RESEARCH ARTICLE

More functional genes and convergent overall functional patterns detected by GEOCHIP in phenanthrene-spiked soils

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Received 31 January 2012; revised 2 May 2012; accepted 8 May 2012.
Final version published online 15 June 2012.

DOI: 10.1111/j.1574-6941.2012.01413.x

Editor: Tillmann Lueders

Keywords
GEOCHIP; soil microcosms; phenanthrene.

Abstract

To explore the effect of phenanthrene on the functional diversity of soil microbial communities, Luvisol and Cambisol spiked with phenanthrene and their corresponding control soils were incubated in soil microcosms. Total community DNA extracted from samples taken at days 0 and 21 was analyzed by GEOCHIP. The number of genes detected by GEOCHIP was unexpectedly higher in spiked soils than in control soils, especially for Luvisol. Enriched genes in the spiked Luvisol were mainly affiliated to proteobacterial and actinobacterial genes involved in the degradation of aromatic compounds, heavy metal resistance, sulfate reduction, nitrogen and carbon cycling, suggesting changes in the relative abundance of these aerobic and anaerobic functional groups after phenanthrene spiking. Interestingly, the overall functional gene patterns in the different soils converged after phenanthrene spiking, indicating the selection of similar functional groups.

Introduction

Anthropogenic activities such as combustion of wood, coal, and petroleum or mining accidents continually pollute soils with polycyclic aromatic hydrocarbons (PAH). PAH or its metabolites might be toxic to soil microorganisms (Eom *et al.*, 2007; Maliszewska-Kordybach *et al.*, 2007), and thus, a reduced bacterial diversity was expected for soils after PAH pollution. Degradative bacterial populations can grow in polluted soils, using the bioavailable PAH substrates, as revealed by the cultivation-independent analysis of genetic markers such as 16S rRNA genes (Gomes *et al.*, 2005; Johnsen *et al.*, 2007; Jones *et al.*, 2011) and/or key degradative genes (Lloyd-Jones *et al.*, 1999; Ni Chadhain *et al.*, 2006; Gomes *et al.*, 2007; Bordenave *et al.*, 2008; Sipila *et al.*, 2008; Ding *et al.*, 2010). The type of amended PAH substrates (Singleton *et al.*, 2005; Gray *et al.*, 2011) but also plant exudates (Sipila *et al.*, 2008; Yrjala *et al.*, 2010; Cebron *et al.*, 2011) were found to selectively enrich bacterial populations related to PAH degradation. The structure of bacterial communities evolved under the same PAH pollutant differed between soils (Peng *et al.*, 2010; Zhang *et al.*, 2011), possibly due to different soil types with different bacterial community compositions and physicochemical properties. However, it is still unclear whether PAH pollutants select similar functional groups from different soils or not. To address this question, a comprehensive analysis of different functional groups is needed. He *et al.* (2007) developed a functional gene microarray named GEOCHIP 2.0, which consists of 24 243 oligonucleotide probes targeting over 10 000 genes in more than 150 functional groups involved in nitrogen, carbon, sulfur, and phosphorus cycling, metal reduction and resistance, and organic contaminant degradation. This approach was already successfully used in other studies to investigate functional groups in polluted soils (Leigh *et al.*, 2007; Liang *et al.*, 2011).

Soil samples from a microcosm experiment with a Luvisol (silt loam) and a Cambisol (clay loam) spiked with or without phenanthrene were previously analyzed for the abundance and diversity of polycyclic aromatic hydrocarbon ring–hydroxylating dioxygenase (*PAH-RHDα*) genes (Ding *et al.*, 2010), which code for key enzymes catalyzing the first step of microbial mineralization of PAH by incorporating molecular oxygen into the aromatic nucleus. Populations carrying *PAH-RHDα* genes were detected from polluted soils but not from the control soils. The diversity

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and abundance of enriched populations carrying *PAH-RHDα* genes differed between both phenanthrene-spiked soils (Ding *et al.*, 2010). The concentration of phenanthrene decreased after spiking, and at day 21, only 60% of phenanthrene on average was detected for both spiked soils. In the present study, total community DNA was amplified by means of the TempliPhi kit, Cy5-labeled, and hybridized with GEOCHIP 2.0 to explore the influence of phenanthrene spiking on the microbial functional diversity and pattern in comparison with their corresponding control. We hypothesized that phenanthrene spiking might reduce the functional diversity of soils and that phenanthrene spiking would select similar functional groups.

