Response of soil bacterial communities pre-exposed to different metals and reinoculated in an unpolluted soil

M. Díaž-Raviña\textsuperscript{a,b,}\textasteriskdash, E. Bååth\textsuperscript{a}

\textsuperscript{a}Department of Microbial Ecology, Lund University, Ecology Building, S-223 62 Lund, Sweden
\textsuperscript{b}Plant Biology and Soil Science Department, Vigo University, Edificio Politécnico, 32004 Orense, Spain

Received 29 June 1999; received in revised form 25 October 1999; accepted 4 July 2000

Abstract

Changes in tolerance levels of soil bacterial communities pre-exposed to different metal concentrations and then reestablished in an unpolluted soil were examined during a one year incubation period using the thymidine incorporation technique. The study was performed with a sterilized agricultural soil, which was reinoculated with bacteria extracted from soils previously contaminated with high doses of Zn, Cu or Cd, and from an unpolluted soil. Bacteria pre-exposed to metal addition initially exhibited a greater tolerance than those non-pre-exposed. The microbial communities responded to the absence of metals in the reinoculated soil with a rapid decrease in community tolerance, losing most of the acquired tolerance (70\textendash90\%) within the first week. After that no changes in community tolerance were detected. Thus, a long-lasting effect of the original community tolerance of the inoculum was detected even 12 months after the metal stress was removed. At this time there was still a dose-response effect left since higher tolerance levels were usually found in soils with higher tolerant inoculum. Changes in tolerance levels over time showed similar trends, irrespective of which metal the bacterial communities were initially tolerant. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Heavy metals; Soil bacterial communities; Metal tolerance; Thymidine incorporation technique

1. Introduction

Microbial adaptation to environmental stress at the community level has rarely been studied, partly because of the lack of available methods. Recently, the thymidine and leucine incorporation techniques have been adapted to study heavy metal toxicity and tolerance of soil bacterial communities (Bååth, 1992a; Díaž-Raviña et al., 1994; Díaž-Raviña and Bååth, 1996b). The methodology is rapid, sensitive, and selective in assessing the metal impact, and is thus attractive for monitoring changes in microbial communities under metal stress. The application of this technique in experimentally metal polluted soils indicated that the bacterial community tolerance changed over time. It has been suggested that there is an immediate tolerance increase due to initial toxicity, followed by a more gradual increase due to the different competitive ability of surviving bacteria and to the physiological and/or genetic adaptation (Díaž-Raviña and Bååth, 1996a).

Previous results of several laboratory and field experiments concerning the long-term heavy metal effects showed an increased bacterial community tolerance to metals present in soil, with the tolerance increase being correlated with the pollution level (Díaž-Raviña et al., 1994; Díaž-Raviña and Bååth, 1996a; Pennanen et al., 1996; Bååth et al., 1998a,b). Shifts in the microbial community structure, as determined by the phospholipid fatty acid analysis (PLFA pattern), were also observed in these polluted environments when compared to the corresponding non-contaminated soil (Frostegård et al., 1993, 1996; Pennanen et al., 1996; Bååth et al., 1998a,b), suggesting that changes in microbial species composition led to a more tolerant population. Similar increases in tolerance have been found due to other toxic compounds, e.g. compounds produced after heating of soil (Díaž-Raviña et al., 1996).

There is however little information available identifying and quantifying the microbiological changes that take place in soil ecosystems once the toxic stress is removed. In principle, after a temporary stress, the reversibility of the process is not assured and therefore the microbial population might either retain or lose the tolerance previously acquired. Although the latter seems the more probable hypothesis, it needs experimental verification. The rate at...
which tolerance is lost is also of interest. The present investigation is a first attempt to quantify the time course of loss of tolerance after a toxic stress has been removed using metals as a model for toxic substances. Thus, the response was studied of soil bacterial communities pre-exposed to different metal concentrations and then established in a non-metal stressed, sterilized soil. This allowed us to estimate the loss of tolerance in two different situations: one where abundant substrate was colonized (directly after inoculation of the sterilized soil) and the other where the soil was colonized and substrate availability was low (after initial recolonization).

