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Microbiological Characterization of a Fuel-Oil Contaminated Site Including Numerical Identification of Heterotrophic Water and Soil Bacteria

Peter Kämpfer, Martin Steiof, and Wolfgang Dott

Fachgebiet Hygiene der Technischen Universität Berlin, Amrumerstrasse 32, 1000 Berlin 65, Federal Republic of Germany

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Abstract. Seven soil samples and seven groundwater samples from a site contaminated with fuel-oil were investigated using several chemical and microbiological techniques. In soil samples, 500 to 7,500 mg/kg of total hydrocarbons were found. These samples contained no n-alkanes but iso- and branched chain alkanes. No polychlorinated biphenyls could be detected. Microbiological investigations included estimations of total cell counts, viable cell counts on different media, and numbers of methylotrophic, denitrifying, sulphate reducing, anaerobic (with the exception of methanogenic organisms), and hydrocarbon degrading bacteria. Viable and hydrocarbon degrading bacteria were found in all samples. A total of 1,366 pure cultures was characterized morphologically and physiologically and identified by numerical identification using a data base of more than 4,000 reference strains. Groundwater samples were dominated by gram-negative bacteria of the genera *Pseudomonas, Comamonas, Alcaligenes,* and *Acinetobacter,* which were also found in soil samples. In addition, more gram-positive bacteria belonging to the genera *Arthrobacter, Nocardia,* and *Bacillus* could be isolated from soil samples.

Introduction

Groundwater is the most important source of drinking water; however, this vital resource is more often found to be contaminated with potentially toxic organic wastes. As organic carbon in soil and groundwater is the most important energy source, heterotrophic bacteria thus dominate, and the degradation potential of microorganisms is often exploited for in situ clean-up of groundwater pollution. Combined chemical and microbiological investigations are a prerequisite for hazard assessment and the prediction of success of in situ cleaning procedures, but microbiological methodologies are often restricted to estimations of viable cell counts on nutrient rich media and qualitative estimations of biodegradation potential. Plate counts are generally accepted as inadequate for counting all naturally occurring bacteria [4, 15]. Alternative methods are based on assessment of active bacteria that take up radioactive organic com-

Offprint requests to: P. Kämpfer

pounds or that respire [24, 33] or on microscopic examination. Recent investigations include biomarkers [35] and gene probe techniques [34] for the detection of bacteria in water and soil. For the characterization and identification of heterotrophic bacteria, however, they have to be isolated. Several media have therefore been developed to optimize plate-counting techniques [32, 38].

An extended study of the culturable microorganisms and their identification within the system of recognized species is often neglected. In most cases, identification of bacteria from natural habitats is difficult, both with conventional methods and with commercially available identification kits. Conventional methods are often time consuming, and the application of commercially available test systems developed for clinical isolates fails to identify isolates from environmental sources because of nutrient rich media, high incubation temperatures, and short incubation times [12, 27]. Commercially available test kits are based on numerical identification procedures [20, 30, 43]. However, although many probability matrices have been published for several heterotrophic gram-positive bacteria [1, 18, 29, 31, 37, 44] and gram-negative bacteria [7, 21, 22], the application of these methods has been restricted to identification of single isolates. We used the principles of numerical identification in combination with miniaturization of biochemical tests and automated reading of test results to identify isolates from water and soil and to assess its suitability for ecological investigations.

Materials and Methods

Habitat and Sampling

The contaminated area is situated in the south of Berlin (West), thus lying on the periphery of the North German Lowland. The subsoil is characterized as a pleistocene aquifer with fine and middle sands.

Figure 1 shows the top view of the contaminated area with the three wells from which samples were taken, the borehole of the special drilling ("rubber sleeve core drilling"), the exploratory boring sites, and the distribution of the organic pollution. The pollution was caused by a leaking pipeline 45 to 50 years ago and is supposed to be due to fuel oil. The amount of leaked fuel oil is estimated at approximately 15,000 to 17,000 liters, and the larger portion of the oil floating on the groundwater was removed at the end of the 1970s. Groundwater samples were drawn from wells W1 (samples G1 and G2), W2 (samples G3 and G4), and W3 (samples G5 and G6) (Fig. 1). Well W4 (sample G7), which was not affected by the pollution, was located approximately 50 m north of well W2. Within three weeks, samples were collected under sterile conditions on two different days.

Soil sampling was done by rubber sleeve core drilling (see PB in Fig. 1). The pipe boring was carried out to a depth of 13 m. The tube core of soil was divided into segments of defined depth. Samples were designated as follows: S1 (4.25–4.55 m), S2 (5.20–5.40 m), S3 (6.30–6.50 m), S4 (7.25–7.50 m), S5 (8.25–8.50 m), S6 (9.25–9.50 m), and S7 (11.30–11.55 m).

Both water and soil samples were carefully transferred to sterile glass containers avoiding any contamination, quickly transported to the laboratory, and either immediately analyzed or stored at 4°C.

For further microbiological investigations, the microorganisms were first extracted from the soil. Thirty grams of soil were mixed with 270 ml 0.2% tetrasodium pyrophosphate solution and shaken at 150 rpm for 30 min. The pH of the supernatant was further adjusted to 7.0–7.1 with 130 μl of 1 M NaH_2PO_4.

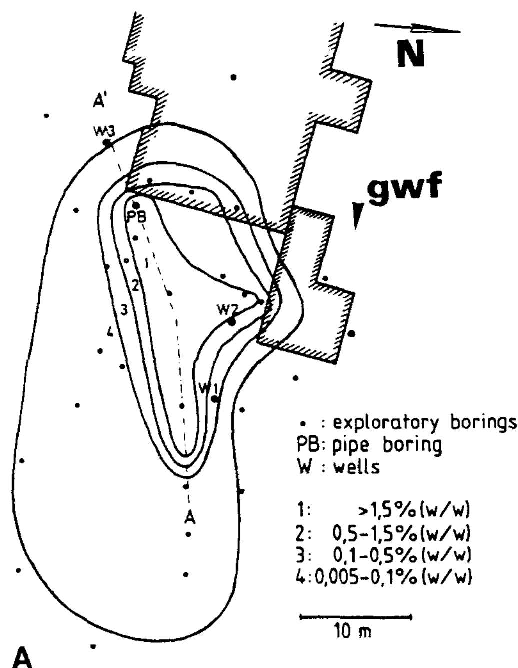
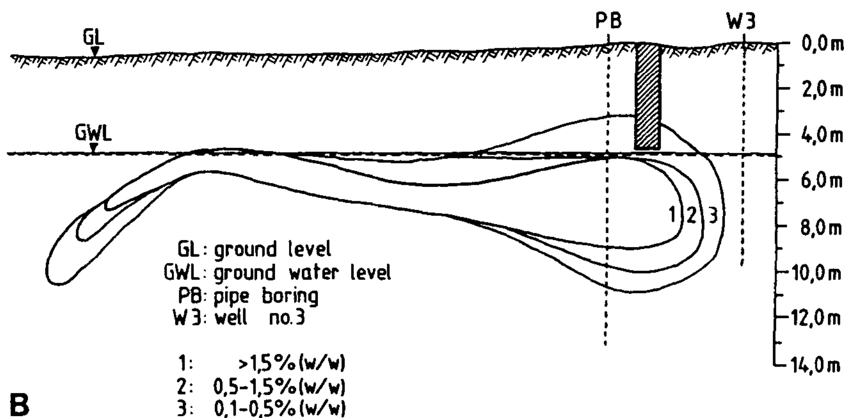


Fig. 1. Top view (A) and side view (B) of the contaminated area illustrating the positions of the three wells from which water samples were taken (W1, W2, W3), the special drilling (PB), exploratory boring sites, and the distribution of the organic pollution, given as percentages. gwf = groundwater flow; N = north. The control well (W4) is located 50 m north of W2.



