Cocontaminant Effects on Degradation of Triazine Herbicides by a Mixed Microbial Culture

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In most pesticide-contaminated agrichemical facilities, atrazine is found in combination with other widely used agricultural chemicals, and remediation strategies must account for the multiple-contaminant environment. The ability of an atrazine-mineralizing culture to degrade other *s*-triazines in liquid culture was evaluated. Cyanazine and simazine, added to liquid culture, either alone or combined with atrazine, were degraded in 6 days. Cyanazine was degraded to persistent metabolite(s), as yet unidentified. Metribuzin was not degraded. The culture completely degraded atrazine in the presence of cocontaminants including alachlor, metolachlor, and trifluralin. Atrazine degradation was not affected when cocontaminant herbicides were introduced singly. Mixtures of cocontaminants increased half-life values of atrazine plus hydroxyatrazine 2−3 times. Nitrate presence did not affect atrazine. These results suggest that our mixed culture could be successfully used to bioaugment *s*-triazine contaminated systems, in the presence of nitrate and commonly detected cocontaminant herbicides.

Keywords: *Biodegradation; atrazine; simazine; cyanazine; cocontaminants*

INTRODUCTION

Atrazine [6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine], cyanazine [2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile], metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one], and simazine [6-chloro-*N*,*N*′-diethyl-1,3,5-triazine-2,4-diamine], triazine herbicides used to control weeds in corn and other crops (Weed Science Society of America, 1994), have been detected in ground water as a result of agricultural production (Ritter, 1990). However, commercial agrichemical facilities (Habecker, 1989) or on-farm agrichemical mixing sites may represent a more serious potential for pesticide contamination, as high levels and mixtures of chemicals are typically encountered at contaminated sites. Krapac *et al.* (1993) reported that atrazine, alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide], metolachlor [2-chloro-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide], and trifluralin [2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine] were the four most common pesticides found at 49 agrichemical facilities. Studies of agrichemical mixing−loading facilities in Illinois showed atrazine, cyanazine, metribuzin, and simazine were frequently detected at elevated concentrations in the surface gravel or fill layer (Krapac *et al.*, 1993). Goetsch *et al.* (1993) also detected atrazine, cyanazine, and metribuzin in water samples taken from four agrichemical facilities in Illinois. In addition to the triazine herbicides, alachlor, metolachor, and trifluralin are also commonly detected in soils and ground water beneath agrichemical facilities (Habecker, 1989; Krapac *et al.*, 1993). The exact magnitude of on-farm soil pesticide contamination resulting from storage or mix-load practices has not been established.

While single organisms and mixed cultures that transform or mineralize atrazine have been isolated (Assaf and Turco, 1994a; Behki *et al.*, 1993; Behki and Khan, 1986, 1994; Khan and Behki, 1990; Mandelbaum *et al.*, 1993; Mougin *et al.*, 1994; Radosevich *et al.*, 1995; Yanze-Kontchou and Gschwind, 1995), the mixed culture of Assaf and Turco (1994a) mineralized 96% of 0.56 mM atrazine in liquid culture. This degradation efficiency exceeds most reported atrazine degradation rates found in the literature, except that described by Yanze-Kontchou and Gschwind (1994). Previously reported isolates were only capable of biotransformation of atrazine to form metabolites (Behki *et al.*, 1993; Behki and Khan, 1986, 1994; Khan and Behki, 1990; Mougin *et al.*, 1994). Moreover, this mixed culture was also able to mineralize 87% of 0.14 mM atrazine in soil (Assaf and Turco, 1994a). To date, only one other study has shown mineralization of atrazine (4.6×10^{-4} mM) in soil samples by introduced organisms (Yanze-Kontchou and Gschwind, 1995). Microbial degradation of simazine and metribuzin has been reported to occur in soil by an isolated fungi (Behki and Khan, 1994; Beynon *et al.*, 1972; Kaufman *et al.*, 1965; Locke and Harper, 1991a,b; Moorman and Harper, 1989; Schilling *et al.*, 1985; Sirons *et al.*, 1973). Sirons *et al.* (1973) reported degradation of cyanazine in soil with formation of metabolites. Beynon *et al.* (1972) also reported transformation of cyanazine in soil but could not directly attribute degradation to soil microorganisms.

