Atrazine Removal Using Aquatic Plants: A Kinetic Approach

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ABSTRACT

Aquatic and terrestrial plants and microorganisms were screened to test their ability to remove the herbicide, atrazine, from the environment. Plants included hornwort (*Ceratophyllum demersum*), switchgrass (*Panicum virgatum*), reed canarygrass (*Phalaris arundinacea*), annual ryegrass (*Lolium multiflorum*), and two varieties of perennial ryegrass (*Lolium perenne*). Microorganisms studied included a fungus of the genus *Penicillium* and an uncharacterized sample of neuston (floating microbial community) dominated by green algae. The atrazine-metabolizing fungus was found to afford a variety of as yet uncharacterized degradation products.

An aquatic plant, hornwort, was found to take up atrazine from dilute aqueous solution (50 μg/L, 0.23 μM), a concentration typical of spring runoff in the northern United States. The rate of uptake was light-dependent, and even in the absence of plants atrazine was lost, due to induced photochemical reactions initiated by nitrate and an iron (III) complex in the nutrient solution used in the experiments. Breakdown products of atrazine in the presence of plants included deethylatrazine, desisopropylatrazine, and a glutathione conjugate of atrazine. Hydroxyatrazine was not detected. Autoradiography indicated that labeled material largely remained within the plant cells, indicating that microorganisms played a minor role in the disappearance of atrazine. The deethyl- and desisopropyl derivatives were also formed by photochemical processes in the absence of plants.
1. INTRODUCTION

Agricultural runoff is well known to contribute to contamination of surface and groundwater. Due to its non-point nature, such contamination is difficult to regulate or even to assess. Nevertheless, recent concerns over the occurrence of atrazine and related herbicides in groundwater, surface water, and public drinking water supplies have led to studies directed toward reducing their concentrations in soils and soil runoff.

A. Phytoremediation - background

A considerable body of knowledge has been accumulated in the past two decades concerning phytoremediation, or the use of plants to remove contaminants (usually heavy metals) from polluted environments. Phytoremediation has many attractive features including low initial cost, relatively low impact, public acceptance, potential for self-sustainability, and potential for immobilization of toxic compounds. Several studies in recent years have shown that some plants not only take up potentially hazardous organic materials, but metabolize them enzymatically to forms that may be less toxic, either to the plant itself or to the environment. Uptake of inorganic nutrients such as fertilizer-derived nitrate has also been observed in laboratory and field studies. It has been demonstrated that atrazine can be partly removed and converted to metabolites by the roots of hybrid poplar trees that could be planted in barrier strips adjacent to streams (Burken and Schnoor, 1997). However, insufficient data have been collected
in these studies to demonstrate the relative contributions of plants, soil microorganisms, and soil constituents to the removal process.

A previous study on the phytoremediation of herbicide-containing waters with aquatic plants was performed by Rice et al. (1997). These authors found that several herbicide-tolerant plants, namely *Ceratophyllum demersum*, *Elodea canadensis*, and *Lemna minor*, were able to reduce the concentrations of metolachlor and atrazine in test-tubes containing the plant and the herbicide. *C. demersum* was the most effective species tested under these conditions.

In other literature reports, eight aquatic plants were examined for their ability to remove metals from wastewater (Rai et al., 1995). *Hydrodictyon reticulatum* and *Ceratophyllum demersum* were shown to be among the most effective species for reducing levels of chromium, iron, manganese, and lead. In another study, *C. demersum* was found to be highly effective at concentrating cadmium from a hydroponic nutrient solution (Gupta and Chandra, 1996).

B. Atrazine environmental fates

Atrazine (structure 1) is a widely-used triazine herbicide that has been used by itself or in combinations for decades. Atrazine is recognized to have considerable environmental persistence, but at the same time its relatively high water solubility (35 mg/L, 1.6 x 10^{-4} M) has led to its detection in a variety of waters, including
groundwater, surface freshwater, and drinking water (Kolpin and Kalkhoff, 1993; Kolpin et al., 1997; Battaglia et al., 1999). The highest levels in surface waters normally occur shortly after it is applied to fields in the spring. Early field studies demonstrated that atrazine was metabolized (or abiotically converted) to various products including deethylatrazine (structure 2), deisopropylatrazine (structure 3), and hydroxyatrazine (structure 4). These products continue to

\[
\text{2} \quad \text{3} \quad \text{4}
\]

be the most frequently identified breakdown products of atrazine in the environment.

Several attempts have been made to model the loss of atrazine from the environment (Armstrong et al., 1967; Gan et al., 1996; Clemente et al., 1998; Jenks et al., 1998). Atrazine loss rates in soil were generally highest near the surface, one study indicating that from 0-30 cm depth the first-order rate constant was about 0.02/day (half-life about 37 days), whereas deeper in the soil (270-300 cm) it was about 0.003/day (half-life 223 days: Jenks et al., 1998). Rates of loss also vary with concentration (Armstrong et al., 1967; Gan et al., 1996). In one study using soil columns the half-lives ranged from 108 days at the lowest concentration studied (5 mg/L) to 250 days at the highest (50 mg/L: Armstrong et al., 1967). In a more recent soil-column study, 9% of atrazine remained after 84 days (which would correspond to a 16-day half-life, though no
rate constants were calculated: Kruger et al., 1993).

Losses of atrazine in water have been reported by several investigators. In Roberts Creek, Iowa, atrazine disappeared rapidly in a light-dependent manner with half-lives ranging from about 1 day in summer to 7 days in winter (Kolpin and Kalkhoff, 1993). In contrast, the half-life of atrazine in a 230-m long wetland mesocosm containing plants, *Daphnia*, frogs, and mosquito larvae ranged from 8-14 days (Detenbeck et al., 1996).

Certain individual microorganisms or consortia of microorganisms are able to mineralize atrazine. Some are able to use it as a sole source of nitrogen. The partial metabolism of atrazine by particular microorganisms often results in the production of hydroxyatrazine (Mandelbaum et al., 1993), although other reports suggest that oxidative metabolites such as side-chain dealkylation products are also formed (Behki and Khan, 1986).

In the case of multicellular organisms, many herbicides are oxidatively metabolized by monooxygenase enzymes such as P450 forms (Frear, 1995). In the case of atrazine, the most electron-rich and easily oxidizable sites would be expected to be the N-alkyl side chains, and numerous investigations have revealed the formation
of deethyl- and deisopropylatrazine. A competing pathway in plants (and perhaps in bacteria) is a nucleophilic aromatic substitution reaction with glutathione, leading ultimately to a dechlorinated adduct (Structure 5: Field and Thurman, 1996).

