**Online resource to: Interaction of minerals, organic matter, and microorganisms during biogeochemical interface formation – What can we learn from artificial soils ?**

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Biology and Fertility of Soils

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Online resource 2: Phenanthrene degradation in artificial soils

# 1. Aim of the experiment

The aim of the experiment was to characterize the effect of soil composition on soil microbial community composition and function. We therefore amended artificial soils of different composition with 13C-labelled phenanthrene and analyzed phenanthrene degradation and mineralization. We also identified microorganisms involved in phenanthrene degradation by tracing the incorporation of phenanthrene-derived carbon into microbial biomass. This was achieved by analysis of the concentration and isotopic composition of phospholipid fatty acids (PLFA) after incubation with phenanthrene for 40 days.

# 2. Materials and Methods

We selected a series of three different artificial soils containing quartz + illite (IL), quartz + illite + ferrihydrite (IL+FH) and quartz + illite + ferrihydrite + charcoal (IL+FH+CH) that were matured for 18 months (Table 1, main text). Material from all three replicates was mixed and spiked with unlabeled or 13C-labelled phenanthrene (9, 10-[13C]-phenanthrene) at a final concentration of 100 mg g‑1 soil. In addition, a blank series receiving only the solvent (dichloromethane) was prepared. The soils were mixed with a glass rod until the solvent was evaporated and incubated in 250 ml Schott bottles containing vials with 5 ml of 1 M NaOH solution and equipped with Oxitop® devices to register respiration. After 5, 9, 20 and 40 days, the NaOH solutions were retrieved from the bottles and analyzed for 13CO2 by gas chromatography-isotope ratio monitoring-mass spectrometry (GC/irmMS), and fresh NaOH solution was placed in each bottle. Samples from day 0 and from day 40 were analyzed for remaining phenanthrene and the amount and isotopic composition of PLFA. All incubation experiments including CO2 quantification were run in duplicate batches, and phenanthrene and PLFA analysis as well as the isotopic analysis of CO2 in each batch were performed in duplicates.

For phenanthrene analysis, the soils were first extracted with ethylacetate and subsequently saponified by methanolic KOH at 95°C (Eschenbach et al. 1994). The saponification products were extracted from the alkaline solution by liquid-liquid extraction with hexane. Phenanthrene in all extracts was analyzed by GC/MS and the concentrations in the ethylacetate extractable fraction and the saponifiable fraction were quantified using phenanthrene-d10 as an internal standard.

The PLFA were extracted according to Bligh and Dyer (1959) and analyzed for their concentrations by GC/MS and for their isotopic composition by GC/irmMS (Miltner et al. 2004). To characterize the total and the phenanthrene-degrading microbial community, the ratio of fatty acids indicative of Gram-negative (monounsaturated fatty acids) and Gram-positive bacteria (terminally branched fatty acids) was calculated as well as the ratio between cyclopropyl fatty acids and monounsaturated fatty acids, which has been described as a marker for stress. The fatty acids were assigned to organism groups and physiological states according to Green and Scow (2000) and Frostegård et al. (2011).

To identify differences in the total and the phenanthrene degrading microbial community in the three artificial soil compositions under study, principal component analysis was performed using the PLFA data of the samples after incubation using the statistics option of SigmaPlot 13.0 (Systat Software, Erkrath, Germany).

# 3. Results and Discussion

3.1. Phenanthrene mineralization

*Fig. ER2-1: Mineralization of phenanthrene in artificial soils of different composition. Data are given as production of 13CO2 in % of the initially added label in phenanthrene.

*After an initial lag phase of about 10 days, 31 to 45% of the added phenanthrene-13C was mineralized rapidly until 40 days of incubation (Fig. ER2-1). The mineralization was significantly lower in the treatment containing charcoal than in the other two treatments.

3.2. Phenanthrene degradation

On day 0, the phenanthrene added to the soils was recovered completely. Most of the compound was extractable with ethylacetate, only a minor fraction was bound in the saponifiable fraction. During the experiment, the phenanthrene concentrations decreased considerably, indicating almost complete degradation of the added phenanthrene during the 40 day-incubation (Fig. ER2-2). At the same time, the relative proportion of the saponifiable fraction increased although the absolute amounts decreased for both fractions. The phenanthrene concentration remaining after 40 days of incubation was highest in the treatment containing charcoal. This is in line with the lower mineralization in this treatment.



Fig. ER2-2: Phenanthrene concentrations in artificial soils of different composition. The columns at 0 day represent the average of all treatments, whereas at 40 days, the individual treatments are shown (n=3).

3.3. Microbial community composition

The composition of the microbial community as determined by PLFA pattern was similar for all treatments at the end of the incubation experiment (Fig. ER2-3). There was also little difference of the PLFA pattern before and after incubation. However, during incubation of the artificial soils, the relative importance of Gram-positive bacteria increased and that of both Gram-negative bacteria and fungi tended to decrease slightly. As the PLFA pattern in the unamended and the phenanthrene-amended treatments were rather similar, these small changes during incubation presumably were not induced by the phenanthrene amendment, but are a consequence of the incubation experiment.

