.71	0.79	1.12	0.90	0.87	1.02
16:1ω7t	12.29	1.05	0.81	1.47	1.25	1.52	1.53
16:1ω5	2.42	0.79	0.90	0.78	1.04	1.33	1.72
16:0	15.24	0.94	0.98	0.97	0.95	0.85	0.98
br 17:0	3.2	0.98	1.05	0.62	0.80	0.67	0.96
10Me16:0	5.23	1.20	0.73	0.88	1.01	0.78	0.87
i17:0	2.27	0.80	1.11	0.77	0.93	0.87	0.85
a17:0	2.56	1.16	1.21	1.10	1.07	0.96	1.16
cy17:0	0.88	0.96	0.92	1.09	0.97	0.71	1.19
17:0	1.34	1.01	1.25	0.70	1.34	0.89	1.03
br18:0	2.84	0.90	0.81	0.65	0.77	0.99	0.88
18:2ω6,9	3.00	0.82	0.78	1.52	1.61	0.96	1.09
18:1ω9	5.42	1.67	1.00	0.89	1.07	1.05	0.97
18:1ω7	7.59	0.94	0.97	1.16	0.89	0.90	0.94
18:1	2.65	0.92	1.31	1.30	0.82	0.81	0.72
18:0	2.63	0.78	1.16	1.21	0.97	1.15	1.08
10Me18:0	4.31	0.98	0.90	0.87	0.96	0.97	1.15
cy19:0	4.57	1.15	0.77	1.11	0.98	0.88	0.73
20:0	2.8	1.14	1.18	0.87	0.69	1.10	1.04

Table 7

Distribution of PLFAs in soils contaminated with Cu, Zn and Cd 60 days after the addition of metals

PLFA	Proportion of PLFA (%) in control soil	Ratio with given metal					
		Cu (μg g^{-1})		Zn (μg g^{-1})		Cd (μg g^{-1})	
		1000	2000	1000	2000	500	1000
i14:0	0.85	0.75	0.87	1.31	0.95	0.95	1.10
14:0	2.37	1.04	1.06	0.64	0.65	1.23	1.14
i15:0	8.01	1.15	0.89	1.30	1.27	0.98	0.81
a15:0	3.78	1.04	0.92	1.04	1.16	0.98	0.79
15:0	1.05	0.98	1.23	1.30	1.06	1.92	1.26
i16:1	0.67	0.81	1.21	0.84	1.08	0.88	0.78
i16:0	3.0	1.37	1.28	1.42	1.56	1.28	1.53
16:1ω9c	6.32	0.95	0.84	0.99	1.25	1.06	0.94
16:1ω7t	2.68	1.20	1.22	1.17	1.24	1.22	1.28
16:1ω5	4.79	1.39	1.07	0.87	0.76	0.94	0.66
16:0	15.55	1.05	1.1	1.00	0.90	1.02	0.97
br17:0	0.70	0.74	1.23	1.13	0.71	0.80	0.92
10Me16:0	3.33	1.02	1.32	1.38	1.53	1.30	1.36
i17:0	2.38	1.08	1.46	1.02	1.08	0.89	0.81
a17:0	2.56	0.92	1.21	1.37	1.17	0.87	0.86
cy17:0	3.07	1.33	0.97	1.13	1.07	0.94	0.82
17:0	1.04	0.97	1.05	0.90	0.73	1.23	1.37
br18:0	0.65	0.99	1.12	1.08	1.16	0.75	1.12
18:2ω6,9	2.89	0.78	0.63	1.14	1.35	1.38	1.12
18:1ω9	6.02	0.94	1.02	1.01	0.93	0.76	0.96
18:1ω7	9.55	1.14	1.12	1.21	1.17	1.15	1.18
18:1	2.65	0.68	1.13	0.75	0.98	0.62	0.97
18:0	6.88	0.99	0.63	0.80	0.95	0.95	1.10
10Me18:0	2.94	0.74	0.97	0.81	0.78	1.24	1.12
cy19:0	4.05	0.97	1.03	0.95	1.18	0.78	0.98
20:0	1.31	0.88	1.06	0.79	0.64	1.05	1.02

terial communities, the ratios between the contents of individual PLFAs found in metal-polluted samples and the means of the control were calculated. This ratio was subsequently named as the metal ratio.

The changes in PLFA patterns depended on which metal was added to the soil. The effect of Cu on the profile of PLFA differed from that of the Zn or Cd. On the first sampling day, the most significant difference in the PLFA patterns was found for fatty acid 18:2ω6,9 that decreased with Cu contamination but the increase in response to Zn, as compared to the control, was noticed. In the Cu-treated soil, a metal ratio for 18:2ω6,9 showed 0.82 and 0.78 for the lower and higher dose of Cu, respectively, whereas in the higher Zn-treated soil 18:2ω6,9 had the ratio of 1.61. In turn, there was not significant difference between the percentage of 18:2ω6,9 in the

Table 8

Distribution of PLFAs in soils contaminated with Cu, Zn and Cd 90 days after the addition of metals

PLFA	Proportion of PLFA (%) in control soil	Ratio with given metal					
		Cu (μg g^{-1})		Zn (μg g^{-1})		Cd (μg g^{-1})	
		1000	2000	1000	2000	500	1000
i14:0	0.84	0.86	0.89	0.73	0.69	0.8	0.82
14:0	1.34	0.67	0.71	0.89	1.02	1.12	1.04
i15:0	6.01	0.86	0.8	0.83	0.9	0.98	0.71
a15:0	3.71	1.37	1.56	0.79	0.85	0.89	1.02
15:0	0.98	0.92	0.88	0.93	0.99	0.75	0.82
i16:1	0.85	1.14	1.21	0.72	0.85	2.39	2.17
i16:0	3.22	1.03	0.98	1.02	1.13	1.23	1.94
16:1ω9c	6.44	1.01	1.11	1.12	1.19	0.8	0.76
16:1ω7t	2.68	1.31	1.36	1.62	1.46	1.25	1.62
16:1ω5	5.13	0.98	1.05	1.28	1.31	0.82	0.84
16:0	15.15	0.96	0.92	0.86	0.9	1.02	0.96
br 17:0	1.25	0.8	0.72	0.67	0.79	0.89	1.11
10Me16:0	3.49	1.13	1.1	1.34	1.35	0.76	0.83
i17:0	3.49	1.21	1.15	1.06	1.31	1.18	1.34
a17:0	2.19	1.31	1.25	1.23	1.12	1.25	1.18
cy17:0	2.16	1.31	1.31	1.14	1.13	1.27	1.87
17:0	1.5	0.83	0.87	0.85	0.86	1.04	0.92
br18:0	0.8	1.29	0.8	0.9	0.71	0.69	0.85
18:2ω6,9	2.24	0.88	0.78	2.02	1.78	1.69	1.73
18:1ω9	5.42	1.03	1.13	1.11	1.2	1.26	0.91
18:1ω7	9.69	1.12	1.01	0.9	0.8	0.87	0.9
18:1	4.65	0.79	0.84	0.71	0.62	1.16	1.12
18:0	6.88	0.69	0.9	0.94	1.02	0.94	0.79
10Me18:0	3.08	0.7	0.78	0.74	0.63	0.87	0.94
cy19:0	4.57	1.32	1.28	0.91	0.73	1.1	0.9
20:0	1.3	0.78	0.81	0.86	0.91	0.9	0.89

Cd-polluted and control soil. In response to Cd addition a decrease was found for 15:0, i16:1, i16:0 (500 μg Cd g^{-1}) and br17:0; the highest increase was observed for fatty acids 16:1ω7t and 16:1ω5. The percentage of 16:1ω7t also increased in the Cu-polluted soil. The reverse effects of the lower and higher concentrations of Cu on PLFA patterns were observed. In the lower polluted soil, the strong decrease was found for i15:0, a15:0 and i17:0, whereas the contents of these fatty acids increased in the soil treated with the higher dose of Cu (Table 6).

A decrease in 18:2ω6,9 in the soil contaminated with Cu was still observed on day 60, what indicated by metal effect ratios (Table 7). In the higher polluted soil with Cu the 18:2ω6 ratio was even lower (0.63) than that on day 15 (0.78). In contrast to the data gathered during the first sam-

pling time, the 18:2ω6,9 ratio increased in Cd-polluted soil, and had similar values to those observed in Zn-polluted soils. The highest increase in PLFA was found for the fatty acid 15:0 in Cd-polluted soil, with ratio up to 1.92. The ratios between 1.2 and 1.5 were found for i16:0 and 10Me16:0 in the polluted soils. The exception was found for the soil contaminated with the lower dose of Cu, in which ratio for 10Me16:0 was 1.02. In response to the metals the amounts of 16:1ω7 increased in all polluted soils. The metal ratio varied from 1.20 to 1.28 in Cu or Cd-polluted soils. The highest decrease was observed for i14:0 and i16:1 in soil portions contaminated with Cu or Cd, respectively. In the soil contaminated with Zn, the decrease of 16:1ω5 and 10Me18:0 was found among the major individual PLFAs. Distinct differences between the PLFA profiles obtained from uncontaminated and metal-polluted soils were also observed 90 days after the metal treatment (Table 8). During the last sampling time, the decrease in branched fatty acids i14:0 and i15:0 was observed in all metal-polluted soils. The highest decrease was found in samples contaminated with Zn, where the metal ratio showed the values of 0.73 and 0.69 for the lower and higher dose of Zn, respectively. Significant changes in the amounts of individual fatty acids were also observed for other branched fatty acids, i.e. i17:0 and a17:0. In contrast to i14:0 and i15:0, the amounts of i17:0 and a17:0 increased in metal contaminated soils. The increase was also observed for cy17:0 in soil contaminated with Cd (1000 mg g^{-1}), where the metal ratio reached the value of 1.87. The amount of another cyclopropane fatty acids cy19:0 also increased in Cu-contaminated soils, whereas in Cd-polluted soils it was similar to that found in the control soil. In the higher Zn-polluted soil, the amount of cy19:0 was significantly lower than that in the unpolluted soil; the metal ratio had the value of 0.73. In response to the metals added to the soil changes in the amounts of methylated fatty acids were also observed. All metals decreased the content of 10Me18:0. The highest decrease was observed in the soil treated with the higher dose of Zn (Table 8). Interestingly, a marked increase in the amount of another methylated fatty acid 10Me16:0 was found in the same soil.

PLFAs have been used for evaluating the changes of major fatty acids groups. Isolated PLFAs were classified into following groups: even-numbered, odd-numbered, branched, monounsaturated, polyunsaturated, methyl and cyclopropane fatty acids. In the soils, the three major groups of PLFAs were: even-numbered, monounsaturated and branched fatty acids. As indicated in Fig. 13–15, the profiles obtained from metal-contaminated and control soils were similar, however, distinct differences in the proportion of the PLFA groups were detected. The changes in the PLFA profiles were different at each sampling time and were dependent on the metal doses. On day 15, the significant changes in the percentages of branched-chain and

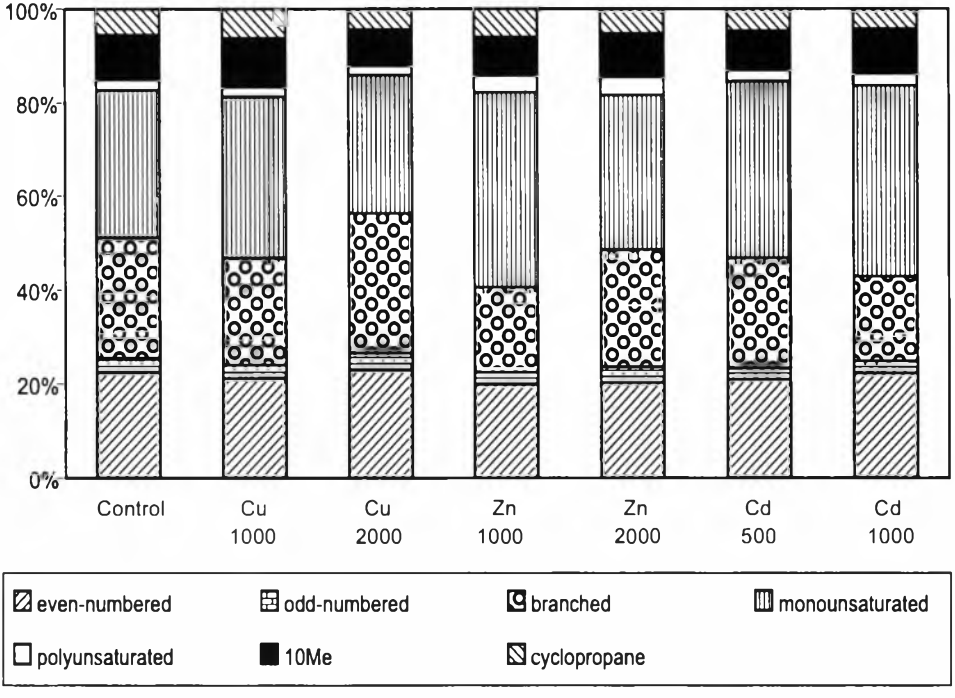

Fig. 13. Proportions of major groups of fatty acids in microbial communities of soil contaminated with Cu, Zn or Cd (in μg g^{-1}) 15 days after the addition of metals

monounsaturated PLFAs were observed in the soils contaminated with higher concentrations of Zn or Cd. The proportion of branched fatty acids constituted about 18% of the total PLFAs and was 30% lower than that in the control soil. In contrast, an increase in the unsaturated fatty acid contents was detected. In Zn- (1000 μg g^{-1}) and Cd-contaminated soils, these fatty acids composed 40% of the total PLFAs, whereas in the control soil their percentage was 30% of the total PLFAs. In Cu-contaminated soil, the percentage of both branched and unsaturated fatty acids was similar to those in the control soil. In turn, the contents of methylated fatty acids decreased in the response to soil contamination with Cu. In the higher polluted soil, the percentage of these fatty acids was 25% lower than in the control soil. The differences between the PLFA profiles obtained from metal-contaminated and control soils were less pronounced over time. On 60 and 90 days, all major PLFA groups were detected at similar amounts. In comparison with control soil, the biggest changes were found for polyunsaturated fatty acid, represented by 18:2ω6,9 fatty acid. During the incubation of soil contaminated with Cu the percentages of 18:2ω6,9 decreased as compared to those in the control soil. In contrast, the amount of this fatty acid was higher in