Studies of abiotic reactions of atrazine in water have focused on attempted remediation methods. Prados et al. (1995) examined several different HO-radical-generating methods such as the Fenton reaction (iron[II]-hydrogen peroxide) and ozone-hydrogen peroxide combinations. They also showed that triazines were removed with varying efficiencies by all such methods. Attack by HO• or ozone on atrazine is selective, leading principally to dealkylation products; addition to the triazine ring is a minor pathway (Acero et al., 2000; Tauber and von Sonntag, 2000). The N-deethylated product predominates over the deisopropyl compound by 4:1 in hydroxyl radical attack and by 19:1 in ozonation. The initial side-chain HO•-derived radicals, after diffusion-controlled addition of molecular O₂, rapidly eliminate HOO• to form Schiff bases. These rapidly hydrolyze to produce, in addition to the dealkylated amine, stoichiometrically equal quantities of acetaldehyde or acetone, respectively (Tauber and von Sonntag, 2000). Suggestions that photolytic pathways for loss of atrazine in the aquatic environment may be important have been made (Kolpin and Kalkhoff, 1993). These authors pointed out a light-dependent relationship between atrazine degradation rate and day length. The rate constant was not significantly correlated with water temperature, but could have reflected biodegradation.
C. Aquatic plants

The majority of reports on the interactions between aquatic plants and herbicides have focused on the attempted elimination of aquatic weeds from lake and pond environments. For example, it was reported that treatment with the herbicide 2,4-D was successful at clearing Eurasian watermilfoil (*Myriophyllum spicatum*) from Beulah Lake, WI (Helsel et al., 1996). However, the same authors also reported that the native plant *Ceratophyllum demersum*, although it partially died back after the treatment, recovered nearly all of its standing crop within 12 weeks after exposure.

*Ceratophyllum demersum*, also known as hornwort or coontail, and its less common relative, *C. echinatum*, are found in ponds and slowly flowing waters throughout Illinois (Winterringer and Lopinot, 1977). Typically, the species is found to inhabit deep pools and to predominate at >3 m depth (Best, 1977). The fast-growing, floating plants are well-known to rapidly take up nitrate (Kelly, 1995). Preliminary work in our laboratory has also indicated that it is capable of removing dinitrotoluene and other nitrogenous organic compounds from water. *C. demersum* is a native plant, but also can be obtained in large quantities from commercial dealers as a decorative adjunct for aquaria. Furthermore, it has a very large leaf area per gram of biomass, a characteristic similar to that of the subtropical aquatic species, parrotfeather (*Myriophyllum aquatica*), that has been studied for phytoremediation by Jeffers and Wolfe (1997).

*Ceratophyllum* has been shown to be metabolically active in cold temperatures (Spencer
and Wetzel, 1993). Even under ice cover in a southeastern Michigan lake, it was able to photosynthesize (winter level was 32% of summer level) and to accumulate phosphorus.

Reed canarygrass (*Phalaris arundinacea*) is a tall, perennial, early-emergent grass, hardy in winter and has a great disease resistance. It grows well in poorly-drained soils, thriving even in standing water, yet it is exceptionally tolerant to drought. In recent phytoremediation studies, it was shown to partially remove aromatic nitro and chloro compounds such as TNT and PCBs (Dzantor et al., 2000).

D. Previous studies from our laboratory

In a preliminary study, we demonstrated that several native Illinois aquatic plants (found in ponds and drainage channels throughout the state) showed promise in taking up atrazine from water without being killed in the process. Water primrose (*Jussinea repens*) and hornwort (*Ceratophyllum demersum*) were particularly efficient. In contrast to earlier model stream experiments that showed a half-life of 8-14 days for atrazine (Detenbeck *et al.*, 1996), our preliminary studies with *Ceratophyllum demersum* indicated uptake of 50 µg/L atrazine with a half-life of 3-5 days (depending on the mass of plant material). Similarly, *Jussinea repens* took up atrazine with a half-life of 6-7 days. (Substrate materials, gravel and pressed clay, also adsorbed some atrazine, but at significantly slower rates.)

In this study, we have extended the work with *Ceratophyllum* to examine some of the
mechanistic details associated with its activity, and also looked quantitatively at some other pathways of loss for atrazine, including uptake by other aquatic organisms and photochemical decomposition.

2. MATERIALS AND METHODS

A. Reagents and analytical methods

"Saturated" aqueous stock solutions of atrazine were prepared by stirring the solid with deionized-distilled water for 24 h and then filtering the undissolved material. The concentration of such solutions were determined by UV, comparing the absorbance to the molar extinction coefficient of atrazine at the wavelength of its maximum absorbance (223 nm). In one typical preparation, it was found that the solution concentration was 11.3 mg/L.; the literature solubility of the compound has usually been reported to be 30-40 mg/L.

Atrazine concentrations in dilute solution experiments were measured using high-performance liquid chromatography (HPLC). An HPLC method used routinely in our laboratory, involving the injection of large volumes of solution on a crosslinked polystyrene column, allows us to measure concentrations as low as 5 µg/L. Analysis of the atrazine concentrations in each sample was completed using HPLC (Beckman 110B solvent delivery module) with an absorbance detector (Spectroflow 757) attached. Injection volumes were 2.0 mL. An Altima C18-3u with a mobile phase of 1 MeCN:1 H₂O and a flow rate of 2 mL/min was used. A more rapid technique was also developed, using a novel HPLC column (Rocket®, Alltech), which
permitted us to analyze atrazine-containing samples in 6 min as opposed to 25 min for a conventional column. The Rocket is a specially designed 53 x 7 mm column that can speed analysis times by 3-5 fold while maintaining the same efficiency as a 250 x 4.6 mm, 5 μm column. Unlike a short, narrow-bore column, the Rocket Column maintains enough column volume to permit the column’s use on conventional HPLC systems, with no modifications.

For kinetic analyses, area counts were used to determine the concentration of atrazine relative to the initial concentration of the experimental control. To compare the results, graphs were made by plotting these values versus the time of exposure using the natural logarithms of the concentration ratios.

Uptake by plants was also monitored by selective extraction and HPLC combined with photodiode array (PDAD) analysis of the parent compound and its known metabolites. In other cases, it was necessary to work with labeled (14C) herbicide material. Atrazine-ring UL-14C was obtained from Sigma (St. Louis, MO). The wavelength for measurements was 223 nm, the absorbance maximum for atrazine.

*Product analysis* — The oxidative degradation products deethylatrazine, deisopropylatrazine, and hydroxyatrazine were determined by HPLC. The elution characteristics of the standards are illustrated in Figure 1. The glutathione conjugate of atrazine was synthesized *in vitro* to be used as a reference standard using methods described by Clay *et al.* (1990) and
Frassanito et al. (1998). A sample of 5.4 mg atrazine with 4 μCi of $^{14}$C atrazine was dissolved in 5 mL of acetone, and then 445 μL of 45% trimethylamine was added. The solution was evaporated to dryness after 18 hr at room temperature (25°C), and 2 mL of an aqueous solution containing 8.4 mg sodium carbonate and 20.3 mg reduced glutathione was added. This solution was shaken for 18 hr on a water bath at 50°C. The solution was diluted to 10 mL with 0.01 M CaCl$_2$ and stored at 2°C to minimize further reaction. A bulk preparation was done with 200 mg atrazine (unlabeled) following the same procedure with 200 mL acetone, 17.8 mL 45% trimethylamine, 24 mL aqueous solution of 200 mg sodium carbonate and 800 mg reduced glutathione, and diluting to 50 mL with 0.01 M CaCl$_2$. SPE was done as for the plant extract (eluted with methanol). The eluate was evaporated to dryness and a solid was obtained. The structure of the atrazine-glutathione conjugate was confirmed by nuclear magnetic resonance (NMR), mass spectrometry (MS, ionization technique: fast atom bombardment), LC-MS (ionization technique: electrospray, combined with MS/MS analysis), and HPLC-PDAD analyses.

