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Density and Sediment Organic Matter Content as Potential Confounding Factors in Sediment Toxicity Tests with *Hyalella azteca*

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Toxicity tests with *Hyalella azteca* are commonly used to assess potentially contaminated sediments with mortality, growth and reproduction used as toxicity test endpoints following exposures of 10 to 42 days (USPEA 2000; ASTM 1999). Confounding factors in sediment toxicity tests such as sediment organic matter content, sediment particle size distributions, and feeding levels have been previously studied (Ankley et al. 1994; Suedal and Rodgers 1994; Ingersoll et al. 1998; Kemble et al. 2000); however, only a limited number of studies have been conducted to determine if *H. azteca* density confounds sublethal endpoints (Kubitz et al. 1996).

Growth has been determined to be a more sensitive endpoint than survival, and better in predicting possible toxic effects of contaminated sediment to invertebrates (Kubitz et al. 1995; Ingersoll et al. 1996; Steevens and Benson 1998). However, there has not been a thorough investigation to determine if the number of individuals surviving influences growth (i.e., density dependence). Kubitz et al. (1996) observed significant decreases in growth of *H. azteca* at densities of 14,000/m^2 compared to 7,000/m^2, however these differences in density are relatively large compared to possible densities differences that may be observed in standard toxicity tests. Borgmann et al. (1989) observed that *H. azteca* density negatively affected reproduction after 6 weeks when various numbers of young (37 to 181/L) were used to start cultures, and also observed that minimum reproductive size was reached sooner at lower densities.

In standard toxicity tests, growth values are compared among sediment treatments regardless of the number of individuals remaining. The possibility of misinterpreting results may exist if amphipods from treatments with low survival experienced greater growth because of reduced competition for resources than amphipods from treatments with high survival. The objectives of this study were to determine if different densities of amphipods or sediment organic matter content affect growth.

MATERIALS AND METHODS

Two liters of sediment were collected from the top 5 cm of Little Crab Orchard Creek,

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Carbondale, Illinois using a stainless steel hand trowel. Samples were placed in sealable plastic bags for transport to the lab and stored in the dark at 4°C until use. Prior chemical and radiochemical analyses of these sediments indicated negligible contamination (Meister 2003).

Humus was purchased from a local garden supply store and mixed with tap water in 19 L buckets. After 2-3 hours, when most of the material had settled, the supernatant was poured off to remove any soluble organic material that may have been present. This process was repeated 4 times over 2 d. Remaining humus was dried at 60°C for 48 h, and pulverized in a blender to create particle sizes comparable to the original sediment (< 2 mm) and to facilitate mixing. The collected sediment was divided and humus was added to half the sediment at a concentration of 10-15% and mixed thoroughly to create a high organic matter sediment. The remaining half of the sediment was unaltered and served as a low organic matter sediment.

Organic matter content was determined by first drying subsamples at 60°C for 24 h in aluminum weigh boats to eliminate moisture. Samples were weighed (0.0001 g) and ashed in a muffle furnace at 550°C for 2 h to eliminate organic matter. Samples were re-weighed and percent organic matter was determined as the difference between the ashed weight and dry weight divided by the dry weight. The high organic matter sediment contained 12.61 ± .44 % organic matter and the low organic matter sediment contained 1.52 ± .11 % organic matter.

A modified version of an inexpensive exposure system described by Leppanen and Maier (1998) was used with 3 plastic laundry sinks (64-cm long x 64-cm wide x 82-cm high) acting as the main compartments. Water was circulated through a water heater and diverted 3 ways using plastic "T" splitters and rubber tubing. An inlet and outlet tube was placed into each sink to ensure a constant temperature of 23°C and to prevent thermal gradients within and among sinks. A plastic ceiling grid (49-cm x 47-cm) was placed in the bottom of each sink to provide a flat surface to support 48 300 mL glass jars. A rectangular notch was cut into each jar, and covered with a stainless steel cloth to prevent test organisms from escaping during water renewal. A PVC stand pipe was glued into the laundry sink to maintain a water level below the lips of the glass jars. Another plastic ceiling grid (56-cm x 55-cm) was placed over the sink and portions removed to allow 60 mL disposable plastic syringes (total volume 75 mL) to rest above each jar. The syringes were fitted with 1 inch, 18-gauge needles to allow steady water flow. One hundred milliliters of high or low organic matter sediment ($n = 60$ each) was added to each jar 2 days before test commencement and overlying water renewal began. Water was renewed twice a day by filling the corresponding syringe body twice and allowing the water to drain into each glass jar by gravity.