Materials and methods

Experimental design and sampling

The setup of the experiment was described previously by Ding *et al.* (2010). Briefly, phenanthrene was dissolved in acetone and spiked to one-tenth of soils which were used as seeding soils to minimize effects of acetone on soil microbial communities. Seeding soil treated only with acetone was used as control. After evaporating acetone in a chemical hood overnight, the seed soils were mixed with 150 g of natural soil. In a pre-experiment, no influence of acetone-spiked seeding soil on bacterial communities was observed (data not shown) but still soil mixed with acetone-treated seeding soil served as control. The final concentration of phenanthrene in both phenanthrene-spiked soil was 2 mg g^{-1} soil. Four replicate microcosms (165 g each replicate) of Luvisol (48°N, 11° E) or Cambisol (60°N, 17°E), either phenanthrene spiked or not, were incubated at room temperature (23 °C) in the dark. Soil parameters, phenanthrene concentration, quantitative real-time PCR (qPCR) for 16S rRNA genes, studies on the abundance and diversity of *PAH-RHDα* genes as well as the soils' sorption ability of phenanthrene were previously reported (Ding *et al.*, 2010). Total community DNA extracted by means of Bio-101 DNA spin kit for soil (QBiogene, Heidelberg, Germany) from samples collected at days 0 and 21 by Ding *et al.* (2010) was used in the present study. The following abbreviations are used in the text for the treatments: T0C, Cambisol soil on day 0; T0L, Luvisol soil on day 0; T21CP, phenanthrene-spiked Cambisol on day 21; T21CA, Cambisol control on day 21; T21LP, phenanthrene-spiked Luvisol on day 21; T21LA, Luvisol control on day 21.

GEOCHIP analysis

GEOCHIP hybridization was performed as follows: Total community DNA was subjected to whole-community genome amplification using TempliPhi kit (GE Healthcare, Piscataway, NJ) for 6 h at 30 °C. The amplified DNA was labeled with Cy-5 (GE Healthcare; Wu *et al.*, 2006). After purification with a QIAquick purification kit (Qiagen) and drying in a SpeedVac (at 40 °C for 1 h), the labeled products were suspended in 130 μL hybridization buffer [50% formamide, 450 mM NaCl, 45 mM trisodium citrate, 10 ug of unlabeled herring sperm DNA (Promega, Madison, WI), and 0.3% SDS] and denatured at 95 °C for 5 min. The hybridization solution, GEOCHIP 2.0 slides, coverslips, and the pipette tips were kept warm in a 50 °C incubator prior to hybridization, which was carried out on HS4800 Hybridization Station (Tecan US, Durham, NC) at 42 °C for 10 h. The scanned images were gridded and quantified using software package IMAGENE 6.0 (Biodiscovery Inc., EL Segundo, CA). Noise data (poor-quality spots, spots with signal-to-noise ration < 2, and outlier) were removed according to He *et al.* (2007). When all corresponding probes for a particular gene were detected, this gene was reported as being present. The hybridization score for each gene was the average signal intensity of all corresponding probes. Three individual hybridizations (technical replicates) for each of the four biological replicates were carried out. But because of the technical problems and quality control procedures, only 46 slides for 21 biological replicates (Table 1) were suitable for further analysis. The signal intensity of a respective gene in each biological replicate was the average of gene hybridization scores from the corresponding technical replicates (1–3 replicates). A report containing the signal intensities of detected genes for the 21 biological replicates analyzed was used as the basis for further analysis.

Statistical analysis

Multiple one-way ANOVA in conjunction with Tukey HSD tests were used to identify genes with significant differences in signal intensity between spiked and control soils and between soil types. The statistical analysis and data summaries were performed using software package R 2.11 (http://www.r-project.org/). To compare the microbial functional patterns between samples, Kruskal's nonmetric multidimensional scaling analysis was carried out based on binary distance between different samples using R package MASS.

