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Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons

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Abstract

Bacterial and protozoan communities were examined in three cores (A, B and C) from an aquifer located at an abandoned refinery near Hünxe, Germany. Cores were removed along a transect bordering a plume containing various monoaromatic hydrocarbons. Monoaromatic hydrocarbons could not be detected in the unsaturated zone in any core but were present in the saturated zones of core C (between 280 and 42 600 μmol kg^{-1} of core material [dry wt.]) and cores A and B (between 30 and 190 μmol kg^{-1} of core material [dry wt.]). Xylene isomers accounted for 50–70% of monoaromatic hydrocarbons in all cores. The number of DAPI-stained bacteria was found to increase from the low-contaminated cores A and B (approx. 0.1×10^8 cells and 0.2×10^8 cells g^{-1} of core material [dry wt.], respectively) to the high-contaminated core C (2.4×10^8 cells g^{-1} of core material [dry wt.]). The higher bacterial numbers in core C were found to coincide with a higher detection rate obtained by in situ hybridization using probe Eub338 to target the domain Bacteria (13–42% for core C as compared to 3–25% for cores A and B, respectively). Proteobacteria of the δ-subdivision (which includes many sulfate-reducing bacteria) were the most predominant of the groups investigated (7–15% of DAPI-stained bacteria) and were followed by Proteobacteria of the γ- and β-subdivisions (4% and 1% of DAPI-stained bacteria, respectively). The total numbers of protozoa and bacteria determined by direct counting occurred in a ratio of approx. $1{:}10^3$, which was independent of depth or core examined. Most probable number analysis combined with a subsequent classification of the culturable protozoa revealed nanoflagellates as the major component of the protozoan community. Naked amoebae became increasingly more encysted with depth, except in the high-contaminated core C where vegetative trophozoites were present in the saturated zone. The co-occurrence of bacteria and protozoa in association with high concentrations of monoaromatic hydrocarbons suggests the involvement of trophic interactions in the process of biodegradation.

Keywords: Bacterial community; Protozoon; In situ hybridization; Monoaromatic hydrocarbon; Bioremediation

1. Introduction

In situ bioremediation is widely considered to be an environmentally friendly and cost effective tech-

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nology for the treatment of hydrocarbon-contaminated sites [17,26]. The technology relies on the collective ability of microorganisms to degrade the contaminants under prevailing environmental conditions. Microbial degradation of organic contaminants such as petroleum-derived hydrocarbons is usually monitored by analyzing changes in chemical parameters including reductions in contaminants and oxidants, and increases in dissolved inorganic carbon and reduced species [20]. In addition, measurements of the $^{13}C/^{12}C$ isotopic ratios in educts and products quite often allow mass balances of the catabolic processes to be established [4,18,23]. Microbiological monitoring of in situ bioremediation has been considered less reliable due to limited information on the requirements and capabilities of the indigenous microorganisms, and a lack of suitable methods for in situ determination of their abundance and activity [6].

Previous studies on bacterial populations in contaminated aquifers have largely been confined to techniques relying on the culturability of organisms as in the case of plate counts or most probable number techniques [2,11,25,43]. However, these techniques only allow a low percentage of the total number of microorganisms to be assessed [2]. Many recent studies have focused on the isolation and characterization of bacteria capable of degrading specific contaminants [12,33,48], on determining their catabolic pathways [7,47], or on determining the catabolic potential of natural populations in laboratory aquifer columns [21,22]. Consequently, the impact of natural communities of bacteria on the degradation of contaminants in the field remains poorly understood.

Within the last few years, it has been suggested that populations of subsurface protozoa might be a good indicator of in situ biodegradation activity [27,42]. Since many protozoa are bacterivorous, it is thought that high numbers of protozoa are indicative of rapidly growing populations of bacteria [30]. Comparatively large populations of sub-surface protozoa have been reported from various organically contaminated sites [27,29,30,41,42]. However, the influence of bacterivorous protozoa on the composition and hence degradative ability of the bacterial community at these sites is currently unknown. Since our knowledge of natural populations of microorganisms is largely derived from culture-dependent studies, it is likely that current information on the composition of bacterial and protozoan communities is biased by the limitations of culturability.

The present study examined the chemical environment in relation to the microbial community at a field site contaminated with monoaromatic hydrocarbons. The in situ hybridization technique (for review see [1]) was used in association with DAPI epifluorescence staining as a culture-independent direct counting method for determining in situ the absolute abundance of bacteria and protozoa. Studies on bacterial community structure initially focused on the analysis of higher phylogenetic groups and on *Azoarcus* sp. which are considered to be involved in the biodegradation of hydrocarbons under oxic and denitrifying conditions [22]. A comparison was made between the enumeration of protozoa by in situ hybridization and by the culture-dependent most probable number (MPN) technique which also provided information on the composition of the protozoan community.

