Microbial attenuation of atrazine in agricultural soils: biometer assays, bacterial taxonomic diversity, and catabolic genes

James F. Douglass^a, Mark Radosevich^b, Olli H. Tuovinen^{a*}

^a*Department of Microbiology, Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, USA*

^b*Biosystems Engineering and Soil Science, University of Tennessee, 2506 E.J. Chapman Drive, Knoxville, TN 37996, USA*

*Corresponding author.

Email: tuovinen.1@osu.edu (O.H. Tuovinen)

Abstract

The purpose of this study was to examine the potential biomineralization of atrazine and identification of atrazine-degrading bacteria in agricultural soils. Different atrazine application histories of soils impacted the kinetics of biomineralization but not the presence of catabolic genes of two atrazine degradative pathways (Trz and Atz). Biomineralization was based on the measurement of $^{14}\text{CO}_2$ from [U-ring- ^{14}C]-atrazine in surface soil (0-7 cm) samples incubated in biometers. Aerobic atrazine biomineralization rate constants (k) varied in the range of 0.004 to 0.508 d^{-1} depending on the specific soil sample and glucose amendment. The corresponding k -values for anaerobic biometers $\pm$ nitrate, ferrihydrite or sulfate were 0.002-0.360 d^{-1} . Glucose enhancement of atrazine biomineralization was not consistent. Aerobic enrichments from soil samples and *in-situ* incubated BioSep beads yielded mixed cultures, four of which were characterized by 16S rRNA gene amplification, cloning and sequencing. Twelve pure cultures were isolated from enrichments and they were primarily *Arthrobacter* spp. (10/12). The presence of eight atrazine catabolic genes representing two degradative pathways was investigated in seven bacterial isolates by PCR amplification and sequencing. Several combinations of atrazine catabolic genes were detected; each contained at least *atzBC*. A complete set of genes for the Atz pathway was not found among the isolates. Our data indicate that atrazine metabolism involves multiple microorganisms and cooperative pathways diverging from atrazine metabolites.

Keywords: Atrazine biodegradation, Atz pathway, BioSep beads, soil microbiology, Trz pathway

1. Introduction

Agricultural pesticide residues and metabolites are commonly found in groundwater as well

as runoff-impacted rivers. Atrazine and metabolites are among the most often detected pesticides in agriculture-impacted streams in the U.S. (Schreiner et al., 2016). Hydroxyatrazine and desethylatrazine as well as deisopropylatrazine are the most commonly detected atrazine metabolites (Mahía et al., 2007; Toccalino et al., 2014). With decades of wide-spread use of atrazine in agriculture, atrazine residuals have been detected even in non-agricultural soils, wetlands, surface waters, river sediments, and aquifers (Mouvet, 2008; Lazorko-Connon and Achari, 2009; Viegas et al., 2012; Sadler et al., 2014; Radović et al., 2015). The vertical and horizontal movement of atrazine in soil makes it available to microbial attenuation in many environmental matrices under diverse conditions (Mudhoo and Garg, 2011; Viegas et al., 2012; Fan and Song, 2014). The application rates in agricultural crop soils in the U.S. are limited to 2 lb acre⁻¹ (2.24 kg ha⁻¹) for any single application and 2.5 lb acre⁻¹ year⁻¹ (2.80 kg ha⁻¹) for total annual application. Repeated annual application of atrazine result in long-term exposure of soil microbes to low levels of residues and metabolites, sometimes leading to gradual enhancement of biodegradation of the target pesticide in soil. In general, microbial attenuation of atrazine in agricultural soils takes place in vadose zone, most actively in the top 15 cm layer, and only a fractional residue of atrazine or metabolites can reach the saturated zone. Atrazine biomineralization activity declines with soil depth (Tuovinen et al., 2015). Drainage of atrazine into saturated zone is enhanced in sandy soils and with shallow water table level. Photodegradation of atrazine is an important mechanism of attenuation in surface water environments including wetlands, but it has a negligible role in soil systems because it is limited to the very surface (Fenner et al., 2013).

Enhanced biodegradation has been reported multiple times as a soil history effect for s-triazines as well as other herbicides (Martin-Laurent et al., 2004; Zablotowicz et al., 2006;

Mueller et al., 2008; Krutz et al., 2012; Sinkkonen et al., 2013; Fang et al., 2015). Krutz et al. (2012) reported enhanced biodegradation of atrazine in history ('adapted') soils. When atrazine application was terminated, atrazine biodegradation activity enhancement gradually declined over four years to a low level, which was comparable to bioattenuation in 'non adapted' soils that had not received atrazine. Kruz et al. (2012) suggested that the numbers of soil borne atrazine-degraders decline in the absence of atrazine to a steady, uncultivable level until atrazine is available again. Although the soil history effect of atrazine application is well documented, its biological basis remains cryptic. It is possible that the genes encoding the biodegradation of atrazine are inducible, maintaining a steady level of transcription in soil borne bacteria in history soils. *S*-triazine monitoring data indicate that residual concentrations in soil may decrease to low ppt levels during the annual cycle through the seasons (Mouvet, 2008). Even after decades of use in agricultural soils, atrazine and aromatic metabolites can still be detected at least at ppt levels, suggesting that soil microbes cannot metabolize trace levels these compounds (Jablonowski et al., 2008; Vonberg et al., 2014; Liu et al., 2016). Atrazine sorption and partitioning into non polar phase and bound residue formation in soil (Koskinen et al., 2008; Prado et al., 2014a,b) retard the biodegradation and thus extend the persistence of the residual fraction.

Of the atrazine-degrading microorganisms, bacteria are the best characterized group. Atrazine-degrading bacteria do not constitute a uniquely specific group and are distributed mostly in diverse phyla of α -, β - and γ -Proteobacteria, Firmicutes and Actinobacteria (e.g., Rousseaux et al., 2001; Smith et al., 2005; Devers et al., 2007; Govantes et al., 2007; Udiković-Kolić et al., 2010; Monard et al., 2011; Alvarez et al., 2017). Atrazine degraders assigned to the phylum Bacteroidetes have also been reported in some soil studies (Martin-Laurent et al., 2006; Udiković-Kolić et al., 2011). Much of the information on atrazine biodegradation is based on soil

borne bacterial studies, although some bacteria have been enriched for or isolated from aquatic environments – wastewater, river water column and suspended and bedded sediments, and wetland samples.

To date, two biochemical pathways specific for atrazine biodegradation have been characterized, Atz and Trz (Shapir et al., 2007; Hofstetter and Berg, 2011; Udiković-Kolić et al., 2012; Fan and Song, 2014; Huang et al., 2017). These are not exclusive to each other as combinations of both pathways genes are found in some soil bacteria. The biochemical steps are similar and the intermediates are identical (Table S1), but the pathways differ in the respective catabolic genes and proteins. N-dealkylation of atrazine by cytochrome P450 enzymes has been described for some Actinobacteria (Meyer et al., 2014). Atrazine-degrading microscopic fungi can also metabolize atrazine but specific pathways and genes have not been elucidated. Atrazine-degrading fungi include members in genera *Aspergillus*, *Penicillium*, *Phanerochaeta*, *Trametes*, and *Tricoderma* among many others as well as some mycorrhizae (Islas-Pelcastre et al., 2013; Chan-Cupul et al., 2016). Atrazine biodegradation by eukaryotic microbes is mediated by laccases, lignin peroxidases, or Mn-peroxidase type reactions involving oxyradicals (Mougin et al., 1994; Chirnside et al., 2011). Several fungi, including *Phanerochaete chrysosporium*, can modify atrazine with their extracellular enzymes such as lignin peroxidase and laccases. Atrazine- or s-triazine-specific enzymes in eukaryotic microbes have not been reported in the literature.

