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Bioremediation strategies for removal of residual atrazine in the boreal groundwater zone

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Abstract Strategies for bioremediation of atrazine, a pesticide commonly polluting groundwater in low concentrations, were studied in two boreal nonagricultural soils. Atrazine was not mineralized in soil without bioremediation treatments. In biostimulation treatment with molasses, up to 52 % of atrazine was mineralized at 10 °C, even though the degradation gene copy numbers did not increase. Incubations with radioactively labeled atrazine followed by microautoradiographic analysis revealed that bioremediation strategies increased the relative proportion of active degraders from 0.3 up to 1.9 % of the total bacterial count. These results indicate that atrazine degradation might not solely be facilitated by *atzA*/*trzN*–*atzB* genes. In combined biostimulation treatment using citrate or molasses and augmentation with *Pseudomonas citronellolis* ADP or *Arthrobacter aurescens* strain TC1, up to 76 % of atrazine was mineralized at 30 °C, and the atrazine degradation gene numbers increased up to 10^7 copies g^{-1} soil. Clone libraries from passive samplers in groundwater monitoring wells revealed the presence of phylogenetic groups formerly shown to include atrazine degraders, and the presence of atrazine degradation genes *atzA* and *atzB*. These results show that the mineralization of low concentrations of atrazine in the groundwater zone at low temperatures is possible by bioremediation treatments.

Keywords Atrazine degradation · Bioremediation · Quantitative PCR · Microautoradiography · ^{14}C-mineralization

Introduction

Intensive use of pesticides has increased the amount of accidental exposure of these harmful substances to the ecosystem. Although many soil bacteria have potential for pesticide biodegradation, several herbicides are frequently detected in soil and groundwater. Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) was banned by the European Union a decade ago due to its recalcitrance, but despite the concerns of environmental impacts and possible risks to human health, it is still one of the most widely used herbicide against broad leaf weeds worldwide today.

Although atrazine is a recalcitrant compound, it is susceptible to microbial degradation in topsoil (Aislabie et al. 2004; Nousiainen et al. 2014; Sagarkar et al. 2013; Sagarkar et al. 2014). Bacteria can degrade atrazine by two different pathways, initiated by dealkylation and dechlorination. The latter route is well described and catalyzed by the enzymes encoded by the *atzABCDEF* genes (de Souza et al., 1996). Instead of *atzA*, the functional homolog, *trzN* gene (Mulbry et al. 2002), may be more commonly observed in the environment (Arbeli and Fuentes 2010). An alternative atrazine degradation pathway, initiated by a dealkylation reaction, is carried out by a P-450 cytochrome system in certain strains of *Rhodococcus* species (Nagy et al. 1995). This reaction is not specific for triazine breakdown, and hence, the presence of this pathway

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cannot directly be linked with atrazine degradation by genomic tools.

High concentration of atrazine can be degraded in topsoil (Wang et al. 2013), but often, it partly leaches into the subsoil, where its degradation is considerably slower (Accinelli et al. 2001). Atrazine removal is economically and technically most difficult to achieve when it is present in subsoil and at low concentrations. Unfortunately, this is the most common type of atrazine pollution. A survey of pesticides in Finnish groundwater revealed atrazine concentrations up to 0.34 μg L^{-1} (Vuorimaa et al. 2007). Similar low values are common in groundwaters globally (Hallberg 1989; Jurado et al. 2012; Stuart et al. 2012). As the European Union has set the maximum limit for pesticides in drinking water to 0.1 μg L^{-1}, even a trace amount of atrazine can cause the groundwater to be unfit for drinking water use. The majority of the studies related to atrazine degradation in subsoil are carried out using agricultural topsoil having high atrazine concentration (Li et al. 2008; Lima et al. 2009; Singh and Cameotra 2014), and little is known on atrazine degradation in nonagricultural subsoil where, despite the low concentrations, it potentially endangers groundwater reserves. Thus, research conducted on high atrazine concentrations may not be applicable in the bioremediation of atrazine in the groundwater zone.

The environmental impacts of herbicides are frequently assessed by evaluating their half-lives under standardized conditions. However, environmental conditions seldom resemble the laboratory conditions, and degradation can be reduced when the herbicide is used in the field (Bromilow et al. 1999). Atrazine is moderately soluble in water, and it therefore leaches into the groundwater, where its half-life can increase up to nine times (Blume et al. 2004). Pesticide degradation rates can be dramatically influenced by soil pH (Mueller et al., 2010), organic material (Cheyns et al. 2012), temperature (Kookana et al. 2010), and soil layer (Willems et al. 1996).

Finnish atrazine-contaminated subsoils are typically nonagricultural, often acidic, and low in organic carbon, and ambient temperatures are low. For these reasons, residual atrazine is still found in the groundwater decades after its use was discontinued (Vuorimaa et al. 2007). In bioremediation, the environmental conditions are modified to improve degradation. Because many bacteria use atrazine as a nitrogen source (Udiković-Kolić et al. 2012), the addition of carbon may enhance atrazine degradation in soils where the amount of available carbon, rather than nitrogen, is limiting growth. Several studies have shown that bioaugmentation with *Pseudomonas citronellolis* ADP harboring the *atzABCDEF* genes enhance atrazine degradation in topsoil (Chelinho et al. 2010; Lima et al. 2009). Bioaugmented *P. citronellolis* is capable of atrazine degradation at 12 °C (Monard et al. 2008), but no studies explored its exploitation in subsoil.

