

Culture methods as indicators of the biological quality of phytostabilized heavy metal-contaminated soil

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ABSTRACT

The short-term effect of aided phytostabilization of heavy metal-contaminated soil on microorganisms under outdoor field conditions was tested. Heavy metal contaminated soil from a mining site was amended with lignite, lime and two commercial fertilizers and vegetated with grass *Festuca arundinacea*. Results demonstrated that the amended phytostabilization approach of Pb-Cd contaminated soil gave a positive change on the native microbial populations evaluated by culturable techniques during the first 28 weeks of the experimental period. In the end of the experiment, the number of bacteria, actinomycetes and fungi

increased in treated soil about 16-fold, 10-fold and 2-fold, respectively. Changes in the biodiversity of bacterial populations were evaluated by the Ecophysiological Index (EP) and the Colony Development Index (CD) for oligotrophs and copiotrophs. During the experimental period slower growing microorganisms (K-strategists) predominated. The application of amendments to the soil led to an increase of the CD index in both copiotroph and oligotroph populations after 28 weeks. EP, CD, bacteria, actinomycetes and fungi increased in the treated soil. Traditional microbiological methods based on culture techniques can be used to evaluate the biological quality of the phytostabilized heavy metal-contaminated soil.

INTRODUCTION

Pollutants such as heavy metals and chemicals in the soil, water and air are affected by various physicochemical, biological, and environmental factors. Bioremediation is a biological process by which environmental pollutants are removed or transformed to less toxic substances. Soil amendments including fertilizer and lime, appropriate moisture levels, and periodic tilling can maximize or improve bioremediation (Brigmon et al. 2002). Phytoremediation specifically utilizes plants for contaminant control and has been combined with soil amendments for increasing or reducing metals uptake (Wilde et al. 2005).

The ecological evaluation of *in situ* biological-based remedial systems requires special attention to be given to the use of soil health bioindicators. Bioindicators are the most important

criterion of soil quality (Alkorta et al. 2010; Markert et al. 2003). Many definitions of soil quality have been suggested. However, a short and comprehensive definition is given by Doran and Parkin (1994) who have defined soil quality/soil health as "The capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health".

One of the major challenges of microbial ecology is to gain more information on the microbial structure and diversity in contaminated soils, sediments and water during and/or after remedial applications. Microorganisms respond quickly to different stressors, both natural and anthropogenic, and they rapidly adapt to environmental conditions. This adaptation potentially allows the use of microbial analyses to assess soil health and changes in microbial populations and activities

may therefore indicate changes in soil health (Nielsen and Winding 2002). Changes in microbial populations or activity including specific population increases or decreases can provide signs of soil improvement or soil degradation (Pankhurst et al. 1995). Thus, microbiological measurements could be used as soil biomarkers for soil remediation efficiency, and as tools for evaluation of soil quality criteria or Ecological Risk Assessment. Microbial indicators have been broadly applied to describe polluted sites and to evaluate the capacity of remediation processes to help restore soil health (Alkorta et al. 2010; Barruti et al. 2009; Castaldi et al. 2004; Dawson et al. 2007; Epelde et al. 2008a, b; Hernandez-Allica et al. 2006; Hinojosa et al. 2010; Kizilkaya et al. 2004; Knox et al. 2008; Kumpiene et al. 2009; Margesin et al. 2011; Mora et al. 2005; Plaza et al. 2010; Wuertz and Mergeay 1997).

Microbial indicators cover a diverse set of microbial measurements, traditional and modern (molecular and -omics technologies), which have become an integral tool for managing and monitoring soil health in contaminated sites and have aided in evaluating environmental remediation technologies (Desai et al. 2010). Traditional microbiological methods based on culture techniques are simple and inexpensive, and are ready to use upon standardization of incubation, media selection, and counting procedures. Molecular methods are more complex, expensive and time consuming. All techniques should be tested before implementation. In recent years progress has been made in molecular methods (Desai et al. 2010). Culture methods such as colony forming units (CFU) or most probable number (MPN) also have some limitations as a large proportion of microorganisms from natural ecosystems are nonculturable and their ecological significance is not clear (Desai et al. 2010).