2. Materials and methods

2.1. Field site and sampling procedure

The field site was an area contaminated with monoaromatic hydrocarbons on the site of an abandoned refinery near Hünxe in the lower Rhine area of Germany [36,37]. The contaminated aquifer consisted of a calcite-rich sand predominantly of grain size 0.2–1.0 mm and a fractional organic carbon content of 0.07%. A porosity of 25% was estimated for the saturated zone [37]. The contaminated zone was partially bioremediated as described in [37].

In March 1997, three cores A, B and C (diameter 3.6 cm; length 7.0 m) were taken along a transect bordering the xylene plume and northwest of the area with xylene in free phase, using a pile-driven coring device. Core A was located outside the plume, core B at the edge of the plume and core C inside the plume. In situ temperature was 12°C and no increase was observed due to the sampling procedure. At the time of sampling the water table was 5.4 m below the surface and the saturated thickness was about 15 m.

Cores A, B and C showed a grey coloration below a depth of 6.6 m, 6.4 m and 6.2 m, respectively. Each core was sampled at 50-cm intervals above 4.5 m depth and at 10-cm intervals below this depth. Samples (approx. 10 g) for the analysis of volatile hydrocarbons were collected on site using gas-tight headspace flasks sealed with teflon coated rubber stoppers. For the analysis of microorganisms, core material (5–15 g) was added on site to 15 ml Falcon tubes and either left unfixed or fixed with 5 ml of 4% paraformaldehyde solution. All samples were transferred on ice to the laboratory and then stored at either 4°C or −20°C prior to analysis.

2.2. Chemical analysis

Pore water was obtained by centrifuging approx. 12 g of core material added to 5-ml plastic syringes which were plugged with silane-treated glass wool (Supelco Inc., Bellefonte, PA, USA). The filled syringes were placed in 15-ml Falcon tubes and centrifuged at 4°C and 2500×*g* for 10 min. Concentrations of NO_3^-, NO_2^-, SO_4^{2-}, PO_4^{3-}, and Cl^- were analyzed in 15-μl samples of pore water by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, CA, USA) using an eluent of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ [21]. Data from ion chromatography were analyzed using Chrom-Card for Windows (Fison Instruments, Rodano, Italy) [21].

For the analysis of volatile hydrocarbons, the volume of pore water in each flask was calculated from the dry weight of the core material, and an equal volume of pentane was injected [16]. After vortex-mixing for 1 min, water and pentane phases were allowed to separate. The pentane phase was transferred to gas-tight glass vials which were then sealed with teflon-lined screw caps. One μl of each extract was injected into a Carlo Erba GC 8000 gas chromatograph equipped with a flame ionization detector (Fison Instruments). A glass column (diameter 3 mm; length 2 m) packed with 5% SP 1200 and 5% Bentone 34 on Supelcoport 100/120 mesh (Supelco Inc.) was used to resolve the hydrocarbons. Conditions were 100°C isothermal for 15 min and N_2 was used as the carrier gas. Data from gas chromatography were analyzed using Chrom-Card for Windows [16].

Less volatile hydrocarbons were analyzed in extracts of mixed material of each core from a depth of 5–7 m. Extracts were obtained from 6-g samples dried with 20% Na_2SO_4 by Soxhlet extraction with 35 ml CCl_4 at 55°C for 15–20 h [5]. The resulting extract was concentrated to a volume of 2 ml under a gentle stream of N_2 at 25°C. Two μl of extract was injected into a Fisons HRGC Mega II GC (Fison Instruments) equipped with a flame ionization detector and a BGB-5 fused silica capillary column (length 3 m, inner diameter 0.32 mm) with a 5% phenyl-methyl polysiloxane (0.25 μm) bonded phase (BGB Analytik AG, Zurich, Switzerland). The conditions for analysis were splitless injection with H_2 as the carrier gas and a column temperature of 40°C for 2 min followed by a temperature increase to 250°C at a rate of 3°C min^{-1}. Data were again analyzed using Chrom-Card for Windows with *o*-terphenyl as a calibration standard [5].

2.3. In situ hybridization

After 36 h of fixation in 4% paraformaldehyde, samples for in situ hybridization were washed twice with phosphate-buffered saline (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.2), resuspended in 98% ethanol to a final density of 0.6 g core material [dry wt.] per ml and stored at −20°C [15]. These suspensions were then diluted 10-fold in 0.1% pyrophosphate and thoroughly mixed. After 10 s sedimentation, 10 μl of each dispersed sample was spotted onto gelatin-coated slides, dried at room temperature for at least 4 h, and finally dehydrated in 50%, 80% and 96% ethanol for 3 min in each.

