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Degradation of Diffuse Pesticide Contaminants: Screening for Microbial Potential Using a Functional Gene Microarray

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Recently, a functional gene array—GeoChip—was introduced, targeting more than 12,000 different microbial functional genes. In addition to microbial genes involved in widespread microbial functions, this array also includes 37 probes for genes involved in atrazine [6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine] degradation. In our study, we evaluated whether this type of microarray is suitable for screening a rural aquifer for native atrazine degraders and their intrinsic degradation capacities. Surprisingly, small amounts of organic contaminants (up to ~10 µg/L) in a well close to a farmyard led to the establishment of a specific degrader community. This means that although contamination at such low concentrations should have only a minor effect on water quality, the microbial community in this oligotrophic environment reacted in a very sensitive manner to the contaminants. Specific atrazine degradation genes were detected in the aquifer showing the highest level of contamination. Their presence was confirmed using a specially developed polymerase chain reaction approach combined with gene sequence analysis. Whether this should be considered an undesired ecotoxicological effect or whether it should be taken as evidence for regular natural attenuation of such diffuse contaminants is a question that remains to be answered by more comprehensive studies.

Abbreviations: PCR, polymerase chain reaction.

The use of the *s*-triazine herbicide atrazine has been banned in Germany since 1991 and in most countries of the European Union since 2005, mainly because the levels of atrazine and its main metabolite hydroxyatrazine in groundwater and drinking water were often found to be above the permitted amount of 0.1 µg/L. Although atrazine has been banned for a considerable period of time, it is still one of the pesticides most often found in groundwater (Tappe et al., 2002). The question arises as to why this is the case and whether atrazine and its metabolites are degraded in situ. Most studies of this question have used chemical and biochemical methods to characterize the degradation of atrazine either in microcosms or in batch experiments with special emphasis on contaminated soils and sometimes groundwater having relatively high levels of contamination between 2 and 30 mg/kg (Agverted et al., 1992; Issa and Wood, 1999; Katz et al., 2000; Jacobsen et al., 2001; Accinelli et al., 2001; Hang et al., 2005). Recent studies have used molecular techniques to study the microbial degradation potential (Shapir et al., 2000; Devers et al., 2004).

A functional gene array called GeoChip was recently introduced by He et al. (2007). The Geochip targets more than 12,000 different microbial functional genes involved in C, N, S, and P metabolism, as well as in organic contaminant degradation and heavy metal resistance. This also includes 37 genes involved in atrazine degradation, mostly atrazine chlorohydrolase (atzA) and *N*-ethylammeline chlorohydrolase (trzA), both of which catalyze the initial stage of atrazine degradation. In this study, we used the GeoChip to screen natural aquifers for native atrazine degraders and thus for the native degradation potential for atrazine. We also determined whether relevant concentrations in these aquifers affected the establishment of specific atrazine degrader communities.

The Jülicher Zwischenscholle area has been the focus of several analyses, particularly within the framework of Pesticides in European Groundwaters (PEGASE, a detailed study of representative aquifers and simulation of possible evolution scenarios), which was funded by the European Union. The Zwischenscholle area, which is located in North Rhine–Westphalia (Germany), was one of six test areas within the PEGASE project. The area is predominantly characterized by agricultural land use (Fig. 1). The main crop is sugarbeet (*Beta vulgaris* L. ssp. *vulgaris*) in annual rotation with winter wheat (*Triticum aestivum* L.), winter barley (*Hordeum vulgare* L.), and winter rye (*Secale cereale* L.). Minor crops are corn (*Zea mays* L.), potato (*Solanum tuberosum* L.), and oat (*Avena sativa* L.). Several pesticides have been found in groundwater wells (e.g., atrazine, diuron [*N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea], and simazine

