

Biominingalization of atrazine and analysis of 16S rRNA and catabolic genes of atrazine degraders in a former pesticide mixing site and a machinery washing area

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Abstract

Purpose The purpose of this study was to determine the first-order rate constants and half-lives of aerobic and anaerobic biominingalization of atrazine in soil samples from an agricultural farm site that had been previously used for mixing pesticide formulations and washing application equipment. Atrazine catabolic genes and atrazine-degrading bacteria in the soil samples were analyzed by molecular methods.

Materials and methods Biominingalization of atrazine was measured in soil samples with a [U-ring-¹⁴C]-atrazine biometer technique in soil samples. Enrichment cultures growing with atrazine were derived from soil samples and they were analyzed for bacterial diversity by constructing 16S rDNA clone libraries and sequencing. Bacterial isolates were also obtained and they were screened for atrazine catabolic genes.

Results and discussion The soils contained active atrazine-metabolizing microbial communities and both aerobic and anaerobic biominingalization of [U-ring-¹⁴C]-atrazine to ¹⁴CO₂ was demonstrated. In contrast to aerobic incubations, anaerobic biometers displayed considerable differences in the kinetics of atrazine mineralization between duplicates. Sequence analysis of 16S rDNA clone libraries constructed from the enrichment cultures revealed a preponderance of *Variovorax* spp. (51 %) and *Schlesneria* (16 %). Analysis of

16S rRNA gene sequences from pure cultures ($n = 12$) isolated from enrichment cultures yielded almost exclusively *Arthrobacter* spp. (83 %; 10/12 isolates). PCR screening of pure culture isolates for atrazine catabolic genes detected *atzB*, *atzC*, *trzD*, *trzN*, and possibly *atzA*. The presence of a complete metabolic pathway was not demonstrated by the amplification of catabolic genes among these isolates.

Conclusions The soils contained active atrazine-metabolizing microbial communities. The anaerobic biometer data showed variable response of atrazine biominingalization to external electron acceptor conditions. Partial pathways are inevitable in soil microbial communities, with metabolites linking into other catabolic and assimilative pathways of carbon and nitrogen. There was no evidence for the complete set of functional genes of the known pathways of atrazine biominingalization among the isolates.

Keywords *Arthrobacter* spp. · *Atz* and *trz* genes · Biodegradation of atrazine · Herbicide biodegradation · Pesticide biodegradation

1 Introduction

Atrazine is in common use as an agricultural herbicide in the U.S.A. As a consequence, atrazine residuals and metabolites and atrazine-degrading microorganisms are widely found in surface soils, rivers and wetlands in agricultural watersheds (Lazorko-Connon and Achari 2009; Mudhoo and Garg 2011). Half-lives of atrazine biodegradation can be shorter than 10 days in agricultural soils that have decades of annual atrazine applications (Pussemier et al. 1997). Soil microbial communities vary greatly in atrazine biodegradation potential. Biodegradation and biominingalization of atrazine involve multiple enzymatic steps, most hydrolytic, and some

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alternatively oxidative, which vary in pathway sequence, metabolites and genes in soil and sediment bacterial communities (Krutz et al. 2010; Lian et al. 2012; Udiković-Kolić et al. 2012; Fenner et al. 2013; Fan and Song 2014; Singh and Singh 2016). The best known biodegradative pathway is the Atz, which was originally elucidated in *Pseudomonas* ADP and involves six proteins (AtzA, AtzB, AtzC, AtzD, AtzE, and AtzF) (de Souza et al. 1995; Boundy-Mills et al. 1997; Sadowsky et al. 1998; Martinez et al. 2001; Cheng et al. 2005). Other degradative enzymes have been characterized, such as TrzN (triazine chlorohydrolase) in *Nocardioideis* sp. strain C190 (Mulbry et al. 2002) and *Arthrobacter aureescens* TC1 (Sajjaphan et al. 2004), and TrzD (cyanuric acid amidohydrolase) in *Pseudomonas* sp. strain NRRLB-12227 (Eaton and Karns 1991) and *Klebsiella pneumonia* strain 99 (Karns and Eaton 1997). To date, analyses of catabolic genes in soil bacterial isolates suggest that the complete Atz pathway is relatively rare and various combinations of *atz* and *trz* genes are more common in environmental isolates (Devers et al. 2007; Vaishampayan et al. 2007; El Sebaï et al. 2011). It is likely that pathway intermediates feed into other degradative pathways in microbial communities and the complete mineralization through the Atz pathway may not be dominant. Examples of cooperative pathways could include the hydrolytic release of the ring side chains ethanolamine and isopropyl amine, both biodegradable by many microorganisms that cannot degrade the parent molecule atrazine, or the ring cleavage metabolites such as urea, an excellent N source for soil microorganisms. Neither the Atz nor Trz pathway requires molecular O₂ and thus they can function in both aerobic and anaerobic environments.

Cooperative pathways and synergistic metabolism of intermediates are central in the attenuation of atrazine by soil microorganisms. Several eukaryotic soil microorganisms in genera such as *Cryptococcus*, *Phanerochaete*, *Pichia*, and *Pleurotus* have also been described that can mediate atrazine biodegradation but these pathways do not proceed beyond cyanuric acid formation (Donnelly et al. 1993; Mougin et al. 1994; Masaphy et al. 1996; Bastos and Magan 2009; Abigail et al. 2012, 2013; Romero et al. 2014). Laccases may be involved, but specific catabolic genes associated with atrazine biodegradation have yet to be characterized in eukaryotic microorganisms.

For this study, the biomineralization potential of atrazine in an agricultural farm setting in central Ohio was examined. Two sites at this farm were of specific interest: (1) a pesticide mixing area that had been abandoned by late 1990s, about 10 years prior to this study, and (2) an adjacent (about 30 m) area that has received spills from washings of pesticide formulations from application equipment in a shed. Previous studies have shown that agricultural farm and agrochemical dealer mixing and spill areas have microbial populations that can actively degrade residual pesticide mixtures (Arthur et al.

