SCSGC-T-96-002 C2

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MICROBIAL COMMUNITY EXPOSURE TO ATRAZINE

IN

ARTIFICIALLY SIMULATED TIDAL CREEKS

BY

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Microbial Community Exposure to Atrazine in Artificially Simulated Tidal Creeks. Marie E. DeLorenzo. 1996. NOAA/NOS. An in-house report

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INTRODUCTION

Agricultural pesticide runoff is a critical issue for many southeastern estuaries. The microbial food web plays an important role in nutrient cycling and transfer of nutrients to higher trophic levels. Microbial communities may serve as sensitive, early indicators of environmental stress due to pesticides.

Primary production in aquatic ecosystems does not follow a strictly linear progression (algae, zooplankton, and fish). Beginning with a landmark paper by Pomeroy (1974), attention turned to another, quantitatively important food web composed of heterotrophic microorganisms. Approximately 50% of phytoplankton cell constituents may be incorporated into microbial pathways rather than passing to higher trophic levels (Fenchel, 1988; Pomeroy and Wiebe, 1988; Berman, 1990; Sherr and Sherr, 1988; 1991). The term "microbial loop" refers to these microbial pathways, in which nutrients are rapidly recycled. The microbial food web has not been extensively studied in tidal creeks, and toxicity data involving microorganisms and pesticides are limited.

Atrazine is an s-triazine herbicide used primarily to control broad-leaf plants and grassy weeds. It one of the most extensively used herbicides in the United States and is routinely detected in aquatic habitats due to runoff from agricultural fields (deNoyelles *et al.*, 1982; Thurman *et al.*, 1992; Pereira and Hostetller, 1993). Atrazine accounts for 60% of the total pesticide volume applied to crops each year, with an estimated 29 million kilograms of atrazine active ingredient applied in 1989 alone (Gianessi and Puffer, 1991). Atrazine is algistatic, inhibiting photosynthesis by blocking electron transport during the Hill reaction of photosystem II (Hull, 1967; Forney and Davis, 1981). Algal responses to atrazine vary widely, depending upon concentrations used, duration of exposure, and algal species tested. In the present study, the effects of atrazine on the microbial food web were examined in artificially constructed tidal creeks.

METHODS

The artificial tidal creek mesocosm system consisted of three chambers, each with three intertidal regions: high, mid, and low marsh, and a subtidal creek channel. The mesocosms were enclosed in a greenhouse. Tidal flow and a salinity gradient were incorporated (See Figure 3).

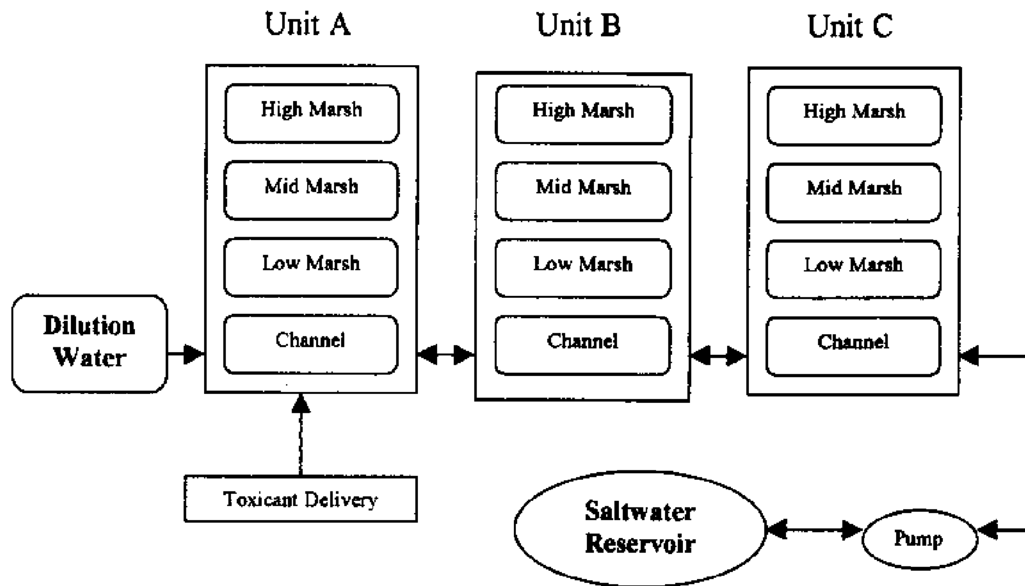

Figure 3. Mesocosm design simulating an estuarine ecosystem (Lauth *et al.* 1996).

The above design allowed for a control plus a low (40 μg/L) and high (160 μg/L) pesticide treatment. Units A, B, and C were treated as replicates, although they did exchange water and thus were not independent. Exposure in the high and low units was designed to simulate non-point source runoff events. Pesticide dosing occurred July 18, 1996. Pesticide was introduced as a pulsed exposure along with freshwater in Unit C at ebb tide and allowed to mix through units B and A on the incoming high tide. Earlier studies (Lauth *et al.* 1996) have found that water pesticide concentrations reached equilibrium in each unit within 24 hours (two tidal exchanges).