Chemical Measurements

In order to extract organic compounds from the water samples, one liter of groundwater was successively shaken three times with 20 ml pentane, each for 5 min. The three organic layers were combined and, following evaporation, were diluted in 1 ml hexane. For soil samples, 100 g of soil were subjected to soxhlet extraction by adding 500 ml pentane for a period of 6.5 hours. Afterwards, the pentane was evaporated and the sample was dissolved in exactly 10 ml pentane. Gas chromatography was done using a Shimadzu GC 9A equipped with a flame ionization detector. The 0.32 mm ID × 50 m fused silica column was coated with SE 54 (Macherey-Nagel, Düren, FRG). A sample volume of 1 μl (pentane or hexane extract) was injected. To monitor the single compounds of the organic contamination, a moderate temperature rise (5°C/min, starting from 40°C) was chosen. For the quantification of the total hydrocarbon concentration, a high temperature program

rate (20°C/min, starting from 68°C) was chosen. In each case, the final temperature of 260°C was held until all compounds were eluted. In addition, n-alkanes and aromatics were injected as reference substances to detect their presence in the samples. The total peak areas were calculated by an integrator (Shimadzu C-R3A) according to a GC distillation analysis method.

In order to check any possible presence of polychlorinated biphenyls (PCB) or pesticides, an additional gas chromatographic examination was made using an electron capture detector and an SE 30-CB (Durabond) coated column. Furthermore, a soxhlet extract of the samples with pentane was evaporated, in order to obtain an extract of all organics (including halogenated organic compounds) present in the soil. These extracts were combusted in the presence of ethylene glycol and sodium peroxide, and the inorganic chloride, if present, was determined using a potentiometric method.

Cell Counts, Colony Counts, Presence-Absence Tests of Physiologically Specialized Groups, and Biomass Determination

Total cell counts were estimated using appropriate dilutions of the samples stained with acridine orange and counted microscopically on 0.2 μm polycarbonate membranes according to Hobbie et al. [19]. Colony counts were determined as colony-forming units (CFU) on the following media: R2A-agar according to Reasoner and Geldreich [38] and DEV agar according to German Standards [8] containing (per liter) meat extract (10.0 g), peptone (10.0 g), NaCl (0.5 g), and agar (15.0 g). The inoculum (0.1 ml from appropriate soil or water dilutions) was spread on the agar surface. Plates were incubated at 20°C for 20 days.

As a measure of biomass, the protein concentration was determined by the Lowry method modified by Herbert et al. [17]. Two parallel dilution series of water samples and soil extracts were studied for the presence of physiologically specialized bacteria. Methylotrophic bacteria were evaluated on mineral salt-agar under an atmosphere of 0.5 ml methanol per liter. The medium contained $Na_2HPO_4 \cdot 2H_2O$, 6.99 g; KH_2PO_4, 0.8 g; $(NH_4)_2SO_4$, 1.8 g; $MgSO_4 \cdot 7H_2O$, 0.123 g; $CaSO_4 \cdot 2H_2O$, 0.017 g; trace element solution SL 8 [36], 1 ml; and distilled water, 1000 ml.

The detection of various physiologically specialized groups was carried out as follows by duplicate serial dilution of samples and recording of positive tubes after an incubation period of 20 days at 20°C: under anaerobic conditions: (a) anaerobic bacteria (not methanogens) in RCM 5410 medium (Merck); (b) denitrifying bacteria in mineral salt medium (as previously described) with 2.0 g/liter sodium acetate as carbon source and 5.0 g/liter KNO_3; (c) sulphate reducing bacteria in medium—containing (g/liter) sodium lactate (6 g), NH_4Cl (1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (1.5 g), Na_2SO_4 (1.5 g), $(NH_4)_2FeSO_4 \cdot 6H_2O$ (0.1 g), yeast extract (0.5 g), SL 8 (10 ml), pH 7.5, with addition of an iron nail; (d) fuel oil degrading denitrifying bacteria in mineral salt medium with 1% (v/v) fuel oil as sole carbon source and 5 g/liter KNO_3; (e) anaerobic fuel oil degrading bacteria in mineral salt medium with 1% (v/v) fuel oil as sole carbon source.

Isolation and Morphological Characterization of Colony-Forming Organisms

From each sample 48 colonies on DEV agar and R2A agar were randomly selected, purified, and morphologically characterized under a phase contrast microscope. Gram-stain was performed using a modified Hucker method [10]. Colony shape and color were noted in addition to cell shape and cell morphological features. Isolates showing morphological peculiarities were further characterized using the methods of Kölbel-Boelke et al. [27] originally described by Dott and Thofern [11]. Isolates from soil sample S3 obtained on R2A agar were tested for their ability to grow on fuel oil (1% v/v) as sole carbon source.

Physiological Characterization and Numerical Identification of Isolates

All isolates were examined with 87 physiological tests based on classical biochemical, carbon source utilization, sugar fermentation, and qualitative enzyme tests using chromogenic substrates [12]. Tests were performed in standard microtitration plates (Greiner, Nürtingen, FRG) and were read visually and photometrically. Classical biochemical tests were done as described elsewhere [12, 26]. These tests were used mainly to identify bacteria of the family *Enterobacteriaceae.* The following tests were performed: tryptophan deaminase (TDA), indol (IND), H_2S-production (H2S), esculin (ESC), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), urease (URE), alkalinization of citrate (CTT), and malonate (MLL), Voges-Proskauer reaction (VP), acid formation from: glucose (GLQ), rhamnose (RHQ), sucrose (SUQ), adonitol (ADQ), inositol (INQ), xylose (XYQ), and sorbitol (SOQ) according to [23].

Carbon source utilization was tested in a mineral medium containing the following constituents (g/liter): K_2HPO_4 (1.74), KH_2PO_4 (1.36), NH_4SO_4 (5.0), $MgSO_4 \cdot 7\ H_2O$ (0.5), $CaCl_2 \cdot 2\ H_2O$ (0.1), NaCl (9.0), yeast extract (Oxoid) (0.02), peptone (Merck) (0.02), vitamin solution (5 ml) and trace element solution (1 ml). The vitamin solution contained (mg/liter): Ca-pantothenate (0.1), nicotinic acid (0.1), biotin (0.005), cyanocobalamin (0.005), folic acid (0.1), pyridoxine (0.1), p-aminobenzoic acid (0.1), thiamine pyrophosphate (0.1), inositol (0.1), thiamine (0.1), and riboflavin (0.1). The trace element solution contained (mg/liter) H_3BO_4 (0.5), $CuSO_4 \cdot 5\ H_2O$ (0.04), KI (0.1), $FeCl_3 \cdot 6\ H_2O$ (0.2), $MnSO_4 \cdot H_2O$ (0.2), $Na_2MoO_4 \cdot 2\ H_2O$ (0.2), and $ZnSO_4 \cdot 7\ H_2O$ (0.4). After adding the different carbon sources at final concentrations of 0.2% (wt/vol) (for aromatic compounds, concentrations of 0.05% (wt/vol) were used) the media were sterilized by filtration. The utilization of the following compounds was tested: N-acetyl-D-galactosamine (AGA), N-acetyl-D-glucosamine (AGL) L-arabinose (LAR), D-cellobiose (CEL), D-fructose (FRU), D-galactose (GAL), α-D-galacturonate (GAK), gluconate (GLK), D-glucose (GLU), glycogen (GCY), D-maltose (MAL), D-mannose (MAN), α-D-melibiose (MEL), L-rhamnose (RHA), D-ribose (RIB), D-sucrose (SAC), salicin (SAL), D-trehalose (TRE), D-xylose (DXY), adonitol (ADO), i-inositol (INO), D-mannitol (MNT), D-sorbitol (DST), acetate (ACE), propionate (PRO), trans-aconitate (ACO), adipate (ADI), citrate (CIT), fumarate (FUM), DL-3-hydroxybutyrate (3HB), DL-lactate (LCT), L-malate (MAT), pyruvate (PYR), suberate (SUB), L-alanine (LAL), L-aspartate (ASP), L-histidine (HIS), L-hydroxyproline (HPL), L-ornithine (ORN), L-proline (PRO), L-serine (SER), putrescine (PUT), 3-hydroxybenzoate (3HY), 4-hydroxybenzoate (4HY), and phenylacetate (PAC).

