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J. J. Crawford · G. K. Sims · R. L. Mulvaney
M. Radosevich

Biodegradation of atrazine under denitrifying conditions

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Abstract Anaerobic biodegradation of atrazine by the bacterial isolate M91-3 was characterized with respect to mineralization, metabolite formation, and denitrification. The ability of the isolate to enhance atrazine biodegradation in anaerobic sediment slurries was also investigated. The organism utilized atrazine as its sole source of carbon and nitrogen under anoxic conditions in fixed-film (glass beads) batch column systems. Results of HPLC and TLC radiochromatography suggested that anaerobic biotransformation of atrazine by microbial isolate M91-3 involved hydroxyatrazine formation. Ring cleavage was demonstrated by $^{14}\text{CO}_2$ evolution. Denitrification was confirmed by detection of $^{15}\text{N}_2$ in head-space samples of K^{15}NO_3 -amended anaerobic liquid cultures. In aquatic sediments, mineralization of uniformly ring-labeled [^{14}C]atrazine occurred in both M91-3-inoculated and uninoculated sediment. Inoculation of sediments with M91-3 did not significantly enhance anaerobic mineralization of atrazine as compared to uninoculated sediment, which suggests the presence of

indigenous organisms capable of anaerobic atrazine biodegradation. Results of this study suggest that the use of M91-3 in a fixed-film bioreactor may have applications in the anaerobic removal of atrazine and nitrate from aqueous media.

Introduction

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] is a widely used herbicide that has been detected in groundwater and surface water (Kolpin et al. 1996). Its presence in these environments, which are often anoxic, has generated interest in the potential for anaerobic biodegradation of atrazine. Information concerning the anaerobic fate of atrazine is limited to kinetic studies (Chung et al. 1996; Jessee et al. 1983; Radosevich et al. 1995a; Wilber and Parkin 1995), and anaerobic metabolite formation has not usually been determined. Characterization and information about the persistence of anaerobic metabolites are important for understanding the fate of atrazine in anoxic aquatic and terrestrial environments, especially since the degradation products deethylatrazine (2-amino-4-chloro-6-isopropylamino-s-triazine) and deisopropylatrazine (2-amino-4-chloro-6-ethylamino-s-triazine) are nearly as toxic as atrazine (Kaufman and Kearney 1970). Chung et al. (1996) detected hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-s-triazine) formation in anoxic sediment slurries. Transformation of atrazine to hydroxyatrazine has also been observed in flooded soils (Armstrong et al. 1967), and is usually attributed to abiotic hydrolysis catalyzed by adsorption to clay and organic matter under saturated (Armstrong et al. 1967; Brown and White 1969; Helling et al. 1971; Stevenson 1972) as well as unsaturated anaerobic (J. W. Stucki, personal communication) conditions. Other studies have reported negligible atrazine degradation under denitrifying conditions (Nair and Schnoor 1992; Topp et al. 1995).

The facultatively anaerobic bacterium M91-3 is unique in its ability to utilize atrazine as its sole C and N

Names are necessary to report factually on available data; however, the United States Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable

J. J. Crawford¹ · R. L. Mulvaney
Department of Natural Resources and Environmental Sciences,
University of Illinois, Urbana, IL 61801, USA

G. K. Sims (✉)
USDA-ARS, 1102 S. Goodwin Ave., Urbana, IL 61801, USA
Tel.: +1 217 333 6099;
Fax: +1 217 333 5251
e-mail: gk-sims@uiuc.edu

M. Radosevich
Department of Plant and Soil Sciences, University of Delaware,
Newark, DE 19717, USA

Present address:

¹ Department of Civil Engineering, 3230 Newmark Laboratory,
205 N. Mathews Ave, Urbana, IL 61801, USA
e-mail: jrcrawfor@uiuc.edu

source under aerobic as well as anaerobic conditions (Radosevich et al. 1993, 1995a). Atrazine degradation does not result in substantial biomass increase unless an additional electron donor (such as glucose) or elevated atrazine levels (approx. 1000 mg l^{-1}) are present. Utilization of the triazine N as a nitrogen source has been reported for this and other environmental isolates (Mandelbaum et al. 1995; Radosevich et al. 1995a,b). Metabolite formation and ring cleavage of atrazine by M91-3 have not been explored under anoxic conditions.