Aided phytostabilization can be used to remediate soil highly contaminated with heavy metals. This method combines

phytoremediation with initial chemical immobilization of heavy metals to restore optimal physicochemical and biological properties of polluted soil (Kumpiene et al. 2011; Margesin et al. 2011). Plants tolerant to heavy metals are able to immobilize metals by accumulation in the roots, adsorption in/onto the roots, and/or precipitation in the rhizosphere. Currently, most of what is known about aided phytostabilization of heavy metal-contaminated soils focuses on analysis of physicochemical soil properties, especially concentration of bioavailable forms of metals/metalloids and their accumulation in plant tissues (Wilde et al. 2005). However, there are only a few reports on how aided phytostabilization affects over the long term the biological features of soil such as microbial biomass, respiration and enzyme activity (Kumpiene et al. 2009; Renella et al. 2008).

The objective of this work was to use traditional microbiological methods based on culture techniques to evaluate the biological quality of heavy metal-contaminated soil that has been remediated with aided phytostabilization.

MATERIAL AND METHODS

Soil characteristics

The soil was collected from the vicinity of the former mine and zinc-lead smelter “Waryński”, which is situated in the Upper Silesia industrial region of southern Poland between the towns Piekary Śląskie and Bytom. In this area Zn, Pb and Cd ores, dolomite and coal were extracted and processed from 1927 to 1990. These activities contaminated the soil with large amounts of heavy metals. Currently most of the area remains non-vegetated which makes it wasteland (Krzyżak et al. 2006). The physicochemical properties of the soil used in the field experiment have been described in detail by Krzyżak et al. (2012) and are summarized in Table 1.

Table 1. Characteristics of soil used in the field experiment.

Property	Value
pH (1:2.5 soil/KCl)	6.38 ± 0.02*
pH (1:2.5 soil/H ₂ O)	6.62 ± 0.16
Organic matter (%)	4.76
Texture	
Sand (1.000–0.050mm), %	37.3
Silt (0.050–0.002 mm), %	56.3
Loam (< 0.002 mm), %	6.8
Total metal content (aqua regia extraction)	
Pb (mg·kg ⁻¹)	1291 ± 66.37
Cd (mg·kg ⁻¹)	85 ± 2.53
Zn (mg·kg ⁻¹)	4506 ± 365.79
As (mg·kg ⁻¹)	36 ± 1.74

*mean ± SD; N = 5

Experiment amendments

Lignite (co-product, dust fraction) from Brown Coal Mine TURÓW S.A. in Bogatynia was used in the experiment. It had the following initial parameters: 5.93 ± 0.05 pH in H_2O , $0.03 \pm 0.01 mg \cdot kg^{-1}$ total Pb, Cd below detection limit, $0.143 \pm 0.09 mg \cdot kg^{-1}$ Zn. Lime (DOLOMIT 50, PPHU DOLPOL, Poland) was added to neutralize soil pH. Since metalliferous wastes are very often deficient in nitrogen, phosphorus and sometimes other macronutrients (Gucwa-Przepióra et al. 2007; Kucharski et al. 2005; Smith and Bradshaw 1979) fertilization with macroelements was done using commercially available fertilizers: Azofoska (Inco VERITAS S.A., Poland: 5.5% NO_3 , 8.1% NH_4 , 6.4% P_2O_5 , 19.1% K_2O , 4.5% MgO, microelements) and ammonium nitrate.