The purpose of this study was to investigate bioremediation in boreal subsoils contaminated with low concentrations of atrazine. The natural degradation potential of one of the soils used in this study has been previously described as low (Nousiainen et al. 2014). We tested the effects of temperature, atrazine concentration, carbon source, and bioaugmentation with atrazine-degrading microbial strains to enhance atrazine degradation in subsoil.

Materials and methods

Sampling

Soil was collected in two different sampling campaigns from two similar sites on the Salpausselkä ice-marginal formation, in Sveitsi natural park (SNP), Southern Finland (60° 38′ 18″ N, 24° 49′ 17″ E) on 15.09.2009, and in Lohjanharju (LOH), Southern Finland (60° 17′ 39″ N, 24° 12′ 46″ E) on 15.12.2011. Both sites were in a coniferous forest, adjacent to waterworks where atrazine and its degradation products had been observed in the raw groundwater. At both sites, the most likely source of atrazine pollution was from nonagricultural use of atrazine, e.g., on railroads.

Soil from site SNP was sandy or silty and had a low organic carbon content (<1 % dry weight). The groundwater level was very high, and the saturated layer was only 20 cm below surface. Saturated soil was collected from site SNP from 2 to 5 m for bioremediation experiments and from 50 cm for microautoradiography (MAR).

The soil from site LOH had similar characteristics as the soil from site SNP in regard to texture and carbon content. The groundwater level in the area was 4–6 m and varied depending on rainfall and season. The soil pore gas composition at the depth of 2.5 m was measured with a Dräger gas meter at site LOH, and the high O_2 concentration (20.4–20.6 %) was found to indicate low microbial activity. Material for testing atrazine degradation for increased atrazine concentration was collected from site LOH from three different depths; bulk samples representing topsoil (1–2 m), unsaturated soil (2–5 m), and saturated soil (6–9 m). All soil samples were transported to the laboratory, sieved through an 8-mm sieve, and mixed well and packed in plastic buckets. Soils were stored at +4 °C in field moisture conditions until use (1 to 5 months). Samples for the determination of gene abundances in situ were frozen and stored at −20 °C.

Preparation of bacterial inocula

Atrazine-degrading strains, *P. citronellolis* strain ADP (DSM 11735) genotype *atzA*/*atzB* and *Arthrobacter aurescens* strain TC1 (ATCC BAA-1386) genotype *trzN*/*atzB*, were obtained

from the culture collections ATCC and DSM, respectively. Prior to microcosm experiments, the bacteria were grown in liquid 0.1× Luria-Bertani (LB) medium amended with 0.5 mg L^{-1} atrazine at 30 °C, 200 rpm. Cells were harvested from overnight cultures by centrifugation and washed with 0.9 % NaCl. A turbid suspension in 0.9 % NaCl was prepared from both strains, and approximately 2×10^5 cells g^{-1} soil were used for bioaugmenting the microcosms.

Construction of microcosms and experimental setup

The atrazine mineralization potential of the autochthonous microbial community was tested by amending the soil with 1.2 mg kg^{-1} commercial atrazine (50 % active ingredient) product (SriZon, Crystal Phosphates, Delhi, India) to samples from three soil layers (topsoil, unsaturated, and saturated) of the LOH site in order to reach the concentration required for the detection by LC/MS/MS. One hundred seventy-five milliliters of the commercial atrazine was mixed with 3.5 kg of soil from each layer in three additions, mixing the soil thoroughly after each addition.

The effect of additional carbon (citrate or molasses) and/or atrazine-degrading bacteria on atrazine mineralization was tested in microcosms using soil samples from site SNP. The carbon sources were added in the microcosm treatments as a component of modified Cai medium (Cai et al. 2003). The carbon components added were an equal mass of carbon atoms in either sodium citrate (3 g L^{-1}) or molasses (3.74 g L^{-1}). Modified Cai medium was also prepared without any additional carbon amendment.

Atrazine mineralization by radiorespirometry

Atrazine mineralization was carried out by radiorespirometry as described elsewhere (Nousiainen et al., 2014) using 10 g of soil and modified Cai medium with or without carbon source, ring-labeled ^{14}C atrazine [specific activity 160 mCi $mmol^{-1}$; 99 % radiochemical purity (Larodan Fine Chemicals Ab, Sweden)] as tracer (6.25×10^{-10} mol g^{-1} soil (freshwater, fw)), or labeled and additional atrazine (0.006 mol g^{-1} soil (fw)) and/or bacteria according to Table 1. The Cai medium containing carbon source and tracer was added in the bottles in 500-μL volumes, drop by drop, while mixing the soil. All microcosms were carried out in triplicate. Negative controls were prepared by autoclaving the soil microcosms three times. Microcosms were incubated at 30 and 10 °C, 100 rpm for 115 days. The parameters of the mineralization kinetics were determined by fitting a modified Gompertz model (Zwietering et al. 1990) to the mineralization curves using Sigma Plot 4.0.

Chemical analysis of atrazine, HA, DIA, DEA, and DEDIA

Parallel samples containing 60 g of soil from Site LOH were set up for chemical analysis. The concentration of atrazine and its degradation products hydroxyatrazine (HA), desethylatrazine (DEA), deisopropylatrazine (DIA), and desethyl-deisopropylatrazine (DEDIA) in soil were examined at the beginning, middle (35 days for 30 °C incubations, 56 days for 10 °C incubations), and end of the experiment (56 days for 30 °C incubations, 112 days for 10 °C incubations) from 60 g of soil by Ramboll Analytics (Lahti, Finland) by gas–liquid chromatography–mass spectrometry. Acetone/hexane was used as the solvent to extract pesticides from soil.

