Effects of heavy metals on substrate utilisation capacity and structure of terrestrial microbial communities

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The Demmerikse polder, in the Ronde Venen, like other peat areas in the central western parts of the Netherlands, was intensively used for digging lumps of peat from the 14th to the 19th century (Rutgers and Bogte, 2002). Parts of the region were used for farming, usually an extensive form of cattle farming. The poor soil quality and hydrological conditions, aggravated by oxidation of the peat, were tackled by applying a mixture of garbage from surrounding cities, dredging sludge, farm-yard manure and sometimes dune sand, to improve the fields. This practice was called 'toemaaken'. Nowadays, the fields are covered by a 15-to-50-cm-thick completely anthropogenic layer, the so-called 'toemaakdek', containing a high fraction of sand particles, and elevated levels of heavy metals, mainly Zn, Cu, Cd and Pb. As part of the SSEO, a research project was undertaken to study the effects of metals on the soil ecosystem in the Demmerikse polder. The basic hypothesis of this study was that the observed correlations between structural and/or physiological changes in communities of soil organisms and metal concentrations, demonstrate that metals directly or indirectly affect the organisms. The aim of this research is to find out whether any effects of these metals can be observed in the 'toemaakdek'

The presence of confounding factors such as pH and organic matter makes it difficult to causally link the effects observed to the presence of contamination. Several techniques were combined in this project. Structural and physiological changes of heterotrophic bacterial communities were investigated. Community-level physiological profiling (CLPP) was used to obtain insight in the physiological changes of the heterotrophic bacterial communities, and denaturing gradient gel electrophoresis (DGGE) was used to investigate the structural changes of the bacterial communities. However, it was not possible to causally link shifts in CLPP and DGGE patterns to the presence of a particular metal. Therefore, pollution-induced community tolerance (PICT) was used to link community effects to the presence of metals. It is generally accepted that observations of PICT provide higher causality in ecological field studies (Blanck *et al.*, 1988; Boivin *et al.*, 2002).

Approximately 90 soil samples were taken from the upper 10 cm surface layer of the Demmerikse polder in October 2001. Of these 90 samples, 30 were selected: 15 samples that showed the lowest Zn, Pb and Cu concentrations and the 15 samples that showed the highest Zn, Pb and Cu concentrations. This grouping was based on total metal concentration. Both total metal concentration and metal concentration in pore water was measured. Previous results have not yet given any indication which type of information provides the best correlation with possible effects on microbial communities in the field. Therefore, no attention will be given to distinguish between the different metal information. The samples were divided into three parts. The first part was used for abiotic measurements (field humidity, pH, dissolved organic carbon (DOC), total metal concentration and pore water), the second part was used for CLPP and PICT analysis (newsletter 4; Garland, 1997; Garland *et al.,* 1999; Lehman *et al.*, 1997;

Rutgers *et al.,* 1998) and the third part was used for DGGE analysis (newsletter 5). Before CLPP and DGGE analysis, the soil was sieved using a sieve of a 4 mm pore size and was brought to WHC50 (water holding capacity of 50%). The samples were then incubated for 2 weeks in the dark at 10^0C. Principal component analysis (PCA) and redundancy analysis (RDA) were used to analyse the CLPP and DGGE data. A PCA analysis is an indirect analysis, which means that all differences measured between samples are addressed (differences in Biolog substrate utilisation capacities per community independent of abiotic parameters). RDA analysis is a direct analysis, which means that only differences explained by the environmental variables are addressed. RDA was used in our case only to calculate the importance of the different abiotic parameters measured on the biotic differences between the samples. A Monte Carlo permutation test was performed using 9999 permutations; the different abiotic parameters were separately tested. For the PICT analysis the same frozen (-70^0C) bacterial suspensions were used as for the CLPP experiments (newsletter 6; Rutgers and Breure, 1999; Blanck, 1988; Boivin *et al.*, 2002). The EC_{50} were finally compared using a T-test ($\alpha = 0.05$).