Hybridizations were carried out with Cy3-labeled oligonucleotide probes as described previously [46]. Probes were used to detect the domains Bacteria (probe Eub338) and Eukarya (Euk516), bacteria of the α- (Alf1b), β- (Bet42a), γ- (Gam42a), and δ- (SRB385 and SRB385Db) subdivisions of Proteobacteria, the high G+C Gram-positive bacteria (HGC69a), the *Cytophaga-Flavobacterium* cluster (CF319a) [46], and hydrocarbon-degrading *Azoarcus* sp. (Azo644) [22]. Hybridizations were performed in 9 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) in the presence of 10–35% formamide, 1 μl of the probe (25 ng μl^{-1}), and 1 μl of a solution of the DNA

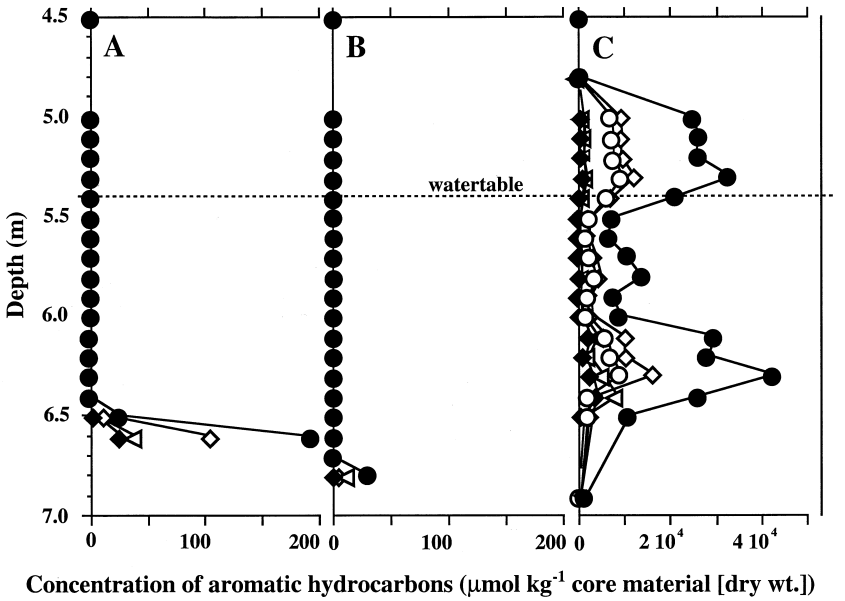

Fig. 1. Concentration profiles showing total aromatic hydrocarbons (●), total trimethylbenzenes (○) and *m*- (▽), *p*- (◆) and *o*-xylene (◇) in cores A, B, and C at depths between 4.5 and 7.0 m. Note the different scales used.

intercalating dye 4′,6-diamidino-2′-phenylindole (DAPI, 200 ng μl^{-1}, Sigma, Buchs, Switzerland) at 42°C for 2 h [46]. After hybridization, the slides were washed in buffer at 48°C for 15 min, rinsed with distilled water and air-dried.

The slides were mounted with Citifluor solution and the preparations were examined with a Zeiss Axiophot microscope fitted for epifluorescence with a high-pressure mercury bulb (50 W) and filter sets 02 (Zeiss, Oberkochen, Germany; G 365, FT 395, LP 420) and HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G 535/50, FT 565, BP 610/75). Organisms were counted at 1000× magnification in randomly selected fields each covering an area of 0.01 mm^2. Twenty fields were examined for probe-conferred signals indicating specific bacterial groups. Hybridization signals obtained with probe Euk516 which was used to detect protozoa were examined in 300 fields. Bacterial and protozoan numbers are expressed per g core material [dry wt.] for DAPI-stained samples or as a percent of DAPI counts for bacteria detected with specific oligonucleotide probes. Numbers were expressed as mean ± standard error.

2.4. MPN enumeration of protozoa

In addition to detection by in situ hybridization, protozoa were also analyzed by a modification of a MPN technique [35]. Samples from 1-m depth intervals in each core (approx. 2 g) were weighed, suspended in a protozoan saline [31] and vortex-mixed. A three-fold dilution series was prepared from each suspension using a sterile soil extract diluted 1:4 with deionized water [35]. Dilution levels from 10^1 to 10^5 with five replicates for each were prepared in a 96-multiwell plate and incubated at 21°C for 28 days. Plates were examined microscopically and each well scored for the presence or absence of ciliates, of flagellates, and of vegetative and encysted amoebae. An MPN for each sample was calculated from the number of positive end-point dilutions [8] and expressed per g of core material [dry wt.]. The lower limit of detection for the technique was 0.8 protozoa g^{-1} of core material [dry wt.] with an estimated standard error of 50% [40].