Analysis of genes involved in metabolic pathways for xenobiotic degradation

The role of significantly responding genes in biodegradation pathways of xenobiotics was studied by the following two approaches. Firstly, all amino acid sequences encoded by significantly enriched genes were downloaded from

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Table 1. Average of genes deteced for phenanthrene-spiked and the control Cambisol and Luvisol

Gene category	Initial Cambisol day 0 (2)	Control Cambisol day 21 (4)	Phenantrene-spiked Cambisol day 21 (4)	Initial Luvisol day 0 (4)	Control Luvisol day 0 (4)	Phenantrene-spiked Luvisol day 21 (3)
Organic pollutant remediation	66 ± 28 bc	271 ± 102 bc	472 ± 250 ab	68 ± 59 c	106 ± 53 c	678 ± 238 a
Heavy metal resistance	34 ± 12 bc	136 ± 39 bc	236 ± 122 b	34 ± 34 c	56 ± 22 bc	346 ± 116 a
Nitrification	36 ± 12 c	129 ± 32 bc	188 ± 85 ab	32 ± 27 c	50 ± 21 c	264 ± 72 a
Denitrification	23 ± 3 c	96 ± 25 ab	147 ± 71 ab	18 ± 20 c	29 ± 14 c	194 ± 79 a
Carbon degradation	14 ± 6 bc	62 ± 24 bc	112 ± 63 ab	12 ± 10 c	20 ± 12 c	180 ± 60 a
Sulfate reduction	18 ± 7 bc	68 ± 23 bc	108 ± 54 ab	16 ± 13 c	26 ± 11 c	153 ± 49 a
Carbon fixation	12 ± 1 bc	36 ± 9 abc	54 ± 29 ab	12 ± 8 c	20 ± 10 bc	74 ± 26 a
Nitrogen fixation	7 ± 3 bc	25 ± 10 abc	39 ± 22 ab	6 ± 6 c	11 ± 5 bc	56 ± 19 a
Methane generation	3 ± 1 b	15 ± 5 ab	27 ± 13 ab	4 ± 5 b	8 ± 5 b	38 ± 18 a
Methane oxidation	8 ± 4 c	22 ± 4 bc	34 ± 15 ab	7 ± 5 c	10 ± 4 c	44 ± 8 a
Phosphoenol-pyruvate	0 ± 0 c	0 ± 0 c	1 ± 1 b	0 ± 0 c	0 ± 0 c	2 ± 1 a
Total	220 ± 78 bc	858 ± 265 bc	1417 ± 719 ab	210 ± 186 c	334 ± 146 c	2028 ± 679 a

Values in parentheses represent numbers of biological replicates analyzed; average of genes detected ± standard deviation.
Different letters behind the number indicated significantly ($P < 0.05$) different gene numbers belonging to each functional group.

NCBI and analyzed by BLAST-P against K genes on Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp). Based on the BLAST-P report, KEGG gene orthology identity numbers of significantly enriched genes were retrieved and mapped against the pathways (http://www.genome.jp/kegg/pathway.html Okuda *et al.*, 2008). Secondly, genes in the upper pathway for PAHs mineralization were retrieved based on BLAST-N reports of 40 known *PAH-RHDα* genes. All closest hits were downloaded, and amino acid sequences for corresponding genes in the upper pathway were selected and checked manually. A local database was constructed with amino acid sequences coded by 218 genes in known upper pathways (*nah, phn, ndo, bph, nar, pdo, dox, nid*) from 40 sequences (accession numbers in Supporting information) in NCBI. The retrieved amino acid sequences for significantly responding organic remediation genes (ORG) were analyzed by BLAST-P against the local database using standalone BLAST-P tools. Those genes with significant hits ($1\ e^{-30}$ and > 75% conserved) in the database were regarded as upper pathway genes.

Results

More functional genes detected in phenanthrene-spiked soils

GEOCHIP 2.0 was used to study changes in the abundance of functional genes of indigenous soil microbial communities after phenanthrene spiking. A total of 4192 genes in 11 functional gene categories were detected from both soils. Most genes detected were related to organic pollutant remediation (ORG), heavy metal resistance (MET), nitrogen cycling, or carbon cycling. More functional genes were detected in phenanthrene-spiked soils (Table 1). A significantly higher ($P < 0.05$) number of genes were detected for the spiked Luvisol than its corresponding control (Table 1). More genes were also detected from the spiked Cambisol but the difference between the spiked Cambisol and the control was smaller and not significant.

