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Response of Genotypes of *Hyalella azteca* to Zinc Toxicity

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Toxicity tests are most frequently conducted on organisms derived from laboratory cultures that have been reared for many generations following standardized protocols. The restricted density of laboratory populations and the possibility of occasional population crashes increases the probability that random genetic drift and inbreeding may decrease genetic variation of the culture below that typically found in natural populations of the species. Likewise, isolation of organisms in different laboratories may contribute to significant genetic differentiation among culture populations. For example, Duan et al. (1997) present evidence for extensive genetic differentiation among laboratory cultures of the amphipod *Hyalella azteca,* a species routinely used in toxicity testing. Duan et al. (1997) suggest consistency among toxicity tests performed in different laboratories can only be assured with a knowledge of the genetic similarity among cultures. The recommendation for monitoring the genetic structure of laboratory populations is further supported by the results of studies that indicate, for some species, differential tolerance of genotypes to an environmental stressor (e.g. Sullivan and Lydy 1998, Guttman 1994). In the situation where certain genotypes are more resistant to a particular stressor, using a culture that is dominated by the resistant genotype may underestimate the toxic effect of a contaminant. Similarly, the inclusion of a greater number of sensitive genotypes in a test may overestimate toxicity. This experiment was conducted to test the null hypothesis that no difference in tolerance to zinc contamination would be noted among distinct genotypes of *H. azteca* obtained from a single laboratory source. Zinc was selected as the contaminant in this study because much of the focus in research conducted to examine genotypic differences in stressor susceptibility has centered on heavy metals. Although zinc is an essential trace element for organisms, it can , at high concentrations, act as an environmental contaminant, particularly in aquatic ecosystems that receive mining effluent. *Hyallela azteca* was chosen because the research of Duan et al. (1997) indicated genetic differentiation among laboratory cultures and this species is commonly used in ecotoxicological studies.

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MATERIALS AND METHODS

Hyallela azteca were obtained from the Great Lakes Environmental Research Laboratory (GLERL) in Ann Arbor, MI. Initial acclimation of amphipods was performed with water obtained from a natural pond that had hardness (302 mg/L $CaCO_3$) and alkalinity (114 mg/L $CaCO_3$) similar to that used in the organisms' prior culture water. After 1 week of acclimation in this water, hardness and alkalinity were reduced each week by exchanging two-thirds of the water volume with EPA hard water, and then EPA reformulated moderately hard water (Smith et al. 1997), which was used in acute toxicity tests; final concentrations of alkalinity and hardness were 60 mg/L $CaCO_3$ and 100 mg/L $CaCO_3$, respectively. Water was changed thereafter as needed. Amphipods were fed twice a week, using approximately 0.3 g of powdered Tetrafin® flake food.

For the acute toxicity test, 21 day old *H. azteca* (n=10) were placed in each of a series of one liter beakers containing aerated moderately hard reconstituted water. No substrate was added to the beakers because substrate makes it difficult to enumerate organisms at the end of the test (Borgmann 1989). Five different concentrations of zinc (50, 100, 200, 400 and 800 µg/L), each with three replicate beakers, were used to determine the LC_{50} value for zinc. A negative control was also run with each experiment. The zinc stock solution was prepared using $ZnSO_4$ as the source of zinc in all tests. Tests were conducted in a Precision Scientific environmental chamber at 20 °C, with a photoperiod of 16L:8D. After 96 hours, the number of dead arnphipods at each concentration was determined by using the lack of locomotion upon gentle prodding as the lethality endpoint (Hoke et al. 1994). Data was entered into a Trimmed Spearman-Karber computer program (Hamilton et al. 1977); the LC_{50} value was determined to be 436 µg/L. Data was graphed on log probit paper to estimate the LC_{70} value (700 µg/L), which was then used in the time-to- death (TTD) test. The LC_{70} value was chosen over lower LC values for the TTD test to increase the numbers of mortalities for statistical testing.

The 96 hour TTD test was conducted under the same conditions at the 96 hour acute toxicity test, with the exception that a concentration of 700 µg/L was the single zinc concentration. Amphipods (n= 185) were randomly and equally placed in ten 1L beakers with 1 L of dosed moderately hard reconstituted water. A control beaker (n=20) with no zinc added was also established. Beakers were checked for mortality every three hours over the 96 hour time period. Dead organisms were removed and placed into appropriately labeled tubes which were then stored at -70 °C prior to electrophoretic analysis. Organisms surviving the 96 hour test were also preserved for genetic analysis. Zinc water concentrations were measured, in both LC_{50} and TTD tests, using a Perkin-Elmer model 2380 Atomic Absorption Spectrophotometer (AAS). A 40 ml water sample was taken from each beaker at the beginning and end of an experiment. Water samples were acidified with concentrated nitric acid (pH < 2) and then run on AAS at a wavelength of 213.9 nm and a slit width of 0.7 nm using an air-acetylene flame.