Porous activated carbon Bio-Sep beads have been tested for enrichment of microorganisms capable of degrading specific pollutants and xenobiotic compounds (Rege et al., 1998; Mehta et al., 2004; Ghosh et al., 2009; Omotayo et al., 2011). The beads can be equilibrated with the target compound and incubated *in-situ* in soil or sediment matrix. In the case of atrazine-

equilibrated beads, atrazine-degrading microbes may sorb onto atrazine-equilibrated beads during *in-situ* incubation and be subsequently processed in the laboratory for cultural or molecular ecological analyses. The sorption is not specific for atrazine-degraders because beads can sorb other microbes, metals and non-polar compounds from soil organic matter pool. Biofilm growth may develop on bead pores that may have no association with atrazine metabolism. Similar beads in a commercial application (Bio-Trap, Microbial Insights, Knoxville, TN) have been employed to capture atrazine-degraders from a groundwater environment (Nousiainen et al., 2015). After four months of exposure, the DNA from the Bio-Trap beads was extracted for further analysis. The technique was successful, perhaps because the subsurface site had such a sparse microbial community that a clone library could not be constructed without the initial pre-enrichment with the beads.

For this study, the potential of biomineralization of atrazine was examined in field soil samples from an agricultural farm (Molly Caren) in Madison County, central Ohio. Pesticide chemistry and intrinsic soil and environmental factors were not included in this study. Atrazine has been used in the farm fields for decades as part of the overall agricultural management system. Using a biometer assay technique soil samples were screened for atrazine biomineralization. Aerobic and anaerobic enrichment cultures with atrazine were initiated with the soil samples. Additionally, Bio-Sep beads were *in-situ* incubated in Molly Caren soils for subsequent biomineralization assays and enrichment of atrazine-degraders. Samples of *in-situ* incubated Bio-Sep beads were examined for 16S rRNA gene sequence-based analysis of atrazine-degrading mixed cultures and bacterial isolates. Along with molecular ecological analysis of bacterial cultures, the presence of *atz* and *trz* catabolic genes was screened in an effort to establish pathway analysis. Thus the scope was to characterize the microbiology of

atrazine biodegradation and biomineralization at Molly Caren field sites.

2. Materials and Methods

2.1. Sampling

The Molly Caren Agricultural Center (<http://fsr.osu.edu/node/77>) is an 850-ha Ohio State University facility located approximately 3 km north of London, OH. In addition to housing a 26-ha conservation area, approximately 566 ha of the facility are farmed for corn, wheat and soybeans (Figure S1). At the time of this study, the agricultural management system adopted at the farm routinely used annual applications of 2.05 kg atrazine ha⁻¹ (1.83 lb acre⁻¹). Atrazine was sprayed as Bicep II MAGNUM formulation (33.0% active ingredient atrazine) in late April for corn fields. The formulation is approved as a restricted use pesticide in the U.S. and also contains S-metolachlor and safener benoxacor (26.1% active ingredient S-metolachlor). The fields were sampled in early September. Fields 1 and 4 had received an atrazine application one year prior, fields 2 and 5 had received two years of prior atrazine applications, and fields 3 and 6 had not received atrazine application for at least the past 3 years prior to being sampled for this study.

Top soil samples (0-7 cm) were removed with a sterile trowel at each site and stored in sterile zip lock bags until processed in the laboratory. The sampling avoided plant roots. Soils 1 and 4, 2 and 5, and 3 and 6 were each paired together upon arrival at the laboratory and were treated as composite soils for further analysis.

Notwithstanding potential problems of recovery of atrazine degraders, the Bio-Sep technique was tested in this study in an effort to probe for soil-borne atrazine-degraders from *in-situ* incubated beads (Ghosh et al., 2009; Omotayo et al., 2011). The shape and size of the activated carbon beads were identical to those used in the commercial Bio-Trap application. The beads

were equilibrated in sterile solution of 2 mg atrazine L⁻¹ or distilled water for 3 days before they were enclosed in sterile nylon mesh bags (~30 beads per bag). The bags were sealed with plastic clips and buried in the top 10 cm layer of soil in fields 1, 2 and 3. The *in-situ* incubations of the Bio-Sep bean bags in soil were carried out in three batches of 40 d (April-May), 57 d (July-September), and 86 d (November-February) of contact time.

2.2. [U-ring-¹⁴C]-atrazine mineralization

Biometer assays (Radosevich et al., 1996; Douglass et al., 2015) were used to measure atrazine mineralization in soil samples under aerobic and anaerobic conditions. The biometers contained 5 g soil samples and 0.064 μmol [U-ring-¹⁴C]-atrazine ± 55 μmol glucose per biometers. Atrazine mineralization by microbes trapped on Biosep beads was also tested using 6-10 diced Bio-Sep beads per biometer. The biometers inoculated with Biosep beads received 5 mL mineral salts solution (Douglass et al., 2015) to alleviate possible nutrient and microelement limitation. The inoculum beads used for the biometers had been incubated *in-situ* for 86 d (November-February) at sites 1, 2 and 3. Anaerobic incubations under N₂ head space involved (per biometer) 25 μmol nitrate as KNO₃, 125 μmol Fe(III) as ferrihydrite, or 15 μmol sulfate as Na₂SO₄ as external electron acceptors, with distilled H₂O used as a blank.

All biometers were incubated at 22±2 °C. Biometer sampling and counting of radioactivity have been previously described (Douglass et al., 2014). ¹⁴CO₂ evolution was calculated as a percentage of the total amount of radioactivity in [U-ring-¹⁴C]-atrazine added to the biometers at the beginning of each time course of biomineralization. Rate constants and half-lives of atrazine mineralization were determined with a pseudo first-order rate expression (Guerin and Boyd, 1992). Where data would not fit to a first-order rate function, a linear regression ($y = mx + b$) of the data was performed to determine the slope ($m = k$), y-intercept (b), and half-life $t_{1/2} =$

$$[(y_{max}/2) - b]/m.$$

2.3. *Enrichment culture conditions*

Soil samples and *in-situ* incubated Bio-Sep beads from sites 1, 2, and 3 were used as inocula for aerobic and anaerobic enrichments. Culture media contained 30 mg atrazine $\pm$ 2 g glucose per L and mineral salts solution (per L medium: K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeCl₃·H₂O, 0.1 mg; CaCl₂·H₂O, 1 mg; MnCl₂, 0.01 mg; ZnCl₂, 1 μ g; pH 6.8). The lack of nitrogen in the mineral salts solution was intended to select for those organisms that could use NH₄⁺ available from atrazine degradation. Aerobic cultures were incubated in shake flasks at 150 rev min⁻¹ at 22 $\pm$ 2 °C. Anaerobic cultures with and without external electron acceptors NO₃⁻ (15 μ mol KNO₃ per biometer), SO₄²⁻ (15 μ mol Na₂SO₄ per biometer) or Fe(III) (125 μ mol ferrihydrite per biometer) were incubated statically at 22 $\pm$ 2 °C in crimp-sealed serum bottles with Ar headspace. The enrichment cultures were screened for residual atrazine concentration using HPLC as previously described (Douglass et al., 2015).

