International Journal of Soil, Sediment and Water
Documenting the Cutting Edge of Environmental Stewardship

Volume 3 | Issue 2 Article 5

7-12-2010

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Recommended Citation
Shaffer, Elizabeth; Sims, Gerald; Cupples, Alison; Smyth, Charles; Chee-Sanford, Joanne; and Skinner, Andrea (2010) "Atrazine Biodegradation in a Cisne Soil Exposed to a Major Spill," *International Journal of Soil, Sediment and Water*: Vol. 3: Iss. 2, Article 5.
Available at: http://scholarworks.umass.edu/intljssw/vol3/iss2/5

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This conference proceeding is available in International Journal of Soil, Sediment and Water: http://scholarworks.umass.edu/intljssw/vol3/iss2/5

ATRAZINE BIODEGRADATION IN A CISNE SOIL EXPOSED TO A MAJOR SPILL

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ABSTRACT

Conventional soil tests, culture-based microbial methods, and the novel method of ^{15}N-DNA stable isotope probing (SIP) were employed to illustrate atrazine biodegradation as related to the physiochemical properties of an atrazine-exposed Cisne soil. The soil exhibited enhanced atrazine degradation and apparently accumulated cyanuric acid. The soil showed elevated ambient concentrations of NO_3^-; however NO_3^- did not suppress atrazine degradation. Atrazine natural attenuation was limited by incomplete distribution through the unsaturated soil matrix. Approximately four moles of inorganic N derived from atrazine were detected for each mole of atrazine carbon mineralized, indicating that at least 80% of the atrazine N was released (less than 20% assimilated). ^{15}N-DNA- SIP experiments were conducted using^{15}N (ring)- and ^{15}N-*ethylamino*-atrazine. The results of these experiments failed to establish a causal relationship between *in-situ* atrazine-degradation and enrichment of DNA associated with soil microorganisms. These results are likely due to isotopic dilution, either as a result of insufficient ^{15}N assimilation or competition by other N sources. Further experiments using ^{13}C-ethyl/isoproylamino-atrazine may yet establish the identities of organisms responsible for enhanced natural attenuation exhibited in the Cisne soil.

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Keywords: Cisne, Atrazine, Stable Isotope Probing, Natural Attenuation

1. INTRODUCTION

Atrazine and other s-triazine herbicides have been used for over 50 years for the control of a variety of weeds in agricultural crops, most notably maize (U.S.Department.of Agriculture, 2004). Triazines are moderately persistent, but also sufficiently water-soluble as to create contamination problems in surface and groundwater (Solomon et al., 1996, Tasli et al., 1996). Atrazine contamination of water resources is a concern because the compound is a suspected endocrine-disruptor (Hayes et al., 2002, Hayes, 2004). Widespread use of these herbicides appears to have promoted the development of bacteria capable of rapidly degrading atrazine as a sole C or N source (Mandelbaum et al., 1995, Topp et al., 2000a, Topp et al., 2000b).

The following experiments characterize atrazine degradation in a soil exposed to high levels of atrazine and metolachlor as a result of a single chemical spill. Large atrazine releases are not uncommon during peak application periods and there is no way to know how many spills go unreported. Therefore, it is important to examine the factors that influence the potential for natural attenuation in soils where known spills have occurred.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Unlabeled atrazine (98%) was purchased from ChemService (West Chester, PA). Uniformly ^{14}C-ring-labeled-atrazine (9.3 mCi per mmol, radiochemical purity ≥95%) was purchased from Sigma-Aldrich (St. Louis, MO). Atrazine-*ethylamino*-^{15}N (99 atom % ^{15}N) was purchased from Isotec (Miamisburg, OH). Uniformly ^{15}N-ring-labeled atrazine was synthesized from ^{15}N-urea according to the method described in (Bichat et al. 1999). Atrazine metabolite standards: deethylatrazine (2-amino-4-chloro-6-isopropylamino-*s*-triazine) (98%), deisopropylatrazine (2-amino-4-chloro-6-ehtylamino-*s*-triazine)(99%), hydroxyatrazine (2-hydroxy-4ethylamino-6-isopropylamino-*s*-triazine)(99%), deethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine)(98%), and deethyldeisopropylatrazine (2-chloro-4,6-diamino-*s*-triazine)(99%), were a gift of W. Roy (United States Geological Survey, Champaign, IL). Cyanuric acid

(99%) was purchased from Alfa Aesar (Ward Hill, MA). Organic solvents were Optima grade (Fisher Scientific, Pittsburg, PA). Scintillation cocktail was Fisher ScintiVerse BD, (Fisher Scientific, Pittsburg, PA).

2.2 Growth and Maintenance of Atrazine-Degrading Microorganisms

Previously researched atrazine-degrading isolates of *Pseudomonas* sp. Strain ADP and *Pseudaminobacter* sp. Strain C147 were generously donated by L. Wackett (University of Minnesota) and E Topp (Agriculture and Agri-Food Canada) respectively. Atrazine-degrading cultures in this study were grown on atrazine mineral salts (AMS) media previously described (Topp et al., 2000a), modified with the addition of 1ml MR2A trace element solution (Atlas, 1997). Post autoclaving, the medium was supplemented with 1ml of a filter-sterilized (0.22 µm, polyethersulfone, Millex-GP,) vitamin solution (Yang and McCarty, 1998) modified with the addition of 0.005 g of thiamine hydrochloride and 0.005 g of nicotinamide L^{-1}, and 1ml of filter sterilized iron stock solution (5g Fe $SO_4 \cdot 7H_2O$ L^{-1}). Solid media preparations consisted of the mineral salts media modified with the substitution of 0.5 g L^{-1} atrazine instead of 0.025 g L^{-1} of atrazine (delivered in 1ml of methanol) and supplemented with 15g of Noble agar (Difco, Sparks, MD). Carbon and nitrogen supplied by the vitamin solution were negligible, thus atrazine represented the sole source of those elements. The concentration of atrazine in the solid media preparations exceeded the solubility limit resulting in a chalky suspension (Mandelbaum et al., 1995) that cleared as isolates began to degrade atrazine. Isolates were stored at -80°C in a 15% glycerol solution.

2.3 Properties and Preparation of Soils

The herbicide impacted Cisne-Darmstadt intergrade (Cisne), consisted of surface material excavated five months prior to this investigation from a spill in Patoka, IL. The Cisne (Fine, smectitic, mesic Mollic Albaqualfs) and Darmstadt (Fine-silty, mixed, superactive, mesic Aquic Natrudalfs) series are generally deep, poorly drained, and slowly permeable soils formed in loess. The site and soil properties observed were more characteristic of the official series description for Cisne, thus the soil is referred to as Cisne herein. The soil was determined to have a cation exchange capacity of 18.9 meq/100g, a pH in water of 6.7, and an organic carbon content of 2.8%. The initial atrazine burden for the Cisne soil is unknown, but exposure was assumed to be significant owing to near complete release of the contents of an applicator truck at the site. The source contamination came from an herbicide pre-mix consisting of Bicep II (Syngenta Crop

Protection) containing S-metolachlor and atrazine at concentrations of 0.29 and 0.37 kg/L respectively. Analysis of an acetone:ethyl acetate (50:50) extract of the soil by gas chromatography-mass spectrometry (performed by E. Pappas, USDA-ARS, W. Lafayette, IN) revealed the presence of 0.85 μg/g residual atrazine and 5.1 μg/g residual metolachlor. Since the original product contains an atrazine/metolachlor ratio of 0.827, the initial atrazine concentration was, minimally 4.2 μg/g, assuming no metolachlor degradation. The site had been excavated to a depth of two meters and the contaminated soil was stored under a tarp for approximately six months prior to land application. To obtain soil with the greatest atrazine exposure, the darkest material, presumed to be from the surface horizon was used for these studies. Properties, exposure histories and taxonomic information for the soils used in this study are described in Table 1.