2000; Chirnside et al. 2007, 2009; Udiković et al. 2003; Udiković-Kolić et al. 2007, 2010). Potential problems of point source pesticide contamination in agricultural farm sites have been mitigated with systems whereby the spray or washing area is covered with rich organic material which supports pesticide retention and microbial degradation. Several configurations and performance data have been discussed in the literature (Grigg et al. 1997; Arthur et al. 2000; de Wilde et al. 2007; del Pilar Castillo et al. 2008; Spanoghe et al. 2009). However, soil-bound residues of atrazine from historic applications or spills can be persist for decades (Liu et al. 2016).

At the farm site in this study, atrazine and other pesticides have been used on and off depending on crop rotation and integrated pesticide management practices. No special remediation was undertaken over the years of practicing pesticide disposal until the late 1990s, at which time the containment and storage of liquid pesticide residues was initiated. A previous study of enrichment of atrazine degraders from the farm site yielded a soil bacterial isolate M91-3 (Radosevich et al. 1995), subsequently identified as *Ralstonia basilensis* (Stamper et al. 2002), obtained originally from samples collected from the pesticide mixing and spill site. The purpose of this study was to determine the half-lives of aerobic and anaerobic biomineralization of atrazine in soil samples from the past pesticide mixing and spill and the machinery washing areas. Biomineralization was measured in biometers using soil samples and [U-ring-¹⁴C]-atrazine. Bio-Sep beads were also tested for trapping atrazine degraders from soil. Soil-borne atrazine degraders have been previously probed with porous Bio-Sep beads (Ghosh et al. 2009; Omotayo et al. 2011). Atrazine enrichment cultures from the soil samples and field-incubated Bio-Sep beads yielded atrazine-degrading mixed cultures and isolates of bacteria. Phylogenetic analyses based on 16S rRNA gene sequences and the screening of known catabolic genes of atrazine metabolism were included in the scope of this study.

2 Materials and methods

2.1 Sampling

The Western Agricultural Research Station of the Ohio Agricultural Research and Development Center is located 5.6 km northwest of South Charleston, Ohio (39°86'38"N, 83°67'22"W). The farm (established in 1958) is a 173 ha agricultural research facility that supports agronomic and specialty crop programs, including corn and soy, in the western part of Ohio. As part of the weed control programs, the management practice has involved the application of atrazine as a pre-emergence weed killer in corn fields. The soil at the site is primarily Strawn–Crosby complex (0 to 2 % slopes) (<http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx>).

The farm site has a shed building where herbicide formulations are stocked and prepared for transportation to the field. Next to the shed was a grassy area (Fig. 1) where pesticides were mixed (site 1), and this area received numerous pesticide spills over the years. A second adjacent grassy area was at the base of the ramp to the shed where pesticide application machinery was rinsed (site 2). About 10 years prior to this study, the practice of allowing spills and rinsing outdoors was discontinued and liquid waste was contained to a special holding tank for further treatment. Both sites are now grass-covered.

Soil samples were collected from site 1 (the former pesticide mixing site) and site 2 (machinery washing area) in mid-May. Several grab samples of top soil (0–7 cm) were removed with a sterile trowel to yield about 500 g composite and stored in sterile plastic bags on ice until processed in the laboratory. The sampling avoided grass roots. Previous studies generally indicate that maximum activity of atrazine biodegradation is usually associated with the soil top layer (e.g., Tuovinen et al. 2015). No effort was made to sample the subsurface.

Bio-Sep beads were equilibrated in solution of 2 mg atrazine/l or distilled water for 3 days before they were closed in sterile nylon mesh bags (~30 beads per bag) and sealed with plastic clips. Bead bags were buried in the top 10-cm layer of soil in site 1 at the former pesticide mixing area and retrieved

after 33 days of contact time with soil (mid-May to mid-June). Only site 1 was sampled with Bio-Sep beads because the thrust of the study was on soil sampling directly for biometers and enrichments of atrazine degraders.

2.2 Biomineralization of [U-ring-¹⁴C]-atrazine in biometers

Aerobic and anaerobic mineralization of atrazine in soil samples and Bio-Sep beads was measured with the biometer technique that has been previously described (Douglass et al. 2014, 2015). The biometers (60-ml volumetric capacity) contained 5 g soil samples or 6–10 diced Bio-Sep beads and 0.064 μmol [U-ring-¹⁴C]-atrazine \pm glucose (11.1 mM). In order to prevent possible microelement limitation, biometers with Bio-Sep beads received 5 ml mineral salts (MS) solution, which contained (per liter): K_2HPO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{FeCl}_3 \cdot \text{H}_2\text{O}$, 0.1 mg; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1 mg; MnCl_2 , 0.01 mg; ZnCl_2 , 1 μg ; pH 6.8. Anaerobic incubations under N_2 head space involved 25 μmol nitrate as KNO_3 , 125 μmol Fe(III) as ferrihydrite, or 15 μmol sulfate as Na_2SO_4 per biometer as external electron acceptors or distilled H_2O as a blank. These additions were intended to approximate e^- -equimolar acceptor conditions. Controls of sterile soil were included in biometer incubations.

The biometers were incubated at 22 ± 2 °C in the laboratory. Biometer sampling and measurement of ¹⁴CO₂ have been previously described (Douglass et al. 2014). ¹⁴CO₂ evolution was calculated as a percentage of the total amount of radioactivity of the [U-ring-¹⁴C]-atrazine added to the biometers at the beginning of the time course. Standard error bars were included for the replicates. Rate constants (k , days⁻¹) and half-lives ($t_{1/2}$, days) of atrazine mineralization were determined with a pseudo first-order rate expression (Guerin and Boyd 1992).