B. Plants and culture conditions
Plants were generally grown in a greenhouse environment, using 6-L polyethylene opentop vessels, containing 5-10% dilutions of Hoagland’s nutrient solution (HNS: see below) and the concentration of atrazine to be tested. During the experiment the water volume was kept constant by addition of reverse-osmosis water as necessary. Control experiments without plants were performed to quantify other loss processes such as uptake by the container. Thermal and light regulation was provided by thermostatic and light source adjustments. Containers included either water alone, water and plants alone, water and sediment alone, or the combination of water, sediment, and plants.

Hoagland’s nutrient solution (Hoagland and Arnon, 1938) has been widely used as a growth medium for terrestrial and aquatic plants. Hoagland’s solution contains a variety of nutrients (Table 1). For all of the nutrients, except for the iron-DTPA complex (Sequestrene-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in 5% HNS</th>
</tr>
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<tbody>
<tr>
<td>MgSO₄×7H₂O</td>
<td>9.98E-5</td>
</tr>
<tr>
<td>Ca(NO₃)₄×4H₂O</td>
<td>2.50E-4</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5.00E-5</td>
</tr>
<tr>
<td>KNO₃</td>
<td>2.97E-4</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>2.31E-6</td>
</tr>
<tr>
<td>MnCl₂×4H₂O</td>
<td>4.60E-7</td>
</tr>
<tr>
<td>ZnSO₄×7H₂O</td>
<td>3.83E-8</td>
</tr>
<tr>
<td>CuSO₄×5H₂O</td>
<td>1.80E-8</td>
</tr>
<tr>
<td>MoO₃</td>
<td>4.17E-9</td>
</tr>
<tr>
<td>Fe-DTPA</td>
<td>5.74E-9</td>
</tr>
<tr>
<td></td>
<td>24.6</td>
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<td>6.8</td>
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<td>0.003</td>
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330, also known as Sprint-330), a stock solution was prepared by dissolving the indicated quantity in deionized water. The iron-DTPA complex was added on the morning of the test. Stock solutions were then stored in a refrigerator for the duration of the tests.

**Hornwort.** -- Hornwort (*Ceratophyllum demersum*) plants were obtained from Maryland Aquatic Nurseries (Jarrettsville, MD), maintained in a greenhouse in Hoagland’s nutrient solution (diluted to 10% strength with deionized water), and kept in large, aerated plastic tanks (stock tubs), 70-L open, shallow plastic containers fitted with aquarium-type aerator-filters. For experiments, plants were removed from the stock tubs and placed in smaller glass or polypropylene vessels together with a solution of atrazine (usually 50 μg/L) and Hoagland’s medium (usually 5% strength).

**Reed canarygrass** – Seeds of reed canarygrass (*Phalaris arundinacea*) were surface sterilized by dipping in sodium hypochlorite (NaClO, 5.25%) plus Tween 80 (0.02%) for 5 min. The sterilized samples were washed twice in 100 mL sterile Hoagland’s nutrient solution (HNS). The seeds were placed on sterile filter paper (4” diameter) which was kept partially dipped in 50 ppb ^14C atrazine (in 100 mL HNS) in 500 mL glass bottles. The bottles were covered with glass lids and incubated in the greenhouse.

Reed canarygrass was also employed in other experiments in which the plant was germinated in foam plugs which were subsequently placed in holes in a vessel lid, with the roots dipping into aqueous solutions of atrazine. Plants were grown under natural daylengths in 2-
liter pots containing 2 L sand and 1.5 L nutrient solution. Plants were supplied with 33 ppb atrazine and a 33% modified Hoagland's solution at the beginning of the experiment.

*Green algae* – Some plant samples became contaminated with one or more unknown filamentous algae, a portion of which was separately removed and tested for its ability to degrade atrazine. The samples contained about 10 g fresh weight algae in 200 mL of HNS containing 50 ppb $^{14}$C atrazine while the sterile controls had no algae. The samples were placed in 1-L Mason jars and incubated in a growth chamber under day/night conditions similar to that of hornwort.

*Penicillium fungus* – A fungal contaminant isolated from “sterilized” reed canarygrass seeds was isolated and a plate culture was obtained on potato dextrose agar (PDA). A sub-culture was obtained directly on microscopic slides by growing spores on PDA pieces placed on slides, for identification. The fungus was identified as a *Penicillium* sp. based on its reproductive structures and hyphae (Figure 2).

**Figure 2.** Photomicrograph of the fungus isolated from reed canarygrass seed.

C. $^{14}$C experiments and autoradiography

In the first $^{14}$C-atrazine exposure experiment with hornwort, a closed Mason jar system was developed to contain uniformly $^{14}$C-labeled atrazine as well as $^{15}$N labeled KNO$_3$. 

17
*Pseudomonas* strain ADP (P. ADP) and *Agrobacterium radiobacter* strain J14a (J14a), previously isolated by workers in G. K. Sims's laboratory, were added to the system. The study was conducted under the conditions: 16 hr light cycle @ 330 μEinsteins/m²s (8hr dark), 18°C, and 25-50% humidity. Water samples were taken every other day for analysis of atrazine and nitrate. The radiolabeled chemicals were tracked using a liquid scintillation counter, HPLC radiodetector, and mass spectrometer. The plants were extracted, combusted, and analyzed for uptake at the conclusion of the experiment. A sample of hornwort plant kept in 1 mg/L ^14^C-atrazine for 30 days was used. All the plant material was alive initially, but certain parts died after about 2 weeks. Both dead and live fractions were analyzed separately. About 1 g of fresh plant material was rinsed twice with 500 mL deionized water; atrazine and metabolites were extracted by homogenization in 2 mL of 99.9% (v/v) methanol using a ceramic mortar and pestle. Dead and live fractions of plant material were pulverized separately. The resulting crude homogenate was centrifuged at 8000 rpm for 5 min, the supernatants were decanted, and the pellets were pulverized and re-extracted with 3 mL of 99.9% methanol using the same procedure. The resulting supernatants were combined with the initial supernatants. Radioactivity in an aliquot of the crude extract was quantified by liquid scintillation spectrometry. The crude extract was analyzed for atrazine and its metabolites using PDAD/HPLC.

In another experiment, the samples contained about 15 g fresh weight hornwort plant in 250 mL of synthetic nutrient solution (HNS, 5%) containing 50 μg/L ^14^C atrazine (uniformly ring-labeled) while the controls had no plants. Controls consisted of both sterile (autoclaved) and nonsterile samples. The samples were placed in 1-L Mason jars and incubated in a growth
chamber with light intensity 145-160 μmol/m²/s⁻¹/μA and temperature around 30°C for day (15 hr) and 25°C for night (9 hr). Weekly aliquots (5 mL) of the samples were removed for analysis. The aliquots were counted for total disintegrations per min (dpm) and analyzed using HPLC (isocratic, mobile phase 60:40 acetonitrile: 0.1% phosphoric acid in HPLC grade water) to obtain the remaining atrazine concentration. Separation of metabolites was done using HPLC gradient analysis (mobile phase: acetonitrile and 95:5 5mM HCl:acetonitrile). PDAD/ HPLC detection was used for identification of the metabolites.