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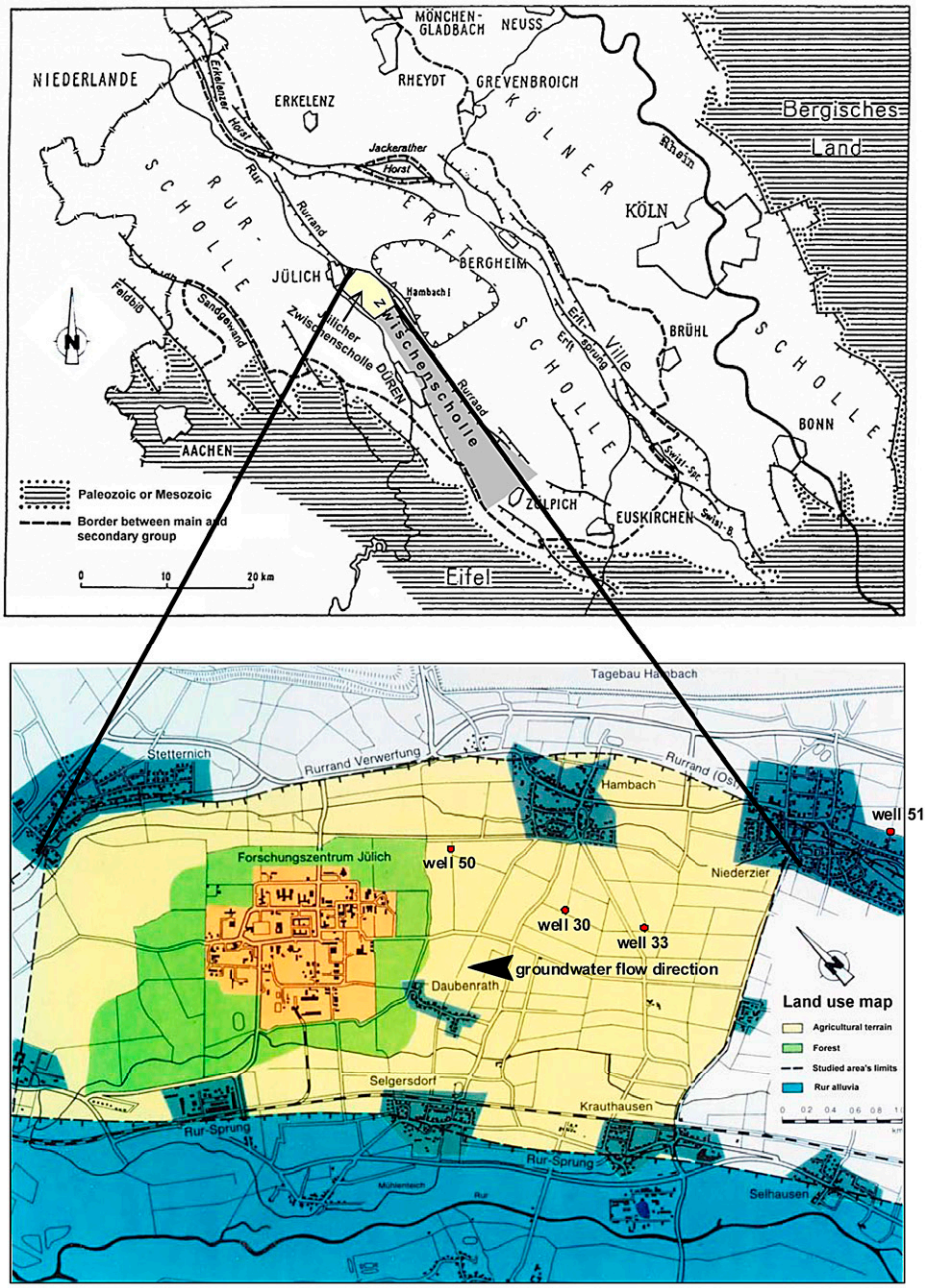

FIG. 1. Site map and location of the wells (modified from Lingelbach, 1996).

[6-chloro-*N*,*N*′-diethyl-1,3,5-triazine-2,4-diamine]), some with concentrations in excess of 100 times the drinking water limits imposed by the European Union (isoproturone, *N*,*N*-dimethyl-*N*′-[4-(1-methylethyl)phenyl]urea, Table 1).

Experimental Procedures

Site Description and Sampling

The four aquifers sampled are located in the Jülicher Zwischenscholle area in the district of Düren, in North Rhine–Westphalia, Germany. As mentioned above, this area has been intensively studied as part of the PEGASE project (for more details, see pegase.c3ed.uvsq.fr/casetu/_ca-docs/Descrition%20site%20Julich.doc, verified 14 May 2009). Since the wells were installed at least 15 yr before our study and had been regularly sampled to monitor groundwater quality, a long time series of data was available for comparison, which also showed that initial artifacts from well installation could be ignored. All analyzed wells are used directly for agricultural purposes, with the exception of Well 51, which is located close to a farm. From each well, sediment and free water samples were collected from the shallow groundwater table at a depth of approximately 7 m below the surface. The groundwater table in all wells was at a depth of between 2.7 and 5.5 m below the surface. Three of the wells had a 15-yr history of atrazine contamination, with one of them (Well 51) showing pesticide concentrations about 10 times higher than the drinking water limit of 0.1 µg/L, while two wells had levels close to this limit (Table 1).

Sampling was performed with a suction pump. For the sediment samples, we first collected a small sample separately from the rest. This first portion contained a small amount of sediment material. The sediment samples were stored overnight at 7°C and carefully decanted. The remaining sediment suspension was centrifuged with 3500 × *g* for 30 min and the sediment was then collected in 1.5-mL reaction tubes. The free water samples were collected after pumping water from the wells for 5 to 10 min until a constant electrical conductivity was measured.

Cultivation of Standard Organisms

The polymerase chain reaction (PCR) was optimized with DNA from test strains *Streptomyces violaceoruber* (DSM 40783, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *Methanothermobacter thermautotrophicus* str. Delta H (DSM 1053, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *Streptomyces violaceoruber* was cultivated in a liquid culture for 72 h at 30°C in GYM medium containing 0.4% glucose, 0.4% yeast extract, and 1.0% malt extract. *Methanothermobacter thermautotrophicus* str. Delta H was purchased as an actively growing culture, and DNA was extracted directly from that culture.

DNA Extraction and Quantification

Before DNA extraction from groundwater, 2 L of the groundwater was filtered (<0.2 µm). A total of 250 mg of material was extracted from the sediments. Liquid bacterial cultures were pelleted. The DNA was extracted with the FastDNA spin kit for soil (Bio101 Systems, Qiagen, Hilden, Germany). The DNA extracts were further purified using the Wizard DNA Clean-Up System (Promega Corp., Madison, WI) and desalted by adding 4% (v/v) 5 mol L^{-1} NaCl and 2 volumes of ice-cold ethanol. The precipitate was pelleted, washed with 70% ethanol, vacuum

TABLE 1. Aquifers sorted by increasing maximum pesticide contamination in recent years; values <0.01, <0.02, and <0.05 are below the detection limit (data analyzed by BRGM, Orleans, France).