Quantification of degradation genes by qPCR

Parallel samples for each mineralization bottle were prepared without the labeled atrazine for molecular analyses. Total DNA was extracted from 0.5 g of soil periodically during the course of the experiments by FastDNA Spin Kit for Soil (MP Biomedicals, OH, USA) according to the manufacturer's instructions. The number of degradation genes *atzA*, *trzN*, and *atzB* were quantified as described elsewhere (Nousiainen et al., 2014).

Estimation of active atrazine degraders by microautoradiography

The numbers of active atrazine-degrading microorganisms in native soil from site SNP, in soil amended with 1 g citrate kg^{-1} and in soil spiked with a turbid mixture of actively growing *P. citronellolis* and *A. aurescens* grown in mineral medium containing 100 mg L^{-1} atrazine, were investigated by microautoradiography (MAR) as described in details elsewhere (Nielsen and Nielsen, 2005) with minor modifications. Briefly, 1.0 g of soil was weighed into three 3-mL glass vials, and 20 μCi of ^{14}C ring-labeled atrazine (specific activity of 160 mCi $mmol^{-1}$) were mixed well by vortexing and incubated under aerobic conditions for 5 days at 100 rpm and 30 °C. Parallel samples were used for the number of degradation genes.

Microbial cells were extracted from 1 g of soil by density gradient centrifugation (Burmølle et al., 2003), using OptiPrep Density Gradient Medium (Sigma-Aldrich, MO, USA). Extracted cells were homogenized and washed by glass tissue grinder (Thomas Scientific®) with 500 μL of sterile tap water three times. Dilutions of the washed cells were filtered on 0.22-μm white polycarbonate filters. The filters were placed on regular microscopy glass slides (24 × 60 mm), air dried on the bench. The slides were then dipped in liquid film emulsion (Kodak autoradiography emulsion type NTB, NY, USA), air dried, and exposed for 2 days at +4 °C. Exposure conditions

Table 1 Experimental setup of the mineralization experiments, and results from mineralization and degradation gene *atzA*, *trzN,* and *atzB* quantifications

								Gene copy number at 42 days		
Temp (°C)	Soil site	Soil layer	Added carbon, bioaugmented strain	C-added atrazine, $t = 0$ (mol g^{-1} soil fw)	Atrazine mineralized in 115 days (%)	Atrazine $t_{1/2}$ (days)	Mineralization rate (% d^{-1})	log *atzA* (g^{-1} soil)	log *trzN* (g^{-1} soil)	log *atzB* (g^{-1} soil)
	SNP	Unsaturated	In situ	0.0	NA	NA	NA	1.3 ± 0.9	2.7 ± 0.12	3.0 ± 0.05
30	SNP	Unsaturated	Citrate, *Pseudomonas*	6.25×10^{-10}	52 ± 30	7.7 ± 2.1	20.2 ± 18.0	7.7 ± 0.3	6.2 ± 0.4	7.7 ± 0.7
	SNP	Unsaturated	Molasses, *Pseudomonas*	6.25×10^{-10}	55 ± 32	12.8 ± 5.0	11.7 ± 3.9	6.9 ± 0.4	<2.4	7.3 ± 0.5
	SNP	Unsaturated	No carbon, *Pseudomonas*	6.25×10^{-10}	65 ± 38	13.3 ± 2.8	11.2 ± 2.3	<2.4	6.4 ± 0.2	6.6 ± 0.3
	SNP	Unsaturated	Citrate, *Arthrobacter*	6.25×10^{-10}	76 ± 44	5.1 ± 0.5	16.6 ± 1.7	2.0 ± 0.4	5.9 ± 0.4	5.6 ± 0.4
	SNP	Unsaturated	Molasses, *Arthrobacter*	6.25×10^{-10}	62 ± 36	7.6 ± 1.4	8.7 ± 2.5	<2.4	5.5 ± 0.3	5.1 ± 0.2
	SNP	Unsaturated	No carbon, *Arthrobacter*	6.25×10^{-10}	39 ± 23	ND	8.7 ± 3.1	<2.4	5.9 ± 0.4	5.6 ± 0.3
10	SNP	Unsaturated	Citrate	6.25×10^{-10}	1 ± 1	ND	ND	3.3 ± 0.9	<2.4	4.7 ± 0.3
	SNP	Unsaturated	Molasses	6.25×10^{-10}	1 ± 1	ND	ND	<2.4	<2.4	4.4 ± 1.5
	SNP	Unsaturated	No carbon	6.25×10^{-10}	21 ± 12	17.6	5.9	<2.4	3.0 ± 0.2	3.0 ± 0.3
	SNP	Unsaturated	Citrate	6.25×10^{-10}	23 ± 13	13.7	8.1	5.4 ± 0.2	3.1 ± 0.7	2.9 ± 0.8
	SNP	Unsaturated	Molasses	6.25×10^{-10}	52 ± 30	28.9 ± 0.1	2.2 ± 0.7	<2.4	<2.4	4.3 ± 1.4
	SNP	Unsaturated	No carbon	6.25×10^{-10}	1 ± 1	ND	ND	3.4 ± 1.1	<2.4	<2.4
	LOH	unsaturated	In situ	0.0	NA	NA	NA	>1.1	2.36 ± 0.6	>1.78
30	LOH	Topsoil	High atrazine	0.006	1.7 ± 1.0	ND	ND	<1.1	<3.58	<1.78
	LOH	Unsaturated	High atrazine	0.006	2.1 ± 1.2	ND	ND	<1.1	<3.58	<1.78
	LOH	Saturated	High atrazine	0.006	4.8 ± 2.8	ND	ND	<1.1	1.3 ± 0.1	<1.78
	LOH	Unsaturated	High atrazine, *Pseudomonas*	0.006	67	3.5 ± 0.01	27.4 ± 1.5	4.3 ± 0.2	<0.5	4.0 ± 0.2
10	LOH	Topsoil	High atrazine	0.006	1.3 ± 0.7	ND	ND	1.5 ± 1	1.0 ± 0.7	2.6 ± 0.2
	LOH	Unsaturated	High atrazine	0.006	0.8 ± 0.5	ND	ND	<1.1	0.9 ± 0.6	3.0 ± 0.3
	LOH	Saturated	High atrazine	0.006	1.7 ± 1.0	ND	ND	1.3 ± 0.5	2.4 ± 0.1	2.8 ± 0.3
	LOH	Unsaturated	High atrazine, *Pseudomonas*	0.006	46	ND	17.1	5.0 ± 0.2	1.8 ± 1.0	3.7 ± 0.2