Marsh plants and sediment were collected from a historically clean reference marsh on the western branch of Leadenwah Creek on Wadmalaw Island, South Carolina and transplanted into the mesocosms. Other investigators in the study deployed and monitored bioassay organisms, such as oysters, grass shrimp, seed clams, fiddler crabs, marsh snails and fish in mesocosms. These test species, also collected from Leadenwah Creek, were used to assess the toxicity of atrazine to higher organisms. After marsh stabilization, microorganisms were colonized in the mesocosms using polyurethane foam substrates positioned in the tidal channel of each mesocosm at the low tide mark. The artificial substrates chosen were polyurethane foam cubes (4x5x6 cm). Polyurethane foam permits colonization by swimming and sessile organisms. Previous use has exhibited a high degree of species overlap among replicates. Colonized sponges are good representatives of the natural microbial community composition and productivity (Cairns *et al.*, 1979).

One substrate was collected from each mesocosm immediately prior to pesticide addition, 24 and 48 hours after pesticide addition. After 24 hours, clean substrates were placed in each mesocosm to examine microbial colonization patterns in an atrazine impacted system. These substrates were removed 9 days after the start of the experiment. Samples were taken by removing substrates from the mesocosms and gently squeezing the contents into individual sterile sample cups. Each substrate yields approximately 60 mL volume. The samples were homogenized by gently stirring the contents and aliquots were removed for the different analyses.

<u>Phototrophic Carbon Assimilation</u>

To measure phototrophic carbon assimilation, 5 mL of sample were spiked with 1 µCi/ml $NaH^{14}CO_3$ (fc) and placed in an incubator set at *in situ* light regime and temperature for 24 hours. After 24 hours samples were filtered through 0.45 µm cellulose nitrate membrane filters and rinsed with 0.2 µm filtered seawater. Filters were placed into scintillation vials and 1 mL of 10% HCl was added. Samples were allowed to fume overnight in the dark. The next day, scintillation fluid was added and disintegrations per minute (DPM) measured after 4 hours (a

stabilization period) using a liquid scintillation counter. This method was adapted from DiTullio, 1993 and Li *et al.*, 1980.

Chlorophyll *a*

Chlorophyll *a* was used to estimate phototrophic biomass. 3 mL of sample were filtered onto glass fiber filters (Type GF/F). After filtering, samples were kept in the dark for the rest of the procedure. Filters were placed in vials with 1 mL $MgCO_3$ and frozen until analysis. Next, 9 mL of acetone were added, shaking well. Samples were refrigerated overnight, shaken the next day, and refrigerated overnight again. The next day, samples were brought to room temperature and read on a Sequoia-Turner Model 450 fluorometer.

Chlorophyll *a* (µg/mL) was determined as:

(light aperture constant*fluorescence reading*gain correction*acetone volume)/volume filtered*gain). (1)

Phototrophic Biovolume

For community composition assessments, 5 mL of sample were preserved in 5% buffered formalin (fc) and another 5 mL were preserved by adding 0.5 mL Lugol's solution (50 mL acetic acid + 200 mL Lugol's). Samples were stored in the dark at 4 °C until analysis. Algal cells were counted by placing 20 µL of diluted, formalin preserved sample on a slide and viewing 4 transects across the coverslip at a total magnification of 500. Algae were identified to genus where possible and the number of cells per taxon, average cell shape and size were recorded for each taxon. Phototrophic biovolumes (μm^3) were estimated for each taxon using cell sizes and simple geometric shapes (Wetzel and Likens, 1991) and then multiplied by the total number of cells per taxon in each sample.

Bacterial Abundance

Bacterial abundance in each sample was determined using epifluorescence microscopy (Kepner and Pratt, 1994). 0.25 mL of chemical dispersant (Na_3 PPi) was added to the formalin-preserved sample and sonicated briefly. The sample was then diluted with 0.2 μm filtered sea water and stained with the fluorochrome, acridine orange, (100 mg/L, fc) for three minutes. The sample was then filtered onto a 0.2 μm black membrane filter and mounted on a slide. Bacterial counts were made using a Zeiss fluorescence microscope with super high-pressure mercury lamp and blue light filter (470-490 nm). A total magnification of 1250 was used, with oil. Ten random grids were counted and then averaged for each sample. Bacterial density was calculated using the formula:

Bacterial density (cells/mL) = (N x Af) / (d x Ag) (2)

where

N = average number of cells counted,

Af = effective area of the filter (mm^2),

Ag = area of the counting grid (mm^2),

d = the dilution factor.

Ciliates and Flagellates

Heterotrophic ciliate and flagellate abundances were determined from Lugol's preserved samples. Diluted samples were placed on a Sedgewick-Rafter slide (1 mL volume) and 10 random grids were counted at a total magnification of 500. At least 200 organisms were counted in each sample. An average of the 10 grids was calculated for each sample. Standard error was less than 10%. Unpigmented protozoa were sorted into size classes of > 20 μm and < 20 μm. They were enumerated from the 24, 48 and 72 hour samples. Taxonomy of the ciliates and flagellates was not determined.

