SOIL MICROBIAL TOXICITY OF EIGHT POLYCYCLIC AROMATIC COMPOUNDS: EFFECTS ON NITRIFICATION, THE GENETIC DIVERSITY OF BACTERIA, AND THE TOTAL NUMBER OF PROTOZOANS

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Abstract—Eight polycyclic aromatic compounds (PACs) were tested for their toxic effect on the soil nitrification process, bacterial genetic diversity, and the total number of protozoans (naked amoebae and heterotrophic flagellates). After four weeks of exposure in a well-characterized agricultural soil, toxic effects were evaluated by comparison to uncontaminated control soils. All PACs affected the nitrification process, and the calculated no-observed-effect concentrations (NOECs) for nitrification were 79 mg/kg for pyrene, 24 mg/kg for fluoranthene, 26 mg/kg for phenanthrene, 72 mg/kg for fluorene, 23 mg/kg for carbazole, 22 mg/kg for dibenzothiophene, 75 mg/kg for dibenzofuran, and 1,100 mg/kg for acridine. For all substances but acridine, nitrification was the most sensitive of the three toxicity indicators evaluated. No effect of the tested substances on bacterial diversity was found, as measured by denaturant gradient gel electrophoresis. In general, weak effects at very high concentrations were found for the protozoans. However, for acridine, protozoan numbers were reduced at lower concentrations than those that affected the nitrification process, that is, with a 5% reduction at 380 mg/kg. For effects on nitrification, toxicity (NOEC values) expressed as soil porewater concentrations (log_{10}(\mumol/L)) showed a significant inverse relationship with lipophilicity (log octanol–water partition coefficient) of the substances (r^2 = 0.69, p = 0.011, n = 8). This finding could indicate that the toxicity of substances similar to those tested might be predicted by a quantitative structure–activity relationship with lipophilicity as the predictor variable.

Keywords—Polycyclic aromatic hydrocarbons Heterocyclic compounds Lipophilicity Toxicity Microorganisms

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a variable group of compounds, all being made up of two or more benzene rings. Both natural and man-made processes (e.g., burning of biomass or fossil fuels) cause release of PAHs, and they are widespread in the environment. Studies of ice cores in Greenland have shown that the atmospheric level of PAHs is now approximately 100 times the level in the period 1500 through 1799 [1]. More than 90% of the PAH burden in the United Kingdom resides in soil [2], and PAH contamination is a serious problem at a large number of sites worldwide. At sites contaminated with coal or tar, heterocyclic compounds (i.e., N, S, or O as part of the ring structure) are typically present at a concentration of about 1 to 10% of the total concentration of polycyclic aromatic compounds (PACs) [3]. The N-, S-, and O-PACs constitute a diverse group with regard to physiochemical properties.

Little is known about the effects of PAHs and N-, S-, and O-PACs on soil microbial ecology. Therefore, new microbial ecotoxicity data for representative compounds from this group of common soil pollutants would improve the scientific basis for the ecological risk assessment of PAC-contaminated soils.

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According to the current guideline for hazard and risk assessment of chemicals from the European Union (the Technical Guidance Document [4]), soil microbial toxicity should preferably be assessed based on information on the effect of a chemical on nitrogen mineralization. However, the bacterial community may be markedly changed even if the overall metabolism appears unaffected [5] because some organisms may be suppressed and others may proliferate in the vacant ecological niches. Therefore, it would be of interest to evaluate the sensitivity of the nitrification test in comparison to other methods of assessing the toxicity of chemicals to microorganisms.

The polymerase chain reaction (PCR) earlier was successfully combined with denaturant gradient gel electrophoresis (DGGE) to study bacterial diversity [6]. In PCR, specific DNA is amplified by using primers that determine the specificity of the reaction. Hence, PCR products (e.g., from bacteria) can be retrieved, and these can be rapidly and reproducibly separated by DGGE. Fragments of DNA with the same length, but different nucleotide sequences, are separated based on differences in the mobility of the molecules in polyacrylamide gels with a linear denaturant gradient. The mobility of each fragment is determined by its base composition and sequence variability [7].