In situ field experimental design and sampling

The studies under outdoor field conditions (7 months; from spring to autumn 2012) were conducted at two experimental plots (each with a surface area of $2m^2$): a control plot labeled as "CS" (control, untreated soil) and a treated plot labeled as "PS" (aided phytostabilized soil). Approximately 3 tons of soil were collected from the heavy-metals contaminated site. CS was mixed with Azofoska ($0.2g \cdot kg^{-1}$ soil) and ammonium nitrate ($0.4g \cdot kg^{-1}$ soil). Doses of soil amendments (lignite and lime) were trialed during the lab scale experiments. Based on these results 10% lignite and 2.5% lime were used. PS was mixed with lignite ($100g \cdot kg^{-1}$ soil), lime ($25g \cdot kg^{-1}$ soil), Azofoska ($0.2g \cdot kg^{-1}$ soil) and ammonium nitrate ($0.4g \cdot kg^{-1}$ soil).

Five soil samples (1kg) were collected from the 0-30cm layer in each plot. Then, they were immediately transported to the laboratory, sieved, and stored at field humidity in polyethylene bags at $4^\circ C$ until microbiological processing. The soil samples were sampled at the beginning of experiment and at 3, 6, 12, 20 and 28 weeks after treatment. All analyses were done in three replicates.

Enumeration of culturable aerobic microorganisms

The number of viable heterotrophic bacteria, copiotrophs, oligotrophs, actinomycetes and fungi were determined by cultivation of microorganisms on selective media. The results are presented as CFUs or Log CFUs and calculated per 1g of dry weight soil.

Culturable soil microorganisms were enumerated by the plate-count method for viable cells. Soil suspensions were prepared by shaking soil samples (10g fresh mass) for 30min at 180rpm with 90mL of sterile (filtered) 0.85% NaCl (pH 7.0-7.2). Appropriate dilutions of soil suspensions were surface spread onto selective agar plates. Colony-forming units (CFU) per gram of dry weight soil were calculated after drying in a $105^\circ C$ oven.

SMA (Standard Methods Agar, BioMérieux) was used to determine numbers of viable heterotrophic bacteria. Cycloheximide ($100mg \cdot L^{-1}$) was added to the medium to inhibit fungal growth. Colonies were enumerated after 72 hours incubation at $22^\circ C$.

Fungi were incubated on MEA medium (Malt Extract Agar, BioMérieux) with $100mg \cdot L^{-1}$ chloramphenicol at $22^\circ C$ for 7 days.

Actinomycetes were isolated on a selective medium, which contained ($g \cdot L^{-1}$): 2.0 sodium caseinate, 0.1 asparagines, 4.0 sodium propionate, 0.5 dipotassium phosphate, 0.1 magnesium sulfate, 0.001 ferrous sulfate, 5.0 glycerol, 20.0 agar. The plates were incubated at $22^\circ C$ for 7 days.

Copiotrophs and oligotrophs were isolated using a carbon rich source medium Nutrient Broth (NB contained ($g \cdot L^{-1}$): 10 peptone, 10 meat extract, 5 NaCl). The Nutrient Broth concentrations were $25g \cdot L^{-1}$ and $0.25g \cdot L^{-1}$ for copiotrophs and oligotrophs, respectively. Additionally, the media were solidified by using Bacteriological Agar (Oxoid) and Agar Noble (Sigma Aldrich), respectively. Three replicates of each dilution were plated. Bacterial colonies were counted after 1, 2, 3, 4, 5, 6 and 10 days of incubation at $25^\circ C$. The number of bacteria were expressed as the number of colonies grown on culture medium (solidified medium) after 2 days of incubation for copiotrophs and after 6 days for oligotrophs.

To characterize the community composition, two indices were calculated. The colony development index (CD) adapted from Kotsou et al. (2004) was calculated as follows:

$$CD = \left[\frac{P_2}{2} + \frac{P_4}{4} + \frac{P_6}{6} \right] \cdot 100$$

where P_2 , P_4 and P_6 represent the proportions of bacterial colonies appearing on days 2, 4 and 6.

