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Effect of Multiple Atrazine Exposure Profiles on Hemocyte DNA Integrity in the Eastern Oyster (*Crassostrea virginica*)

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Atrazine is a triazine herbicide commonly used to control broad leaf weeds in a variety of crops including Christmas trees, maize, sorghum and sugar cane. Atrazine is one of the most commonly used herbicides globally with approximately 76.4 million pounds used annually (EPA 2002). It is still manufactured in large quantities in the United States; approximately 50,000 tons per year. Atrazine's mode of function is through inhibition of electron transport in photosystem II of a plant's photosynthetic pathway. This narrow target mechanism produces an effective broad leaf herbicide, while minimizing toxicity to animals (DeLorenzo et al. 2001). Studies of watersheds and reservoirs in the Midwest showed the presence of atrazine in numerous streams and 92% of sampled reservoirs (Solomon et al. 1996). Despite the prevalence of atrazine in these water bodies, atrazine contamination was determined to pose only a minor risk to the marine environment at concentrations measured (up to 20 μg/L) (Solomon et al. 1996).

Despite the existing reports of little or no effect of atrazine concentrations up to 20 μg/L on non-target organisms (Hubner et al. 1993; Solomon et al. 1996), additional data indicates that prolonged exposure to atrazine concentrations as low as 1 μg/L could lead to increased sensitivity of phytoplankton to subsequent acute exposures (Hamala et al. 1985; Nelson et al. 1999). By directly affecting the phytoplankton community, atrazine has the potential to produce a series of indirect effects on the dependent ecosystem, particularly herbivores such as the Eastern oyster, *Crassostrea virginica* (Pennington 2002). In addition to the potential indirect effects mentioned, conflicting data has been published on the ability of atrazine to cause DNA damage (Clements et al. 1997; Ribas et al. 1995; Tennant et al. 2001). The single cell gel electrophoresis (Comet) assay has been used previously to examine atrazine genotoxicity in female C57B1/6 mice (Tennant 2001), bullfrog tadpoles (*Rana catesbeiana*) (Clements 1997) and human lymphocytes (*Homo sapiens*) (Ribas et al. 1995). The objective of this study was to evaluate the potential genotoxicity of atrazine in the Eastern oyster (*Crassostrea virginica*) using the single cell gel electrophoresis (Comet) assay following three different exposure profiles.

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MATERIALS AND METHODS

Oysters were exposed to atrazine in a series of estuarine mesocosm exposures. The mesocosms used in this experiment are of a similar design to those described previously by Lauth et al. (1996) with a few minor changes. The mesocosms used for this exposure consisted of 4 sets of 3 tanks linked with 7.6 cm diameter conduits, as described by Lauth et al. (1996), with the addition of flow gates between each tank. When closed, the gates allowed each tank to act as an independent replicate. This tank separation required that each tank be supplied with its own sump to accommodate water for the tidal exchange. The mesocosm units were housed in a greenhouse with thermostat-controlled fans behind the National Oceanic and Atmospheric Administration's (NOAA) Center for Coastal and Environmental Health and Biomolecular Research (CCEHBR) building at Ft. Johnson, Charleston, SC, USA (Latitude 32.747° N; Longitude 79.903° W).

Adult oysters (>7.82 cm) were collected from Leadenwah Creek (Latitude 32.6333° N; Longitude 80.2° W) and were acclimated in the lab for 2 weeks in 90 L holding tanks with adequate aeration and food. Immediately prior to the atrazine exposure, the oysters were measured for height and total weigh. Technical grade atrazine (96% purity) was procured from Novartis Crop Protection (Greensboro, NC, USA). Twelve mesocosm units were used to evaluate the genotoxicity of 3 different atrazine treatment regimens as compared to a carrier control (0.01% acetone) as listed in Table 1. Briefly, Treatment A received no atrazine for the first 4 week period (Phase I) or during the 2 week Phase II, but received 200 µg/L for the 96 hour Phase III. Treatment B was dosed with 20 µg/L atrazine for Phase I, then allowed to recover with no addition of atrazine during Phase II. During Phase III, Treatment B was dosed with 200 µg/L for 96 hours. Mesocosms in Treatment C were dosed with 20 µg/L for a total of six weeks during Phase I and Phase II followed immediately with 200 µg/L atrazine for Phase III. The chronic concentration of 20 µg/L was chosen based on the ecosystem level NOEC published by Solomon et al. (1996), as well as previously reported observations in South Florida, Texas and South Carolina. Atrazine concentrations were analyzed as detailed by Pennington (2002) using enzyme-linked immuno-sorbant assay (ELISA), with gas-chromatography ion-trap mass spectrometry (GC-ITMS) to validate the ELISA. Atrazine detection limits for ELISA and GC-ITMS were 0.100 µg/L and 0.046 µg/L respectively. Renewal of the atrazine dose during Phase I and Phase II was performed based on the measured atrazine concentration. When measured atrazine concentrations fell below 4 µg/L, the target dose of 20 µg/L was added at the following morning's low tide (Pennington 2002).