Denaturant gradient gel electrophoresis previously was used in several studies to assess the effect of pesticides on...
soil bacterial diversity [8–10]. Engelen et al. [9] compared the ability of the nitrification assay and temperature gradient gel electrophoresis (which is analogous to DGGE) to rank the ecotoxicity of various pesticides. Both methods showed marked effects of one of the pesticides, and the results of the two methods agreed very well. These results, and many other DGGE analyses of microbial communities in soil, demonstrate the ability of DGGE to reflect changes in community composition. However, other reports have shown that DGGE in some cases may be insensitive to community changes [11], possibly because PCR will amplify DNA from active as well as dormant bacteria.

In the present study, eight PACs that commonly are found in high concentrations at PAC-contaminated sites were selected for toxicity testing. For each substance, toxic effects on soil nitrification, bacterial genetic diversity (DGGE), and the total number of protozoans (naked amoebae and heterotrophic flagellates) were determined after four weeks of exposure. The aim of the study was to generate new ecotoxicity data for this important group of soil contaminants, to compare the sensitivity of the three microbial methods used, and to compare the toxicities of the various substances both on the basis of total soil concentrations and estimated pore-water concentrations.

MATERIALS AND METHODS

Experimental soil

All tests used an agricultural soil from Askov, Denmark, which was sieved (2 mm) and dehumidified by heating to 80°C for 24 h. The dehumidification of the soil was performed as part of the sample preparation procedure, to allow for a homogeneous distribution of test substances. From 10 kg of homogeneously blended soil, 20-g subsamples for each replicate were collected in 100-ml glass vessels. The Askov soil is a sandy loam, and has the following particle size distribution: coarse sand (200–2,000 μm) 38.4%, fine sand (63–200 μm) 23.6%, coarse silt (20–63 μm) 10.0%, fine silt (2–20 μm) 12.3%, and clay (<2 μm) 13.0%. The humus content of the soil was 2.8%, and the total content of organic carbon was 1.6%. The soil pH was 5.0, density, and total cation exchange capacity were 6.2, 1.135 g/cm³ dry soil, and 8.14 meq/100 g, respectively.

Sample preparation

Fluoranthene (purity >99%), fluorene, pyrene (desiccate), phenanthrene (purity >96%), dibenzo thiophene, acridine (purity 97%), carba zole (purity 95%), and dibenzofuran (purity >99%) were from Sigma-Aldrich (St. Louis, MO, USA). Relevant physicochemical properties for the tested chemicals are included in Table 1. Acetone (high-performance liquid chromatography quality, J.T. Barker, Phillipsburg, NJ, USA) was used as spiking solvent. The PACs were dissolved in acetone in a stock solution corresponding to the highest test dosage, and a dilution series, corresponding to the test concentrations used, was prepared from this stock solution. Acetone solutions of the test substances (4 ml) were added to each glass vessel (20 g soil), and thoroughly mixed into the soil. The solvent evaporated for 24 h. Glass vessels were weighed before and after evaporation to assure that the solvent was totally evaporated.

The following treatments were used for each test substance: controls with acetone; controls without acetone; and test concentrations of 1, 3, 10, 30, 100, 300, 1,000, and 3,000 mg/kg dry soil (nominal values). Three replicates were used per concentration of test substance.

Test procedure

The test set-up was similar to a method proposed by the International Organization for Standardization [12]. In this method, a nitrogen source is added to the soil samples at the beginning of the test, and the effect of various concentrations of a chemical on soil nitrification is determined by comparison with uncontaminated controls after four weeks of exposure. In the tests for this study, some deviations from this standard were considered necessary because dehumidified soil samples were used and a solvent (acetone) was used in the spiking process. Both the temperature treatment (to dehumidify the soil) and the solvent addition were likely to reduce soil microbial diversity and biomass. An inoculum, prepared from fresh soil samples of the Askov soil type, therefore was added after solvent evaporation and addition of the nitrogen source. The nitrogen source used was horn meal (Solsikken, Linå, Denmark) at 1.00 ± 0.05 g/kg soil dry weight, as recommended in the standard [12]. The nitrogen content of the horn meal batch was 9.1% (w/w; standard deviation = 0.1%, n = 3), and the C:N ratio was 3.48 (mol/mol).