The ecophysiological index (EP, a modification of the Shannon diversity index) was calculated as described by De Leij et al. (1993).

$$EP = - \sum (P_i \cdot \log_{10} P_i)$$

where P_i represents the proportion of bacterial colonies in class i , i.e. the proportion of colonies appearing on day i ($i = 2, 4, 6$). The more even the distribution of the classes, the higher the EP index, with $EP_{max} = 0.477$ and $EP_{min} = 0.0$.

To determine relative differences between the number of designated microbial groups in PS and in CS soils statistical significance was calculated by the unpaired Student t -test. Hypothesis H_0 , which assumes no statistically significant differences in the number of microorganisms in soils PS and CS was set at $p > 0.05$. All data results were checked with the χ^2 test for normality.

RESULTS AND DISCUSSION

The differences in numbers of colonies of heterotrophic bacteria, actinomycetes and fungi as a function of time are illustrated in Figure 1. In both the treated and untreated soil all measured populations increased during the 28 week experiment (Figure 1). For example, at the beginning of the

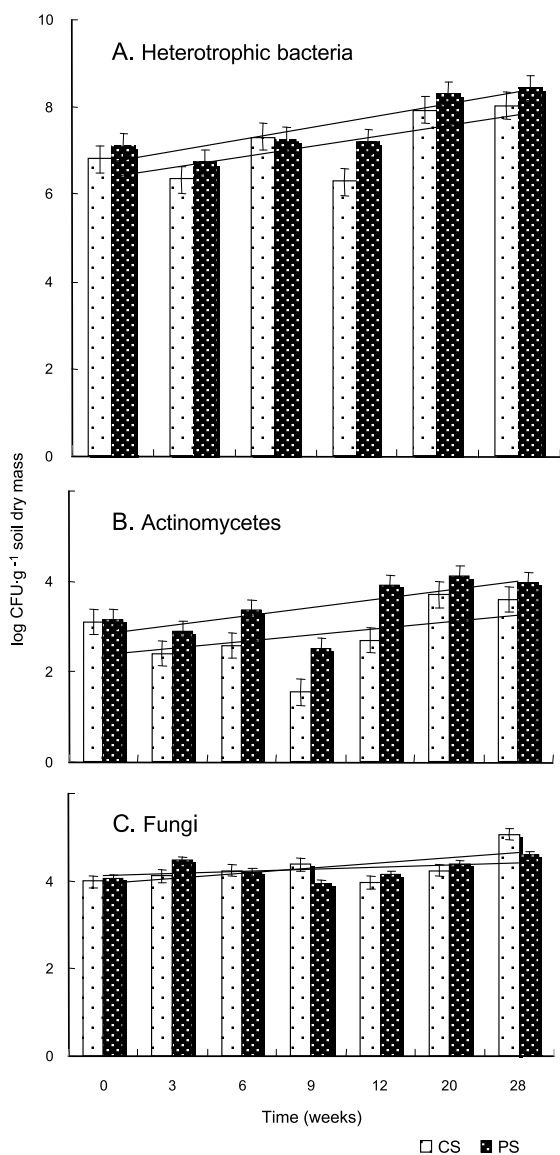


Figure 1. Numbers of culturable heterotrophic bacteria, actinomycetes and fungi in treated and control soils. Trend lines for microbes are presented. CS - control soil; PS - treated soil.

experiment the number of heterotrophic bacteria was $1.35 \cdot 10^7$ CFU·g⁻¹ soil dry mass. During the first 3 weeks after treatment a decrease of 59% was observed. Over the following weeks, the bacteria numbers insignificantly increased and reached $2.21 \cdot 10^8$ CFU·g⁻¹ soil dry mass in week 28. This was about a 94% increase from the beginning of the experiment. In the control soil, the numbers of heterotrophic bacteria were about 60% lower than in the treated soil at the end of the experiment.