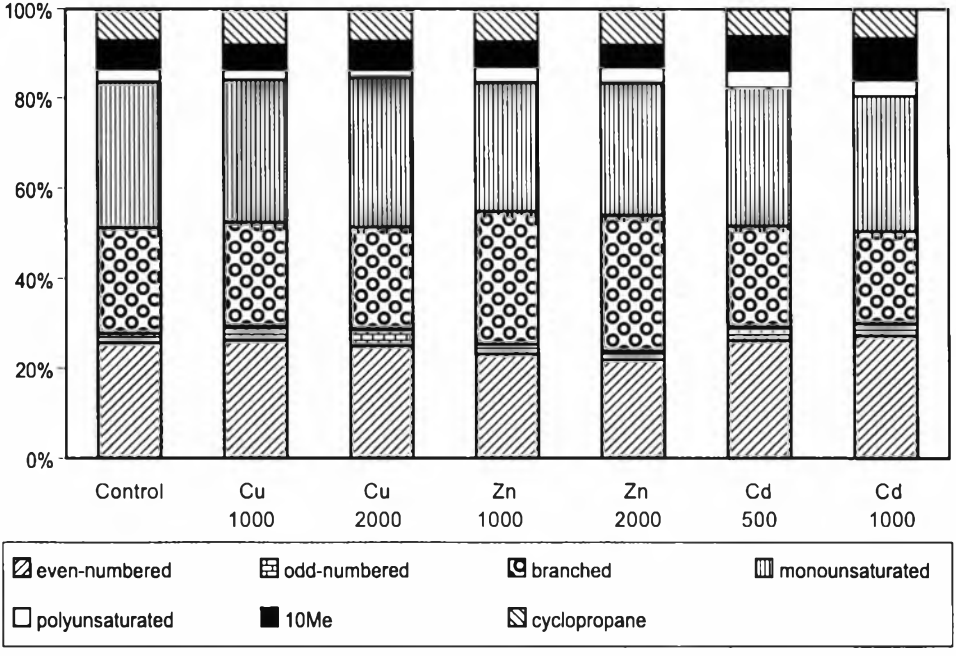

Fig. 14. Proportions of major groups of fatty acids in microbial communities of soil contaminated with Cu, Zn or Cd (in $\mu g\ g^{-1}$) 60 days after the addition of metals

Zn- and Cd-polluted soils as compared to the control. Generally, the impact of heavy metals on the PLFA profiles was more pronounced for individual fatty acids than for groups of PLFAs.

Cluster analysis of the PLFA profiles confirmed differences between the structure of microbial communities in metal-polluted soils and control soils (Fig. 16). The dendrograms created from the data of PLFAs obtained after 15, 60 and 90 days of soil exposure to the metals were similar. Therefore, the dendrogram showing the similarities between the PLFA profiles 90 days after the addition of metals to soil was the only presented. This analysis showed that the microbial populations reacted in different ways to Cu, Zn or Cd-contamination, because contaminated soils grouped into three separate clusters.

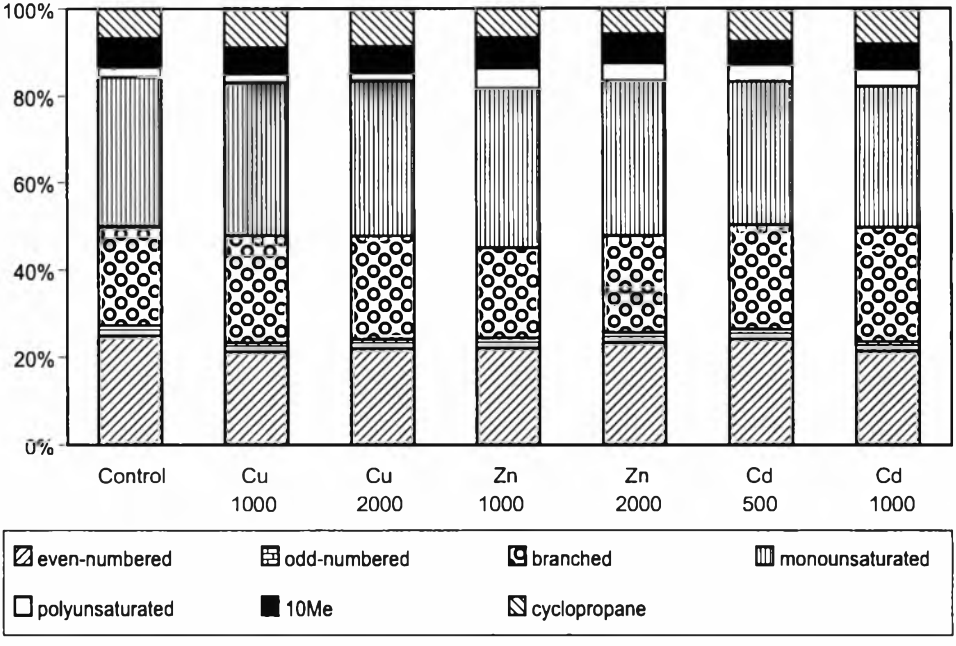

Fig. 15. Proportions of major groups of fatty acids in microbial communities of soil contaminated with Cu, Zn or Cd (in μg g^{-1}) 90 days after the addition of metals

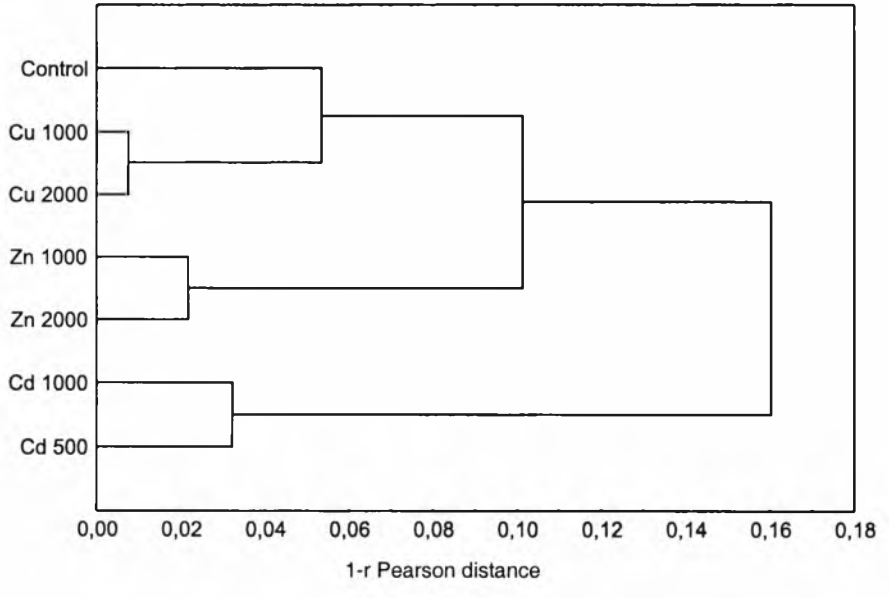

Fig. 16. Dendrogram representing similarities of PLFA profiles of soil contaminated with Cu, Zn or Cd (in μg g^{-1}) 90 days after the addition of metals

4.7. MIDI-FAME analysis

FAME analyses were performed considering bacterial communities that were able to grow on agar plates. In total, 25 different FAMEs were detected in the bacterial biomass isolated from all soil samples. On overage, from 10 to 25 different FAMEs were identified in cells of culturable fractions of bacteria isolated from each soil sample depending on the pollution and time of sampling. In general, the highest numbers of FAME were found in the bacterial cells cultured from the uncontaminated soil, whereas the lowest FAME numbers were detected in the bacterial biomass isolated from Cu- or Cd-contaminated soils (i.e. 10 and 12, respectively). Specific differences among the soils were clarified by the comparison of FAME structural classes. All soil samples contained a variety of the FAMEs comprising of saturated, unsaturated and branched fatty acids. Cyclopropane fatty acids were detected in uncontaminated soil and some metal-polluted soils, whereas the methyl-branched (10Me17:0) fatty acid was only found in a few metal-polluted soil samples (Fig. 17–19). The MIDI-indistinguishable fatty acid 18:1 w9c/ω12t/ ω7c, an indicator for *Pseudomonas* that was presented in almost all soil samples, was excluded from the group of unsaturated fatty acids.

At each sampling time, the distinct differences between the profiles of FAMEs obtained from the culturable fraction of bacteria isolated from the control and metal-treated soils were observed (Fig. 17–19).

For all bacterial communities, the FAME profiles were dominated by branched-chain fatty acids (e.g. i12:0, i14:0, a14:0, i15:0, a15:0). However, their percentages were always higher in Cu-, Zn- or Cd-polluted soils when compared to the control (Fig. 17–19).

On day 15, the amount of branched fatty acids increased from 47% in the control soil to about 70% in the soil contaminated with Zn or Cd (Fig. 17). In turn, the percentages of saturated fatty acids (i.e. 12:0, 14:0, 16:0, 18:0) decreased in the soil polluted with heavy metals as compared to the control. The highest decrease was observed in Cu-contaminated soil portions in which the amounts of these fatty acids were 4.5- and 5.8-fold lower than those in the control soil. The similar situation was observed for unsaturated fatty acids (i.e. 16:1ω7c, 16:1ω11c, 18:1ω9c) in samples polluted with Zn or Cd. However, the effect in Cd-polluted soils was strongly dependent on the dose of metal added to the soil. In the lower polluted soil, the percentage of unsaturated fatty acids (4.8%) was 3-fold lower than that (15.22%) in higher polluted soil. The metal-treatment also resulted in the lower proportion of 18:1ω7c/ω9t/ω12t in comparison with the control soil. This fatty acid composed of 8.8% of the total FAME profile in the uncontaminated soil, and its percentages decreased to 2.0, 4.2 and 4.1% in the soils

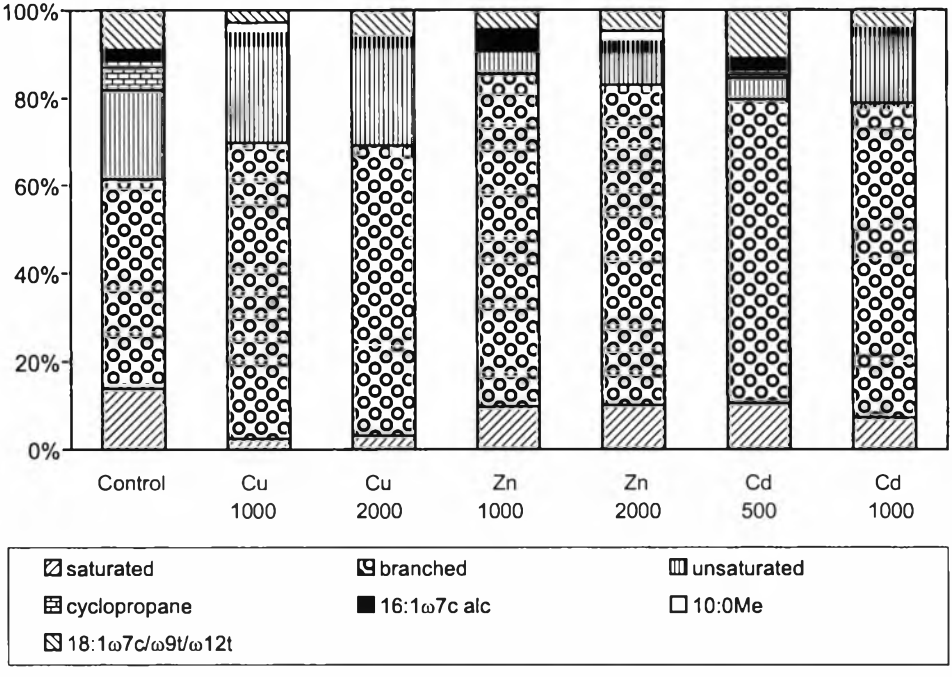

Fig. 17. Proportions of major groups of fatty acids in the communities of culturable bacteria isolated from soils polluted with Cu, Zn or Cd (in μg g^{-1}) 15 days after the soil contamination

contaminated with Cu (1000 μg g^{-1}), Zn (1000 μg g^{-1}) and Cd (1000 μg g^{-1}), respectively. During the first sampling time, cyclopropane fatty acids were only found in the control samples, and small amounts (2.4%) of methylated FAMEs were detected in the soil polluted with the lower dose of Cu and the higher dose of Zn.