Each oyster was measured for height and total weight immediately following conclusion of Phase III, after which a hole was chipped in the shell of the oyster on the posterior end. A 21-gauge needle and syringe were used to extract hemolymph from the adductor muscle sinus, which was immediately transferred to a 1.5 ml Eppendorf tube and placed on ice. The cells were centrifuged at

Table 1. Experimental design for Atrazine exposures in the salt marsh mesocosm.

Control	Treatment A	Treatment B	Treatment C	Details
No Dose	No Dose	Chronic	Chronic	Phase I: 4 Weeks Chronic Dose 20 ppb (µg/L)
No Dose	No Dose	No Dose (Recovery)	Chronic	Phase II: 2 Weeks Chronic Dose 20 ppb (µg/L)
No Dose	Acute	Acute	Acute	Phase III: 96 Hour Acute Dose 200 ppb (µg/L)

3000 x g for 2 minutes, after which the supernatant was discarded. The pellet was resuspended in twice the hemolymph volume of 0.65% low melting point agarose (LMAgarose) in Kenny's Salt Solution (23.5 g NaCl, 0.7 g KCl, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$ in 1 L deionized water, pH 7.5, filter sterilized) at 30°C.

Prior to the experiment, fully frosted microscope slides were coated with 1% normal melting agarose in Kenny's Salt Solution and allowed to dry. Samples were processed according to the protocol produced by Singh et al. (1988) with minor alterations. Briefly, 50 µl of the suspension was rapidly pipetted onto a previously coated fully frosted microscope slide, covered with a 20 x 60 mm cover slip and placed on a chilled metal slide tray for 5 minutes. Once the agarose had solidified, the coverslip was carefully removed, and 75 µl of LMAgarose was pipetted onto the slide. The cover slip was replaced and the slide was again kept on a chilled slide tray for 5 minutes. Once the top coat of agarose had solidified, the cover slip was removed, and the slides were placed in freshly prepared chilled lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris-HCl, 10% DMSO, 1% Triton X-100, pH 10) for 2 hours at 4°C.

The slides were removed from the lysis solution after 2 hours, and rinsed three times for two minutes in cold distilled water, then placed in a horizontal electrophoresis chamber filled with cold unwinding buffer (54 ml 10 N NaOH, 9 ml 0.2 M EDTA, 1737 ml distilled water). The slides were allowed to sit in this solution for 15 minutes, after which the power supply was turned on for 10 minutes (25 volts, 300 mAmps). Following electrophoresis, the slides were soaked three times for two minutes in chilled 0.4 M Tris pH 7.5 for neutralization of the alkaline solution, followed by 5 minutes in ethanol. Following removal from ethanol, the slides were allowed to air-dry and were stored in a cool and dark location until evaluation was possible.

Slides were transported in a cooler to Skidaway Institute of Oceanography in Savannah, GA, for image analysis in the laboratory of Dr. Richard Lee, using a

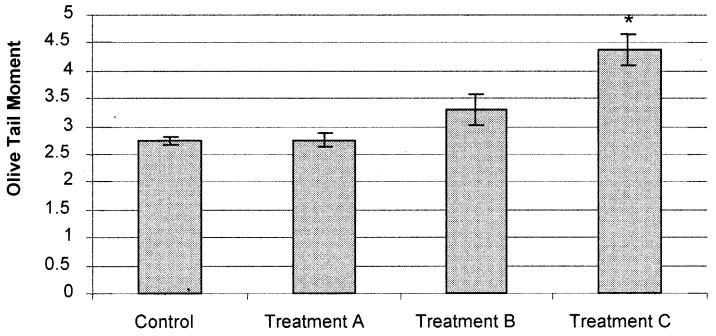

Figure 1. Mean Olive tail moments of oysters exposed to atrazine (± S.E.).

Nikon Eclipse E400 inverted fluorescent microscope (magnification x200), digital camera and the image analysis software Komet v.4.01 (Kinetic Imaging Ltd). Twenty µl of 20 µg/ml ethidium bromide was pipetted onto each slide individually, immediately prior to evaluation on the microscope. Fifty comets were measured from each individual oyster. Several metrics measured by the Komet program were considered for use in analysis including tail length, tail extent moment (length of tail * % DNA in tail), and Olive tail moment ((mean tail length – mean head length) * % DNA in tail/100) (Olive et al. 1990). The Olive tail moment was found to produce the most consistent results, and was used for the duration of these experiments.