2. Material and methods

2.1. Experimental set-up

The study was performed with an agricultural sandy loam soil (4.4% organic matter, pH 7.8). The soil was initially artificially contaminated in the laboratory with high concentrations of Zn (8, 16 and 32 mmol kg$^{-1}$ dry weight (d.w.) as a ZnSO$_4$ solution), Cu (16 and 32 mmol kg$^{-1}$ d.w. as a CuSO$_4$ solution) and Cd (8 and 16 mmol kg$^{-1}$ d.w. as a CdSO$_4$ solution) and incubated at room temperature (approximately 22°C) and constant moisture level during different storage times.

Microbial inoculum was prepared from these soils by homogenizing 10 g of soil in 50 ml distilled water in a Sorvall Omnimixer for 1 min at 80% full speed, followed by centrifugation for 10 min at 750g and the supernatant was collected. Five ml of the resulting supernatant bacterial solution were inoculated into 100 g dry weight of unpolluted sterilized soil, which then was kept at constant conditions of moisture and temperature (75% of water holding capacity, 22°C). The unpolluted soil had previously been sterilized by autoclaving for 3 h at 121°C on two consecutive days.

Two separate experiments with different lengths of incubation were carried out using two replicates of each treatment. First, the tolerance of soil bacterial communities pre-exposed to 16 and 32 mmol Zn kg$^{-1}$ d.w. for 28 months and established in the sterilized soil was examined on a short time-scale. Measurements were made 4, 8, 16 and 32 days after soil reinoculation. In the second experiment, the tolerance of soil bacterial communities pre-exposed to Zn, Cu and Cd for 14 months, and to Zn for 3 months, was studied on a long time-scale. Measurements were made 1, 3 and 12 months following soil reinoculation. Sterilized soil samples reinoculated with bacteria from unpolluted soils were also included in each experiment and used as controls (not pre-exposed to metals). In both experiments tolerance measurements at day 0 were made on the contaminated soils, since the extremely low activity values detected initially in the reinoculated sterilized soil made it impossible to estimate the bacterial tolerance. In both experiments bacterial activity (thymidine and leucine incorporation rate) was examined in the same bacterial solutions that were used for the tolerance measurements.

In addition, for the long time-scale experiment, the PLFA pattern was examined at the end of the experiment. This was only made on treatments pre-exposed to the highest metal levels. Soil samples contaminated at the highest levels (after 2 years incubation), originally used for preparing the inoculum, as well as unpolluted samples from both fresh and 2 years stored soil, were also included in the analyses.

2.2. Bacterial activity measurements

Bacterial activity was simultaneously determined by the incorporation of thymidine (Tdr) and leucine (Leu) into bacteria, following the procedure previously described (Bååth, 1992b, 1994). On each measurement occasion, 10 g of soil, obtained by mixing 5 g of each treatment replicate, was homogenized with 200 ml distilled water, centrifuged at 750g for 10 min and the supernatant was collected. The extracted bacterial suspension (2 ml) was incubated with 100 nM [²H]-thymidine (925 Gbq mmol$^{-1}$, Amersham, UK) and 395 nM L-[¹⁴C]-Leucine (11.9 Gbq mmol$^{-1}$, Amersham, UK) at 20°C for 2 h and then the reaction was stopped by adding 1 ml of 5% formalin. Filtration, washing of the filters and scintillation counting were performed as described by Bååth (1994). Analyses were carried out in duplicate because the coefficient of variation between replicate measurements was usually <5%.