Incubation of sediments with M91-3 enhanced mineralization of atrazine in both sterile and nonsterile sediments, though indigenous organisms were also involved (Radosevich et al. 1996). Anaerobic biodegradation of atrazine by M91-3 and its potential for bioremediation have not been explored in anaerobic systems.

The purpose of this study was to characterize further the anaerobic transformation of atrazine by the bacterial isolate M91-3 under denitrifying conditions. Studies to confirm denitrification were carried out using nitrogen-15 tracer techniques. The bacterium was used as a tool in two bioremediation situations: as a fixed film on glass beads in the decontamination of aqueous medium, and in situ by M91-3 amendment to atrazine-contaminated sediment.

Materials and methods

Chemicals

Uniformly ring-labeled [*ring*-U- ^{14}C]atrazine ($9.25 \times 10^8 \text{ Bq mmol}^{-1}$) (purity of 99%) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Unlabeled atrazine (purity of 99%) was obtained from Chem Service (West Chester, Pa.). Metabolite standards employed in this study and their respective chemical nomenclature and purity were as follows: deethylatrazine (98%), deisopropylatrazine (99%), hydroxyatrazine (99%), deethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine; 98%), deethyldeisopropylatrazine (2-chloro-4,6-diamino-*s*-triazine; 99%), and ammeline (2-hydroxy-4,6-diamino-*s*-triazine; 99%). The metabolite standards were provided by W. R. Roy (United States Geological Survey, Champaign, Ill.). ^{15}N -labeled KNO_3 (99.9 atom % ^{15}N) was obtained from Isotec (Miami, Ohio). Solvents were Optima grade (Fisher Scientific, Pittsburgh, Pa.). All other chemicals used were at least A. C. S. reagent grade.

Test bacterium and medium

The isolate M91-3 (ATCC 55512) was routinely cultured in anoxic atrazine nitrate (AN) medium that contained 0.1 mM atrazine (21.6 mg l^{-1} , C and N source) and 1 mM nitrate (terminal electron acceptor). The medium also contained (l^{-1}): 0.5 g K_2HPO_4 , 1.5 g PIPES (dipotassium 1,4-piperazine-diethanesulfonate buffer), 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 ml trace metal stock solution. The trace metal stock solution contained (l^{-1}) 2.0 g nitrilotriacetic acid, 0.9 g CaSO_4 , 1.0 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.8 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.23 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g Na_2SeO_4 , and 0.02 g Na_2WO_4 , (pH 6.0). The medium was prepared by first dissolving atrazine, K_2HPO_4 , and PIPES in double-distilled H_2O , and adjusting the pH to 7.2. Solutions were made anoxic by heating to the boiling point and transferring, under O_2 -free N_2 , to O_2 -free bottles before autoclaving (20 min, 121°C). Before use, the

remaining components of the AN medium were added aseptically via a syringe from anoxic sterile stock solutions. The medium used in the mineralization and metabolite studies contained 0.1 mM [^{14}C]atrazine ($6.29 \times 10^6 \text{ Bq mmol}^{-1}$), 1 mM NO_3^- as KNO_3 , and 0 mg l^{-1} dissolved O_2 (dissolved O_2 probe 084010, meter 840, Orion, Boston, Mass.).