Addition of inoculum to soil samples

Fresh Askov soil was sampled and stored at 5°C for a maximum of 3 d. The inocula were prepared according to a procedure described by Lindahl and Bakken [13]: 20 g of soil and 180 ml of deionized water were mixed with a kitchen blender for 1 min and then cooled on ice for 5 min. This procedure was repeated once, then the slurry was mixed again for 1 min and cooled for 30 s. The slurry was then used for inoculation: 3.5 ml was added to each glass container containing 20 g of soil and mixed thoroughly into the soil. Hence, the resulting population density of microorganisms added was 2% of that in the fresh soil material if the extraction efficiency of microbes from the original soil was assumed to be 100%. The amount of water (inoculum) added to the microcosms corresponded to 57% of the water-holding capacity of the soil.

Test conditions

The inoculated soil samples were transferred to test cylinders (diameter 3.5 cm, height 5.0 cm), which were sealed with plastic lids. The upper lid was perforated (five small holes made by a needle) to allow for exchange of gases. Test cylinders were incubated for four weeks at 20°C in the dark. Twice a week during the incubation period, test cylinders were weighed and water was added corresponding to the weight loss. For all replicates but one, the water loss was within the validity range defined by the test standard (57 ± 5% of the water-holding capacity).

At the end of the tests, soil within each replicate was roughly homogenized by use of a spatula, and subsamples (10.0 ± 0.05 g) were taken for chemical analysis (NO₃⁻), DGGE measurements (0.55 ± 0.05 g), and analysis of the protozoan community (5.0 ± 0.1 g). Samples collected for chemical analysis and DGGE were immediately frozen (−18°C). Samples collected for analysis of the protozoan community were stored at 5°C until analysis.

Nitrate analysis

To each sample, 45-ml of 1 M KCl was added, after which the resulting mixture was shaken in a mechanical shaker at
Table 1. Octanol water partition coefficients (log \(K_{\text{ow}}\)), soil water partition coefficients (\(K_d\)) in the Askov soil, and structural formulas for the eight substances used in this study

<table>
<thead>
<tr>
<th>Substance</th>
<th>Log (K_{\text{ow}})</th>
<th>Estimated or measured (K_d)</th>
<th>Water solubility (mg/L)</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>4.56(^a)</td>
<td>304(^b)</td>
<td>1.290(^c)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Pyrene</td>
<td>4.88(^a)</td>
<td>731(^b)</td>
<td>0.135(^c)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>5.16(^e)</td>
<td>667(^b)</td>
<td>0.260(^c)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Fluorene</td>
<td>4.18(^a)</td>
<td>142(^d)</td>
<td>1.980(^c)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Carbazole</td>
<td>3.51(^e)</td>
<td>101(^d)</td>
<td>1.8(^f)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>4.49(^e)</td>
<td>189(^d)</td>
<td>1.47(^c)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>4.12(^e)</td>
<td>110(^d)</td>
<td>3.11(^f)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
<tr>
<td>Acridine</td>
<td>3.27(^e)</td>
<td>977(^d)</td>
<td>38.4(^f)</td>
<td><img src="image" alt="Structural formula" /></td>
</tr>
</tbody>
</table>

\(^a\) Güsten and Sabljic [23].
\(^b\) Estimated \(K_d = K_{OC}\) (as reported by Güsten and Sabljic [23]) \(\times 0.016\) (fraction of organic carbon in the Askov soil).
\(^c\) Experimental values quoted by Mackay et al. [24].
\(^d\) Sverdrup et al. [21].
\(^e\) Nielsen et al. [25].
\(^f\) Experimental values quoted in the Syracuse Research Corporation PHYSPROP database [26].