2.3 Enrichment of atrazine degraders

Aerobic enrichment cultures were set up with 2.5 g soil or 6–10 Bio-Sep beads in MS media containing 30 mg/l atrazine \pm glucose amendment (2 g glucose/l) and were incubated in shake flasks (150 rpm) at 22 ± 2 °C. Aliquots of enrichment cultures were transferred to the corresponding fresh media at intervals of 2–4 weeks. After at least six transfers to fresh MS media with 30 mg/l atrazine, enrichment cultures were screened for residual atrazine concentration using HPLC with a UV detector at 220 nm as previously described (Radosevich et al. 1996). Samples of cultures were mixed with mobile phase (65 % HPLC-grade acetonitrile in HPLC-grade distilled water) followed by centrifugation at $16,000 \times g$ for 15 min. The supernatants were transferred to HPLC vials in an autosampler and pumped through an Adsorbosphere C18 HPLC column at a flow rate of 1.0 mL/min.

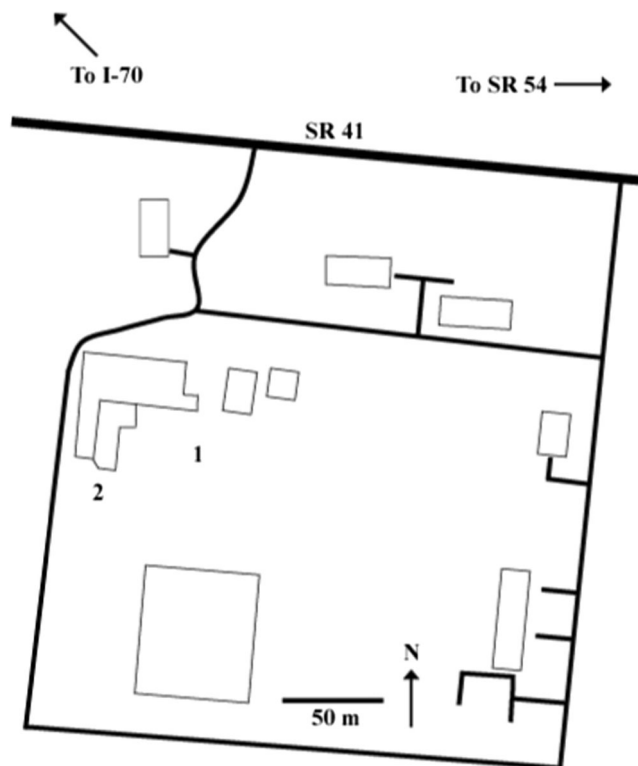


Fig. 1 Sampling sites at the Western Agricultural Research Station. 1 = former pesticide mixing site, 2 = former machinery washing area. The sites were about 200 m² each. Letter symbols: SR 41, state route 41 (South Charleston Pike); SR 54, state route 54 (Urbana Lisbon Road); I-70, Interstate 70 highway

Reference samples containing uninoculated sterile MS + atrazine media and standards with varying concentrations of atrazine dissolved in mobile phase were included with every HPLC run. Enrichments that exhibited at least 80 % decrease in atrazine concentration were deemed to contain atrazine-degrading mixed cultures.

2.4 Isolation of atrazine-degrading pure cultures

Prospective enrichment cultures of atrazine degraders were inoculated to solid MS + atrazine media. Dilutions were spread-plated in MS + atrazine media solidified with 1.5 % Noble agar. All plates were incubated at 22 ± 2 °C for up to 2 weeks. Discreet colonies from the spread plates were subcultured twice on streak plates before transferring them into 5 ml of corresponding liquid MS media. Changes in atrazine concentration were monitored with HPLC during subsequent incubation in liquid media. Pure cultures were stored at 4 °C. Liquid cultures were stored as 20 % glycerol stocks at -55 °C.

2.5 DNA isolation, 16S rRNA gene amplification, and denaturing gradient gel electrophoresis (DGGE)

Genomic DNA was isolated from pure and mixed cultures with MoBio UltraClean Microbial DNA Isolation Kits per manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). The V3 region of the 16S rRNA gene from atrazine-degrading mixed cultures was amplified in 50 µl total volume, consisting of 1X TaKaRa Premix Taq (1.25 U *Taq*; 0.2 mM dATP; 0.2 mM dTTP; 0.2 mM dCTP; 0.2 mM dGTP; 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂), 1 µM primers 357fGC and 518r (Muyzer et al. 1993), and 20 ng of template DNA. The PCR amplification followed a previously established protocol (Douglass et al. 2015). PCR products were resolved with DGGE using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA). Gels consisted of 8 % acrylamide and a 40–60 % denaturant gradient. Gels were electrophoresed at 83 V for 16 h in 0.5X TAE buffer solution at 60 °C. After electrophoresis, gels were stained in 1X SYBR Gold (Molecular Probes, Eugene, OR) for 30 min on a rocking platform and visualized on a UV transilluminator. Mixed cultures corresponding to unique DGGE banding patterns were selected for further analysis via 16S rDNA clone library sequencing.

2.6 16S rDNA clone library formation and analysis

Genomic DNA from atrazine-degrading mixed cultures and isolates was amplified for the approximately 1,500-bp region of the 16S rDNA using 1 µM primers 27f and 1525r (Lane

1991) and 20 ng of template DNA. The PCR conditions followed previously published protocols (Douglass et al. 2015). PCR products were used to construct 16S rDNA clone libraries. The clones were made using the Promega pGEM-T vector system and JM109 high-efficiency competent cells (Promega, Madison, WI). Overnight cultures of clones were plated onto 96-well plates and sent to the Clemson University Genomics Institute for sequencing. For each pure culture, three clones were sequenced; for each mixed culture, 25 clones were sequenced. Sequences were edited in Geneious (<http://www.geneious.com/>). A consensus sequence was generated for each set of three clones resulting from pure cultures when possible, and these consensus sequences were entered into the NCBI-BLAST and Ribosomal Database Project (RDP) databases. If a consensus sequence of three clones was not available, individual edited clone sequences or consensus sequences constructed from two clones were entered as queries into the aforementioned databases. Each individual edited clone sequence resulting from mixed cultures was searched in these databases.