Autoradiography was also used to investigate uptake by hornwort plant and its epiphytes. An initial atrazine concentration of 1 ppm was used for identification (tracking) purposes, while 50 μg/L was used for degradation studies. For autoradiography, hornwort plants were kept in 5% Hoagland's solution containing approximately 1 mg/L ¹⁴C atrazine for 3 weeks (in the growth chamber under day/night conditions). The control plant sample was kept in non-radioactive atrazine (1 mg/L, in HNS). A sample of the plant was dipped in distilled water to remove excess atrazine, fixed in 10% neutral buffer formalin (24hr), and dehydrated using sequentially 70% ethanol, 95% ethanol, 100% ethanol and xylene. Specimens were then embedded in paraffin, chilled, and cut at 4-8 microns on a rotary microtome. The plant cross sections were placed on charged slides, air dried and then put in the lab oven at 60°C for 20 min for fixing. Slides were cooled, deparaffinized and rehydrated by dipping for about 5 min in each of xylene (Histoclear), 100% ethanol, 95% ethanol, 70% ethanol and deionized water (DIW). The slides were air-dried, and, in a dark room, dipped in emulsion (Kodak # 1654433) and air-dried for several minutes. The dried slides were placed into a slide box, which was wrapped with aluminum foil and stored.
at 4°C for 3 weeks. The slides were then dipped in developer (Kodak, Sigma, P-5670) and in fixer (sodium thiosulfate, Sigma S-1648), for 5 min in a dark room, and washed by immersing in a basin of DIW for about 5 min. The slides were air-dried and observed under the microscope for black colored silver granules.

*Differential filtration:* Differential filtration was used to investigate whether atrazine was taken up by fractions other than the hornwort/epiphyte system in water. A plant segment (with epiphytes) was dried at 60°C for 3 days and ground into a powder and then combusted and oxidized to count the 14C. The solutions of the samples, which contained the plant and the nonsterile control (no plant), were filtered through 8 μm pore size Millipore filters (to separate plant debris etc) followed by 0.45 μm (to separate microbes). Material remaining on the filter papers was also counted for radioactivity (dpm).

**D. Light exposure techniques**

Experiments were conducted using a variety of light sources. The laboratory lamp (“solar simulator”) was a 2000-watt xenon arc, filtered to AM-1 (sea level radiation), and maintained at a distance of 30 cm from the sample to approximate midsummer midday solar intensity. Greenhouse illumination was unaugmented natural sunlight, diminished by passing through ordinary window glass panes which were (during the summer months) whitewashed. Outdoor experiments were conducted during the late summer, early autumn, and midwinter months at
latitude 40° N. In autoradiography and growth chamber experiments, the samples were incubated with light intensity (from fluorescent bulbs) of 145-160 μmol/m²/s/μA and temperatures around 30°C for day (15 hr) and 25°C for night (9 hr). “Dark” samples were kept in complete darkness at similar temperatures. Light intensities in the visible (400-800 nm) range were measured using a Li-Cor meter with a PAR probe; UV-A (320-400 nm) and UV-B (290-320 nm), with an UVX meter having separate probes for the two ranges. Some uptake experiments were performed in which the plants were kept in the dark for several days.

For photochemical kinetic experiments, test tubes were placed on a rotator under the solar simulator described above. The distance from the lamp to the samples was set up to simulate the conditions of a midsummer midday solar intensity. The solar simulator was turned on 20 minutes prior to the start of the experiment to allow it to come to a steady light state. Light readings in the photosynthetically active region (PAR) and UV-B range were taken using Li-Cor probes for PAR and UV-B and a datalogger. For each hour that the tests were run, one of the test tubes was removed from the rotator and stored in dark conditions until analysis. A control that received no light exposure was also taken. All of the tests lasted for either 4 or 5 hours.

On all of the testing dates, except for the sterilized test, the Hoagland’s solution was prepared immediately before exposure to the light source. A 100% solution was made initially according to a modified recipe (Table1) with deionized water as the solvent. A 5% solution was then prepared by taking the appropriate volume of the 100% solution and diluting to 1.0 L using deionized water and the atrazine stock solution. Adjustments were made for tests in which the
concentration of one or more of the nutrients was increased. The final concentration of atrazine in each of the final solutions was 50 ug/L. In one of the tests, the Hoagland's solution was sterilized in order to determine microbial effects. Sterilization was achieved by autoclaving the prepared 5% solution at a pressure and temperature of 25psi and 121 °C for 20 minutes. In each case, after all the nutrient solutions were prepared, they were poured into 10 mL borosilicate test tubes. For the sterilized test, transfer took place within a laminar flow hood.

E. Kinetic analyses

Rate constant estimation was done by plotting the natural logarithm of the quantity $C/C_0$ versus time in days, where $C_0$ was the initial molar concentration of atrazine and $C$ the concentration at the time of analysis. The slope of this line was the first-order rate constant for atrazine loss. For most experiments, a first-order approximation fit the data well (there was no appreciable lag period before loss ensued, for example).

3. RESULTS AND DISCUSSION

A. Losses of atrazine in microcosms

Controls. -- The relative insensitivity of the atrazine degradation to temperature is consistent with an earlier report (Grollier et al., 1997) in which it was found that the degradation of the phenylurea herbicide isoproturon by Elodea densa and Ludwigia natans was
Figure 3. Loss of atrazine from microcosms containing hornwort plants. Solid line, control kept in dark. Dotted line, plants present but kept in dark. Dashed line, plants present and exposed to light.

Figure 4. $^{14}$C counts after differential filtration and in the oxidized plant material. The fraction larger than 8 μM consisted mostly of plant debris, whereas that between 8 and 0.45μM consisted largely of microorganisms.
largely unaffected over the range from 12-28 °C. We performed two experiments on the rate of
uptake of atrazine by two minerals used in the greenhouse. One mineral was a fairly large,
smooth gravel and the other was a smaller, rougher pressed-clay medium. The loss of atrazine in
the gravel-only experiment was negligible. The clay substrate was somewhat more active, but
only an 11% decline in the initial 50 ug/L concentration was observed after 15 days.

Plants: temperature and light effects -- Loss of atrazine from containers having
hornwort plants was faster (half-life typically 4-7 days) than that in the controls without plants,
although the rate was greater in higher-light conditions. (Abiotic or photochemical loss of
atrazine was also light-dependent.) Rate constants varied from 0.09/day in low-light conditions to
as much as 0.21/day at high light intensities (Figure 3). There was a slow degradation in the
presence of plants under dark conditions. That the atrazine loss was principally due to the plants
(or to its epiphytic microorganisms) was discerned by a 14-day experiment with radiolabeled
atrazine. At the conclusion of the study, roughly half of the label remained in the water and half
with the plant material. Little or no mineralization occurred.