Analytical Chemistry

Pesticide Recovery

Water samples were taken from the mesocosms immediately after dosing and after 48 hours to measure pesticide concentration. Pesticide residues were extracted onto C18 cartridges, using two column volumes of methanol followed by one column of distilled, deionized water to condition the cartridge. Sample extraction was followed by one column of distilled, deionized water. Columns were then dried (pulling air through under vacuum for approximately 5 minutes), wrapped in aluminum foil and stored frozen until elution. Pesticides were then eluted off the C18 columns with ethyl acetate (two column volumes). Samples were transferred to TurboVap tubes, the ethyl acetate was evaporated to ~ 0.5 mL, isooctane was added (~ 10 mL) and evaporated again to 0.5 mL. Using isooctane, samples were diluted to 1 mL in a graduated tube and then transferred into gas chromatography (GC) vials, capped, and frozen until analysis on the gas chromatograph.

Determination of Pesticide Concentrations

Samples were analyzed GC-Nitrogen -Phosphorus Detection (NPD). A Hewlett Packard HP 5890 series II gas chromatograph equipped with an HP 7673 liquid autosampler tower, a capillary inlet system, and a PC-based data handling system was employed. The atrazine standard was 120 ng/mL. Standards were injected at the beginning of each sample string and every five injections thereafter. The carrier gas was helium, and detector makeup gases were nitrogen (ECD) and helium (NPD). The analytical column was a J&W Scientific DB-5, 5% phenyl-, 95% methyl- polysiloxane, 30 m in length (0.25 mm x 0.25 mm). The injection volume used was 2 mL in the splitless mode and the average linear velocity was 33 cm/sec.

Statistical Analyses

Three replicates were used in each treatment. The values were averaged and plotted in bar-graph form with error bars to indicate standard deviation. An asterisk denotes those

treatments which were statistically different from the control. Statistics were calculated using a MINITAB® software package. One-Way Analysis of Variance (ANOVA) was used to test for significant differences among treatments for each endpoint measured (e.g. chlorophyll, bacterial abundance, etc). The validity of using parametric procedures was confirmed by first testing for equal variances, normality and homeoscadicity (F-test). Where ANOVA revealed a significant difference among treatments, Dunnett's procedure for multiple comparisons was used to determine which treatments differed significantly from the control (Zar, 1984). The family error rate was set at 0.05.

RESULTS

Atrazine targeted the phototrophic variables measured. After 24 hours there was a two-fold difference in chlorophyll *a* between the control and atrazine treated mesocosms, with the 40 and 160 μg/L treatments being reduced to approximately the same degree. Chlorophyll *a* remained reduced throughout the experiment. Chlorophyll *a* content was also impaired on substrates colonized 24 hours after dosing in the treated mesocosms (Figure 1).

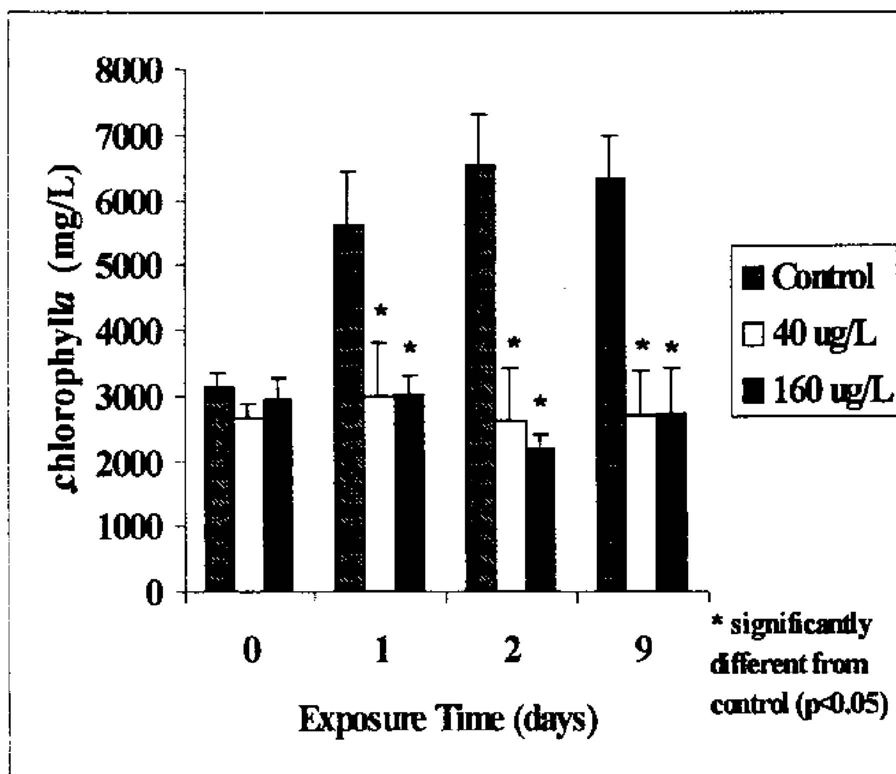

Figure 1. Atrazine effects on chlorophyll *a* measured from artificial substrates.