Atrazine degradation gene copy numbers in triplicate at the beginning (in situ) and at 42 days of the experiment, with standard deviations

NA not applicable, *ND* not determined

and development procedures were performed as described elsewhere (Nielsen and Nielsen, 2005).

The exposed and developed filters were studied with a laser scanning microscope (Zeiss LSM 510 Meta). Microscopic evaluations of active cells were determined from 30 random images taken from each filter. The number of active cells was estimated using the threshold criterion of minimum five silver grains within a circle (Ø = 4 μm) covering a cell. Pasteurized (10 min at 80 °C) control samples or samples without addition of radioactive substrate did not result in formation of silver grain assemblages passing the applied threshold criterion. The total number of bacterial cells at the end of the 5 days incubation in MAR samples was determined by DAPI (4′,6-diamidino-2-phenylindole) staining as described elsewhere (Tuomi et al., 2004). Enumeration of the total DAPI count and the number of MAR-positive cells were performed with a custom-made macro for the ImageJ software (version 1.43u) (detailed information about the software can be found at www.cmc.aau.dk).

Microbial community in groundwater

Due to the very low cell number and DNA content in the soil samples, we were not able to obtain a reliable clone library from soil. Therefore, we chose to use Bio-Trap® samplers (Microbial Insights, TN, USA) to capture bacteria from the groundwater zone. Bio-Trap® samplers, containing activated carbon granules as the trapping material, were used to obtain a sample of the groundwater bacterial community. The samplers were installed in two groundwater monitoring wells at approximately 5 m depth at the LOH sampling area. Monitoring well 608 was situated on the edge of a forest, at a sandy ridge next to the waterworks, whereas monitoring well PM was located approximately 100 m away on a slight slope, surrounded by small-scale industries. After 4 months, the samplers were retrieved, transported to the laboratory, and opened in sterile conditions. The bacterial DNA was extracted directly from the sampler material using the same protocol as for extracting DNA from soil.

The number of *atzA* and *atzB* genes in the DNA was quantified as described above. The bacterial community composition was investigated by constructing a clone library of 16S ribosomal RNA (rRNA) genes in the extracted DNA pool (Yli-Hemminki et al., 2014). One clone library was constructed from each groundwater well, each consisting of 100 clones. Cloned inserts were sequenced using T7 and T3 sequencing primers at Macrogen (Seoul, South Korea). The sequences were deposited in the GenBank with the accession numbers KJ670501-KJ670677. The sequences were compared with those found in the NCBI Sequence Database using the BLAST tool on 19.5. 2011. Species richness was estimated by calculating Colwell rarefactions with EstimateS software version 9.1 and plotting the rarefaction curves.

Results

Atrazine mineralization

Atrazine mineralization was marginal in the microcosms where no bacteria or carbon was added, indicating low natural degradation (Fig. 1b). The addition of atrazine-degrading bacteria markedly increased atrazine mineralization (Fig. 1a, Table 1), and maximum mineralization in SNP samples using biostimulation at 10 °C was at the same level as in the bioaugmented LOH samples incubated at 30 °C (Fig. 1d, Table 1). However, the most efficient mineralization was achieved by a combination of additional carbon source and atrazine-degrading bacterium (Fig. 1c, Table 1). Amendment by *A. aurescens* and citrate mineralized 76 % of labeled atrazine (Fig. 1c, Table 1). The highest atrazine mineralization rate, 27 ± 1.4 % day^{-1}, was at 30 °C in unsaturated soil amended with atrazine and augmented with *P. citronellolis* (Table 1).

Quantification of atrazine degradation genes by qPCR

The degradation genes *atzA*, *trzN*, and *atzB* genes were quantified at the beginning, in the middle, and at the end of the experiment (day 42). The gene copy levels did not show a marked change after the midpoint of the experiment (21 days). The copy numbers in situ (representing the beginning of the experiment), and at the end of the experiment (42 days), are displayed in Table 1.

The gene copy numbers in situ were low, but increased when bioaugmentation treatments were used (Table 1). One-way ANOVA revealed a statistically significant increase in degradation gene copy numbers in the bioaugmented microcosms ($p < 0.001$, <0.000, and <0.001 for *atzA*, *trzN*, and *atzB* gene copy numbers, respectively). Citrate amendment supported higher *atzA* gene copy numbers, irrespective of bioaugmentation with bacteria ($p = 0.031$), suggesting that the native bacterial population can be activated with citrate amendments. The smallest changes of gene copy numbers were observed in microcosms where commercial atrazine was added without carbon amendments.