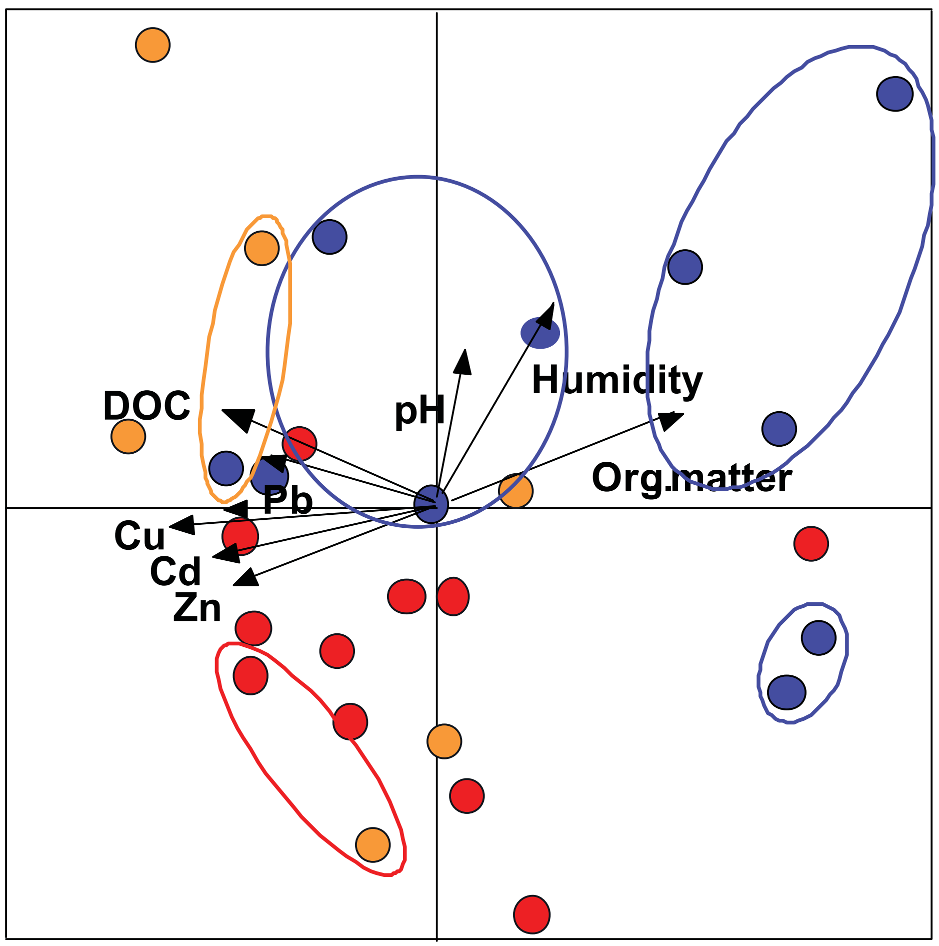

Fig. 1. Principal component analysis (PCA) of CLPP of different soil samples from the Demmerikse polder. Colours represent the degree of pollution (based on Zn, Pb, Cu and Cd). Bleu: relatively clean; Orange: relatively polluted; Red: polluted. Axis1: 16.8%; Axis2: 13.9% and Axis3: 9.8%

To investigate the influence of metals on the substrate utilisation capacity of heterotrophic bacterial communities, CLPPs were made. Figure 1 shows a PCA based on CLPPs of different samples taken in the Demmerikse polder. The first two axes explain 30.7% of the variation between the different samples. The pattern suggests that there is a correlation between the CLPP of the heterotrophic bacterial communities and the metals present in the field. Polluted samples (red markers) and cleaner samples (bleu markers) tend to group together. When RDA is performed, Zn ($p = 0.008$) and Cd ($p = 0.013$) are shown to have a significant influence on the substrate utilisation capacity of the bacterial community. Field humidity, pH, DOC and organic matter did not show any significant influence on the substrate utilisation capacity of the community ($p > 0.05$). What is also interesting to note, is that samples taken closer together showed more metabolisation similarities than samples taken further apart (encircled symbols indicate samples taken within 2 metres from each other).