Water column chlorophyll *a* was also reduced in the presence of atrazine. Water samples from treated mesocosms were reduced five-fold from control values, again with the 40 and 160 μg/L treatments being reduced to approximately the same degree (Figure 2). Atrazine was also found to inhibit phototrophic carbon assimilation in the artificial tidal creeks. After 24 hours of exposure, carbon assimilation was reduced in a dose-response manner. Productivity was further inhibited after 48 hours (Figure 3).

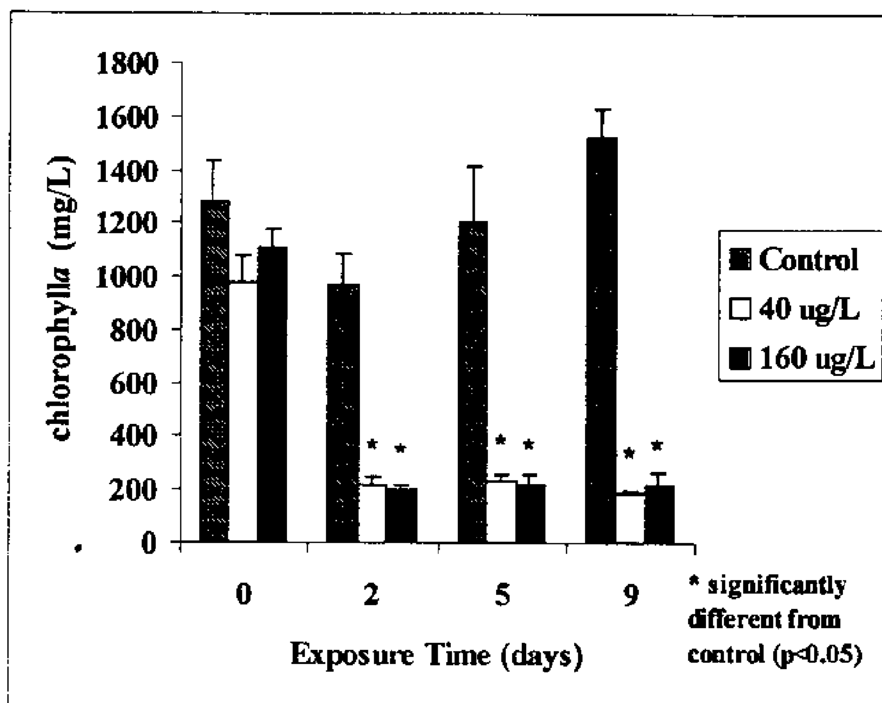

Figure 2. Atrazine effects on chlorophyll *a* measured from the water column.

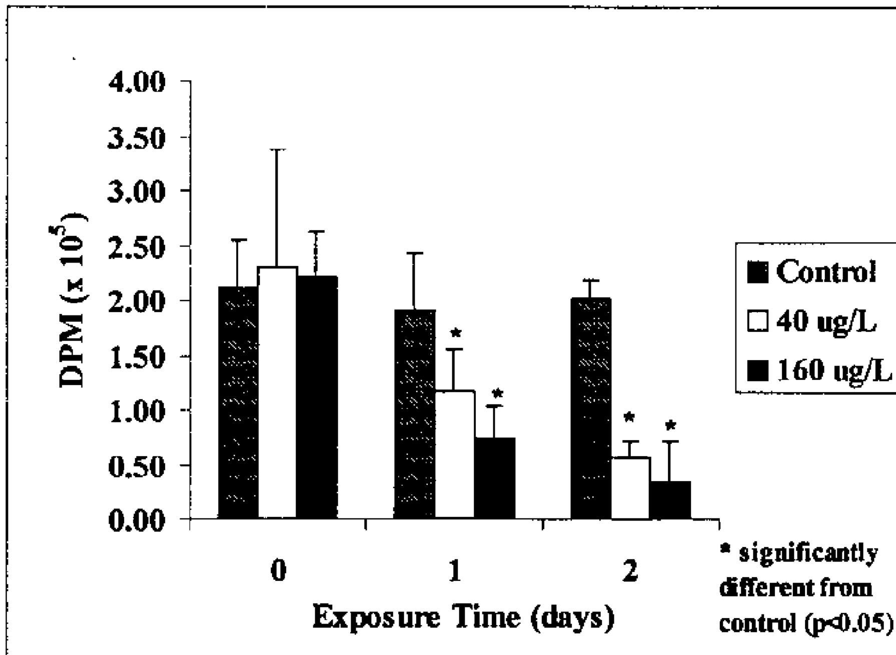

Figure 3. Atrazine effects on phototrophic carbon assimilation.

Phototrophic biovolume was significantly reduced in atrazine treatments (Figure 4). Atrazine exhibited a change in structure of the algal assemblages. There was a shift away from dominance of *Chlamydomonas* and *Cryptomonas* genera and toward dominance of a small *Cymbella* species and many coccoid blue-green algae. One diatom genus, *Cylindrotheca,* had fewer, smaller and more curved cells in the atrazine treatments. Total taxa number decreased from 28 in the control mesocosm to 20 in the atrazine treated mesocosms. Dissolved oxygen was not significantly affected by atrazine in the mesocosms (Figure 5). No recovery in phototrophic responses was noted after 9 days of atrazine exposure (phototrophic carbon assimilation was not measured beyond 48 hours). A total of 28 algal taxa were identified from the colonized substrates (Table 1).