Table 1. Physical characteristics, soil taxonomy, atrazine exposure history, and inorganic N content for soils used in this experiment.

Soil Name	**USDA soil series name**	**Taxonomy**	**Atrazine History**	**Extractable Inorganic Nitrogen**	
Cisne	Cisne-Darmstadt intergrade	Fine, smectitic, mesic Mollic Albaqualfs / Fine-silty, mixed, superactive, mesic Aquic Natrudalfs	extensive recent exposure	NO_3^- mg/kg	NH_4^+ mg/kg
				161	3
Drummer (field)	Drummer	Fine-silty, mixed, superactive, mesic Typic Endoaquolls	unknown	47	11
Drummer (chemical loading)	Drummer	Fine-silty, mixed, superactive, mesic Typic Endoaquolls	exposure 10 years prior	61	13
Flanagan	Flanagan	Fine, smectitic, mesic Aquic Argiudolls	continuous exposure (>10years)	14	7
Thorp	Thorp	Fine-silty, mixed, superactive, mesic Argiaquic Argialbolls	exposed to glyphosate, atrazine, manure	27	6
Clarksdale	Clarksdale	Fine, smectitic, mesic Udollic Endoaqualfs	exposed to glyphosate, atrazine, manure	34	8

The primary focus of the experiments was the Cisne soil impacted by an herbicide spill. However, a preliminary study was performed comparing this soil to a collection of five reference soils, taken from agricultural production sites throughout Illinois with a known history of atrazine use. Each of the reference soils was collected from a depth of 0-15cm. These soils included: material from a former pesticide mixing-loading facility (Drummer-chemical loading) and the alfalfa field adjacent to the mixing-loading facility (Drummer-field), two manure-amended agricultural production areas (Thorp and Clarksdale), and the zero nitrogen treatment from the University of Illinois Morrow plots long-term fertility experiment (Flanagan) with a long history of atrazine exposure. Each of the soils was thoroughly homogenized, sieved to pass through a 2-mm screen, and stored at 4°C.

2.4 Atrazine Mineralization in Cisne and Reference Soils

The atrazine mineralization rate for the contaminated Cisne soil was compared to reference soils from Illinois using aerobic laboratory incubations. Owing to the potential for inorganic N sources to suppress atrazine degradation (Bichat et al., 1999; Sims, 2006), and a very high residual nitrate concentration in the Cisne soil (Table 1), a leaching treatment (Mulvaney et al., 2001) was included to remove excess nitrogen from the study soils. Unleached controls were also included for Cisne, Drummer-field, and Drummer-chemical loading (which had the highest background NO_3^- concentrations). Mineralization of ^{14}C-ring-labeled-atrazine was monitored over a period of 84 days for soils incubated in 473-mL mason jar microcosms (Mervosh, 1995b) at 19°C. Each replicate (three per treatment) received field moist soil (3.5 g on a dry weight basis), which was adjusted to 40% water-filled pore space using an aqueous solution containing uniformly ^{14}C-ring-labeled-atrazine and unlabeled atrazine to deliver a concentration of 30 µg atrazine and 300 Bq ^{14}C / gram soil (dry weight basis). Physical mixing of the treatment solution in the soil was avoided to prevent the collapse of the relatively weak Cisne soil structure and ensure proper aeration at the relatively high water content. Instead, atrazine was allowed to disperse through the soil via advection. Microcosms also contained a 10-mL 0.2M NaOH trap and a 90mm qualitative filter wetted with 0.5 ml buffer (0.575mM KH_2PO_4) to maintain the proper headspace humidity. At 3-7 day intervals the microcosms were aerated and the evolved $^{14}CO_2$ in the NaOH trap was measured in a 2-mL aliquot using liquid scintillation spectrometry (LSS) in a Packard model 1600TR-Tri-Carb instrument (Packard Instruments, France).

2.5 Soil Incubations with $^{15}N/^{14}C$-Labeled-Atrazine

Two mass balance incubations were performed using microcosms as described for mineralization studies above. In these incubations, Cisne soil was treated with combinations of atrazine variously labeled with ^{15}N and/or ^{14}C. In both incubations, DNA extraction was performed using the Powersoil kit (Mobio Laboratories, Carlsbad, CA) according the manufacturer's instructions. Evolved $^{14}CO_2$ was measured using LSS of 1-ml aliquots from CO_2 traps. Inorganic N was extracted from the remaining soil with 2 M KCl (soil:solution = 0.1) and quantified as described by Sims et al. (1995). Nitrogen isotope-ratio analysis was performed as described by Mulvaney et al. (1997).

An aerobic incubation study was performed in which samples of the Cisne soil (5 replicates/treatment) were amended twice (Day 0 and 26) with either ^{14}C-ring-UL- atrazine (1110 Bq) and unlabeled atrazine (30µg g^{-1}), or ^{14}C-ring-UL-atrazine (1110 Bq) and uniformly ring labeled ^{15}N atrazine (99 atom%, 30µg g^{-1}). This was accomplished by delivering atrazine to empty scintillation vials in ethyl acetate, which was then allowed to evaporate. Sufficient deionized water was added to dissolve the atrazine and bring the soil to 40% water-filled pore space. Finally, 4 grams (dry weight basis) of the study soil were added to each vial, the vials were placed in sealed microcosms containing a 10-ml 0.1M NaOH trap, and a 90mm qualitative filter wetted with 0.5 ml (0.575 mM) KH_2PO_4 was added to maintain proper humidity. The microcosms were stored at 19°C in the dark. Soils (unlabeled and labeled with ^{15}N-atrazine) were destructively sampled (for DNA and HPLC analysis) on days: 5, 10, 15, and 41. The procedure described above was used to respike the samples on day 26; however the samples were transferred to new scintillation vials. Between days 15 and 41 the microcosms were opened at 3-4 day intervals and the NaOH trap was sampled for analysis and replaced.

An additional incubation study was performed using side ^{15}N chain-labeled atrazine under the conditions described above for the ring labeled study. The conditions were modified as follows. Replication number was reduced to 3 replicates/treatment, and the herbicide amendment was introduced only once (Day 0). The following forms of atrazine were introduced at 30µg atrazine g^{-1} soil: unlabeled atrazine (control), atrazine-*ethylamino*-^{15}N (treatment), or uniformly ring-labeled ^{14}C-atrazine (53 Bq g^{-1} soil), the latter treatment was included to facilitate radiochemical analysis of atrazine fate. Atrazine solutions were prepared in methanol (3.2-3.5 µg atrazine/µl methanol) and added to the volume of water necessary to bring the water-filled pore space of the soil to 40%. The

atrazine/water solutions were deposited onto the bottom of aluminum weigh boats (57mm, Life Science Products, Fredrick, CO) and six grams of the study soil were placed on top of the solution allowing the solution to diffuse through the soil pore space. The weigh boats were placed in sealed microcosms fitted with filter paper and a NaOH trap as described above and incubated at 19°C in the dark. One gram of soil was removed from each replicate on days 5, 10, 15, 21, 26 and sacrificed for DNA extraction and analysis. NaOH samples were also collected, as previously described, from the ^{14}C-atrazine treatments on these dates to estimate the percent of compound mineralized in the control and treatment samples. DNA samples from each replicate taken on day 15 were individually separated by ultracentrifugation and purified as described above.