3. Results

3.1. Chemical characterization of the aquifer

Monoaromatic hydrocarbons could not be detected in any of the cores A, B and C above a depth of 4.8 m. Below this depth, monoaromatic hydrocarbons were detected in large amounts only in core C (between 280 and 42 600 μmol kg^{-1} of core material [dry wt.]) and in much lower amounts in cores A and B (up to 190 μmol kg^{-1} of core material [dry wt.]) (Fig. 1). In core C, they comprised in order of decreasing concentrations *o*-xylene, 1,3,5-trimethylbenzene and/or 1,2,4-trimethylbenzene (not resolved on the gas chromatograph), 3-ethyltoluene, *m*-xylene, *p*-xylene, 4-ethyltoluene, 1,2,3-trimethylbenzene, ethylbenzene, toluene, and benzene. Xylene isomers accounted for 90 ± 1% of monoaromatic hydrocarbons in core A, 77% in core B, and 50 ± 5% in core C (with $< 50\%$ at a depth above 6 m and $> 50\%$ further down this core). Less volatile hydrocarbons were only detected in trace amounts in all three cores after Soxhlet extraction of a mixture of soil samples from below 5 m.

Nitrate concentrations in pore water from the saturated zones (Fig. 2) decreased from core A (between 6 and 135 μM) at the border of the plume towards core C (between 4 and 15 μM), lying inside the plume. Along the depth profile of each core concentrations of nitrate and sulfate were generally found to decrease with depth. The concentrations of sulfate as well as of nitrite were highest in core C and lowest in core B (Fig. 2). Concentrations of sulfate ranged between 70 and 1090 μM in core A, between 210 and 490 μM in core B, and between 600 and 1800 μM in core C. Concentrations of nitrite ranged between 1 and 22 μM in core A, between 2 and 10 μM in core B, and between 10 and 58 μM in core C.

3.2. Bacterial community structure

After DAPI staining, bacteria were detected in numbers between 4 and 8×10^8 cells g^{-1} of core material [dry wt.] in the non-contaminated surface layers in all cores (Fig. 3). Numbers decreased within the first meter of each core to values between 0.1 and 1×10^8 cells g^{-1} of core material [dry wt.] and did not change significantly toward the saturated zone. In the saturated zone, a significant increase in bacte-

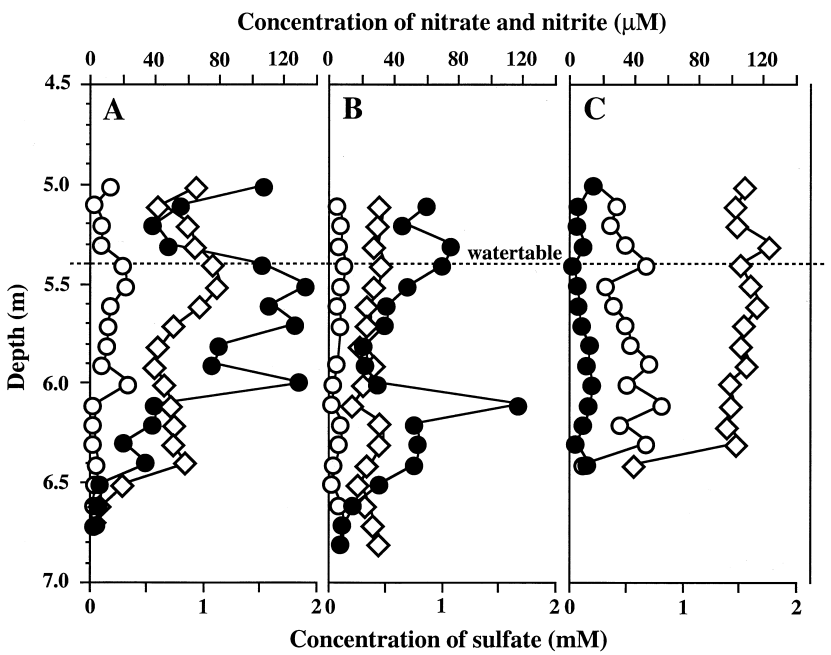

Fig. 2. Concentration profiles showing nitrate (●), nitrite (○), and sulfate (◇) in cores A, B and C at depths between 4.5 and 7.0 m.