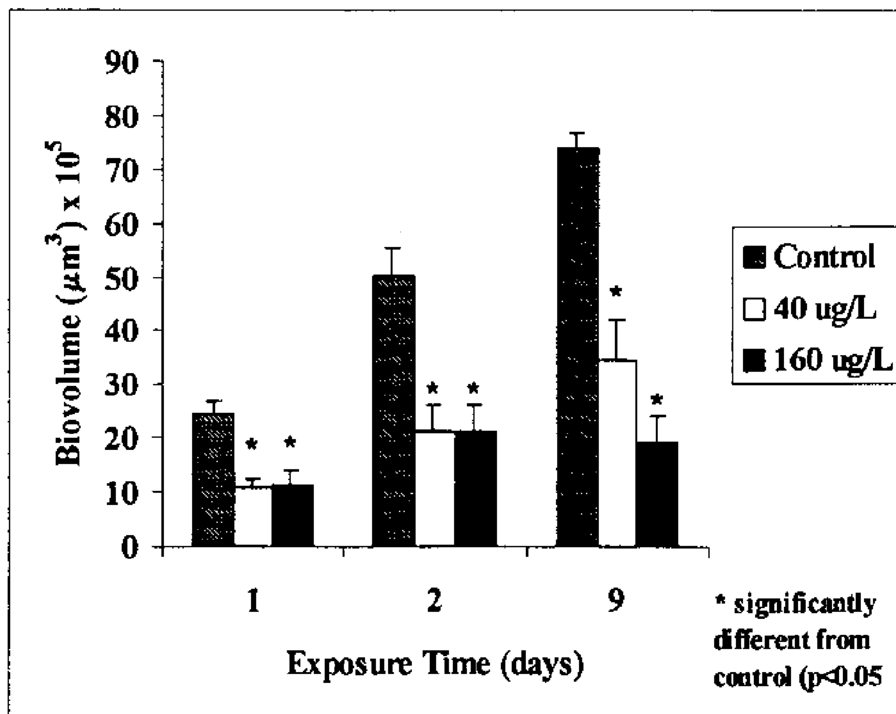

Figure 4. Atrazine effects on phototrophic biovolume.

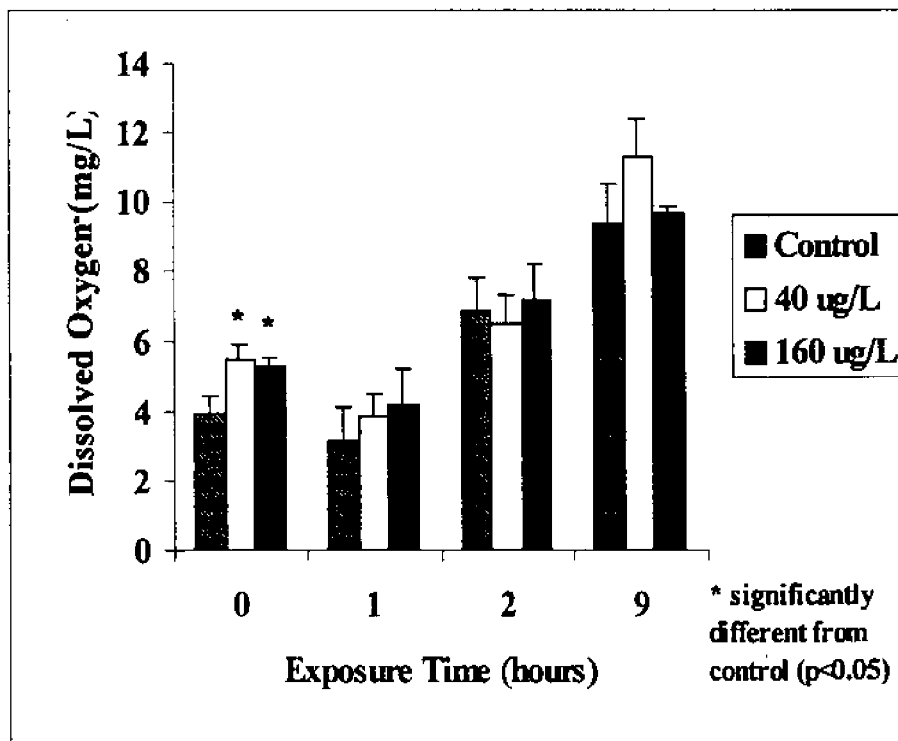

Figure 5. Atrazine effects on dissolved oxygen.

Table 1. Algal taxa identified from the artificial substrates during the artificial tidal creek mesocosm experiment. Grouped according to phylum (Prescott, 1978).