2.6 Atrazine Extraction and HPLC Analysis

After removing one gram dry weight equivalent of soil per replicate for DNA extraction, the remaining 3 grams of soil were stored at -20°C for chemical analysis. Soil samples (2.5 gram) were transferred to 50–ml Teflon centrifuge tubes and extracting solutions were introduced sequentially to recover atrazine in pools assumed to represent decreasing degrees of microbial bioavailability. Soils were initially extracted with 0.01M $CaCl_2$ (4ml), followed by two methanol extractions (4ml each). Extractions in 0.01M $CaCl_2$ were mixed for 1.5 hours and methanol extractions were mixed for 2 hours on a horizontal shaker and then centrifuged at 17,200*g* for 15min. The supernatant was removed from the 0.01M $CaCl_2$ extraction and 1ml was filtered (PTFE, Alltech Associates, Deerfield, IL) prior to reverse phase high performance liquid chromatography (HPLC, Hewlett Packard Series 1050, San Fernando, CA) analyses to measure reversibly-sorbed, bioavailable atrazine. A similar procedure was followed for the first methanol extraction for analysis of the irreversibly-sorbed, potentially bioavailable atrazine. Prior to bound residue analysis, the second methanol extraction was used to remove residual extracting solution (containing atrazine) that remained trapped in the interstitial pore space. This extract was discarded. HPLC conditions were: injector volume, 100µl; mobile phase flow rate, 1.0mL min^{-1}; UV detector wavelength 215 nm; reverse phase C_{18} column (150mm × 4.6mm, Alltima column, Alltech Associates, Deerfield, IL) and an isocratic mobile phase (methanol:water, 65:35). ^{14}C was detected with a Flow Scintillation Analyzer 500TR Series (Packard Instruments Company, Meridien, CT). An apparent K_d value was determined for atrazine and each of the detected metabolites (where the substance was detected in both phases) from the ratio of potentially bioavailable (sorbed) to bioavailable (solution) phase material. After the extraction procedure the soil samples were air dried and combusted according to the method described in Cupples et al.(2000). Finally, to account for any residual radioactivity due to

precipitated atrazine (a precautionary measure owing to relatively high solution concentrations at the onset of the study), the incubation containers were washed with 1ml of MeOH and the radioactivity in the liquid was measured using LSS.

2.7 PCR Amplification of 16S rRNA gene

DNA isolated from the Cisne soil was used as template for the amplification of the following genes using the referenced primers: 16S rRNA gene (Liu et al., 1997), *atz*A, *atz*B, *atz*C (Costa et al., 2000), *atzD*, *atz*E, *atz*F (Piutti et al., 2003), *trz*D (Rousseaux et al., 2001), *and trz*N (Mulbry et al., 2002). PCR conditions for atrazine-degrading genes were as follows: 94°C (10 min); 94°C, 58°C for *atz*A, *atz*D, and *trz*N / 68°C for *atz*B / 62°C for *atz*C, *atz*D, *atz*E, *atz*F, and *trz*D, 72°C (1 min) (30 cycles); 72°C (10min). PCR conditions for amplifying the 16S rRNA genes were as follows: 94°C (10 min); 94°C (1.5 min), 55°C (1.5 min), 72°C (1.75 min) (25 cycles); 72°C (10min). 25µl PCR reactions were performed according the manufacturer's protocol.

2.8 ^{15}N-DNA-SIP of Pure Cultures of Atrazine-Degraders

The most common aerobic pathway for atrazine degradation includes cyanuric acid as the substrate for ring fission, and since the carbon atoms in cyanuric acid are discontiguous and in a plus four oxidation state, aerobic atrazine degraders do not derive energy or assimilate C from the atrazine ring, though ring-N is assimilated (Bichat et al., 1999). Some atrazine degraders, such a *Pseudomonas* sp. Strain ADP, have been shown to completely degrade atrazine to release both C and N from the ring as CO_2 and NH_3 respectively (Bichat et al., 1999), whereas others, such as *Nocardiodes* sp. strain C190, may utilize only side chain C and N (Topp, et al., 2000b). In order to determine whether different organisms would dominate the processing of atrazine N from the ring or side chain, we included stable isotope probing to look for incorporation of ^{15}N into microbial DNA formed during the ^{15}N mass balance studies described above. Stable isotope probing (SIP) involves the use of stable isotopes to label phylogenetically informative biomolecules (e.g., DNA) to provide evidence that a detected population is active in a specific process, if that process results in assimilation of C or N into cellular constituents. Carbon labeling is considerably more sensitive than N labeling, and has been used extensively (Sims, 2008). The use of ^{15}N labeled substrates for this purpose is theoretically possible (Cupples et al., 2007) but has not yet been applied to measure biodegradation of herbicides in soil. Since it was not clear that ^{15}N-SIP could be used to identify atrazine-degrading organisms in environmental samples, we preceded the environmental SIP analysis

with preliminary SIP studies using known strains of atrazine-degraders in pure culture.

Two isotopically labeled forms of atrazine were used in pure culture experiments with bacterial isolates known to utilize all of the N atoms in the atrazine molecule. Using atrazine-*ethylamino*-^{15}N as a treatment, two cultures of *Pseudaminobacter* strain sp. C147 were grown (rotating at 25°C) on AMS media (5mL) supplied with either ^{15}N-(100 atom%) or unlabeled atrazine (25mg/L) and then transferred (5% v/v) to media of the same type. To explore the use of uniformly ^{15}N ring-labeled-atrazine as a treatment, cultures of *Pseudaminobacter* strain sp. C147 and cultures of *Pseudomonas* strain sp. ADP were grown under the conditions described above on AMS media supplied with either ^{15}N(ring)- or unlabeled atrazine. Following growth, cells were harvested from the culture suspension in late exponential growth stage by centrifugation (3000*g*) and the cell pellets were frozen at -20°C for subsequent DNA extraction.

2.9 DNA Extraction and CsCl Density Gradient Ultracentrifugation

DNA from cell pellets was extracted using the DNeasy tissue system (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions for Gram-negative bacteria. DNA was added to a solution of CsCl and Tris-EDTA (TE, pH 8.0). The CsCl/TE starting BD was adjusted to approximately 1.71 g/mL. Ultracentrifugation of samples was performed in Quick-Seal polyallomer tubes (13 X 51 mm, 5.1 ml, Beckman Coulter) in an Optima LE-80K Preparative Ultracentrifuge (Beckman Instruments) outfitted with a VTi 65.2 vertical tube rotor for 48 h, 184 000g (20°C). Buoyant densities (BD) were measured with a model AR200 digital hand-held refractometer (Leica Microsystems Inc. Depew, NY). Following ultracentrifugation, water was injected with a precision pump (model PHD 2000, Harvard Apparatus, Holliston, MA) into the headspace of the centrifuge tube and fractions (75µl) were collected at the bottom as previously described (Cupples et al., 2007, Cupples and Sims, 2007, Lueders et al., 2004). After fractionation, DNA was dialyzed using a 0.025-µm Millipore mixed cellulose ester dialysis filter (Bedford, MA) as previously described (Gallagher et al., 2005). Fractions and purified DNA were stored at -20°C.

Purified DNA from the atrazine-*ethylamino*-^{15}N experiment was used as template for PCR amplification of the 16S rRNA gene using the conditions described above. PCR products from labeled and unlabeled incubations were paired according to buoyant density of the template, and separated by electrophoresis on a 1% agarose gel. The effect of the treatment on template buoyant density was determined by comparing the buoyant densities of the

heaviest fractions that produced a PCR product. DNA collected in the experiments using uniformly ^{15}N ring-labeled atrazine as a treatment was fluorometrically quantified using the PicoGreen nucleic acid quantification dye (Molecular Probes, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Fluorometry was performed on an Opticon 2 Real Time Thermal Cycler (MJ Research, Bio-Rad Laboratories, Hercules, CA) as previously described (Cupples et al., 2006, Tian and Edenberg, 2004). The effect of the treatment on template buoyant density was determined by comparing the buoyant densities of the first significant fluorometric peak.