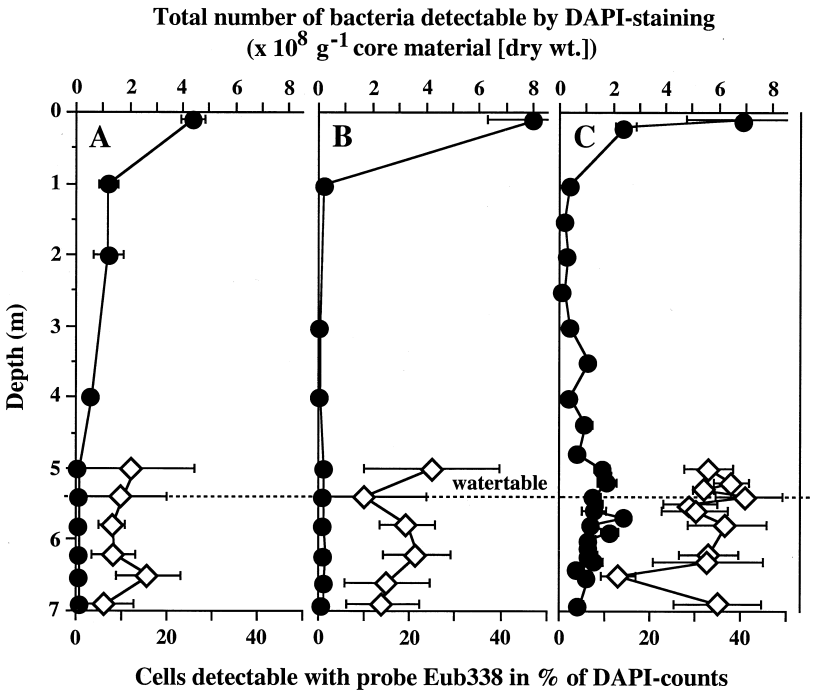

Fig. 3. Total number of bacteria determined after DAPI staining (●) and the percentage of cells detectable after hybridization with the Cy3-labeled probe Eub338 (◇) in cores A, B, and C at depths between 0.1 m and 7.0 m.

rial numbers was only detected in contaminated core C with up to 2.4×10^8 cells g^{-1} of core material [dry wt.]. Numbers of bacteria in the low-contaminated cores A and B remained low (approx. 0.1×10^8 cells and 0.2×10^8 cells g^{-1} of core material [dry wt.], respectively).

In situ hybridization with probe Eub338 targeting the domain Bacteria detected percentages of DAPI-stained cells of 3–16%, 10–25%, and 13–42% in cores A, B, and C, respectively. Low numbers of bacteria in cores A and B were reflected in a lower detection rate by in situ hybridization, which rendered the use of oligonucleotide probes difficult. Consequently, only spot checks were carried out for higher phylogenetic groups in these cores. During these checks, only members of the δ-subdivision of Proteobacteria were occasionally detected.

In the saturated zone of contaminated core C, 10–20% of DAPI-stained bacteria could be assigned to the phylogenetic groups investigated which represented up to 50% of the bacteria detectable by in situ hybridization (Fig. 4). Some 7–15% of DAPI-stained bacteria were detected with a combination

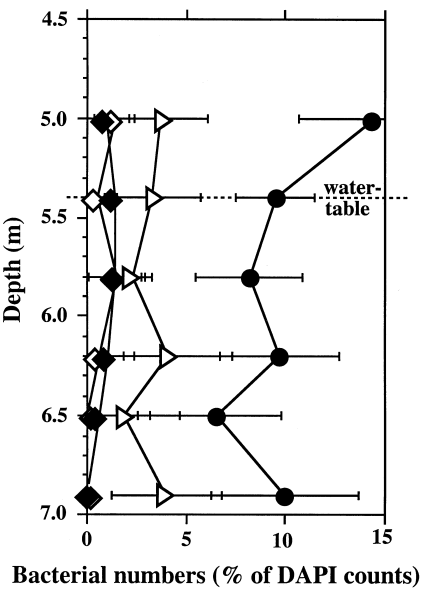

Fig. 4. Prevalence of bacterial groups belonging to the β-subdivision (◆; Bet42a), γ-subdivision (△; Gam42a), and δ-subdivision of Proteobacteria (●; SRB385 and SRB385Db) and the hydrocarbon-degrading *Azoarcus* sp. (◇; Azo644) in core C at depths between 4.5 and 7.0 m.

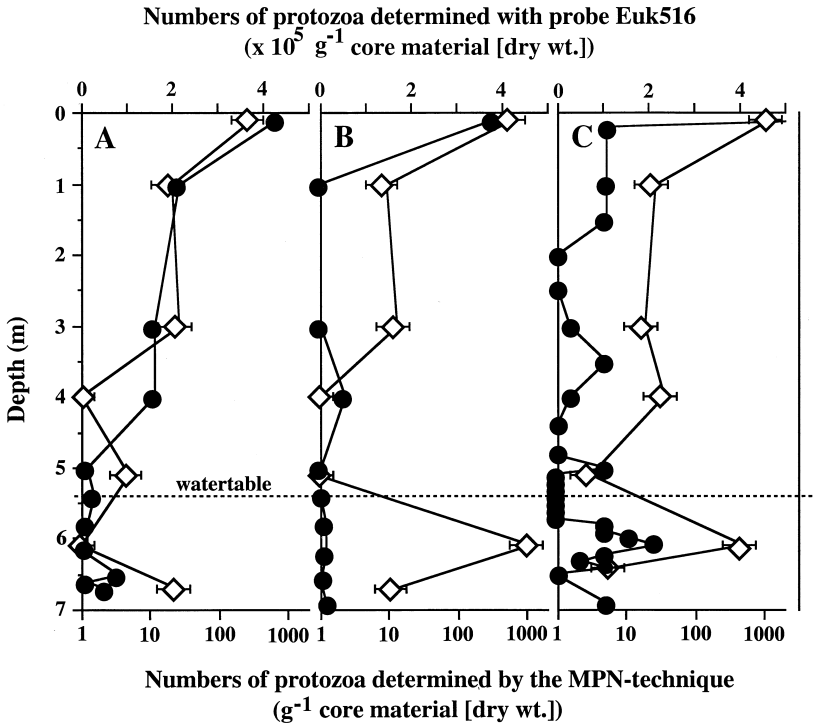

Fig. 5. Total number of protozoa as determined by in situ hybridization with the Eukarya-specific probe Euk516 (●) and by the MPN technique (◇) in cores A, B, and C at depths between 0.1 m and 7.0 m.