Microbial remediation systems have been proposed as a cost effective method for cleanup of contaminated materials (Koybayashi and Rittmann, 1982). Forsyth *et al.* (1995) suggested solid-phase, slurry-phase, and *in situ* and *ex situ* systems for cleanup of contaminated soil and ground water. *In situ* and *ex situ* bioremediation have been suggested for soils and ground water contaminated with atrazine (Habecker, 1989; Risatti and Zagula, 1993). Bioaugmentation, the introduction of microorganisms capable of degrading pesticides, can be used in both *in situ* and *ex situ* remediation systems. Shin and Crawford (1995) described bioremediation of TNT (2,4,6-trinitrotoluene)-contaminated soil in a bioreactor after bioaugmentation with *Clostridium bifermen-*

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tans. Baud-Grasset and Vogel (1995) reported biodegradation of over 95% of 2,4-D [(2,4-diphenoxy)acetic acid] from sandy soils contaminated with 710 mg kg^{-1} 2,4-D after bioaugmentation with 2,4-D-degrading microorganisms.

Little data have been presented on the degradation of herbicides present as mixtures or on the impact of inorganic nutrient sources on mixed herbicide degradation. The ability of our mixed culture to mineralize atrazine in liquid and soil environment (Assaf and Turco, 1994a) suggests its potential use as a tool for bioremediation of contaminated soils. The objective of this research was to determine the potential of our mixed microbial culture to degrade *s*-triazine herbicides, in the presence of herbicide mixtures and NO_3-N. Successful degradation of multiple herbicides in the presence of cocontaminants would increase the value of the mixed microbial culture as a remediation tool.

MATERIALS AND METHODS

Culture Isolation, Medium Composition, and Sample Preparation. Enrichment of the mixed culture capable of degrading atrazine was previously reported (Assaf and Turco, 1994a). The mixed culture was isolated from soil subjected to repeated applications of atrazine. Liquid cultures were prepared in minimal basal salts medium supplemented with 0.5% glycerol (C source) and atrazine at 0.23 mM (N source). The minimal basal salts medium was composed of 0.1 M K_2HPO_4 and KH_2PO_4 (pH 7.0), 0.8 mM $MgSO_4$, 0.18 mM $CaCl_2$, 5 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $FeCl_3$, 1 μM $CoCl_2$, 1 μM $NiSO_4 \cdot 6H_2O$, 1 μM $Al_2(SO_4)_3 \cdot 18H_2O$, 1 μM H_3BO_3, 1 μM $FeSO_4$, and 8 μM sodium molybdate.

Cultures were grown in the minimal basal salts medium (0.5% glycerol) supplemented with atrazine. The degradation rate of atrazine in mixed cultures was monitored, and a target atrazine degradation rate of 0.12 mmol L^{-1} day^{-1} was confirmed prior to use.

Microbial population size in liquid cultures was determined by direct counts using acridine orange staining (0.03% acridine orange in $NaHCO_3$). Cells were counted by epifluorescence microscopy (standard microscope 16, Zeiss, West Germany) under UV light (Zeiss, West Germany). Mixed cultures were diluted in the minimal basal salts medium to achieve the desired population size of 1.1×10^9 cells mL^{-1}. Cultures (25 mL) were maintained in 125 mL Erlenmeyer flasks plugged with cotton at 22 ± 2 °C on a rotary shaker (150 rpm) (G10 gyrotory shaker, New Brunswick Scientific Co., Inc., Edison, NJ).

Herbicides and NO_3. Cyanazine, metribuzin, and simazine, technical grade (ChemService, West Chester, PA) with 98% or greater purity, were added to liquid culture at 0.23 mM, 0.23 mM, and 12.6 μM, respectively. Atrazine (technical grade, 99% purity; ChemService, West Chester, PA) was added at 0.23 mM.