2.10 Environmental ^{15}N-SIP

DNA samples taken from the replicates on day 41 (chosen based on the results of preliminary mineralization curves) were individually separated by ultracentrifugation and purified as described above. After fractionation and recovery of DNA, TRF profiles were generated from the 12 heaviest fractions of each sample tube. PCR primers (Operon Biotechnologies) used were 27F-FAM (5' AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescein) and 1492R (5' GGTTACCTTGTTACGACTT). PCR mixtures (100µl) included the TaKaRa Ex Taq mixture (Takara Bio), primers (45 pmol each), and 1µl whole cell suspension. The PCR conditions were: 94°C (10 min); 94°C (1.5 min), 55°C (1.5 min), 72°C (1.75 min) (25 cycles); 72°C (10min). PCR products were purified using the AMPure PCR purification system (Agencourt Bioscience Corp., Beverly, MA) according to the manufacturer's instructions. Purified PCR products were quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 150ng of the purified PCR products were separately digested using the restriction endonucleases: HaeIII, RsaI, and MspI (New England Biolabs, Ipswich, MA) and the subsequent TRF profiles were generated as described by Liu et al. 1997. DNA fragments were separated by capillary electrophoresis (model 3730xl Genetic Analyzer, Applied Biosystems, Foster City, CA) at the Keck Center. The ROX 1000 (Applied Biosystems) internal standard was used to size terminal restriction fragment (TRF) lengths. Data were analyzed with GeneMapper V3.7 software (Applied Biosystems). A separate profile was generated from each sample and restriction endonuclease combination.

Each TRF had a unique fragment length, and a reported peak area of fluorescently labeled product, in arbitrary fluorescence units (FU). Percent abundance of each TRF was determined by dividing the FU under each TRF by the total FU under all the TRFs in the profile as described previously (Abdo et al., 2006, Yu and Chu, 2005). Values given for the percent abundance of each FU are

reported as relative fluorescence units (RFUs). Data sets were constructed of TRFs that were between 50bp and 1000bp in length and greater than 50 FU in height. Each TRF was identified with a sample name, isotopic treatment, buoyant density (determined during fractionation), and RFU value.

2.11 Statistical Analyses

Statistical analyses were performed using the statistical functions in Excel (Microsoft Corp, Seattle, WA) and SAS (SAS Institute Inc, Cary, NC). The continuous and quantifiable nature of the CsCl density gradient necessitated the use of a covariance model for detecting upward shifts in TRF buoyant density due to isotopic treatment. If the buoyant densities of individual fractions were the same across test tubes then an Analysis of Variance would be appropriate. However, since they are not, it was necessary to include TRF buoyant density as a continuous parameter in the model. An Analysis of Covariance using SAS Proc Mixed was performed on the TRF data generated from the day 15 atrazine-ethylamino-^{15}N incubations and the day 41uniformly ring labeled ^{15}N atrazine incubations. Sample dates were chosen based on the results of preliminary mineralization curves and the order of release of N from the side chains versus ring for pure culture studies. Analysis was performed using the model:

$$Y_{ijkl} = \text{Isotope}_{\,i} + \text{Test tube (Isotope)}_{\,j(i)} + \text{Density}_{l} + \text{Error}_{\,k(ijl)}$$

Error = Sample (test tube, isotope, density)

Sample information was entered in the following categories: tube (enzyme), fraction, isotope, density, peak, and RFU. Results were considered significant if the analysis of the effect of isotope produced F values correlating to an α error rate less than 0.25. This error rate is fairly liberal and allows for the detection of TRF buoyant density shifts that may not be due to isotopic treatment; however this liberal error rate allows for the screening and detection of possibly enriched TRFs. TRFs that have been identified as possibly enriched require further analysis using the remaining two enzymes. TRFs with the same relative abundance in TRFLP profiles generated with the remaining enzymes must be examined using the same method. TRFs that comprise similar relative abundances and show similar shifts in buoyant density would be considered enriched. This process could be accelerated through the use of Multivariate Analysis of Covariance to detect concurrent buoyant shifts using all three enzymes simultaneously.

3. RESULTS AND DISCUSSION

3.1 Atrazine Mineralization in Cisne and Reference Soils

Mineralization of atrazine observed for the six soils is presented in Figure 1**.** When compared to the five Illinois reference soils as well as previous reports in the literature, the Cisne soil appeared to exhibit enhanced atrazine degradation. Leaching did not significantly increase mineralization rates for the Cisne soil, indicating the relatively high inorganic N concentration was not likely rate limiting. The unleached Cisne soil exhibited the most rapid initial degradation rate (1.822×10^{-5} mmoles atrazine mineralized day$^{-1)}$ and cumulatively mineralized >82% of the atrazine applied, apparently meeting criteria for enhanced degradation (Zablotowicz et al., 2006). These findings are consistent with atrazine mineralization potential observed for other soils with prior exposure to atrazine (Pussemier et al., 1997, Barriuso and Houot, 1996, Martin-Laurent et al., 2003, Yassir et al., 1999, Vanderheyden et al., 1997). The rapid mineralization of atrazine suggested that the soil was a good candidate for natural attenuation.

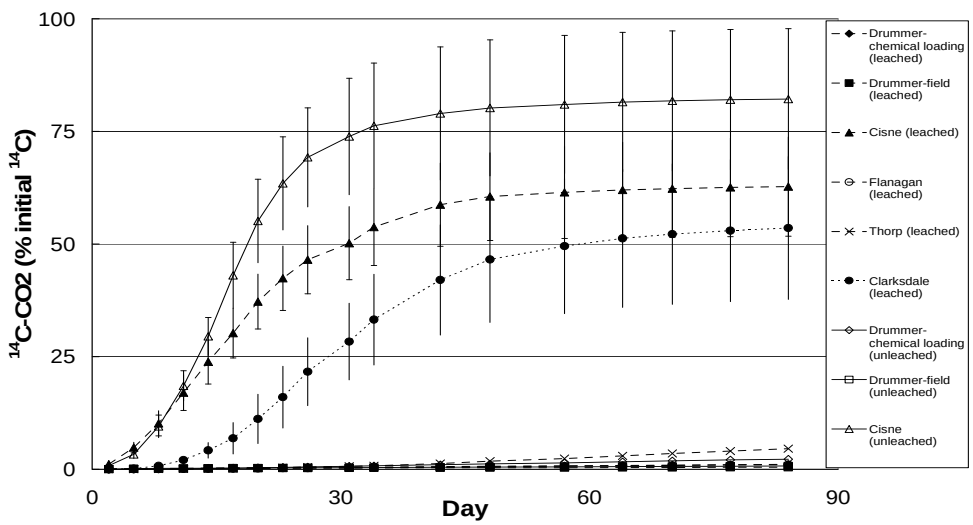

Figure 1. ^{14}C(UL)ring-labeled atrazine mineralization in six Illinois soils previously exposed to atrazine. Samples included leached treatments and unleached controls. Leaching was performed to reduce inorganic N concentrations.

A significant portion of the atrazine applied to the Cisne soil (18%) was not mineralized, and may have been present in the soil as atrazine, metabolites, or bound residues. Incomplete degradation of herbicides has been attributed largely

to bioavailability limitations resulting from sorption (Sims and Cupples, 1999) or diffusion limitation through tortuous paths in unsaturated soils (Johnson et al., 1998). Owing to the large carbon content (2.8 %) in the Cisne soil (and care taken to maintain soil structure during the incubation), both of these mechanisms are likely. Mineralization studies were thus followed with more detailed degradation studies in which a mass balance was performed on the C and N added as atrazine to provide a better understanding of material flow in the Cisne soil.