The 16S rRNA gene sequences were deposited with GenBank. The accession numbers for aerobic mixed culture clones are KP779907–KP779962. The accession numbers for aerobic isolates are KP779963–KP779997.

2.7 Atrazine catabolic gene detection in atrazine-degrading bacterial isolates

Genomic DNA from atrazine-degrading isolates was amplified for known atrazine catabolic genes *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF*, *trzD*, and *trzN*. *Escherichia coli* genomic DNA was also amplified as a negative control for all catabolic genes. *Pseudomonas ADP* was used as a positive control for *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, and *atzF*, and as a negative control for *trzD* and *trzN*. *Arthrobacter aurescens* TCI was used as a positive control for *trzN* as well as an additional positive control for *atzB* and *atzC* and a negative control for *atzA*, *atzD*, *atzE*, and *atzF*. *Klebsiella pneumoniae* 99 was used as a positive control for *trzD*.

PCR reactions were conducted with 1 µM forward primer and 1 µM reverse primer as specified in Table 1 and 20-ng template DNA as described by Douglass et al. (2015). PCR products were loaded onto agarose gel, and after electrophoresis the gel was stained with ethidium bromide and visualized with a UV gel imager. Detection of a band of anticipated size was treated as preliminary evidence of the presence of the given catabolic gene, and a random assortment of these PCR reactions were used to create clone libraries as outlined in section 2.6. For each PCR reaction, three clones were sequenced. When possible, a consensus sequence for these three clone sequences was generated and used for bioinformatics analysis; otherwise, a consensus sequence of two clones was

Table 1 Genes and PCR primers used in the study

Gene	Gene size (bp)	Protein	Primers	PCR product size (bp)	Annealing temperature (°C)	Reference
<i>atzA</i>	1,425	Atrazine chlorohydrolase	atzAf-CCATGTGAACCAGATCCT atzAr-TGAAGCGTCCACATACC	500	55	de Souza et al. (1998)
<i>atzB</i>	1,446	Hydroxyatrazine ethylaminohydrolase	atzBf-TCACCGGGATGTCGGGGC atzBf-CTCTCCCGCATGGCATCGGG	500	68	de Souza et al. (1998)
<i>atzC</i>	1,212	N-isopropylammelide isopropylamidohydrolase	atzCf-GCTCACATGCAGGTACTCCA atzCi-GTACCATATCACCCGTTTGCCA	600	61	de Souza et al. (1998)
<i>atzD</i>	1,092	Cyanuric acid amidohydrolase	atzDf-TCCCACTGACATCACAAC atzDi-GGGTCTCGAGGTTTGATTG	200	61	Devers et al. (2004)
<i>atzE</i>	1,374	Biuret hydrolase	atzEf-GAGCCTCTGTCCGTAGATCG atzEe-GATGGCGTGTACCGTTTACC	200	63	Devers et al. (2004)
<i>atzF</i>	1,818	Allophanate hydrolase	atzFf-ACCAGCCCTTGAATCAATCAG atzFr-TATTGTCCCAGTACCCCAACG	200	63	Devers et al. (2004)
<i>trzD</i>	1,125	Cyanuric acid amidohydrolase	trzDf-CCTCGGTTCAAGGTCTACT trzDi-TCGAAGCGATAAC-TGCAITG	800	63	Rousseaux et al. (2001)
<i>trzN</i>	1,371	Atrazine chlorohydrolase	trzNf-CACCAGCACCTGTACGGAAGG trzNi- GATTGGAACCATTCCAAACG	420	64	Mulbry et al. (2002)

generated, or a single edited clone sequence was used. Query sequences were entered into NCBI-BLAST and aligned with their corresponding catabolic gene sequences. Catabolic gene sequences were deposited with GenBank, and the accession numbers are KP863790-KP863798.

3 Results and discussion

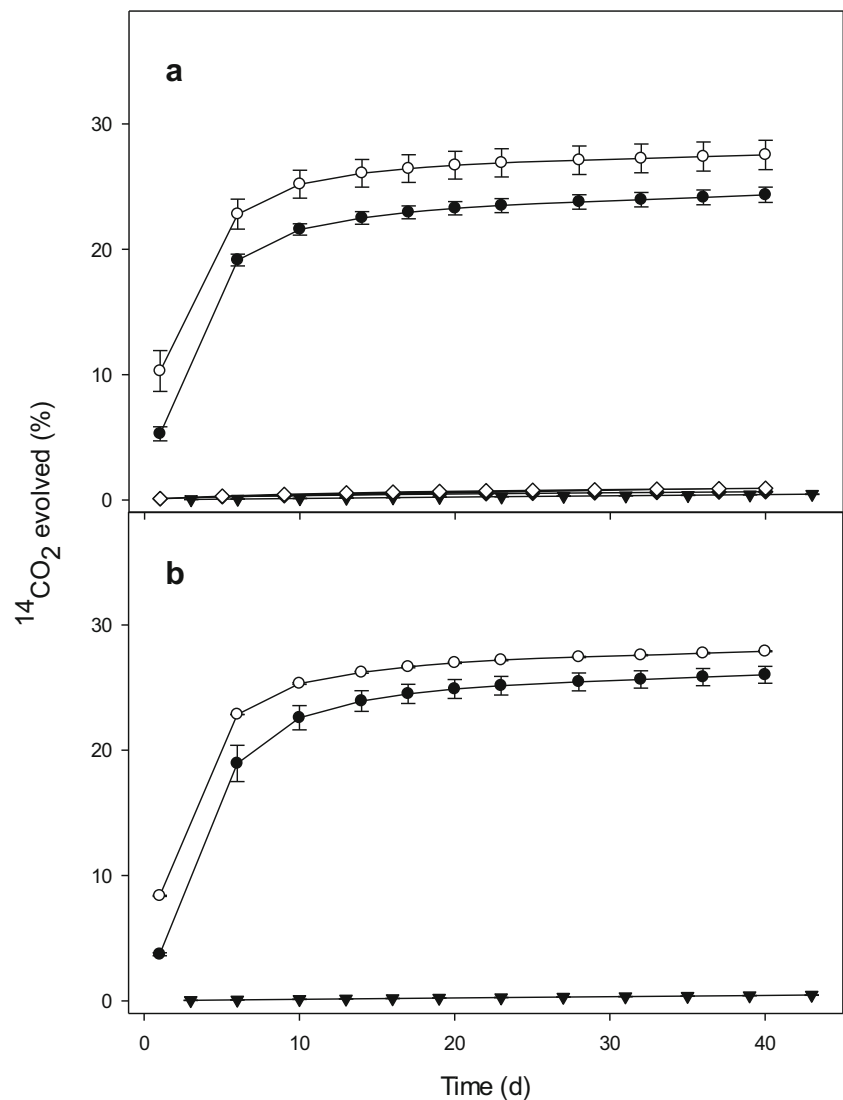
3.1 Measurement of atrazine mineralization in biometers with [U-ring-¹⁴C]-atrazine