Considerable changes in the number of actinomycetes were also noted (Figure 1B). In the first weeks of the experiment, actinomycetes decreased from $1.44 \cdot 10^3$ CFU·g⁻¹ soil dry mass to $7.69 \cdot 10^2$ CFU·g⁻¹ soil dry mass (about 47%). However, in subsequent weeks they increased and in week 20 the number of actinomycetes reached $1.36 \cdot 10^4$ CFU·g⁻¹ soil dry mass and was stable over the following weeks. In the treated soil the number of actinomycetes had increased about 89% at the end of the experiment. Throughout the experiment the number of actinomycetes increased from 40 to 60% in comparison to untreated soil.

Fungi were the third group of microorganisms that were evaluated. The differences over 28 weeks were not as pronounced as with heterotrophic bacteria and actinomycetes (Figure 1C). In the beginning of the experiment, the number of fungi was $1.13 \cdot 10^4$ CFU·g⁻¹ soil dry mass and this increased to $1.86 \cdot 10^4$ CFU·g⁻¹ soil dry mass in week 20. The number of fungi remained relatively low compared to bacteria. However, in week 28 of the experiment, they increased to $3.74 \cdot 10^4$ CFU·g⁻¹ soil dry mass and $1.16 \cdot 10^5$ CFU·g⁻¹ soil dry mass in treated and control soils, respectively.

By week 28, the bacteria, actinomycetes and fungi had increased in treated soil about 16-fold, 10-fold and 2-fold, respectively. In turn, in the control soil the increases for heterotrophic bacteria, actinomycetes and fungi were lower: 13-fold, 4-fold and 2-fold, respectively. The stimulation of microbial growth is a positive effect of this amended phytostabilization. Culturable bacteria were found to be the largest and most active part of autochthonous microflora in treated soils. There were no significant differences between populations in treated and control soils (Table 2).

Table 2. Results of Student t-test to determine differences between numbers of designated microorganism groups in treated and control soils.

Culturable microorganisms	Statistical significance
Heterotrophic bacteria	0.3555
Copiotrophs	0.2566
Oligotrophs	0.1661
Actinomycetes	0.1251
Fungi	0.6900

Differences statistically not significant ($p > 0.05$)

The bacterial colony development method was used to determine eco-physiological differences in microbial communities in soil under aided-phytostabilization. In Table 3, changes in the biodiversity of bacterial populations as evaluated by EP and CD indices for 28 week period of aided phytostabilization are presented. The EP and CD indices can be used to characterize soil bacterial communities (De Leij et al. 1993; Kotsou et al. 2004) as a measure of the number and abundance of species in a population. The EP index can be used to indicate changes in microbial community structure, including microbial shifts from r-strategists (copiotrophs) to K-strategists (oligotrophs). The concept of r/K strategy is derived from evolutionary ecology and attempts to explain genetic differences between organisms that can exploit and survive in different kinds of environments (De Leij et al. 1993). R-strategists are more competitive in nutrient-rich environments, more sensitive to environmental toxins, and they grow better in uncrowded conditions; conversely, K-strategists are characterized by slow growth in response to enrichment, but are

more successful in stable and crowded environments (De Leij et al. 1993; Kotsou et al. 2004; Margesin et al. 2011). In general, r-strategists are characteristic in unstable environments, while K-strategists grow better in stable environments (Sarathchandra et al. 1997).

Application of the EP-index to calculate diversity showed a more than twofold increase in diversity of oligotrophs, from EP=0.16 on day 1 to EP=0.35 on week 28. This can be explained by the fact that amendments applied in the experiment had an enriching effect on the soil microorganisms.

Similar values of EP-index for oligotrophs and copiotrophs were obtained for the control soil. During the experiment the predominance of K-strategists was observed (Figure 2). The ratio of oligotrophs to copiotrophs in treated and control soils during the experiment is presented in Figure 3. In the treated soil the ratio of oligotrophs to copiotrophs was generally higher than in the control soil. A high ratio, e.g., predominance of oligotrophs, may indicate stable environmental conditions with low substrate availability (Nielsen and Winding 2002).