50 rpm for 60 min. Then samples were centrifuged (5,000 rpm for 10 min), and the supernatant was filtered through 50 µm-filter. Particle-free extracts were then stored at −18°C. Samples were thawed and placed at 5°C the day before analysis of their nitrate content. Nitrate was measured spectrophotometrically with a LaChat QuikChem\(\textsuperscript{a}\) analyzer (LaChat Instruments, Milwaukee, WI, USA). Nitrate was reduced to nitrite by passage of the sample through a copperized cadmium column. The nitrite (reduced nitrate plus original nitrite) was then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. The resulting watersoluble dye has a magenta color that was read at 520 nm. A calibration curve was then used to quantify nitrite concentrations in the samples. Two samples of known NO\(_3\) concentrations and one 0.1 mM NO\(_3\) sample were run for every 12 test samples, and the samples were reanalyzed if the measured values of the known concentrations were different from the actual concentrations by more than 5%. The 0.1 mM NO\(_3\) sample was run to check the performance of the NO\(_3\)-reducing cadmium column. If the measured concentration for this sample was below 95% of its original concentration, the cadmium column was replaced with a new one. All samples were measured in duplicate. If the divergence between duplicate measurements were larger than 5%, the sample was reanalyzed.

The mean nitrate content of each replicate was used for the statistical analysis. No-observed-effect concentration (NOEC) values and lowest-observed-effect concentration values were assessed by analysis of variance (ANOVA) and Dunnett’s procedure (on a 5% significance level). Estimation of the 10% and 50% reduction of nitrate production (10% effective concentration [EC10] and 50% effective concentration [EC50] values, respectively) was done by linear interpolation, and the confidence intervals were calculated by the inhibition concentration method [14].
Protozoans

The total protozoan population (naked amoebae and heterotrophic flagellates) as well as the flagellate subpopulation was determined by a most probable number method [15] and by the use of a dilute (0.3 g/L) tryptic soy broth medium. Because the population of naked amoebae is difficult to estimate exactly for statistical reasons [16], no attempt was made to estimate this subpopulation. For each of the 64 treatments (eight compounds at eight concentrations), eight replicate dilution series (three factor dilutions) were prepared in a Costar® 96-well microtiter plate (Costar, Corning, NY, USA). Microtiter plates were stored at 10°C in the dark and inspected for the presence of protozoans after one and three weeks with a Leica DM IRB inverted microscope (×300 magnification, phase contrast; Leica, Solms, Germany). Data from the inspection of microtiter plates were converted to protozoan numbers by a computer program as described by Rønn et al. [15]. Because only one most probable number was available for each of the concentrations tested, the NOEC for the protozoan method could not be performed with ANOVA. Instead, the dose–response relationships were fitted to a simple three-parameter relationship \( y = a/[1 + (x/x_a)^b] \) by using SigmaStat® 2.0 (SPSS, Chicago, IL, USA). This provided an estimate of the concentrations that caused a 5% reduction (EC5) in the total protozoan population, as well as the subpopulation of heterotrophic flagellates, for each substance.

Bacterial DGGE

The DNA was extracted by using the FastPrep System bead beater (BIO101, Vista, CA, USA) [17] following the manufacturer’s recommendations. The V3 region of 16S rDNA was amplified by PCR by using the bacteria-specific primer PRBA338f (5’ ACT CCT ACG GGA GGC AGC AG 3’) [18,19] and the universal PRUN518r primer (5’ ATT ACC GGC GCT GCT GG 3’) [6]. The PRBA338f was supplemented with a GC-clamp on the 5’ end (5’ CGC CCG CCG CGG GCG GGG GGG GGC GCA CGG GGG G G 3’) [6]. The PCR conditions were 10 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; 6 min at 72°C; and soak at 4°C. A hot start procedure was used. Each PCR tube contained a total volume of 46 µL, comprising 33.4 µL of twice-distilled water, 4.5 µL of 10× Ampli Taq PCR buffer (Perkin-Elmer, Norwalk, CT, USA), 0.2 µL of each of the two primers (in 0.1 mM), 4.5 µL of 1% DNase-free bovine serum albumin (Pharmacia Biotech, Uppsala, Sweden), 2 µL of Gene Amp 10 mM deoxynucleoside triphosphate mix (Perkin-Elmer), 0.2 µL of Ampli Taq Gold polymerase (Perkin-Elmer), and 1 µL of template DNA.