These results indicate that the presence of the hornwort plant considerably enhanced the
degradation process in a light-dependent manner. Results of differential filtration confirmed
these observations showing that, in the solid fraction of the samples, the plant/epiphyte system
retained the major portion of radioactivity. However, some radioactivity was present also in the
free-living 'microbial fraction' and other debris in the solution (Figure 4). Therefore, fractions
such as bacteria and other possible organisms could be playing an important role in the
Figure 5. Radio-TLC chromatograph of $^{14}$C-atrazine (lower trace) and products released into solution by hornwort (upper trace). The principal metabolite was deethylatrazine (2). X-axis = time in minutes, Y-axis = radioactive counts.
Figure 6. Conversion of $^{14}$C- atrazine (squares) to atrazine-glutathione conjugate (diamonds) and other products (triangles) by Ceratophyllum demersum. Points represent fraction of total radioactivity (which did not change significantly over the course of the experiment) in the methanol extract of the plant tissue.
degradation process. Further evidence of differential contributions of the plant and its epiphytes came from separate analyses of the solution surrounding the plant and extracts of the plant material.

From the solution, the major degradation product found was diethyltrazine (2: Figure 5). If the plant was contributing to the formation of 2, it should have a pool of the metabolite within the tissue. However, it is also widely known that 2 may be produced by other means such as microbial degradation (Behki and Khan, 1986; Roberts, 1998). If the 2 present in HNS was produced by microorganisms, it is very probable that they are epiphytes, because the control without plants (at the light intensity of 145-160 imol m$^{-2}$s$^{-1}$) did not have such a significant production of 2, indicating that the process cannot entirely be due to the microorganisms unattached to the plant or to a photochemical process.

Parallel analysis of extracted plant material indicated that atrazine and a major metabolite (which was not 2) were present in the plant. When the plant was exposed to 20.05 mg L$^{-1}$ atrazine in HNS, the extract contained 11.9 mg L$^{-1}$ (0.0188 mg atrazine per g of plant fresh weight), so a total of approximately 7% of the atrazine was taken into the plant. The fraction of the major metabolite was 53% (of total radioactivity) after 7 days of exposure (Figure 6). In a separate experiment, the fraction of the major metabolite when hornwort was exposed to 1 mg L$^{-1}$ $^{14}$C atrazine for 30 days was 22% (atrazine: 70%, of total radioactivity). This indicated that a larger production of the major metabolite may occur in the plant with increased atrazine concentration in the solution. The major metabolite had the same retention time (HPLC
radiolabel analysis) and mass spectrum as the authentic atrazine-glutathione conjugate 5. The LC-MS analysis indicated that a compound having a mass of 486 and fragment ions at 244 and 214 was present in the metabolite peak (these masses were not present in the control plant extract at the same retention time), with at least one other compound (having masses 478 and 316, these masses were present also in the control plant extract) co-eluting. The evidence further suggests that the metabolite is 5. Metabolism of atrazine in higher plants by conjugation with glutathione has been repeatedly demonstrated (Gimenez et al., 1996; Hatton et al., 1996; Jachetta and Radosevich, 1981; Shimabukuro et al., 1970), and hornwort has shown glutathione-S-transferase activity as a mechanism of detoxification of xenobiotics (Pflugmacher and Steinberg, 1997).

The ability of hornwort to remove atrazine concentrations greater than 50 ppb was examined. Initial concentrations of atrazine were 100, 300, and 500 ppb. HPLC analysis showed atrazine losses of 44%, 47%, and 27% respectively after 4 days. These higher concentrations of atrazine did not yield observable damage to the plant. The first half-life for 100 ppb atrazine was 5.3 days (calculated rate constant was 0.13/day); 300 ppb was 3.9 days (calculated rate constant was 0.18/day); and 500 ppb was 6.9 days (calculated rate constant was 0.10/day). We concluded that atrazine could be removed at higher concentrations without apparent damage to Ceratophyllum demersum.

The concentration of atrazine removed by Ceratophyllum demersum (hornwort) within the first 90 minutes after plant addition was determined under indoor laboratory conditions. Initial concentration of atrazine was 50 ppb. The HPLC results showed an atrazine loss of 10 ppb.
within the first 90 minutes. This experiment was replicated three times; hornwort removed an average of 8 ppb of atrazine within the 90 minute period. We concluded that removal of atrazine by Ceratophyllum demersum occurred rapidly upon plant introduction into the water, which suggests that atrazine-degrading microorganisms were either not important in the initial uptake (since no lag phase to build up their activity was observed), or that they were present on the plant from the beginning of exposure.

**Effects of temperature on atrazine uptake by hornwort.** -- To continue to understand interactions between hornwort and atrazine uptake/loss, an experiment was conducted under three temperature regimes and no light. The temperatures represented warm greenhouse conditions (37°C), ambient room temperature (22°C), and cold winter conditions (7°C). The plants were grown for 12 days in 500 ml beakers and supplied with 5% modified Hoagland’s solution and 50 ppb atrazine at the beginning of the experiment. Plants grown in all three temperature regimes behaved similarly with half-lives ranging only from 24 days in the room temperature treatment to 28 days in the cold treatment. The half-life in the warm treatment was 27 days. These results suggest that temperature has little effect on atrazine loss in the presence of hornwort plants.

On the other hand, the controls (without plants) did have disappearance rate variations indicating atrazine loss increased as the temperature increased. The cold control treatment had the longest half-life at 173 days as compared to 51 days for the warm control treatment. These data suggest either that microorganisms present in the medium degrade atrazine by a light-
independent pathway, or (less likely) that there is some thermal, perhaps hydrolytic, route for atrazine degradation in the medium.

*Experiments with reed canarygrass.* -- Experiments were performed using *Phalaris arundinacea* (reed canarygrass) to determine its effect in atrazine degradation. *P. arundinacea* is a species that can tolerate a wide range of soil moisture conditions and is commonly found in stands on the edges of lakes and in wetlands (Bernard and Lauve, 1995). These authors studied nutrient uptake and growth of reed canarygrass at a site receiving landfill leachate, concluding that the species was effective for filtering landfill leachate and able to remove a significant amount of N, Fe, and Mn.

Five experiments were performed to determine the ability of *P. arundinacea* to remove atrazine from waters. The grass was grown for two weeks and, in the first experiment, had a significant effect on reducing atrazine with a half-life of 5.8 days compared to 21.4 days for the control (no plants). The second experiment was performed under similar conditions, but the atrazine loss was less significant. The difference in atrazine loss may have been due to the age of seedlings and/or light conditions. In the first experiment, seedlings were 7.5 weeks old, whereas in the second experiment, the seedlings were only 4 weeks old. Also, during the first experiment 11 of the 14 days were sunny as compared to only 3 in the second experiment.