Cellulose acetate gel electrophoresis (CAGE) was used to examine allozyme variability in the amphipods included in the TTD test. Electrophoretic methods developed for *H. azteca* by Duan et al. (1997) were followed in this experiment. A preliminary survey of enzyme systems indicated the following polymorphic systems for our arnphipod culture: phosphoglucomutase (PGM, E.C. # 5.4.2.2), glucosephosphate isomerase (GPI, E. C. # 5.3.1.9), and acid phosphatase (ACP, E.C.# 3.1.3.2). Organisms were homogenized with 20 µl of distilled water and centrifuged at 1200 RPM at 4 °C. Ten µl of the supernatant from each sample was applied to the cellulose acetate strip (Sepraphore ® III, Gelman Sciences Inc.). The buffer systems used for PGM, GPI and ACP were a 0.15 M Tris-EDTA-borate-$MgCl_2$ (pH 7.8), a 0.1 M Tris-EDTA-maleate-$MgCl_2$ (pH 7.4), and a 0.05 M phosphate (pH 7.0) buffer, respectively, described by Duan et al. (1997). Gels were run at 165 volts for approximately 60 minutes. Three alleles were noted in amphipods for each polymorphic locus. Alleles were identified based on their speed of migration relative to the gel origin. The fastest migrating allele was designated "F", the slowest migrating allele "S", and the intermediate form "M". Each organism was assigned a heterozygosity value based on the number of heterozygous loci observed for the individual; heterozygosity values ranged between 0 and 3 heterozygous loci.

Chi-square homogeneity tests were used to determine if there were significant differences between the genotypic and allele frequencies of amphipods that died prior to the first 48 hours of the TTD test and those that survived past that time. The LIFETEST procedure of the Statistical Analysis System (1985) was used to estimate Kaplan-Meier survival function curves for each genotype class of each genetic locus. Survival functions were determined for each collection period at which dead organisms of a particular genotype were collected; individuals that survive the test are not included in these estimates. The survival function at any time point t is an estimate of the probability that an individual survives longer than time t (Dixon and Newman 1991). The LIFETEST procedure provides:1) a log-rank test, a non-parametric test that has an approximate Chi-square distribution and tests the hypothesis of equality of survival function curves, and 2) estimates of mean survival and standard errors. In all tests, statistical significance was accepted if the probability level p was ≤ 0.05. The statistical power $(1-\beta)$ of the chi-square homogeneity tests was determined by the methods of Murphy and Myors (1998).

RESULTS AND DISCUSSION

No significant differences were noted between the actual concentrations of zinc in the acute toxicity test, as determined by atomic absorption analysis, and the nominal concentrations. Therefore, the LC_{50} value of 436 µg/L and the estimated LC_{70} value for the TTD test were based on nominal concentrations. Water chemistry parameters including dissolved oxygen (6.8 - 7.7 mg/L) , pH (7.8 - 8.2) and conductivity (350 - 400 µs/cm^3) were monitored and found to be within EPA guidelines throughout the TTD test (USEPA 1991). The percent mortality at

Table 1. Number of dead organisms, number of survivors, percent mortality, average time-to-death in hours (TTD) and standard error for nonsurvivors for specific genotype classes of each polymorphic locus.

Locus	Genotype	Dead	Alive	% Mortality	TTD (SE)
PGM	FF	5	5	50	85.7 (1.5)
	MF	14	11	56	87.3 (1.9)
	MM	11	15	42	79.0 (2.3)
	SF	24	16	60	79.0 (2.0)
	SM	22	11	67	67.0 (3.8)
	SS	30	14	68	82.6 (1.8)
GPI	FF	45	33	58	85.7 (1.5)
	MF	23	18	56	87.3 (1.9)
	MM	33	15	69	79.0 (2.3)
	SF	2	0	100	^
	SM	2	3	40	^
	SS	3	2	60	^
ACP	FF	11	6	65	74.8 (3.5)
	MF	20	10	67	86.3 (2.3)
	MM	44	31	59	84.0 (1.8)
	SF	10	4	71	81.1 (4.3)
	SM	19	12	61	83.9 (2.3)
	SS	5	8	39	91.2 (2.3)

^ Sample sizes too low for calculation of mean survival functions.

the conclusion of the TTD test was 61% (113 non-survivors and 72 survivors). The first mortality occurred at 33 hours and increased to only 21.6% mortality at 73 hours. A toxicity threshold appeared to be reached at approximately 73 hours, after which mortality increased from 21.6% to 49.2% in a 12 hour period of time.