Chlorophyta
Chlamydomonas

Chrysophyta
Amphiprora
Asterionella
Bacillaria
Biddulphia
Chaetoceros
Coscinodiscus
Cylindrotheca
Cymbella
Dinobryon
Diploneis
Fragilaria
Gomphonema
Licmophora
Melosira
Navicula
Nitzschia
Pleurosigma
Rhizosolenia
Sellaphora
Stauroneis

Cryptophyta
Cryptomonas

Cyanophyta
Cylindrical blue-green
Filamentous blue-green
Oscillatoria
Synechococcus

Euglenophyta
Euglena

Pyrrhophyta
Gymnodinium

Bacterial abundance was significantly increased in the high dose at 48 hours (Figure 6). While there was no significant effect on small ciliates (Figure 7), at 40 and 160 μg/L atrazine, large ciliates and small flagellates increased significantly in number after 48 hours and remained elevated after 9 days (Figures 8-9). 160 μg/L atrazine significantly reduced large flagellate density throughout the experiment (Figure 10).

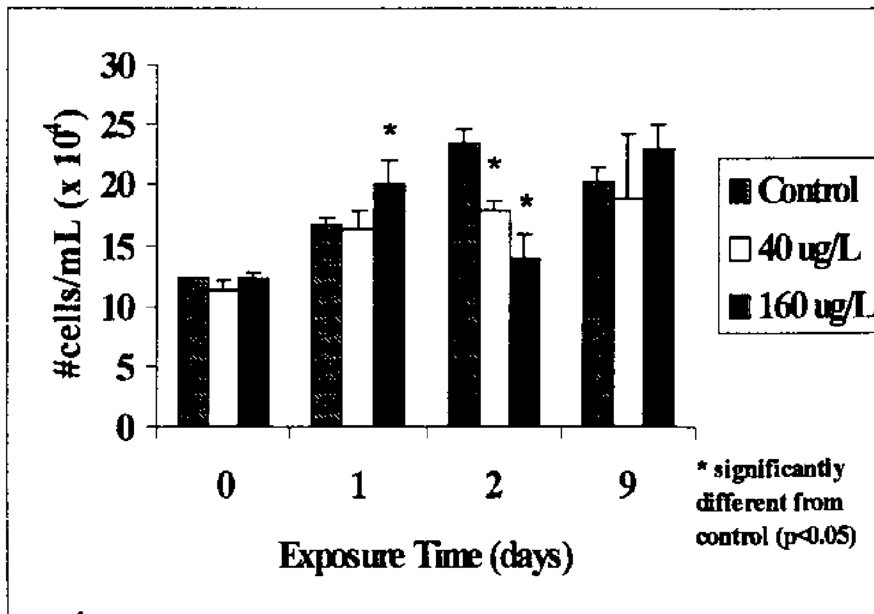

Figure 6. Atrazine effects on bacterial abundance.

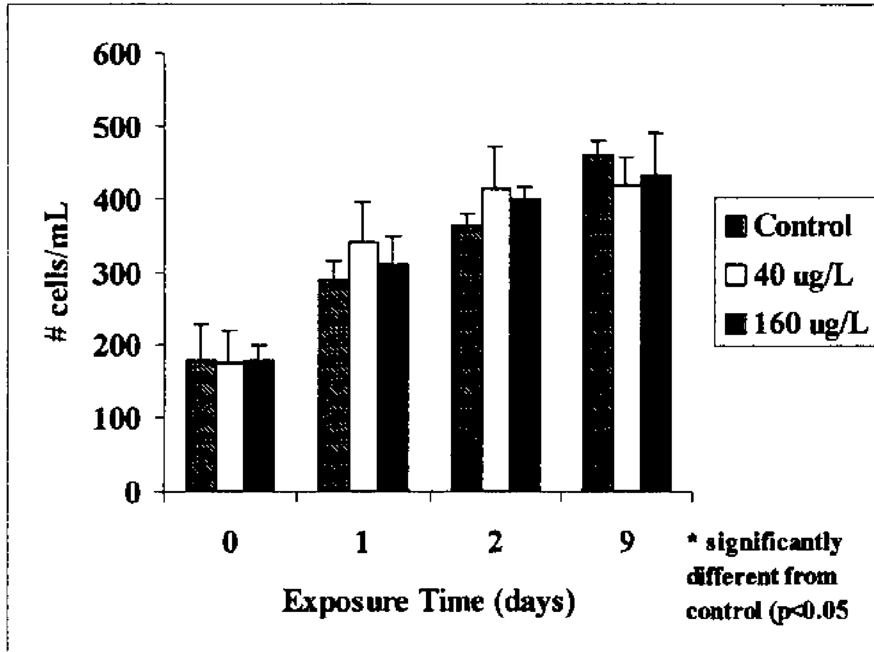

Figure 7. Atrazine effects on abundance of heterotrophic ciliates (< 20 μm).