2.3. Tolerance measurements

Metal tolerance of soil bacterial communities was determined by means of the thymidine incorporation technique, as previously described (Bååth, 1992a; Díaz-Ravíña et al., 1994; Díaz-Ravíña and Bååth, 1996b). Bacteria were first extracted from soil by homogenization–centrifugation as described above. The bacterial solutions (1.8 ml) thus obtained were amended with different amounts of heavy metals (0.2 ml) in a range of concentrations giving no inhibition to total inhibition of incorporation. These suspensions were incubated with the labelled substrate and the incorporated radioactivity was measured. The data were expressed as a percentage of the treatment with no metal added (control). A plot of these percentage inhibition values versus log metal concentration in the bacterial suspension has earlier been shown to follow the logistic model (Díaz-Ravíña et al., 1994), which then could be used to calculate the values of IC$_{50}$ (the logarithm of the metal concentration resulting in a 50% reduction compared to the control) applying nonlinear regression. In addition, it has also been stated that IC$_{50}$ can be calculated from the slope of the decreasing linear part of the plot of percentage of inhibition versus log metal concentration, when not enough data points are available for stable nonlinear regression. This latter approach was therefore used in the present study. Since the calculation was made using log metal
concentrations in the bacterial suspension, we used logarithmic values for IC$_{50}$. This IC$_{50}$ value is a relative measure of the metal tolerance of the bacterial community and should not be compared with metal concentrations in the soil. Changes in the bacterial community tolerance level were estimated from the difference between IC$_{50}$ values in soils reinoculated with bacteria pre-exposed and non-pre-exposed to heavy metals ($\Delta$IC$_{50}$ = IC$_{50}$ pre-exposed − IC$_{50}$ non-pre-exposed). Examples of plots showing percentage of inhibition versus log metal concentration and a description of the whole methodology are found in Dõaz-Ravinäa et al. (1994).

2.4. Lipids extraction and PLFA analysis

The phospholipid extraction and phospholipid fatty acid (PLFA) analysis were performed following the procedure described by Frostegård et al. (1993). Lipids were extracted with a chloroform–methanol–citrate buffer mixture and fractionated into neutral, glyco- and phospholipids on columns containing silicic acid. The phospholipids were subject to a mild alkaline methanolsysis to obtain methyl esters of the fatty acids before analysing on a gas chromatograph. The PLFA data were expressed as mole percent and logarithmically transformed before principal-components analyses (PCA) were performed.

3. Results

The thymidine (TdR) and leucine (Leu) incorporation values for bacteria extracted from non-sterilized unpolluted soil were $9.7 \times 10^{-14} \pm 2.7 \times 10^{-14}$ mol h$^{-1}$ ml$^{-1}$ and $20.2 \times 10^{-13} \pm 3.3 \times 10^{-13}$ mol h$^{-1}$ ml$^{-1}$, respectively (mean ± standard error, $n = 2$). The development of the bacterial activity after reinoculation of the sterilized soil was very similar irrespective of whether the inoculum came from polluted or unpolluted soil. Therefore, the mean values for each measurement occasion were calculated for TdR and Leu incorporation rate, irrespective of the inoculum origin (Fig. 1). Initially after reinoculation, TdR and Leu incorporation values in the sterilized soil were 1–2% of those in the corresponding non-sterilized soil (see above), which probably was due to the activity of the inoculum. This was followed by a rapid recovery within the first week of incubation. After 4 days of incubation, TdR and Leu incorporation values had already increased by a factor of 5 and 20 compared to the non-sterilized unpolluted soil, respectively. The activity increased until day 16 for the
TdR incorporation values, while for the Leu incorporation rate the high values were maintained between 4 and 16 days of incubation. After that incorporation values decreased slowly, but they were still slightly higher than the initial values in the non-sterilized soil by the end of the incubation period (one year).

Although the pattern of incorporation was similar for TdR and Leu incorporation, the response of the Leu incorporation to reinoculation was more rapid and pronounced than that detected for the TdR incorporation. Thus, the Leu/TdR incorporation ratio, which in the non-sterilized soil was around 20–25, increased to a maximum value of 90 four days after reinoculation (Fig. 1). Then the Leu/TdR incorporation ratio declined until the end of the incubation period, when values comparable or even lower than those in the non-sterilized soil were found.