Concentrations of atrazine and its degradation products

The concentration of atrazine and its degradation products in atrazine-spiked soil at the beginning (0 day), in the middle (35 days for 30 °C and 56 days for 10 °C), and the end (56 days for 30 °C and 112 days for 10 °C) of the experiments are presented in Fig. 2. At 30 °C, atrazine concentration was reduced by 17–61 % in all soil layers while at 10 °C, the greatest atrazine removal was 34 %. The accumulation of degradation products, especially HA and DIA, was observed. At the beginning of the experiment, no HA was detected, but it started

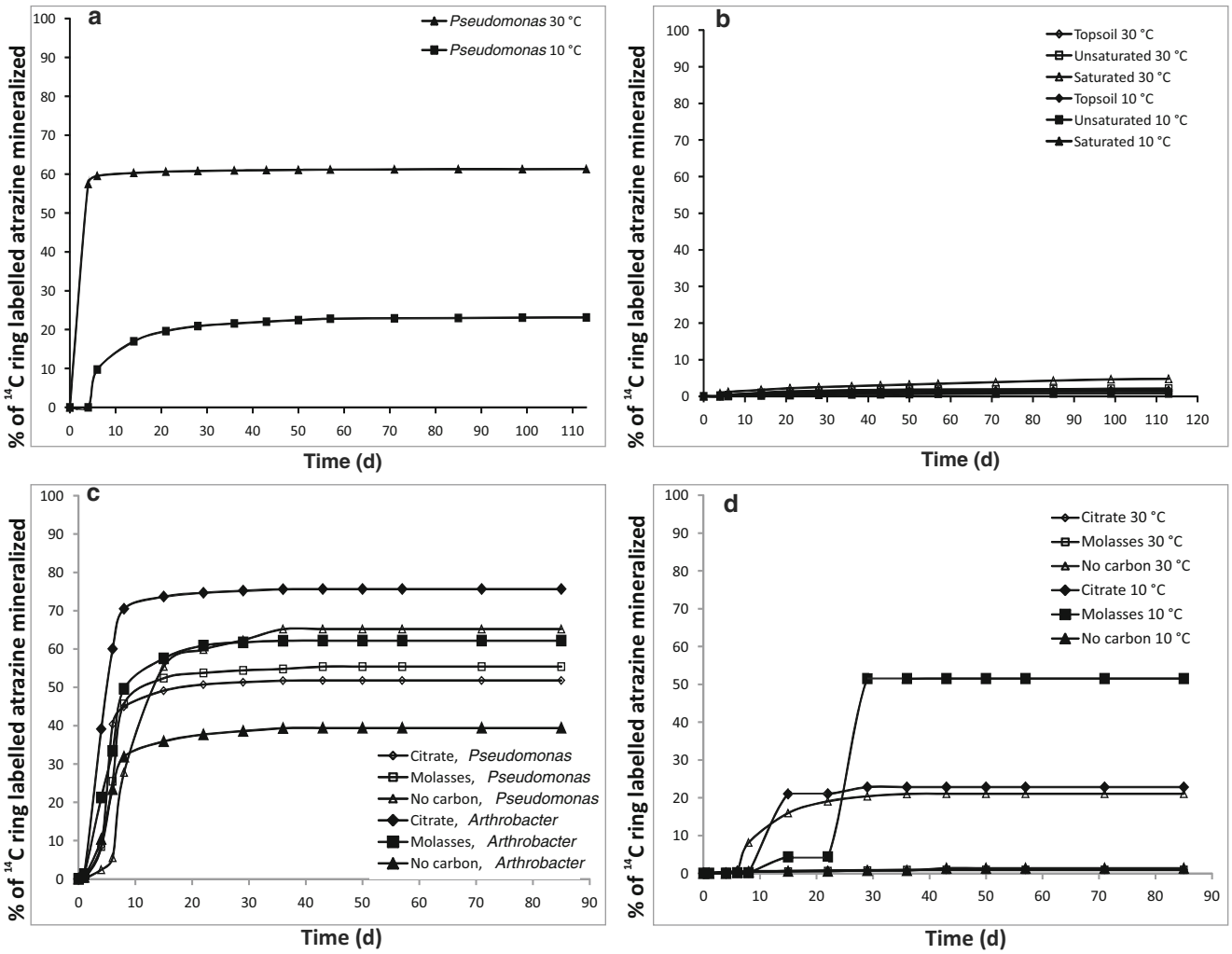

Fig. 1 Mineralization of ^{14}C-ring-labeled atrazine in LOH soil bioaugmented with *Pseudomonas citronellolis* at 30 and 10 °C (**a**) and in three LOH soil layers at 30 and 10 °C (**b**). Mineralization in bioaugmented (*Pseudomonas citronellolis* (atzAB) and *Arthrobacter aurescens* (trzN/atzB)) and biostimulated microcosms at 30 °C, site SNP (**c**). Mineralization in biostimulated (BS) microcosms at 10 and 30 °C, site SNP (**d**). For standard deviations, see Table 1

to accumulate at the midpoint of the experiment indicating atrazine degradation, even though the degradation genes *atzA* and *trzN* were not detected by quantitative PCR (qPCR). The commercial atrazine used to spike the microcosms contained DIA as an impurity, which was found in the beginning of the experiment at approximately 0.35 mg kg^{-1} soil (dw). It is therefore difficult to estimate the proportion of DIA formed out of degraded atrazine. The maximum concentration of DEA in soil was 0.01 mg kg^{-1}; the concentration of DEDIA was below detection limit (0.005 mg kg^{-1}) (data not shown).