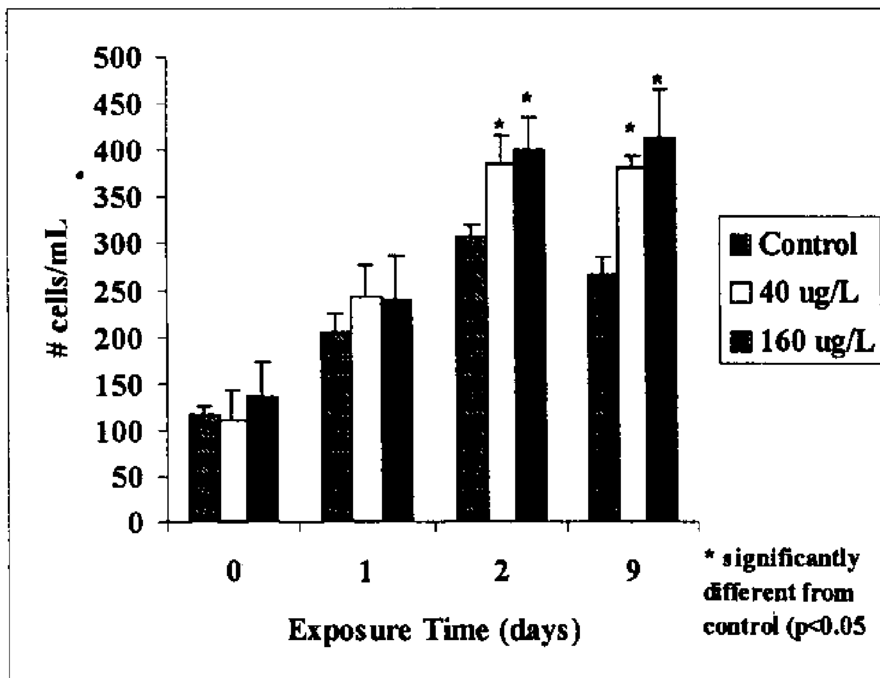

Figure 8. Atrazine effects on abundance of heterotrophic ciliates (> 20 μm).

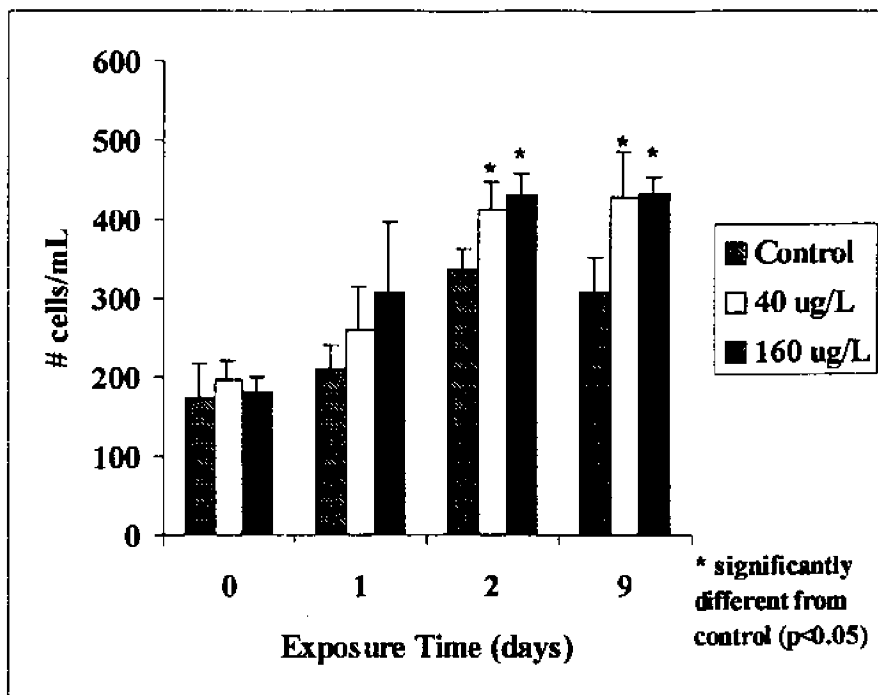

Figure 9. Atrazine effects on abundance of heterotrophic flagellates (< 20 μm).

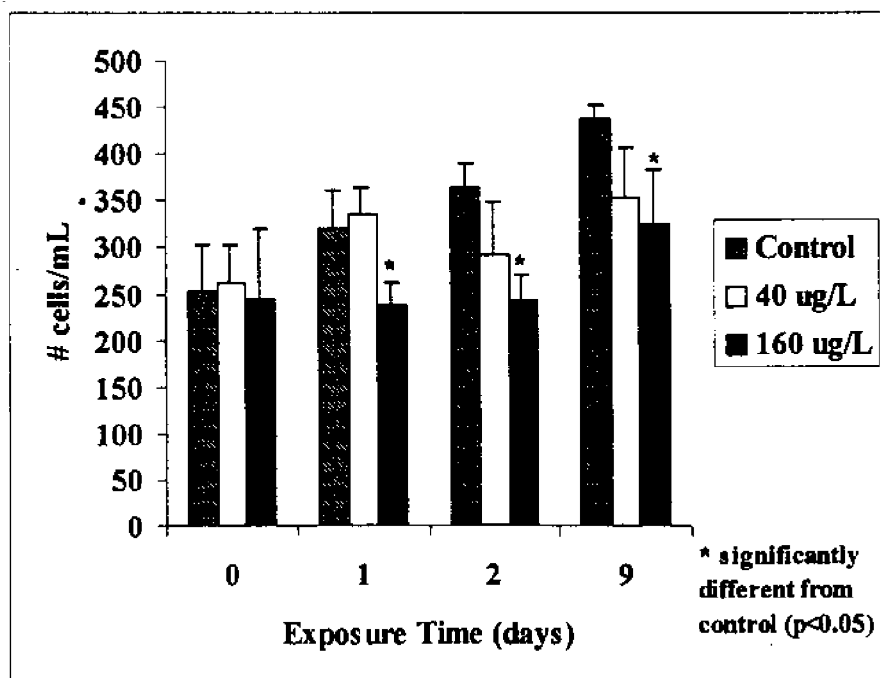

Figure 10. Atrazine effects on abundance of heterotrophic flagellates (> 20 μm).