The FAME profiles obtained on day 60 showed that changes in the community structure of the culturable fraction of bacteria were similar to those observed on day 15 (Fig. 18). The domination of branched-chain fatty acids in the FAME profile was similar to that found at the first sampling time. These fatty acids constituted almost 67%, 73% and 69% of the total fatty acids detected in the higher polluted soils, while they represented 54% in the control sample. As compared to the control soil (i.e. 15.1%), the proportion of saturated fatty acids significantly decreased in Zn-polluted soils (7.1% and 6.0% for the lower and higher dose, respectively), whereas no differences among the amount of saturated fatty acids in the control, Zn- or Cu-polluted soils were found. In contrast to the first sampling time, the amount of unsaturated fatty acids extracted from the bacterial community in Zn-contaminated soils was similar to that in the control. In addition, this

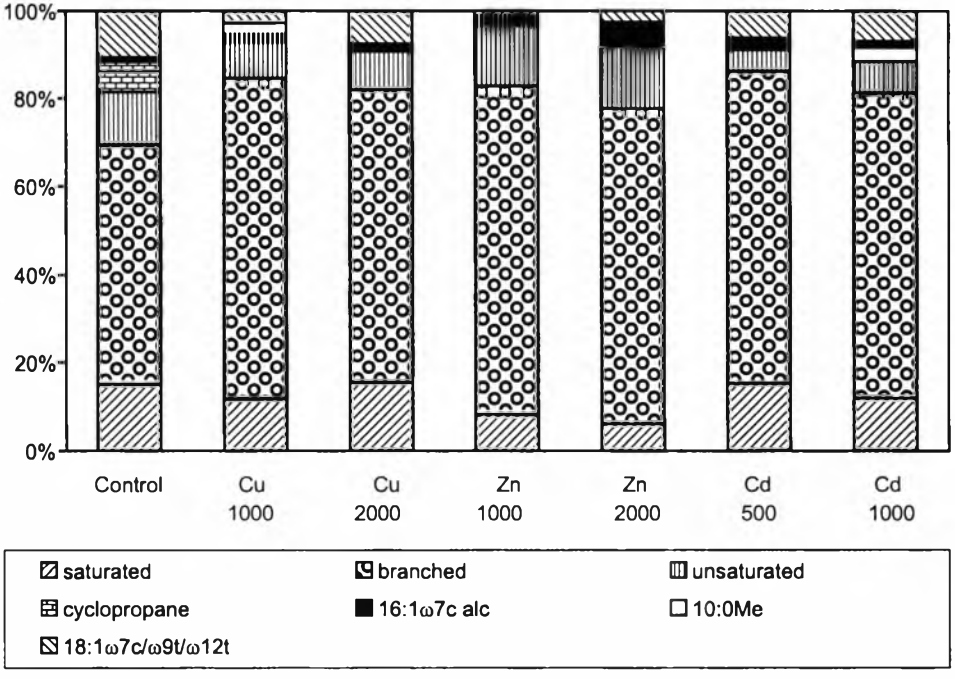

Fig. 18. Proportions of major groups of fatty acids in the communities of culturable bacteria isolated from soil polluted with Cu, Zn or Cd (in μg g^{-1}) 60 days after the soil contamination

amount was even higher than that observed in Cu-contaminated soils. When compared to the soil polluted with Cu or Cd, the bacterial community cultured from the soil contaminated with Zn (2000 μg g^{-1}) contained significantly higher concentrations of 16:1ω7c alcohol. The percentage of this FAME was 5.6%, whereas it was about 2% in the bacterial biomass isolated from soils contaminated with Cu. In the soil contaminated with Cd the amount of 16:1ω7c alcohol depended on the dose of metal added. In the soil polluted with 1000 μg Cd g^{-1} the amount of this FAME was 2.6-fold higher than compared to the lower polluted soil. The cyclopropane fatty acids were only detected in the bacterial biomass isolated from the control and higher Cd-polluted soil; their content in Cd-contaminated soil was 2-fold lower than in the control soil.

The analysis of the FAME profiles from the last sampling indicated that after 90 days differences in bacterial community structures for different metal treatments persisted. However, the differences in the FAME patterns between the control and metal-contaminated soils were lower when compared to the first and second sampling times. The proportion of branched fatty acids in the bacterial biomass cultured from soils contaminated with Cd decreased

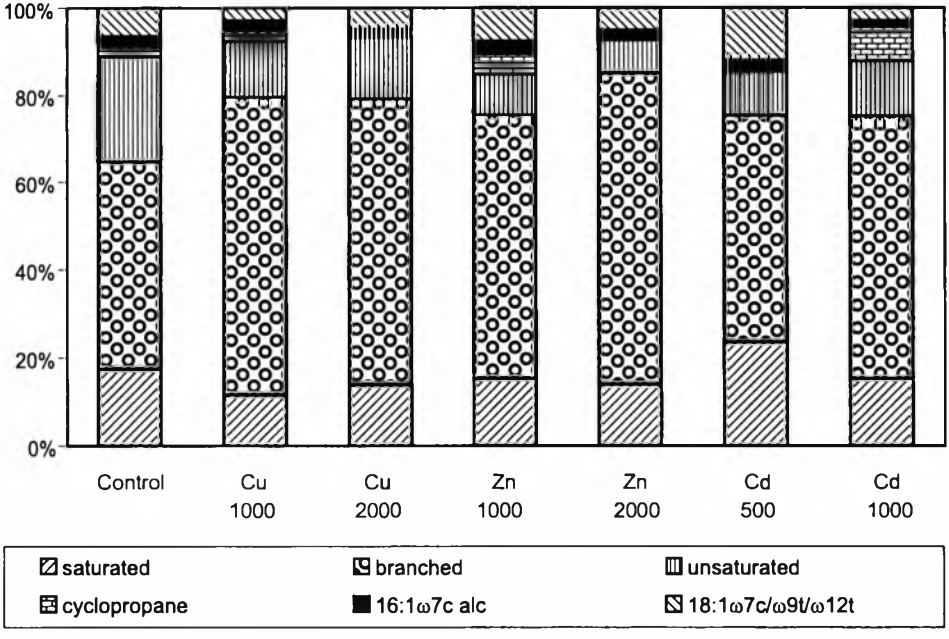

Fig. 19. Proportions of major groups of fatty acids in the communities of culturable bacteria isolated from soils polluted with Cu, Zn or Cd (in μg g^{-1}) 90 days after the soil contamination

from 70% on day 15 to 51.8 and 59.7% on day 90, for the lower and higher dose of Cd, respectively. However, the percentages of branched fatty acids detected in bacterial cells isolated from soil polluted with Cu or Zn soils were similar to those found during the first sampling time. On day 90, the contents of saturated fatty acids obtained from the bacteria isolated from metal-treated soils were higher than those found on day 15. In addition, the amounts of these fatty acids were similar in all samples on day 90, with one exception. For the bacterial cells isolated from the soil contaminated with Cd at the dose of 500 μg g^{-1}, the amount of these fatty acids was significantly higher (23.7%) than in the biomass from the control soil (16.9%). The FAME profiles also showed changes in the percentage of unsaturated fatty acids over time. On day 90, the contents of unsaturated fatty acids found in the bacterial cells isolated from both Cu-polluted soils were significantly lower than those on day 15. In contrast, the concentration of these fatty acids in bacteria from Zn (1000 μg g^{-1}) and Cd (500 μg g^{-1}) polluted soils were about 2-fold higher than those on day 15. On day 90, the contents of unsaturated fatty acids were present at the similar percentage in bacterial biomasses isolated from the metal-polluted soils. During the last sampling time, cyclopropane fatty acids were detected in the bacterial fraction cultured from

the control (2.0%), the soils contaminated with the lower dose of Cu (2.4%) and Zn (4,5%), and the soil polluted with the higher dose of Cd (7.7%). By contrast, the methylated fatty acids were not found in any samples.

The cluster analysis of the FAME profiles, considering the quantities of all fatty acids obtained, confirmed the changes observed between the community structures of the culturable fraction of bacteria isolated from the uncontaminated and metal-contaminated soils. On day 15, close similarity between the communities cultured from soils contaminated with Cu or Zn at the same dose was found (Fig. 20).

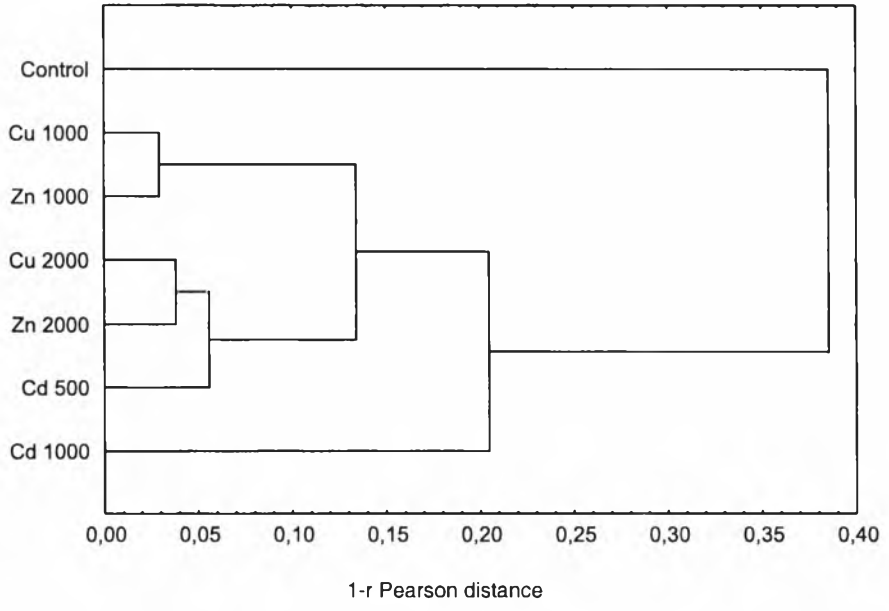

Fig. 20. Dendrogram representing similarities of the culturable bacterial fraction profiles obtained with MIDI-FAME analysis in soil contaminated with Cu, Zn or Cd (in $\mu g\ g^{-1}$) 15 days after the addition of metals

In contrast, the concentrations of Cd formed separate lineages indicating the most distinct changes, especially in the FAME profiles of the community exposed to the higher dose of the metal. The bacterial response to the metals changed over time and close similarity was found between soils contaminated with the same metals (except Cd) at both concentrations on day 90 (Fig. 21).