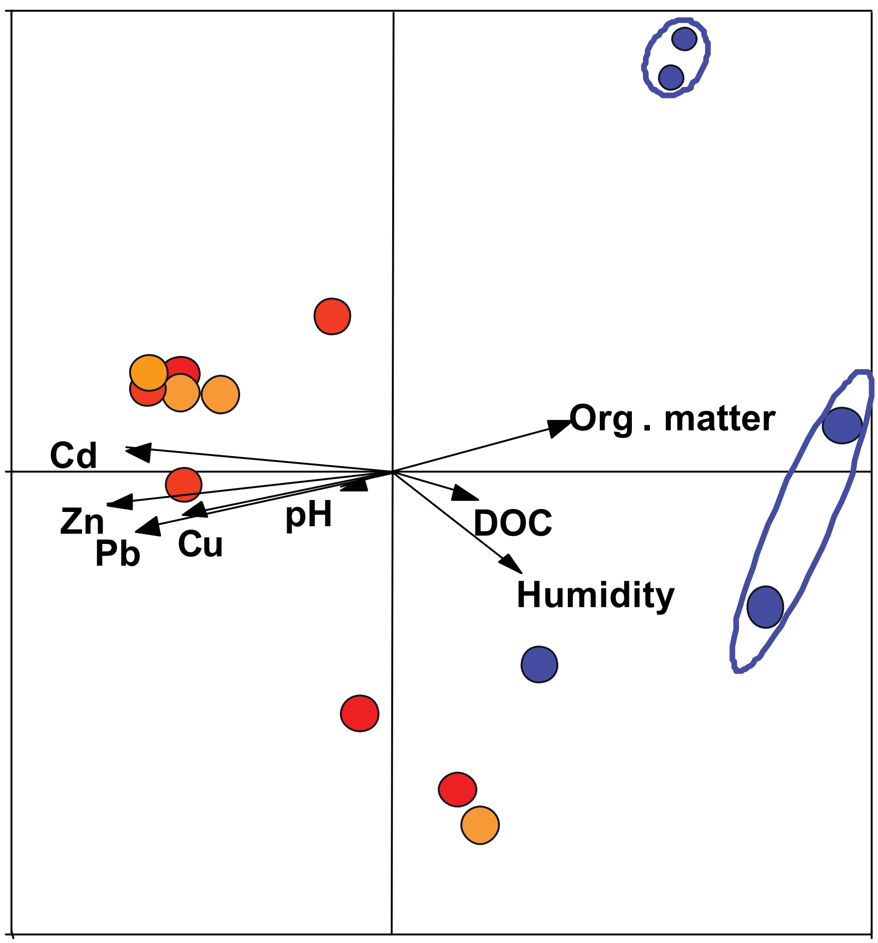

Fig. 2. Principal component analysis (PCA) of DGGE patterns of different soil samples from the Demmerikse polder (the densiometric data were used for comparison). Colours represent the degree of pollution. Bleu: relatively clean; Orange: relatively polluted; Red: polluted. Axis1: 42.6%; Axis2: 19.7% and Axis3: 8.4%.

The influence of metals on the bacterial community structure in the Demmerikse polder was also investigated using DNA extraction and DGGE. The same trends were observed for the structure of the different bacterial communities (Fig. 2) as for the CLPPs. This means that metals apparently also have an influence on the structure of the bacterial communities. The first two axes explain 62.3% of the differences between the different samples. The Monte Carlo Permutation test indicates that Zn ($p = 0.012$), Cd ($p = 0.03$) and Cu ($p = 0.03$) apparently affect the structure of the bacterial communities significantly. Other abiotic parameters, such as field humidity, organic matter, pH and DOC did not significantly affect the structure of the community ($p > 0.05$). The two samples taken within 2 metres of each other were more structurally similar than those taken further apart.

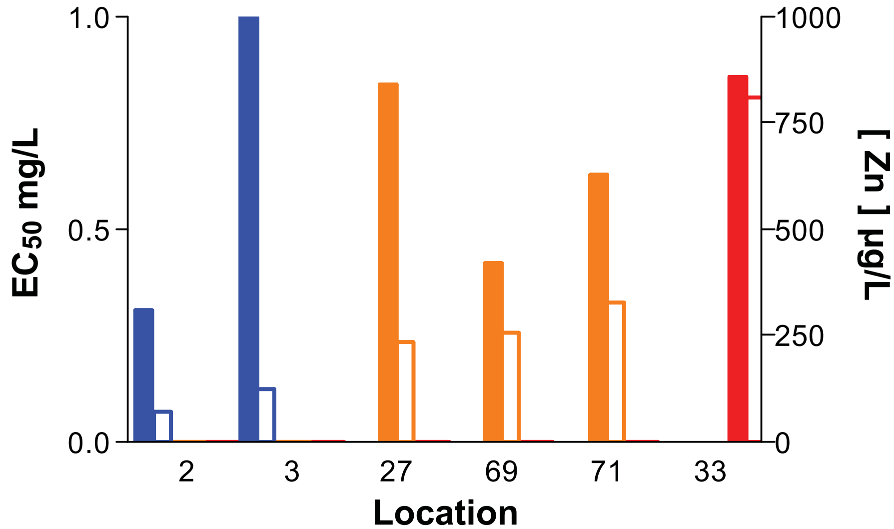

Fig. 3. Histogram representing zinc concentrations in pore water (open bars) and EC_{50} values (solid bars) from those locations.

To expand the links between metal contamination, substrate utilisation capacities and structure of the bacterial communities in the Demmerikse polder, PICT was investigated. Figures 3 and 4 represent the sensitivity shifts of the different heterotrophic bacterial communities. When we look at the data for Zn (Fig. 3), PICT was observed between the sample having the lowest Zn concentration (location 2) and the sample showing the highest Zn concentration (location 33). However, the samples with in-between Zn concentrations did not show trends of PICT. On the other hand, when we look at the data for Pb (Fig. 4), the PICT responses showed logical trends. The samples taken from the most heavily Pb-polluted area showed a development of tolerance to Pb compared to samples taken from cleaner areas. All these samples (2-3-71-27 and 69) were not significantly different from one another, but significantly different from sample 33, which also shows the highest Pb concentration (pore water).