Qualitative enzyme tests were done in filter sterilized medium (pH 7.2) containing 0.05 M Tris-HCl buffer and 0.05% (wt/vol) each of yeast extract (Oxoid) and bio-lactysat (bio-Merieux). Filter sterilized solutions of chromogenic substrates (p-nitrophenyl-linked substrates) were added at final concentrations of 2 mM. For para-nitroanilides, concentrations of 1 mM were used. Hydrolysis of the following 42 chromogenic substrates was tested: (pnp = p-nitrophenyl, pna = p-nitroanilide) pnp-N-acetyl-β-D-galactosaminide (CAK), pnp-N-acetyl-β-D-glucosaminide (CBG), pnp-α-L-arabinopyranoside (CAP), pnp-β-D-cellobioside (CAC), pnp-β-D-galactopyranoside (CGB), pnp-β-D-glucuronide (CGL), pnp-α-D-glucopyranoside (CAU), pnp-β-D-glucopyranoside (CBU), pnp-β-D-lactoside (CLA), pnp-α-D-mannopyranoside (CMN), pnp-α-D-maltoside (CML), pnp-β-D-xyloside (CBX), bis-pnp-phosphate (CBP), pnp-phenyl-phosphonate (CPP), pnp-phosphorylcholine (CPC), 2-deoxythymidine-5′-pnp-phosphate (CDH), L-alanine-pna (CAL), γ-L-glutamate-pna (CGM), L-glutamate-γ-3-carboxy-pna (CGB), glycine-pna (CCY), L-leucine-pna (CLE), L-lysine-pna (CLY), L-proline-pna (CPR), and L-valine-pNA (CVA). All test media were added in 100 μl amounts to the wells of the microplates. Prior to inoculation of the tests, all isolates were cultivated on DEV agar on R2A agar for 5 days at 20°C. After growth, the microplate wells were inoculated with 50 μl portions of the bacterial suspension in 0.9% NaCl (wt/vol) at a MacFarland Standard Tube No. 5. Test plates were covered with plastic sealers (Flow Laboratories, Meckenheim, FRG) and incubated at 20°C for 7 days, unless stated otherwise.

Reading of test results was done photometrically using a Multiscan MCC340 photometer (Flow Laboratories, Meckenheim, FRG). A carbon substrate utilization test was considered positive if E_{414}(test) $-$ E_{414} (assimilation control) > 0.05, a qualitative enzyme test was considered positive if E_{414}(test) $-$ E_{414} (chromogenic substrate control) > 0.3, while a sugar fermentation test was

considered positive if E_{620} (fermentation control) − E_{620} (fermentation test) > 0.25. All test results were checked visually. All of the characters were scored plus (1) or minus (0) and then compared with a data base by calculating the different coefficients of the MATIDEN program [40]. The identification coefficients determined were the likelihood (L_{wJ}) of congruence with taxon within the data base, Willcox probability (*P* according to Willcox et al. [42]), the taxonomic distance (d) and standard error of taxonomic distance. Isolates showing low scores of L_{wJ} (<1:1,000,000) and *P* (<0.7) in addition to high scores of d (>3.0) were grouped into the category of not-identified bacteria. In vitro physiological activities of each isolate, given as the number of positive tests, was determined for all isolates of one sample dependent on the isolation medium [13] (Formula (a) of reference 28). In vitro physiological activities of all isolates of the 14 samples were calculated for each of the 87 physiological tests according to Dott and Trampisch [13], (Formula (b) of reference 28).

Results

Chemical Investigations

In all groundwater samples investigated no alkane or isoalkane profiles typical for a fuel-oil contamination could be detected by gas chromatography. The peaks obtained in the analysis could not be identified. No groundwater sample contained more than 250 μg/liter organic compounds, given as total organic carbon. The investigated soil samples contained (mg/kg dry weight): S1 (400), S2 (1,300), S3 (3,700), S4 (2,500), S5 (800), S6 (500), S7 (400). Gas chromatographic profiles showed no typical alkanes and aromatic compounds, thus indicating that these compounds had been already degraded. No polychlorinated biphenyls or other chlorinated organic compounds could be detected.

Cell Counts, Colony Counts, Presence-Absence Tests of Physiologically Specialized Groups and Biomass Determination

Results of total cell counts of the soil samples are given only as estimates, because small fluorescent particles, which could not be unambiguously identified as bacterial cells, were present in varying numbers. Numbers of cells per gram of soil ranged from 10^9 (S6) to 10^{10} (S2). Numbers of countable bacteria (including fluorescent particles) decreased with the depth of the soil sample. Cell count in groundwater samples G1 to G6 ranged from 4.8×10^6 (G3) to 7.4×10^6 (G5) per ml. In sample G7, originating from the well that was not affected by the pollution, 4.0×10^7 bacteria could be counted, due possibly to contamination with bacteria from the surface during the installation of this well. Colony counts of groundwater samples were 100- to 1,000-fold lower, whereas counts on R2A agar gave 3 to 10 times higher counts than the nutrient rich medium (DEV agar) for water samples. These results were also obtained with the soil samples, but with fewer differences in colony counts between the two media. All samples contained anaerobes (not methanogenic bacteria), denitrifiers, sulphate reducing bacteria, and methylotrophic organisms (Tables 1 and 2). In addition, all samples investigated contained aerobic fuel oil degrading

Table 1. Cell counts, colony counts, and presence-absence test results for physiologically specialized groups from groundwater samples

Sample	Total cell count per ml	Colony counts DEV-agar per ml	Colony counts R2A-agar per ml	Most probable numbers of denitrifying bacteria/ml	Most probable numbers of methylotrophic bacteria/ml	Minimum volume (ml) in which bacteria detected: Anaerobic bacteria	Minimum volume (ml) in which bacteria detected: Sulphate reducing bacteria	Minimum volume (ml) in which bacteria detected: Fuel-oil degraders
G1	5.9×10^6	7.9×10^3	2.1×10^4	4.5×10^2	5.5×10^3	0.1	0.1	0.01
G2	5.1×10^6	3.6×10^3	7.8×10^3	2.0×10^2	5.5×10^2	0.1	0.1	0.01
G3	4.8×10^6	9.5×10^3	1.3×10^4	4.5×10^2	3.3×10^3	0.1	0.1	0.1
G4	7.3×10^6	1.9×10^3	5.4×10^3	2.5×10^2	7.9×10^2	0.1	0.1	0.01
G5	7.4×10^6	5.1×10^3	1.9×10^4	1.1×10^5	7.9×10^3	0.01	0.1	0.01
G6	4.5×10^6	6.9×10^3	7.0×10^3	2.5×10^3	2.4×10^3	0.01	0.1	0.01
G7	4.0×10^7	1.1×10^6	2.8×10^6	2.5×10^1	3.3×10^2	1.0	0.1	0.01

bacteria, which could be detected in volumes of 0.1 ml (in the majority of water samples) up to 0.0001 ml (extracts from soil samples S2 and G4). Even in the unpolluted water from the well W4, fuel oil degraders were present in 1 ml. No fermentative or dentrifying fuel oil degrading organisms could be enriched from the investigated samples. Protein content of the soil samples ranged from 114.4 μg/g dry weight (S3) to 661.6 μg/g dry weight (S4). These data correlated with cell counts of the soil sample. The water samples contained 1.7 mg/ml (G3) up to 9.0 mg/liter protein (G4).