2.4. *Isolation of atrazine-degrading pure cultures*

Prospective cultures of atrazine-degraders in enrichment media $\pm$ glucose were spread plated to atrazine-mineral salts media solidified with 1.5% Noble agar. Discreet colonies were subcultured twice on streak plates before transfer to 5 mL aliquots of the corresponding liquid media. Changes in atrazine concentration were monitored with HPLC during subsequent incubation in liquid media. All liquid cultures were stored as 20% glycerol stocks at -70 °C and pure cultures on solid media at 4 °C.

2.5. *DNA isolation and 16S rRNA gene amplification for DGGE*

MoBio UltraClean Microbial DNA Isolation Kits (MoBio Laboratories, Carlsbad, CA) were used to extract and isolate DNA from the test cultures. The V3 region of the 16S rRNA gene was

amplified with PCR reactions using the primer pair 357FGC and 518r (Muyzer et al., 1993) as previously described (Douglass et al., 2016). PCR products were analyzed with denaturing gradient gel electrophoresis (DGGE) using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA). DGGE gels consisted of 8% acrylamide and a 40%-60% denaturant gradient (100% = 7 M urea in 40% formamide). As previously described (Douglass et al., 2016), after electrophoresis the gels were stained in SYBR Gold (Molecular Probes, Eugene, OR) before examining the DGGE banding patterns. Unique bands were chosen for 16S rDNA clone library sequencing.

2.6. 16S rDNA clone library and analysis

For phylogenetic analysis, the 16S rRNA gene from four mixed cultures and bacterial isolates was amplified with primers 27f and 1525r (Lane, 1991) using a previously described protocol (Douglass et al., 2016). 16S rDNA clone libraries were constructed from PCR products using the Promega pGEM-T vector transformation system and JM109 high efficiency competent host cells (Promega, Madison, WI). The 16S rDNA fragments of three clones for each isolate and 25 clones for each mixed culture were sequenced at the Clemson University Genomics Institute. After editing the sequences in Geneious, consensus sequences were produced for the three clones of each isolate and entered as queries in the NCBI-BLAST and RDP databases. Where a complete set of three sequences was not available, individual sequences or consensus sequences constructed from two clones were used as queries. Clone sequences from mixed cultures were searched individually in the databases.

All 16S rRNA gene sequences were deposited with GenBank. The accession numbers for aerobic mixed culture clones are KT225023-KT225026, KT225028-KT225067, KT225069, KT225071, KT225077-KT225079, KT225086-KT225091, KT225093-KT225094, KT225097-

KT225098, KT225100, KT225103-KT225105, and KT225109. The accession numbers for aerobic isolates are KT225120-KT225155, KT225159-KT225182, KT225186-KT225216, KT225220-KT225228, and KT225232-KT225258.

2.7. Atrazine catabolic genes

The presence of atrazine catabolic genes *trzD*, *trzN*, *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, and *atzF* were screened in atrazine-degrading isolates. The corresponding proteins are listed in Table S1 in the supplementary section. Genomic DNA was amplified with the appropriate primer pairs specific for each catabolic gene (Table S1) as previously described (Douglass et al., 2016). Genomic DNA *Klebsiella pneumoniae* 99 (Karns and Eaton, 1997) was used as a positive control for *trzD*. *Arthrobacter aurescens* TCI (Strong et al., 2002) was used as a positive control for *trzN*, *atzB* and *atzC* and as a negative control for *atzA*, *atzD*, *atzE*, and *atzF*. *Pseudomonas* ADP (de Souza et al., 1998; Shapir et al., 2007) was used as a positive control for *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF*, and as a negative control for *trzD* and *trzN*. *Escherichia coli* DNA was also amplified as a negative control for all catabolic genes. Following the electrophoresis, PCR products on agarose gels were stained ethidium bromide. Single band of the anticipated size on the gel was construed as preliminary evidence for the given catabolic gene. Clone libraries were constructed as an assortment of the respective PCR products as described for the 16S rDNA libraries. Three clones were sequenced for each selected PCR reaction. They were edited with Genious and used to generate a consensus sequence. If three sequences were not obtained after editing, a consensus from two clones or an individual clone sequence was used. The sequences were entered into the NCBI-BLAST data base and aligned with their corresponding catabolic gene sequences. Catabolic gene sequences were deposited with GenBank with the accession numbers KT346377- KT346394.

3. Results and Discussion

3.1 Mineralization of atrazine

Under aerobic conditions, mineralization of atrazine in soils from fields 4/1 and 2/5 reached ~25-30% (Figure 1). Atrazine mineralization was less than 1% in all sterile soil samples from each soil combination. Glucose amendment had a minimally positive effect on the biomineralization. Microbes in soil from field 2/5 mineralized slightly more atrazine than did soil from field 4/1. Aerobic mineralization in soil from field 3/6 was minimal, reaching a maximum of ~7% in 84 d. As fields 4/1 and 2/5 had received atrazine applications in the year and two years, respectively, prior to sampling, and fields 3/6 had not, the biometer data suggest that an enrichment of the soil microbial communities of fields 4/1 and 2/5 for atrazine degraders had occurred.

Mineralization in biometers inoculated with Bio-Sep beads was negligible and comparable to sterile controls. The lack of $^{14}\text{CO}_2$ evolution from Bio-Sep bead biometers was attributed to insufficient number of atrazine-degrading soil microorganisms in the *in-situ* incubated beads. Biometer assays were not performed with the two other BioSep batches. However, after an enrichment phase in liquid medium, mixed cultures derived from all three batches of Bio-Sep beads readily degraded atrazine as confirmed via HPLC analysis. Atrazine-degrading pure cultures were also successfully isolated from mixed cultures initially derived from Bio-Sep beads.

In anaerobic biometer assays, soil samples from fields 4/1 and 2/5 achieved mineralization levels of approximately 25-30% regardless of electron acceptor amendments, whereas samples from fields 3/6 were almost comparable to the sterile control (Figure 2). Unlike in our previous

study of anaerobic biometer assays of wetland sediments (Douglass et al., 2014), the differences between duplicates was minimal. The distribution and activity of atrazine-degraders appeared to be relatively uniform in anaerobic biometer assays as also observed in aerobic assays.

The half-lives of aerobic mineralization of atrazine calculated from the first order rate constants indicated relatively fast atrazine attenuation in soils from fields 4/1 and 2/5, ranging from about 1 to 3 days (Table 1). Although the biometer assays were extended to 80 d, the rate constant and half-life calculations were based on the initial increases in $^{14}\text{CO}_2$ from [U-ring- ^{14}C]-atrazine. The $^{14}\text{CO}_2$ evolution levelled off with time, indicating atrazine or metabolite sequestration due to sorption in clay fraction and partitioning in soil organic matter such as humus. The half-lives indicate that soil microorganisms at these sites have previously acclimated to atrazine, likely as a result of prior applications within the past two years. In contrast, the half-lives of aerobic mineralization in soils from fields 3/6 ranged from ~40-190 d, reflecting the lack of enriched atrazine-mineralizing microbial communities as in fields 4/1 and 2/5. The half-lives of anaerobic biomineralization in soil samples from fields 4/1 and 2/5 were about a week or less regardless of the electron acceptor. Soils from fields 3/6 had anaerobic half-lives of atrazine biomineralization ranging from approximately a month (ferrihydrite) to a year (nitrate).

