Some observations on the copper tolerance of bacterial communities determined by the (³H)-thymidine incorporation method in heavy metal polluted humus

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Abstract

Changes in pH after filtration of bacterial suspensions are important when applying the radioactive thymidine incorporation method to heavy-metal polluted soils with low microbial activity. In the original method (Bååth 1992; Soil Biology and Biochemistry 24, 1157–1165) the blended and centrifuged suspension was filtered through glass wool to remove humus particles from the suspension. When we filtered the bacterial suspension through glass wool the pH increased by 2 units and the thymidine incorporation rate decreased. This made the community copper tolerance measurement ambiguous. When using soil samples with very low activity, we recommend the use of acid-washed glass wool or polyester net filtration which eliminates changes in pH.

Keywords: Copper; Heavy metals; Microbial activity; Thymidine

The bacterial growth rate in soil has been studied on the basis of the incorporation of radioactive thymidine into the macromolecules of bacteria extracted from soil after homogenization-centrifugation (Bååth, 1992a). The technique has been modified for determining the heavy-metal tolerance of soil bacterial communities (Bååth, 1992b; Diaz-Raviña et al., 1994; Diaz-Raviña and Bååth, 1996; Pennanen et al., 1996; Pennanen et al., 1998). For the assay, the bacterial suspension was mixed with a range of heavy metal concentrations that gave no to complete inhibition of bacterial growth. Growth was estimated by adding (³H)-thymidine and measuring its incorporation into the bacteria during a 2-h period. The IC₅₀ value, i.e. the metal concentration resulting in 50% inhibition, was then calculated. The higher the IC₅₀ value, the greater is the tolerance of the bacterial community to the metal in question. An overview of the suitability of the method for studying heavy-metal pollution has been given by Bååth et al. (1998).

We applied the (³H)-thymidine incorporation method for measuring the heavy-metal tolerance of bacteria in a study on the microbial remediation of heavy-metal polluted humus. The polluted humus, which had a very low microbial activity, gave low mean incorporation values with high standard deviations. This made it difficult to assess a change in the IC₅₀ values of Cu due to the addition of an organic top layer onto polluted humus. We therefore decided to reinvestigate the preparation of the bacterial suspension in order to raise the (³H)-thymidine incorporation rate of coniferous humus with a high organic matter content and high metal concentrations.

Forest humus (F+H layer) from two different sites...
were used: unpolluted humus from a forest site in southern Finland, and heavy-metal polluted humus from a site located 2 km from a Cu-Ni smelter in southwestern Finland (Pennanen et al., 1996). The unpolluted and polluted humus contained 69% and 33% organic matter (loss in weight on ignition), and the respective basal respiration rates (14C), describing microbial activity, were 26 and 5 µg CO2 g−1 d.m. h−1. The total copper concentration in the unpolluted humus was less than 100 mg and in the polluted humus 3000 mg kg−1 d.m. The pHH2O of the samples was ca 4. The samples were sieved (mesh size 2.8 mm) and stored at 4°C until analyzed. Copper tolerance measurement was determined as described by Bååth (1992a, b) using 1.0 g d.m. of fresh unpolluted humus or 3.0 g d.m. of polluted humus. The radioactivity was counted in a Wallac 1411 liquid scintillation counter using the fine-tuned external standard method. Two homogenization techniques for preparing the bacterial suspension and three filtration techniques were tested. The homogenization techniques were blending in a Sorvall Omnimixer at 80% of top speed for 1 min as used by Bååth (1992a, b) (B), and rotary shaking at 250 rpm for 1 h at 4°C (S). The filtration techniques were glass wool (Pyrex fiber glass, Sliver 8 micron; W), acid-washed (2% HCl) glass wool (AW), and a polyester net (mesh size 0.2 × 0.8 mm; N). We investigated the following four combinations: B–W, B–AW, B–N, and S–N, the B–W technique being the one originally published by Bååth (1992a).

The filtration technique affected the Cu tolerance value, whereas homogenization had only a minor effect. As an example, the results for the polluted humus are shown in Fig. 1. The difference in the IC50 values was mainly attributable to that between the B–W and the other treatments, the IC50 value for B–W being 0.00003 M Cu and for B–N 0.035 M Cu. For the unpolluted humus, the effect of the techniques was similar but smaller. Since replicates were not used, no statistical analysis could be performed on the data, but in repeated measurements the result was reproducible.