Under aerobic conditions, mineralization of atrazine in both sites reached ~20–25 % in biometers (Fig. 2). Glucose amendment had no discernible effect on mineralization. Enhancement of glucose amendment could indicate that atrazine-mineralizing bacteria are limited by carbon rather than other nutrients. Whether glucose is co-metabolized with atrazine or only increases the amount of active biomass is not known. In this case, the lack of glucose effect suggests that bacteria were not limited for carbon and glucose did not specifically increase the biomass or activity of atrazine-mineralizing bacteria.

Mineralization in biometers inoculated with Bio-Sep beads was comparable to sterile controls (Fig. 2). The lack of ¹⁴CO₂ evolution from Bio-Sep bead biometers was attributed to negligible size of atrazine-degrading microbial community associated with the beads used in the assay. This assertion is supported by the fact that after an enrichment phase, mixed cultures derived from Bio-Sep beads degraded atrazine as confirmed via HPLC analysis. Subsequently, atrazine-degrading pure cultures were successfully isolated from mixed cultures enriched from Bio-Sep beads.

Under anaerobic conditions, pesticide mixing area soil (site 1) outperformed machinery washing area soil (site 2) in biometers with Fe(III) amendment (~20 vs. 6 %) and no electron acceptor amendment (~18 vs. 7 %). The opposite trend was true in the nitrate-amended biometers (~26 vs. 22 %). Microbial communities in both soils mineralized atrazine to a similar extent (~20 %) under sulfate amendment (Fig. 3). The soil sample from the washing area (site 2) mineralized less than 10 % of the added atrazine in the absence of external electron acceptors as well as in ferrihydrite-amended biometers. The reduction of the external electron acceptors nitrate, Fe(III), and sulfate was not tested in the ¹⁴C-labeled atrazine biometer experiments. In general, biometers constructed with samples from the former pesticide mixing area (site 1) had large variation between duplicate anaerobic biometers. The variation was due to different lag times and extent of mineralization between the duplicates. In general, the variation reflects spatial heterogeneity in microscale in soil microbiology that has been reported numerous times in terms of

Fig. 2 Aerobic mineralization of [U-ring- ^{14}C]-atrazine in biometers in the absence (a) and presence (b) of glucose amendment. Symbols: (black circle), site 1, soil from the former machinery washing area; (white circle), site 2, soil from the former pesticide mixing area; (black down-pointing triangle), sterile soil control; (white diamond), water-equilibrated Bio-Sep beads retrieved from site 1; (black diamond), atrazine-equilibrated Bio-Sep beads retrieved from site 2. Bio-Sep beads were only used to inoculate biometers without glucose. Data points represent mean cumulative percentages obtained from duplicate biometers, with the exception of those representing Bio-Sep biometers, which represent results from single biometers. Bars indicate the standard error for the replicates, and in some cases are smaller than the data point symbols



microbial numbers and activity as well as community structure and composition (e.g., Ettema and Wardle 2002; O'Donnell et al. 2007).

Thus the anaerobic biometer results indicated that nitrate and sulfate enhance the biomineralization of atrazine. Atrazine and nitrate especially are common pollutants in surface runoff from agricultural fields. If the runoff is trapped in a riparian zone or a wetland system, nitrate flux may promote the concurrent biodegradation of atrazine. Whether it is due to denitrification is not clear because atrazine can also be metabolized via hydrolytic steps. However, metabolites such as the ethyl and isopropyl side chains and the lower pathway intermediates from atrazine biodegradation may link into other degradative pathways involving respiratory activities in microbial consortia.

The half-lives of aerobic mineralization of atrazine calculated from the first-order rate constants indicated relatively

fast atrazine attenuation, about 3 days in the soil samples from the washing area (site 2) and 2 days in the pesticide mixing area (site 1) (Table 2). These half-lives clearly indicate that soil microorganisms at these sites have previously acclimated to atrazine. The half-lives of anaerobic mineralization in site 2 soil were 4 days under nitrate amended conditions, but were extended to 11–34 days in the absence of external electron acceptors or in the presence of Fe(III) or sulfate. In contrast, the half-lives of anaerobic mineralization in soil samples from site 1 were in the range of 3 to 5 days (Table 2). For reference, the half-lives of 2–3 days for aerobic biomineralization estimated in this study are in the general range of atrazine attenuation determined for agricultural soils with histories of atrazine application (Kruz et al. 2010, 2012; Mueller et al. 2010). The half-life of 1.7 days ($k=0.397\text{ days}^{-1}$) is among the shortest ever reported for a soil sample in the literature. If the soil from this site were used for crop