Table 3. Colony development (CD) and eco-physiological (EP) indices for bacterial populations in treated and untreated soils.

Week of experiment	Oligotrophs		Copiotrophs	
	CD	EP	CD	EP
Treated soil				
0	34.22 ± 1.76*	0.16 ± 0.02	59.41 ± 2.33	0.23 ± 0.009
3	27.64 ± 0.76	0.20 ± 0.01	47.90 ± 1.09	0.33 ± 0.071
6	29.38 ± 2.03	0.25 ± 0.09	56.73 ± 0.65	0.29 ± 0.080
9	39.70 ± 1.34	0.28 ± 0.067	47.59 ± 0.79	0.27 ± 0.005
12	46.70 ± 0.98	0.28 ± 0.07	48.67 ± 1.57	0.26 ± 0.006
20	46.67 ± 3.01	0.24 ± 0.013	47.75 ± 2.11	0.29 ± 0.01
28	54.87 ± 1.22	0.35 ± 0.007	74.26 ± 1.54	0.26 ± 0.007
Untreated soil				
0	31.30 ± 0.89	0.21 ± 0.09	64.50 ± 0.99	0.21 ± 0.004
3	27.17 ± 1.06	0.22 ± 0.01	47.48 ± 1.11	0.26 ± 0.006
6	31.56 ± 1.76	0.24 ± 0.004	60.55 ± 2.34	0.21 ± 0.021
9	35.24 ± 0.99	0.24 ± 0.06	38.78 ± 1.89	0.28 ± 0.003
12	33.73 ± 2.56	0.23 ± 0.02	41.15 ± 3.21	0.26 ± 0.03
20	58.49 ± 1.88	0.20 ± 0.01	46.15 ± 2.38	0.21 ± 0.007
28	63.04 ± 1.61	0.28 ± 0.012	57.56 ± 0.89	0.29 ± 0.04

*mean ± SD; N = 3

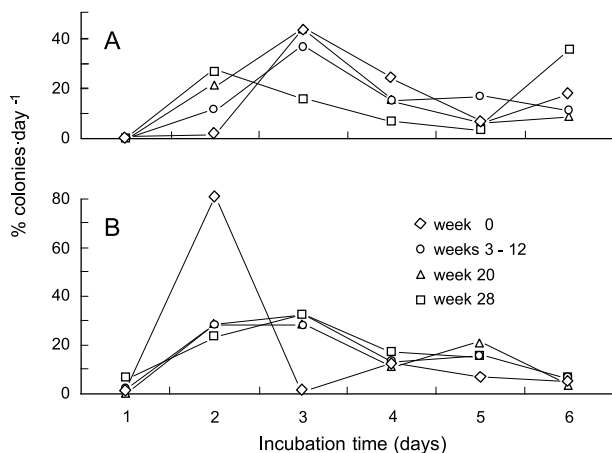


Figure 2. Changes in the oligotroph (A) and copiotroph (B) populations in treated soil.

Literature was not found concerning how aided phytostabilization changes the ratio of r- to K-strategists. Margesin et al. (2011) obtained high EP indices for both copiotrophs and for oligotrophs, which indicated high bacterial diversities in heavy-metal contaminated soils. The application of amendment materials to the soil led to an increase of the CD index in both copiotroph and oligotroph populations after 28 weeks of the experiment. However, in our experiment copiotrophs reached higher CD values at the end of the experiment (Table 3).

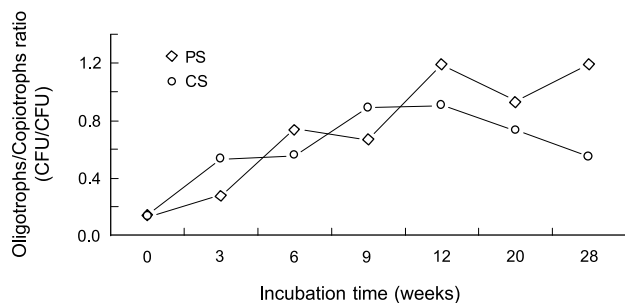


Figure 3. Ratio of oligotrophs to copiotrophs during aided phytostabilization. PS - treated soil; CS - control soil.