Technical grade (>98% purity; ChemService, West Chester, PA) alachlor, metolachlor (both of the acetanilide chemical family), and trifluralin (dinitroaniline chemical family) were used to evaluate cocontaminant effects on microbial degradation of atrazine. In addition to atrazine (0.23 mM), cocontaminants were added to liquid culture singly and in combination at concentrations of 0.59 mM, 1.1 mM, and 0.72 μM for alachlor, metolachlor, and trifluralin, respectively. In another experiment, the effect of NO_3 on atrazine degradation was evaluated by adding KNO_3 (0.12 and 0.23 mM) to cultures containing 0.23 mM atrazine.

Triazine herbicides, and cocontaminant herbicides when used, were added to atrazine-degrading cultures at the concentrations indicated previously. Herbicides were dissolved in filter-sterilized ethanol to form stock solutions. These solutions were briefly sonicated prior to use to insure adequate mixing and lysis of possible contaminant microorganisms. Herbicides were directly added to mixed cultures, and in no case did ethanol addition exceed 1% of culture volume. No atrazine precipitates were observed in these cultures; however, atrazine was possibly suspended in media rather than being fully dissolved. No other herbicide precipitates were observed, as herbicides other than atrazine were added at levels below their maximum water solubility (Weed Science Society of America, 1994). Uninoculated controls were prepared by adding the herbicides to sterile liquid medium and were monitored for possible abiotic degradation. No abiotic degradation of atrazine or other triazine herbicides was noted in the duration of this study. Treatments were prepared in duplicate. Herbicide degradation was monitored using HPLC as described below.

Herbicide Analysis of Liquid Cultures. Herbicide degradation was monitored by sampling 1 mL from each culture. Samples were centrifuged (Marathon 21K, Fisher Scientific, Pittsburgh, PA) for 20 min at 16000*g* to precipitate cells. The supernatant was removed and analyzed using HPLC (Varian Model 5000 liquid chromatograph, Varian Instrument Group, Walnut Creek, CA) to determine the concentration of parent triazine herbicides and metabolites.

The HPLC was equipped with a Gilson holochrome UV−vis detector (230 nm). Triazine herbicides and metabolites were separated with a 250 × 4.6 mm C_{18} column (Spherisorb 10 ODS 2, Phenomenex, Torrance, CA) using a 60:40 (v:v) methanol:50 mM ammonium acetate (pH 7.4) mobile phase at 22 ± 2 °C and a flow rate of 1 mL min^{-1}. Triazine herbicides and hydroxyatrazine concentrations were determined by integration of peak areas, which were equated to solution concentration using external standards.

With this method, it was not possible to resolve cyanazine and the only detectable atrazine metabolite, hydroxyatrazine [2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. An alternative HPLC method was developed to allow quantification of hydroxyatrazine and cyanazine. The methanol:ammonium acetate mobile phase was replaced with 50:50:1 (v:v:v) acetonitrile:50 mM potassium phosphate (pH 5.0):acetic acid. All other HPLC conditions were the same as described above.

Previous research (Assaf and Turco, 1994a) indicates 96% mineralization of atrazine in liquid culture, indicating no accumulation of atrazine or metabolites in cell material. In addition we have conducted trials where the remaining cell pellet was lyzed by sonication and the supernatant from that process analyzed for atrazine and hydroxyatrazine content. These trials indicate that there is no significant atrazine associated with cell material immediately following complete removal of atrazine from liquid medium.

RESULTS AND DISCUSSION

Triazine Degradation in Culture. Atrazine (0.23 mM), simazine (12.6 μM), and cyanazine (0.23 mM) were degraded by the mixed culture in 2, 0.5 and 8 days, respectively (Figure 1a−c). Metribuzin was not degraded by the mixed culture (Figure 1d). Hydroxyatrazine was formed as the intermediate product in the atrazine-supplemented cultures but was degraded in all cultures by day 3. No other atrazine metabolites were detected using either HPLC method. Atrazine degradation and hydroxyatrazine formation and mineralization by our culture were unaffected by the presence of other triazine herbicides. However, simazine degradation was slowed when atrazine was combined with simazine in the cultures, requiring 1 day for complete degradation of simazine. No simazine metabolites were detected.