Well no.†	Atrazine	Desethyl-atrazine	Desethyl-simazine	Simazine	Diurone	Isoproturone	Total‡
				μg/L			
50 (20250)	<0.05	<0.02	<0.02	<0.01	<0.05	<0.05	<0.05
30 (20230)	0.09	0.08	<0.02	0.12	NA§	NA	0.24
33 (20233)	0.18	0.11	0.14	0.43	0.17	NA	0.84
51 (82295)	1.99	0.07	0.06	<0.05	<0.05	11.8	13.9

† Numbers in parentheses indicate well no. in the original Pesticides in European Groundwaters (PEGASE) study.
‡ Sum for 1 yr.
§ NA = not analyzed.

dried, and redissolved in 25 μL of sterile deionized water. The DNA concentrations were determined by absorption at λ = 260 nm with a NanoDrop ND-1000 spectral photometer (NanoDrop Technologies, Wilmington, Germany).

Whole-Community Genome Amplification

Before labeling with fluorescent dyes, whole-community genomic DNA was amplified using the procedure described by Wu et al. (2006), with minor modifications. Briefly, 1 μL of the DNA extract containing 10 to 35 ng of extracted DNA was mixed with 10 μL of sample buffer. After incubation at room temperature for 10 min, 10 μL of reaction buffer, 1 μL of enzyme mixture, 1 μL of 1 mmol L^{-1} single-stranded DNA binding protein (Epicentre Biotechnologies, Madison, WI), and 1 μL of 1 mmol L^{-1} spermidine (Amersham Biosciences, Piscataway, NJ) were added. The mixture was then incubated for 5 h at 30°C. The reaction was heat inactivated for 10 min at 65°C (all reagents were obtained from the Templiphi Amplification Kit, Amersham Biosciences, Piscataway, NJ, unless otherwise stated).

Microarray Design and Experiment

Amplified DNA was labeled with fluorescent dyes and hybridized. The 37 probes on the GeoChip related to the initial stages of atrazine degradation are listed in Table 2. Oligonucleotide probes were synthesized by MWG Biotech (High Point, NC) and printed in duplicate spots (Schadt et al., 2005) onto aminopropyl silane-coated glass slides (UltraGAPS, Corning, Corning, NY) at Oak Ridge National Laboratory (Oak Ridge, TN). Slides were prehybridized immediately before use according to the manufacturer's protocol.

The DNA was labeled with Cy5- or Cy3-dUTP (Amersham Pharmacia, Piscataway, NJ) using the BioPrime Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA) and subsequently hybridized for 16 h at 50°C in 50% formamide with the microarray according to the protocol by Schadt et al. (2005). Hybridization solutions of 51.5 μL per hybridization contained 50% (v/v) formamide, 3× NaCl–sodium citrate (SSC) buffer, 0.3% SDS (v/v) (all Ambion, Austin, TX), 3.6 μg salmon sperm DNA (Invitrogen, Carlsbad, CA), 0.9 mmol L^{-1} dithiothreitol (Roche Diagnostics Corp., Indianapolis, IN), and 9 μmol L^{-1} spermidine. After hybridization, the slides were immersed briefly in Buffer 1 (2× SSC, 0.1% sodium dodecyl sulfate [SDS], 50°C), washed for 5 min in fresh Buffer 1, for 10 min in Buffer 2 (0.1% × SSC, 0.1% SDS, room temperature), four times for 1 min each in Buffer 3 (0.1% × SSC, room temperature), and for 1 min in Buffer 4 (0.01% × SSC, room temperature), after which they were immediately air dried using compressed air.

Microarrays were scanned with a GenePix 4000 (Molecular Devices, Sunnyvale, CA) using a pixel resolution of 10 μm. The slides were scanned with 100% laser power and a photo multiplier tube gain of 80% or higher. The scanned images were saved as 16-bit TIFF files, and each spot was quantified using ImaGene 6.0 (Biodiscovery, El Segundo, CA). Signals with a signal-to-noise ratio >2 were considered positive hybridizations and further verified by visual inspection. Since no significant differences were observed between groundwater and sediment samples, the results of two groundwater extractions and two sediment extractions were each combined with two technical replicates to increase the overall significance of the results.

Specific Polymerase Chain Reaction Approach for Detection of Atrazine Degradation Genes

To validate the microarray results, the occurrence of the two genes detected in the wells with the highest atrazine contamination had to be confirmed. The PCR approaches were designed based on the specific probe set consisting of three different probes from *Methanothermobacter thermautotrophicus* and *Streptomyces violaceoruber* (Table 3), which were originally designed using the CommOligo program of Li et al. (2005) and which are both gene specific and species specific according to criteria proposed by Liebich et al. (2006). Primers were designed after comparing nucleotide sequences of genes from probes with a significantly higher signal intensity in the contaminated well with nucleotide sequences of homologous genes from other organisms (NCBI Genbank, National Center for Biotechnology Information, Bethesda, MD). For the atzA gene of *Streptomyces violaceoruber*, only a nested PCR approach was revealed to be specific. Primer sequences and references are given in Table 3. Polymerase chain reaction was performed in 50-mL reactions according to the following protocol. The reaction buffer contained 75 mmol L^{-1} Tris-HCl (pH 8.8), 20 mmol L^{-1} $(NH_4)_2SO_4$, 0.01% (v/v) Tween, 1.5 mmol L^{-1} $MgCl_2$, 200 μmol L^{-1} each of dNTP (all ABgene, Epsom, UK), 5 μg BSA (New England Biolabs, Ipswich, MA), 25 μmol L^{-1} of each primer (MWG Biotech, Ebersberg, Germany), 0.4 U Thermo-Start DNA polymerase (ABgene), and 1 μL of the original DNA extract. Amplification was conducted after an initial hot start at 94°C for 15 min in 35 cycles, each of which consisted of 1 min at 94°C, 1 min at the annealing temperature (Table 3), and 1.5 min at 72°C, followed by a final extension period of 8 min at 72°C (iCycler, Biorad, Hercules, CA). Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gels. The PCR products from Well 51 were removed from the agarose gels and purified using the QIAex II Kit (Qiagen, Hilden, Germany). The DNA was reamplified using the same set of primers and protocol as before. Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and directly sequenced by MWG-Biotech (Ebersberg, Germany) using either primer atzA_259_F or trzA_626_F.