The Demmerik area shows a very patchy and heterogeneous distribution of heavy metals. Within about 5 metres, relatively clean spots are found as well as relatively polluted spots (Rutgers and Bogte 2002). However, if we take heterogeneity into account, the location showed patterns of metal distribution. The centres of the fields are generally more polluted than the sides of the ditches. This might be due to the fact that sediment from the ditches was dredged and layered along the sides of the ditches. Another explanation might be a relatively higher peat oxidation in the middle of the field, this could be due to differential hydrological conditions. Furthermore, the patterns of substrate utilisation capacities and also the genetic structures of the microbial communities are more similar for samples taken close together than for samples taken further apart.

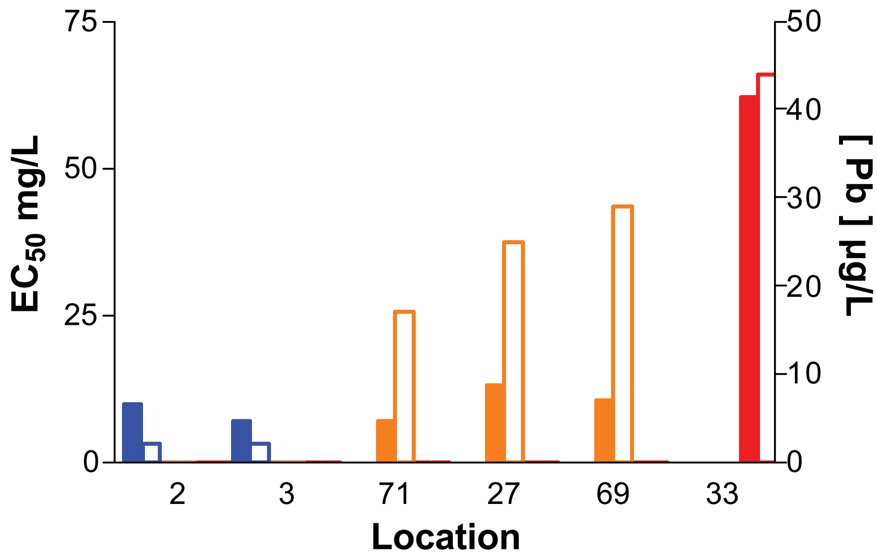

Fig. 4. Histogram representing Pb concentrations in pore water (open bars) and EC_{50} values (solid bars) from those locations

The main objective of the research described in this paper was to investigate the influence of heavy metals on microbial communities. The research was performed in collaboration with teams of the Free University of Amsterdam (VU) and Wageningen University and Research Centre focussing on Terrestrial Model Ecosystems (TMEs) and the nematode communities in the Demmerikse Polder, respectively. Their results will be presented elsewhere. The main outcomes of the present research are the apparent CLPP and DGGE shifts and the correlation of PICT, with the presence of metals. The PCA, which was made from the CLPPs, shows two major clusters representing clean and polluted locations. Based on statistical test (Monte Carlo permutation), it seems that Zn and Cd have the highest correlations with CLPP shifts, especially compared to other abiotic parameters such as pH and organic-matter contents. These outcomes are in agreement with Bosveld *et al*. (2000) who observed that Zn, Cd and Pb affected CLPP shifts most. They did not observe any impact of pH or organic-matter content on CLPP from Demmerik samples either, which is in agreement with our results. These results should be further investigated to find out whether a decrease in biodiversity can be observed on the basis of DGGE and CLPP patterns, and also to find out which functions and species are actually disappearing or are stimulated in locations that are polluted with metals.

The occurrence of PICT in the Demmerik area is premature. The pattern observed for Zn is not what we expected. The EC_{50} values are variable except for the lowest and highest Zn concentrations. On the other hand, PICT-Pb was observed for the highest soil Pb concentration. Apart for the limited amount of experimental data, these vague results may be due to method limitation: were the bacteria extracted and preserved properly, was the laboratory metal exposure adequate, was data treatment correct? The impact of pH and organic matter on the availability of metals in the field may also influence the results.

Except for method limitation, it could be difficult to quantify relevant field exposure to metals, simply because it may be too difficult to estimate this from standard methods such as total destruction, pore water or $CaCl_2$ extraction, and model calculations. In that case, PICT will provide more direct clues to metal effects than any metal concentration determined in field samples. More samples should be investigated to better understand the PICT observations in the Demmerikse polder.

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