Cyanazine was degraded to persistent metabolite(s) in 8 days by the mixed culture. In the presence of atrazine, cyanazine degradation increased, requiring only 6 days for degradation; however, the cyanazine metabolite(s) were still evident. An additional study of cyanazine degradation with and without atrazine present was conducted to determine long-term persistence of cyanazine metabolite(s) in culture (data not shown). After 100 days and weekly supplementation with atra-

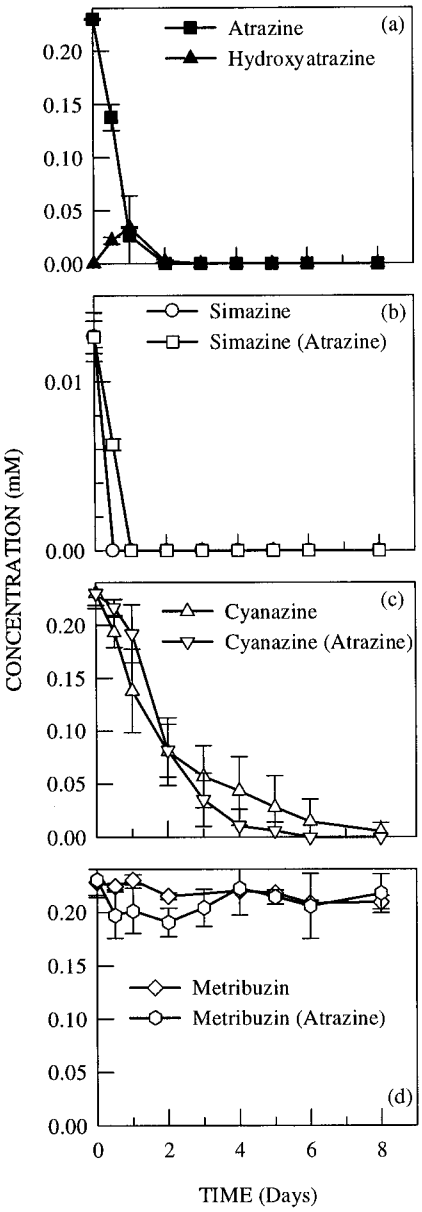

Figure 1. Degradation of triazine herbicides, as single triazines and mixtures, by our culture: (a) atrazine and hydroxyatrazine degradation by the cultures, with atrazine as the sole triazine; (b–d) degradation of simazine, cyanazine, and metribuzin by the culture, both as the sole source of triazine and coapplied with atrazine.

zine, the cyanazine metabolite(s) were still detectable in culture at the highest concentration previously observed. Formation of the cyanazine metabolite(s) was not affected by the presence of atrazine.

The rapid degradation of atrazine and simazine by this mixed culture can be explained by the chemical similarity between the two molecules. The only difference between atrazine and simazine is at the 6-position which is an isopropylamino group for atrazine and an ethylamino group for simazine. Since this ethylamino group is already present at the 4-position of atrazine, the enzyme system for removal of this group was likely active in our atrazine-degrading culture. Conversely, the inability of our culture to fully degrade cyanazine may be explained by molecular differences between cyanazine and atrazine. The presence of the large methylpropanenitrile group of cyanazine as well as the nitrile group may inhibit its removal from the triazine ring. The inability to remove the methylpropanenitrile group from cyanazine may prevent triazine ring cleavage and subsequent mineralization. Mass spectroscopic examination of the persistent cyanazine metabolite(s) is ongoing; however, to date the metabolite(s) are unidentified.

Using similar reasoning, metribuzin, a 1,2,4-triazine, is chemically dissimilar to both atrazine and simazine, which are 1,3,5-triazines. This difference could prevent ring cleavage of the 1,2,4-triazine ring via previously proposed pathways for 1,3,5-triazine compounds (Cook, 1987; Mulbry and Kearney, 1991). In addition, metribuzin contains a methylthio group at the triazine ring carbon, whereas the other *s*-triazines evaluated were chlorinated at the 2-position of the triazine ring. This along with the occurrence of hydroxyatrazine implies that dechlorination is the key first step in degradation of triazine herbicides by our culture. This probability was previously noted by both Skipper and Volk (1972) and Assaf and Turco (1994a).