Culture conditions

Biphasic column systems containing the ^{14}C -labeled AN medium were used in atrazine metabolism studies. Multi-side-port Pyrex columns (The Ohio State University Glass Blowing Shop, Columbus, Ohio) were filled with 5-mm (diameter) Pyrex beads (approx. 340 g), fitted with rubber sleeve septa, a stopper, and Tygon tubing, and autoclaved (121°C) (Fig. 1). Each column received 130 ml anaerobic AN medium that had been inoculated with a 10^6 log-phase culture of M91-3. Anaerobic conditions were maintained in the column by continuous sparging with O_2 -free N_2 ($62 \pm 2 \text{ ml min}^{-1}$) through port 6. $^{14}\text{CO}_2$ was collected in a culture tube by directing the effluent gas stream through 1 M KOH. Column experiments were initiated when microbial colonization of the glass beads was evident through atrazine and nitrate disappearance without a lag phase upon addition of fresh AN medium.

The aqueous medium from the column was sampled (1 ml) via a syringe from ports 1 or 3. Samples were filtered ($0.2 \mu\text{m}$, polyvinylidene difluoride) before HPLC analysis. An aliquot of the KOH trap was transferred to a scintillation vial containing 15 ml scintillation cocktail (Bio-Safe II, Research Products International, Mount Prospect, Ill.), and the $^{14}\text{CO}_2$ collected was measured by liquid scintillation counting using a Packard 1900TR liquid scintillation spectrophotometer (Meriden, Conn.). At the conclusion of

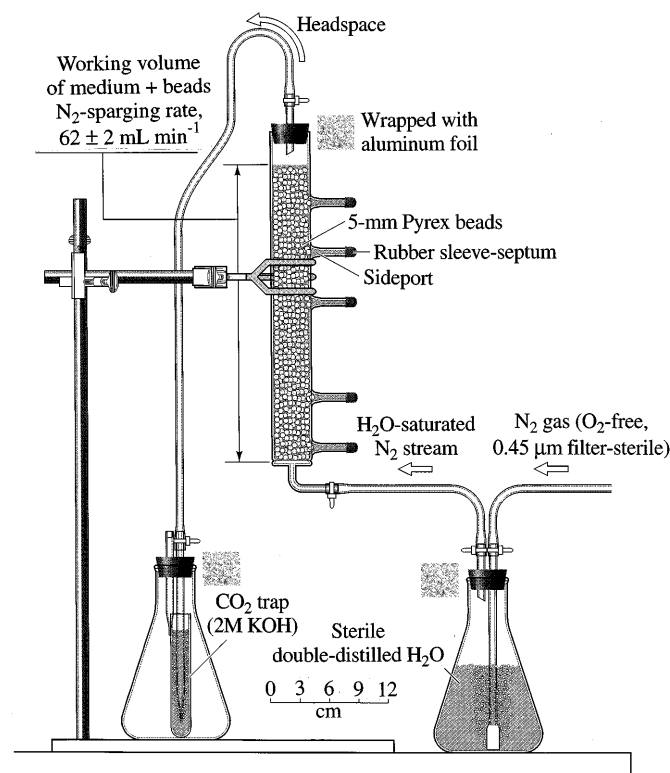


Fig. 1 Biphasic column used for the study of atrazine biodegradation by M91-3. The column contained glass beads (5 mm) and ^{14}C -labeled AN medium, and was colonized with M91-3. It was sparged with O_2 -free N_2 from port 6. An in-line base trap was used for $^{14}\text{CO}_2$ collection. Port numbering is from the top

the study, cells were washed from the glass beads with 0.1% $\text{Na}_4\text{P}_2\text{O}_7$, and ^{14}C -labeled biomass was determined with the method of Mans and Novelli (1961).

Determination of denitrification by M91-3

The bacterium M91-3 was inoculated into 125-ml conical flasks that had been modified with stopcocks and tubing to allow manipulation and sampling of headspace gas (Mulvaney 1988). The anoxic medium contained atrazine (0.1 mM), glucose (0.1 mM), and 2.5 mM K^{15}NO_3 (0.57 g l^{-1} 99.9 atom % ^{15}N diluted with 0.175 g l^{-1} unenriched KNO_3 to yield a final enrichment of 79.2 atom %). Upon inoculation, flasks were made anaerobic by sparging with N_2 , and headspace gas was replaced with N_2 (741.5 mmHg; 98.8 kPa). The headspace volumes were recorded for each of six replicate flasks (including three sterile controls). Flasks were incubated in the dark at $25 \pm 2^\circ\text{C}$. After 8 days, the headspace gas was displaced into evacuated gas-collection tubes using N_2 . The samples were subsequently analyzed by isotope-ratio mass spectrometry for $^{15}\text{N}_2$ and $^{15}\text{N}_2\text{O}$ with on-line data processing as described by Mulvaney et al. (1997).