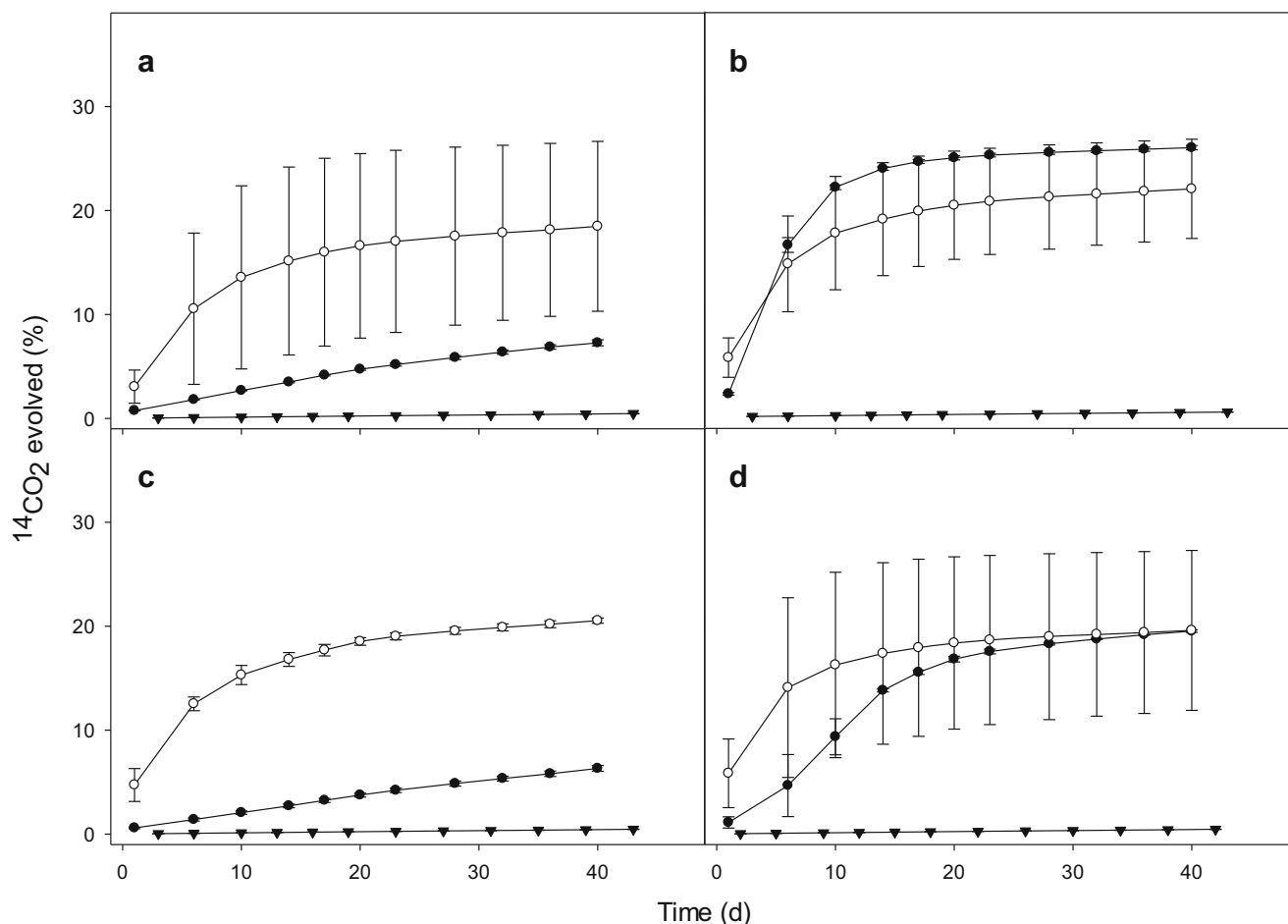


Fig. 3 Anaerobic mineralization of [U-ring-14 C]-atrazine in biometers. The biometers were amended with **a** no electron acceptor, **b** K-nitrate, **c** ferrihydrite, and **d** Na-sulfate. Symbols: (*white circle*), site 1, soil from the former pesticide mixing area; (*black circle*), site 2, soil from the former

machinery washing area; (*black down-pointing triangle*), sterile soil control. Data points represent mean cumulative percentages obtained from duplicates. Bars indicate the standard error for the replicates, and in some cases are smaller than the data point symbols

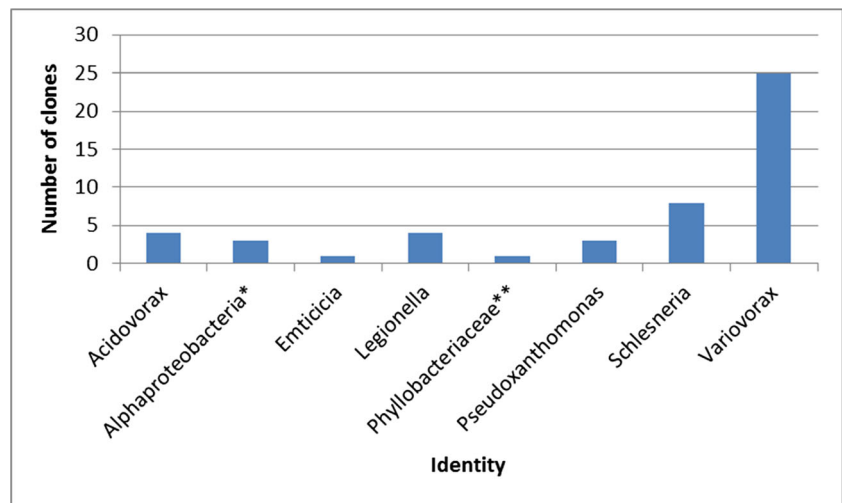
production, atrazine application would have little impact on weed control because its concentration would attenuate in less than a week to non-herbicidal levels based on the estimated half-life. As a point of reference, given the wide

use of atrazine over several decades, verifiable information on rate constants or half-lives of atrazine biomineralization in U.S. soils with no previous environmental exposure to atrazine is not available in the literature.