of probes SRB385 and SRB385Db designed to detect members of the δ-subdivision of the Proteobacteria. Their number decreased slightly with increasing depth from 15% at a depth of 5 m to 10% at 5.4 m, and remained quite stable at this level (8% at 5.8 m, 10% at 6.2 m, 7% at 6.5 m, and 10% at 6.9 m). The analysis of bacteria belonging to the family Desulfobacteriaceae using probe SRB385Db and unlabeled probe SRB385 as a competitor showed a more pronounced decrease of cells with increasing depth. Their numbers decreased from 11% at a depth of 5 m to 7% (5.4 m), 6% (5.8 m), 5% (6.2 m), 3% (6.5 m) and finally 4% at a depth of 6.9 m. Populations of other higher phylogenetic groups remained relatively unchanged along this depth profile. Bacteria detected with probe Gam42a designed to detect members of the γ-subdivision of Proteobacteria accounted for 2–4% of DAPI-stained bacteria (Fig. 4). Those detected with probe Bet42a designed to detect members of the β-subdivision of Proteobacteria accounted for about 1% of DAPI-stained bacteria occurring between 5 and 6 m depth and decreased in prevalence below this depth (Fig. 4). Comparable numbers of bacteria were detected with probe Bet42a and with probe Azo644 which targeted hydrocarbon-degrading *Azoarcus* sp. belonging to the β-subdivision of Proteobacteria. In situ hybridization with probes designed to detect other higher phylogenetic groups such as the Gram-positive bacteria with a high DNA G+C content, the *Cytophaga-Flavobacterium* cluster, the α-subdivision of Proteobacteria, and the planctomycetes did not result in significant detection yields ($<1\%$ of DAPI-stained bacteria).

3.3. Protozoan community structure

Numbers of protozoa were detected in each core A, B, and C by in situ hybridization with probe Euk516 and by the MPN technique (Fig. 5). Although the actual numbers of protozoa detected by both methods differed considerably (between 2 and 5 orders of magnitude higher using in situ hybridization), a similar trend was found between both sets of data. Numbers of protozoa were highest

Table 1
Prevalence of protozoa in cores A, B and C as determined by the MPN technique

Core	Depth (m)	Total protozoa MPN (g^{-1} [dry wt.])	Prevalence (%)				
			Ciliates	Flagellates	Amoebae		
			Total	Total	Total	Trophozoites	Cysts
A	0.1–0.3	300	2	56	42	23	19
	1.0–1.6	21	0	21	79	38	41
	3.0–3.5	26	0	60	40	16	24
	4.0–4.5	1	0	50	50	0	50
	5.0–5.2	5	0	84	16	0	16
	6.0–6.2	1	0	100	0	0	0
	6.6–6.8	25	0	100	0	0	0
B	0.1–0.3	640	4	27	69	6	63
	1.0–1.6	9	0	8	92	38	54
	3.0–3.5	13	0	78	22	22	0
	4.0–4.5	1	0	100	0	0	0
	5.0–5.2	1	0	0	100	0	100
	6.0–6.2	1100	0	99	1	0	1
	6.6–6.8	12	0	100	0	0	0
C	0.1–0.3	1200	1	55	44	30	14
	1.0–1.6	27	0	6	94	7	87
	3.0–3.5	19	0	88	12	5	7
	4.0–4.5	35	0	96	4	0	4
	5.0–5.2	3	0	77	23	0	23
	6.0–6.2	490	0	97	3	1	2
	6.4–6.5	6	0	89	11	11	0

in the surface layer in each core (up to 10^5 cells g^{-1} of core material [dry wt.] as detected by in situ hybridization) and decreased sharply below about 1 m depth. Elevated numbers of protozoa approaching those found in the surface layer were only detected in the saturated zone in contaminated core C (Fig. 5). The ratio between total numbers of bacteria determined after DAPI staining and protozoa detected with probe Euk516 was quite constant (core A: $0.7 \pm 0.4 \times 10^3$; core B: $1.7 \pm 0.9 \times 10^3$; core C: $1.2 \pm 0.7 \times 10^3$; average of cores A to C: $1.1 \pm 0.7 \times 10^3$).