Possible explanations for the differences in IC50 values between the different homogenization and filtration techniques are (i) bacteria with different Cu tolerance were retained selectively in the suspension by the different filtration techniques, (ii) the isotope dilution increased with increasing copper concentrations due to the lysis of living cells, (iii) the copper was partially complexed with the humus, thereby lowering the effective metal concentration, or (iv) the pH values of the incubation suspension following filtration were different.

All four hypotheses were tested using polluted humus and the contrasting B–W and B–N treatments. If glass wool filtration retained different subsets of bacteria in the suspension compared to polyester net filtration, this should be revealed by microbial community structure analysis using, for instance, phospholipid fatty acid analysis (PLFA) (Frostegård et al., 1993). The bacteria for the PLFA analyses were harvested from the suspension by centrifugation (20 min; 47,000 × g). No clear differences between the treatments were detected. All the microbial PLFAs were at the same mol% level except for the fungal PLFA, 18:2ω6,9, which was slightly more abundant (2.0 mol%) in the B–N than in the B–W treatment (1.6 mol%). Fungi in general do not take up thymidine (Bååth, 1990) and thus do not contribute to these values. This hypothesis was therefore rejected.

The second hypothesis that the differences in the

![Fig. 1. Inhibition of bacterial growth by increasing copper concentrations as measured by the incorporation of 3H-thymidine into bacterial macromolecules. Extraction of the bacterial population from the heavy-metal polluted soil was performed either by blending with a Sorvall Omnimixer at 80% of top speed for 1 min (B) or rotary shaking at 250 rev min−1 for 1 h at 4°C (S). The filtration techniques were glass wool (Pyrex fiber glass, Sliver 8 micron; W), acid-washed (2% HCl) glass wool (AW), and a polyester net (mesh size 0.2 × 0.8 mm; N). The following four combinations were investigated: B–W, B–AW, B–N, and S–N, of which the B–W technique is the one in the original (Bååth, 1992a).](image-url)
amount of isotope dilution were due to the lysis of living cells in the presence of high Cu concentrations was not valid. Isotope dilution was measured using unlabelled thymidine as described by Pollard and Moriarty (1984). The degree of isotope dilution was about the same irrespective of the copper concentration in the incubation suspension. At high copper concentrations the degree of isotope dilution was highly variable, but no trend could be detected.

The third hypothesis, the inactivation of added Cu due to complexation with humus, was tested by adding excess Cu to the B–W and B–N filtered suspensions. The B–W suspension had less humus (index 1.3) than the B–N suspension (index 1.5) when the color index of the liquid scintillation spectrometer was used to indicate the amount of dissolved humus. The amount of complexed copper in the suspension was determined by using an Amberlite IR-120 (plus) ion exchange resin. The amount of Cu in the solution, before and after passing the resin, was measured by atomic absorption spectrophotometry. The amount of complexed copper, 0.89 mg l\(^{-1}\) for B–W and 1.10 mg l\(^{-1}\) for B–N, was on the same level for the two techniques and the observed degree of complexation was so low, under 0.2% of the Cu added, that it cannot have had an influence on the IC\(50\) value.

The last hypothesis was based on the difference in the pH of the suspension following filtration. In the original method by Bååth (1992a) the blended and centrifuged suspension was filtered through glass wool to remove humus particles from the suspension. However, our Pyrex fibre glass wool increased the pH of the suspension from ca 5 to 7, and gave a lower thymidine incorporation rate (0.8 × 10\(^{-11}\) mol TdR g\(^{-1}\) d.m.) than the unfiltered suspension (6.1 × 10\(^{-11}\) mol TdR g\(^{-1}\) d.m.). A high pH is a stress factor for bacteria adapted to pH 4 (Bååth, 1996), and thus the rate of thymidine incorporation remained low.

In conclusion we found one pitfall in the technique: the filtration of the soil suspension. The change in the pH of the soil suspension caused by the glass wool must be avoided when using soil samples of very low activity. We recommend the use of acid-washed glass wool or polyester net filtration.

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References