Active atrazine-degrading cells

The total numbers of bacteria after 5 days of incubation, based on DAPI count, were 5.3×10^6 cells g^{-1} in unamended soil, 7.1×10^6 cells g^{-1} in soil amended with citrate, and 2.0×10^6 cells g^{-1} in soil inoculated with *P. citronellolis* and *A. aurescens*. The mean numbers of active atrazine-degrading cells, based on MAR incubation, were 1.8×10^4, 1.3×10^5, and 3.8×10^4 cells g^{-1} soil at 30 °C, respectively. The mean proportions of the atrazine-degrading bacteria estimated by MAR in different treatments were thus 0.3 % of the total cell count in native soil, 1.9 % in bioaugmentation treatment, and 1.8 % in biostimulation treatment. An example of representative MAR image is shown in Figure S2. Atrazine mineralized poorly in this experiment: radiorespirometric measurements parallel to the MAR experiments showed that <1 % of the labeled atrazine mineralized.

In the unamended soil, the silver grain densities around MAR-positive cells were generally smaller than in amended soils, indicating less active atrazine incorporation. When citrate- or atrazine-degrading bacteria were added, the differences in silver grain densities varied between the MAR-

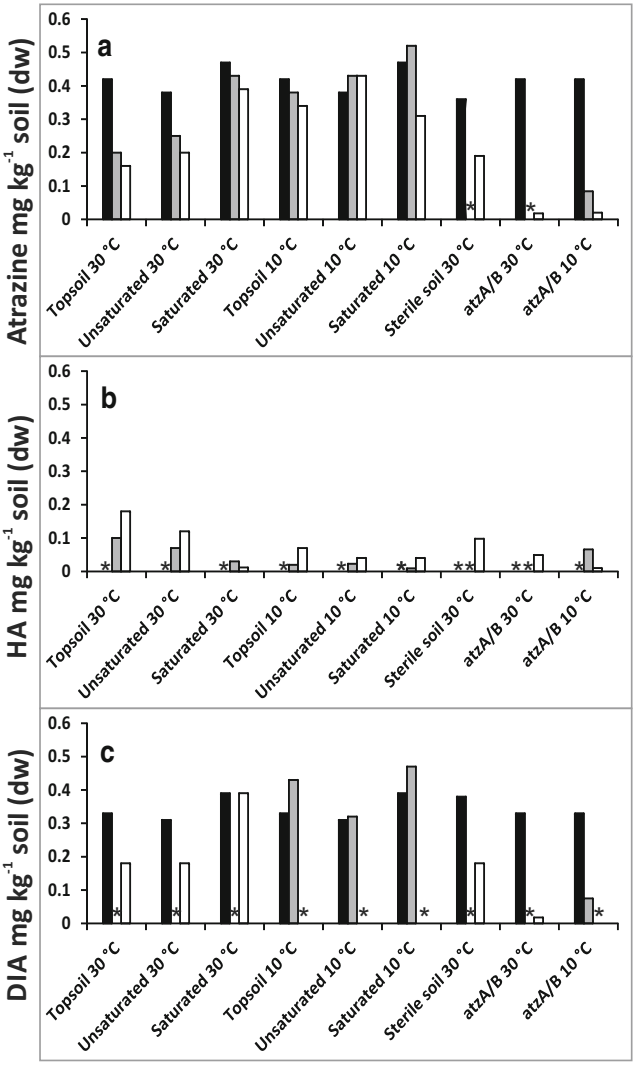

Fig. 2 Atrazine (**a**), hydroxyatrazine (HA) (**b**), and deisopropylatrazine (DIA) (**c**) concentrations in spiked microcosms from site LOH soil at the beginning (*black*), middle (35 days for 30 °C incubations, 56 days for 10 °C incubations) (*gray*), and end (56 days for 30 °C incubations, 112 days for 10 °C incubations) (*white*) of the experiment. *Asterisk* indicates below detection limit (0.005 mg kg^{-1}). DEA and DEDIA were measured, but not detected

positive cells, indicating that some of the atrazine degraders were stimulated by the presence of citrate and that the amended degraders were actively degrading atrazine.

Microbial community in groundwater

In groundwater monitoring well 608, the most common bacterial phyla were identified as α-, β-, and γ-proteobacteria and *Actinobacteria* (Table 2). Sequences of strains belonging to *Rhodococcus*, *Pseudomonas*, and *Arthrobacter* were found on this site. Seventy-seven percent of the clones were most closely related to members of cultured species; the most numerous of these clones were identified as *Acinetobacter*, *Aminobacter*, and *Rhodococcus*. The bacterial community composition in groundwater well PM differed from that of well 608 (Table 3). All clones belonged to either α-, β-, or γ-proteobacterial phyla, and *Rhodobacter* were commonly observed. Several matches to clones originating from activated carbon filters used for drinking water purification were found. As in well 608, many clones (84 %) in well PM were identified as close relatives of the members of cultured species. The PCR assay used for constructing the clone library amplifies 590-bp-long fragments of the 16S rDNA gene.

The copy numbers of *atzA* and *atzB* in well 608 were 6.3×10^3 and 2.7×10^4 g^{-1} activated carbon used as sampler material, respectively, and in well PM 1.3×10^4 and 4.6×10^4 g^{-1} sampler material, respectively.

Rarefaction analysis revealed that the size of the clone libraries adequately represented the bacterial community inhabiting the Bio-Trap® sampler (Figure S1).

Discussion

We explored the potential of two bioremediation techniques for soils with low atrazine concentration. Atrazine did not naturally mineralize in the boreal subsoils, most likely due to the low organic content and low number of degradation genes (Nousiainen et al., 2014). However, atrazine mineralization could be enhanced significantly by bioremediation treatments.