The numbers of dead and surviving organisms in each genotype class at the conclusion of the TTD test are depicted in Table 1. Chi-square homogeneity tests indicated no significant differences in the relative frequencies of genotypes observed for the surviving and nonsurviving amphipods at any genetic locus. Similarly, no differences in the frequencies of individuals in different heterozygosity classes or the frequencies of alleles were noted between dead and surviving organisms. Survival function curves for amphipod genotypes at each locus are presented in Figure 1. Survival curves for the SF, SM and SS genotypes of GPI were not determined due to low sample sizes (see Table 1). Although there was variability among genotype classes for both survival function curves and mean time-to-death in hours (Table 1), these differences were not significant, as determined by log-rank Chi-square tests (GPI: $X^2 = 3.82$, d.f.=2, $p = 0.15$; PGM: $X^2 = 4.99$, d.f.=5, $p = 0.42$; ACP: $X^2 = 4.56$, d.f. = 5, $p = 0.47$).

These results suggest that differential susceptibility to zinc toxicity does not occur among genotypes of *H. azteca*, at least at the particular genetic loci examined and

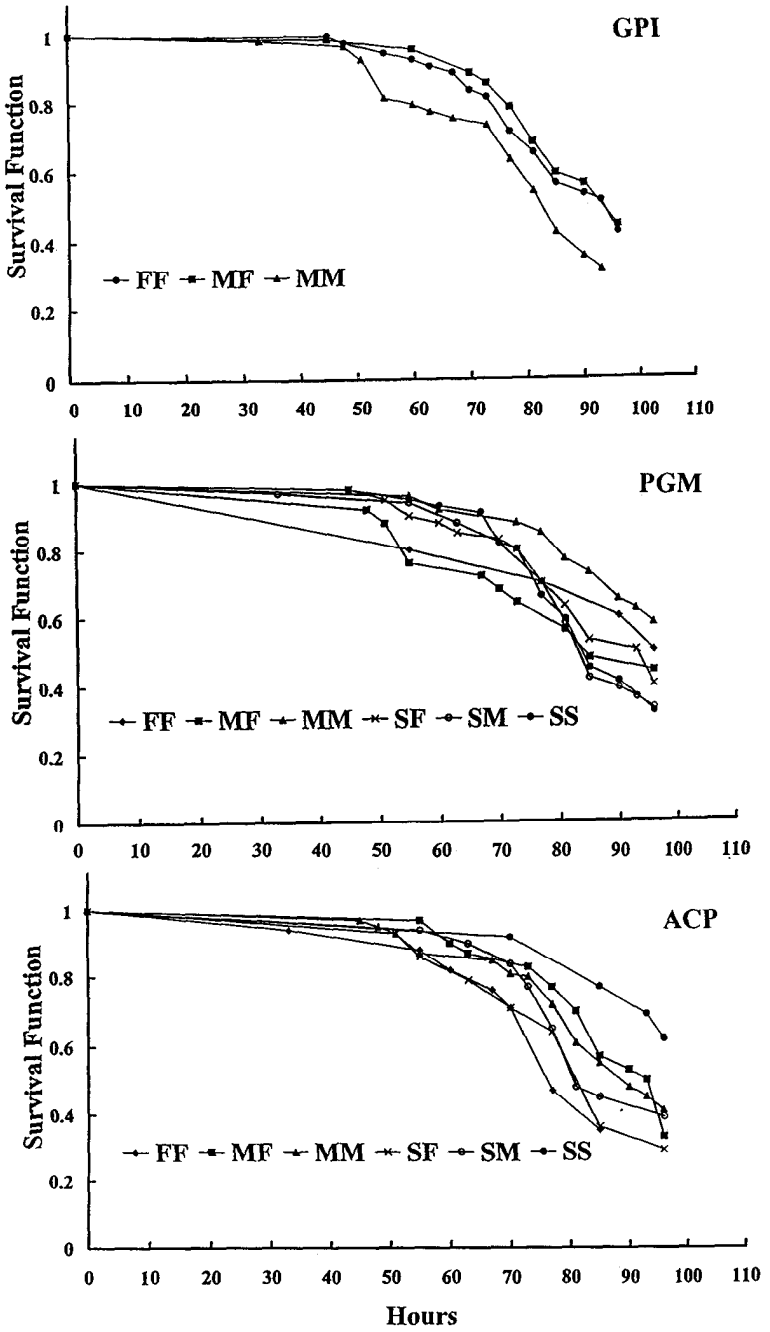

Figure 1. Survival function curves for Hyalella azteca exposed to zinc during a 96 hour TTD test. Results are presented separately for observed genotypes of the ACP, GPI and PGM genetic loci.