H. azteca were cultured in 80 L glass aquaria following EPA methods (USEPA 2000). Newly-produced amphipods were isolated from the culture (Tomasovic et al. 1995) and held for one week before exposure. Amphipods were added to replicate jars in groups of 2, 4, 6, 8, and 10 to simulate possible survival outcomes in toxicity tests and in groups of 20 to observe what may happen when the system was exacerbated (Table 1). Replicates for each of the 12 treatments (6 stocking densities x 2 sediment types) were fed 1.0 mL of YCT [yeast-cerophyl®-trout chow®, 1,800 mg/L stock solution (USEPA 2000)] daily.

Table 1. Densities of *Hyalella azteca* used in sediment toxicity tests.

No. *H. azteca*/beaker	Equivalent density (no./m^2)
2	620
4	1240
6	1860
8	2480
10	3100
20	6190

After 28 d, the contents of the glass jars were sieved through a 500 µm sieve and *H. azteca* survival was determined. Amphipods were then preserved in 8% sugar formalin (USEPA 2000) for growth determination. Growth of *H. azteca* was determined using a photo microsystem equipped with a Leica MZ9.5 microscope (Leica Microsystems, Wetzlar, Germany) and Fuji 6800 Zoom digital camera (Elmsford, NY, USA). Digital photos were take of each amphipod and length was determined using ImageJ 1.29 image analysis software (National Institute of Health, Washington D.C., USA). Amphipod length was measured on the dorsal side from the base of the first antenna to the end of the third uropod (Ingersoll and Nelson 1990).

A two-way analysis of variance (ANOVA) was used to test effects of amphipod density and organic matter content on growth of amphipods. A Tukey's HSD multiple-comparison procedure was used to test differences among individual means ($\alpha = 0.05$). Nonparametric tests were used to test effects of organic matter content and amphipod density on survival of amphipods. A Pearson correlation was used to determine the relationship between growth and survival. All statistical analyses were conducted using the program JMP IN (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Sediment organic matter content did not significantly ($p = 0.613$) affect survival (Table 2), however, there was a marginally significant ($p = 0.049$) difference in survival due to initial amphipod density (Table 3). The mean survival for all treatments was 77.7%. Amphipod treatments with initial densities of 4, 8, 10, and 20 amphipods per replicate had survival less than 80%. Treatments with 2 and 6 amphipods per replicate had greater than 80% survival.

Both amphipod density ($p < 0.001$) and organic matter treatment ($p < 0.001$) had highly significant effects on amphipod growth. There was an inverse relationship between amphipod density and mean length (Table 3). Mean length was significantly ($p < 0.001$) greater for sediment with high organic matter content (3.84 ± 0.05 mm) compared with mean length for sediments with low organic matter content (3.48 ± 0.05 mm; Table 2). No significant interaction was observed between amphipod density and percent organic matter content ($p = 0.100$).

Within both organic matter treatments, there was a significant decrease ($p < 0.001$) in growth as initial amphipod density increased (Figure 1). For each density treatment there was a trend towards greater length in amphipods exposed to the high organic matter content relative to those exposed to the low organic matter content. However, organic matter content only had a significant effect on growth at densities of 2, 4, and 8 amphipods per replicate. This indicates that sediment organic matter content has a greater effect on amphipod growth at lower densities than higher densities. There was a significant negative correlation between mean length and number of survivors in a replicate ($r^2 = -0.41$, $p < 0.001$, Figure 2). Dissolved oxygen, pH, and conductivity were within normal ranges and did not vary significantly throughout the test.