The overall IC50 values (log metal concentration) for Cu, Cd and Zn of the bacterial community in the sterilized unpolluted soil reinoculated with inoculum from the unpolluted soil were 2.627 ± 0.06, 2.513 ± 0.04 and 2.438 ± 0.03 (mean and SE, n = 3), respectively. These values were similar to those in the unpolluted non-sterilized control soil (2.616 to 2.665 for Cu, 2.528 to 2.551 for Cd and 2.483 to 2.517 for Zn, range taken from Díaz-Ravinía et al., 1994 and Díaz-Ravinía and Bäath, 1996a,b, studying the same soil as in the present work). This indicates that bacteria did not change their natural characteristics in terms of metal tolerance when established in a sterilized soil.

Changes in bacterial community tolerance (ΔIC50) over time in the short-time scale experiment using an inocula of bacteria pre-exposed to 16 and 32 mmol Zn kg⁻¹ d.w. for 28 months are shown in Fig. 2. Initially, Zn tolerance had increased by 1.98 and 2.38 logarithmic units as a consequence of the earlier exposure to 16 and 32 mmol Zn kg⁻¹ d.w., respectively. These tolerance levels declined quickly with most of the changes in tolerance already occurring during the first 4 days following soil reinoculation. Then, after a slight decrease between 4 and 8 days, tolerance levels hardly changed over the rest of the incubation period.

Changes in tolerance measurements of soil bacterial communities, pre-exposed to Zn, Cd or Cu stress, on a long-time scale are reported in Fig. 3. Similar results were obtained for bacterial communities irrespective of both metal and concentration of the earlier exposure. Bacterial community tolerance obtained as a consequence of the earlier heavy metal exposure was lost quickly, since only 15–31% (mean 22%) of community tolerance was retained 1 month after reinoculation. The average percentage of tolerance retained after 3 and 12 months ranged from 13 to 34% (mean 19%) and from 7 to 32% (mean 18%), respectively. No major changes in tolerance between 1 and 12 months were therefore observed.

Although most of the tolerance disappeared in a short time, some metal effect was still evident after 1 year of incubation (Fig. 3). At this time the bacterial community tolerance also appeared to be correlated with the concentration of the previous exposure. Thus, higher tolerance levels were usually found in treatments initially inoculated with bacteria from the most polluted soils compared to treatments inoculated with a less tolerant community. This was found irrespective of whether the tolerance was induced by Cd, Cu or Zn, although the differences were small for Cd and Cu. This was also found in both short- and long-term experiments after 1 month incubation (Figs. 2 and 3, respectively).

The PLFA patterns from the sterilized and recolonized soils after one-year incubation were compared with those of the polluted soils originally used to obtain bacterial
inoculum (Fig. 4). Only samples contaminated at the highest levels were included in the principal component analyses. The results showed that PLFA pattern clearly differentiated between the reinoculated sterilized samples and the other treatments. Thus, in the first principal component, accounting for 49.9% of the variation in the data, the recolonized soils were all found to the left while the originally polluted soils were situated to the right. Little variation was found in the second principal component (13.6%), which appeared to differentiate between non-polluted and polluted samples in the original soils (non-reinoculated), although an influence of storage time was also indicated. However, this may not hold for the reinoculated sterilized soils since the treatment inoculated with bacteria from initially unpolluted soil did not differ from those inoculated with metal tolerant communities.