Table 2. Cell counts, colony counts, and presence-absence test results for physiologically specialized groups from soil samples

Sample	Total cell count per g	Colony counts DEV-agar per g	Colony counts R2A-agar per g	Most probable numbers of denitrifying bacteria/g	Most probable numbers of methylotrophic bacteria/g	Minimum sample volume (g dry weight) in which bacteria detected: Anaerobic bacteria	Minimum sample volume (g dry weight) in which bacteria detected: Sulphate reducing bacteria	Minimum sample volume (g dry weight) in which bacteria detected: Fuel-oil degraders
S1	counts	2.9×10^6	3.4×10^6	2.2×10^3	6.9×10^4	0.01	0.01	0.1
S2	ranged	8.1×10^6	4.8×10^6	2.7×10^3	4.5×10^4	0.01	0.01	0.0001
S3	from 10^9	9.6×10^5	1.4×10^6	n.d.[a]	n.d.	0.01	n.d.	n.d.
S4	to 10^{10}	8.4×10^6	7.4×10^6	4.9×10^2	1.6×10^4	1.0	1.0	0.0001
S5	bacteria	8.7×10^6	2.5×10^7	9.9×10^1	2.3×10^3	0.1	1.0	1.0
S6		7.2×10^7	1.6×10^7	1.0×10^2	1.1×10^4	0.01	1.0	0.01
S7		6.1×10^6	8.3×10^7	none	2.9×10^3	0.1	1.0	0.01

[a] n.d. = not determined

Table 3. Identification results of 1366 isolates from different soil and groundwater samples isolated on different media

	Soil samples (no. of strains)													
	S1 (96)		S2 (96)		S3 (96)		S4 (127)		S5 (96)		S6 (96)		S7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Gram-negative bacteria:														
Acinetobacter baumannii	—	—	—	—	—	—	—	—	1	—	—	1	—	—
Acinetobacter calcoaceticus	—	—	1	1	—	—	—	—	—	—	—	—	—	—
Acinetobacter johnsonii	—	—	1	—	1	—	—	—	—	—	—	1	—	1
Acinetobacter lwoffii	1	—	—	1	—	2[a]	—	—	—	1	—	—	5	10
Acinetobacter species	—	1	—	—	—	—	1	—	—	—	—	—	—	1
Aeromonas hydrophila	—	1	—	—	4	1	—	—	1	—	—	—	—	2
Agrobacterium tumefaciens	—	—	—	—	—	—	—	1	—	1	—	—	2	—
Alcaligenes faecalis	—	—	1	—	2	1[a]	1	1	1	1	—	—	—	—
Alcaligenes xylosoxidans subsp. *denitrificans*	—	—	1	1	5	—	3	4	3	—	1	3	—	3
Chromobacterium violaceum	—	—	—	—	—	—	—	—	—	—	—	—	—	2
Chryseomonas luteola	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Comamonas acidovorans	—	—	—	—	—	2	—	—	—	1	—	—	—	—
Comamonas testosteroni	—	1	2	3	8	6[a]	17	5	7	5	—	1	15	1
Enterobacter agglomerans	—	—	1	—	—	—	—	—	—	—	—	—	—	—
Flavimonas oryzihabitans	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Flavobacterium breve	—	—	—	—	—	—	—	—	—	—	—	—	1	—
Flavobacterium group IIb	—	—	—	—	—	—	1	3	—	1	—	—	—	—
Flavobacterium meningosepticum	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Flavobacterium multivorum	—	—	—	1	—	2	—	—	—	1	—	—	—	—
Hydrogenophaga flava	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Hydrogenophaga pseudoflava	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Methylobacterium mesophilicum	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Plesiomonas shigelloides	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pseudomonas aeruginosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—
[*Pseudomonas*] *cepacia*	15	2	4	8	4	12[a]	5	12	10	17	18	20	2	3

Table 3. Continued

	Groundwater samples (no. of strains)													
	G1 (97)		G2 (99)		G3 (91)		G4 (93)		G5 (91)		G6 (96)		G7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Gram-negative bacteria:														
Acinetobacter baumannii	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Acinetobacter calcoaceticus	—	—	—	1	2	—	2	—	—	—	—	—	—	—
Acinetobacter johnsonii	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Acinetobacter lwoffii	4	—	—	—	4	1	—	1	—	—	1	—	—	—
Acinetobacter species	—	—	—	—	3	—	1	1	—	—	—	—	—	—
Aeromonas hydrophila	1	—	—	1	5	—	—	1	1	—	—	—	—	—
Agrobacterium tumefaciens	2	4	—	—	—	1	3	1	1	3	1	—	1	1
Alcaligenes faecalis	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Alcaligenes xylosoxidans subsp. *denitrificans*	—	1	—	—	—	1	1	—	1	—	—	1	—	1
Chromobacterium violaceum	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Chryseomonas luteola	—	—	1	—	—	1	—	—	2	3	1	2	—	—
Comamonas acidovorans	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Comamonas testosteroni	—	—	—	—	—	—	—	—	1	—	—	—	—	—
Enterobacter agglomerans	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Flavimonas oryzihabitans	—	—	1	—	—	2	—	—	—	—	—	—	—	—
Flavobacterium breve	—	1	1	2	—	7	3	4	1	6	3	1	—	—
Flavobacterium group IIb	—	1	—	1	—	—	—	—	2	—	2	—	3	—
Flavobacterium meningosepticum	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Flavobacterium multivorum	—	—	—	—	—	—	—	1	—	—	—	—	—	—
Hydrogenophaga flava	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Hydrogenophaga pseudoflava	1	1	2	—	—	—	—	—	—	—	—	1	—	—
Methylobacterium mesophilicum	—	—	—	—	—	—	—	1	—	—	—	—	—	—
Plesiomonas shigelloides	—	—	—	—	—	—	—	1	—	—	—	—	1	—
Pseudomonas aeruginosa	—	—	—	—	—	—	—	—	—	—	1	—	—	—
[*Pseudomonas*] *cepacia*	7	5	3	8	1	1	3	5	1	2	—	2	—	—

Table 3. Continued

	Soil samples (no. of strains)													
	S1 (96)		S2 (96)		S3 (96)		S4 (127)		S5 (96)		S6 (96)		S7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Pseudomonas fluorescens	12	2	6	4	6	4[a]	23	25	7	12	8	8	4	7
[Pseudomonas] paucimobilis	–	–	–	–	–	–	–	–	–	–	1	–	–	1
[Pseudomonas] pickettii	–	–	–	–	–	1[a]	–	–	1	–	–	–	2	3
Pseudomonas putida	2	–	–	1	4	–	3	1	4	1	–	–	–	–
Pseudomonas stutzeri	–	–	–	1	3	1[a]	–	1	1	1	–	1	1	–
Pseudomonas alcaligenes	–	–	–	–	–	–	–	–	–	–	–	–	–	–
[Pseudomonas] diminuta	–	–	1	–	1	–	–	–	1	–	–	–	5	3
Pseudomonas pseudoalcaligenes	–	–	1	–	3	10[a]	4	2	1	4	–	1	–	3
[Pseudomonas] vesicularis	–	–	–	–	–	–	1	–	1	1	–	–	–	–
Shewanella putrefaciens	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xanthomonas maltophilia	–	–	1	1	3	3	1	2	–	–	–	–	1	–
gram-negative, not identified	–	3	1	–	4	–	5	1	2	–	–	4	1	7
Gram-positive bacteria:														
Arthrobacter globiformis	8	10	–	2	–	–	–	–	2	–	1	2	–	–
Arthrobacter oxydans	3	14	1	–	–	–	–	–	1	–	6	4	–	–
Arthrobacter pascens	3	–	–	–	–	–	–	–	–	–	2	–	–	–
Arthrobacter picolinophilus	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arthrobacter ramosus	–	–	–	1	–	–	–	–	1	–	–	–	–	–
Aureobacterium flavescens	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aureobacterium testaceum	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus brevis	–	–	–	1	–	–	–	–	–	–	–	–	–	–
Bacillus coagulans	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus macerans	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bacillus megaterium	–	–	1	1	–	–	–	–	–	–	1	–	–	–