Sediment study

Anaerobic sediment slurries (1:2, wet weight/vol.) were prepared in 120-ml serum bottles using river-bed sediments designated S1 (silty) or S2 (sandy) and ^{14}C -labeled AN medium. Both sediments were of the Sawmill series (fine-silty mixed mesic Cumulic Haplaquolls) and were obtained from the Sangamon River, University of Illinois Allerton Park (Monticello, Ill.). The medium contained 0.1 mM [^{14}C]atrazine ($0.073 \mu\text{Ci } \mu\text{mol}^{-1}$) and 1 mM KNO_3 . Each bottle was sealed with a butyl rubber septum that had an attached vial containing 1 ml 1 M KOH for $^{14}\text{CO}_2$ collection. Inoculated treatments were prepared by adding an aliquot of an M91-3 culture to slurries. Incubations were performed using 15 replicates for each of four treatments, including inoculated S1, uninoculated S1, inoculated S2, and uninoculated S2.

Three replicates per treatment were sacrificed at 3, 15, 30, 60, and 108 h. At sampling, microcosms were acidified (approx. pH 2) with 2 M H_2SO_4 to evolve aqueous CO_2 . After trapping evolved CO_2 for 8 h it was quantified by liquid scintillation counting.

HPLC analysis

Atrazine, nitrate, and metabolites were quantified with high-performance liquid radiochromatography using a Waters 510 or Hewlett-Packard 1050 series instrument and HPLC as described by Vermeulen et al. (1982). The HPLC mobile phase (isocratic or gradient-controlled) was delivered at 1.0 ml min^{-1} to a C_8 RP Econosil column ($5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$; Alltech Associates, Deerfield, Mich.). The mobile-phase components were fortified with 50 mM ammonium acetate and adjusted to pH 7.4 prior to use. The isocratic composition was 40:60 methanol:water. When a gradient was used, the mobile phase composition (water:methanol, by vol.) was as follows (linear gradient): 0–25 min, 60:40, 25–35 min, 20:80, 50–60 min 60:40. Both ^{14}C (Packard Radiomatic Flo-one/beta detector) and UV (220 nm) detectors were employed.

Samples and standards for HPLC analysis were adjusted to pH 7.4 before diluting 1:1 with mobile phase before analysis. Standards were prepared in the appropriate mobile phase and stored at -20°C . Samples were quantified using external standards.

TLC analysis

Ascending normal-phase thin-layer radiochromatography was performed with Adsorbosil Plus 1 P plates (catalog no. 16381, Alltech) and a mobile phase composed of 8:1:1 isopropanol:30% NH_4OH :water by vol. (Thompson et al. 1971). Radiochromatograms were produced by scanning plates with an Ambis 4000

Radioanalytic Imaging Detector and Ambis version 4.31 software (Scanalytics, Inc., Billerica, Mass.).

Results

Respective retention times of atrazine, hydroxyatrazine, and nitrate were 10.6, 6, and 3.1 min in HPLC. TLC was also used to separate atrazine and hydroxyatrazine. Atrazine was depleted in anaerobic columns to 50% of the amount applied within 144 h (6 days), and a radiolabeled metabolite accumulated over time (Fig. 2). The radiolabeled metabolite co-chromatographed with a hydroxyatrazine standard in two different HPLC solvent systems and the TLC solvent system, and is henceforth referred to as hydroxyatrazine.