3.2 Soil Incubations with Uniformly $^{15}N/^{14}C$ Ring-Labeled-Atrazine

A mass balance of applied atrazine C indicated that overall recovery of added radiocarbon ranged from 75 to 110 %, depending on sampling date. Initially, a significant portion of the atrazine (up to 58% of applied) was recovered from the bottom of the incubation vessel as a precipitate, however this fraction decreased rapidly to 6.2 % of applied. ^{14}C-mineralization was calculated as described above, and the distribution of radioactivity among mineralized, aqueous ($CaCl_2$ extractable), sorbed (MeOH extractable), and bound (non-extractable) fractions was determined at the end of the incubations. A relatively small portion of the recovered material (< 4%) was found in bound residue (Figure 2 inset). The bulk of the recovered ^{14}C was initially associated with the aqueous phase, which was depleted thereafter to supply the other phases (Fig. 2 inset). Mineralization did not differ significantly between ^{15}N-labeled and unlabeled atrazine treatments. Additional atrazine and water (as described above) were introduced on day 26 (respike) to promote degradation. A sharp increase in $^{14}CO_2$ evolution detected on day 26 indicates a response to the respike, and suggests that much of the atrazine present at day 25 was not bioavailable (Fig. 2). A considerable pool (14%) of aqueous and sorbed phase atrazine remained intact at 41 days.

The concentration of atrazine used initially exceeded the solubility limit in this study. Soil moisture content prior to the respike was maintained at or below 40% water-filled pore space to ensure proper aeration, and was increased to 50% water-filled pore space as a result of the respike process. Thus it is likely that atrazine availability was increased as a consequence of a higher water content and more complete redistribution of the compound through the soil matrix to the active atrazine-degraders. This hypothesis is supported by several observations. Both the aqueous and sorbed atrazine pools were preferentially depleted to a relatively constant value in the first ten days of incubation with a corresponding release of radiocarbon as CO_2 (Figure 2 inset). A resurgence of mineralization was observed at 41 days (after the 26-day respike), even though a considerable

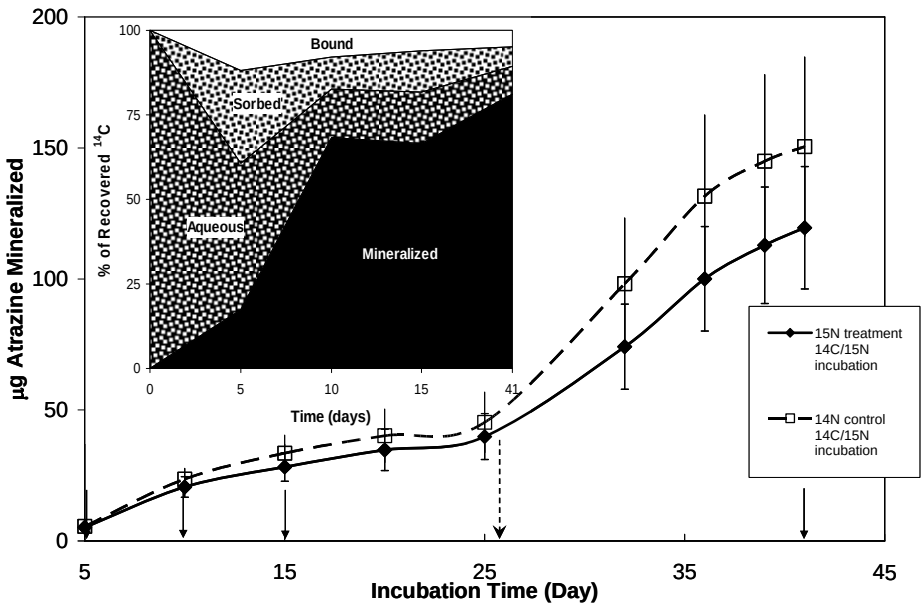

Figure 2. Atrazine mineralization in the Cisne soil incubated with ring labeled $^{14}C/^{15}N$ atrazine and data from initial screening . ^{15}N series includes treatment replicates. Unlabeled series includes control replicates. Solid arrows indicate destructive sampling points for DNA extraction and HPLC analyses. The broken arrow indicates the respike of 30µg atrazine g-1 soil on day 26. Inset shows Distribution of $^{15}N/^{14}C$(UL)ring-labeled atrazine radiocarbon among pools recovered as CO_2 and parent compound.

amount of atrazine remained present in the system. This increase in mineralization coincided with a decrease in precipitated atrazine, presumably as a result of improved dissipation and redistribution of the chemical due to the additional water added with the respike. These findings are consistent with previous work showing increased utilization of an aromatic substrate present in soil solution as water content reached a threshold expected to result in greater continuity of pore space (Johnson et al., 1998). Based on that work, also performed with a Cisne soil, a significant portion of the atrazine present in a bioavailable form would be expected to reside in discontinuous pore space at the lower water content initially used.

When combined, the two extractable fractions accounted for 6.5-18.2% of the initial ^{14}C-atrazine, whereas the non-extractable bound residues only accounted for 2-3% of the initially applied atrazine. The relatively little bound residue

detected in the extracted soil is consistent with the unavailability of atrazine ring carbon for incorporation into biomass (Bichat et al., 1999), which would be expected to appear as bound residue in the analysis scheme used here. In a similar study by Houot et al.(2000), soils showing accelerated degradation tended to equally partition residual radioactivity between the extractable and non-extractable fractions, which combined to equal 5-10% of the initial atrazine application. The same study showed that in soils showing minimal atrazine degradation, approximately 50% of the initial radioactivity remained extractable and 30% remained as non-extractable bound residues. The fractioning of radioactivity in the Cisne soil more closely resembled the pattern displayed in soils with accelerated degradation than the less active soils described in the Houot study.

Figure 3 depicts the dissipation of atrazine radiocarbon and formation of degradation products in Cisne soil incubated with $^{15}N/^{14}C$(UL)ring-labeled atrazine. Three labeled metabolites were detected in the Cisne soil, with HPLC peaks that corresponded to the retention times for hydroxyatrazine (3.8-4.0min), deethylatrazine and deethylhydroxy atrazine (3.3 min), and the unresolvable peaks of deethyldeisopropylatrazine, deisopropylatrazine, and cyanuric acid (2.3-2.5 min). The extent of release of ^{15}N from side chain labeled atrazine (Fig. 5) is consistent with cyanuric acid as the identity of the latter peak (and likely precludes both deethyldeisopropylatrazine and deisopropylatrazine), however the identity has not been confirmed via other methods. The metabolites represented approximately 50% of the extracted radioactivity in the aqueous fraction, but only 20-40% of the extracted radioactivity in the sorbed fraction, likely owing to the more polar nature of the metabolites. Atrazine appears to be transiently converted to hydroxyatrazine, DEA and other unknown metabolites, whereas cyanuric acid accumulated. Accumulation of cyanuric acid in soils exhibiting accelerated degradation has been reported previously (Zablotowicz et al., 2008). Batch sorption isotherms (24 hour equilibration, 5:1 ratio of 0.01M $CaCl_2$:soil) are compared to apparent K_d values calculated from solution and sorbed phase measurements in Fig. 4. Apparent K_d values increased over time for each of the compounds measured, however none exceeded the batch sorption values over the course of the incubation. Hydroxyatrazine proved the most sorptive in the Cisne soil, however the compound was no longer detected in the sorbed phase after 15 days.

Mineralization kinetics in the Cisne soil underestimated the rate of atrazine dissipation demonstrated by the accumulation of several metabolites. The results of metabolite analysis supported the presence of an active population of atrazine-degraders in the Cisne soil comparable to other sites from which such organisms

have been successfully isolated (Mandelbaum et al., 1995, Topp et al., 2000a, Topp et al., 2000b Aislabie et al., 2005).