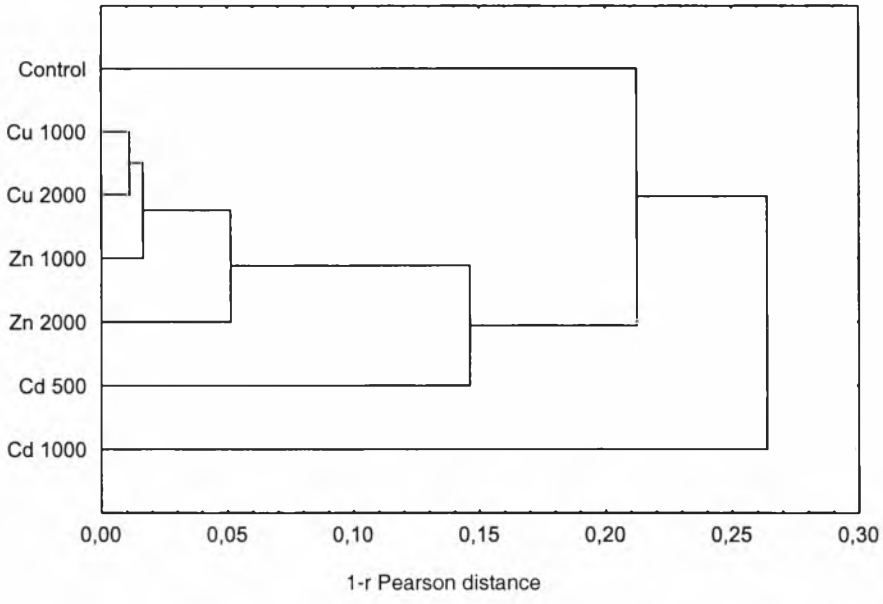

Fig. 21. Dendrogram representing similarities of the culturable bacterial fraction profiles obtained with MIDI-FAME analysis in soil contaminated with Cu, Zn or Cd (in $\mu g\ g^{-1}$) 90 days after the addition of metals

5. Discussion

5.1. Metal bioavailability and soil pH

The effect of heavy metals on soil microorganisms depends on their availability in the soil solution. In this study, the bioavailable fractions of Cu, Zn or Cd were estimated as the amounts of these metals in water extracts of contaminated soil. Generally, the concentrations of water-soluble Cu, Zn or Cd were low and represented a few percentages of the total metal contents. The strong decrease of water-soluble Zn was also observed by Leita et al. (1995), who found that the water-extractable fraction of Zn represented 2.8% of total Zn in soil contaminated with 600 μg Zn g^{-1} and 200 μg Pb g^{-1}. They found that the concentration of water-exchangeable Zn decreased over time and constituted 1.9% of total Zn after 6 weeks of soil incubation. The low concentration of water-soluble fraction of Cd was reported by Renella et al. (2004), who studied the bioavailability of Cd in long-term Cd-contaminated soil. They found that in soil containing 0.36 mmol Cd kg^{-1} the percentage of water-extractable Cd ranged from 0.5 to 9.5% in different plots, indicating a low availability of Cd. Using the BIOMET biosensor they did not detect bioavailable Cd in the soil amended with 0.18 mmol Cd kg^{-1}, whereas the response was detected in soils containing 0.36 mmol Cd kg^{-1}. As a result, it was proved that enough Cd bioavailable to evoke a bacterial response encoded by a specific resistance mechanism was presented only in this soil. Pandeya et al. (1998) reported that the water-soluble and exchangeable fraction of Cd in different soils accounted for 4.3 to 7.2% of total Cd. In contrast, Ahnstrom and Parker (1999) found that Cd in the soluble-exchangeable fraction obtained from a specific sequential extraction procedure accounted less than 20% of total Cd. Such big differences in the amount of exchangeable Cd found in various studies on metal availability should be ascribed to differences in soil properties and different techniques used for extraction (Harter and Naidu, 2001). The

water-soluble Cu also represented a small fraction of total soil Cu content. Tom-Petersen et al. (2003) studying the effect of copper amendment on the bacterial community found that less than 0.5% of the Cu added was biologically available for *P. fluorescens* DF57-Cu15 – a Cu-specific reporter strain. In their study, the bioavailable Cu fraction tended to be higher in low-Cu field plots than that compared to high-Cu field plots, what was not observed in presented study. The lower concentrations of Cu in highly contaminated samples were explained by them by a relative decrease in water solubility of Cu in increasing levels of contamination. Similarly to this study, they observed the decrease in bioavailable Cu fraction during the experimental period. They suggested that the mechanism leading to a progressive reduction in water solubility might be associated with diffusion of Cu into inaccessible pores in soil matrix.

There appears to be no simple relationship between the total metal content, number of microorganisms and their level of activity in soil contaminated with heavy metals. Total metal concentrations in soil are inaccurate for predicting any of their effects on activity and composition of the soil microbial communities, since soils vary considerably in their capacity to bind metals in biologically unavailable forms. Ullrich et al. (1999) measured the total and bioavailable contents of Pb, Cd and Zn in soils of extensive historical mining and smelting of lead and zinc at the Upper Silesia. To assess the potential availability of Pb, Zn and Cd to plants they measured the exchangeable fraction (i.e. in 0.5 M $MgCl_2$) of metal ions. They found that the levels of available fractions were generally low for Pb (less than 1%, maximum 15.6% of total metal content), moderate for Zn (less than 10%, maximum 32.4% of total metal) and high for Cd (mean 35%) and were strongly influenced by soil pH. Similarly, Ivask et al. (2004) using both chemical and biosensor analysis for the estimation of Cd, Zn and Pb bioavailability, observed that only $\mu g\ l^{-1}$ levels of metals were extracted from the soil containing up to 20 mg/kg of Cd, 1390 mg/kg of Zn and 1050 mg/kg of Pb in the water phase. They extracted only 0.1%, 0.5% and 0.07% of total cadmium, zinc and lead, respectively.

It has been well recognized that metal availability is controlled by soil physico-chemical properties and for this reason, similar heavy metal inputs to contrasting soil types may have different impacts on the number of bacteria, biomass and microbial activity (Hattori, 1992; Khan and Scullion, 2000; Frostegård et al., 1993a). Hattori (1992) observed that metals inhibited CO_2 evolution more in a soil with low organic matter and cation exchange capacity than that with higher organic matter and cation exchange capacity. Similarly, Maliszewska et al. (1985) found that metals were, in general, less toxic to microorganisms in fine-attributed alluvial than in sandy loams. They explained the differences in the metal toxicity by

variations in sorption capacity of soils used in their study. These findings have been supported by results obtained by Khan and Scullion (2000), who studied the microbial responses to metal contamination in five soils with varying clay and organic contents. They observed that both metal bioavailability and microbial responses to heavy metals differed significantly between soils. In soils contaminated with higher concentrations of Zn, Ni and Cu the increase in water-soluble metal concentrations was much more pronounced in soils with lower organic matter contents. In this study, heavy metals added to the soil did not strongly affect the total number of heterotrophic bacteria for long period of time what might be connected with the low availability of Cu, Zn and Cd in the soil solution. Since the impact of metals on soil microorganisms depends on their availability in soil solution, the adverse effect of metals may partially decrease or completely disappear if they are removed from the soil solution by binding to clay particles or to organic materials, precipitating at an alkaline pH and complexing or interacting with other chemicals (Ross, 1994b; Krishnamurti and Naidu, 2000). Shi et al. (2002a) showed the role of soil particle in the protection of bacterial cells against heavy metals toxicity by comparing the metal resistance of bacteria present in soil and bacterial cells extracted from the soil. They found that adverse effect of Cr and Pb occurred at much lower concentrations in soil bacterial extracts than in the soil system. The metal toxicity might also be reduced by specific bacterial properties of some soil microorganisms. Kunito et al. (2001a) in long-term field studies observed that Cu-resistant bacteria, which produced a large quantity of exopolymers, reduced the toxicity of Cu in soils and thus enhanced the growth of other microorganisms and plants. However, Jensen-Spaulding et al. (2004) observed 2 to 4-fold increase in dissolved copper and lead in the presence of polymer, indicating that naturally present bacterial polymers in soil may have an influence on metal mobility.

The results of this study show that concentrations of water-soluble Cu, Zn and Cd decreased over time. In recent study, lasting over 180 days, Vig et al. (2003) found that the concentration of Cd in soil solution of freshly contaminated soil (3 mg Cd kg^{-1}) decreased exponentially with time to < 0,00006 mg l^{-1} within 50 days of aging. They suggested that most of the short-time laboratory experiments have presumably overestimated the effect of Cd on soil microbial biomass and activity. The decrease in metal bioavailability with time was also reported by Turpeinen et al. (2004). They found that As compounds that have aged in soils were less available than when freshly added to soil.

The contamination of soil with the highest concentrations of Cu, Zn or Cd salts was accompanied by a one-unit decrease in soil pH in this study. It has been observed that a one-unit decrease in soil pH resulted in a two-

fold increase in the concentration of Cd, Ni and Zn in the soil solution (Sanders et al., 1986). The highest decrease in the soil pH was found in soil with the higher Cu amendment. The cation Cu^{2+} has a greater tendency to hydrolyse in solution and is also more strongly adsorbed by soils than Zn^{2+} and Cd^{2+} (Percival et al., 1999). This results in the higher concentration of H^+ in the solutions of soil contaminated with Cu when compared to Zn or Cd. It has been shown that the decrease in the pH solution for a given amount of metal added followed the sequence Cu = Pb > Cd = Ni = Zn (Basta and Tabatai, 1992; Speir et al., 1999). Because soil pH influences the availability of metals and their toxicity, in ecological studies the role of organic matter and clay content for the maintenance of pH stability should also be considered (Ross, 1994b). Speir et al. (1999) reported the greatest declines in pH in the soil that contained more than 80% of sand-size particles and had the lowest C content and cation exchange capacity of the soils under investigation. As a consequence, this soil had a much lower buffer capacity against the acidifying effect of the pollutants. Since pH has a large effect on metal solubility and mobility, there is often difficult to separate the influence of heavy metals from the effects of pH and other environmental factors.

5.2. Bacterial numbers

Copper, zinc or cadmium treatment had a strong effect on the size of microbial communities only at the beginning of soil incubation, as was indicated by viable counts. On day 15, the numbers of bacteria were about 80% lower in highly metal-contaminated soil samples than those compared to the control. However, after 60 days no significant differences were observed among the numbers of total heterotrophic bacteria cultured from Cu-, Zn-, or Cd-polluted and the control soils. The similar short-time effect of Cd and Zn on microbial population was observed by Kozdrój (1995) and Kelly et al. (1999a). Kozdrój (1995) found that Cd applied to soil even at very high concentration (5000 $\mu g\ g^{-1}$) significantly decreased the total number of heterotrophic bacteria only for 2 weeks. Also Kelly et al. (1999a) observed the toxic effect of Zn (6538 $\mu g\ g^{-1}$) on viable counts for a few weeks. For zinc microcosms, they found that two weeks after soil contamination viable counts decreased to 86.3% when compared to the uncontaminated control. From day 45 to the end of the experiment (420 days), the total number of heterotrophic bacteria was equivalent to the control soil. In the same study, they observed that the same high Zn concentrations had different effects on viable counts in the laboratory and in the Zn smelter field

soils. While in the laboratory study the viable counts were not affected by Zn-treatment after the first sampling day, in the field study the effect of metal contamination on total bacterial number was noticed. They explained this difference by the fact that laboratory soils were treated with Zn only, whereas the field soils had additionally highly elevated loadings of nickel, lead, chromium and copper. Contrary to short-time studies, the total number of heterotrophic bacteria was not affected in soil contaminated with heavy metals for many years (Turpeinen et al., 2004). Renella et al. (2004) studying the impact of Cd on microbial community structure in long-term Cd-contaminated soil did not find significant changes in bacterial counts. Cadmium did not affect the total cfu on rich media, however, a decrease in numbers on a minimal medium amended with glucose as a sole C source was observed for the soil containing 0.18 and 0.26 mmol Cd kg^{-1}. Moreover, an increase in the viable counts of bacteria was found in the soil polluted by emissions from the iron and steel work (Bååth et al., 1992).

Microbial numbers and functions in soils are typically quite resilient towards metal contamination, they are often found to recover after an initial inhibition by high metal inputs (Kelly et al., 1999a). In this study, heavy metals did not significantly affect the viable counts longer than 15 days what might be attributed to decreasing availability of Cu, Zn or Cd added due to immobilization reactions in the soils. Indeed, a gradual decrease in the percentages of water-soluble fraction was observed over time. The second factor that might contribute to bacterial recover was the replacement of metal sensitive microorganisms by tolerant ones within each functional group, thus changing the structure of microbial community and increasing bacterial metal tolerance. In this study, the tolerance of bacterial populations to heavy metals was not determined. Nevertheless, the high numbers of bacteria in soils polluted with Cu, Zn or Cd indicated their natural tolerance. It is well recognized that many soil bacteria are intrinsically resistant to high concentrations of heavy metals (Duxbury and Bicknell, 1983; Hassen et al., 1998; Kunito et al., 2001a).