Table 3. Continued

	Groundwater samples (no. of strains)													
	G1 (97)		G2 (99)		G3 (91)		G4 (93)		G5 (91)		G6 (96)		G7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Pseudomonas fluorescens	10	8	7	5	9	15	14	9	13	4	4	7	—	—
[*Pseudomonas*] *paucimobilis*	1	6	—	5	—	—	—	4	2	4	3	1	—	2
[*Pseudomonas*] *pickettii*	—	1	—	—	—	1	—	—	2	1	1	1	4	—
Pseudomonas putida	3	1	—	2	—	—	—	—	1	—	—	1	—	—
Pseudomonas stutzeri	1	—	—	—	—	—	1	—	—	—	1	—	—	—
Pseudomonas alcaligenes	—	—	4	—	—	—	—	—	—	—	—	—	14	27
[*Pseudomonas*] *diminuta*	—	—	—	—	—	—	—	—	—	1	—	—	—	—
Pseudomonas pseudoalcaligenes	—	—	—	—	—	—	—	1	—	—	—	—	—	—
[*Pseudomonas*] *vesicularis*	—	1	3	3	—	1	1	—	—	—	3	—	—	—
Shewanella putrefaciens	—	—	—	1	3	—	—	1	—	—	1	2	1	—
Xanthomonas maltophilia	4	1	—	—	—	—	—	—	—	—	—	—	1	—
gram-negative, not identified	3	4	—	9	5	10	7	8	4	12	7	11	7	4
Gram-positive bacteria:														
Arthrobacter globiformis	2	—	—	1	—	3	—	—	—	—	—	2	1	—
Arthrobacter oxydans	1	1	—	—	—	—	—	1	—	—	1	1	—	3
Arthrobacter pascens	—	2	—	1	—	1	1	—	1	—	—	—	—	1
Arthrobacter picolinophilus	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Arthrobacter ramosus	1	—	—	—	—	—	1	—	—	—	—	—	—	—
Aureobacterium flavescens	—	—	—	—	—	—	—	—	2	—	1	—	—	—
Aureobacterium testaceum	—	—	—	—	—	—	—	—	—	—	—	—	—	2
Bacillus brevis	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bacillus coagulans	—	—	—	1	—	—	—	1	—	—	—	—	—	—
Bacillus macerans	1	—	—	—	—	—	—	—	4	—	5	—	1	—
Bacillus megaterium	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 3. Continued

	Soil samples (no. of strains)													
	S1 (96)		S2 (96)		S3 (96)		S4 (127)		S5 (96)		S6 (96)		S7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Bacillus species	—	1	3	2	—	—	—	—	—	—	—	—	—	1
Bacillus sphaericus	—	—	10	3	—	—	—	—	—	—	—	—	—	—
Cellulomonas cellulans	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Cellulomonas fimi	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Clavibacter michiganense	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Micrococcus species	—	—	—	2	—	—	—	—	—	—	—	—	5	3
Nocardia species	—	5	8	9	—	2[a]	—	1	3	—	4	—	1	—
Pimelobacter simplex	—	3	1	—	—	—	—	—	—	1	6	1	—	—
Rhodococcus species	—	—	1	—	—	—	—	—	—	—	—	—	—	—
gram-positive, not identified	4	5	2	4	1	—	1	2	—	—	—	—	—	—

Pseudomonas species in [ ] do not belong to the true pseudomonads according to De Vos and De Ley [9]

[a] A total of 39 from 48 strains (81.25%) isolated on R2A agar from sample S3 were able to grow in mineral medium with 0.1% (v/v) fuel oil as sole carbon source

Table 3. Continued

	Groundwater samples (no. of strains)													
	G1 (97)		G2 (99)		G3 (91)		G4 (93)		G5 (91)		G6 (96)		G7 (96)	
	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A	DEV	R2A
Bacillus species	4	1	3	1	2	—	—	4	3	1	4	1	5	2
Bacillus sphaericus	—	—	—	—	—	1	2	—	1	—	—	—	1	—
Cellulomonas cellulans	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cellulomonas fimi	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Clavibacter michiganense	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Micrococcus species	—	—	—	—	—	—	1	—	—	—	—	—	2	—
Nocardia species	2	4	2	2	2	1	—	2	2	—	4	8	2	1
Pimelobacter simplex	—	—	—	—	—	—	—	—	—	—	—	2	—	—
Rhodococcus species	—	—	—	—	1	—	—	—	—	—	—	—	1	—
gram-positive, not identified	1	5	24	2	3	2	2	—	1	8	5	1	6	1

Identification Results and In Vitro Activities

The results of the identification of isolates obtained from DEV agar and R2A agar are summarized in Table 3. Generally, more gram-positive bacteria could be isolated from soil samples than from groundwater samples, most of them belonging to the genera *Arthrobacter, Nocardia* and *Bacillus.* In general, the number of gram-positive bacteria decreased with the depth of soil. In addition, gram-positive bacteria were almost absent from soil samples S3, S4, and S5, which contained the highest amounts of hydrocarbons. From these samples, the majority of isolated bacteria belonged to the genera *Pseudomonas* and *Comamonas* and could be assigned to the species *P. cepacia, P. fluorescens,* and *C. testosteroni.* Only slight differences were found in the numbers of identified organisms isolated from DEV agar and R2A agar. A total of 39 bacteria (81.25%) isolated from soil sample S3 were able to grow on fuel oil as sole carbon source (see Table 3). The majority of isolates from groundwater samples G1 to G6 also belonged to the gram-negative genera *Pseudomonas* and *Comamonas.* In contrast to soil samples, more isolates of *Flavobacterium* species could be identified. All groundwater samples contained endospore-forming bacteria assigned to the genus *Bacillus,* which could not be identified to species level. From water sample G7 (unpolluted water), no isolates of *P. fluorescens* or *P. cepacia* could be obtained. This sample contained large numbers of physiologically inactive pseudomonads belonging to *P. alcaligenes.* The majority of samples contained bacteria which could not be identified, because their test profile did not correspond with any taxon in the data base or because of their physiological inactivity.

Activities of all strains and all samples are shown in Table 4 and total activities are given in Figs. 2 and 3. Distributions of total activities of isolates from soil reveal characteristic profiles (Fig. 2). Soil sample S1 (4.25–4.55 m) contained high numbers of physiologically versatile organisms (45% to 65% positive test results). This number decreased with increasing depth of soil, and in soil sample S7 (11.30–11.55 m depth) most bacteria showed positive test results between 20 and 30%. Samples S3 and S4, containing the highest amounts of hydrocarbons, contained bacteria with low and high in vitro activities in nearly equal amounts. The in vitro activities of groundwater samples differed significantly from those obtained with the soil samples. Samples G1 to G6 contained diverse bacteria with respect to their total in vitro activities, which ranged from 0 to 65%. In contrast, sample G7 from the unpolluted well contained large amounts of inactive bacteria, most of them showing only 3 to 15% positive test results (Fig. 3). Significant differences between soil and groundwater samples and between polluted and unpolluted samples were obtained, demonstrated by the distribution of physiological in vitro activities.