Functional gene patterns in both soils converged after pollution

To study the functional gene patterns, nonmetric multidimensional scaling was performed based on pairwise binary distance between samples. Microbial functional patterns differed between spiked and the corresponding control soil (Fig. 1). Interestingly, all samples of the phenanthrene-spiked soils closely grouped together except one replicate from the spiked Cambisol, indicating that in general similar functional groups from both soils were selected (Fig. 1). Compared to the control soils, the variation in functional gene patterns in both spiked soils was much smaller, especially for the Luvisol (Fig. 1). Changes in functional gene patterns with time were also observed for the control Cambisol, suggesting that other factors such as mechanical mixing might have also caused changes in the microbial community in the Cambisol, which has higher clay content than the Luvisol (Fig. 1).

More genes with increased abundance in the Luvisol than in the Cambisol after phenanthrene spiking

To identify genes with increased abundance in response to the phenanthrene spiking, the signal intensities were tested by multiple one-way ANOVA in conjunction with Tukey HSD tests ($P < 0.01$). Signal intensities of more

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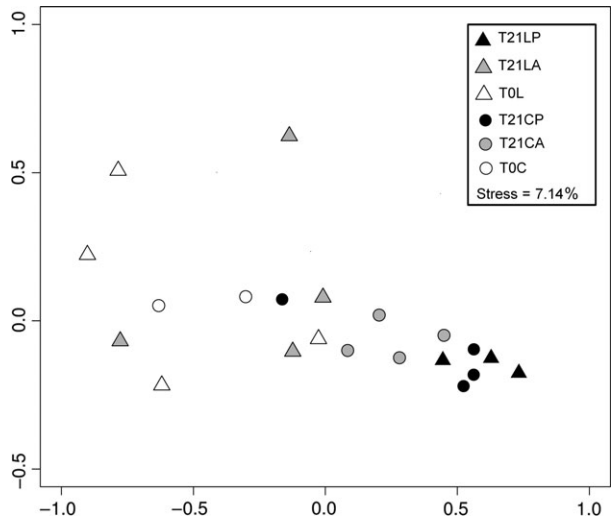

Fig. 1. Nonmetric multidimensional scaling based on all functional genes detected for soils (L, C) at day 0 (T0) and soils spiked with phenanthrene (LP, CP) or not (LA, CA) at day 21 (T21).

Table 2. Number of genes with significant difference ($P < 0.01$) in signal intensity between soil types and treatments

Gene category	T0L–T0C	T21CP–T21CA	T21LA–T21CA	T21LP–T21LC	Total*
ORG	0	5	1	158	1396
MET	0	0	0	104	685
NIT	1	1	3	75	499
NRED	0	0	0	47	445
CDEG	0	2	1	49	377
DSR	0	0	0	35	301
CFIX	0	0	0	11	179
NFIX	0	0	0	18	132
Methane generation	0	0	0	8	96
Methane oxidation	0	0	0	12	73
PER	0	0	0	2	3
Total	1	8	5	519	4192

ORG, organic pollutant remediation; MET, metal resistance; NIT, nitrification; NRED, denitrification; CDEG, carbon degradation; DSR, sulfate reduction; CFIX, C fixation; NFIX, nitrogen fixation; PER, phosphoenolpyruvate.
*Total number of detected genes belonging to each functional group.

than 12% of the detected genes (4192) were significantly higher in phenanthrene-spiked Luvisol than in the untreated control (Table 2). In addition to genes involved in organic pollutants remediation, genes of other categories such as nitrogen cycling, heavy metal resistance, carbon cycling, or sulfate reduction were enriched in the spiked Luvisol. For most of these functional groups, the proportion of significantly enriched genes varied between 6% to 16% of the genes detected for the corresponding functional group. Two of three genes in the phosphoenolpyruvate (PER) group were of significantly higher signal intensity in the spiked Luvisol than in the control. This was followed by methane oxidation genes where 16.4% of the detected genes were enriched. Only *c.* 11% of the detected ORG were enriched, although in this gene category the highest number of responding genes was found. The lowest percentage of responding genes of only 6% was found for genes involved in carbon fixation (CFIX). Only few responding genes were detected for the Cambisol (0.2%).