The analysis of the protozoan community using the MPN technique is based on the assignment of culturable protozoa to the morphologically distinct groups of ciliates, flagellates, and amoebae (Table 1). Ciliates accounted for up to 4% of the protozoan population and were confined to the surface layer above 1 m depth in each core. Flagellates and naked amoebae were found to co-occur in variable proportions to a depth of about 1.6 m below which the former were the predominant group in each core. Amoebae became increasingly more encysted with depth, except in contaminated core C, where vegetative trophozoites were also detected in the saturated zone (Table 1).

4. Discussion

4.1. Chemical characterization of the aquifer

The chemical data confirmed that cores A, B, and C were sampled in the border zone of the plume since large differences for monoaromatic hydrocarbons and for nitrate were observed between cores A and C. The highest concentrations of monoaromatic hydrocarbons (up to 40 mmol kg^{-1} of core material [dry wt.]) were found in core C where they exceeded the individual solubilities of the constituent hydrocarbons. The maximum solubility of the most prominent monoaromatic hydrocarbons found in core C ranged between 0.4 mM (trimethylbenzenes) and 1.7 mM (xylenes) [39]. One kilogram of water-

saturated core material was found to contain only about 0.14 l of water which corresponded to a water content of approx. 25%. This means that only a very small fraction of the hydrocarbons detected in core C can be solved in the pore water (approx. 200 μmol kg^{-1} of core material [dry wt.] and that a free xylene phase still exists in core C which probably serves as a source for further contamination [36]. Core C contained high concentrations of *o*-xylene, trimethylbenzene and ethyltoluene, in contrast to earlier findings which reported mainly *p*- and *m*-xylene in the plume [36]. The high *o*-xylene concentrations could reflect the close proximity of the sampling sites to the former *o*-xylene production plant.

The concentration of nitrate in each core was found to be inversely related to concentrations of monoaromatic hydrocarbons, suggesting that nitrate reduction and degradation of aromatics are coupled. Furthermore, dissolved oxygen concentrations were previously shown to be high outside the plume but low inside the plume [37]. These observations together with the accumulation of nitrite in core C suggest that the resident microbial population uses monoaromatic hydrocarbons as their carbon and energy source under aerobic and nitrate reducing conditions. A decrease in sulfate concentrations was observed in the lower parts of cores A and C where the sediment also appeared greyish-black. This suggests that sulfate reductive assimilation may be taking place at a depth below about 6.5 m. This suggestion, however, is not supported by the sulfate profile in core B in which a decrease in sulfate concentration was not observed. Because earlier analyses performed at the site gave no indication for the occurrence of high concentrations of sulfate and neither of sulfate reduction [36,37], sulfide analysis was not considered necessary in our sampling program. Additional supporting data for sulfate reduction are therefore not available.

4.2. Bacterial community structure

The number of DAPI-stained bacteria increased from core A to core C and corresponded with increased levels of contaminants. The higher numbers of bacteria detected after DAPI-staining in the saturated zone of contaminated core C were found to coincide with a higher detection rate obtained with probe Eub338. Detection rates after hybridization with probe Eub338 are used as an indication for the presence of metabolically active cells which contain sufficient amounts of rRNA coupled with a sufficient cell permeability or permeabilization to permit their detection [1], though recent studies have shown that probe Eub338 does not detect all members of the domain Bacteria [46]. The detection rate showed no direct correlation with the growth rate or activity of bacteria and varies according to the species examined and their consumption of nutrients and oxidants (for review see [1]). Nevertheless, it is likely that the increase in detectability of bacteria in the saturated zone of contaminated core C (as compared with the low-contaminated cores A and B) is due to the availability of monoaromatic hydrocarbons as growth substrates. The correspondingly low level of oxidants detected in core C provides support for this assumption.

Proteobacteria of the δ-subdivision comprising many sulfate-reducing bacteria were predominant in core C and provided support for the assumption of sulfate-reductive conditions. Due to the presence of sulfate-reducing bacteria in core C, the contribution of sulfate-reductive degradation of hydrocarbons [3,32] might have been underestimated. However, further investigations would be necessary to quantify their contribution. A high proportion of DAPI-stained bacteria (2–4%) was represented by the γ-subdivision of Proteobacteria. Since the γ-subdivision of Proteobacteria is physiologically very heterogeneous, a higher phylogenetic resolution would be necessary to attribute certain physiological activities to its constituent bacterial groups. Bacteria of the β-subdivision of Proteobacteria only accounted for approx. 1% of DAPI-stained cells. The latter findings were in contrast to those obtained in a laboratory aquifer column which was set up in order to simulate the aerobic and denitrifying remediation processes during degradation of petroleum-derived hydrocarbons. In this column up to 90% of DAPI-stained cells belonged to the β-subdivision of Proteobacteria [22]. Though many of the organisms detected in connection with aerobic [13,14] or denitrifying [22] degradation of monoaromatic hydrocarbons belong to the β-subdivision of Proteobacteria, their impact on hydrocarbon degradation at the field site in Hünxe may be impeded by adverse environ-

mental factors such as low redox potentials. Lower numbers of bacteria belonging to the β-subdivision of Proteobacteria could also occur if these bacteria are less tolerant to high concentrations of aromatic hydrocarbons [14] as, for example, those of the γ-subdivision of Proteobacteria [24].