In summary, all groundwater and soil samples contained methylotrophic, denitrifying, sulphate reducing, anaerobic, and hydrocarbon degrading bacteria. Significant differences in the culturable bacterial communities from soil and groundwater samples could be obtained. Soil samples contained more gram-positive bacteria, whose numbers decreased with increasing depth. In vitro activities of soil samples revealed nearly equal amounts of bacteria with low and high in vitro activities. In groundwater samples from the polluted area,

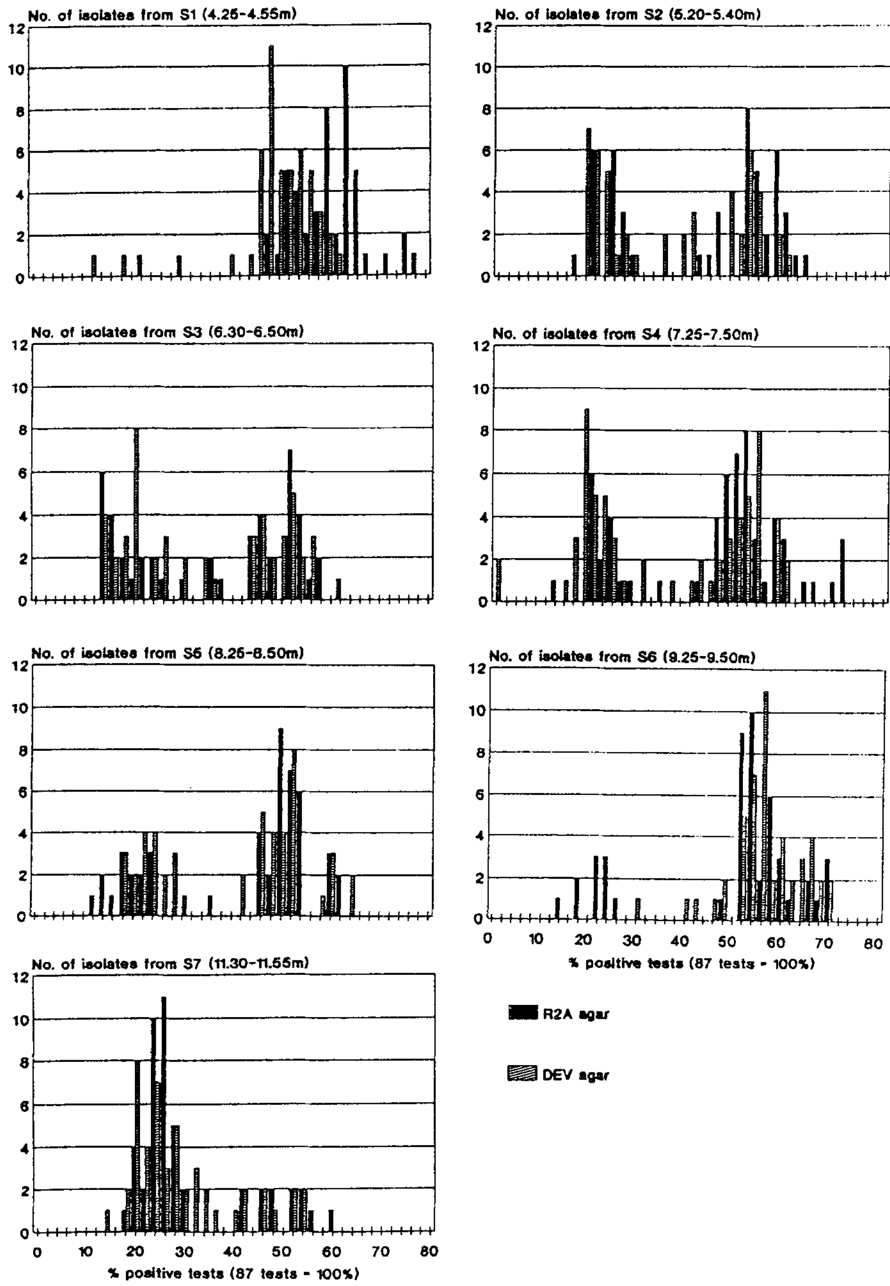


Fig. 2. Distribution of percent positive test results of the isolated bacteria from the seven soil samples (S1 to S7).

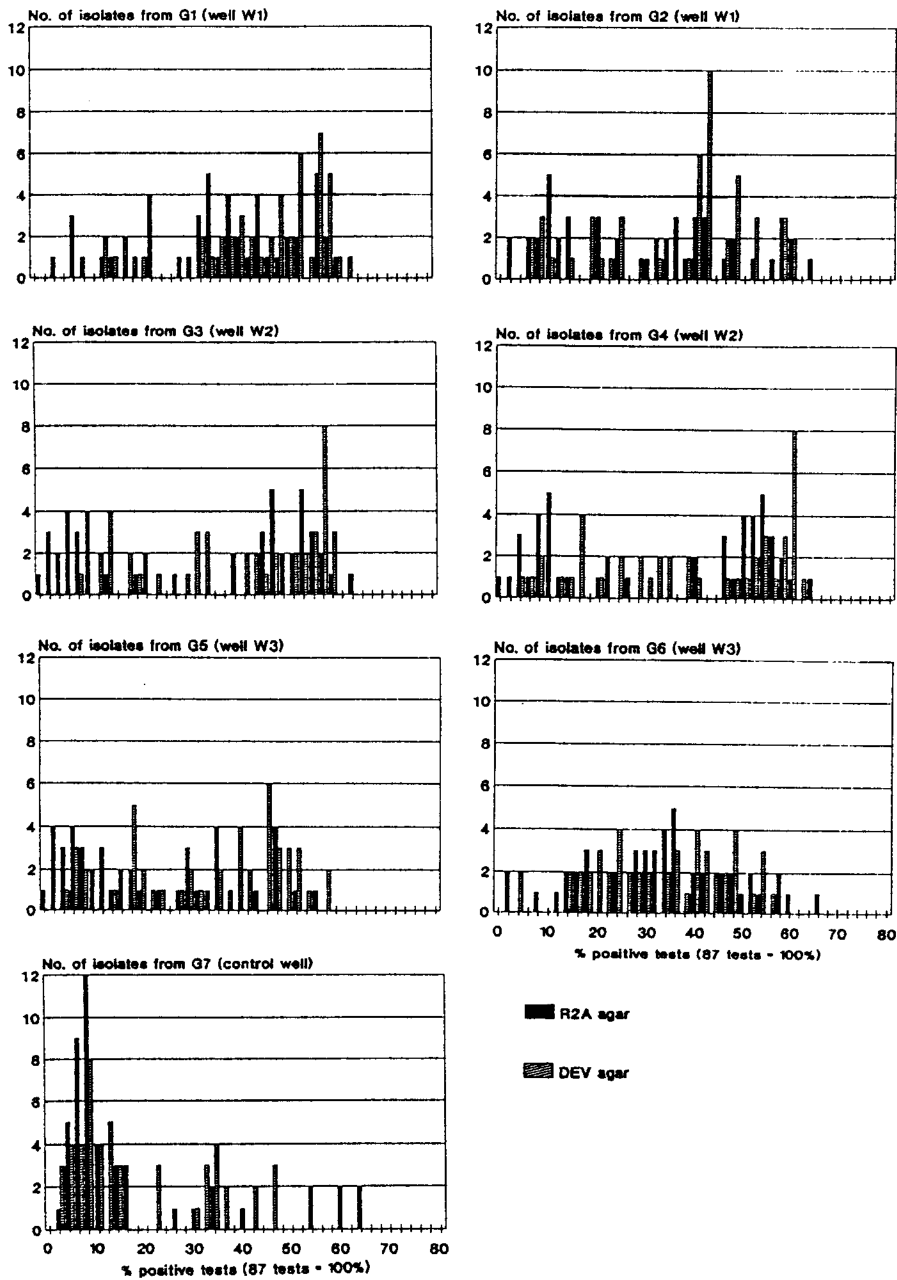


Fig. 3. Distribution of percent positive test results of the isolated bacteria from the seven groundwater samples (G1 to G7).

high numbers of diverse isolates were obtained, whereas unpolluted samples contained higher numbers of physiologically inactive bacteria.

Discussion

Cell Counts

A comparison of total cell counts (acridine orange) of the groundwater samples with colony counts on DEV and R2A agar showed that only 0.1 to 1% of all bacteria of all samples could be isolated. These results are in accordance with other investigations [16, 32, 45]. Total cell counts of soil samples were difficult to interpret. Although microscopic methods have also been recommended for soil, differentiation of bacterial cells, cell fragments, and soil particles was very difficult, so that the total cell counts in soil of 10^9 to 10^{10} are only estimates, similar to those from other studies [5, 32]. An assignment of cells to different size distributions was difficult, as reported by Olsen and Bakken [32]. Furthermore, it was difficult to distinguish cocci from small rods and even from cell fragments. Total cell counts of soil samples must be interpreted with care.