Table 2 Kinetic parameters and half-lives of atrazine in soils collected from site 1, the former pesticide mixing area, and site 2, the former farm machinery washing area at the Western Agricultural Research Station

Sampling site	Additional amendment	Incubation conditions	k (day ⁻¹)	R^2	$t_{1/2}$ (day)
Site 1 soil	None	Aerobic	0.397	0.971	1.7
	Glucose	Aerobic	0.317	0.990	2.2
	Nitrate	Anaerobic	0.195	0.973	3.5
	Sulfate	Anaerobic	0.235	0.961	2.9
	Iron	Anaerobic	0.155	0.972	4.5
	None	Anaerobic	0.139	0.993	5.0
Site 2 soil	None	Aerobic	0.256	0.994	2.7
	Glucose	Aerobic	0.208	0.995	3.3
	Nitrate	Anaerobic	0.172	0.991	4.0
	Sulfate	Anaerobic	0.065	0.973	11
	Iron	Anaerobic	0.020	0.995	34
	None	Anaerobic	0.031	0.995	23

Fig. 4 Identification of 16S rDNA clones from atrazine-degrading aerobic mixed cultures. Forty-nine clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. All identifications are made at the genus level except for *Alphaproteobacteria* (one asterisk) at the class level and *Phyllobacteriaceae* (two asterisks) at the family level



3.2 16S rRNA gene sequence based analysis of atrazine-degrading enrichment cultures and bacterial isolates

Aerobic enrichments of atrazine degraders from the soil samples of sites 1 and 2 and Bio-Sep bead samples from site 1 yielded mixed cultures which consistently depleted atrazine in liquid media upon successive subcultures. The HPLC chromatograms indicated the presence of multiple metabolites that varied in time course samples, but metabolite analysis was outside the scope of the study.

Three mixed cultures were selected for further analysis based upon the 16S rDNA gene banding patterns in DGGE profiles: two enrichments created with soil from site 1, the

former pesticide mixing area (one amended with glucose, one not amended with glucose) and an enrichment created with soil from site 2, the former farm machinery wash area. Amplification of 16S rRNA genes from the three mixed cultures yielded 49 clones for sequencing; the remainder of the 75 clones failed to produce usable sequence data. Sequence data from 16S rDNA clones was queried in the RDP database for nearest matches. As shown in Fig. 4, most clones ($\geq 90\%$ similarity match) were consistent with *Variovorax* (51 %) and *Schlesneria* (16 %) spp. Other clusters included *Acidovorax* and *Legionella* spp. (8 % each). Some clones could only be matched at a family (*Phyllobacteriaceae*, 2 %) or class (*Alphaproteobacteria*, 6 %) level, due to the lack of cultured representatives in the RDP database matching the query

Fig. 5 Identification of 16S rDNA clones from atrazine-degrading aerobic pure cultures. Ten consensus sequences in total are represented. All sequence queries returned similarity scores of 0.97 or greater. All identifications are made at the species level

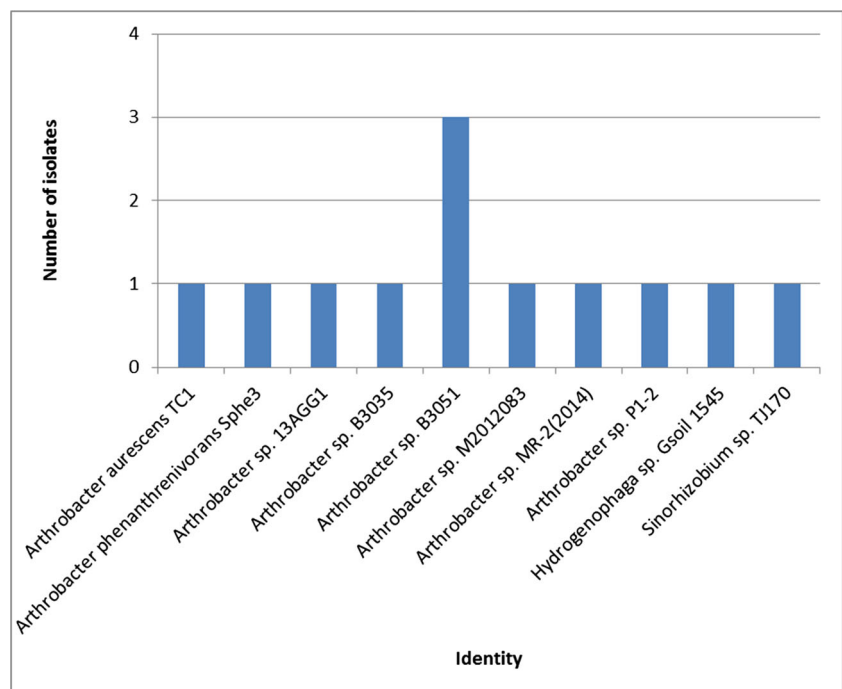


Table 3 Occurrence of atrazine catabolic genes in a selection of atrazine-degrading pure cultures from the Western Agricultural Research Station samples, based on PCR amplification of genomic DNA followed by agarose gel electrophoresis