The DGGE was performed as described by Øvreås et al. [19]. In short, the polycrylamide gels were made with a denaturant gradient from 30 to 70% (where 100% denaturant corresponds to 7 M urea and 40% formamide). After electrophoresis, the gels were stained with GelStar (FMC Bioproducts, Rockland, ME, USA) and photographed in ultraviolet light.

Chemical analysis

All test substances are somewhat volatile and could evaporate from the soil during sample preparation (i.e., the 24 h in a fume hood used to evaporate acetone). Therefore, verification of initial exposure concentrations was considered to be necessary. Soil samples from the 10-mg/kg and 1,000-mg/kg nominal test concentrations were collected at the start of each test, and the mean recovery value for these two analyses (i.e., measured concentrations divided by nominal ones) were used to correct nominal concentrations for these losses. The samples with 100 mg of soil (high PAC content) or 1,000 mg of soil (low PAC content) were added an equal amount of sodium sulfate. Known amounts of internal standards \( d_{10}^- \)-fluorene, \( d_{12}^- \)-dibenzothiophene, \( d_{10}^- \)-phenanthrene, \( d_{12}^- \)-acridine, \( d_{10}^- \)-fluoranthene, and \( d_{12}^- \)-pyrene) were added to the soil–sodium sulfate mixture. The soils were extracted ultrasonically six times with a 50:50% mixture of dichloromethane (proanalysis quality, Merck, Darmstadt, Germany) and acetone (proanalysis quality, Merck). Each extraction lasted 30 min. The combined extracts were concentrated to 5 to 10 ml in a rotary evaporator and to 1.5 ml by gentle evaporation with purified N₂.

Identification and quantification of compounds were carried out with a gas chromatography–mass spectrometric system consisting of a Varian 3400 Star gas chromatograph and a Varian Saturn III ion trap mass spectrometer (Varian, Walnut Creek, CA, USA), by using temperature-programmable splitless injection and a 30-m XTI-5 fused silica capillary column coated with 95% methyl-siloxane:5% phenyl-siloxane (Restek, Bellefonte, PA, USA).

RESULTS AND DISCUSSION

Soil nitrification

No significant difference was found in soil nitrification rates between acetone-treated control samples and control samples without acetone amendment for any of the substances tested. Therefore, the control replicates within each test (substance) were pooled. All of the tested substances reduced soil nitrate production, but the dose–response relationship differed strongly between substances (Fig. 1). Summary statistics for the effects of the PACs on nitrification (NOEC, EC10, and EC50 values) are reported in Table 2. Soil nitrification generally was affected at PAC concentrations of 13 to 130 mg/kg (Table 2), but for acridine, nitrification was only inhibited at the highest concentration tested (3,300 mg/kg), indicating a lower toxicity of this substance. We do not know of any earlier studies on the effect of PAH or PAC substances on soil-nitrifying bacteria relevant for a comparison with these results. The soil–pore-water partitioning for the substances in question previously was investigated with the same batch of soil as the one used in the present study [20]. By using these partitioning coefficients (measured or estimated distribution coefficient \([K_d]\) values) [21], pore-water concentrations could be estimated from the measured total soil concentrations as: pore-water concentrations = total soil concentrations/\([K_d]\).

The use of estimated pore-water concentrations revealed some new aspects of the results. First, use of estimated pore-water concentrations could explain the presence of a plateau for toxic effects, found at high total soil concentrations of carbazole, dibenzofuran, dibenzothiophene, phenanthrene and fluorene, by the fact that the pore water was saturated (i.e., calculated pore-water concentrations equaled the water solubility of these substances at the lowest concentration of the plateau). This observation suggests that only the fraction of the substance that is dissolved in the pore water is available to the nitrifying bacteria.