Data were analyzed using Sigma Stat version 2.0 (Jandel Scientific). Comet assay data were analyzed using the non-parametric Kruskal-Wallis ANOVA on Ranks, followed by all pairwise multiple comparisons using Dunn's Method to evaluate the effects of different atrazine exposure durations.

RESULTS AND DISCUSSION

Analysis of the metrics produced by the Komet program revealed that the Olive Tail Moment produced the most consistent and conservative results when compared with tail length and extent tail moment. Oysters in Treatment A, which were only exposed to an acute 96-hour acute exposure (Phase III) of this experiment (200 µg/L atrazine), showed no alteration in levels of DNA damage compared to the carrier control oysters (Figure 1). Oysters from Treatment B, which were exposed to an initial 4-week chronic exposure to atrazine (20 µg/L), followed by a 2-week recovery period and a final 96-hour acute exposure (200 µg/L) had a slight non-statistically significant increase in mean Olive Tail Moment when compared to carrier controls. Oysters in Treatment C, which were exposed to 6 weeks of continuous chronic atrazine exposure (20 µg/L) followed immediately by a 96-hour acute exposure of 200 µg/L, had a significant increase

in DNA damage compared to controls as well as oysters from other treatment groups (Treatment A and B) (Figure 1).

Our experiment utilized the ability of the mesocosm design to simulate various exposure profiles that might be encountered in estuarine environments adjoining agricultural communities. Previous work has established that atrazine exposures of 50 and 250 μg/L decreased the abundance of indicators of photosynthetic phytoplankton, concurrent with an increase in bacterial populations (DeLorenzo et al. 1999). This shift in microbial populations may further affect larger organisms by lowering dissolved oxygen concentrations, decreasing viable food supply for filter feeders such as oysters, and possibly an increase in potentially infectious strains of bacteria.

Lawton (2001) reported that chronic atrazine exposure affected bivalve growth in clams, via indirect effects on the microbial loop community. Chronic atrazine exposure reduced the abundance of phytoplankton, reducing the potential food supply for bivalves. As phytoplankton abundances were reduced, bacterial abundances were increased. Scott and Lawrence (1982) demonstrated that increased bacterial abundance might reduce oyster assimilation efficiencies, due to increased maintenance metabolism (e.g. mucous production), which reduces energy available for growth. Pennington (2002) reported similar reductions in oyster growth following chronic atrazine exposure. Lawton (2001), Pennington (2002) and this study were all conducted as atrazine mesocosm experiments, as an integrated assessment of chronic atrazine exposure to estuarine herbivores. What the results of these experiments indicate is that chronic atrazine exposure affects food supply and assimilation efficiency, which leads to reduced growth and increased damage to DNA.

Atrazine exposure may not necessarily lead to lasting DNA damage in oysters, as illustrated in Figure 1 with treatments A and B. Previous reports have shown that marine organisms have the ability to remain relatively unaffected or recover quickly from a variety of atrazine exposure profiles (Solomon, 1996). The results of this study indicate that *Crassostrea virginica* is also capable of surviving both chronic and acute exposures to atrazine with no significant lasting DNA damage, but a biphasic atrazine insult is capable of causing a decrease in DNA integrity in the Eastern oyster.

The sample collection timetable limited the possibilities for broader investigation of the effects of atrazine on oyster DNA integrity. No oysters were collected immediately following Phase I or Phase II, so it is uncertain as to how the oysters reacted to each of those exposures, though we were able to establish that a prolonged and biphasic exposure was able to significantly increase levels of DNA damage in circulating hemocytes of the Eastern oyster. As indicated by the lack of apparent DNA damage in Treatment groups A and B following the conclusion of Phase III, the oysters were able to either prevent DNA damage during the

atrazine exposure, or were able to recover from any damage induced by the initial exposure, and prevent damage during the acute exposure.

The results of this experiment indicate that while it is not certain that atrazine is directly genotoxic, it is apparent that atrazine release into the aquatic environment can lead to increases in DNA damage, though the mechanisms of this damage remain to be elucidated. Future experiments should investigate DNA damage during the exposure as well as at intervals following the conclusion of the exposure. This will allow for a better understanding of atrazine's genotoxic capabilities as well as the ability of marine organisms to recover from different exposures. Previous work has established that the Comet assay can be used successfully to monitor the rate of DNA damage repair in cells (Tice and Strauss 1995), a technique that might be useful in such situations as presented here. By repeatedly sampling the same individuals during the course of such a long term exposure, it would be possible to evaluate the effects of atrazine on DNA damage in oyster hemocytes both during and following the completion of the exposure. It may also be worthwhile to investigate the effects of atrazine on microbial communities concurrently with the application of the Comet assay; in order to clarify what possible role the alteration in microbial communities by atrazine may play in the observed genotoxicity.

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