Bacteria can adapt to toxic levels of heavy metals by means of intrinsic properties of the cell, including those related to their cell wall structure, extracellular polysaccharide production, and the ability to bind or precipitate metals inside or outside the cell. In addition, in response to heavy metals bacteria can evolve specific mechanisms of detoxification that include processes such as reduction (Misra, 1992), sequestration (Cooksey, 1993) and active efflux of some metals (Nies and Silver, 1995). The estimation of bacterial community tolerance can be a direct way of detecting toxic effects of heavy metals in soil, since an altered tolerance should reflect a selection pressure due to a toxic substance. The presence of elevated metal concentration can change the microbial community leading to the increase

in the numbers of metal-tolerant and metal-resistant species (Kelly and Tate 1998b; Bååth et al., 1998; Renella et al., 2004). The increase in heavy metal resistance caused by heavy metals was observed by Diaz-Raviňa and Bååth (1996), who used the thymidine incorporation technique to measure the metal tolerance of soil microbial communities experimentally exposed to elevated heavy metal concentrations. They found significant increases in metal tolerance in highly polluted soils and the degree of metal tolerance continued to increase throughout the time of study. Comparisons between microbial communities originating from unpolluted and heavy metal polluted sites can give information on changes that have occurred in communities as a result of metal contamination, but such studies do not provide information on the time course of these changes. It may have taken some time for soil microbial communities to adapt to the stress caused by elevated metal concentrations and for metal-resistant members of the community to increase the numbers in metal-contaminated soils (Pennanen et al., 1996). An increase in Zn-resistant populations over time was showed in the study conducted by Kelly et al. (1999a). Although in all samples Zn-tolerant microorganisms comprised a small proportion of the bacterial communities over 420 days of incubation, the Zn-tolerance of the microbial populations caused by Zn amendment changed significantly over time. Thirty-five days after soil contamination, the Zn-tolerance for the Zn-polluted soil was 0.75%, compared to 0.08% for the control. The Zn-tolerance of microorganisms from Zn-treated microcosms continued to increase to 2.31% by day 420 (Kelly et al., 1999a). Renella et al. (2004) found that the proportion of bacteria growing on selective media containing 0.2 and 0.4 mmol of Cd significantly increased in the most contaminated soils when compared to the control soil. However, they suggested that large inputs of Cd in these soils rather induced some physiological adaptations than a selection process, since no genetically determined resistance mechanisms were identified from the screening of culturable bacterial populations. In contrast, Angle et al. (1993) found that most bacteria isolated from soil were resistant to very high concentrations of heavy metals, regardless of whether the soils were contaminated with metals or not. They showed that the average Zn tolerance of bacteria isolated from highly polluted and unpolluted sites was 75 and 26 mg l^{-1}, respectively, despite very small content of extractable forms of zinc. In studies reported by Ellis et al. (2001), the proportions of metal-resistant bacteria in soils under investigation were highly variable. They assumed that proportions of resistant bacteria might only be useful for differentiating polluted from unpolluted sites but not for the quantitative assessment of pollution degree. The development of metal tolerance of soil bacteria is strongly dependent on the concentrations of heavy metals that are present in available forms. Almås et al. (2004), studying the Zn- and Cd-tolerance

of soil microbial community by the incorporation rate of [^{3}H] thymidyne at different metal concentrations, found that the metal tolerance was correlated with the concentrations of heavy metals in pore water. Similarly, Rasmusen and Sørensen (2001) reported that the microbial response to Hg(II), observed as development of Hg-resistant bacteria, was correlated with the concentrations of bioavailable Hg. Turpeinen et. al. (2004), studying the microbial community structure in soil contaminated for many years with chromated cooper arsenate (CCA), revealed that the total numbers of bacteria were not affected by metal contamination and the proportion of As(III)-resistant strains was dependent on the concentration of As in soils. A significant positive relationship was found between the bioavailability of As and the proportion of As(III)-resistant bacteria. In contrast, the ratio of As(V)-resistant bacteria to the total heterotrophic population was relatively independent of the concentration of As(V). They suggested that As(III)-resistant bacteria were selected due to the high toxicity of As(III). Although the presence of As-resistant bacteria in the soils was assessed by culturing and may not be representative of the entire microbial community, terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA analysis showed a shift within the structure of microbial community (Turpeinen et al., 2004).

Giller (1998), studying the metal resistance of bacteria in soil amended with sewage sludge containing heavy metals, found an increase in numbers of plasmids, mediating metal resistance in *Rhizobium leguminosarum* bv. *viceae* with increasing metal contamination. Recently, the important contribution of mobile genetic elements (MGE) such as plasmids, phages, transposons and gene cassettes to bacterial adaptability to environmental stress has been fully appreciated (Smalla and Sobecky, 2002). The significance of horizontal gene transfer was first recognized when multiple antibiotic resistant pathogens occurred. MGE are undoubtedly an important and essential component that promotes bacterial diversification. MGE play a primary role in the spread of many specific bacterial genes, e.g. those responsible for metal-resistance. These elements allow bacterial species rapidly adapt to a strong selective pressure of contaminants (Tschäpe, 1994).

The studies concerning the impact of heavy metals on viable counts of bacteria and their biomass showed that even with a high amount of metal contamination bacterial populations in metal-polluted soil can reach the same size as the population in the control over time. The results obtained by Turpeinen et al. (2004) supported the thesis that microbial community is able to compensate reduced numbers with time. According to T-RFLP results, they revealed that this compensation was not due to a reversion towards the pre-exposure community but mainly due to the appearance of new dominating species. They found that As(V) stimulated the proliferation of certain groups of microorganisms in soil resulting in a shift of the microbial

community that comprised only a few tolerant species. Therefore, these results support the conclusion suggested by Bååth et al. (1998) that population size may not be the most sensitive indicator for assessing the impact of heavy metal on soil microbial community dynamics. In contrast, Ellis et al. (2003) suggested that the bacteria that form colonies on laboratory media may be considered an ecologically relevant parameter. Their study proved that the effect of different heavy metal concentrations on the dominant bands in denaturing gradient gel electrophoresis (DGGE) analysis of DNA extracted directly from soil was minimal when compared to the effects on the culturable fraction of the community. They assumed that the metals had a detrimental effect on certain bacteria by affecting their ability to replicate on artificial media. Although only a small fraction of total bacteria population can form colonies on agar plates, they constitute a large proportion of the total microbial biomass. Culturable bacteria usually represent the bacterial largest size group and ecologically important fraction of soil bacterial community (Hattori et al., 1997). It is also possible that these bacteria represent a large proportion of metabolically active bacteria, since increases in specific thymidine incorporation rate were found in soil polluted with dust from an iron steel work (Bååth et al., 1992).

5.3. Microbial biomass

Soil microbial biomass is closely related to soil fertility and environmental quality: it serves as a pool of nutrients and often is used as an indicator of microbial changes in soils. Most attention is given to fungi and bacteria because these two groups of microbes are the most important for energy flow and nutrient transfer in terrestrial ecosystems.

In this study, the decrease in soil bacterial biomass, expressed as amount of PLFAs of bacterial origin, was strongly affected by all metals used. In Cu-, Zn- or Cd-polluted soils a marked decrease in the microbial biomass was found during the first sampling time, and then the biomass increased in all metal-polluted soils throughout the study. However, microbial biomass remained significantly lower than in the control in soils amended with the higher dose of Cu or Zn and both doses of Cd at each sampling time. A significant decrease in microbial biomass in short time after soil amendment was also observed by Kelly et al. (1999a), who studied the effects of high Zn-contamination on soil microbial community. On day 15, they found that the microbial biomass was 50% lower in Zn-amended soil than that in the control. However, in contrast to the present study, they observed that the biomass for Zn-treatment was statistically equivalent after day 15 (till day 420).

Considerable numbers of evidence documenting the decrease in soil microbial biomass as a result of long-term exposure to heavy metals have been reported (Brookes and McGrath, 1984; Knight et al., 1997; Speir et al., 1999). Yao et al. (2003), studying microbial biomass in paddy soils with different metal concentrations in the vicinity of Cu-Zn smelter, observed nearly 10-fold decrease in the microbial biomass C_{mic} in a soil close to the source of contamination when compared to the soil located 5 km from the smelter. They observed a consistent decrease in the microbial biomass C with an increase of heavy metal concentrations. A significant decrease in microbial biomass was also observed in soil treated with the high-Cu sludge amendment (Bååth et al., 1998). For this soil, the detrimental effect of sewage sludge containing heavy metals also caused a significant decrease in soil respiration rate and microbial activity. Chander and Brookes (1991) also observed a net decrement in the soil microbial biomass as a response to treatments with a sludge heavily contaminated with Zn and Cu. However, the soil respiration remained at the same level. They explained the results suggesting that metals may disturb the division of energy between the growth and maintenance of soil microorganisms which require greater amounts of C for maintenance under metal stress. As a result, a decrease in microbial biomass measured as C_{mic} was observed. Chander and Brookes (1993) reported that amendment of soil with single-metal (Cu, Ni or Zn) sludge had only minor effects on soil microbial biomass, whereas the application of bimetallic sludge (Ni + Zn or Cu + Zn) decreased the total bacterial biomass by 41% and the ratio of microbial biomass to organic C by 53% when compared to the control. Konopka et al. (1999), studying the microbial biomass size in Pb-contaminated soils, found that the biomass estimated by phospholipid phosphate was 8- to 30-fold lower in the soil with high level of Pb (48 mmol of Pb kg^{-1}) than in the control soil. Leita et al. (1995) reported the high negative impact of Zn on microbial biomass C. In particular, a 32% decrease in microbial biomass C was observed after the first week of incubation, and 46% after the third week. The parameter values remained constant till the end of the experiment lasting 8 weeks. They found that bacterial biomass was not such closely related to content of soil organic matter as other microbial indices such as respiration or metabolic quotient (qCO_2). The analysis of soils in the vicinity of metal-contaminated sites confirms that a decrease in soil microbial biomass occurs at a relatively modest, and sometimes even at a surprisingly low metal load (Kuperman and Carreiro, 1997). On the contrary, Shi et al. (2002a) did not observe significant decrease in microbial biomass in soils that were contaminated with Pb and Cr for more than 40 years. On the other hand, many studies reported that habitats subjected to long-term metal stress, even at modest exposure, are not able to maintain the same overall biomass as in uncontaminated

habitats (Roane and Kellog, 1996; Kuperman and Carreiro, 1997; Kandeler et al., 1996).

In some studies, soil microbial biomass measured by the fumigation extraction method, was not adversely affected by Cd added to soil even at a very high concentration ranged from 500 to 1000 μg Cd g^{-1} (Fritze et al., 2000; Landi et al., 2000). Also Renella et al. (2004) reported that microbial biomass was lower in soils contaminated with Cd for two years at the concentration from 0.09 to 0.36 mmol Cd kg^{-1} when compared to that in the control soil, but the differences were not significant. Ekelund et al. (2003) found a significant decrease in microbial biomass, determined by substrate-induced respiration (SIR) method, in soils contaminated with Cu at the concentrations of 400 and 1000 μg Cu g^{-1}. However, similar to the results of this study, their study proved that Cu-contamination had a short-time effect on some microbial parameters. The reduction in microbial biomass was observed 7 days after metal contamination, whereas the level of microbial biomass was similar to that measured in the unamended control on day 14 and 70 (Ekelund et al., 2003).

Soil microbial biomass is difficult to measure and different methods are applied in studies on environmental factors that affect the microbial biomass. Beside such methods as substrate-induced respiration (SIR) (Anderson and Domsch, 1978) and ATP content (Balkwill et al., 1988), the phospholipid fatty acid (PLFA) analysis (Frostegård and Bååth, 1996) and the chloroform-fumigation extraction (CFE) procedure (Jenkinson and Powlson, 1976) are now routinely used to estimate soil microbial biomass. Bailey et al. (2002) found a strong relationship between these two measures for mineral soils and proposed the equation to convert PLFAs to CFE – the more common measure of microbial C. Theoretically, only one or a few PLFAs could have been used to estimate bacterial biomass. As it has been reported by Frostegård and Bååth (1996), the pattern of measured biomass was similar when only i15:0 was used as a bacterial indicator and when the sum of bacterial PLFA was regressed against the microscopical counts. Since the proportions of individual PLFA differ between bacterial species, they recommended the use of several bacterial PLFAs, because this is likely to obtain less variable results as compared to using only single PLFA. However, application of different methods makes it often difficult to compare the results obtained for different soils. Murata et al. (2002), studying the relationship between soil bacterial community structure and the amount of bacterial biomass, found that bacterial biomass estimated by the contents of PLFAs was directly proportional to biomass calculated from FAME profiles obtained from soil samples. They revealed that larger amount of bacterial biomass in soil was correlated with higher diversity of culturable soil bacterial community growing on agar medium.

In this study, fungal biomass was estimated by the amount of PLFA 18:2ω6,9 which is regarded as a reliable indicator of fungal biomass (Frostegård et al., 1993a). In several studies, ergosterol, the fungal-specific component of the cell membrane has been used as fungal indicator (Grant and West, 1986; Frostegård and Bååth, 1996; Khan and Scullion, 2000). Frostegård and Bååth (1996) found the concentration of 18:2ω6,9 to be positively correlated with the ergosterol in different agricultural and forest soil that supported application of this PLFA as an indicator of fungal biomass. However, they stressed that PLFA analyses had an advantage since the same technique was adopted for both fungal and bacterial biomass measurements.