While efforts to identify the atrazine-degrading organisms are ongoing, mixed culture and initial isolate cultures have been evaluated for their genetic similarity to other atrazine-degrading organisms (see De Souza *et al.*, 1996). Our mixed culture was shown to contain the gene sequence for the *Ava*I DNA fragment (M. J. Sadowsky, personal communication) previously identified from *Pseudomonas* sp. strain ADP (De Souza *et al.*, 1996). This sequence is responsible for production of atrazine chlorohydrolase, an enzyme enabling transformation of atrazine through the formation of the initial metabolite hydroxyatrazine. These findings along with degradation patterns further support hydroxyatrazine as the principal metabolite formed by our triazine-degrading culture and support our discussion of reasons for the lack of metribuzin degradation.

A conservative population estimate of atrazine degraders can be extrapolated, utilizing the rate of hydroxyatrazine formation (3×10^{-6} μmol mL^{-1} min^{-1}, calculated from day 1 hydroxyatrazine concentrations) at the 0.23 mM initial atrazine concentration, a specific activity of 0.208 μmol min^{-1} mg of protein^{-1} (atrazine chlorohydrolase; De Souza *et al.*, 1996) from *Escherichia coli*, and our initial population size. Normalizing the rate of hydroxyatrazine formation to the specific activity, 1.5×10^{-5} mg of protein mL^{-1} is associated with atrazine-degrading microorganisms. Assuming that 10% of living cells are crude protein (Luria, 1960), this protein level indicates approximately 1.5×10^{-4} mg of cell mL^{-1}. Using the value of 10^8 cells mg^{-1} biomass (Turco and Konopka, 1990), approximately 1.5×10^4 cells mL^{-1}, of the microbial population of 1.1×10^9 cells mL^{-1}, are responsible for the conversion of atrazine to hydroxyatrazine and probably the subsequent mineralization of the metabolite.

Cocontaminant Effect on Triazine Degradation in Culture. To assess the impact of cocontaminants on triazine degradation, we monitored atrazine degradation in the presence of alachlor, metolachlor, trifluralin, and NO_3-N, all commonly detected at agrichemical mixing–loading facilities. Half-life ($t_{1/2}$) values for atrazine (plus hydroxyatrazine) were calculated assuming first-order kinetics (Table 1) and are statistically compared using analysis of variance (Steel and Torrie, 1980).

The presence of single cocontaminant herbicides in the mixed culture did not generally affect atrazine degradation when compared to atrazine alone (Figure 2a–d). In all cases of single cocontaminant herbicide

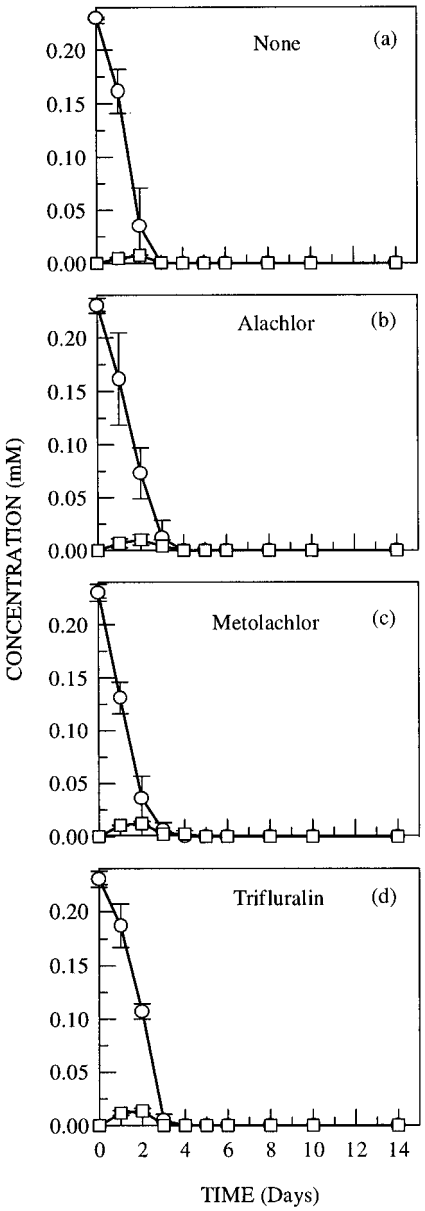

Figure 2. Atrazine (○) and hydroxyatrazine (□) concentration in culture as affected by the presence of cocontaminant herbicides: atrazine and hydroxyatrazine degradation by the culture when no cocontaminant was present; (b−d) effect of a single-cocontaminant presence on atrazine and hydroxyatrazine degradation in culture.