Table 2. Probes related to atrazine degradation.

Probe name indicating NCBI Gene ID	Species	Protein function†	Probe sequence
10640374_325	*Thermoplasma acidophilum*	TRZ/ATZ	GTGGGCATAAGGGCTTTTCTCTCATGGGTTACACTGGACAGGGAATTCAC
11498160_64	*Archaeoglobus fulgidus*	trzA	GTTGAAGGGAAAAAGATTTCCGAAATAGGCGGTAAGGCTGTGAAGAGCGA
11498602_604	*Archaeoglobus fulgidus*	trzA	AAGCACATACACGTCTCTGAAACTCTGTGGGAGGTAAAGGAGGTTAGGGA
11499364_943	*Archaeoglobus fulgidus*	atzA	CTTGTGAGGAGGGCCGGGGTTAAGGACATTGAGAGGGTTTTAATCCTATG
15606034_116	*Aquifex aeolicus*	trzA	AAGCAAAGTACACGATAAACGGGAAGGGCAAGATAGCTTTTCCTTCCTTT
15613309_872	*Bacillus halodurans*	trzA	ACATTCCCGGTTTGTTAGATCAAGCAATCCCCGTTAGTTTAGGAGCGGAC
15668880_615	*Methanocaldococcus jannaschii*	atzA	GCATAGGGGGGCTGTGGAATACAGCTTAAACAAATATGGTATGACAGAGG
15669736_817	*Methanocaldococcus jannaschii*	trzA	GGAGTAGCTCCAATTCCAAAACTCTTGGCTGAGGGAATAAACGTTACCTT
15679012_679	*Methanothermobacter thermautotrophicus*	trzA	GTTGAAAGGGCCCTCAACGCAGGATTCAAACTCCTTGTACACCTCACAAA
15679502_1101	*Methanothermobacter thermautotrophicus*	trzA	GAGGCCCCACCTGACTCCCTGGAGAAACCCTCCGTCACACACGGTATATT
15790043_851	*Halobacterium* sp. NRC-1	atzA	CGATCGGGATCACTGAGCTCCGCGCACACTCGGTGGCGTTGACGTGGTAC
15791066_613	*Halobacterium* sp. NRC-1	trzA	GTGCCGGTGCATTTCCACGCCAACGAGACCGAGCAGGAGGTCGAGCCGAT
15897332_1012	*Sulfolobus solfataricus*	trzA	GAAGCTGGGTTACTAGTCCTTGAGGGAGATAGCACGGGCATATTAAACTC
15897586_127	*Sulfolobus solfataricus*	trzA	GAGGGAGATGAGGTAGATTGCTCAGAATACATTGCAATACCAGGGCTAGT
15903257_339	*Streptococcus pneumoniae*	trzA	AGCTGAGCAGATTATAGACTATCAGGGAGCTTGGATTATGCCTGGTTTGG
16081385_457	*Thermoplasma acidophilum*	atzA	ATACTCTCCGCCATTGAAGTTATAAAGAACACAGGCCCCATAAGCGTTGT
16262740_1251	*Sinorhizobium meliloti*	trzA	ACACCCTGCGGTTCAATCGATCTTCCCTCCCAGCTATGAAGTGACAAGAA
17546838_806	*Ralstonia solanacearum*	atzB	AATACGCTGAAGACCTCGGCTGGGTCGGCCACGACGTCTGGCATGCGCAC
18977910_1051	*Pyrococcus furiosus*	trzA	GAGGGGTATATTGCAGATTTAGCGCTGATAAACCTAAGAAGGCCGCACTT
19113705_869	*Schizosaccharomyces pombe*	TRZ/ATZ	GCCCGGTATCAAATTCCAAACTAGGTAGTGGAATCGCCCCACTAAAAGAA
20516599_702	*Thermoanaerobacter tengcongensis*	TRZ/ATZ	GGATTTGGGAATTTTTGAAGTGCCTACTGTAGCTGCTCACTGTGTACACC
21220467_307	*Streptomyces coelicolor* A3(2)	TRZ/ATZ	CCCACCGAGGAGTGGTTCAACGACGTCGTCTGGCCCGTCGAGTCCAACCT
21221512_459	*Streptomyces coelicolor* A3(2)	atzA	TGTCCGCATCACCCTCCTCGACACCTGCTACCTGTCCTCCGGCTTCGGCG
21231035_1139	*Xanthomonas campestris* pv. *campestris*	atzA	GGATCTGCGTCGGTGCATCGGCCGACTTGCTGGAGCTGGACCACACTCAT
21242389_535	*Xanthomonas axonopodis* pv. *citri*	atzA	ATCGACGGCTTCGCGCGCTTGCTGGACGACTGCAGACACAGCCTGAAAGC
22126223_10	*Yersinia pestis* KIM	atzA	CTGCGGGGCGCGTTTGACTCTGAACAAAAATATTGGGATATCGCTATGCC
228:13022196_1045	*Enterobacter cloacae*	trzC	CGCATGCTGGGATTCCCCTCACTTTTAGGCGTCGTGGAAGGGGCAGTCGC
254:11890745_1306	*Acidovorax avenae subsp. citrulli*	atzA	CGCTTGAGCTTTCTTCCCCCTGAACGTGAGTTGGCGTTCCTTGAGGAAGC
27382310_15	*Bradyrhizobium japonicum*	trzA	AATCTTCGGCAGCTATGTGCTGTCACGGAAAGACGGCGCGCAGGATGTGC
28199370_372	*Xylella fastidiosa Temecula1*	trzA	CTATTTTTTCCCTGATGTCCAAGCCGCCGTTTACAAACAGCACGGTTTCC
28211827_176	*Clostridium tetani*	atzA	GTTGTGCTATTCCGGGATTAGTAAATGCACATACTCATGCCGGAATGACC
28867993_10	*Pseudomonas syringae* pv. *tomato*	atzB	ACCCGTATCTGGTTGAAAAACCCTCTTGCTGTATTTACCGCCAATGACCT
294671_739	*Gordonia rubripertinctus*	trzA	GATCCCATCGAGGCCCGAGTGCACTCCATGAACGCCCCGGAATATTTACA
29833194_349	*Streptomyces avermitilis*	trzA	GAGAGCGTACGGGCCGGGGTGACGTCCGCGCTGGACATGTACTGGTTCCA
32475246_682	*Pirellula* sp. 1	trzA	TCATTGCGAACTCTAGGATTGCCTATCGAGGCGTTCTTTCCCTGGACAAA
34496487_15	*Chromobacterium violaceum*	atzA	TTATGAAAAGATTATCTCCGCCCGCTGGATCATCACCGTGGAAACTGACG
63:17938388_561	*Rhizobacterium radiobacter*	trzA	GGGCCGTATGTTTCCGGTTTTTGATGAGGAGCGTGGTTTTCAGGGACTGA