In this study, it has been observed that fungi differed in their response to heavy metals stress. The fungal biomass increased significantly in the soils contaminated with Zn or Cd, whereas the biomass markedly decreased in Cu-contaminated soils. Also, Frostegård et al. (1993b) observed the significant decrease in fungal biomass in soils heavily polluted with Cu. They found that 18:2ω6,9 had a metal ratio of 0.78 in the arable soils polluted with the highest dose of Cu (128 mmol Cu kg^{-1}). The decrease in fungal biomass in samples contaminated with Cu might be explained by the fact that copper is known to be very toxic to fungi, and that is why Cu is commonly used as a fungicide. However, Frostegård et al. (1993a) did not observe any effect of Cu-contamination on fungal biomass in a forest soil. The explanation might be that 18:2ω6,9 is also found in plant residues of *Pinus sylvestris* L. Therefore, the effect of Cu on the abundance of 18:2ω6,9 extracted from fungi in forest soils might be masked by a large amount of this PLFA derived from the plant material (Frostegård et al., 1993a). In contrast, an increase in fungal biomass was reported by Khan and Scullion (2000), who observed a progressive increase in ergosterol content with higher metal input after the soil contamination with Cu, Zn and Ni. The increase in fungal biomass was associated with the decrease in bacterial: fungal ratio of PLFAs.

Other authors have also found an increase in fungal counts as a response to heavy metal presence (Ohya et al., 1988; Hattori, 1992). This proportional increase in fungi at Zn- and Cd-contaminated sites is most likely due to the fact that fungi are thought to be more resistant to some heavy metals than bacteria. The added metal compounds decreased the soil pH, but Pennanen et al. (1998b) showed that fungal biomass appeared not to suffer from acidification to the same extent as bacterial biomass. They reported that the amounts of both fungal fatty acid 18:2ω6,9 and ergosterol were unaffected by soil acidification, whereas basal respiration was significantly lower than in the control soil. The decrease observed in respiration took place due to pH affecting mainly the bacterial fraction of total microbial commu-

nity. The results of this study for Zn or Cd treatment were in accordance to previous studies showing that soil acidification and heavy metals did not affect or even increased the fungal biomass and fungal/bacterial biomass ratio (Bååth et al., 1992, 1998). Similarly, Rost et al. (2001) observed that the effects of Zn contamination on microbial biomass were relatively small in comparison with the effects on the CO_2 production and mineralization of nitrogen. These results suggested that metabolically active microorganisms were more sensitive to toxic substances than dormant microorganisms (Ohya et al., 1988). For this reason a dramatic reduction of certain groups of microorganisms might have led to a marked decrease in a specific microbial activity.

5.4. Microbial community structure – r/K strategy

The impact of heavy metals on microbial community structure studied by the concept of r/K strategy is based on differentiation of bacterial populations into r- and K-strategists that differ in their ability to grow in soils under different nutrient conditions. The concept of r/K strategy is derived from evolutionary ecology and states that there are genetic differences between organisms in their ability to survive in different environments (De Leij et al., 1993).

The differences in the occurrence of r- and K-strategists during the study were observed in both the metal-contaminated soils and the control and also in the soils contaminated with different metals. Significant changes in the growth of bacterial populations were also observed over time. Generally, the bacteria that formed visible colonies on the third and successive days of incubation dominated in Cu-, Zn- and Cd-contaminated soils when compared to the bacteria isolated from the control soil. Therefore, the addition of the metals shifted bacterial populations from r-strategists toward K-strategists. The contradictory shift, from K-strategists toward r-strategists in metal-polluted soils could be expected as a result of the addition of heavy metals because the organic compounds released from killed bacteria increased the content of available nutrients. The bacteria forming colonies during the two first days of incubation dominated only in soil contaminated with 1000 μg Zn g^{-1} on day 15. However, these results may not be attributed to the increase of easily available nutrients but to the low toxicity of this concentration of Zn on the soil bacterial populations. In comparison to K-strategists, r-strategists are more active and multiply faster but are thought to be more sensitive to toxic substances (De Leij et al., 1993). This could be the explanation why mainly K-strategists could survive in the presence of

observed contents of available heavy metals. Bacterial community distribution curves indicate that different subpopulations of K-strategists probably evolved in response to the metals and their concentration in the soil samples (Kozdrój, 1995).

The effect of metal-treatments on culturable fraction of bacterial populations in soil was also evident when diversity indices (EP) were calculated. In Cu-, Zn- or Cd-polluted soils, the biodiversity, expressed as the EP indices, was lower as compared to the control soil. The highest decrease in the EP values was found 90 days after soil contamination, suggesting that bacterial communities needed much more time to change their composition and diversity. On 15 day the EP indices were not significantly lower in comparison with those of the control soil, however, the shift in the structure of bacterial community was found. A community that shifts from domination by, for example, r-strategists to domination by K-strategists, can have a similar diversity index, although some changes could have taken place (De Leij et al., 1993). The reason is that EP-index condenses a community into one value and may not differentiate between the dominance of r- or K-strategists. In this study, the values of EP indices supported the findings derived from the microbial community structure data, among which there was stated that the microbial community in metal-contaminated soils had an uneven distribution of K-strategists. It has been recognized that environmental stress caused by pollution generally decreases the diversity of microbial population and changes an ecological balance of population interactions within the community (Bååth, 1989; Atlas et al., 1991; Kelly et al., 1999a, b; Ellis et al., 2003; Turpeinen et al., 2004).

The uneven distribution of classes within the culturable bacterial community in contaminated soils was also confirmed by CD indices. The high CD values indicated greater proportion of r-strategists while the low values indicated higher proportion of K-strategists. For eight classes, $CD_{max} = 100$ and $CD_{min} = 10$. Sarathchandra (1997) found that the CD-index was of greater relevance and related better to the r/K concept than the EP-index. Also, the correlation between the high CD value and dominance of a given bacteria group was observed in this study. The highest values of CD were found in the uncontaminated soil where r-strategists dominated. Similarly, the lowest CD values were found in the soils contaminated with Cd, and the strong dominance of K-strategists was observed simultaneously. Generally, both indices directly showed that the culturable fraction of bacteria was highly affected by the addition of metals, and CD index appeared to be sufficiently sensitive to detect significant differences in the rate of colony formation of the bacteria isolated from different samples.

Also, Hattori (1983) proposed plate counts to describe naturally occurring microbial communities that were isolated from different environ-

ments. His concept was based on the fact that the time required for a bacterial cell to form visible colonies on agar plates is its intrinsic characteristic and is related to the physiological state of the bacterial cell. The starved cells and cells that occur in the dormant state take longer to form colonies than those of growing cells. Hattori (1985) reported that contamination of water increased the value of parameter λ, indicating the rate of colony formation. Kozdrój (1995) used this method for characterization of soil bacterial populations under heavy metal stress. He did not find such correlation, however, differences in λ values between the modes of the metal additions were observed. The significant differences in the values of tr parameter, reflecting the growth rate, between the same doses of different metals, indicated that bacterial groups evolved in response to Cu or Cd. In other studies, it has been observed that the addition of Cu at the dose of 1500 or 2500 μg Cu g^{-1} prolonged the retardation time of colony appearance of indigenous Gram-negative bacteria (Kozdrój and Piotrowska-Seget, 1995). The fact that these bacteria needed remarkably longer periods to divide in Cu-treated samples proved that they required some changes in gene expression to begin a new cycle of cell replication.

Several studies have shown that metal stress decreased microbial diversity (Maliszewska et al., 1985; Kandeler et al., 1996; Smit et al., 1997; Kelly et al 1999a; Sandaa et al., 1999a). The negative impact of heavy metals on soil bacterial diversity was reported earlier by Kozdrój and van Elsas (2000), who observed the lower value of the EP in highly contaminated soil as compared to the EP value for bacteria cultured from the soil with low contents of Zn, Cd and Pb. The decrease in bacterial diversity was clearly confirmed by using molecular methods for characterization of soil bacterial populations. Smit et al. (1997) found relatively large differences in amplified ribosomal DNA restriction analysis (ARDRA) between unpolluted and Cu-polluted soils indicating that microbial community reduced their diversity in response to Cu-contamination. The decrease in bacterial diversity with increasing soils contamination was also observed by Sandaa et al. (1999b), who studied the impact of heavy metals on soil bacterial communities in soils amended with sewage sludge containing different concentrations of heavy metals for many years. They found a significant decrease in bacterial diversity from 16 000 bacterial genomes in the uncontaminated soil to 6400 bacterial genomes in soil with low metal concentrations and only 2000 bacterial genomes in soil with high metal levels. Moffett et al. (2003) used ARDRA to compare the diversity of the extractable bacterial fraction in Zn-contaminated soil (400 mg kg^{-1}) to that in the control soil (57 mg kg^{-1}). The comparison of the restriction fragment length polymorphisms of 236 clones from each soil proved that the stress caused by zinc toxicity reduced bacterial diversity. They found 120 opera-

tional taxonomic units (OTUs) in the control soil, and only 90 OTUs in Zn-contaminated soil, indicating a 25% decrease in the bacterial diversity. The most dominant OTUs in the contaminated soil accounted for the higher proportions of clones than those found in the control.

5.5. Fatty acid analyses of microbial community

Fatty acid methyl ester (FAME) and phospholipid fatty acid (PLFA) techniques provide complementary data on the response of soil microbial community to heavy metals. These methods allow generating the communities fingerprints based on fatty acids, originating from microbial cells isolated from soil as well as extracted directly from the soil samples. These fingerprints can be used to detect changes in the microbial community structure. In this study, each soil sample was characterized by PLFA and FAME to estimate the effect of Cu-, Zn- or Cd-contamination on the total and culturable fraction of the bacterial communities, respectively.

The FAME analysis showed that Gram-positive bacteria dominated culturable fractions of bacterial communities in unpolluted and polluted soils, because the branched-chain fatty acids, characteristic for these groups of bacteria were presented at the highest amounts. However, their percentages in the metal-polluted soils were higher than those compared to the control soil. In some samples, they constituted even 80% of the total fatty acids isolated from the bacterial biomass. It was mainly caused by a significant increase in the content of fatty acids i15:0 and a15:0 in the bacterial biomass obtained from the metal-contaminated soils. Similar results were reported by Kozdrój and van Elsas (2001b), who studied the structural diversity of microbial communities in arable soils of a heavily industralised area. They also found that the FAME profiles, obtained from the bacteria isolated from the metal-polluted samples, were dominated by branched-chain fatty acids that together constituted from 51 to 82% of total fatty acids.

The dominance of Gram-negative over Gram-positive bacteria was found in metal-contaminated soils (Frostegård et al., 1993b; Kelly et al., 2003). In this study, the evidence for a similar shift was observed 90 days after the soil contamination when PLFA profiles were analyzed. On that sampling day, the increase in 18:1ω9 and cy17:0, characteristic of Gram-negative bacteria (Zelles, 1999a) and the decrease in i15:0, i14:0 and 10Me18:0, characteristic of Gram-positive bacteria (Zelles, 1999a; Frostegård et al., 1993b), in the soils polluted with Cu, Zn or Cd were detected, when compared to the control. These changes indicated the shift in the abundance of these two bacterial groups due to the metal pollution. However, this inter-

pretation is not straightforward because the amounts of some branched fatty acids (i.e. i17:0, a17:0), which are typical for phospholipids in Gram-positive bacteria, also increased in response to the metals. The PLFA 18:1ω7 that is commonly found in Gram-negative bacteria showed variable responses. The similar shift was found by Bååth et al. (1992), who studied the microbial community structure in an area polluted with alkaline dust deposition. In contrast, Pennanen et al. (1998a) observed the increase in branched fatty acids in soil artificially polluted by a simulated acid rain at the higher levels of acid application. The dominance of branched fatty acids was also observed in this study in whole-cell fatty acid profiles obtained from the culturable fraction of the total bacterial populations. Pennanen et al. (1996), who studied the effects of metals in forest soil around the smelters, reported that metal toxicity always increased in the relative abundance of some PLFAs and decreased in others. They suggested that it is possible to predict the changes in PLFA pattern obtained from forest soil. However, such specific changes in the PLFA profiles appear not to be so obvious when other soil types were studied. Frostegård et al. (1993b) and Bååth et al. (1998) observed different changes in PLFA patterns obtained from different types of arable soils. For example, the concentration of cy17:0 increased in the high-Ni and high-Zn treatments at Lee Valley but decreased in the same treatments at Luddington when compared to those in the control soil. Similarly, the concentration of 16:1w5 that increased with the metal concentration at Luddington, decreased at Lee Valley. They pointed out that PLFAs, which were found in both soils, might represent different groups of microorganisms, thus changes in the abundance of certain PLFAs might represent changes in different microbial groups in the two soil types.