Results of the current study indicate that differences in amphipod density or sediment organic matter content in standardized sediment toxicity tests have a significant effect on amphipod growth. Amphipod survival was marginally affected by density but not by sediment organic matter content. Decrease in growth associated with an increase in initial amphipod density likely results from an increased competition for food and/or space. Though amphipods are fed daily in sediment toxicity tests, food resources may limit growth in sediment with low organic matter content. This effect was not as severe for amphipods in high organic matter sediments. Amphipods exposed to sediment with high organic matter content were consistently larger than amphipods exposed to sediment with low organic matter content for all densities tested. Significant growth differences between organic mater treatments at lower densities may indicate that organic matter content can be limiting.

It appears that overcrowding at high densities may affect amphipods to a greater degree than effects of organic matter content of the sediment. The significant negative correlation between number of amphipods surviving per replicate and growth also suggests that amphipod density in sediment toxicity tests may influence growth endpoints.

Although initial amphipod density had a significant effect on survival, no clear trends were established between percent survival and amphipod density. However, 4 of the 6 density treatments did exhibit survival less than the 80%. Survival below 80% is lower than the acceptability criteria established by the U.S.EPA for sediment toxicity tests (USEPA 2000). Failure of some treatments to meet the acceptability criterion suggests that water quality may have been a factor in amphipod survival. Tap water was dechlorinated by aeration for 24-48 hours prior to exposure. Dissolved oxygen, pH, hardness and alkalinity of the tap water were within acceptable ranges, however, residual chemicals used during water treatment processes may still have been present.

Table 2. Percent survival and mean length (± SE) of *Hyalella azteca* in sediment toxicity tests containing high (12.61%) and low (1.52%) organic matter content.

Organic matter treatment	*n*[a]	Percent survival[b]	Mean length (mm)[c]
High	73	76.3 ± 0.02	3.84 ± 0.05 A
Low	83	79.1 ± 0.03	3.48 ± 0.05 B
p-value		0.613	< 0.001

[a] Number of replicates per treatment

[b] Wilcoxon nonparametric test

[c] T-test

Table 3. Percent survival and mean length (± SE) of *Hyalella azteca* in sediment toxicity tests at different initial amphipod densities.

Initial amphipod density	*n*[a]	Percent survival[b]	Mean length (mm)[c]
2	30	81.8 ± 0.04	4.03 ± 0.07 A
4	31	74.2 ± 0.04	3.80 ± 0.07 AB
6	24	82.6 ± 0.05	3.80 ± 0.08 AB
8	23	70.3 ± 0.05	3.62 ± 0.08 BC
10	24	78.0 ± 0.05	3.43 ± 0.08 C
20	24	79.2 ± 0.05	3.07 ± 0.08 D
p-value		0.049	< 0.001

[a] Number of replicates per treatment

[b] Kruskal-Wallis nonparametric test

[c] ANOVA

Kubitz et al. (1996) observed significant growth decrease in *H. azteca* at densities of 14,000/m^2 compared to a density of 7,000/m^2. Borgman et al. (1989) reported that *Hyalella* density negatively affected reproduction after 6 weeks when various numbers of young were used to start cultures, and that minimum reproductive size was reached sooner at lower densities. Elmgren et al. (2001) reported that growth of the marine amphipod *Monoporeia affinis* was density dependent when juvenile and subadult densities varied from 580 to 38,000 individuals/m^2. They did not observe a density dependent effect on survival.

Growth is an important sub-lethal endpoint in sediment toxicity tests because *H. azteca* exhibits size- specific fecundity (Strong 1972; France 1992) and may be indicative of ecologically relevant changes in the environment. Larger amphipods produce larger broods than smaller amphipods and growth inhibition could be indicative of reproductive impairment in the environment. Reproductive endpoints may therefore be confounded due