Enriched functional genes involved in the degradation of aromatic compounds

Only eight genes had significantly higher signal intensity in phenanthrene-spiked Cambisol than in the control. Five of them (NCBI gi: 40890267, 40890329, 14289342 from *Rhodococcus* sp. 19070; 24575091 from *Streptomyces globisporus*; 26991304 from *Pseudomonas putida* KT2440) were related to the degradation of one-ring aromatic hydrocarbons such as styrene or benzoate. The remaining three genes (NCBI gi: 3298347, 19570948, and 401315) code for cellulase, chitinase, and guanylate kinase, respectively.

In phenanthrene-spiked Luvisol, 158 of 519 detected genes involved in aromatic compound degradation were significantly increased in abundance compared to the control. Under aerobic conditions, dioxygenases catalyze the key step for PAH degradation by incorporating molecular oxygen into the aromatic nucleus. Four genes encoding the alpha- or beta-subunit of dioxygenases had significantly higher signal intensities in the spiked Luvisol than in the control. These genes were derived from *Pseudomonas aeruginosa* (*nahAa,* NCBI gi 1255667), *Ralstonia* sp. U2 *(nahAc,* NCBI gi: 2828018), *Neptunomonas naphthovorans* (*nahAc*, NCBI gi: 3170519), and *Mycobacterium gilvum* (*rhda*, NCBI gi: 26080263). The three other genes (*Pseudomonas aeruginosa*, *nahQ*, NCBI gi 6226946; *Rhodococcus opacus*, *narR*-like, 38524451, 38524452) enriched in the spiked Luvisol were also commonly found as upper pathway genes for PAH degradation. The remaining enriched ORG detected with increased abundance were related to the degradation of one-ring aromatic compounds. The KEGG-based analysis confirmed that 87 ORG coded for enzymes involved in the degradation pathway for one-ring aromatic hydrocarbons, such as benzoate, nitrotoluene, atrazine, aminobenzoate, styrene, toluene, xylene, fluorobenzoate, dioxin, or chlorohydrocarbons like chlorocyclohexane or chloroalkane. Many more enriched ORG were involved in the degradation of one-ring aromatic compounds, suggesting that bacterial populations carrying

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these genes might benefit indirectly from phenanthrene. Detected ORG with increased abundance were derived from *Pseudomonas* (31 ORG), *Ralstonia* (11 ORG), *Rhodococcus* (9 ORG), *Mycobacterium* (9 ORG), *Burkholderia* (9 ORG), *Streptomyces* (5 ORG), *Xanthomonas* (5 ORG), *Sphingomonas* (4 ORG), or *Yersinia* (4 ORG).

Phenanthrene spiking also enriched populations carrying genes involved in heavy metal resistance, sulfate reduction, nitrogen and carbon cycling

Bacterial populations carrying genes involved in heavy metal resistance were also enhanced in phenanthrene-spiked Luvisol. Altogether, 104 heavy metal resistance (MET) genes were enriched in the spiked Luvisol. Most of them were involved in the resistance or reduction of arsenic (20%), tellurium (16%), mercury (16%), and copper (12%). MET genes with significantly increased abundance were derived from *Pseudomonas* (10 MET), *Mycobacterium* (6 MET), *Streptomyces* (5 MET), and *Ralstonia* (5 MET) and in addition to genera of *Geobacter* (6 MET), *Salmonella* (6 MET), *Escherichia* (6 MET), *Shewanella* (5 MET), and *Staphylococcus* (5 MET). Thirty-five sulfate reduction genes (19 *dsrA* genes and 16 *dsrB* genes) were also significantly increased in the phenanthrene-polluted Luvisol, suggesting that anaerobic bacteria might have also benefited from phenanthrene spiking.

Populations carrying genes involved in nitrogen fixing (18 *nifH* genes), denitrification (NRED; 14 *nirK*, 11 *narG*, 9 *nirS*, 4 *nasA*, 4 *norB*, 4 *nosZ*, and 1 *narB*), and nitrification (NIT; 75 genes) were enhanced in the Luvisol after phenanthrene spiking. Most enriched *nifH* genes (67%) and genes involved in NRED (83%) were derived from uncultured bacteria. Most enriched genes (76%) belonging to the gene category of NIT code for urease (57), followed by *amoA* (11). The signal intensities of genes coding for cellulase (19 genes), chitinase (13 genes), laccase (10 genes), and polygalacturonase (4 genes) were also higher in the spiked Luvisol than in the control.