Second, EC10 values for effects on nitrification, expressed on the basis of µM pore-water concentrations...
Fig. 1. Dose (nominal concentrations)–response (mean ± standard deviations) relationship for the effect of eight individual polycyclic aromatic compounds on nitrogen mineralization in soil.

Fig. 2. Toxicity of eight polycyclic aromatic compounds (10% effective concentration [EC10] values), expressed as soil pore-water concentrations, in relation to the lipophilicity of the substances (log octanol–water partition coefficient [log $K_{ow}$]).

$(\log_{10}[EC10(\mu mol/L)])$, showed a significant negative correlation with the lipophilicity (log octanol–water partition coefficient [log $K_{ow}$]) of the substances tested (Fig. 2), and after this recalibration of toxicity, acridine was no longer found as a nontoxic outlier (Fig. 2). Such a correlation between lipophilicity and toxicity is expected for substances acting by a nonspecific mode of toxic action (narcosis or baseline toxicity) [20]. Narcotic chemicals do not bind to specific molecules within the cell, but elicit toxicity by dissolving in biological membranes and disturbing their fluidity and function. Therefore, narcosis is considered to be a nonspecific mode of toxic action. The degree to which a substance is accumulated in biological membranes depends on the lipophilicity, and thus a causal link occurs between the log $K_{ow}$ and toxicity for nonpolar narcotic substances. The strong correlation between lipophilicity and toxicity found in this study therefore suggests that the substances in question do not have very specific modes of toxic action. A similar relationship has been found earlier for effects of these substances on the reproduction of two soil-dwelling invertebrates [21,22].

Protozoans

Overall, the effect of PACs on protozoan numbers was surprisingly weak. Low PAC concentrations generally led to somewhat increased protozoan abundance, which gradually leveled off with increasing concentrations, most pronounced for the flagellates. Estimated EC5 values for the individual substances are summarized in Table 2.

Note that the low sensitivity found for the protozoans does not suggest that these organisms are insensitive to chemicals on a general basis. In the method used, the structure of the protozoan community was not examined, and robust oppor-

### Table 2. Effective toxic concentrations (ECs) determined on the basis of concentrations measured at the start of each test

<table>
<thead>
<tr>
<th>Substance</th>
<th>NOEC (mg/kg)$^a$</th>
<th>EC10 values (95% CI) (mg/kg)$^a$</th>
<th>EC10 values (μmol/L)$^b$</th>
<th>EC50 values (95% CI) (mg/kg)$^a$</th>
<th>Total number of protozoa (mg/kg)$^c$</th>
<th>Heterotrophic flagellates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrene</td>
<td>79</td>
<td>130 (58–170)</td>
<td>0.880</td>
<td>NE$^c$</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>24</td>
<td>13 (1.5–66)</td>
<td>0.096</td>
<td>NE</td>
<td>NE</td>
<td>2,200</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>26</td>
<td>42 (0–70)</td>
<td>0.776</td>
<td>250 (220–380)</td>
<td>2,400</td>
<td>250</td>
</tr>
<tr>
<td>Fluorene</td>
<td>72</td>
<td>33 (0–72)</td>
<td>1.400</td>
<td>190 (160–220)</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Carbazole</td>
<td>23</td>
<td>33 (0–49)</td>
<td>1.956</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>22</td>
<td>24 (11–45)</td>
<td>0.690</td>
<td>180 (160–190)</td>
<td>2,000</td>
<td>1,100</td>
</tr>
<tr>
<td>Dibenzofuraran</td>
<td>75</td>
<td>74 (0–86)</td>
<td>4.004</td>
<td>200 (190–210)</td>
<td>1,300</td>
<td>600</td>
</tr>
<tr>
<td>Acridine</td>
<td>1,100</td>
<td>1,400 (1,100–1,500)</td>
<td>8.005</td>
<td>2,800 (2,600–3,600)</td>
<td>380</td>
<td>490</td>
</tr>
</tbody>
</table>

$^a$ NOEC = no-observed-effect concentration. Soil effects concentration based on measured initial concentrations (mg/kg dry wt).