Viable counts or counts of colony forming units (CFU) cannot replace total cell count, and for 20 years plate counts have generally been considered inadequate for counting all naturally occurring bacteria [4, 15]. However, alternative methods, based on an assessment of active bacteria that take up radioactive organic compounds or respire or methods based on microscopic investigations, also have their disadvantages and do not give quantitative and qualitative information about non-culturable or culturable microorganisms. The recently introduced methods of gene probe and immunofluorescent techniques for detection of bacteria in water and soil [4, 34, 35, 41] are quite important. However, in order to study details on the microorganisms that can be detected, they still have to be isolated.

Identification Results and Bacterial Activities

The isolation of 40 to 60 strains from one sample has been recommended in previous studies [3, 28], although this value became the upper practicable limit in our study. All isolates obtained from DEV agar and R2A agar were characterized using 87 miniaturized physiological tests. Media compositions with respect to nutrient content can be regarded as a compromise between the needs of groundwater and soil bacteria and practicable growth conditions. The majority of organisms isolated from low nutrient containing habitats cannot be easily characterized by using the media described for characterization of medically important bacteria. Apart from few classical biochemical tests, which were mainly used for differentiating bacteria tolerant to high nutrient concentrations, we chose carbon substrate utilization tests and qualitative enzyme tests using chromogenic substrates for setting up a data base with reference organisms and for testing physiological capabilities of groundwater bacteria. Nearly all of the isolated bacteria were able to utilize organic acids, i.e., acetate,

Table 4. Percent positive test results of strains isolated from soil and water samples

		Soil samples (no. of strains)							Groundwater samples (no. of strains)						
		S1 (96)	S2 (96)	S3 (96)	S4 (127)	S5 (96)	S6 (96)	S7 (96)	G1 (97)	G2 (99)	G3 (91)	G4 (93)	G5 (91)	G6 (96)	G7 (96)
Conventional tests															
Tryptophan deaminase	TDA	0	0	0	1	0	5	5	0	0	5	2	0	1	0
Indol	IND	0	0	0	0	0	4	5	0	1	3	2	0	0	0
H_2S production	H2S	5	5	3	17	6	1	0	11	1	1	2	3	0	0
Esculin	ESC	66	25	14	36	28	21	0	21	4	1	3	3	2	0
Lysine decarboxylase	LDC	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ornithine decarboxylase	ODC	40	4	20	59	25	11	9	25	34	34	1	0	1	1
Arginine dihydrolase	ADH	43	11	21	62	30	35	10	13	16	56	50	23	23	3
Urease	URE	40	13	10	30	22	21	20	25	43	20	30	20	45	2
Citrate (alkalinization)	CTT	31	46	53	53	56	44	27	25	13	35	29	21	11	0
Malonate (alkalinization)	MLL	33	72	61	80	68	49	48	34	13	43	32	22	12	0
Voges-Proskauer Reaction	VP	3	46	48	7	33	19	48	13	7	19	17	2	2	0
Acid formation from															
D-Glucose	GLQ	31	34	33	45	29	29	22	79	57	38	45	42	39	19
Rhamnose	RHQ	4	3	3	9	6	17	3	25	10	3	4	2	10	1
Sucrose	SUQ	20	20	14	17	16	16	17	23	12	13	18	5	11	3
Adonitol	ADQ	2	0	1	3	2	9	62	12	1	59	53	62	47	56
i-Inositol	INQ	7	5	3	2	6	25	44	32	19	45	61	53	32	24
D-Xylose	XYQ	40	46	47	64	44	41	33	31	12	20	37	29	19	7
Sorbitol	SOQ	0	0	1	0	1	38	3	27	14	8	5	2	11	0
Utilization of															
N-Acetyl-D-galactosamine	AGA	61	40	32	44	44	46	2	55	48	3	3	8	5	16
N-Acetyl-D-glucosamine	AGL	53	44	38	53	51	60	41	52	40	43	47	27	30	22
L-Arabinose	LAR	59	42	24	46	47	56	19	61	47	55	55	47	57	22
D-Cellobiose	CEL	69	19	20	9	20	61	4	38	34	15	27	29	38	20
D-Fructose	FRU	96	51	44	61	65	90	23	72	70	56	59	56	69	28
D-Galactose	GAL	93	43	34	57	53	72	17	67	44	55	49	56	48	25

Table 4. Continued

		Soil samples (no. of strains)							Groundwater samples (no. of strains)						
		S1 (96)	S2 (96)	S3 (96)	S4 (127)	S5 (96)	S6 (96)	S7 (96)	G1 (97)	G2 (99)	G3 (91)	G4 (93)	G5 (91)	G6 (96)	G7 (96)
α-D-Galacturonate	GAK	34	22	24	39	29	35	9	45	26	35	32	24	18	2
Gluconate	GLK	96	49	51	63	74	95	38	73	75	57	61	48	60	34
D-Glucose	GLU	96	76	68	76	75	99	39	90	84	63	70	63	74	38
Glycogen	GCY	97	95	86	85	77	100	29	57	70	32	30	25	32	20
D-Maltose	MAL	95	68	70	43	59	89	15	65	63	25	51	34	47	31
D-Mannose	MAN	96	76	80	71	80	95	32	84	59	68	63	58	66	29
α-D-Melibiose	MEL	68	14	11	6	10	24	3	32	29	11	14	24	26	9
L-Rhamnose	RHA	32	7	4	16	12	32	1	30	15	8	16	21	28	1
D-Ribose	RIB	76	47	45	66	64	84	23	47	39	52	46	36	39	15
D-Sucrose	SAC	88	38	21	40	35	91	10	69	66	35	54	43	55	31
Salicin	SAL	76	49	40	31	30	98	5	42	33	21	26	23	30	23
D-Trehalose	TRE	95	57	56	45	51	91	68	73	70	64	67	44	62	38
D-Xylose	DXY	83	74	83	55	59	76	19	71	52	51	54	57	51	17
Adonitol	ADO	34	31	34	9	17	54	11	9	9	18	9	11	4	3
i-Inositol	INO	73	25	12	32	30	65	5	55	40	44	43	33	32	5
D-Mannitol	MNT	80	42	31	54	56	86	21	67	60	55	56	48	64	22
D-Sorbitol	DST	60	25	12	36	40	78	4	62	52	45	46	35	53	20
Acetate	ACE	100	99	97	97	97	100	86	75	67	67	59	44	57	24
Propionate	PRO	98	98	97	96	95	100	62	64	52	62	54	40	44	22
trans-Aconitate	ACO	82	65	72	64	72	93	39	54	30	63	53	35	28	22
Adipate	ADI	68	68	80	46	73	86	47	24	20	30	16	8	9	6
Citrate	CIT	97	83	76	89	74	96	56	68	60	68	60	44	43	24
Fumarate	FUM	100	95	76	93	83	96	76	88	66	73	69	55	74	24
DL-3-Hydroxybutyrate	3HB	100	93	86	93	96	97	97	75	72	77	63	48	71	74
DL-Lactate	LCT	100	98	94	92	96	98	90	74	76	80	66	59	74	76
L-Malate	MAT	100	97	97	96	94	100	85	94	81	77	73	66	80	31
Pyruvate	PYR	100	99	98	95	98	100	99	87	79	84	73	58	78	48
Suberate	SUB	54	57	51	41	48	94	34	37	32	33	22	12	29	12
L-Alanine	LAL	98	97	93	90	89	99	92	82	64	81	62	42	57	45