The mass balances of applied radiocarbon in the inoculated and sterile control columns were 65% and 95% respectively. The 35% of applied radiocarbon unaccounted for in the column that had been colonized with the bacterium M91-3 was presumably associated with the biofilm present on glass beads or had diffused into cells but was not assimilated into biomass. Neither biofilm-sorbed nor intracellular unassimilated radiocarbon was recovered with the sampling methods used. A portion of unaccountable radiocarbon is a function of HPLC and radiometric detection (95% efficiency).

Mineralization of triazine-ring C provided evidence of ring cleavage under anaerobic conditions (Fig. 2). After 264 h, $^{14}\text{CO}_2$ evolution represented 19% of the added label (up to 1% of which may have been derived from labeled impurities) in the M91-3-inoculated column compared to 0.3% in the sterile control (Fig. 2). Analysis of cells washed from glass beads demonstrated that ^{14}C from the triazine ring was not assimilated into biomass (data not shown), which is consistent with results of previous studies (Radosevich et al. 1995a; Wolf and Martin 1975) and was expected, owing to the oxi-

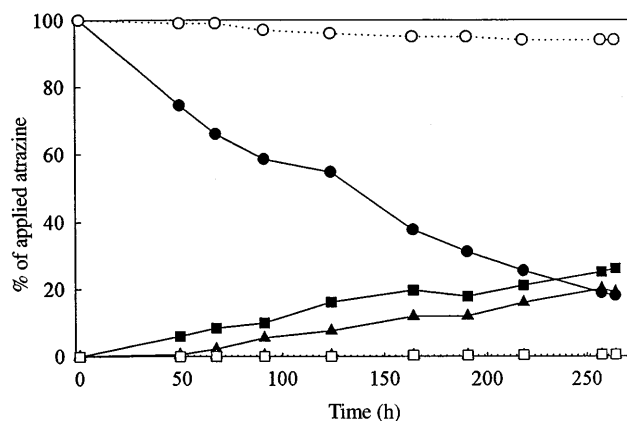


Fig. 2 Disappearance of [^{14}C]atrazine and concomitant ^{14}C -labeled metabolite formation and $^{14}\text{CO}_2$ evolution in biphasic columns containing ^{14}C -labeled AN medium and M91-3. Atrazine: ● M91-3, ○ sterile control. Metabolite: ■ M91-3, □ sterile control. CO_2 : ▲ M91-3, △ sterile control

dized status of the triazine ring C (4^+ , Erickson and Lee 1989). The side-chain C's atoms were unlabeled, thus their assimilation could not be determined by liquid scintillation counting. ^{14}C -labeled metabolites represented 29% of the applied radiocarbon at experiment termination. Of the ^{14}C -labeled metabolites, only one was consistently detected, [^{14}C]hydroxyatrazine, representing 90% of all the metabolite peaks, or 26% of the applied radiocarbon. Atrazine losses in the sterile control may have been due to abiotic $^{14}\text{CO}_2$ formation (0.3% of applied), abiotic hydrolysis to hydroxyatrazine (0.5% of applied) and inefficiency of HPLC detection.

Nitrate depletion concurrent with atrazine disappearance was demonstrated in column samples (Fig. 3). In a subsequent study, denitrification of $^{15}\text{NO}_3^-$ by M91-3 was confirmed by quantifying the evolution of gaseous denitrification end-products $^{15}\text{N}_2$ and $^{15}\text{N}_2\text{O}$ after 8 days of anaerobic incubation. Results demonstrated that $0.87 \pm 0.30\%$ of $^{15}\text{NO}_3^-$ -N was evolved as $^{15}\text{N}_2$, and $^{15}\text{N}_2\text{O}$ was not detected. $^{15}\text{N}_2$ or $^{15}\text{N}_2\text{O}$ enrichment did not occur in sterile controls.

Biodegradation of atrazine in sediment slurries incubated for 5 days under denitrifying conditions was not significantly enhanced by inoculation with M91-3 (Fig. 4a, b). Atrazine mineralization occurred in anaerobic sediments in both the presence and absence of M91-3. The more rapid atrazine mineralization rate in the silty soil compared with the sandy soil may be due to higher microbial numbers in the silty soil due to the increased bioavailability of organic carbon (Tate 1995).