$^b$ Equilibrium soil pore-water concentrations estimated from soil concentrations by using the soil–water partition coefficient ($K_{ow}$) values given in Table 1, and the molecular weight of the compounds.

$^c$ NE = could not be estimated.
Soil microbial toxicity of polycyclic aromatic compounds

**Comparison of method sensitivity**

In the present study, nitrogen mineralization was generally the most sensitive of the three endpoints measured. All substances affected nitrate production, typically at soil concentrations above 20 mg/kg soil. The absence of effects on the genetic diversity of bacteria was not expected based on several studies documenting a changed bacterial diversity under influence of chemicals [9,10]. In particular, the results are in contrast to the study of Engelen et al. [9], which showed a good agreement in results between nitrification and bacterial diversity assays. A possible explanation for the observed results could be the fact that PCR amplifies DNA from active as well as dormant bacteria, and if PACs induce dormancy of bacteria, rather than killing them, the nitrification process will be inhibited, whereas genetic diversity, on a strictly qualitative basis, remains the same.

For the total number of protozoans (or the flagellate subpopulation), the results show that this endpoint is more sensitive than the genetic diversity of bacteria, but generally less sensitive than the soil nitrification process. However, for one substance (acridine), protozoans were more sensitive than the nitrifying bacteria by a factor of 2.9 (EC5 of 380 mg/kg compared to a NOEC of 1,100 mg/kg for soil nitrification), confirming the rule that no species or method is the most sensitive to all substances.

**Chemical analysis**

The results of the chemical analyses showed that average measured concentrations for the various substances compared to nominal values were 0.79 for pyrene, 0.80 for fluoranthene, 0.88 for phenanthrene, 0.72 for fluorene, 0.78 for carbazole, 0.73 for dibenzothiophene, 0.75 for dibenzofuran, and 1.09 for acridine (Table 3). As expected, most values were lower than nominal because of evaporation of the substances during sample preparation.

**Conclusion**

Polycyclic aromatic hydrocarbons and N-, S-, and O-PACs are toxic to soil bacteria involved in the mineralization of nitrogen, typically at concentrations above 20 mg/kg for the soil used in the present study. A comparison of the sensitivity

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**Table 3. Results of the chemical analysis**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Nominal concentration (mg/kg dry wt)</th>
<th>Measured concentration (mg/kg dry wt)</th>
<th>Mean value for measured/nominal concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>10</td>
<td>9.6</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>10</td>
<td>7.9</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>780</td>
<td></td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>10</td>
<td>8.3</td>
<td>0.80</td>
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*Fig. 3. Results of the denaturant gradient gel electrophoresis (DGGE) measurements for the substance fluoranthene. This DGGE gel pattern, typical for all substances tested, indicated that the diversity of bacteria detected with this method did not change under the influence of polycyclic aromatic compounds. The numbers to the right indicate the concentration of fluoranthene (mg/kg) in the soil samples from which the different bacterial DNA samples were extracted. PAH = polycyclic aromatic hydrocarbon.*

*tunicistic species possibly could dominate by numbers in the contaminated soil samples. The relatively long exposure period used (four weeks) would typically allow for such a recovery to occur (i.e., so that the sample would contain a lower number of species, but high population densities of one or a few of these robust species).*

*Bacterial DGGE*

All samples yielded PCR products, which were separated on gels. Surprisingly, we saw no effects of any treatments on bacterial diversity. The resulting gel pattern for fluoranthene (Fig. 3) was typical for all substances tested.
of the three microbial endpoints nitrification, protozoan number, and bacterial genetic diversity shows that the nitrification process generally is the most sensitive. No effect of the selected substances could be found on bacterial diversity, and only weak effects at very high concentrations could be found for the total number of protozoans, as well as the flagellate subpopulation. For effects on nitrification, toxicity expressed as soil pore-water concentrations showed a significant inverse relationship with the lipophilicity of the substances. This finding indicates that the toxicity of substances similar to those tested might be predicted by using a quantitative structure-activity relationship.

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