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

Previous studies have shown considerable diversity of atrazine-degrading bacteria in soil enrichment cultures (De Souza et al., 1998; Smith et al., 2005; Martin-Laurent et al., 2006; Ros et al., 2006), suggesting cooperative and divergent pathways and interspecies catabolism of atrazine and metabolites in the soil environment. Aerobic enrichment of atrazine-degraders from Bio-Sep bead samples yielded mixed cultures growing with atrazine. Based upon distinctly

different PCR-DGGE profiles, four of the mixed cultures were selected for further analysis. Amplification of their 16S rRNA genes yielded 64 clones for sequencing. Sequence data from 16S rDNA clones was queried in the Ribosomal Database Project (RDP) database for nearest matches. Most clones ($\geq 90\%$ similarity match) were consistent with *Mucilaginibacter* and *Variovorax* spp. (Figure 3). An atrazine-degrading anaerobic mixed culture amended with sodium sulfate and inoculated with Biosep beads was also recovered. Ten clones were sequenced from this culture, all of which were identified as various *Ralstonia* species with s_ab scores of 1 (KT225110-KT225119). *Ralstonia* is a basonym because the species have been re-classified into the genus *Cupriavidus* (Vandamme and Coenye, 2004).

Aerobic atrazine-degrading mixed cultures were plated onto the corresponding solid media. Forty-six pure cultures were isolated from these plates. DNA was extracted and amplified for 16S rRNA genes; amplification products were cloned and sequenced. Figure 4 summarizes the RDP nearest matches ($\geq 97\%$ similarity) of the resulting 16S rDNA consensus sequences. *Arthrobacter* spp. dominated the pure culture collection, with only one isolate each of *Hydrogenophaga* and *Variovorax* spp. to round out the collection. The remaining 35 isolates did not have a $\geq 97\%$ match in the RDP database. Their sequences were deposited with GenBank but they were not pursued further in this study.

Clones identified from the mixed cultures were notably absent from the isolations. None of the bacteria identified in the clones from the mixed cultures were isolated in pure culture, indicating a bias in the culture-based analysis. 10 of the 12 pure cultures were *Arthrobacters* but none was found among the clones of the four mixed cultures. The explanation for the lack of clones consistent with *Arthrobacter* spp. is not clear. *Arthrobacters* may have been missed in this limited volume of cloning due to their low occurrence or slow growth. 16S rRNA gene

amplification bias in mixed culture samples is also possible.

Previous studies of atrazine-degrading soil microbial communities have yielded numerous *Arthrobacter* isolates in spite of the lack of selective culture-based techniques specific for these Actinobacteria (Mongodin et al., 2006; Huang et al., 2017). *Arthrobacter* spp. have been isolated from different geographical regions of the world from soils that have been exposed to atrazine (Aislabie et al. 2005; El Sebaï et al., 2006; Vaishampayan et al. 2007; Vibber et al. 2007; Krutz et al. 2010; Zhang et al. 2011; Alvarez et al., 2017; Huang et al., 2017). In general, Arthrobacters are among the most numerous soil bacteria that are cultivable on solid media, perhaps because they are nutritionally versatile and not fastidious, and tend to survive extremes of desiccation, dehydration and starvation in the environment (Busse and Wieser, 2014; Alvarez et al., 2017). Their adaptability and tolerance to cold and chemicals have also been noted (Mongodin et al., 2006; Hennessee and Li, 2010). Arthrobacters are also known for their ability to degrade a wide range of aliphatic and aromatic compounds and chloroorganics (Alvarez et al., 2017).

3.3 Detection of *atz* and *trz* catabolic genes

The presence of atrazine catabolic genes was investigated in 15 pure culture isolates by PCR amplification and agarose gel visualization (Table 2). All of the isolates investigated were *Arthrobacter* spp. and the typical set of atrazine catabolic genes was *trzN* and *atzBC*, consistent with the previous results of atrazine-degrading *Arthrobacter* isolates (Mongodin et al., 2006; Wang and Xie, 2012; Sagarkar et al., 2014; Douglass et al., 2015; Huang et al., 2017). No isolates were shown to contain *trzD* or *atzADEF*. Previous reports (Shapir et al., 2000; Tyler et al., 2013) suggest that *atz/trz* gene detection from soil DNA extracts can be enhanced by using nested PCR but this was not tested in the present study of mixed and pure cultures. Six of the successfully amplified catabolic genes were chosen for subsequent cloning and sequencing

(Table 3). BLAST searches against respective database catabolic gene sequences yielded similarity scores in the range of 99-100% and query coverage values of 100%.

The catabolic gene data for the pure cultures track atrazine biodegradation potential from the parent compound to cyanuric acid. Ring cleavage (*atzD* and *trzD*) was not affirmed for the pure cultures. The biometer assays with soil samples measure $^{14}\text{CO}_2$ formation from [U-ring- ^{14}C]-atrazine, which would require a pathway at least to biuret formation (*atzD* and *atzE* in the Atz pathway) even if biuret and allophanate would not mineralized. Indigenous soil microbial communities in biometer assays proved atrazine biomineralization activity beyond the catabolic gene amplicon data obtained for pure cultures. Metabolites from cyanuric ring cleavage require less substrate-specific enzymes and can disperse in synergistic pathways in soil microbial communities. Thus the important role of the upper Trz and Atz pathways in this study is in the biodegradation of atrazine to the ring cleavage. The lower Atz and Trz pathway has less role in soil microbial communities because of the competition for metabolites as C and N sources by microbes that may not have enzymes of either degradative pathway. This is consistent with the catabolic gene data invariably showing the presence of *trzN-atzBC*, whereas the lower pathway genes were not detected in bacterial isolates in this work and have been found sporadically in previous molecular ecological studies of soil samples (Shapir et al., 2000; Omotayo et al., 2013; Tyler et al., 2013).

4. Conclusions

The application history of atrazine affecting its attenuation was demonstrated in this study of soils that were of similar type and within the same location but under different crop management practices. Aerobic biomineralization atrazine was relatively fast in soils that had received

atrazine in previous years. In contrast, the biomineralization of atrazine was almost negligible in soil samples from fields that had not had atrazine application in the past few years. Anaerobic incubation conditions slowed down atrazine biomineralization in biometer assays. Neither the Atz nor Trz pathway enzymes mediating the dechlorination, dealkylation, hydroxylation, and ring cleavage require molecular oxygen. The reduction of nitrate, sulfate or Fe(III) was not monitored in anaerobic biometer assays of ^{14}C -atrazine biomineralization. The results of anaerobic biomineralization of atrazine showed differential responses to electron acceptor conditions. The aerobic and anaerobic incubations displayed negligible differences between duplicates, indicating that atrazine-degraders were relatively evenly distributed in soil samples. Among aerobic bacteria, *Variovorax* spp. in enrichment cultures and isolates of *Arthrobacter* spp. were particularly dominant. Arthrobacters may have been selectively isolated from enrichments due their resistance to adverse environmental conditions -- desiccation and nutrient deprivation in soil, and sample handling and stringent isolation medium. There was no evidence for the complete Atz or Trz pathway among the *Arthrobacter* isolates. *TrzN* with *atzBC* was a common combination of the upper pathway genes. Genes for the lower pathway enzymes were not detected in the isolates, suggesting that atrazine metabolism in soil microbial consortia involves microorganisms that have cooperative pathways diverging from atrazine metabolites.