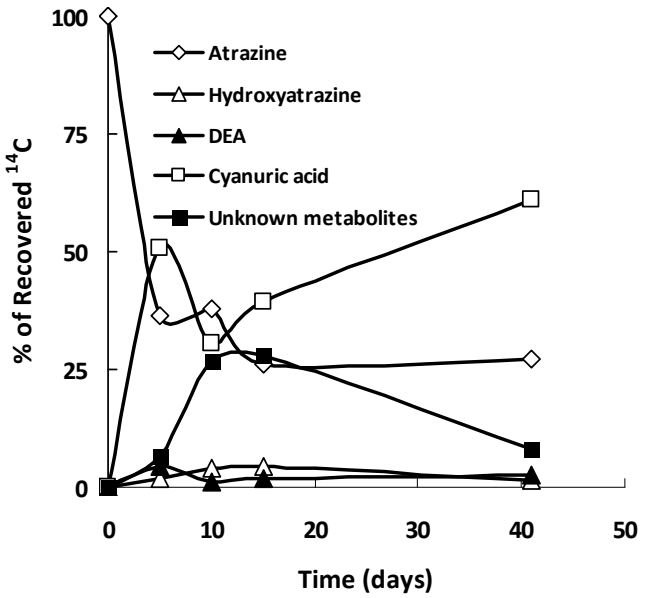

Figure 3. Dissipation of atrazine radiocarbon and formation of degradation products in Cisne soil incubated with $^{15}N/^{14}C$(UL)ring-labeled atrazine. Unknown metabolites represents the sum of all unidentified peaks. Data are expressed as percentage of recovered radiocarbon.

Isotopic analysis of inorganic N (NH_4^+ and NO_3^-) detected in the ring and side chain labeled studies established the concentration of atrazine N mineralized (as μmol NH_4^+ plus NO_3^- per g soil). The relationship between mineralization of atrazine ^{14}C (as CO_2) and inorganic ^{15}N is shown in Figure 5. Clearly, both ring N and side chain N were released, though the patterns differed, and the individual relationships to ^{14}C were not quite linear. However, when the inorganic N data from both incubations are extrapolated to the same sampling dates and combined, a highly significant correlation is obtained ($R^2 = 0.997$) with a slope indicating the net release of approximately 4 moles of N per mole of atrazine. Equations for the side chain and ring N gave 1.2 and 2.8 moles of N per mole of atrazine, respectively. These results would suggest that a higher portion of the side chain N was incorporated into biomass or other N forms. This is consistent with the earlier release of side chain N expected for known degradation pathways (Shapir et al., 2007) and the transient accumulation of cyanuric acid observed in this study.

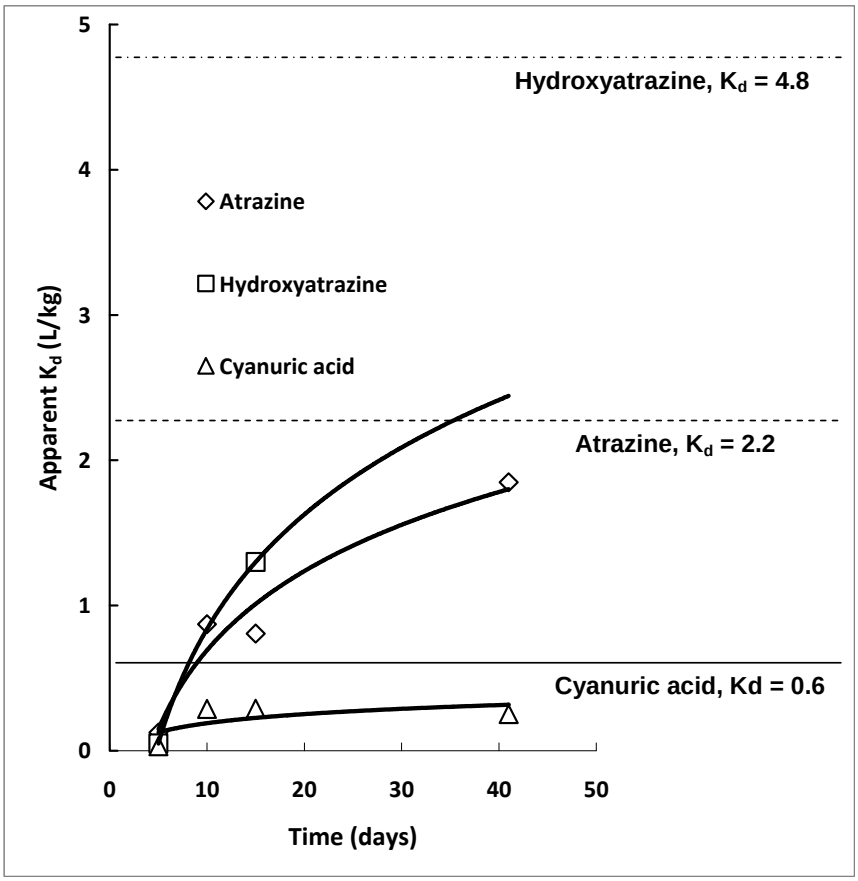

Figure 4. Change in apparent K_d values for atrazine, hydroxyatrazine, and cyanuric acid as a function of incubation time in a Cisne soil. Horizontal lines at 0.61, 4.8, and 2.2 L/kg represent batch sorption isotherm values (24hr) for cyanuric acid, atrazine, and hydoxyatrazine respectively. Hydroxyatrazine was no longer detectable in the sorbed phase after 15 days.

3.3 Pure culture ^{15}N-DNA-SIP

DNA based SIP is a relatively new microbial tool used to examine microbial interactions in the environment. Previous research in our lab demonstrated that ^{15}N enriched compounds can be used as substrates for SIP (Cupples et al., 2007, Cupples and Sims, 2007). SIP experiments herein were conducted to examine if two different forms of ^{15}N-labeled atrazine, atrazine-*ethylamino*-^{15}N and uniformly ^{15}N ring-labeled atrazine, could also serve as suitable substrates for SIP-based investigations. Fine fractions were collected and sensitive DNA detection methods were employed to detect small changes in buoyant density. Results from the atrazine-*ethylamino*-^{15}N experiment indicated that the buoyant

density of *Pseudaminobacter* strain C147 DNA increased from approximately 1.733220 g/ml to 1.736735 g/ml, an increase of 0.003515 g/ml (Figure 6). Results from the uniformly ^{15}N ring-labeled atrazine experiment indicated that the buoyant density increase of *Pseudomonas* strain ADP DNA was 0.007040 g/ml and the average buoyant density increase of *Pseudaminobacter* strain C147 DNA was 0.006143 g/ml +/- 0.001663 g/ml. Figure 7 shows how the results of these experiments compare with the buoyant density increases demonstrated in Cupples et al. (2006) and Meselson and Stahl (1958). The buoyant density shifts found in these previous investigations were reported for substrates that were enriched to 100 atom% ^{15}N. The buoyant density increase published in Meselson and Stahl (0.014 g/ml) was used to calculate the theoretical buoyant density increases expected at other enrichment levels. The two N-labeled forms of atrazine used herein were selectively labeled at 100 atom % ^{15}N at the label positions. Incorporation of all five N atoms from atrazine would thus result in enrichment of DNA equivalent to 20 atom % ^{15}N for the ethylamino-labeled atraine and 60 atom % ^{15}N for the ring labeled material. Based on that assumption, the data from our experiments closely follows the trend projected by the Meselson and Stahl data. Our results demonstrate that addition of substrates with known enrichment levels of ^{15}N will result in reliable increases in DNA buoyant density in pure culture.

The consistent relationship between substrate enrichment and increases in DNA buoyant density makes it possible to anticipate the amount of substrate incorporation necessary to result in a detectable increase in buoyant density. In our experiments, the decrease in buoyant density from one fraction to the next is approximately 0.00136 +/- 2.074 E-4 g/ml. Using this data, it is possible to extrapolate that at least 13.6% of the nitrogen atoms in the target organism's nucleic acids must be labeled with the ^{15}N isotope in order for the separation of light and heavy DNA to be observed. Nucleic acid enrichment levels below this threshold will not be detectable in our study system.