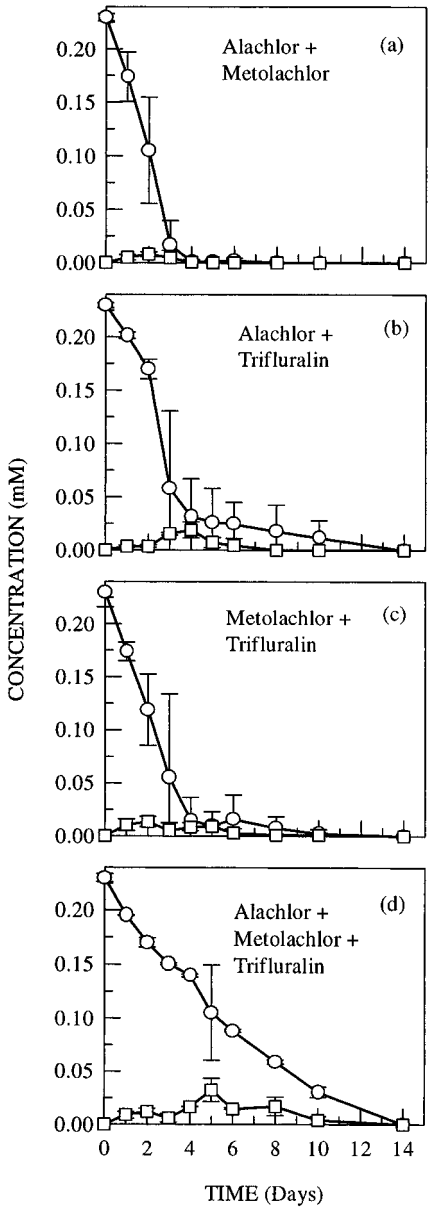

Figure 3. Atrazine (○) and hydroxyatrazine (□) concentration in culture as affected by the presence of cocontaminant herbicides: (a−c) effect of a two-part cocontaminant presence on atrazine and hydroxyatrazine degradation; (d) shows the effect of a three-part cocontaminant mixture on atrazine and hydroxyatrazine degradation by the culture.

Table 1. Half-Life of Atrazine in the Presence of Herbicide Cocontaminants

treatment	atrazine half-life (days)[b]
no cocontaminant	1.0c
alachlor	1.2c
metolachlor	1.0c
trifluralin	1.4bc
alachlor + metolachlor	1.4bc
metolachlor + trifluralin	2.0bc
alachlor + trifluralin	2.3b
alachlor + metolachlor + trifluralin	3.6a
LSD	1.1

[a] Values followed by the same letter are not significantly different at the $P = 0.05$ level.

addition, 95% of atrazine was degraded by day 3. However, trifluralin did increase the atrazine half-life to 1.4 days. Hydroxyatrazine formation and degradation were also unaffected by the presence of single cocontaminants. Hydroxyatrazine was not present after day 5 in any single cocontaminant treatment (Figure 2). Mixtures of two cocontaminants resulted in an increased half-life of atrazine, including hydroxyatrazine, when compared to the presence of a single-cocontaminant herbicide (Table 1).