† atzA, atrazine chlorohydrolase; atzB, hydroxydechloratrazine ethylamonhydrolase; trzA, N-ethylammeline chlorohydrolase; trzC, ammelide aminohydrolase; TRZ/ATZ, unspecified (chloro-)hydrolases from the atrazine degradation pathway; acronyms represent also isologs, "probable", and "putative" annotations.

Results

Microarray Analysis

Using the functional array approach as described by He et al. (2007), DNA samples from groundwater and aquifer material were analyzed. Overall, between 70 and 141 hybridizations were detected for the 8800 probes on the GeoChip (Table 4). The majority of hybridized probes were found in the DNA extract from Well 50, which had no history of pesticide detection since at least 1992 (141 probes hybridized). The second highest number of hybridized probes was detected in Well 51, which had the highest level of pesticide contamination (103 probes). This is also the well with the highest number of positive hybridizations of probes with genes related to the degradation of specific organic compounds (a total of 28 positive probes) as well as the highest number of positive hybridizations with genes related to the degradation of industrial organic compounds (21 probes; Table 4). Compared with the total amount of hybridized probes for these wells, a clear relationship with pesticide contamination is obvious. In the DNA extract with the highest level of pesticide contamination (Well 51, Table 1), 20% of all positive probes can be ascribed to the degradation of xenobiotics or other compounds of industrial significance. Well 52 was followed by Well 33 with moderate contamination, in which positive genes represented 15% of the compounds. A total of only 81 probes hybridized in Well 33, however, and the rather high percentage of genes involved in organic contaminant degradation resulted from only 12 probes. The latter is equal to only about half of the probes related to xenobiotic degradation in Well 51, and is comparable to the absolute number of positive probes of genes involved in organic contaminant degradation in the more pristine wells. These samples taken from wells with no pesticide contamination or with a level of contamination below the drinking water limits contained <10% of the total hybridizations

of probes related to genes involved in the degradation of organic compounds of industrial origin.

The pristine Well 50 was found to have the highest number of positive genes related to the degradation of the natural organic polymers cellulose and chitin. This was also the only well where genes for pectin degradation had been detected. In total, 49 probes from this category hybridized, making up more than one-third of all the probes that hybridized in the DNA of Well 50. This explains the surplus of total positive probes compared with Well 51, where only 15 positive genes were detected within this category. This represents 15% of all hybridized probes. Similar proportions were detected (17 and 10%) in two other wells (Wells 30 and 33, respectively).

With regard to atrazine contamination, genes related to atrazine degradation were detected in Wells 30, 50, and 51 (Table 5); however, some of these genes were detected in <50% of the eight replicates. Probe 11499364_943, which targeted the atrazine chlorohydrolase of Archaeoglobus fulgidus (NCBI Genbank accession NC 000917), hybridized with samples from all three wells, while Probe 18977910_1051, which targeted the N-ethylammeline chlorohydrolase of Pyrococcus furiosus (NCBI Genbank accession NC 003413), hybridized with samples from Wells 50 and 51. Furthermore, Probe 15668880_615 hybridized with all samples including the negative controls (DNA extract from *Escherichia coli*), and thus was excluded from further analysis.