3.4. 13C in the microbial biomass

The 13C-label in the PLFA was used to estimate the amount of label incorporated into the microbial biomass, based on the conversion factor given by Green and Scow (2000). This incorporation amounted to 3.7 ± 0.2% of the initially added label in the treatment IL, 2.9 ± 0.2% in the treatment IL+FH, and 3.9 ± 0.6% in the treatment IL+FH+CH (Fig. ER2-4). Therefore, the incorporation into the biomass was not significantly different for the different artificial soil compositions.

Fig. ER2-3: Total PLFA pattern of the three investigated artificial soils before (average of the three treatments) and after incubation in the absence or presence of 100 mg phenanthrene kg-1 soil.



Fig. ER2-4: Isotope mass balance for phenanthrene degradation in artificial soils of different composition.

3.5. Mass balance

During the incubation experiment, extractable phenanthrene decreased to <10% of the initial amount, but only 30-45% of the labelled C was mineralized and about 3% incorporated into the microbial biomass, indicating the formation of non-extractable residues (NER). NER were not quantified in this experiment, but were estimated by assigning the unidentified label remaining in the artificial soil samples to NER. In all treatments, about 50% of the initial label was assigned to NER (Fig. ER2-4). The main difference between the three treatments thus was the lower mineralization and the lower decrease of the phenanthrene concentration in the charcoal-containing soil composition. A potential explanation for this is that charcoal is a good sorbent for phenanthrene, which is thus protected from degradation. Both microbial biomass and NER accounted for similar amounts of the added label in all three treatments.

3.6. Composition of the microbial degrader community

Microorganisms involved in productive phenanthrene degradation were identified by analysis of the 13C label in the PLFA, i.e. PLFA-stable isotope probing (PLFA-SIP). All bacterial phospholipid acids <C20 were enriched in 13C in the treatments which had received the 13C-labeled phenanthrene (Fig ER2-5). This suggests that a large variety of microorganisms in the artificial soils were able to use phenanthrene as a carbon source. Interestingly, the fungal-derived PLFA 18:2 was the only PLFA which was not labeled. This, however, does not exclude degrading activity of fungi, because these are known to co-metabolically degrade PAHs. Co-metabolically degraded substrates are neither used as C nor as energy source. The degraders will therefore not produce 13C-labelled biomass; co-metabolic activity thus cannot be detected and quantified by SIP.



Fig. ER2-5: PLFA-SIP pattern of the phenanthrene degrading microbial community in the three different artificial soil compositions.

As mentioned above, there were no significant differences between the artificial soil treatments in the composition of the total microbial community, neither before nor after the incubation experiment. However, the difference in the community active in phenanthrene degradation between the treatments was larger than for the total microbial community as assessed by PLFA-SIP analysis (Fig. ER2-6). This also becomes obvious when comparing the ratio between the PLFA indicative of Gram-negative and Gram-positive bacteria and a stress indicator (Table ER2-1). The data suggest that in the treatment IL+FH, Gram-negative bacteria were particularly active in phenanthrene degradation, compared to both the total community in this treatment and to the phenanthrene degrading communities in the two other treatments. In contrast, in the presence of charcoal (IL+FH+CH), stressed bacteria are more involved in phenanthrene degradation. It is unclear, however, if the altered community composition is the reason for or the consequence of the phenanthrene degrading activity. In the treatment IL the phenanthrene-degrading community seems to be slightly more similar to the total community than in the presence of ferrihydrite and/or charcoal. The mineralogical composition of the soil therefore has an important influence on particular microbial activities such as phenanthrene degradation and on the subcommunity involved.

Fig. ER2-6: Principal component analysis of the PLFA patterns of the total and the phenanthrene degrading microbial community in artificial soils of different composition. A: loadings of the individual PLFA on the principal components, B: scores of the treatments.



# 4. Conclusions

Phenanthrene was degraded rapidly in all investigated artificial soils. Charcoal reduced phenanthrene degradation compared to the treatments without charcoal. Although both the total microbial community and the extent of phenanthrene degradation were similar in all treatments, we detected differences in the composition of the phenanthrene degrader community, indicating that soil constituents have different effects on different subcommunities of soil microorganisms.

# References:

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| --- | --- | --- | --- | --- |
|  |  | IL | IL+FH | IL+FH+CH |
| monounsat/term. branched | total community | 0.74 | 0.88 | 0.74 |
|  | phenanthrene degraders | 0.71 | 1.09 | 0.55 |
|  |  |  |  |  |
| cyclo/monounsat | total community | 0.45 | 0.33 | 0.38 |
|  | phenanthrene degraders | 0.56 | 0.19 | 1.28 |

Table ER2-1: Composition of the total and the phenanthrene-degrading microbial communities in artificial soil after incubation with 13C-phenanthrene, based on PLFA patterns

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