Genes with increased abundance in spiked Luvisol affiliated to diverse taxa

Based on NCBI gi numbers, the sequences of all enriched genes in the spiked Luvisol were retrieved from GenBank. Altogether, 498 sequences were acquired. The distribution of these genes among different taxonomic groups was summarized based on NCBI taxonomy (Table 3). Only few genes were affiliated to Fungi, such as *Fusarium*. The majority (69%) of genes with increased abundance were affiliated to *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Firmicutes*. ORG and MET genes were mainly affiliated to *Gammaproteobacteria, Alphaproteobacteria*, and *Betaproteobacteria*. The majority of NIT genes were affiliated to *Proteobacteria* (62.7%) and *Cyanobacteria* (14.7%). Half of sulfate reduction genes were affiliated to *Deltaproteobacteria*. Phenanthrene spiking probably influenced several taxonomic groups in the Luvisol. To analyze the proportion of functional groups enriched, which most likely are affiliated to the same genus, cluster analysis based on Pearson correlation indices was performed using the data matrix (Supporting information, Table S1) in which each row contains the numbers of enriched genes of different gene categories for the same genus. In general, a low proportion (<10%) of enriched genes of different functional groups was derived from the same genus (Fig. S1), except for those belonging to ORG, MET, and NIT genes. More than 45% of ORG, MET, and NIT were derived from the same genus.

Discussion

PAH could be toxic to many bacterial populations (Eom *et al.*, 2007; Maliszewska-Kordybach *et al.*, 2007). Sipila *et al.* (2008) found that the relative abundance of the genus of *Acidobacteria GP1* was lower in the PAH-polluted bulk soil and rhizosphere samples compared to the nonpolluted soil. Therefore, a reduced microbial diversity was assumed for unpolluted soils after PAH contamination. Indeed, using GEOCHIP 2.0 as in the present study, Liang *et al.* (2011) reported on a lower number of functional genes detected in oil-contaminated soils than in noncontaminated soils. In the present study, two different unpolluted soils were spiked with phenanthrene and genes involved in key biogeochemical processes were analyzed by GEOCHIP 2.0. In contrast to the study by Liang *et al.* (2010), a higher number of genes were detected for both phenanthrene-spiked soils compared to the control soils. Oil is a mixture of several components, some of which can be more toxic than phenanthrene. But in general, microbial diversity in soils is extremely high, assuming thousands to half a million bacterial species present in 1 g of soil (Curtis *et al.*, 2002; Hong *et al.*, 2006). Only the most dominant populations are likely to be detected except when specific taxonomic groups were targeted. The increased number of genes detected by GEOCHIP in the present study is most likely due to an increased relative abundance of certain populations. That populations were enriched in the spiked Luvisol is supported by at least two lines of evidence. Firstly, the increased abundance of some populations in spiked Luvisol was evidenced in the DGGE fingerprints (Fig. S2a) by bands of increased intensity for both phenanthrene-spiked soils. Secondly, *PAH-RHDα* genes could be detected in

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Table 3. Number of genes with increased abundance in spiked Luvisol and their taxonomic affiliation

Domain	Phylum	Class	Order	Family	ORG	MET	NIT	CDEG	NRED	DSR	Other	Total
Bacteria	*Proteobacteria*	*Alphaproteobacteria*			27	10	14	1	2	0	4	58
		Betaproteobacteria	*Burkholderiales*		28	9	4	1	2	0	3	47
			Other		1	1	7	0	2	0	0	11
		Gammaproteobacteria	*Pseudomonadales*	*Pseudomonadaceae*	31	11	6	1	0	0	0	49
				Enterobacteriales	10	18	6	2	1	0	0	37
				Other	11	12	0	5	0	0	2	30
		Other			1	10	2	0	0	14	0	27
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	9	0	0	0	0	0	0	9
				Mycobacteriaceae	9	6	5	1	0	0	0	21
				Other	9	5	3	3	0	0	0	20
			Bifidobacteriales	*Bifidobacteriaceae*	0	1	0	0	0	0	0	1
	Cyanobacteria				3	2	11	1	1	0	3	21
	Firmicutes				0	9	4	3	0	0	2	18
	Other				3	4	0	3	0	1	0	11
Archaea					2	0	1	0	0	0	4	7
Others					14	5	12	28	27	13	32	131
Total					158	103	75	49	35	28	50	498

ORG, organic pollutant remediation; MET, metal resistance; NIT, nitrification; CDEG, carbon degradation; CDEG, carbon degradation; NRED, denitrification; DSR, sulfate reduction.

the contaminated Luvisol but not in the control (Ding *et al.*, 2010).