Low signal intensities provided reliable site-specific and repeatable differences in the hybridization of gene probes related to atrazine degradation only in Well 51. This led to the identification of specific marker genes for the groundwater well with the highest level of contamination in our study (Fig. 2). Probes 15679502_1101 and 21221512_459 hybridized specifically with DNA from this well (Table 6). Probe 15679502_1101 was based on the nucleotide sequence of *N*-ethylammeline chlorohydrolase (trzA) of *Methanothermobacter thermautotrophicus* str. Delta H (NCBI

TABLE 3. Primers and specific polymerase chain reaction assays for site-specific *Atz* and *Trz* genes.

Target gene	Primer	Nucleotide sequence (5′→ 3′)	Annealing temp.	Reference
			°C	
atzA	atzA_outer_241_F	ACCTCCTGGACCTGGCGCGA	59.8	this study
	atzA_outer_536_R	AAGCGGCGCTGGTGGGTGTT		
	atzA_259_F	GACCCCGGACACCTACCACG	63.3	
	atzA_459_R	GCGTAGTGGGAGGAGCTGTG		
	atzA1:1	CCATGTGAACCAGATCCT	55.0	Shapir et al. (2000)
	atzA1:2	TGAAGCGTCCACATTACC		
	atz_inner_F	GCACGGACGTCAATTCTA		
	atz_inner_R	CGCATTCCTTCAACTGTC		
atzB	AtzB1:1	CATGGCGGCGGCAGGTGTG	55.0	Shapir et al. (2000)
	AtzB1:2	CCCGGGCCAGCTCCATTTCA		
atzC	AtzC1:1	AGTCAGCGAAGGGCGTAGGTATCA	55.0	Shapir et al. (2000)
	AtzC1:2	GACAAATCCGGGAGACACAAGGTT		
trzA	trzA_626_F	GGTCAGTGAAGTCTCTAGGTCCC	54.0	this study
	trzA_1101_R	TGTGTGACGGAGGGTTTCTC		

TABLE 4. Summary of hybridizations with functional gene probes; italics highlight specific differences in single wells.

Gene probe	Homologous gene probes with positive hybridizations†			
	Well 50	Well 30	Well 33	Well 51
Organic polymer degradation				
Cellulase	20	6	4	3
Chitinase	19	4	3	5
Laccase	5	1	1	6
Mannase	2	1		1
Pectinase	3			
Total organic polymer degradation	49	12	8	15
Degradation of natural organic compounds				
Aminocyclopropane	2	2	2	1
Benzoate, protocatchuate	9	4	7	9
Homogentisate	1			2
Limonene	1	1	1	1
Phenylpropionate	1	1	2	2
Urea/cyanuric acid	12	10	8	12
Total specific organic compounds	26	18	18	28
Organic compounds of industrial significance‡				
Acrylonitrile	1	1		
Atrazine				2
Benzene, toluene	1			2
Biphenyl, PAHs, phthalate	1	1	3	4
Catechol, phenol, salicylate	2	3	2	5
Cyclohexanol	1			1
Halogenated alkanes and organic acids	2	1	2	3
MTBE	1			
Nitroglycerin			1	
Thiocyanate	1			4
Total industrial organic compounds	11	6	12	21
Alternative respiration pathways				
Denitrification	7	3	3	4
Methanogenesis	1	4	2	5
DMSO reduction		1	1	
dissimilatory sulfite reduction	6	3	5	3
Total alternative respiration pathways	14	11	11	12
Others				
Nitrification	3	3	3	2
N2 fixation	3	1	1	1
Methanotrophy	3	4	1	2
CO2 fixation/Rubisco	4		1	2
Cytochrome c	2	2	4	1
Formate-tetrahydrofolate ligase	1		2	
Metal resistance	25	12	20	19
Total others	40	23	30	27
Total all gene probes	141	70	81	103

† Number of different homologous gene probes with positive hybridizations in >50% of the replicates above a signal-to-noise ration of 2 (n = 8).

‡ According to the Biocatalysis/Biodegradation Database of the University of Minnesota (Ellis et al., 2006).

Table 5. Positive hybridizations with atrazine-related genes.

Well	Probe 11499364	Probe 15679502	Probe 18977910	Probe 21221512
51	++†	++	(+)‡	(+)
50	(+)	–§	(+)	–
30	(+)	–	–	–
33	–	–	–	–

† ++, gene detected.
‡ (+), gene detected in <50% of replicates.
§ –, no hybridization.

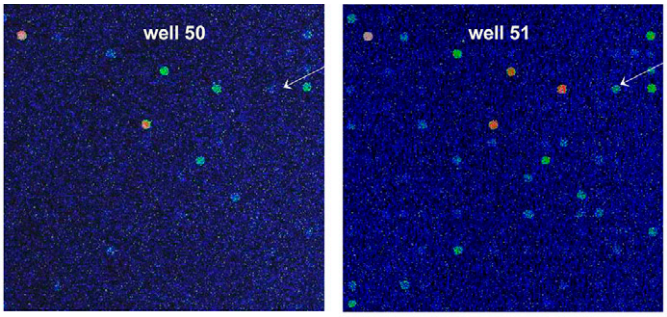

Fig. 2. Excerpt from the hybridization of DNA from Wells 50 and 51; arrows indicate the position of specific probe 15679502 target.