Mixtures of two non-triazine cocontaminants resulted in an increased half-life of atrazine, including hydroxyatrazine, when compared to the presence of a single-cocontaminant herbicide (Table 1 and Figure 3a−c). Atrazine half-life in the alachlor/metolachlor cocontaminant treatment increased to 1.4 days, which was greater than the half-life for either cocontaminant alone. In the two-cocontaminant mixtures of either alachlor or metolachlor with trifluralin, the atrazine half-life increased to 2.3 and 2.0 days, respectively. The presence of all three cocontaminants, alachlor, metolachlor, and trifluralin, resulted in an atrazine half-life of 3.6 days (Table 1 and Figure 3d). This represents a 2−4-

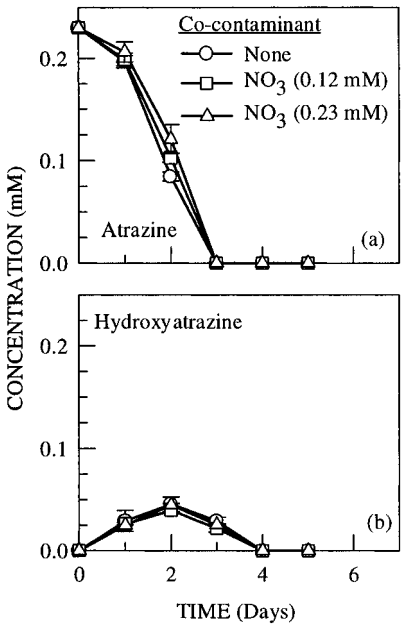

Figure 4. (a) Atrazine degradation by the culture was not affected by NO_3-N presence. (b) Presence of NO_3-N in culture also did not affect hydroxyatrazine formation and subsequent degradation is shown.

fold increase in half-life when compared to cocontaminant herbicide application, either as single- or two-cocontaminant systems. Stojanovic *et al.* (1972) reported that mixtures of two to four pesticides were more easily degraded in soil than the pesticides applied singly. They attribute this finding to the pesticide supplying a broader range of substrates as mixtures compared to single pesticides (Stojanovic *et al.*, 1972). Results of our study, however, indicate that mixtures of herbicides of different chemical families can greatly decrease the rate of degradation of atrazine and its metabolite, hydroxyatrazine, by an adapted population.

Atrazine catabolism as a N source has been proposed (Cook, 1987; Mulbry and Kearney, 1991). Mandelbaum *et al.* (1993) and Radosevich *et al.* (1995) reported the use of atrazine as the sole source of nitrogen. Therefore, strategies for triazine remediation must account for readily available N in the contaminated environment found at agrichemical facilities (Krapac *et al.*, 1993). Nitrate-N did not impact atrazine degradation compared to cultures with atrazine as the only N source (Figure 4a). Atrazine was degraded by day 3 at all NO_3-N concentrations. Likewise, hydroxyatrazine mineralization was not affected by the presence of NO_3-N (Figure 4b). In all cases hydroxyatrazine was degraded by day 4 of the study. This corresponds with the findings of Assaf and Turco (1994a) who found that in culture, initial N supplementation to growth media was needed but no subsequent N was required.

Assaf and Turco (1994b) also found that atrazine degradation by native soil microorganisms was unaffected by initial concentrations of urea-N or C amendment, up to 80 mg kg^{-1} in soil. Our culture was capable of degrading multiple *s*-triazine herbicides, singly or in mixtures. Our results, and other studies (Assaf and Turco, 1994a; Skipper and Volk, 1972), suggest that dechlorination is the key first step in triazine degradation and that the atrazine chlorohydrolase enzyme may be constitutively produced.

Reports of microbial degradation of pesticides in mixed contaminant systems are limited. Behki and Khan (1994) showed degradation of triazine herbicides, atrazine, propazine, and simazine by a *Rhodococcus* strain. Degradation of atrazine, metolachlor, and trifluralin in soil by *Kochia* species has also been described (Anderson *et al.*, 1994). The potential for successful bioremediation by bioaugmentation of triazine-contaminated soils and waters is great if cultures capable of rapid degradation in a mixed chemical environment are available and proven to work in soils.

As indicated by atrazine degradation, the culture was capable of degrading *s*-triazine herbicides in the presence of cocontaminants commonly detected at agrichemical facilities. These characteristics suggest the suitability of our culture for remediation of systems contaminated with multiple *s*-triazine herbicides and associated herbicide and fertilizer N cocontaminants.

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