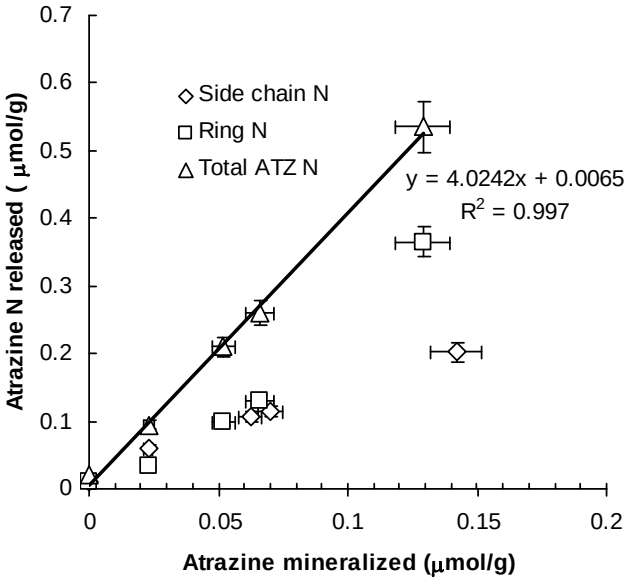

Figure 5. Relationship between mineralization of atrazine ^{14}C (as CO_2) and ^{15}N (as NH_4^+ plus NO_3^-). Averaged data are plotted for the relationship between $^{14}CO_2$ released and ^{15}N released from the atrazine side chain (diamonds), the atrazine ring (squares), and the whole atrazine molecule (triangles) extrapolated from the other two data sets. A simple correlation (solid line) and regression equation are presented for the combined data.

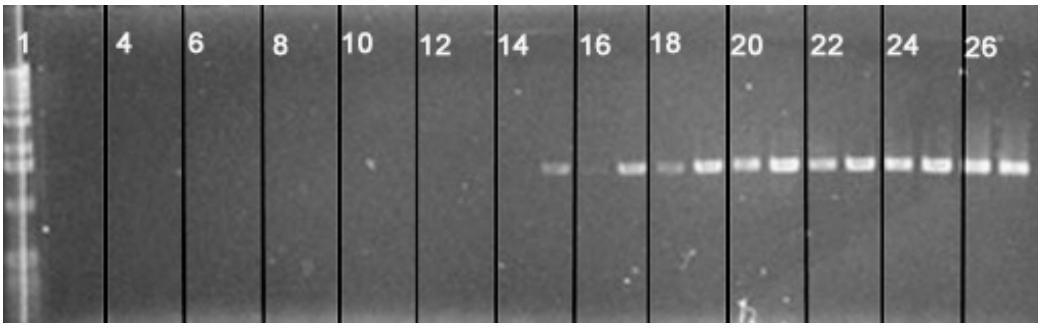

Figure 6. Detection of *Psuedaminobacter* sp. C147 in fractions from a buoyant density gradient using 16S rRNA gene PCR products. Even lanes contain PCR products using template from cells grown on unlabeled atrazine and odd lanes contain PCR products using template from cells grown on ^{15}N-labeled atrazine. Lanes are paired according to closest buoyant density fractions. Lane 1- 1kb ladder. Lane 15- first PCR product detected using ^{15}N enriched *Pseudaminobacter* sp C147 DNA as a template (BD= 1.736735 g/mL), Lane 18- first PCR product detected using , unenriched *Pseudaminobacter* sp C147 DNA as a template (BD = 1.733220).

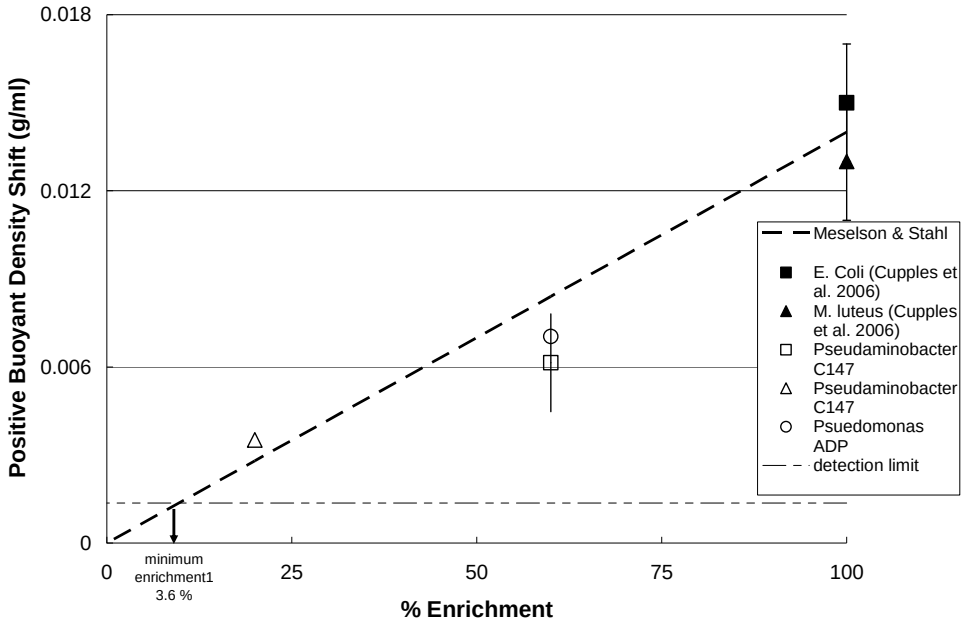

Figure 7. Effect of enrichment with ^{15}N on DNA buoyant density. Closed data symbols show effect of enrichment observed by Cupples et al. (2007) and Meselson and Stahl (1958). Open symbols report buoyant density increases demonstrated in the current study. The buoyant density increase published in Meselson and Stahl (0.014 g/ml) for 100% ^{15}N enrichment was used to calculate a theoretical relationship between buoyant density and enrichment indicated by the dashed line.

3.4 *^{15}N-SIP of Environmental Microbial Communities*

Most practitioners of stable isotope probing rely on the visible separation of "heavy" and "light" nucleic acids. As our lab demonstrated earlier, enrichment with ^{15}N does not result in a sufficient buoyant density increase to visually resolve enriched and un-enriched nucleic acids from one another (Cupples et al., 2007). In such instances, fine fractionation of the buoyant density gradient must be collected and compared to control samples using tools such as quantitative PCR or TRF profiles. Comparison of control and treatment TRFs can be tedious if the effect of the treatment is not pronounced. In such instances, a sensitive detection method is needed. To our knowledge, we have developed the first statistical model capable of distinguishing enriched and un-enriched TRF profiles from one another.