Acknowledgements

The study was funded through the USDA National Research Initiative, Grant no. 2004-35107-14884. JFD gratefully acknowledges a Fellowship from the Robert H. Edgerley Environmental Toxicology Fund, Division of Natural and Mathematical Sciences of the College of Arts and

Sciences, Ohio State University. We thank the two anonymous reviewers for feedback that was helpful in improving the manuscript.

References

- Aislabie, J., Bej, A.K., Ryburn, J., Lloyd, N., Wilkins, A., 2005. Characterization of *Arthrobacter nicotinovorans* HIM, an atrazine-degrading bacterium, from agricultural soil New Zealand. FEMS Microbiol. Ecol. 52, 279-286.
- Alvarez, A., Saez, J.M., Costa, J.S.D., Colin, V.L., Fuentes, M.S., Cuozzo, S.A., Benimeli, C.S., Polti, M.A., Amoroso, M.J. 2017. Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals. Chemosphere 166, 41-62.
- Busse, H.-J., Wieser, M., 2014. The Genus *Arthrobacter*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes: Actinobacteria, fourth ed. Springer, Berlin, 105-131.
- Chan-Cupul, W., Heredia-Abarca, G., Rodríguez-Vázquez, R. 2016. Atrazine degradation by fungal co-culture enzyme extracts under different soil conditions. J. Environ. Sci. Health B51, 298-308.
- Chirnside, A.E.M., Ritter, W.F., Radosevich, M. 2011. Biodegradation of aged residues of atrazine and alachlor in a mix-load site soil by fungal enzymes. Appl. Environ. Soil Sci. 2011, 658569, doi: 10.1155/2011/658569.
- de Souza, M.L., Newcombe, D., Alvey, S., Crowley, D.E., Hay, A., Sadowsky, M.J., Wackett, L.P., 1998. Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. Appl. Environ. Microbiol. 64, 178-184.
- de Souza, M.L., Wackett, L.P., Sadowsky, M.J., 1998. The *atzABC* genes encoding atrazine

- 410 catabolism are located on a self-transmissible plasmid in *Pseudomonas* sp. strain ADP. Appl.
411 Environ. Microbiol. 64, 2323-232.
- 412 Devers, M., El Azhari, N., Kolic, N.-U., Martin-Laurent, F., 2007. Detection and organization of
413 atrazine-degrading genetic potential of seventeen bacterial isolates belonging to divergent
414 taxa indicate a recent common origin of their catabolic functions. FEMS Microbiol. Lett.
415 273, 78-86.
- 416 Douglass, J.F., Radosevich, M., Tuovinen, O.H., 2014. Mineralization of atrazine in the river
417 water intake and sediments of a constructed flow-through wetland. Ecol. Eng. 72, 35-39.
- 418 Douglass, J.F., Radosevich, M., Tuovinen, O.H., 2015. Molecular analysis of atrazine-degrading
419 bacteria and catabolic genes in the water column and sediment of a created wetland in an
420 agricultural/urban watershed. Ecol. Eng. 83, 405-412.
- 421 Douglass, J.F., Radosevich, M., Tuovinen, O.H., 2016. Biomineralization of atrazine and
422 analysis of 16S rRNA and catabolic genes of atrazine-degraders in a former pesticide mixing
423 site and machinery washing area. J. Soils Sedim. 16, 2263-2274.
- 424 El Sebaï, T., Devers-Lamrani, M., Changey, F., Rouard, N., Martin-Laurent, F., 2011. Evidence
425 of atrazine mineralization in a soil from the Nile Delta: Isolation of *Arthrobacter* sp. TES6,
426 an atrazine-degrading strain. Int. Biodeter. Biodegr. 65, 1249-1255.
- 427 Fan, X., Song, F., 2014. Bioremediation of atrazine: recent advances and promises. J. Soils
428 Sedim. 14, 1727-1737.
- 429 Fang, H., Lian, J., Wang, H., Cai, L., Yu, Y., 2015. Exploring bacterial community structure and
430 function associated with atrazine biodegradation in repeatedly treated soils. J. Haz. Mater.
431 286, 457-465.
- 432 Fenner, K., Canonica, S., Wackett, L.P., Elsnér, M., 2013. Evaluating pesticide degradation in

the environment: blind spots and emerging opportunities. *Science* 341, 752-758.

Ghosh, D., Roy, K., Srinivasan, V., Mueller, T., Tuovinen, O.H. Sublette, K., Peacock, A., Radosevich, M., 2009. *In-situ* enrichment and analysis of atrazine-degrading microbial communities using atrazine-containing porous beads. *Soil Biol. Biochem.* 41, 1331-1334.

Govantes, F., Porrúa, O., García-González, V., Santero, E., 2007. Atrazine biodegradation in the lab and in the field: enzymatic activities and gene regulation. *Microb. Biotechnol.* 2, 178-185.

Guerin, W.F., Boyd, S.A., 1992. Differential bioavailability of soil-sorbed naphthalene to two bacterial species. *Appl. Environ. Microbiol.* 58, 1142-1152.

Hennessee, C.T., Li, Q.X., 2010. Micrococcineae: *Arthrobacter* and relatives, In: Timmis, KN (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology*. Springer Verlag, Berlin, 1853-1864.

Hofstetter, T.B., Berg, M. 2011. Assessing transformation processes of organic contaminants by compound-specific stable isotope analysis. *Trends Anal. Chem.* 30, 618-627.

Huang, X., He, J., Yan, X., Hong, Q., Chen, K., He, Q., Zhang, L., Liu, X., Chuang, S., Li, S., Jiang, J., 2017. Microbial catabolism of chemical herbicides: Microbial resources, metabolic pathways and catabolic genes. *Pestic. Biochem. Physiol.*, in press, doi:10.1016/j.pestbp.2016.11.010.

Islas-Pelcastre, M., Villagómez, I.J.R., Madariaga, N.A., Castro, R., González, R.C.A., Acevedo, S.A.A. 2013. Bioremediation perspectives using autochthonous species of *Trichoderma* sp. for degradation of atrazine in agricultural soil from the Tulancingo Valley, Hidalgo, Mexico. *Trop. Subtrop. Agroecosyst.* 16, 265-276.