Discussion

In the present study, degradation of atrazine was studied in liquid culture and fixed-film systems with the isolate M91-3 to determine mineralization of ring C, metabolite formation, and denitrification under anaerobic conditions. Also, the atrazine degrader was inoculated into sediment slurries, and atrazine mineralization was quantified to ascertain whether amendment of sediments with M91-3 would enhance atrazine degradation.

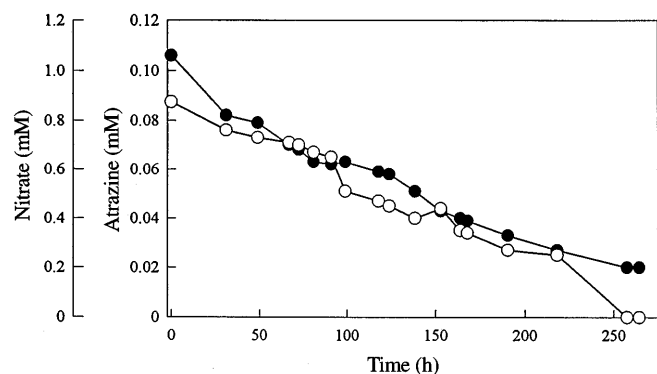


Fig. 3 Disappearance of atrazine and nitrate in biphasic columns colonized with M91-3 and containing ^{14}C -labeled AN medium. ● Atrazine, ○ nitrate

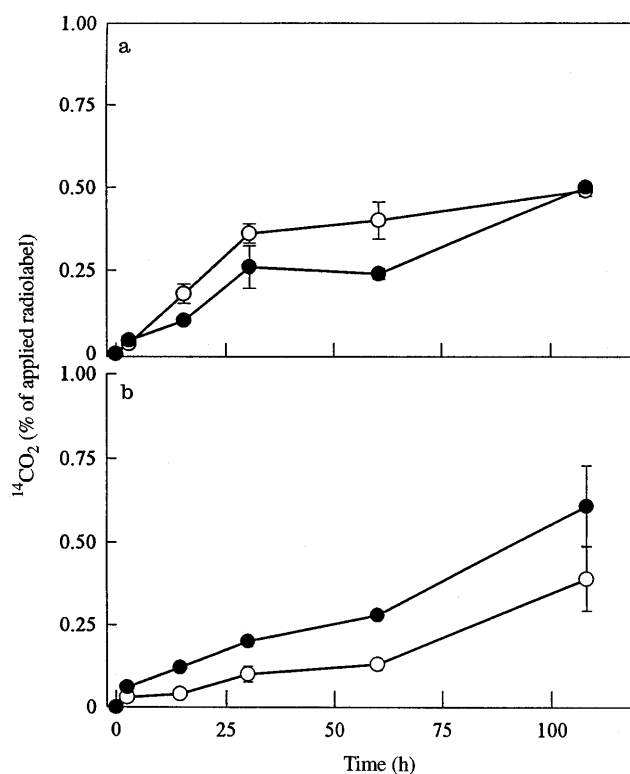


Fig. 4a, b Mineralization of [^{14}C]atrazine under denitrifying conditions in nonsterile aquatic sediments in the presence and absence of M91-3. a S1 (silty) sediment results. b S2 (sandy) sediment results. ● Inoculated with M91-3, ○ Uninoculated