The results of Analysis of Variance tests conducted for each variable are summarized in Table 2.

Table 2. Summary of results from statistical analyses, mesocosm experiment (p-values, which treatment(s) were significantly different from control, and nature of the change). Low = 40 μg/L atrazine, High = 160 μg/L atrazine, ND = not determined.

Variable	24 hours	48 hours	9 days
Chlorophyll *a* (substrates)	0.002 Low - D High - D	0.000 Low - D High - D	0.000 Low – D High – D
Chlorophyll *a* (water column) Measured at 48 hours, 5 days and 9 days	0.000 Low - D High - D	0.000 Low - D High - D	0.000 Low – D High – D
Phototrophic Carbon Assimilation	0.032 Low - D High - D	0.000 Low - D High - D	ND
Phototrophic Biovolume	0.001 Low - D High - D	0.001 Low - D High - D	0.000 Low – D High – D
Dissolved Oxygen	0.409	0.690	0.085
Bacterial Abundance	0.035 High - I	0.001 High - I	0.379
Ciliate Abundance (< 20 μm)	0.388	0.010 Low - I High - I	0.003 Low – I High – I
Flagellate Abundance (<20 μm)	0.257	0.013 Low - I High - I	0.021 Low – I High – I
Ciliate Abundance (>20 μm)	0.375	0.338	0.489
Flagellate Abundance (>20 μm)	0.022 High - D	0.023 High - D	0.055

Pesticide analyses results of water samples taken from each mesocosm are found in Table 3.

Table 3. Atrazine concentrations determined using gas chromatography during the mesocosm experiment. See Figure 3 for a picture of the mesocosm design. The average concentration found in the mesocosms for each treatment is indicated. The percent decrease indicates the average decrease in concentration in the mesocosms after 48 hours.

Nominal Conc.	Measured Conc. (μg/L)		Average Measured Conc. (μg/L)		% Decrease
	0 hour	48 hour	0 hour	48 hour	
Control	0.097	0.223			
B-10 μg/L	0.243	4.101			
B-20 μg/L	**9.072**	**3.861**	**19.286**	**10.548**	**54.69**
B-40 μg/L	48.544	23.681			
A-40 μg/L	0.103	5.677			
A-80 μg/L	**0.609**	**20.725**	**83.971**	**16.928**	**20.16**
A-160 μg/L	251.202	24.382			

Average pesticide levels measured in the mesocosms reflect the average of the target concentrations. This occurred because there was open flow among the three chambers of each treatment. There was a greater percent decrease in atrazine concentration in the low dose mesocosms than in the high dose mesocosms.

DISCUSSION

It is clear from the results that the herbicide atrazine has a significant effect on the phototrophic component of the microbial food web. In most cases, significant inhibition occurred within 24 hours of pesticide exposure. Recovery was not observed after 9 days. Atrazine inhibited chlorophyll *a*, phototrophic carbon assimilation, and phototrophic biovolume in the mesocosms at the concentrations tested of 40 and 160 μg/L. Dissolved oxygen content was not impaired in this study, indicating that the system as a whole was able to compensate for changes in the phototrophic microorganisms and retain function.

Compositional shifts in the algal taxa were observed in the mesocosms. There was a shift away from the dominance of *Chlamydomonas* and *Cryptomonas* genera and toward the dominance of a small *Cymbella* species and coccoid blue-green algae (possibly *Synechocococcus* sp.). One diatom genus, *Cylindrotheca,* had fewer, smaller and more curved cells in the atrazine treatments. The total number of taxa decreased from 28 in the control mesocosms to 20 in the atrazine treated mesocosms.

The model tidal creeks contained a variety of habitats and organisms, creating a sensitive monitoring system for pesticide exposure. Mesocosm tests can be valuable in predicting pesticide toxicity, provided that reliable estimates of the probable exposure of the aquatic organisms can be made. This was demonstrated with the pyrethroid insecticide, cypermethrin in outdoor pond mesocosms (Crossland, 1994). The mesocosms used in the present study yielded results consistent with field data when employed in a study of the organophosphate insecticide azinphosmethyl (Lauth *et al.*, 1996).

Based on the results of this study, impairment of the phototrophs can lead to changes in the other microbial organisms. Bacterial abundance may be altered due to the competition between bacteria and phytoplankton for nutrients. Also, heterotrophic ciliate and flagellate populations may be affected. Decreases in the number of phototrophic organisms may lead to reductions in food availability for certain grazers. Other protozoans may benefit by shifts in the phototrophic community. By directly affecting the phototrophs, atrazine will disrupt the microbial loop, possibly decreasing the flow of nutrients to higher trophic levels in the estuary. Since atrazine is so commonly used, and because it is a fairly persistent compound, the photosynthetic inhibition may affect higher trophic levels.