Genbank accession NC 000916), which catalyzes the dechlorination of deisopropylatrazine to deisopropylhydroxyatrazine. Probe 21221512_459 was designed against the nucleotide sequence of the putative atrazine chlorohydrolase (atzA) of *Streptomyces violaceoruber* [formerly *S. coelicolor* A3(2), NCBI Genbank accession NC 003888], which catalyzes the first step in atrazine degradation.

Validation of Microarray Results by Polymerase Chain Reaction

In Well 51, which had the highest level of atrazine contamination, two specific genes were detected. While all of the other genes detected were also found in the pristine Well 50, these two specific genes could be related to the corresponding contamination in this well. To verify the existence of the atrazine degradation genes, we developed a special PCR approach. The single PCR approach using the primer pair atzA_259_F and atzA_459_R led to a positive and specific amplification product of DNA from *Streptomyces violaceoruber*, but only to an unspecific product in the environmental samples. By extending the PCR approach by an outer primer set atzA_outer_241_F and atzA_outer_536_R, which results in an unspecific product from the standard organism when used as a stand-alone primer set, a specific product was reproducibly obtained from Well 51 (Fig. 3). The trzA primer set (Table 2, Fig. 4) also revealed a positive amplification, which was rather weak and had (even weaker) unspecific products. The length of the amplification product just below 500 bases matched the expected length of 476 bases, however, and provided good evidence for a gene-specific product.

In addition, PCR was performed with the primer sets for the atzA, atzB, and atzC genes from *Pseudomonas* strain ADP, as published by Shapir et al. (2000). Specific products were obtained in all samples using the nested approach targeting the atzA gene of *Pseudomonas* strain ADP, but not for the atzB and atzC genes. This could be due to the fact that the procedure for the atzB and atzC genes only includes a single amplification step, in contrast to the nested approach for the atzA genes, which makes them less sensitive.

Sequence Analysis of Polymerase Chain Reaction Products from Groundwater Well 51

To verify that PCR products from the contaminated wells are indeed related to atrazine chlorohydrolase and *N*-ethylammeline chlorohydrolase, they were eluted from the agarose gels, reamplified, and sequenced. In the case of atrazine chlorohydrolase, sequence analyses revealed the highest similarity with the putative chlorohydrolases of *Streptomyces violaceoruber* (91% sequence identity across 123 bases of 136 total bases sequenced) and of *S. avermitilis* MA-4680 (90% sequence similarity across 122 bases, gi: 29607147, NCBI protein database). In the case of *N*-ethylammeline chlorohydrolase, 95% similarity was found across 121 bases with the homologous gene *Methanothermobacter thermautotrophicus* str. Delta H, which initially served as a template in designing this probe.

Discussion

From the 8800 functional gene probes on the GeoChip, between 70 and 141 (corresponding to 0.8 and 1.6% of all spots on the GeoChip) hybridized with the DNA samples from the four groundwater samples with an agricultural background. While this number appears to be a relatively poor yield, it simultaneously represents a reasonable hybridization effort because the hybridizations were performed under very stringent conditions (50°C plus 50% formamide). Only highly homologous genes can be detected under such hybridization conditions. Thus, even when technological improvements of the last 5 yr are taken into consideration, a recovery of about 1% of the immobilized probes is realistic. Interestingly, two wells stood out, containing, in total, more positive probes. Pristine Well 50 was characterized by genes involved in natural organic polymer degradation, such as cellulose, chitin, and pectin. One possible explanation for this could be the higher, and possibly local, nutrient input into this oligotrophic environment. Such a nutrient input could trigger the overall metabolism of the microbial community in this well, which would result in higher proportions of gene probes from the categories "natural organic compounds" and "others" being hybridized. No difference was observed concerning alternative respiration pathways, however. Therefore, we did not expect an

Table 6. Comparison of signal intensities of significant atrazine-related genes in the pristine Well 50 and in Well 51, which is located close to a farmyard.

Probe	Species	Normalized signal intensity			
		Gene type	Well 50	Well 51	Difference in signal intensity
11499364	*Archaeoglobus fulgidus*	atzA	0.23840	0.5388	unchanged
15679502	*Methanothermobacter thermautotrophicus*	trzA	–1.4121	0.4323	significant (up)
18977910	*Pyrococcus furiosus*	trzA	–0.1391	–0.0335	unchanged
21221512	*Streptomyces coelicolor*	atzA	–1.2969	0.6827	significant (up)

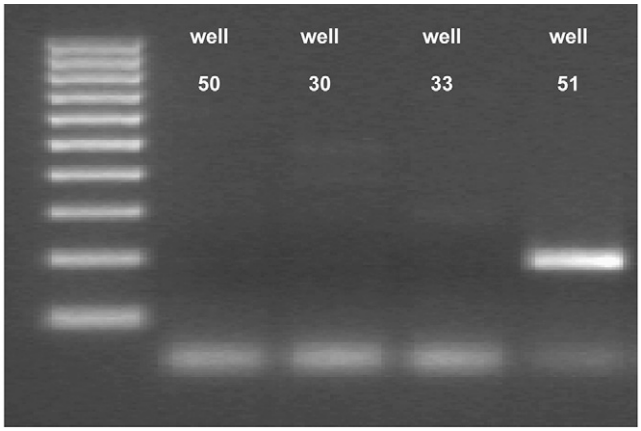

FIG. 3. Polymerase chain reaction products after specific amplification of the atzA gene of Streptomyces coelicolor (left lane: 100 bp marker).