for randomly selected representatives from the culture maintained at the GLERL. Amphipods from the GLERL were not included in the survey of genetic variation in laboratory cultures of *H. azteca* conducted by Duan et al. (1997). However, our preliminary survey conducted on the GLERL amphipods indicated genetic variation at the same loci as observed by Duan et al. (1997). Duan (1997) has reported significant differences in tolerance to zinc contamination among genotypes of *H. azteca*. His toxicity tests were conducted on 1 month old amphipods originally derived from a strain at the USEPA Laboratory in Cincinnati, Ohio. Using $ZnCl_2$ 5600 µg/L caused 73% mortality in 54 hours, which is almost eight-fold less than the toxicity noted in this study (700 µg/L caused 61% mortality in 96 hours). Tests were conducted in reformulated moderately hard reconstituted water, as in this study. Duan (1997) used approximately 800 amphipods in the TTD test. Unlike our results, genotypic differences in zinc tolerance were noted for the ACP and PGM loci. The lack of consistent genotypic responses noted between these two studies may have important implications for conducting standardized toxicity tests, as discussed below.

There are several alternate explanations for the inconsistent results reported by this study and that of Duan (1997). In both studies, the TTD tests were conducted in a similar manner using amphipods of similar age. One distinction between the two studies is that the amphipods derived from the Cincinnati USEPA culture had been maintained for approximately 7 years prior to the TTD study, whereas our amphipods had been acclimated to laboratory conditions for only a few months prior to the TTD test. Although our organisms were eating well and appeared healthy, we cannot totally discount the possibility that shipping had caused stress in the animals. However, it is unlikely that acclimation stress alone would account for such large differences in zinc toxicity. A second distinction is that Duan (1997) used $ZnCl_2$, while we used $ZnSO_4$. Earlier studies in our laboratory conducted on other aquatic species suggested that there should be little difference in toxicity between these two chemical forms. We were unable to find any published studies that addressed possible differences in toxicity for these compounds.

An important distinction between these TTD tests is a four-fold increase in the sample size used in Duan's TTD test compared to the sample size used in this study. Had our results led to a rejection of the hypothesis of no difference in genotype proportions among dead and surviving animals, sample size would not be a possible limitation in the experimental design. However, since the data collected in this study did not lead to rejection of the null hypothesis, we tested for the statistical power of the chi-square homogeneity tests. Statistical power is the probability that a particular experiment will allow rejection of a null hypothesis when it should, in fact, be rejected; low statistical power indicates a high probability of a Type II statistical error. The power of the homogeneity tests conducted to compare genotype frequencies between dead and alive amphipods was approximately 50% for each analysis. Thus, the particular experimental

design employed in this study allowed for a 50% probability of not being able to reject a hypothesis that was incorrect. Sample size and effect size are two major factors that affect statistical power. Effect size, the degree of difference among treatments or groups in the response variable, cannot be manipulated by the researcher. In this study, effect size would be the difference in genotype frequencies among dead and alive animals. While a small effect size may be biologically important, it may be difficult to assign statistical significance to a small effect, especially if sample sizes are low. Determination of power allows estimation of a sample size that would be sufficient to reject a null hypothesis for a particular experiment (Murphy and Myors, 1998). In this experiment, more than 500 amphipods would have needed to be analyzed to alleviate the effect of small sample sizes on statistical power. That Duan (1997) included 800 amphipods in his TTD experiments may explain the inconsistency in results noted between these studies. Finally, another explanation for these distinct outcomes is that genetic differences in tolerance exist among these *H. azteca* cultures. That genotypes of the same two loci exhibited tolerance differences in one culture, but not another, may suggest that these loci are linked to other genetic loci that are responding more directly to the stressor among organisms in the USEPA Cincinnati culture. Thus, there may be genetic differentiation between these laboratory cultures that, while not directly evident from allozyme analysis, significantly affects performance in toxicity tests.

Changes in population genetic structure, as measured with allozyme analysis, have been promoted as potential biological indicators of environmental stress (Gillespie and Guttman 1993). An increasing number of studies have demonstrated significant differences in genotypic responses to pollutants, particularly for aquatic species exposed to metals (e.g., Diamond et al. 1989, Keklak et al. 1994; Sullivan and Lydy 1998). Chagnon and Guttman (1989) have suggested that genetic biomarkers will be useful only if predictable changes in genetic structure (eg., allozyme and genotype frequencies) occur with exposure to a particular pollutant. The results of this study indicate that ecotoxicologists need to examine the degree of predictability of genetic responses to pollutants. If the inconsistent responses noted here are common and not attributable to sample size differences among tests, reevaluation of laboratory protocols for conducting toxicity tests and risk assessment should be undertaken. Finally, the results of this study also suggest that the use of population genetic characters as biomarkers may, in certain situations, be limited. Where a particular pollutant exerts a biologically important but small effect on genetic structure, analysis of very large sample sizes may be either too time consuming or expensive for some types of environmental assessments.

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