4. Discussion

Sterilization by autoclaving kills the soil microbial community and provides a readily available pool of substrate derived from the dead cells (Salonius et al., 1967; Jenkinson and Powlson, 1976b). In our study this allowed rapid recolonization after inoculation, with high bacterial activities, which peaked after only about 4–16 days (Fig. 1). During this period a selective pressure may have favoured fast-growing bacteria. Although this is a very unnatural situation, it may occur where a large part of the microflora is killed, e.g. by fumigation (Jenkinson and Powlson, 1976a; Díaz-Raviña et al., 1989) or addition of large quantities of heavy metals. This latter treatment resulted in a soil recolonized with bacterial communities showing activities several times higher than that of the corresponding unpolluted control (Díaz-Raviña and Báath, 1996a,c). However, as a consequence of the toxic metal impact, the recovery of bacterial activity was slower than that found in the present study and peak activities at the highest metal levels were detected after more than 16 days of incubation. A similar or even slower recovery in TdR and Leu incorporation rates was found after soil sterilization by heating at 200°C and reinoculation with fresh soil irrespective that organic substrates were added (Díaz-Raviña et al., 1996). This behaviour was explained by the presence of a water-soluble toxic substance produced during soil heating at this high temperature.

As well as a slower recovery of bacterial activity in metal treated soils (Díaz-Raviña and Báath, 1996a,c) compared
with the sterilized non-polluted soil studied here, a further difference is the existence of selection pressures for both fast-growing and metal tolerant bacteria. When the metal selection pressure was removed as in the present study, metal tolerance of the inoculum community disappeared very rapidly (Fig. 2). Two explanations can be put forward, which are not mutually exclusive. Firstly, metal-tolerant bacteria are not as fast growing as at least some of the metal-sensitive ones. Secondly, metal-tolerant bacteria have an energetic burden which will be a competitive disadvantage during conditions of normal soil metal concentrations. The latter explanation is similar to lower competitive ability of antibiotic resistant bacteria explaining the decrease in resistant bacteria when the use of antibiotics was stopped (Baquero and Blázquez, 1997). The fact that metal tolerance can be a disadvantage in an unpolluted situation was also discussed for other groups of organisms (plants, animals) by Tyler et al. (1989). Furthermore, the rapid response of the non-tolerant microorganisms during recolonization indicated that they were already present, although in low numbers, in polluted soils.

After the fast decline in bacterial community metal tolerance during the first week, very little subsequent change in metal tolerance was found. Thus, after one year there was still higher metal tolerance in soils initially inoculated with a metal tolerant bacterial community compared to a non-tolerant one and higher levels of tolerance were usually observed when the inoculum emanated from heavily polluted soils (Fig. 3). The presence of high soil–metal concentrations added with the inoculum is an unlikely explanation for this remaining community metal tolerance. For example, assuming the very unrealistic situation that the whole total metal content became water soluble, the inoculum from the soil with the highest metal concentration, 32 mmol Zn kg$^{-1}$ d.w., would not increase the metal concentration in the unpolluted autoclaved soil by more than 0.32 mmol kg$^{-1}$ d.w. Earlier studies with the same soil indicated that significant effects on tolerance of bacterial communities to Zn were only found at metal levels higher than 1 mmol Zn kg$^{-1}$ d.w. (Díaz-Raviña and Bååth, 1996a).

It must be emphasized that the remaining bacterial metal tolerance measured after one year cannot have been due to surviving dormant organisms or spores. This could have been the case of tolerance measurements by means of either plate count techniques (Doelman et al., 1994) or multiwell plates with individual carbon sources (Rutgers et al., 1998) to measure bacterial community tolerance, since the use of these methods required several days of incubation. Instead, the remaining bacterial community metal tolerance detected using the thymidine incorporation approach must be due to actively growing cells, since growth rate is measured during a short time period (2 h) and hence no breaking of dormancy or spore germination could have occurred to such an extent as to affect the measurements.