Biostimulated and bioaugmented (BS/BA) microcosms mineralized up to 76 % of labeled atrazine (Fig. 1). At elevated temperature (30 °C), atrazine mineralization was directed by the bioaugmented strains, likely because the incubation temperature was close to growth optimum of the strains used. In the combined treatments, additional carbon sources did not seem to have a pronounced effect on mineralization. At temperature similar to the ambient temperature in the boreal zone (10 °C), biostimulation of the indigenous, psychrophilic bacteria present in soil was a sufficient treatment method, with up to 52 % of available atrazine mineralized (Fig. 1d). Surprisingly, biostimulation was not successful in the elevated temperature, and based on the methods used in this study, it was not possible to deduce the how biostimulation treatment was dependent on the temperature. It is possible that the psychrophilic, indigenous bacteria suffered from the higher temperature used in this study, but in order to understand which organisms are responsible for the degradation in the boreal soil, more research should be carried out.

The survival of bioaugmentation strains is a key problem, and the persistence of *Pseudomonas* ADP in triazine bioremediation has previously been disputed (Morán et al., 2006; Zhao et al., 2003). Other studies using *Arthrobacter* different from the one used in the present study have shown that additional carbon decreases the density of the bioaugmented strain during bioremediation (Xie et al., 2012). In our study,

Table 2 Closest identities of the most abundant ($n > 2$) clones enriched in the Bio-Trap® samplers in ground water well 608

Closest match	Phylogenetic affiliation	Lowest similarity (%)	No. of clones	Accession no.	Clone origin
Acinetobacter sp.	γ-Proteobacteria	98	21	AJ551148.1	Antarctic/deep sea
Uncultured *Herminiimonas* sp.	β-Proteobacteria	96	16	HQ132390.1	Heavy metal contaminated estuarine sediment
Aminobacter aminovorans	α-Proteobacteria	97	10	NR025301.1	Azo dye-degrading consortium
Rhodococcus qingshengii	Actinobacteria	98	6	HQ439600.1	Data not available
Aminobacter sp.	α-Proteobacteria	98	5	FJ711220.1	Carbonate cave
Acinetobacter sp.	γ-Proteobacteria	98	3	AJ551155.1	Antarctic/deep sea
Aminobacter sp.	α-Proteobacteria	92	3	DQ401867.1	Dichlorobenil-treated soil
Herminiimonas saxobsidens	β-Proteobacteria	97	3	AB512141.1	Rock colonized with lichen
Aminobacter sp.	α-Proteobacteria	97	2	DQ401867.1	Dichlorobenil-treated soil
Aminobacter lissarensis	α-Proteobacteria	99	2	NR041724.1	Terrestrial environment
Rhodococcus sp.	Actinobacteria	98	2	FN646613.1	Soil from Himalayan glacier
Uncultured *Comamonadaceae* sp.	β-Proteobacteria	97	2	AF523046.1	Bottled natural mineral water

A. aurescens genotype *trzN*/*atzB* was always present with augmentation. The *P. citronellolis*-genotype *atzA*/*atzB* was only detected when additional carbon was used, indicating that *P. citronellolis* cannot compete with the autochthonous population in the absence of an additional carbon source. This effect was pronounced when citrate was used, as the net mineralization rate of atrazine was higher in citrate-amended microcosms than in treatments with molasses or controls without carbon. It is noteworthy that in all the microcosms where genotype *atzA*/*atzB* was added, also the *trzN* gene was observed. The prevalence of *trzN* over *atzA* gene in the soil environment reported by Arbeli and Fuentes (2010) might explain why it was often encountered in our experiment. Overall, the measured degradation gene numbers were high in bioaugmented treatments, but often below detection limit in the biostimulated treatments. Despite the low gene copy numbers, a significant amount of atrazine disappeared, e.g., 55 % when molasses was used (Table 1). It is possible that carbon addition directed degradation towards dealkylation, rather than dechlorination of atrazine (Ngigi et al., 2013).

It is not possible from the observations made in this study to estimate the significance of the different degradation pathways and to differentiate between the degradation efficiencies of bioaugmented strains and autochthonous bacteria. Despite this, our results suggest that the gene pool provided by the autochthonous bacteria is sufficient for degrading trace amounts of atrazine, and it possibly contains multiple and unidentified pathways for atrazine degradation. We used microautoradiography (MAR) to estimate proportions of atrazine-degrading bacteria from the total bacteria. This method measures the ability to assimilate atrazine at the cellular level and thus the presence and degradation efficiency of microbial cells capable of degrading the compound. The applied

Table 3 Closest identities of the most abundant ($n > 2$) clones enriched in the Bio-Trap® samplers in ground water well PM

Closest match	Phylogenetic affiliation	Similarity (%)	No. of clones	Accession no.	Clone origin
Rhodobacter sp.	α-Proteobacteria	99	16	HM156123.1	Glacier forefield
Rhodobacter sp.	α-Proteobacteria	98	14	GU441680.1	Glacier cryoconite
Uncultured *Herminiimonas* sp.	β-Proteobacteria	95	11	HQ132390.1	Heavy metal contaminated estuarine sediment
Polaromonas sp.	β-Proteobacteria	96	9	AB426569.1	Freshwater bacterioplankton
Methylotenera sp.	β-Proteobacteria	97	6	NR074693.1	Lake sediment
Pseudomonas sp.	γ-Proteobacteria	98	5	GQ345341.1	PAH containing river sediment
Methylotenera mobilis	β-Proteobacteria	98	4	DQ287786.1	Lake sediment
Hydrogenophaga sp.	β-Proteobacteria	98	3	EU130968.1	Activated carbon filters in water treatment
Polaromonas sp.	β-Proteobacteria	97	3	EU130980.1	Activated carbon filters in water treatment
Polaromonas sp.	β-Proteobacteria	98	3	EU130985.1	Activated carbon filters in water treatment
Pseudomonas putida	γ-Proteobacteria	97	2	DQ178233.1	Phenol- and creosol-degrading species