The overall functional patterns of both spiked soils converged, suggesting that phenanthrene spiking selected similar functional groups from both soils. By GEOCHIP analysis, Liang *et al.* (2011) also reported that five oil-contaminated soils shared similar organic remediation gene pattern but not the overall functional gene pattern. In that study, the polluted and unpolluted soils were collected from sites varying substantially in concentrations of oil components but also nutrient levels (N, P), water content, and soil texture as well as the climate. The type of PAH, physicochemical characteristics, and biotic factors have been reported to influence bacterial populations selected (Ni Chadhain *et al.*, 2006; Yrjala *et al.*, 2010; Cebron *et al.*, 2011; Jones *et al.*, 2011). In the present study, soil samples were collected from a microcosm experiment with four replicates per treatment which allowed us to study the response to phenanthrene while keeping other factors constant.

The response of bacterial communities to phenanthrene spiking was stronger in the Luvisol than in Cambisol, as only few significantly enriched genes by GEOCHIP analysis were observed for the Cambisol and less deviation of community structure by DGGE (Fig. S2b). Previously, *PAH-RHDα* genes were only detectable in the polluted Luvisol at this sampling time. Different responses of microbial communities to phenanthrene spiking might be related to the indigenous bacterial community (Fig. S2) or soil physicochemical characteristics (Ding *et al.*, 2010). Both soils have a contrasting soil texture, which can influence, for example, the exchange of O_2 into the porous soil matrix (Young & Crawford, 2004). Compared with Luvisol (14%), the clay content of the Cambisol (37%) is much higher, which could result in low pore connectivity (Carson *et al.*, 2010). Moldrup *et al.* (2001) showed that air permeability of soils is positively related to pore connectivity. Thus, the exchange of O_2 into the soil matrix could be easier for the Luvisol than for the Cambisol, favoring the aerobic populations.

Interestingly, anaerobic populations in the Luvisol probably also benefited from phenanthrene spiking because populations carrying genes involved in sulfate (*dsrA* and *dsrB*) or nitrite reduction were found to be enriched in the spiked Luvisol. Under anaerobic conditions, sulfate-reducing and nitrite-reducing bacterial isolates can degrade phenanthrene or naphthalene (Meckenstock *et al.*, 2000; Rothermich *et al.*, 2002; Eriksson *et al.*, 2003; Davidova *et al.*, 2007). Sulfate-reducing bacteria are important for the degradation of organic matter in anaerobic environments (Leloup *et al.*, 2009). Phenanthrene or its secondary metabolites probably diffused into microsites occupied by these anaerobic bacteria, or the aerobic

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degradation resulted in more microsites with depleted oxygen, extending the suitable niches for anaerobes.