The proportion of *Azoarcus* sp. detected in the saturated zone of contaminated core C was similar to that found in a laboratory aquifer column (1–2%) designed to simulate the aerobic and denitrifying remediation processes occurring during degradation of petroleum-derived hydrocarbons [22]. The genus *Azoarcus* has already been implicated in the biodegradation of monoaromatic hydrocarbons in a number of studies [13,14,22]. The percentage of hydrocarbon-degrading *Azoarcus* sp. detected accounted for up to 1.4% of the total bacterial community which indicates that they are members of the indigenous microbiota. Indeed, these *Azoarcus* sp. accounted for more cells in the contaminated aquifer (2×10^6 g^{-1}) than the biogeochemistry model [36] previously estimated for the total number of bacteria (max. 1.3×10^6 g^{-1}) present. Similar to the attribution of significant denitrification activity to *Paracoccus* sp. based on their 3.5% abundance [28], these results suggest that *Azoarcus* sp. may play an important role during bioremediation of hydrocarbon-contaminated aquifers. However, confirmation of this assumption depends on the availability of more information on the catabolic activity of the hydrocarbon-degrading *Azoarcus* populations both in column studies and in the field.

4.3. Protozoan community structure

Protozoa are integral members of the microbial community in groundwater aquifers [27,29,40,42]. Numerically, they are second in importance only to bacteria [30]. Evidence from the present study confirmed the discrepancy between culture-dependent and -independent methods with MPN counts between two and five orders of magnitude less than those using direct counting with eukaryotic probe Euk516. Interestingly though, both the MPN technique and probe Euk516 detected elevated numbers of protozoa in the surface layer in each core, which declined sharply with depth but again increased in the saturated zone in contaminated core C. These findings corresponded with published data from other hydrocarbon-contaminated aquifers [27,40,42].

The covariance between results from the MPN technique and probe Euk516 suggests some relative confidence in each approach. The MPN technique will probably remain an important alternative to direct counting methods until the latter have been sufficiently developed to provide information on protozoan taxa [35]. In a recent study, oligonucleotide probes were used to enumerate a particular nanoflagellate species in a mixed population [34]. This demonstrates the possibility for developing molecular tools to investigate both the taxonomy and ecology of protozoa as well as of bacteria and their mutual influence in a variety of habitats by the same methods. Future studies in our laboratory will focus on the development of such tools.

Protozoan taxa comprising ciliates, flagellates and naked amoebae were identified during the present study. Ciliates formed a minor proportion of the protozoan community ($\leq 4\%$) and were confined exclusively to the surface layer (<1 m depth) in each core. Similar findings from other aquifers have been attributed to straining by sediment on large protozoa over 20 μm in diameter [18,40]. The major component of the community was comprised of flagellates which increased in prevalence with depth and thereby confirmed earlier findings from other aquifers [29,30,40]. The predominance of flagellates (particularly nanoflagellates of 2–3 μm in diameter) has been attributed to their optimal size for transport through the aquifer matrix [19]. Naked amoebae became increasingly more encysted with depth except in core C where vegetative trophozoites were present in the saturated zone. Presumably, this was in response to elevated numbers of bacteria associated with contaminants in the saturated zone.

The role of bacterivorous protozoa in contaminant biodegradation is considered to be an indirect result of their ability to selectively graze on and control the biomass of aquifer bacteria [27,30,42]. This in turn creates a nutritional loop in which protozoa rapidly remineralize nutrients which sustain further bacterial growth [9]. The ratio of protozoa to bacteria has variously been reported as $1{:}10^1$–10^5 [41] and $1{:}10^3$ [42] from other aquifers and corresponds with a ratio of $1{:}10^3$ found during the present study. Estimates for feeding rates from batch culture studies suggest

that flagellates require 10^2 bacteria whereas amoebae require 10^3 bacteria per cell division [38,49]. A similar growth requirement of 10^2 bacteria per division has been observed for the flagellate *Spumella* sp. isolated from core C (unpublished data).

The growth of bacteria in porous media supplied with nutrients has frequently been observed to cause reductions in hydraulic conductivity due to bioclogging [10,44,45]. Perhaps bacterivorous protozoa in contaminated aquifers are capable of limiting bacterial bioclogging. The role of bacterivorous flagellates in limiting bacterial bioclogging in model aquifer columns is currently being investigated in our laboratory. Further studies should therefore evaluate the merits of incorporating a protozoan dimension into bioremediation.

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