Several explanations could be proposed for the bacterial community metal tolerance remaining even after one year in these unpolluted soils. The initial presence of high nutrient levels due to killed microorganisms will have been replaced by a situation of nutrient shortage, with lower bacterial activities and turnover rates in the later stages of the experiment. It is probable that the takeover of a more competitive community takes longer at lower turnover rates than at higher ones. Another possibility would be that the metal tolerant bacteria that initially colonized the soil did not show any decreased competitive abilities due to their metal tolerance and thus could persist in the soil during the subsequent incubation time. A third explanation could be the fact that the initial situation after soil sterilization and reinoculation, where no niches will be occupied and thus empty spaces will be colonized, will change later on to a situation where all niches will be occupied by bacteria. The presence of bacteria in a place might be a competitive advantage per se, compensating for any metabolic burden due to the metal tolerance. This could be compared with plants, where it is well known that invasion of new plant species can be promoted by soil disturbance (Hobbs and Huenneke, 1992). This is similar to our study, where the initial sterilization is a disturbance facilitating the establishment of non-tolerant organisms even from the metal tolerant inoculum, while without any disturbance (that is the period after the initial recolonization), new or less dominant species will have difficulties in invading, resulting in a low but maintained
metal tolerance despite the absence of increased soil metal concentrations.

The PLFA profiles, in part, indicated similar changes in the microbial communities, as did the tolerance measurements (Fig. 4). The differences in the PLFA pattern, that were found in the originally polluted soils, compared with unpolluted ones, in the second principal component, had disappeared in the sterilized and reinoculated soils after one year of incubation. This indicated that most of the metal-tolerant community had been replaced with a non-tolerant one, which was similar irrespective of the inoculum origin. However, the PLFA technique did not appeared sensitive enough to detect any significant remaining metal tolerance after one year. This could partly be attributed to the larger differences due to the sterilization and recolonization compared with the original soil samples (see first principal component). Changes in the PLFA pattern can normally be attributed to several variables, which can interact, and therefore the contribution of a specific variable is difficult to discern. As a consequence, the influence of important factors may confuse the effect of less important variables, e.g. the inoculum origin effect was masked by the sterilization/recolonization treatment. Therefore, compared with PLFA pattern, the tolerance measurements are a more sensitive index for detecting heavy metal impact since environmental factors other than the presence of metal are not taken into consideration. This has been discussed more thoroughly by Bååth et al. (1998b).

Although the TdR and Leu incorporation technique gave similar results for the development of bacterial activity after reinoculation (Fig. 1), there were significant changes in the Leu/TdR incorporation ratio over time (Fig. 1). A peak in this ratio was found after a few days of incubation, after which the ratio returned to its initial values. In aquatic batch recolonization cultures higher Leu/TdR incorporation rates are also found shortly after inoculating the cultures (Chin-Leo and Kirchman, 1988, 1990). This was explained by unbalanced growth (Chin-Leo and Kirchman, 1990). However, altered species composition, where different communities have somewhat different abilities to incorporate TdR and Leu, cannot be ruled out. This explanation was put forward as the reason for the long-term changes in Leu/TdR incorporation ratios observed in heavy metal polluted soils (Díaz-Raviña and Bååth, 1996c).

In the present laboratory experiment, the late phase showing low bacterial turnover times and a possible competitive advantage for those organisms that are already present is probably more similar to the natural situation than is the initial phase with fast recolonization. Thus, the rapid disappearance of tolerance during the first week will not be similar to the field conditions where a selection pressure due to some toxic compound is removed by a soil remediation procedure. Instead, the present study suggests the existence of a slow process, where a remaining altered tolerance can probably persist for a long time. The data of Suett et al. (1996) showing an enhanced pesticide degrading ability of soil microorganisms long after the pesticide has been metabolised seem to confirm this hypothesis. However, in other cases soil remediation procedures, e.g. by removing toxic substance using chemical methods, will certainly disturb the microbial community to a large extent, and thus being more similar to the situation in the initial part of our experiment. Further studies should be focused on the response and adaptation of microbial communities to changing environmental conditions and evaluates the capacity of the soil to restore itself after both temporary and permanent disturbances.

Acknowledgements

This study was supported by grants from European Environmental Research Organization (EERO) and the Ministerio Español de Educación y Ciencia to M.D.-R. and from the Swedish Natural Science Research Council to E.B.

References

Díaz-Raviña, M., Bååth, E., Frostegård, A., 1994. Multiple tolerance of soil bacterial communities and its measurement by a thymidine
incorporation technique. Applied and Environmental Microbiology 60, 2238–2247.