Under aerobic conditions, the common microbial degradation of PAH is initiated by dioxygenases. The GEOCHIP analysis suggested that the upper pathway genes *nahAc* and *narA were* enriched in the polluted Luvisol. In contrast, the PCR-based *PAH-RHDα* gene analysis detected in the same total community DNA only the *phnAc* gene in spiked Luvisol (Ding *et al.*, 2010). Similar *phnAc* genes were detected by GEOCHIP hybridization but with equal signal intensities for spiked and control soils. Most likely, the increase in relative abundance of *phnAc* genes in spiked Luvisol was still very low, and thus, these genes detected by GEOCHIP were not identified as significantly enriched. *In silico* analysis suggested that both *nahAc* and *narA* genes should have been amplified by the primer targeting *PAH-RHDα* gene but they were not detected among the cloned amplicons from DNA of spiked Luvisol from day 21. These *nah* genes were previously often reported to be located on IncP-9 plasmids from *Pseudomonas* (Dennis & Zylstra, 2004; Lambertsen *et al.*, 2004; Izmalkova *et al.*, 2006; Sota *et al.*, 2006). The *narA* genes were typically found in strains of the genus *Rhodococcus* (Andreoni *et al.*, 2000; Di Gennaro *et al.*, 2010). Andreoni *et al.* (2000) reported that the *narA* gene was chromosomally located in *Rhodococcus* sp. strain 1BN. To improve the gene detection, the total community DNA was subjected to whole-community genome amplification in the present study using TempliPhi (RCA), which was assumed more efficient for circular DNA templates because of 'infinite' length of template. But in the study of Tate *et al.* (2011), the amplification efficiency of RCA was comparable between linear and circular templates. In the study by Rodrigue *et al.* (2009), the sequencing coverage varied > 1000-fold over the whole genome when the RCA amplicons of a single *Prochlorococcus* cell were sequenced. In that study, the coverage of regions also related to their position in the genome. Therefore, it was assumed that RCA could preferentially amplify certain regions of the genome which might have facilitated the detection of *nahAc* or *narA*. In the previous study (Ding *et al.*, 2010), *nahAc* or *narA* were not detected among the 33 clones sequenced from cloned *PAH-RHDα* amplicons obtained from T21LP DNA probably due to their lower relative abundance.

Many of the organic pollutant remediation genes with increased signal intensities in spiked Luvisol are potentially involved in the degradation of aromatic compounds. Interestingly, only few genes were found from the upper pathway for PAH degradation, while the majority of enriched ORG detected are linked with the degradation of one-ring aromatic hydrocarbons and chlorohydrocarbons. Initial PAH degraders might be only a small proportion of the total bacterial populations enriched after phenanthrene spiking in the present study. Most enriched populations probably benefited from the metabolic intermediates. Head *et al.* (2006) suggested that those populations that control the flux of metabolic intermediates might be important for improving bioremediation. Similar effects of PAH amendments on the soil bacterial community were also suggested by the study of Sipila *et al.* (2008) as dominant enriched bacteria were affiliated to *Burkholderia* (*Betaproteobacteria*) by analyzing 16S rRNA genes, while dominant extradiol dioxygenase genes were affiliated to *Sphingopyxis* (*Alphaproteobacteria*).

Heavy metals inhibit the biodegradation of organic pollutants (Sandrin & Maier, 2003). Bacterial populations carrying genetic systems for PAH degradation and resistance to heavy metals might have an advantage to thrive in industrial sites where soils are co-contaminated with PAH and heavy metals (Campbell *et al.*, 1995). In the present study, the Luvisol was collected from an agricultural field with no known history of PAH and heavy metal pollutions. But populations carrying heavy metal resistance genes were still enriched after phenanthrene spiking. Correlation analysis of the distribution of different functional groups among bacterial genera suggested that a part of enriched ORG and MET genes were possibly carried by similar hosts (Fig. S1). Thus, enriched bacterial populations in spiked Luvisol possibly also harbor heavy metal resistance genes. Metatranscriptomic analysis might help to identify those genes actually involved in response to phenanthrene spiking.

In summary, more functional genes were detected in the phenanthrene-spiked soils which might be misinterpreted as increased functional diversity in these soils. However, more likely the increased number of functional genes detected in spiked soils was caused by the selection of some populations (see Fig. S2) and their subsequently improved detection. In addition, functional gene structure in both soils converged after pollution. Various functional groups related to distant taxonomic groups were found to be enriched after phenanthrene spiking, suggesting a complex mineralization network of PAH in soils.

Acknowledgements

This work was supported by DFG SPP1315 (SM59/8–1) and the BMBF grant, U.S. Department of Energy through the Environmental Remediation Science Program (ERSP), and a Scientific Focus Area Program, ENGIMA (DE-AC02-05CH11231).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Furthest-neighbor-joining cluster of different gene groups based on Pearson's correlation distance using numbers of enriched genes belonging to different bacterial genera (Table S1).

Fig. S2. Bacterial DGGE fingerprint (a) and the corresponding UPGMA clusters (b) for soil spiked with phenanthrene (LP, CP) or the control (LA, CA) at day 21 (T21), a–c indicate the bands with increased intensity in the spiked soils.

Table S1. Number of significant enriched genes belonging to different genus and gene categories.

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