Jablonowski, N.D., Koepchen, S., Hofmann, D., Schaeffer, A., Burauel, P., 2008. Spatial

- 456 distribution and characterization of long-term aged ^{14}C -labeled atrazine residues in soil. J.
457 Agr. Food Chem. 56, 9548-9554.
- 458 Karns, J.S., Eaton, R.W., 1997. Genes encoding *s*-triazine degradation are plasmid-borne in
459 *Klebsiella pneumoniae* strain 99. J. Agr. Food Chem. 45, 1017-1022.
- 460 Koskinen, W.C., Banks, P.A., 2008. Soil movement and persistence of triazine herbicides. In:
461 Homer, M., LeBaron, H.M., McFarland, J.E., Burnside, O.C. (Eds.), The Triazine
462 Herbicides: 50 Years Revolutionizing Agriculture. Elsevier, Amsterdam, 355-385.
- 463 Krutz, L.J., Shaner, D.L., Weaver, M.A., Webb, R.M.T., Zablotowicz, R.M., Reddy, K.N.,
464 Huang, Y., Thomson S.J., 2010. Agronomic and environmental implications of enhanced *s*-
465 triazine degradation. Pest Manag. Sci. 66: 461-481.
- 466 Krutz, L.J., Zablotowicz, R.M., Reddy, K.N., 2012. Selection pressure, cropping system, and
467 rhizosphere proximity affect atrazine degrader populations and activity in *s*-triazine-adapted
468 soil. Weed Sci. 60, 516-524.
- 469 Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.),
470 Nucleic Acid Techniques in Bacterial Systematics. Wiley, Chichester, 115-175.
- 471 Lazorko-Connon, S., Achari, G., 2009. Atrazine: its occurrence and treatment in water. Environ.
472 Rev. 17, 199-214.
- 473 Liu, X., Hui, C., Bi, L., Romantschuk, M., Kontro, M., Strömmer, R., Hui, N., 2016. Bacterial
474 community structure in atrazine treated reforested farmland in Wuying China. Appl. Soil
475 Ecol. 98, 39-46.
- 476 Mahía, J., Martín, A., Carballas, T., Díaz-Raviña, M., 2007. Atrazine degradation and enzyme
477 activities in an agricultural soil under two tillage systems. Sci. Total Environ. 378, 187-194.
- 478 Martin-Laurent, F., Cornet, L., Ranjard, L., López-Gutiérrez, J.-C., Philippot, L., Schwartz, C.,

- 479 Chaussod, R., Catroux, G., Soulas, G., 2004. Estimation of atrazine-degrading genetic
480 potential and activity in three French agricultural soils. FEMS Microbiol. Ecol. 48, 425-435.
- 481 Martin-Laurent, F., Barrès, B., Wagschal, I., Piutti, S., Devers, M., Soulas, G., Philippot, L.,
482 2006. Impact of the maize rhizosphere on the genetic structure, the diversity and the atrazine-
483 degrading gene composition of cultivable atrazine-degrading communities. Plant Soil 28, 99-
484 115.
- 485 Mehta, C., Sublette, K.L., Peacock, A.D., Davis, G., Harrison, M.C., White, D.C., 2004. A
486 comparison of in-situ Biosep[®] bead traps and ex situ microcosms in the evaluation of
487 potential remediation amendments in a PCE-contaminated aquifer. In: Gavaskar, A.R., Chen,
488 A.S.C. (Eds.), Remediation of Chlorinated and Recalcitrant Compounds – 2004. Battelle
489 Press, Columbus, OH, 551-558.
- 490 Meyer, A.H., Dybala-Defratyka, A., Alaimo, P.J., Geronimo, I., Sanchez, A.D., Cramer, C.J.,
491 Elsner, M., 2014. Cytochrome P450-catalyzed dealkylation of atrazine by *Rhodococcus* sp.
492 strain NI86/21 involves hydrogen atom transfer rather than single electron transfer. Dalton
493 Trans. 43, 12175-12186.
- 494 Monard, C., Vandenkoornhuysen, P., Le Bot, B., Binet, F. 2011. Relationship between bacterial
495 diversity and function under biotic control: the soil pesticide degraders as a case study. ISME
496 J. 5, 1048-1056.
- 497 Mongodin, E.F., Shapir, N., Daugherty, S.C., DeBoy, R.T., Emerson, J.B., Shvartzbeyn, A.,
498 Radune, D., Vamathevan, J., Riggs, F., Grinberg, V., Khouri, H., Wackett, L.P., Nelson,
499 K.E., Sadowsky, M.J., 2006. Secrets of soil survival revealed by the genome sequence of
500 *Arthrobacter aurescens* TC1. PLoS Genet. 2, e214, doi:10.1371/journal.pgen.0020214.
- 501 Mougin, C., Laugero, C., Asther, M., Dubroca, J., Frasse, P., Asther, M., 1994.

- 502 Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete*
 503 *chrysosporium*. Appl. Environ. Microbiol. 60, 705-708.
- 504 Mouvet, C., 2008. Pesticides in European groundwaters: Biogeochemical processes,
 505 contamination status and results from a case study. In: P. Quevauviller (Ed.) Groundwater
 506 Science and Policy: An International Overview. RSC Publishing, Cambridge, UK, 545-583.
- 507 Mudhoo, A., Garg, V.K., 2011. Sorption, transport and transformation of atrazine in soils,
 508 minerals and composts: A review. Pedosphere 21, 11-25.
- 509 Mueller, T.C., Steckel, L.E., Radosevich, M., 2010. Effect of soil pH and previous atrazine use
 510 history on atrazine degradation in a Tennessee field soil. Weed Sci. 58, 478-483.
- 511 Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations
 512 by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified
 513 genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.
- 514 Nousiainen, A.O., Björklöf, K., Sagarkar, S., Nielsen, J.L., Kapley, A., Jørgensen, K.S., 2015.
 515 Bioremediation strategies for removal of residual atrazine in the boreal groundwater zone.
 516 Appl. Microbiol. Biotechnol. 99, 10249-10259.
- 517 Omotayo, A.E., Ilori, M.O., Amund, O.O., Ghosh, D., Roy, K., Radosevich, M., 2011.
 518 Establishment and characterization of atrazine degrading cultures from Nigerian agricultural
 519 soil using traditional and Biosep bead enrichment techniques. Appl. Soil Ecol. 48, 63-70.
- 520 Omotayo, A.E., Ilori, M.O., Radosevich, M., Amund, O.O., 2013. Metabolism of atrazine in
 521 liquid cultures and soil microcosms by *Nocardioides* strains isolated from a contaminated
 522 Nigerian agricultural soil. Soil Sedim. Contam. 22, 365-375.
- 523 Prado, B., Duwig, C., Hidalgo, C., Müller, K., Mora, L., Raymundoe, E., Etchevers, J.D. 2014a.
 524 Transport, sorption and degradation of atrazine in two clay soils from Mexico: Andosol and

- 525 vertisol. *Geoderma* 232-234, 628-639.
- 526 Prado, B., Fuentes, M., Verhulst, N., Govaerts, B., De Léon, F., Zamora, O., 2014b. Fate of
527 atrazine in a soil under different agronomic management practices. *J. Environ. Sci. Health*
528 B49, 844-855.
- 529 Radosevich, M., Traina, S.J., Tuovinen, O.H., 1996. Biodegradation of atrazine in surface soils
530 and subsurface sediments collected from an agricultural research farm. *Biodegradation* 7,
531 137-149.
- 532 Radović, T., Grujić, S., Petković, A., Dimkić, M., Laušević, M., 2015. Determination of
533 pharmaceuticals and pesticides in river sediments and corresponding surface and ground
534 water in the Danube River and tributaries in Serbia. *Environ. Monit. Assess.* 187:4092. doi:
535 10.1007/s10661-014-4092-z.
- 536 Rege, M.A., Mills, D.R., Petersen, J.N., Sun, Y., Yonge, D.R., Apel, W.A., 1998. Estimation of
537 mass transport parameters in a column packed with BioSep beads. *Biotechnol. Techn.* 12,
538 913-918.
- 539 Ros, M., Goberna, M., Moreno, J.L., Hernandez, T., Garcia, C., Insam, H., Pascual, J.A., 2006.
540 Molecular and physiological bacterial diversity of a semi-arid soil contaminated with
541 different levels of formulated atrazine. *Appl. Soil Ecol.* 34, 93-102.
- 542 Rousseaux, S., Hartmann, A., Soulas, G., 2001. Isolation and characterisation of new Gram-
543 negative and Gram-positive atrazine degrading bacteria from different French soils. *FEMS*
544 *Microbiol. Ecol.* 36, 211-222.
- 545 Sagarkar, S., Nousiainen, A., Shaligrama, S., Björklöf, K., Lindström, K., Jørgensen, K.S.,
546 Kapley, A., 2014. Soil mesocosm studies on atrazine bioremediation. *J. Environ. Manag.*
547 139, 208-216.