The relative abundance of the methyl-branched PLFAs 10Me16:0 and 10Me18:0 varied in response to the soil contamination with heavy metals. This study showed an initial decrease in 10Me16:0, an indicator for actinomycetes (Frostegård et al., 1993b) in the soils contaminated with both doses of Zn or Cd and a higher dose of Cu as compared to the control soil. After 60 days of incubation, significant differences in the amount of 10Me16:0 between the heavy metal treatments and the control samples were not found. However, the decrease in 10Me18:0, another indicator fatty acid for actinomycetes was found in Zn-polluted soil during that sampling time. On day 90, the decrease in 10Me16:0 was only observed in the soil contaminated with Cd, however Cd-treatment increased the content of 10Me18:0. In contrast, the decrease in 10Me18:0 was observed in Cu- or Zn-polluted soils. The decrease in 10Me16:0 in soil amended with Zn was previously reported by Kelly et al. (1999a). However, the decrease in 10Me16:0 in Zn-polluted microcosmos lasted longer in contrast to this study. After 420 days, the Zn-treatments still showed lower amounts of 10Me16:0. The mixed

results observed in this study might result from different responses of different members of an actinomycetes population to heavy metals. Other results obtained by Frostegård et al. (1993a) and Turpeinen et al. (2004) also revealed that actinomycetes reacted differently to heavy metals. Different members of the actinomycetes population could respond differently to elevated metal concentrations (Kelly et al., 1999a). In addition, Frostegård et al. (1993b) found that methyl-branched 10Me16:0, 10Me17:0 and 10Me18:0 reacted differently to the metals in arable soil as compared to forest soil. All three 10Me-branched PLFAs increased in the forest soil. In arable soils, not all PLFAs responded similarly, and in some cases, they responded in a different way to different metals. The number of actinomycetes either decreased or was unaffected in arable soils when compared to the forest soil. Actinomycetes varied in their metal tolerance from other bacteria and fungi. It was shown by Hiroki (1992) that the tolerance degree in microorganisms isolated from a follow paddy field contaminated with Cd, Cu and Zn was as such: fungi > bacteria > actinomycetes. Also Jordan and Lechevalier (1975) reported that the Zn-tolerance of actinomycetes isolated from a forest close to a smelter increased as compared to isolates from uncontaminated soil. However, actinomycetes were generally less tolerant than bacteria and fungi.

Frostegård et al. (1993a) reported that different metals (Cd, Ni, Pb and Zn) induced similar changes in the PLFA profile within one soil. In the study conducted by Bååth et al. (1998), the same alterations in the PLFA patterns were found for the Zn- and Ni-treated samples in two arable soils. However, the high Cu-treatment differed from other treatments, indicating that the Cu-treatment had different effect on the PLFA pattern from that of Zn or Ni. They found that Cu decreased in relative amounts of the fungal PLFAs 18:2ω6,9 and 20:4, whereas Ni and Zn increased in both of them. Similarly, Cu showed different effect on the PLFA profiles from those of Zn and Cd in this study. The contents of 18:2ω6,9 in the Cu-contaminated soils were significantly lower than in the control. In contrast, an increase in this fatty acid was found in soils contaminated with Zn or Cd.

Fatty acid 18:2ω6,9 was regarded as a marker acid for fungi (Guckert et al., 1986), and it is often used for estimation of fungal biomass in soil samples. Thus, the present results support earlier laboratory results of Frostegård et al. (1993b) suggesting that Cu affects the fungal part of the microbial community differently from other metals. Dahlin et al. (1997) also reported that a high Cu content in soil was correlated with low content of 18:2ω6,9. In turn, similar proportional increase in 18:2ω6,9 was observed by Kelly et al. (1999a) in highly Zn-polluted soil. Interestingly, the increase in 18:2ω6,9 that was observed in soils experimentally contaminated with Zn or Cd, was contrary to the data collected by Pennanen et al. (1996) in

the field experiment. They found a decrease in 18:2ω6,9 in the forest soil samples collected in the vicinity of a metal smelter in Finland. Pennanen et al. (1996) suggested that the decrease in 18:2ω6,9 might have been connected with the negative effect of heavy metals on tree roots colonized by mycorrhizal fungi. The study conducted in a forest localized in the vicinity of a smelter showed that heavy metal contamination damaged fine roots of trees (Helmisaari et al., 1995). In addition, Koomen et al. (1990) reported that elevated heavy metal concentrations could decrease the mycorrhizal infection of plant roots. A lower amount of mycorrhizal fungi may have accounted for the observed decrease in 18:2ω6,9 (Pennanen et al., 1996). This may also explain the contrasting results observed between laboratory and field studies. Since there were no plants in the laboratory soil used in this study, mycorrhizal fungi were not dominant members of the fungal communities in the soils. If fungi were more resistant to Zn and Cd than bacteria in non-planted soil, then fungi might increase their percentage in total microbial communities in Zn- and Cd-polluted soils. Indeed, the significant increase in 18:2ω6,9 was found in Zn- and Cd-amended soils. The higher contents of 18:2ω6,9, that increased with the increase in Zn-concentrations in metal-contaminated arable soils was reported earlier by Frostegård et al. (1996) and Kelly et al. (1999a).

In this study, the microbial community structure changed over time as was indicated by the differences in the amount of single PLFAs obtained directly from the soil. The results showed that the individual fatty acids are more responsible for the changes in the fatty acid profiles than the sum of fatty acids that are regarded as characteristic for actinomycetes, Gram-negative and Gram-positive bacteria. Frostegård et al. (1993b) stated that some of fatty acids varied in their response to metals addition, and the differentiation between Gram-negative and Gram-positive bacteria in soil on the basis of fatty acid patterns was not straightforward. This might be due to the fact that no significant differences were observed between heavy-metal contaminated and uncontaminated soils at the end of the study when PLFAs were presented as even-numbered, odd-numbered, branched, monounsaturated, polyunsaturated, methylated and cyclopropane classes of the fatty acids. The PLFA pattern could be altered not only due to the shift in the species composition, but also the changed environmental conditions might affect the phospholipids profile of the bacterial cells. For example, an increased ratio of *trans* to *cis* isomers of the fatty acids 16:1ω7 and 18:1ω7 has been shown for several bacteria to indicate increased environmental stress, including nutrient starvation and pH changes (Frostegård et al., 1993a; Heipieper et al., 1995; Sajbidor, 1997).

The changes in the structure of bacterial communities were observed also by Sandaa et al. (2001), who studied the effect of long-term heavy metal

contamination on soil microbial community using dot blot hybridization with group specific probes and restriction fragment length polymorphism (RFLP). They revealed that the differences in the community structure in soils with low- and high-metal amendments were more pronounced when the total bacterial community was investigated in comparison to the culturable fraction of the bacterial community. The application of phylogenetic probes provided some information on the groups of microorganisms which were affected by heavy-metal contamination in the soils. The main changes were associated with the increase in the number of bacteria belonging to the α-subdivision of *Proteobacteria* with increased heavy metals content. These results were confirmed by *in situ* hybridization, in which substantial increase in the α-subdivision of *Proteobacteria* was detected with increased heavy-metal contamination (Sandaa et al., 1999b). Tom-Petersen et al. (2003) revealed changes in microbial community structure caused by copper. T-RFLP analyses using two different primers proved a more significant effect of the high Cu-treatment (150 mg Cu kg^{-1}) than low Cu-treatment (50 mg Cu kg^{-1}), as the decreased value of Jaccard similarity coefficient (Jc) displayed. They showed that Cu-induced effects on the bacterial community structure could be detected at concentrations close to the EU safety limits of 50–150 mg Cu kg^{-1}, corresponding to a bioavailable Cu concentration as low as 0,3 mg Cu kg^{-1}. Significant changes in the abundance of some terminal restriction fragments (T-RFs) between treatments were found. In the field experiment, 18% of the T-RFs were affected by the high Cu-treatment. However, the number of affected T-RFs decreased throughout the experimental period, suggesting a short-term effect of Cu on bacterial populations.

The changes of microbial community structure measured by PLFA and T-RFLP profiles of 16S rDNA were also observed by Turpeinen et al. (2004) in As-, Cr-, and Cu-contaminated soils. They found that at the sites, differing in the level of contamination, the response of microbial populations to metals was different. For example, fatty acid 16:1ω5 had the reduced proportion at one site as compared to the control and other metal-contaminated soils. Similar situation was observed in this study. After 15 days of the metals exposure the amount of 16:1ω5 decreased in the soil contaminated with the lower dose of Cu or Zn, whereas this PLFA was unaffected in the soil treated with higher doses of these metals. Turpeinen et al. (2004) revealed distinct shifts in the structures of microbial communities exposed to As, Cr and Cu for many years, that were further supported by characterization and identification of As-resistant bacteria. Corresponding to T-RFLP results, the observed shift was not due to a reversion towards the community found in unpolluted soil, but the shift was mainly caused by the appearance of new dominating species. This idea was supported by the fact that the community of culturable bacteria was considerably different at the

highly contaminated site as compared to less contaminated sites. Their study, combining complementary culture-dependent and independent methods, suggested that microorganisms were able to respond to the metals and they maintained metabolic activity apparently through changes in microbial community structure and selection for resistant species. Moreover, the results demonstrated the adaptation of microbial communities to toxic effects of heavy metals in As-, Cu- and Cr-contaminated soils. Although long-term exposure to high concentrations of heavy metals can change microbial community structure permanently, the soil system appears to compensate for the reduced population diversity. This was supported by the finding that overall metabolic activity measured as glucose mineralization did not change. Also, Rasmusen and Sørensen (2001) found that microbial diversity decreased immediately after exposure to Hg, and adaptation to Hg-stress might result in a recovery of diversity due to the shift in the community structure.

Ranjard (2000) observed modifications in the ribosomal intergenic spacer analysis (RISA) profiles for unfractioned soil, the outer and inner aggregate, and the clay size fractions. Changes in the profiles were mainly due to the appearance of new bands and an increase in relative intensity of bands previously existing in unspiked soils. These results supported the thesis that soil contamination with heavy metals could result in the disappearance of sensitive population and increase in the number of well adapted populations, leading to a bacterial community more tolerant to the metals. Torsvik et al. (1998) claimed that shift in microbial populations and decrease in bacterial diversity revealed by reduction in genetical diversity index values may reflect reduced species richness or reduced evenness due to some bacterial types becoming dominant. Recently, changes in microbial community structure were also reported by Gremion et al. (2004), who studied the impact of heavy metals and phytoextraction practices on the microbial community structure using DGGE and community level physiological profiles (CLPP). In addition, they also measured the rate of ammonia oxidation. The results obtained with these three approaches showed the impact of Zn and Cd on different parts of microbial community. Independently of the marker gene used, analysis of DGGE-profiles revealed consistent differences between contaminated and uncontaminated soils. The CLPP profiles, reflecting the potential activity of culturable fraction of bacterial community growing on the substrates provided on BIOLOG microtitre plates, showed that the potential degradation capability of the microbial community was reduced in Zn- and Cd-contaminated soils as compared to uncontaminated soil. The significant reduction in the potential activity of microbial communities in metal-polluted soils, estimated by the CLPP method, was previously revealed in other studies (Kelly et al., 1999a; Sandaa et al., 2001; Kandeler et al., 1996).

To estimate the environmental risk caused by heavy metals, it is important to measure such parameters of soil microbial community that could reflect the metal toxicity. The results of this study showed that both culture-dependent approach and culture-independent analysis of PLFA profiles seem to be suitable for determination of changes in the structure of microbial community exposed to heavy metals or other pollutants. However, the less sensitive and reliable parameter appeared to be bacterial counts because of quick recovery of bacterial populations. The present understanding of genetic and functional diversity of microbial communities forces investigations of modern methods, based on cell biomarkers, such as lipids and nucleic acids. Future research should combine different complementary techniques, involving a broad spectrum of indicators that allow us to understand better the mechanisms of heavy metal toxicity on soil microbial communities.

6. Conclusions

1. The soil used in this study was characterized by higher ability to bind metals as indicated by the amounts of water-soluble fraction of metals that constituted only a small proportion of the total metal added.

2. The significant decrease in the number of heterotrophic bacteria in the metal-polluted soils, observed shortly after contamination, indicated that the metals showed short-term effect on microbial number.

3. The bacterial populations in the metal-polluted soils reached similar numbers to the population in the control sample over time, suggesting that bacteria could recover after an initial reduction caused by high metal inputs.

4. The bacterial biomass was strongly affected by the metals added and the lower biomass of bacteria was observed by the end of this study, showing that biomass might be a sensitive indicator of heavy-metal toxicity.

5. The fungal biomass was significantly lower in Cu-contaminated soil and the biomass was either unaffected or higher in Zn- or Cd-polluted soils as compared to the control, showing that fungi were more sensitive to Cu than to Zn or Cd.

6. The addition of the metals decreased the diversity of culturable bacterial communities as the reduced values of ecophysiological (EP) and colony development (CD) indices indicated. In addition, the metals shifted bacterial communities from r- to K-strategists.

7. Both FAME and PLFA techniques used to study respectively, culturable and total fractions of microbial communities, revealed that the metals significantly effected the structures of the microbial communities. However, the differences between FAME and PLFA profiles indicated that these methods characterized the reactions of different parts of microbial communities.