approach using density gradient centrifugation most likely secures quite similar cell extraction efficiencies of all soil microorganisms, and thus, a relative estimate of the MAR-positive fraction should allow an unbiased evaluation of the effect provided during the incubation. MAR has predominantly been used in aqueous environments, in which soil colloids provide excellent conditions for single-cell resolution and easy visualization of the produced silver grains. In soils and sediments, extraction of the cells is necessary but may lead to significantly reduced cell recovery (Pascaud et al., 2012). Despite the challenges created by the soil particles, MAR is a robust method for circumventing the need to study specific functional genes, because the activities of the cells are determined by the assimilation of the applied radioactive substrate. In our study, the proportion of atrazine-degrading bacteria was small in all treatments, but it increased when bioremediation treatments were used. The results correlated with degradation gene copy numbers, as the numbers in situ are several orders of magnitude smaller than in biostimulated and bioaugmented treatments. In 1 gram of biostimulated soil, both the number of MAR-positive cells calculated to actual numbers from the total DAPI count and the copy numbers of *atzA* and *atzB* genes estimated by qPCR were approximately 10^4 cells and copies g^{-1} soil, respectively, after 5 days of incubation. The correlation depends on plasmid copy number. If the catabolic plasmid is present in single copy, the gene copy numbers correlate to the MAR-positive cell numbers, whereas multiple copy plasmids will result in the number of degradation genes exceeding the number of MAR-positive cells. The first scenario was true for in situ conditions, where the number of MAR-positive cells (10^3 cells g^{-1} soil) surpass the number of degradation genes (10^2 copies *atzA*, 10^3 copies *atzB* g^{-1} soil). It is also likely that some of the MAR-positive bacteria used an unknown degradation pathway we could not target by the applied PCR approach. The highest proportion of known atrazine degraders was expected and observed in the bioaugmentation treatments. Biostimulation treatments also increased the number of degraders, supporting our hypothesis that carbon addition stimulates the native atrazine-degrading bacterial population in carbon-limited soil. To our knowledge, this is the first attempt to visualize and enumerate indigenous and bioaugmented active atrazine-degrading bacteria by MAR.

Knowledge about the composition of the autochthonous bacterial community is needed when the bioremediation strategy is chosen. Often the factor limiting degradation is not the absence of contaminant-degrading bacteria or degradation genes, but rather the environmental conditions, which do not favor degradation (Thompson et al., 2005). If potential degraders are absent, bioaugmentation may be the only remediation option. Clone libraries revealed that bacteria belonging to known atrazine-degrading species were residing in the groundwater zone. In well 608, the most common clones were identified as *Acinetobacter* (24 %), which are known to be versatile degraders and efficient atrazine degraders in high concentration (Singh et al. 2004). Other clones related to potential atrazine degraders included *Microbacterium* species (Morohoshi et al. 2011), as well as *Polaromonas* (Devers et al. 2007), *Aminobacter* (McDonald et al. 2005), *Rhodococcus* (Behki et al. 1995), *Pseudomonas*, and *Arthrobacter*, species extensively studied because of their atrazine-degrading capabilities. As the 16S rRNA gene used for constructing the clone library does not reflect the metabolic functions the bacteria carry out, it is a poor indication of atrazine degradation as such. This is why the presence of degraders was also confirmed by the amplification of degradation genes by qPCR.

Many of the clones in our library were close matches to cultured bacteria. A likely reason is that only the actively growing fraction of the bacterial community is able to colonize the carbon beads of the Bio-Trap® sampler (Sublette et al., 2006). Despite this bias, the clone libraries identified several organisms that belong to phylogenetic groups known to contain atrazine degraders. The prerequisite of growth and the presence of degradation genes on sampler material indicate that the *atzA* and *atzB* containing bacteria are actively growing.

The positive effect of carbon additions on atrazine mineralization is probably a result of cometabolic processes (Willems et al., 1996). Contrary findings on carbon-promoted degradation have been observed in agricultural soils having high atrazine concentrations (Silva et al., 2004; Xie et al., 2012). These contradicting results highlight the importance of understanding the effect of environmental conditions, as they heavily impact bioremediation treatment. We hypothesize that in our soil, the low carbon content was the factor limiting bacterial growth, and our results show that changing this parameter allowed the removal of residual atrazine in soil.

The addition of atrazine-degrading bacteria is a powerful tool to remediate contaminated soil. Residual atrazine persisted in nutrient-poor subsoil because the nutritional conditions did not favor its degradation, even though potential degraders were present. In the boreal subsoil examined in this study, the combination of biostimulation and bioaugmentation efficiently removed atrazine. Furthermore, the addition of carbon was an efficient aid in atrazine degradation in itself, suggesting that simple biotechnological applications may be sufficient to remediate atrazine from nutrient-poor environments.

In conclusion, bioremediation of low concentrations of atrazine is problematic, since a limit concentration is required for bacterial degradation of any compound. In the case of carbon-poor, boreal subsoil, where atrazine is most problematic, a simple solution may be provided by balancing the microbially available carbon and nitrogen so that atrazine becomes a lucrative source of nitrogen. However, if applicable, the addition of atrazine-degrading bacteria in soil is a fast and efficient method of removing low atrazine concentrations from boreal soil.

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