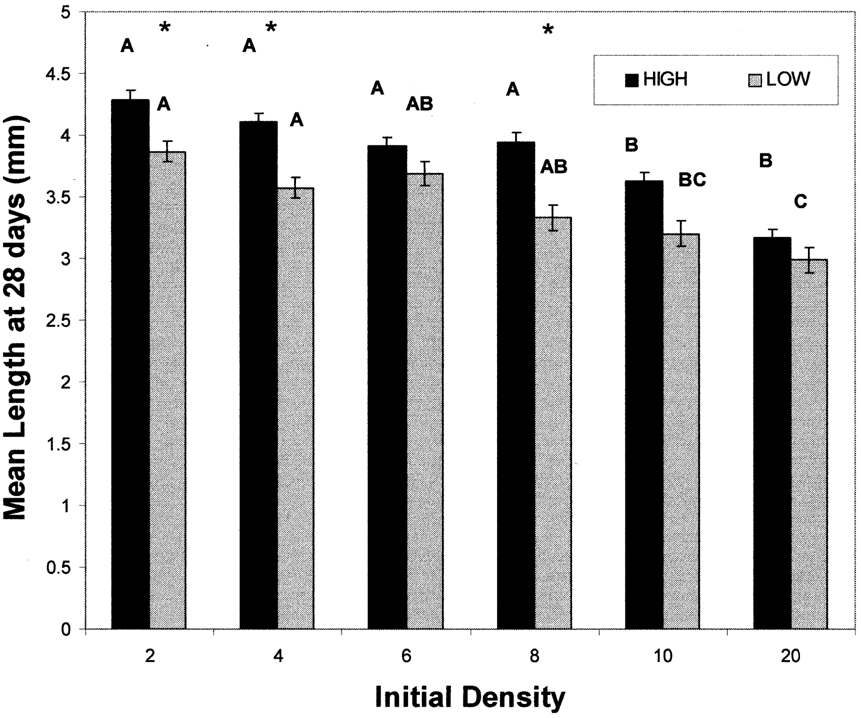

Figure 1. Mean lengths of *Hyalella azteca* at 28 days for both high and low organic matter treatments. Within both organic matter treatments, there was a significant decrease ($p < 0.001$) in growth as initial amphipod density increased. Initial densities marked with an asterisk have mean lengths that are significantly different between high and low organic matter treatments.

to density dependent differences in growth rather than toxic effects.

The data presented in the current study indicate that differences in survival of test organisms, and therefore test organism density, and sediment organic matter content could directly affect growth endpoints and indirectly affect reproduction endpoints. These effects would therefore confound interpretation of test results. It is recommended that survival differences be compared with growth and reproductive endpoints in sediment toxicity tests to ensure that these sublethal endpoints are not confounded as a result of differences in mortality among treatments.

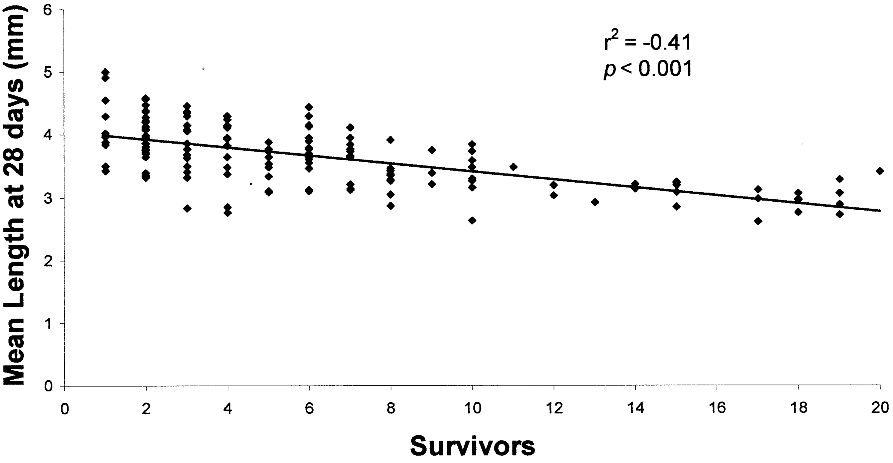

Figure 2. Pearson correlation between the mean length of *Hyalella azteca* at 28 days and number of survivors in a test chamber.

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