- 548 Sadler, E.J., Sudduth, K.A., Lerch, R.N., Baffaut, C., Kitchen, N.R., 2014. A simple index
549 explains annual atrazine transport from surface runoff-prone watersheds in the north-central
550 USA. *Hydrol. Process.* 28, 210-217.
- 551 Schreiner, V.C., Szöcs, E., Bhowmik, A.K., Vijver, M.G., Schäfer, R.B., 2016. Pesticide
552 mixtures in streams of several European countries and the USA. *Sci. Total Environ.* 573,
553 680-689.
- 554 Shapir, N., Goux, S., Mandelbaum, R.T., Pussemier, L., 2000. The potential of soil
555 microorganisms to mineralize atrazine as predicted by MCH-PCR followed by nested PCR.
556 *Can. J. Microbiol.* 46, 425-432.
- 557 Shapir, N., Mongodin, E.F., Sadowsky, M.J., Daugherty, S.C., Nelson, K.E., Wackett, L.P.,
558 2007. Evolution of catabolic pathways: Genomic insights into microbial *s*-triazine
559 metabolism. *J. Bacteriol.* 189, 674-682.
- 560 Sinkkonen, A., Kauppi, S., Pukkila, V., Nan, H., Płociniczak, T., Kontro, M., Strömmer, R.,
561 Romantschuk, M., 2013. Previous exposure advances the degradation of an anthropogenic *s*-
562 triazine regardless of soil origin. *J. Soils Sedim.* 13, 1430-1438.
- 563 Smith, D., Alvey, S., Crowley, D.E., 2005. Cooperative catabolic pathways within an atrazine-
564 degrading enrichment culture isolated from soil. *FEMS Microbiol. Ecol.* 53, 265-273.
- 565 Strong, L.C., Rosendahl, C., Johnson, G., Sadowsky, M.J., Wackett, L.P., 2002. *Arthrobacter*
566 *aureus* TC1 metabolizes diverse *s*-triazine ring compounds. *Appl. Environ. Microbiol.* 68,
567 5973-5980.
- 568 Toccalino, P.L., Gilliom, R.J., Lindsey, B.D., Rupert, M.G., 2014. Pesticides in groundwater of
569 the United States: Decadal-scale changes, 1993-2011. *Groundwater* 52, 112-125.
- 570 Tuovinen, O.H., Deshmukh, V., Özkaya, B., Radosevich, M., 2015. Kinetics of aerobic and

- 571 anaerobic biomineralization of atrazine in surface and subsurface agricultural soils in Ohio. J.
572 Environ. Sci. Health B50, 718-726.
- 573 Tyler, H.L., Khalid, S., Jackson, C.R., Moore, M.T., 2013. Determining potential for microbial
574 atrazine degradation in agricultural drainage ditches. J. Environ. Qual. 42:828–834.
- 575 Udiković-Kolić, N., Hršak, D., Devers, M., Klepac-Ceraj, V., Petric, I., Martin-Laurent, F.,
576 2010. Taxonomic and functional diversity of atrazine-degrading bacterial communities
577 enriched from agrochemical factory soil. J. Appl. Microbiol. 109, 355-367.
- 578 Udiković-Kolić, N., Devers-Lavrani, M., Petric, I., Hršak, D., Klepac-Ceraj, V., Martin-Laurent,
579 F., 2011. Evidence for taxonomic and functional drift of an atrazine-degrading culture in
580 response to high atrazine input. Appl. Microbiol. Biotechnol. 90, 1547-1554.
- 581 Udiković-Kolić, N., Scott, C., Martin-Laurent, F., 2012. Evolution of atrazine-degrading
582 capabilities in the environment. Appl. Microbiol. Biotechnol. 96, 1175-1189.
- 583 Vaishampayan, P.A., Kanekar, P.P., Dhakephalkar, P.K., 2007. Isolation and characterization of
584 *Arthrobacter* sp. strain MCM B-436, an atrazine-degrading bacterium, from rhizospheric
585 soil. Int. Biodeter. Biodegr. 60, 273-278.
- 586 Vandamme, P., Coenye, T., 2004. Taxonomy of the genus *Cupriavidus*: a tale of lost and found.
587 Int. J. System. Evol. Microbiol. 54, 2285-2289.
- 588 Viegas, CA, Chelinho, S., Moreira-Santos, M., Costa, C., Gil, F.N., Silva, C., Lima, D. Ribeiro,
589 R. Sousa, J.P., Fialho, A.M., 2012. Bioremediation of soils contaminated with atrazine and
590 other s-triazine herbicides: Current state and prospects. Advan. Environ. Res. 6, 1-49.
- 591 Vibber, L.L., Pressler, M.J., Colores, G.M., 2007. Isolation and characterization of novel atrazine-
592 degrading microorganisms from an agricultural soil. Appl. Microbiol. Biotechnol. 75, 921-
593 928.

- 594 Vonberg, D., Hofmann, D., Vanderborght, J., Lelickens, A., Köppchen, S., Pütz, T., Burauel, P.,
595 Vereecken, H., 2014. Atrazine soil core residue analysis from an agricultural field 21 years
596 after its ban. J. Environ. Qual. 43, 1450-1459.
- 597 Wang, Q, Xie, S., 2013. Isolation and characterization of a high-efficiency soil atrazine-degrading
598 *Arthrobacter* sp. strain. Int. Biodeter. Biodegr. 71, 61-66.
- 599 Zablotowicz, R.M., Weaver, M.A., Locke, M.A., 2006. Microbial adaptation for accelerated
600 atrazine mineralization/degradation in Mississippi Delta soils. Weed Sci. 54, 538-547.
- 601 Zhang, Y, Jiang, Z., Cao, B., Hu, M., Wang, Z., Dong, X., 2011. Metabolic ability and gene
602 characteristics of *Arthrobacter* sp. strain DNS10, the sole atrazine-degrading strain in a
603 consortium isolated from black soil. Int. Biodeter. Biodegr. 65, 1140-1144.
- 604

Captions to Figures 1-4

Fig. 1. Aerobic mineralization of [U-ring-¹⁴C]-atrazine in biometers in the absence (A) and presence (B) of glucose amendment. Symbols: (●), composite soil from fields 4 and 1; (○), composite soil from fields 2 and 5; (▽), composite soil from fields 3 and 6; (□), water-equilibrated Bio-Sep beads retrieved from field 1; (▼), sterile soil control. Data points represent mean cumulative percentages obtained from duplicate biometers. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

Fig. 2. Anaerobic mineralization of [U-ring-¹⁴C]-atrazine in biometers. The biometers were amended with (A) no electron acceptor, (B) K-nitrate, (C) ferrihydrite, and (D) Na-sulfate. Symbols: (●), composite soil from fields 4 and 1; (○), composite soil from fields 2 and 5; (▽), composite soil from fields 3 and 6; (▼), sterile soil control. Data points represent mean cumulative percentages obtained from duplicates. Bars indicate actual percentages obtained from each replicate, and in some cases are smaller than the data point symbols.