Two other experiments were performed using reed canarygrass seedlings of different ages and with greater numbers of plants, but it was not possible to reliably distinguish the observed loss
of atrazine in the presence of the plants from that of the no-plants controls. We conclude that, at
least under these conditions, reed canarygrass was not effective at removing atrazine from solution.
In a fifth experiment, in which it was attempted to grow the plant from sterile seed, one of the
specimens became contaminated by a fungus, which was studied separately.

B. Role of abiotic (light-induced) processes

Atrazine disappearance rates from abiotic “controls” having no plants were significant and
light-dependent. In darkness, atrazine concentrations showed little or no decrease over a period of
up to two weeks. Sunlight, greenhouse, or xenon lamp exposure, however, did result in significant
losses of atrazine. Rate constants varied from about 0.03/day for low-light exposures (PAR = 400
μmol/m²/sec) to about 0.09/day for high-light periods (PAR = 1800). Since atrazine has no
absorption of light in the solar UV or visible region, an indirect mechanism for photodegradation
must be occurring. The principal sunlight-absorbing constituents of the Hoagland medium are
nitrate (Σ = 7.2 at 302 nm) and the micronutrient iron(III) complex, Sequestrene-330 (Σ = 7500 at
its absorbance maximum, 278 nm; measurable extinction was observed out to ca. 450 nm). Nitrate
is well-known to produce hydroxyl radical on illumination (Zepp et al., 1987), and indeed,
experiments in which solutions of atrazine and nitrate were exposed to xenon-arc UV did lead to
disappearance of atrazine (half-life ~30 hr). The reaction for this process is:

\[ \text{NO}_3^- + \text{H}_2\text{O} + \text{hv} \rightarrow \cdot\text{NO}_2 + \text{HO}^- + \text{HO}^- \] (Larson and Weber, 1994).

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Hydroxyl radicals are known to be highly reactive with most organic compounds, including atrazine (Zepp et al., 1987; Pratap and Lemley, 1998; Balmer and Sulzberger, 1999; Acero et al., 2000; Tuber and von Sonntag, 2000), and could conceivably account for the observed loss in the pesticide. However, nitrate has no absorption above 320 nm, and accordingly it could not be responsible for the loss of atrazine that was observed in greenhouse experiments, since the glass windows remove all UV-B wavelengths. Other light-absorbing constituents, however, are also present in the recipe for the nutrient solution that we are currently using: salts of iron, copper and zinc. The other principal sunlight absorbing component of the Hoagland's nutrient solution, as mentioned previously, is the iron complex, Sequestrene 330. Similar to nitrate, ferrous (Fe²⁺) ions have also been found to produce hydroxyl radicals (Pratap and Lemley 1998) with the reaction being:

\[ \text{Fe(OH)}^{2+} + \text{hv} \rightarrow \text{Fe}^{3+} + \text{•OH} + \text{HO}^- \] (Larson and Weber 1994).

Therefore, this too could be contributing to the overall loss in atrazine when it is exposed to sunlight. Previous work in our laboratory has also shown that various iron salts (in the ferric form) are efficient photosensitizers (Larson et al., 1991), and act by a mechanism involving hydroxyl radical.

The form of iron in the Hoagland's solution is ferric (Fe³⁺) sequestered by a chelating agent [sodium ferric diethylenetriaminepentaacetate (Sequestrene 330™, Novartis)]. A series of aqueous solutions of Sequestrene 330 (10% ferric iron) and atrazine were exposed to solar-
Figure 7. Atrazine photolysis in water (control).

Figure 8. Atrazine photodegradation in 5% Hoagland’s nutrient solution.
Figure 9. Atrazine degradation under low-light conditions. Illumination: winter greenhouse sunlight (100µmol/m²/sec). [Atrazine]: 50 ppb (2.3 x 10⁻⁷ M). [Nitrate]: 500µM. [Fe(III)] = 0.17 µM.

Filled circles: Nitrate only. Filled triangles: iron(III) complex + nitrate. Open circles: iron(III) complex only, no nitrate. Open triangles: Complete 5% Hoagland’s nutrient solution.
simulated light (PAR: 1300 μmol; UV-B: 3 x 10^{-4} kW). In all cases, a fast initial disappearance of atrazine occurred, followed by a slower decay. However, the rate constants of the fast reaction were quite variable, with a range of 0.117/hr - 0.599/hr; the rate constants for the subsequent, slower reaction were more consistent, averaging 0.059/hr. The slower reaction is likely to represent competition for the hydroxyl radicals between atrazine (at a reduced concentration) and the less-reactive organic side chains of the iron complex.

A control test in which atrazine was illuminated in solution with only water and no nutrients showed no loss in concentration over the 5-hour testing period (Figure 7). This correlates with atrazine’s lack of absorbance within the solar UV region. The 5% HNS resulted in a 22% reduction in the amount of atrazine after 5 hours (Figure 8). The largest decrease in concentration occurred within the first hour of exposure to light. Sterilization of the 5% HNS resulted in about the same percentage decrease (20%) in atrazine concentration compared to the non-sterilized solution. This indicates that the role of microorganisms in the degradation of atrazine was not significant in these experiments.

In order to assess the feasibility of photochemical reactions at the low concentrations used in the 5% HNS (nitrate = 500 μM, ferric iron = 0.17 μM) an experiment was performed in the greenhouse under low light conditions. Ambient light was in the range of 1300 μmol (sunny) to 100 μmol (cloudy), however, the samples were placed in sealed borosilicate tubes always in a shaded area so that during the daylight hours the average PAR was 100-150 μmol. The loss of atrazine was slow with the complete 5% HNS (t ½ = 20 days: Figure 9), but comparable to data
previously obtained under similar light levels in the greenhouse during the summer (sunny, but with whitewash on glass). In the presence of nitrate only, there was some loss of atrazine; with the addition of the iron complex or the iron complex and nitrate, acceleration of loss occurred. The results indicate that, under greenhouse conditions, both nitrate and iron contribute to the photochemical loss of atrazine.

Forms of ferrous and ferric iron, as well as nitrate, are well known to be sources of hydroxyl radicals when illuminated. One way to demonstrate the generation of hydroxyl radicals is to compare the relative reactivity of probe compounds, such as anisole (methoxybenzene) and nitrobenzene, in the test system (Zepp et al., 1987). Kinetic analysis of hydroxyl radical reactions with these compounds have been well characterized, showing that reactivity is dependent upon the electron-donating/withdrawing nature of the functional group attached to the benzene ring; that is, anisole should be more reactive than nitrobenzene.

When dilute solutions of anisole and nitrobenzene were exposed to solar simulated light in the presence of Sprint 330, the first order rate constant for anisole loss was 0.435/hr, and for nitrobenzene, 0.136/hr (Figure 10). The difference of reactivity by a factor of 3.2 is comparable to that observed in other •OH-producing systems and is also consistent with other light-induced ferric iron reactions. However, the reactions slowed considerably after the first hour. In the presence of the complete HNS medium (5%) the initial rates for the two compounds were quite slow, but did continue without significantly decreasing. The reasons for the different rate behavior in HNS between atrazine (faster in HNS than in iron-only solution) and the probe compounds
Figure 11. Atrazine photodegradation in different concentrations of Hoagland’s solution. Upper line, 5%; lower line, 50%.