Table 4. Continued

		Soil samples (no. of strains)							Groundwater samples (no. of strains)						
		S1 (96)	S2 (96)	S3 (96)	S4 (127)	S5 (96)	S6 (96)	S7 (96)	G1 (97)	G2 (99)	G3 (91)	G4 (93)	G5 (91)	G6 (96)	G7 (96)
L-Aspartate	ASP	99	100	98	98	99	92	96	43	30	82	72	62	65	43
L-Histidine	HIS	97	54	52	62	66	85	33	54	42	58	48	33	45	11
L-Hydroxyproline	HPL	45	41	34	54	43	39	60	32	20	42	32	32	26	8
L-Ornithine	ORN	40	43	36	54	53	89	24	38	44	57	42	27	27	3
L-Proline	PRO	97	97	94	94	91	97	89	84	79	69	63	58	71	38
L-Serine	SER	92	50	43	61	62	93	32	58	37	55	44	31	29	10
Putrescine	PUT	96	55	43	62	65	98	21	62	64	69	58	46	51	26
3-Hydroxybenzoate	3HY	94	40	49	20	53	90	10	34	33	24	17	18	19	21
4-Hydroxybenzoate	4HY	96	72	74	66	79	97	48	65	62	67	56	43	43	22
Phenylacetate	PAC	95	54	50	46	65	83	16	41	38	30	25	19	14	21
Hydrolysis of															
pNP-N-Acetyl-β-D-galactosaminide	CAK	2	10	17	9	2	0	25	12	5	8	8	10	5	10
pNP-N-Acetyl-β-D-glucosaminide	CBG	21	61	51	51	60	53	73	34	18	58	49	27	27	21
pNP-α-L-Arabinopyranoside	CAP	21	3	0	2	8	8	1	13	9	14	9	13	21	20
pNP-β-D-Cellobioside	CAC	23	12	14	9	6	24	10	28	22	9	23	24	33	23
pNP-β-D-Galactopyranoside	CGB	41	14	12	11	10	14	1	23	7	11	18	12	21	9
pNP-β-D-Glucuronide	CGL	57	5	3	4	9	21	2	24	13	10	11	14	7	12
pNP-α-D-Glucopyranoside	CAU	43	17	14	13	16	44	7	46	57	22	37	36	52	29
pNP-β-D-Glucopyranoside	CBU	71	19	14	13	16	41	3	31	25	16	27	29	45	27
pNP-β-D-Lactoside	CLA	9	10	12	8	4	2	2	13	4	1	4	13	7	5
pNP-α-D-Mannopyranosid	CMN	68	15	14	9	11	41	2	36	21	11	15	14	8	18
pNP-α-D-Maltoside	CML	43	18	12	13	16	45	4	39	45	27	34	34	46	25
pNP-β-D-Xyloside	CBX	31	16	12	12	12	25	2	37	31	16	19	23	31	25
Bis-pNP-Phosphate	CBP	59	79	69	71	77	89	62	64	62	67	71	44	65	45
pNP-Phenyl-phosphonate	CPP	34	51	34	50	47	73	47	44	42	54	48	44	61	45
pNP-Phosphoryl-choline	CPC	28	17	16	12	10	21	18	19	29	24	24	31	45	18

Table 4. Continued

		Soil samples (no. of strains)							Groundwater samples (no. of strains)						
		S1 (96)	S2 (96)	S3 (96)	S4 (127)	S5 (96)	S6 (96)	S7 (96)	G1 (97)	G2 (99)	G3 (91)	G4 (93)	G5 (91)	G6 (96)	G7 (96)
2-Deoxythymidine-5′-pNP-phosphate	CDH	28	35	41	28	47	45	30	26	34	33	41	20	36	9
L-Alanine-pNA	CAL	97	100	99	97	100	99	100	93	86	88	85	71	97	93
γ-L-Glutamate-pNA	CGM	66	57	67	70	75	79	57	60	53	65	73	51	68	31
L-Glutamate-γ-3-carboxy-pNA	CGB	49	55	67	72	74	80	56	52	48	59	66	47	50	11
Glycine-pNA	CCY	92	92	89	91	96	97	100	71	62	75	69	40	76	76
L-Leucine-pNA	CLE	92	95	86	72	94	94	89	79	68	65	54	65	78	41
L-Lysine-pNA	CLY	90	100	98	98	99	97	100	90	76	91	80	68	92	75
L-Proline-pNA	CPR	86	48	42	48	54	84	30	62	53	63	56	29	55	23
L-Valine-pNA	CVA	23	10	14	13	7	15	11	20	31	25	32	12	41	11

pNP = p-nitrophenyl, pNA = p-nitroanilide

pyruvate, propionate, or 3-hydroxybutyrate (Table 4). The majority of pseudomonads and other gram-negative bacteria are able to utilize these compounds. Sugars and aromatic compounds were assimilated less frequently. It should be pointed out that many soil and water isolates are non-fermentative organisms, growing well on several different sugars without producing acids, or on organic acids without producing alkalinization. Carbon substrate utilization tests based on color changes of indicator substances, extensively used in recent studies [27, 28], may therefore lead to false results. Even commercially available identification kits created for gram-negative non-fermentative bacteria are largely based on carbon-substrate utilization tests, for which results are based on growth measurements [1, 25]. Growth tests were judged insufficient because many strains are able to grow in a pure mineral medium without carbon source [28], but these effects can be minimized using the pure mineral base as a control medium for growth. Carbon substrate utilization tests can also be used to differentiate various gram-positive bacteria such as bacilli or the heterogeneous groups of coryneforms and nocardioforms. For the isolation of bacteria, we used the nutrient-rich DEV agar and the R2A agar, which contained less nutrient and which was successfully used for isolation of bacteria from drinking water [38]. However, it is impossible to obtain complete insight into the microbial community of groundwater and soil, because of the selectivity of isolation and test media, test conditions, and incubation [2, 28, 45].

Calculation of in vitro activities as percentages of positive tests revealed differences between the samples (Table 4, Fig. 2). Physiologically versatile species like *P. cepacia* and *P. fluorescens* and the *Arthrobacter* species are responsible for the high values of positive percentages in many tests. In contrast, groundwater sample 7 (G7), which had not been contaminated by fuel oil, contained mainly physiologically inactive *Pseudomonas alcaligenes* isolates and demonstrated low activities in many tests, due to the in vitro inactivity of this species. Differences in the culturable bacteria of contaminated and uncontaminated groundwater samples were clearly demonstrated by the activities and species spectrum.

Differences in total activities of bacteria from soil samples are given in Fig. 2. Bacteria from S1 showed the highest proportion of active bacteria, with positive tests ranging from 45% to 65% of the total. The majority of isolates of this sample were *P. cepacia, P. fluorescens,* and the gram-positive species *Arthrobacter globiformis* and *Arthrobacter oxydans.* In samples S2 to S6 other species were obtained (Table 3, Fig. 2). Soil sample S7, taken at 11.30–11.55 m depth, contained *Acinetobacter lwoffii* and *Comamonas testosteroni* in higher proportions than all other soil samples. It has been pointed out [13, 28] that in vitro activities demonstrate only the properties of single isolates under in vitro conditions, but they show clearly the different physiological properties and changes in the compositions of culturable bacterial communities, which cannot be described by mere assignment of organisms to different morphologically sized groups. Nearly all samples contained bacteria that could not be unambiguously identified. These bacteria did not show positive results in any test, or the test profile could not be assigned to any taxon in the data base.

Chemical investigations revealed no typical fuel oil compounds, like n-alkanes, indicating that these compounds were already degraded. This is consis-

tent with studies that have shown that contamination of subsurface sandy clays increases the biodegradative activity of the microbiota [46]. All soil samples contained gram-positive bacteria, thus confirming previous observations [39, 45]; however, gram-negative bacteria were also common.

It is clear that in microbial ecology various methods have to be applied for studying complex communities. In the light of the excellent criticism of Brock [4], our study was mainly concerned with the application of pure culture studies to obtain information on culturable bacteria from soil and groundwater. This cannot replace the use of techniques like respiration measurements or microscopic investigations and gene probe and immunofluorescent methods, which are generally recommended. However, to obtain more information about respiring or microscopically detectable bacteria, they have still to be isolated, and the use of numerical identification procedures, including miniaturization and standardization of test conditions, provides many advantages compared to conventional techniques. Bacteria that cannot be identified by simple physiological tests should be investigated more extensively by using chemotaxonomic and genetic methods [6].

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