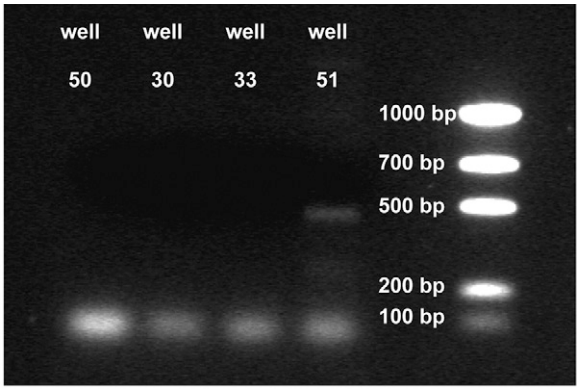

FIG. 4. Polymerase chain reaction products after specific amplification of the *trzA* gene of *Methanothermobacter thermautotrophicus* (right lane: molecular mass ruler).

extremely high nutrient supply here, which would otherwise have led to a depletion of O_2 and thus to a promotion of microbes adapted to growth under anaerobic conditions.

The same appears to be true for Well 51. Although this well showed the highest level of pesticide contamination, the corresponding number of contaminants was still rather low considering the O_2 consumption during their degradation. Therefore, alternative respiration pathways do not seem to play a dominant role in this well either. The levels of pesticide contamination, however, which resulted from inappropriate agricultural practices (as was proven by the chemical analyses), still appeared to be large enough to impact the degrader populations and to enhance their microarray detectability. This can be seen from the high proportion of genes involved in the degradation of organic compounds of industrial (or here agricultural) origin. Again, the total metabolism may have been triggered, as suggested by the higher number of genes detected in the DNA from this well compared with the other two wells with lower contamination levels.

The main objective of this study, however, was to screen for the degradation potential of atrazine in pristine groundwater and in groundwater contaminated with concentrations that can be found in many regions worldwide (Tappe et al., 2002). Regardless of the level of atrazine contamination, genes related to atrazine degradation could be detected in most samples. Additional genes were also detected exclusively in the groundwater of Well 51, however, where concentrations of approximately 2 μg/L had been found regularly during the preceding years. Since the array is gene specific (He et al., 2007), we can deduce that a potential for atrazine degradation exists in shallow groundwater that is used for agricultural purposes regardless of its application and detectability. The widespread, if not ubiquitous, distribution of the atrazine degradation potential has been described elsewhere (de Souza et al., 1998).

The microarray approach can be considered successful. Our study has shown the strengths and the potential of this technique for use in environmental studies. From the 37 probes of genes related to atrazine degradation, a total of five probes hybridized. Two of these probes hybridized with the sample containing atrazine contamination of approximately 2 μg/L only. The occurrence of these two genes was validated by PCR approaches specifically designed for this study, and subsequently by sequence analysis.

While the functional gene specificity of the probes was ascertained by this study, the organism specificity cannot be given. There is a 90 to 91% similarity between the atrazine chlorohydrolase detected and the sequence of the putative chlorohydrolase of *S. violaceoruber* and *S. avermitilis*. *Streptomyces* strains are quite commonly found in ambient environments (e.g., soils) and their occurrence in shallow groundwater is not surprising. The second probe, however, which hybridized specifically with DNA from the contaminated well, was designed from the *N*-ethylammeline chlorohydrolase of *Methanothermobacter thermautotrophicus*, which was isolated from sewage sludge, compost, biogas facilities, and other comparably hot, anaerobic, and copiotrophic environments. It is therefore very unlikely that *Methanothermobacter thermautotrophicus* could survive and actively grow in the aquifer analyzed in this study. This shows that there is still a great lack of genetic information in environmental microbiology. This information is required for the comprehensive description of microbial structures and functions. Another piece of evidence that leads us to believe that it was not actually *M. thermautotrophicus* that was detected is the fact that only Probe 15679502_1101 hybridized, while Probe 15679012_679, which targets another section of the trzA gene from *M. thermautotrophicus*, did not. Another organism harboring a gene with a high sequence similarity to the trzA gene from *M. thermautotrophicus* was probably detected here. The high sequence similarity in the PCR product, however, suggests that the gene detected was homologous to *N*-ethylammeline chlorohydrolase.

No information can be obtained by our approach about the actual degradation of atrazine. Most studies concerning the degradation potential of atrazine and other pesticides have focused on triggering the degradation using rather high concentrations of the compound. Natural aquifers only contain nanomolar concentrations, however, which make it more difficult to assess degradation. Furthermore, the detection of atrazine degradation genes in the well with the highest contamination of atrazine (∼2 μg/L = ∼10 nmol/L) suggests that the directed degradation of atrazine, in contrast to coincidental cometabolic degradation, does indeed occur at these concentrations.

Conclusions

Using the example of the GeoChip designed by He et al. (2007), we showed that functional gene microarrays are an excellent tool for screening the degradation potential of diffuse

atrazine contaminants. Surprisingly, even minor levels of organic contamination (~2 μg/L) in the well close to the farmyard led to the enhanced detection of specific degrader populations. This means that although contamination in this concentration range should have only a minor effect on water quality, the microbial community in these oligotrophic environments appears to be sensitive to relatively small quantities of contaminants. Whether this should be considered as an undesired ecotoxicological effect or whether it should be taken as evidence for regular natural attenuation of such diffuse contaminants is a question that remains to be answered by more comprehensive studies.

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