Figure 12. Atrazine photodegradation in Hoagland’s solution with 2x iron.
(slower in HNS) are not understood. However, a second technique for examining hydroxyl radical importance gave less ambiguous results; addition of the selective quenchers, D-mannitol and t-butanol. These compounds are widely used hydroxyl radical scavengers that have little activity toward other oxidants (Buxton et al., 1988). In separate tests, 0.8 mM of D-mannitol and 3 mM of t-butanol were added to 5% strength Hoagland’s solutions containing 50 ppb of atrazine and illuminated. In both cases, the result was that there was almost no loss in atrazine for the duration of the test. This indicated that there were hydroxyl radicals present in the system.

Increasing the concentration of all the nutrients resulted in a greater reduction in atrazine for the duration of the test (Figure 11). The 10% and 50% HNS experiments indicated a 35% and 48% loss in the concentration of atrazine at the completion of the test, respectively. The shape of both these degradation curves was the same as that for the 5% solution. For at least these concentrations of HNS, an increase resulted in a greater amount of atrazine loss.

*Effect of changes in iron-DPTA and nitrate concentration* -- The HNS with twice the concentration of iron-DTPA had approximately the same amount of atrazine loss as the original 5% HNS solution (Figure 12). The solution containing five times the amount of iron-DTPA, however, resulted in an increase in atrazine reduction. The loss for this test after 5 hours was 34% of the initial atrazine concentration. This is probably due to consumption of •OH by the DTPA side chains of the iron complex. Both of the solutions containing increased nitrate concentrations resulted in a greater amount of atrazine being lost compared to the original 5% HNS (Figure 13). The loss for the solution containing twice the nitrate resulted in a 35% reduction in atrazine, with a
Figure 13. Atrazine degradation in 5% HNS with increased [NO₃⁻]. Filled circles: 2x [NO₃⁻]. Open circles: 5x [NO₃⁻].

Figure 14. Atrazine photodegradation in 5% HNS without Fe(III)DTPA. (Sequestrene)
Figure 15. Atrazine photodegradation in HNS with increased [micronutrients]. Filled circles: 5% HNS without amendments. Open circles: with 100x [Mn]. Filled triangles: with 100x [Zn]. Open triangles: with 100x [Cu].
51% loss for the 5-fold increase.

From the previously listed results, it was expected that the iron complex and nitrate components of the solution were the ones responsible for the majority of the atrazine loss. To determine if any other constituents were also causing atrazine breakdown, the iron and nitrate were not added to the Hoagland’s solution. In the test in which only the iron complex was removed from the solution, there was a decrease in the percentage of atrazine lost, to 20% of the initial concentration. The decrease occurred in a linear fashion (Figure 14) with a rate constant of -0.0418/hr. The test in which both the iron complex and nitrate components of the HNS were removed showed no loss in atrazine concentration for the duration of the test. These results show that there were no other major contributors to atrazine loss, at least in their current concentrations.

Effect of changes in the micronutrients copper, zinc, and manganese — The nutrients copper, zinc, and manganese are only present in small amounts within HNS. To test if there were any effects on atrazine loss caused by these micronutrients, their concentrations were increased in the final solution. For the tests in which zinc and manganese were increased by a factor of 10, there was a slightly greater loss in atrazine after five hours, with values of 27% and 24%, respectively. The copper test was only run for four hours and showed about the same reduction (23%) as that for the 5% HNS at the same time. Increasing the concentration of these micronutrients 100 times resulted in a decrease in atrazine loss for both zinc (23%) and copper (18%) relative to the test with the 10-fold increase (Figure 15). When manganese was increased by a factor of 100, an increase in the percentage of atrazine lost (29%) occurred.
Although copper and zinc both resulted in a decreased loss in atrazine, this was probably not the action of the metals themselves. Instead, both of these components were sulfate species. In a comparison of rate constants of various species with hydroxyl radicals, compounds containing sulfate have been found to slow down reactions involving hydroxyl radicals, possibly by quenching them (Madden, 1996) to produce the less reactive sulfate radical. Therefore, this may have been the cause of the decrease of the amount of atrazine lost.

C. Roles of plants and microorganisms

A continuing key question in this project concerns the relative role of plants and plant-associated microorganisms for the observed uptake and degradation of contaminants. Previous studies have shown that hornwort and/or its associated microbes are capable of degrading atrazine in aquatic systems, producing mainly deethylatrazine along with other currently unidentified products.

*Studies on microorganisms: surface sterilization attempts* -- Little is known about techniques to surface-sterilize aquatic plants. We attempted to apply techniques that have been successful for terrestrial species. Hornwort samples (about 10 g fresh weight per treatment) from the stock were surface sterilized using one of the following: sodium hypochlorite (NaOCl, 5.25% or 1.05%) plus Tween 80 (0.02%), 18 h under ultraviolet (UV) radiation, 70% ethanol, or hydrogen peroxide (3% or 1.5%). For sodium hypochlorite treatment, the plants were taken out
quickly after dipping (5 sec) because they became bleached within 15 sec of dipping in the sterilant. The sterilized samples were washed twice in 500 mL sterile minimum nutrient medium (HNM, same as for stock plants, prepared in de-ionized water). For UV radiation, the plant samples in HNM were placed under UV lamps in a laminar flow cabinet for 18 h. The samples were kept still (no shaking). For 70% ethanol and hydrogen peroxide treatments, plant samples were dipped in the sterilant for one, two, five or fifteen min and then washed in 500 mL sterile HNM twice. The control sample contained nonsterile plants. The treatments were in duplicate. The sterile or nonsterile samples were placed in 1-L Mason jars containing 250 mL HNM with lids loosely closed for aeration. The jars were placed in a growth chamber, at an angle so that the glass surface (not the metal lid) was exposed to light. Light intensity in the growth chamber was 145-160 μmol/m²/s⁻¹/μA which is approximately similar to the outside sunlight condition on a cloudy day with some light penetration. Temperature in the growth chambers was maintained around 30°C. To obtain the bacterial plate-count after sterilization, a sterile (or nonsterile – positive control) plant piece of about three inches in length was shaken in 50 mL sodium phosphate buffer, and a 1 mL sample of the buffer was spread on an agar plate and incubated at 30°C for 24 h. Survival of the sterile plants was investigated for about a week.

The results showed that hornwort plants could not survive any of the surface sterilization methods. Sodium hypochlorite caused bleaching of the plant, possibly because the leaves do not have a wax coating as many terrestrial plants do. All the treated plants lost chlorophyll and died within about five days. UV sterilization led to development of apparent mutations (tumors) and subsequent death of the plant during incubation. Ethanol turned a bright yellow color after dipping.
Figure 16. Loss of atrazine in a growth chamber with light-dark exposure. Filled circles: control (no plants or algae). Open circles: 10 g (wet weight) of unidentified filamentous algae present. Filled triangles: 10 g (wet weight) of hornwort present.