7. Abstract

The effects of Cu, Zn or Cd added to soil on numbers, biomass and structure of the total and culturable fraction of the microbial community were investigated performing a 90-day experiment under laboratory conditions. Both Cu and Zn were added to soil at concentrations of 1000 or 2000 $\mu g\ g^{-1}$, whereas Cd at 500 or 1000 $\mu g\ g^{-1}$. Water-soluble concentrations of the metals and soil pH were determined. The numbers of culturable bacteria were estimated by a soil-dilution plate method. The bacterial and fungal biomass was calculated by the sum of the fatty acids considered to be of bacterial and fungal origin, respectively. Phospholipid fatty acid (PLFA) analysis was used to determine the structure of the entire soil community irrespective of culturing. The structure of culturable bacteria community was determined by fatty acid methyl ester (FAME) profiling as well as using the concept of r- and K-strategists. To determine the changes in diversity of the culturable fraction of the bacterial community EP and CD indices were calculated.

The concentrations of bioavailable Cu, Zn or Cd represented a few percentages of the total metal contents and decreased with time. The soil contamination with the highest concentrations of Cu, Zn or Cd resulted in a one-unit decrease in soil pH. The results showed that the metal-treatment had a negative effect on the numbers of bacteria only shortly after the soil contamination. After 60 days of the exposure to the metals, significant differences were not observed among the numbers of total heterotrophic bacteria cultured from polluted and unpolluted soil samples. The soil bacterial biomass was strongly affected by the metals at each concentration during the first sampling time. In the soil portions treated with the higher dose of Cu or Zn, and both doses of Cd, the microbial biomass remained significantly lower than in the control over time. The distribution of the bacterial classes (i.e. majority of colonies appeared after 48 h), the values of EP and CD indices showed that the metals influenced the structures of bacterial communities. Cu, Zn or Cd reduced the bacterial diversity towards domination of K-strategists. The reduction in the diversity was the highest in the soil contaminated with Zn or Cd, in which the values of EP-indices (from 0.518 to 0.553) were significantly lower as compared to that of the

control (i.e. 0.707). The culturable fraction of bacterial community, presented by FAME profiling, was dominated by Gram-positive bacteria both in the metal-polluted and the unpolluted soils. Cu, Zn or Cd did not significantly change the structure of this fraction. However, the percentages of branched-chain fatty acids, known as a marker of Gram-positive bacteria, were higher in the metal-polluted samples at each sampling time. The culturable fraction of soil bacteria was less diverse than the total microbial community determined by PLFA. The analyses of soil PLFA profiles demonstrated distinct differences in the composition of fatty acids among the metal treated and the control soils, indicating differences in their microbial community structures. The changes in PLFA patterns varied depending on the metal added and sampling time. During the last sampling time, the decrease in branched-chain fatty acids i14:0 and i15:0, and increase in i17:0 and a17:0 were found in all metal-polluted soils as compared to the control. The increase was also observed for 16:1ω7t, whereas the contents of other unsaturated fatty acids as well as cyclopropane fatty acids cy19:0 and cy17:0 varied among metal-polluted soils. The PLFA cy19:0 increased in Cu-contaminated soil, whereas it decreased in the soil treated with Zn. Cu strongly decreased the content of 18:2ω6,9, whereas this PLFA content was unaffected or increased in Cd- or Zn-contaminated soil as compared to the control. The relative abundance of 10Me16:0 and 10Me18:0 varied in response to the soil contamination with the metals over time, indicating that different members of actinomycetes population responded differently to elevated metal concentrations. The cluster analysis of PLFA grouped the contaminated soils into three separate clusters that confirmed that the microbial populations reacted in different ways to Cu-, Zn- or Cd-contamination of the soil.

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Zofia Piotrowska-Seget

Struktura zespołów bakterii w glebie skażonej metalami ciężkimi oznaczana na podstawie analizy kwasów tłuszczowych i metod hodowlanych

Streszczenie

Badano wpływ Cu, Zn i Cd na liczebność, biomasę oraz strukturę ogólnej i zdolnej do wzrostu na podłożach hodowlanych populacji bakterii w warunkach laboratoryjnych. Glebę skażono Cu lub Zn w dawce 1000 lub 2000 μg g^{-1} oraz Cd w dawce 500 lub 1000 μg g^{-1} gleby. Po 15, 60 i 90 dniach od skażenia gleby określono w niej zawartość wymywalnych wodą jonów wprowadzonych metali, ogólną liczebność bakterii na podłożu TSA oraz biomasę bakterii i grzybów na podstawie zawartości wybranych fosfolipidowych kwasów tłuszczowych (PLFA). Strukturę zdolnej do wzrostu frakcji bakterii określano na podstawie profili komórkowych kwasów tłuszczowych (FAME) oraz na podstawie koncepcji r- i K-strategów. Do oceny bioróżnorodności populacji bakterii zastosowano współczynnik ekofizjologiczny (EP) i rozwoju kolonii (CD). Strukturę całego zespołu bakterii glebowych oceniano, opierając się na profilach PLFA izolowanych bezpośrednio z gleby.

Zawartość wymywalnych wodą jonów Cu, Zn i Cd stanowiła jedynie kilka procent wprowadzonych do gleby metali i zmniejszała się w czasie trwania doświadczenia. Wykazano, że metale ciężkie mają jedynie krótkotrwały negatywny wpływ na ogólną liczebność bakterii. Po 60 dniach inkubacji nie stwierdzono istotnych różnic pomiędzy liczebnością mikroorganizmów w glebach skażonych a kontroli. Obserwowano natomiast istotny wpływ wprowadzonych metali na biomasę mikroorganizmów. W glebach skażonych wyższymi dawkami metali biomasa bakterii była zawsze niższa w porównaniu z biomasą bakterii w glebie kontrolnej. Wartości parametrów EP i CD wskazują na wyraźne zmiany w strukturze zespołów bakterii zdolnych do wzrostu w glebach skażonych Cu, Zn i Cd. Wszystkie metale powodowały przebudowę struktury zespołów bakterii w kierunku dominacji K-strategów. Największą redukcję bioróżnorodności stwierdzono w glebach skażonych Zn i Cd, w których wartość parametru EP (od 0,518 do 0,553) była istotnie niższa od wartości EP w kontroli (0,707). Profil komórkowych kwasów tłuszczowych (FAME) izolowanych z wyrosłych komórek wskazuje na dominację bakterii Gram-dodatnich zarówno w glebie kontrolnej, jak i skażonej metalami. Nie zaobserwowano istotnych zmian w profilach FAME

badanych zespołów, jakkolwiek procentowy udział rozgałęzionych kwasów tłuszczowych w glebach zanieczyszczonych Cu, Zn i Cd był wyższy niż w glebie kontrolnej.

Zespoły wyrosłych na podłożu TSA bakterii były mniej zróżnicowane w porównaniu z zespołami mikroorganizmów określanymi za pomocą fosfolipidowych kwasów tłuszczowych, izolowanych bezpośrednio z gleby. Analiza profili tych kwasów wskazuje na wyraźne zmiany w obrębie struktury badanych zespołów bakterii w glebach potraktowanych różnymi metalami i kontrolą. Zmiany w profilu PLFA zależały od rodzaju metalu, jego dawki i czasu, w którym pobierano próbki. Po 90 dniach inkubacji w glebach skażonych Cu, Zn i Cd stwierdzono spadek zawartości rozgałęzionych kwasów tłuszczowych i14:0 i i15:0 oraz wzrost ilości kwasów i17:0 i a17:0 w stosunku do kontroli. W glebach skażonych metalami wzrosła również ilość kwasu 16:lω7t, natomiast zawartość innych kwasów nienasyconych, jak i kwasów cyklopropanowych zmieniała się w zależności od rodzaju metalu wprowadzonego do gleby. Analiza dendrogramów, utworzonych na podstawie uzyskanych profili PLFA, obrazujących podobieństwa między zespołami bakterii w badanych glebach, wskazuje, że mikroorganizmy w różny sposób reagują na skażenie gleby Cu, Zn i Cd.

Zofia Piotrowska-Seget

Die Struktur der Bakterienkolonien in dem mit Schwermetallen verseuchten, und anhand der Analyse von Fettsäuren und mit Zuchtmethoden markierten Boden

Zusammenfassung

Untersucht wurde der Einfluss von Cu, Zn und Cd auf die Mengee, die Biomasse und auf die Struktur der allgemeinen und zum Wachstum auf Zuchtböden in Laborverhältnissen fähigen Bakterienpopulation. Der Boden wurde mit Cu oder Zn in der Dosis von 1000 oder 2000 $\mu g\ g^{-1}$, und mit Cd in der Dosis 500 oder 1000 $\mu g\ g^{-1}$ verseucht. Nach 15, 60 und 90 Tagen von der Verseuchung hat man den Gehalt von den mit Wasser auswaschbaren Jonen der eingeführte Metallen, die Gesamtzahl von Bakterien auf dem TSA-Boden und die Biomasse von Bakterien und Pilzen auf der Grundlage von dem Gehalt der ausgewählten Phosphatiden (PLFA) bestimmt. Die Struktur der zum Wachstum fähigen Bakterienfraktion wurde auf Grund der Zellenprofile von Fettsäuren (FAME) und mit Hilfe der Konzeption von r- und K-Strategen bestimmt. Zur Beurteilung der Biovielfalt von Bakterienpopulationen wurde der ökophysiologische Faktor (EP) und der Kolonienentwicklungsfaktor (CD) angewandt. Die Struktur der gesamten Gruppe von Bodenbakterien beurteilte man anhand von den direkt aus dem Boden abgesonderten PLFA – Profilen.

Der Prozentsatz der mit Wasser auswaschbaren Cu-, Zn- und Cd- Jonen in den Boden eingeführten Metallen war niedrig und wurde während des Experimentes immer niedriger. Es ist nachgewiesen worden, dass die Schwermetalle nur kurz die Gesamtzahl von Bakterien negativ bewirken können. Nach 60 Inkubationstagen wurden keine wesentlichen Unterschiede zwischen der Menge von Mikroorganismen in verseuchten Böden und der Kontrollprobe festgestellt. Es wurde dagegen beobachtet, dass die in den Boden eingeführten Metalle die Biomasse der Mikroorganismen stark beeinflussten. In den mit höheren Metalldosen verseuchten Böden war die Bakterienbiomasse immer niedriger als die Biomasse von Bakterien im Kontrollboden. Die Werte von EP- und CD-Parametern sind in der Struktur der, zum Wachstum in den mit Kupfer, Zink und Cadmium verseuchten Böden fähigen Bakteriengruppen deutlichen Veränderungen unterworfen. Alle Metalle verursachten die Umwandlung von Bakterienstrukturen bis zur Dominanz der K-Strategen. Die größte Reduktion der Biovielfalt wurde in den mit Zn und Cd verseuchten Böden festgestellt, wo

der EP-Parameterwert (von 0,518 bis 0,553) wesentlich niedriger von den EP-Werten im Kontrollboden (0,707) war. Das Profil von den aus gewachsenen Zellen abgesonderten Zelltettsäuren (FAME) zeigt auf die Dominanz von Gramm-negativen Bakterien sowohl im Kontrollboden als auch in dem mit Metallen verseuchten Boden. Es wurden dagegen keine wesentlichen Veränderungen in den FAME-Profilen der untersuchten Gruppen beobachtet, obwohl der Prozentsatz von verzweigten Fettsäuren in den mit CU, Zn und Cd verseuchten Böden höher war, als der im Kontrollboden.

Die Gruppen der auf dem TSA-Boden gewachsenen Bakterien waren nicht so differenziert als die Mikroorganismusgruppen, die mit Hilfe von den, direkt aus dem Boden abgesonderten Phosphatiden bestimmt wurden. Wenn man die Profile der Säuren analysiert. beobachtet man deutliche Veränderungen innerhalb der Struktur von den untersuchten Bakteriengruppen sowohl in den mit verschiedenen Metallen verseuchten Böden, wie auch im Kontrollboden. Veränderte PLFA-Profile hingen von der Metallart, dessen Dosis und von der Zeit ab, in der die Proben entnommen wurden. Nach 90 Inkubationstagen hat man in den mit Cu, Zn und CD verseuchten Böden die niedrigeren Gehalte von verzweigten i14:0 und i15:0 – Fettsäuren, und die höheren Gehalte von i17:0- und a17:0 – Säuren im Vergleich zur Kontrollprobe festgestellt. In den mit Metallen verseuchten Böden stieg auch die Menge der 16:1ω7t – Säure; der Gehalt von anderen ungesättigten Säuren und Zyklopropansäuren veränderte sich je nach der Art des in den Boden eingeführten Metalls. Die Analyse der Dendrogramme, die anhand von den, die Ähnlichkeiten zwischen den Bakteriengruppen in untersuchten Böden darstellenden PLFA-Profilen gebildet wurden weist darauf hin, class die Mikroorganismen sehr unterschiedlich auf die mit Cu, Zn und Cd verseuchten Böden reagieren.

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