Results of this study demonstrated that, under denitrifying conditions, biodegradation of atrazine by M91-3 involved hydroxyatrazine formation and ring cleavage. Accumulation of other metabolites, namely deaminated or N-dealkylated compounds, was not evident. On the basis of the results of this study, we suggest that denitrification-coupled atrazine degradation by M91-3 proceeds through hydroxyatrazine ultimately to CO_2 and NH_3 (Fig. 5). Ring cleavage between C-2 and N of the triazine ring seems likely due to (i) the electrophilic character of the hydroxylated carbon, and (ii) less steric hindrance associated with the N-ethylamino-substituted side of the molecule (Fig. 5). Stoichiometric balance of this transformation was not possible without further investigation into the completeness of denitrification, triazine-N assimilation, and side-chain-C assimilation. However, the ratio of nitrate to atrazine utilized, approx. 8:1 (Fig. 3), was reasonable owing to the electron (e^-) balance of denitrification ($5 e^-/\text{mmol NO}_3^- \times 0.87 \text{ mmol NO}_3^- = 4.3 e^-$ accepted), and side-chain-C oxidation ($8 e^-/\text{mmol C} \times 5 \text{ oxidizable C}/\text{mmol atrazine} \times 0.085 \text{ mmol atrazine} = 3.4 e^-$ transferred), the remaining e^- being associated with triazine- and amino-N utilization, or incomplete reduction of nitrogenous intermediates in denitrification. Ring cleavage and subsequent utilization of ring N are reasonable reactions for this bacterium considering that, under aerobic conditions, the release of amino and ring N has been observed

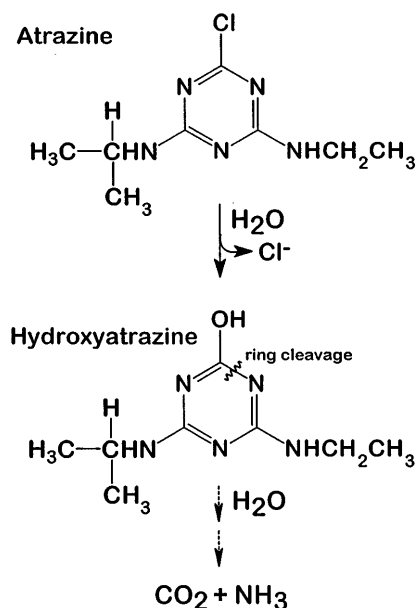


Fig. 5 Suggested pathway of atrazine metabolism by M91-3 under denitrifying conditions

in M91-3 culture medium (Radosevich 1995a). In the previous study, M91-3 aerobically transformed atrazine via ring cleavage, dechlorination, deamination, and dealkylation.

Hydroxyatrazine formation is associated with anaerobic or oxygen-limited conditions, and has been documented in flooded soil (Armstrong et al. 1967; Helling et al. 1971) and atrazine-degrading aerobic cultures (Bouquard et al. 1997; Mandelbaum et al. 1995). Our research results demonstrate the microbially catalyzed formation of hydroxyatrazine under denitrifying conditions. This metabolite is nonphytotoxic (Kaufman and Kearney 1970).

Conclusive evidence that M91-3 is a denitrifying bacterium was obtained by demonstrating the formation of ¹⁵N₂. Nitrate disappearance in the presence of M91-3 was also described. The isolate is similar to *Agrobacterium radiobacter* and a *Xanthobacter* species based on the basis of biochemical and fatty acid methyl ester analyses respectively (Radosevich et al. 1995a).

In the present study, inoculation of contaminated sediment with M91-3 did not enhance atrazine degradation during a 5-day incubation; however, mineralization of the triazine ring in uninoculated sediment slurries suggested that indigenous microorganisms had the ability to degrade atrazine under denitrifying conditions. The inability of M91-3 to enhance anaerobic [¹⁴C]atrazine mineralization contrasts with a previous report of enhanced [¹⁴C]atrazine mineralization in aerobic soils (Radosevich et al. 1996).

In summary, under denitrifying conditions, atrazine served as the sole C and N source for M91-3. The pathway of atrazine degradation included hydroxyatrazine formation and ring cleavage. As a fixed film on glass beads, the bacterium removed both atrazine and

nitrate from aqueous medium, and may therefore be useful in a pump-and-treat bioremediation effort or a wastewater treatment context. Further research is needed to determine the prevalence of this degradative activity under denitrifying conditions in the environment.

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