Figure 17. Loss of atrazine in samples containing reed canarygrass. Filled circles: sterile plants. Open circles: nonsterile plants. Filled triangles: Plants contaminated by fungus (*Penicillium* sp.)
the plants into it, presumably because it dissolved colored organic compounds from the plant tissues. Ethanol treated plants became brownish and died within five days. Hydrogen peroxide (1.5%) seemed to be a better surface sterilant and did not show apparent damage to the plant immediately after the sterilization and also reduced microbial numbers to a great extent. However, new shoots of hydrogen peroxide treated (1 min only) plants showed poor growth while other parts were dead. Those new shoots did not develop well during the incubation.

Algae and fungi – About 10 g fresh weight hornwort plant or 10 g fresh weight algae in 200 mL of 5% HNS containing 50 ug/L $^{14}$C (uniformly ring labeled) atrazine were set up. The controls had no plants and consisted of both sterile and nonsterile samples. The samples were placed in 1-L Mason jars and incubated in a growth chamber with light intensity 142-150 $\mu$mol/m$^2$/s$^{-1}$/µA or in complete darkness at a temperature around 30°C. Weekly aliquots (10 mL) of the samples were taken for analysis. The aliquots were counted for total dpm and also analyzed using HPLC (isocratic mobile phase 60:40 acetonitrile: 1% w/v acetic acid) to obtain the remaining atrazine concentration. In preliminary experiments, samples that contained plants had shown marked pH increases during incubation, therefore pH was buffered at 7.0 using a phosphate/carbonate buffer.

The samples containing hornwort plant and algae degraded atrazine while no significant degradation was observed in controls (Figure 16). Samples containing hornwort plant had a first half-life of about five days, and degraded atrazine almost completely within three weeks. In the presence of algae, half the atrazine was gone after about 11 days, and about 65% of atrazine
disappeared in three weeks. There was a slow degradation (35% after three weeks) in the presence of plants under dark conditions. Dark controls behaved the same way as light controls. Despite the presence of a buffer in these experiments, pH increased during the experiment.

Reed canarygrass was not a good degrader of atrazine (Figure 17). There was no clear difference in the loss of atrazine in sterile and nonsterile samples. However, a fungus found as a contaminant in a sterile replicate (we could not obtain 100% sterility of seeds by the detergent used, even though it was the best out of several sterilants we tested) was capable of degrading atrazine by about 80% within a week with an initial lag phase of about two weeks. Fungal metabolites included compounds 2, 3, possibly 4, and other more polar substances for which we had no reference standards. Further studies with the fungus demonstrated that it was a member of the genus *Penicillium* (Figure 2).

Analysis of hornwort plant extract showed that atrazine and certain metabolites were present in the extract. Extract of the live fraction of the plant contained 70% atrazine and 30% metabolites from total radioactive chemical content, while the dead fraction contained 80% atrazine and 20% metabolites. Composition of metabolites were similar between live and dead materials. A major metabolite in both live and dead extracts had a similar retention time as the standard of atrazine-GSH conjugate. The chemicals present in dead plant samples may have been taken up when the plant was alive, or there can be reactions continuing in the dead material as well. It also is noteworthy that the extract is not free of epiphytic microbes, but having proven that radioactivity is present in the plant tissues (by autoradiography), we assume that the hornwort
Another experiment was conducted using $^{14}$C uniformly ring-labeled atrazine to investigate to what extent atrazine is taken up by hornwort and its associated (epiphytic) microbes. In the first experiment, initial atrazine concentration used was 1.4 mg/L (with $1.4 \times 10^3$ dpm per mL) to facilitate analysis and identification. The samples contained about 20 g fresh weight hornwort plant in 300 mL of 5% Hoagland's solution containing atrazine while the controls had no plants. After exposure, the plants were removed on a weekly basis. Plant samples were sonicated in Hoagland's diluted medium to separate surface microbes prior to filtration. Then the solution was filtered successively through an 8 μm pore size filtration disc (to separate plant debris, etc.) followed by an 0.45 μm disc (to separate microbes) prior to counting. The samples containing hornwort degraded atrazine faster than the control. The majority of the radioactivity was found on the 0.45-μm filter, indicating that it was most likely associated with microorganisms (Figure 4), although plant cells could have lysed and contributed some small particles. It is evident that atrazine was taken up both by epiphytic microbes and plants. Although sonication could not completely separate surface microbes from the plant, the evidence indicates that their activity was important. The major metabolite in solution was deethylatrazine.

**Autoradiography** -- Microscopic autoradiography was used to localize $^{14}$C atrazine in plants and their associated microbes. Microtomal leaf sections were applied to radiosensitive film and exposed for three weeks. In the developed images (Figure 18), silver grains were concentrated inside the plant tissues. Atrazine was more densely distributed in mature parts,
including the stem, than in tender tissues. It is very likely that a greater portion of absorbed atrazine is either getting metabolized, or immobilized in the mature parts. The slides prepared without dehydration and rehydration processes in embedding showed higher density of silver granules compared with the slides undergone the complete procedure. This indicates that dipping in various solvents may have removed a part of $^{14}$C atrazine from the tissues. From the radioautographic pattern, there was no clear indication that atrazine is taken up by surface microorganisms. Certain areas of the plant surface contained dense distribution of silver granules, but the inconsistency of that surface-distribution prevented making conclusions using light microscopy (microbes were almost the size of silver granules).

Figure 18. Autoradiogram (cross-section) of a hornwort leaf treated with $^{14}$C-atrazine for 3 weeks.

4. CONCLUSIONS AND RECOMMENDATIONS

Extensive work on “constructed wetlands” has indicated promise for this approach for the
management of agricultural runoff and other aquatic contamination. Less work has been reported on the management of channels containing aquatic vegetation for agricultural phytoremediation. Although overgrowth of channel plants can reach the point where drainage is impeded (especially in southern locations where plants such as *Hydrilla* and *Eichhornia* have reached nuisance levels), this may not be as much of a problem in colder states like Illinois.

Hoagland's nutrient solution has an active role in the degradation of atrazine when it is illuminated. The activity present mainly comes from two of the light absorbing components of the solution, the iron complex and nitrate. Increasing the concentration of these two nutrients increases the amount of atrazine that is broken down in a given time period. In addition, when both of these components are removed, there is no loss in atrazine concentration. The role of microorganisms in this system is very minute or non-existent as was indicated by sterilization. Other constituents of the solution have no apparent role in atrazine loss, except for the possible quenching of hydroxyl radicals by sulfate when it is present in

Having shown that atrazine is taken up by plants such as hornwort, the mechanism of degradation/metabolism of the compound inside the plant is an interesting area to investigate. The autoradiography technique does not distinguish between the parent compound and metabolites. Therefore other techniques should be practiced to investigate the fate of atrazine in the plant. Analysis of extracts of plants exposed to labeled atrazine is a potential approach to identify the compounds present in the plant tissues. Reactions of agrochemicals mediated by enzymes such as glutathione S-transferase and benzo[a]pyrene hydroxylase have been investigated in various
plants, animals and microorganisms. It is possible that atrazine is conjugated with compounds like glutathione at the sites of entry (mature areas) in the resistant plants (Field and Thurman, 1996) preventing translocation of the compound into photosynthetically active areas.

5. REFERENCES


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