Table 2. BLAST comparison of sequence identities for atrazine-degradation genes amplified using Cisne DNA as template

Source	Strain	Target Gene	Closest identity in BLAST accession number	Similarity (%)	# Bases used for search	% similarity to corresponding genes encoded by P. ADP catabolic plasmid (U66917.2)
Cisne Soil	predominant soil clone	*atzA*	DQ089655.2 *atzA* from *Herbaspirillum* sp. B601 (smzA) gene	99	531	99
Cisne Soil	predominant soil clone	*atz* B	AY456696.1 *atzB* from *Arthrobacter aurescens* strain TC1	100	523	100
Cisne Soil	predominant soil clone	*atz* C	AY456696.1 *atzC* from *Arthrobacter aurescens* strain TC1	99	628	99
Cisne Soil	predominant soil clone	*trz* D	AF086815 from *trzD Acidovorax avenae* subsp. citrulli	100	854	n/a
Cisne Soil	predominant soil clone	*trz* N	AY456696.1 *trzN* from *Arthrobacter aurescens* strain TC1	99	237	n/a

TRF profiles from soil SIP studies were examined for fragments to determine if the addition of ^{15}N-labeled atrazine resulted in an apparent increase in one or more organisms' DNA buoyant density. Table 2 shows atrazine degradation genes detected in the Cisne soil DNA extracts. BLAST comparison sequences identities using Cisne DNA as a template revealed close identities to various atzA, atzB, atzC, trzD and trzN genes. Tables 3 and 4 report statistical anlysis of three of the most common TRF fragments. The results indicate that DNA from Cisne soil was not enriched for any of these fragments in either the uniformly ring-labeled ^{15}N atrazine incubations or the atrazine-*ethylamino*-^{15}N incubations. It is expected that treatments of uniformly ring-labeled ^{15}N atrazine would not produce enrichment of DNA in some organisms, since some are known not to cleave the ring structure (Topp et al., 2000b). It is likely that the failure to produce a significant treatment effect in the atrazine-ethylamino-^{15}N incubations is a result of insufficient enrichment of the target nucleic acids. To avoid enrichment-bias atrazine was added to the microcosms at 30µg g^{-1}soil. This is an environmentally relevant concentration of atrazine comparable to the concentrations seen in some agricultural surface soils, and certainly was initially exceeded at this spill site. As demonstrated in the pure culture SIP experiments, target organisms must utilize the substrate in sufficient quantities to ensure that at least 13.6% of the nitrogen atoms in nucleic acids are ^{15}N-labeled. In pure culture experiments, this parameter is easily controlled; however in the soil environment there are many competing sources of nitrogen. Though the spill occurred in a non-cropped area, the Cisne contained nitrate in excess of many fertilized agricultural soils in the area and approximately an order of magnitude more inorganic N than would be expected for an uncropped Cisne (Mulvaney et al., 2006). It appears likely that this nitrate accumulated as a result of mineralization of atrazine- and metolachlor-

N; however the presence of NO_3^- did not suppress atrazine degradation. Since the soil was stored under a tarp for a number of months before it was remediated the NO_3^- generated from atrazine degradation would not have been leached away. The high nitrate load in the Cisne thus soil could have competed with atrazine resulting in a dilution of the treatment and a decreased likelihood of detection in SIP.

Table 3. Statistical analyses of three prominent TRFLP profiles in the atrazine-ethylamino-^{15}N SIP experiments. Results indicate probability of BD increase due to treatment. Analysis were conducted using 72 observations.

Atrazine-ethylamino-^{15}N SIP							
			mean relative fluorescence of trf				
restriction endo-nuclease	terminal fragment length (trf)	α level associated with isotope effect	control	standard error	treatment	standard error	treatment mean signficantly > control
HaeIII	229	0.7575	5.51E-04	3.49E-04	7.19E-04	3.49E-04	no
RsaI	465	0.356	4.60E-05	8.20E-05	1.70E-04	8.10E-05	no
MspI	229	0.5288	4.72E-04	7.00E-05	4.01E-04	7.00E-05	no

Table 4. Statistical analyses of three prominent TRFLP profiles in the uniformly ring-labeled ^{15}N atrazine SIP experiments. Results indicate probability of BD increase due to treatment. Analysis were conducted using 120 observations.

Uniformly ring-labeled ^{15}N atrazine SIP							
			mean relative fluorescence of trf				
restriction endo-nuclease	terminal fragment length (trf)	α level associated with isotope effect	control	standard error	treatment	standard error	treatment mean signficantly > control
HaeIII	229	0.3317	6.32E-03	2.68E-03	1.02E-02	2.68E-03	no
RsaI	465	0.1107	2.38E-03	6.79E-04	6.57E-04	6.79E-04	no
MspI	229	0.9543	3.66E-03	2.14E-03	3.49E-03	2.14E-03	no

Despite the high concentrations of NO_3^--N in the study soil it is reasonable to expect some organisms could incorporate significant amounts of ^{15}N into biomass. In the atrazine-*ethylamino*-^{15}N incubations, 180µg of atrazine were added to each sample equaling approximately 12.5µg ^{15}N sample^{-1}. Organisms that only incorporated N from the side chain moieties of atrazine would have accumulated equal amounts of ^{14}N and ^{15}N from the herbicide. If we assume that all measured forms of nitrogen (including inorganic N) are equally incorporated

into biomass then the ratio of ^{15}N:^{14}N is 1:80. If the organisms preferentially utilized the atrazine and NH_4-N over NO_3-N, the ratio of ^{15}N:^{14}N in the sample is 1:2.4. To achieve a 13.6% ^{15}N enrichment in DNA approximately 1 in 8 N atoms in DNA must be labeled with ^{15}N. The ratio of 1 ^{15}N atom to 2.4 ^{14}N atoms in the readily assimilable nitrogen pool would be within the requirements for ^{15}N SIP.

Only about 55% of the atrazine added in the *ethylamino*-^{15}N incubations was mineralized and metabolite analysis of the uniformly-ring-labeled incubation indicates that approximately 7% of the atrazine applied was incompletely degraded; therefore a conservative estimate would indicate that approximately 62% of the atrazine applied could have been degraded and used for biosynthesis. Using this estimate, the effective ratio of ^{15}N:^{14}N in the sample is reduced to 1:4 assuming only native soil NH_4^+ competed as an N source for biosynethesis. This ratio is still within the requirement for effective isotopic incorporation, however these estimates assume that all the available ^{15}N was utilized by a single group of atrazine-degrading organisms. It is more likely that the ^{15}N label was distributed among several taxa preventing any individual from assimilating enough ^{15}N to significantly increase its BD. Although the ratio of enriched : unenriched readily assimilable nitrogen was favorable, the presence of multiple atrazine-degrading bacteria would have diluted the treatment effect. Any use of nitrate as an N source would have exacerbated this limitation. This explanation is supported by the presence of multiple atrazine dechlorination genes in the Cisne soil (Table 2). If the *atz*A and *trz* D genes detected code for functional enzymes, then there are several populations of atrazine-degrading bacteria thereby minimizing the incorporation of ^{15}N into the DNA of a particular organism.

Though the failure of ^{15}N SIP to identify dominant degraders in this study was disappointing, it does not preclude the utility of ^{15}N-DNA-SIP for other applications. The conditions that occurred herein, however should be carefully considered when applying SIP to spill sites. In future experiments these problems could potentially be addressed with use of dual-labeled treatments. Cupples et al. (2007) and coworkers demonstrated that the use of ^{13}C and ^{15}N-dual labeled substrates produced a buoyant density increase of 0.045g/ml when supplied as the sole sources of carbon and nitrogen. A buoyant density increase of that magnitude would simplify detection visually or by comparison of TRFLP profiles. The use of dual-labeled substrates in environmental samples would greatly improve the probability of detection when the treatment is applied at low concentrations.

4. CONCLUSION

Mineralization kinetics appeared to be a conservative estimate of atrazine degradation rates for determining the suitability of this soil for natural attenuation. Natural attenuation of atrazine appeared to be primarily limited by incomplete distribution of the compound through the unsaturated soil matrix, as has been reported for other aromatic compounds under similar conditions. Mineralization kinetics underestimated the rate of atrazine dissipation owing to the accumulation of several metabolites. The Cisne soil appeared to accumulate cyanuric acid, as has been observed in other soils exhibiting enhanced atrazine degradation. The feasibility of ^{15}N-DNA-SIP was verified in pure cultures of atrazine-degrading bacteria. A statistical analysis approach was developed to examine DNA buoyant density shifts in fine fractions from density gradients. Coupled with traditional SIP techniques, this approach will hopefully allow for the detection of microorganisms responsible for the degradation of nitrogen-containing compounds such as herbicides and explosives.

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