Isolate and accession nos.	Nearest match and accession no.	S-ab score	atzA	atzB	atzC	atzD	atzE	atzF	trzD	trzN
TN134, KP779963-5	<i>Arthrobacter</i> sp. M2012083, KF893303	1.00	–	+	+	–	–	–	–	+
TN151, KP779972-4	<i>Arthrobacter</i> sp. B3051, JX266390	0.99	–	+	+	–	–	–	–	–
TN154, KP779981-3	<i>Arthrobacter</i> sp. B3035, JX266392	0.99	–	+	+	–	–	–	–	–
TN155, KP779984-5	<i>Arthrobacter</i> sp. B3051, JX266390	0.99	–	+	+	–	–	–	–	–
TN159, KP779986-8	<i>Sinorhizobium</i> sp. TJ170, AJ505297	0.96	–	+	+	–	–	–	–	–
TN161, KP779989-91	<i>Arthrobacter phenanthrenivorans</i> Sphe3, NR_074770	0.98	–	+	+	–	–	–	–	–
TN164, KP779995-7	<i>Hydrogenophaga</i> sp. Gsoil 1545, AB271047	0.99	+/-	+	+	–	–	–	+	+/-

(+) indicates the presence of a band of the expected size for the catabolic gene being amplified; (–) indicates the absence of a band of the expected size for the catabolic gene being amplified; (+/-) indicates the presence of a band of the expected size for the catabolic gene being amplified, the intensity of which is approximately less than one quarter that of the positive control band for the corresponding gene

sequences. *Variovorax* spp. have been previously isolated from atrazine-degrading mixed cultures constructed from agricultural soils (Smith et al. 2005; Zhang et al. 2012), whereas *Schlesneria* spp. have been previously associated with N-cycling (Kulichevskaya et al. 2007). It is conceivable that some clones were incidental and not relevant in the biodegradation of atrazine. Bacteria closely related to the clones were not recovered by cultivation in atrazine media, signaling bias in methods and media of isolation.

Atrazine-degrading mixed cultures were also plated onto solidified MS media. Two mixed cultures, one from the soil sampled at site 1 and the other from site 2, were selected for constructing clone libraries. Additionally, clone libraries were also created from two glucose-amended enrichments of Bio-Sep beads that had been originally incubated in the soil at site 1. One Bio-Sep contained atrazine-soaked beads and the other water-soaked beads. A total of 12 pure cultures were isolated from the plates. DNA was extracted and amplified for 16S rRNA genes; amplification products were cloned and sequenced. Figure 5 summarizes the RDP nearest matches (≥96 % similarity) of the resulting 16S rDNA consensus sequences. *Arthrobacter* spp. dominated the pure culture collection (80 %; 8/10), with isolates similar to *Arthrobacter* sp. B3051 constituting the largest number (25 %; 3/12). A single match each was also made with *Hydrogenophaga* sp. Gsoil 1545 and *Sinorhizobium* sp. TJ170. Similar to the findings in this study, previous efforts to isolate bacteria from atrazine-degrading soil microbial communities have yielded numerous *Arthrobacter* strains in spite of the lack of selective culture-

based techniques specific for these Actinobacteria. *Arthrobacter* spp. have been isolated from around the world from soils that have been exposed to atrazine (Aislabie et al. 2005; Vaishampayan et al. 2007; Vibber et al. 2007; Zhang et al. 2011). The reasons for the cultural bias for *Arthrobacter* remain unclear. This bias is particularly noteworthy because *Arthrobacter* was not detected through cloning. Conversely, clones detected in enrichment cultures were notably absent from the isolations.

3.3 Detection of atz and trz catabolic genes

The presence of atrazine catabolic genes was investigated in seven of the 10 pure culture isolates by PCR amplification and agarose gel visualization (Table 3). All isolates were shown to contain *atzBC*. Additionally, an isolate consistent with *Arthrobacter* sp. M2012083 was demonstrated to contain *atzBC-trzN*, a pattern which has been previously reported in the literature (Devers et al. 2004; Sajjaphan et al. 2010; Zhang et al. 2011).

An isolate matching with *Hydrogenophaga* sp. Gsoil 1545 was shown to contain *atzBC-trzD*, and potentially also *atzA* and/or *trzN*. *Hydrogenophaga* spp. have previously been isolated from river sediment samples containing atrazine degraders (Vargha et al. 2005). A *trzN-atzABC-trzD* pattern of catabolic genes has also been reported for *Hydrogenophaga* spp. isolated from soil sampled in an agrochemical factory (Udiković-Kolić et al. 2010).

Table 4 Sequence similarities of catabolic genes cloned from atrazine-degrading pure cultures to respective database sequences. Query culture numbers correspond to those in Table 3

Gene and subject accession no.	Query culture	Query length (nt)	Similarity (%)	Query accession nos.
<i>atzB</i> , U66917.2	TN164	454	100	KP863793-5
<i>atzC</i> , U66917.2	TN155	574	99	KP863790-2
<i>trzD</i> , AF086815.2	TN164	584	100	KP863796-8

All query lengths reflect the lengths (in nucleotides) of consensus sequences generated from triplicate clones

None of the isolates contained the *atzDEF* combination. Three of the successfully amplified catabolic genes were chosen for subsequent cloning and sequencing (Table 4). BLAST searches against respective database catabolic gene sequences yielded similarity scores in the range of 99–100 % and query coverage values of 100 %.

4 Conclusions

The biomineralization of atrazine was comparable in soil samples from both sites, demonstrating that the soils contained active atrazine-mineralizing microbial communities. While the reduction of external electron acceptors was not confirmed in ¹⁴C-atrazine-containing soil biometers, the data showed atrazine biomineralization anaerobic conditions. Some replicate anaerobic biometers showed considerable variation in the time course of atrazine biomineralization. This indicated that the response to external electron acceptors was variable. Some anaerobic electron acceptors may not necessarily stimulate the activity. *Variovorax* spp. in enrichment cultures and isolates of *Arthrobacter* spp. were particularly dominant. There was no evidence for the functional genes of the complete Atz pathway among the isolates. Partial pathways are inevitable in soil microbial communities, with metabolites linking into other catabolic and assimilative pathways of carbon and nitrogen. In contrast to the identification of isolates predominantly as *Arthrobacter* spp. in the phylum *Actinobacteria*, the 16S rDNA sequences from the clones from enrichment cultures matched the phylum *Planctomycetes* and classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*.

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