Fig. 3. Identification of 16S rDNA clones from aerobic, atrazine-degrading mixed cultures. Sixty four clones in total are represented. All sequence queries returned similarity scores of 0.90 or greater. All identifications are made at the genus level.

Fig. 4. Identification of aerobic, atrazine-degrading bacterial isolates by analysis of their 16S rDNA sequences. Forty six 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.

Figure 1.

630 Figure 2.

631

Figure 3.

636

637

638 Figure 4.

639

640 **Table 1**

641 Kinetic parameters and half-lives of atrazine mineralization in combined soil samples from the Molly Caren Agricultural Center.

642 Calculations are based on the biometer assays shown in Figures 1 and 2.

Sample	Amendment	Incubation conditions	k (d ⁻¹)	R ²	$t_{1/2}$ (d)
4/1	None (water)	Aerobic	0.238	0.9682	2.92
4/1	Glucose	Aerobic	0.508	0.8082	1.37
4/1	None (water)	Anaerobic	0.113	0.973	6.13
4/1	Sulfate	Anaerobic	0.122	0.9734	5.69
4/1	Nitrate	Anaerobic	0.230	0.9524	3.02
4/1	Ferrihydrite	Anaerobic	0.360	0.9281	1.92
2/5	None (water)	Aerobic	0.204	0.9654	3.40
2/5	Glucose	Aerobic	0.425	0.8672	1.63
2/5	None (water)	Anaerobic	0.101	0.9807	6.86
2/5	Sulfate	Anaerobic	0.090	0.9881	7.68
2/5	Nitrate	Anaerobic	0.128	0.9753	5.42
2/5	Ferrihydrite	Anaerobic	0.274	0.9517	2.53

3/6	None (water)	Aerobic	0.085 ^a	0.999	42.2 ^a
3/6	Glucose	Aerobic	0.004	0.9994	191
3/6	None (water)	Anaerobic	0.005	0.9842	129
3/6	Sulfate	Anaerobic	0.014	0.9917	51.3
3/6	Nitrate	Anaerobic	0.002	0.9844	354
3/6	Ferrihydrite	Anaerobic	0.018	0.9889	37.7

^aThe rate constants and half-lives were calculated from linear regression.

645 **Table 2**

646 Occurrence of atrazine catabolic genes in a selection of atrazine-degrading pure cultures from the Molly Caren Agricultural Center
 647 samples, based on PCR amplification of genomic DNA followed by agarose gel electrophoresis. The designation “n/a” indicates the
 648 given column heading is not applicable. (+) indicates the presence of a band of the expected size for the catabolic gene being
 649 amplified; (-) indicates the absence of a band of the expected size for the catabolic gene being amplified; (+/-) indicates the presence
 650 of a band of the expected size for the catabolic gene being amplified, the darkness of which is approximately less than one quarter that
 651 of the positive control band for the corresponding gene; (?) denotes a prominent band of an unexpected size for the catabolic gene
 652 being screened.

Isolate and accession no.	Nearest match and accession no.	Similarity (%)	<i>atzA</i>	<i>atzB</i>	<i>atzC</i>	<i>atzD</i>	<i>atzE</i>	<i>atzF</i>	<i>trzD</i>	<i>trzN</i>
TN69, KT225238-40	<i>Arthrobacter</i> sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN70, KT225241-3	<i>Arthrobacter</i> sp. NI2(2012), JQ435704	98	-	?	+	-	-	-	-	+
TN2, KT225128-9	<i>Arthrobacter</i> sp. LM1KK, LC008302	98	-	+	+	-	-	-	-	+
TN3, KT225151-3	<i>Arthrobacter</i> sp. NI2(2012),	99	-	+	+	-	-	-	-	+

	JQ435704									
TN67, KT225235-7	<i>Arthrobacter</i> sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN58, KT225212-3	<i>Arthrobacter</i> sp. MG-2011-9-BV, FR872498	98	-	+	+	-	-	-	-	+
TN53, KT225201-2	<i>Arthrobacter</i> sp. MG-2011-9-BV, FR872498	97	-	+	+	-	-	-	-	+
TN83, KT225247-9	<i>Arthrobacter</i> sp. NI2(2012), JQ435704	99	-	+	+	-	-	-	-	+
TN26, KT225148-50	<i>Arthrobacter</i> sp. JSPB6, JQ308619	99	-	+	+	-	-	-	-	+
TN85, KT225253-5	<i>Arthrobacter aurescens</i> , JQ435703	97	-	+	+	-	-	-	-	+
TN43, KT225168-70	<i>Arthrobacter</i> sp. MG-2011-9-BV, FR872498	97	-	+	+/-	-	-	-	-	+
TN81, KT225244-6	<i>Arthrobacter</i> sp. NI2(2012), JQ435704	99	-	+	+/-	-	-	-	-	+
TN61, KT225223-5	<i>Arthrobacter aurescens</i> , JQ435703	99	-	+	+	-	-	-	-	+
TN84, KT225250-2	<i>Arthrobacter</i> sp. MG-2011-9-BV, FR872498	98	-	+	+	-	-	-	-	+

TN253, KT225146-7	<i>Arthrobacter</i> sp. RKS6-6, GQ477173	100	-	+	+/-	-	-	-	-	+
<i>Escherichia coli</i> , n/a ^a	n/a	n/a	-	-	-	-	-	-	-	-
<i>Pseudomonas</i> ADP, n/a	n/a	n/a	+	+	+	+	+	+	-	-
<i>Arthrobacter aurescens</i> TCI, n/a	n/a	n/a	-	+	+	-	-	-	-	+
<i>Klebsiella pneumoniae</i> 99, na	n/a	n/a	-	-	-	-	-	-	+	-

Table 3

Sequence similarities of catabolic genes cloned from atrazine-degrading pure cultures to respective database sequences. Query culture numbers correspond to those in Table 2. All query lengths reflect the lengths (in nucleotides) of consensus sequences generated from triplicate clones.

Gene and accession no.	Query culture	Query length (nt)	Similarity (%)	Query accession no.
<i>atzB</i> , U66917.2	TN43	469	100	KT346377-9
<i>atzB</i> , U66917.2	TN67	469	100	KT346380-2
<i>atzB</i> , U66917.2	TN69	454	99	KT346383-5
<i>atzB</i> , U66917.2	TN81	466	99	KT346386-8
<i>atzC</i> , U66917.2	TN67	625	100	KT346389-91
<i>trzN</i> , CP000475.1	TN43	432	100	KT346392-4