Finally, an increase of ecological indices values (EP and CD) and microbial population density at week 28 was observed in the treated soil. Based on these results, phytostabilization of heavy metals benefited from the addition of amendments. There was a positive effect on the native microbial populations evaluated by culturable techniques. The diversity of opportunistic and non-opportunistic species, as formalized in the r/K theory developed for macroecology, can be applied to microbial ecology (Sarithchandra et al. 1997).

In most studies, only plant growth and metal uptake have been monitored to evaluate the efficiency of remediation technologies. However, a few studies have made use of biological indicators to assess the effects of remediation techniques on the functionality, composition and diversity of the soil microbial community (Garau et al. 2007; Kumpiene et al. 2009; Lombi et al. 2002; Mench et al. 2006; Renella et al. 2008). Several measurements of microbial and biochemical parameters are used to assess the effect of different amendments on soil biological quality.

Different approaches, e.g., culture-dependent and culture-independent methods should be combined and tested for a better insight into microbial populations in order to be able to effectively evaluate the short- and long-term effects of remediation technologies. In accordance with this, additional research is needed to assess other microbiological indicators and tests, such as: respiration, enzyme activities, Biolog™ assay and molecular methods such as: FAMES and PLFAs fatty acids analysis, *in situ* PCR, DGGE or FISH.

Our previous results also indicated a positive effect of aided phytostabilization of Pb-Cd contaminated soil on enzyme activity, respiration and substrate-induced respiration (SIR) (Krzyżak et al. 2012). Activities of β-glucosidase, acid and alkaline phosphomonoesterase and dehydrogenase were significantly higher in treated soil than in the control (untreated) soil. Also, soil respiration and SIR were significantly higher in the treated than the control. On the basis of the obtained results we have shown that aided phytostabilization improved soil biological properties.

Information on both short- and long-term effects of phytostabilization on microbial community structure and diversity is as important for understanding the relationship between environmental factors, remedial technologies, and ecosystem functions.

CONCLUDING REMARKS

Soil functions are directly and indirectly related to soil microbiota. Microorganisms play a major role in many specific soil biochemical processes (Bardgett 2005). In order to assess the efficiency of aided phytostabilization for improving the health status of a heavy metal-contaminated soil, biological methods should be more frequently used (Bloem et al. 2006; Hinojosa et al. 2010).

Our results suggest that traditional microbiological indicators based on cultivation techniques may be useful tools in the assessment of changes in microorganism populations and can provide valuable information on the impact of amended phytostabilization on soil biological quality. However, our results show the microbial population changes in the first weeks of the experiment, i.e., the short-term effects of aided phytostabilization. The CFU method is a simple and inexpensive approach and can be ready to use upon standardization of incubation and counting procedures. The results of our study support the use of microbiological methods

for monitoring aided phytostabilization. Microorganisms respond quickly to environmental stress and they rapidly adapt to it; changes in diversity and activity of microbial populations are excellent indicators of soil health, providing an early sign of soil changes.

The use of amendments for the transformation of inorganic contaminants in soils/sediments offers considerable potential for long-term environmental cleanup. Microorganisms have proven valuable as amendments for reducing soil contaminant availability (Knox et al. 2008). Natural autochthonous microorganisms are highly sensitive bioindicators of soil health and they perform many specific soil processes. The number of microorganisms, their activity and biodiversity are very important and necessary for the proper functioning of soil, including nutrient cycling and moisture retention (Alkorta et al. 2010; Kandeler 2007; Markert et al. 2003). Microbial measurements are recommended for use in soil health monitoring programs (Nielson and Winding 2002).

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