Use of Neutral Red to Assess Survival of Juvenile Freshwater Mussels (Bivalvia: Unionidae) in Bioassays[1]

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Abstract. The effectiveness of vital staining for assessing lethal and sublethal responses of juvenile mussels was examined. Neutral red was used to quantify survival of juvenile *Villosa iris* and *Anodonta grandis* after exposures to aqueous copper in 24-hour static bioassays. Live juveniles readily incorporated the stain, but dead individuals did not. Variation in stain intensity was associated with behavioral responses, permitting diagnosis of alive, dead, and sublethal responses of juvenile *V. iris*. The amber coloration of juvenile *A. grandis* prevented detection of variations in stain intensity, thus allowing only living-versus-dead determinations to be made.

Responding to precipitous declines in populations of freshwater mussels (Unionidae), several workers recently conducted laboratory tests to measure sensitivity of juvenile stages to various pollutants (Johnson et al., 1990; Keller & Zam, 1991; Lasee, 1991). Both Johnson et al. (1990) and Keller & Zam (1991) determined post-exposure mortality from observations of internal anatomy, but did not detail any sublethal effects of the exposures. By contrast, Lasee (1991) assessed both post-exposure mortality and sublethal responses by individual inspection of the juvenile mussels. Juveniles were recorded as alive (active and moving), stressed (no foot movement but cilia beating), or dead (no foot or cilia movement).

Toxicity tests depend on an accurate assessment of post-exposure condition and are complicated by the small size (<1 mm) of juvenile mussels. Healthy juveniles are typically active, extruding the foot and gaping (opening) their valves. If immobile or ungaped, their condition is not as apparent. Because juveniles of many species possess transparent valves, with visible internal structure, the reduction or absence of movement by the foot or cilia may be used to assess responses. This requires close, individual inspections, and the effort is time-intensive. A more rapid and equally precise means of assessing post-exposure condition of juvenile mussels thus was desirable.

Vital staining has been used successfully to distinguish living from dead

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invertebrates. Dressel et al. (1972) used vital staining to sort copepods, and Crippen & Perrier (1974) used neutral red to determine mortality among marine plankters. Platter-Rieger & Frank (1987) successfully used neutral red to assess post-exposure effects of tributyltin on mussel larvae (*Mytilus edulis*). They defined three levels of staining: darkly stained (healthy), lightly stained (stressed and inactive), or not stained (dead). They found increasing percentages of lightly stained larvae in treatments with higher toxicant concentrations and assumed that staining intensity was related to the level of stress. The purpose of our investigation was to evaluate the effectiveness of vital staining with neutral red in determining post-exposure survival of juvenile freshwater mussels. Copper was chosen as the toxicant because it is a known molluscicide, highly toxic to invertebrates, and a common pollutant in riverine systems (U.S.E.P.A., 1985; Van Hassel & Gaulke, 1986).

Materials and Methods

Juveniles of *Villosa iris* (I. Lea, 1829) and *Anodonta grandis* Say, 1829 were obtained following metamorphosis of glochidia encysted upon largemouth bass, *Micropterus salmoides* (Lacepede, 1802). We conducted 24-h, static exposures at eight concentrations ranging from approximately 0 to 200 μg Cu/L. Absolute metal concentration was determined by inductively coupled, argon-plasma-emission spectroscopy. We used two replicates of each toxicant concentration for *V. iris* and three for *A. grandis*, with 10 juveniles per replicate. Test containers were held in an incubator at 20°C; after exposure, juvenile response was determined by visual inspection and vital staining.

Prior to vital staining, we examined juveniles at 12–50× magnification with a stereomicroscope. Three classes of response were established: (1) gaped and alive, (2) gaped and dead, or (3) ungaped. Dead juveniles were characterized by their rigid, immobile foot and the absence of beating cilia. We stained juveniles after the initial inspection by the technique of Crippen & Perrier (1974) using a 1-h exposure to a 1:100,000 concentration of neutral red in water. Stained juveniles were stored overnight in a refrigerator at approximately 4°C prior to examination. The degree of staining was assessed using a stereomicroscope at 12–20× magnification. We defined three levels of staining: (1) brightly stained, (2) lightly or partially stained, and (3) unstained. Classes 2 and 3 were combined to yield a total number affected both by visual inspection and vital staining.

Results and Discussion

Vital staining with neutral red permitted rapid quantification of mortality in juveniles of *Villosa iris* and *Anodonta grandis*. Both species responded to copper exposure, as judged by visual inspection and vital staining. As copper concentration increased among treatments, the number of gaped juveniles decreased. This corresponded with increasingly lighter staining in tests with *V. iris*. At successively higher toxicant concentrations, the intensity of staining decreased, grading from intense red to faint pink. By contrast, all juveniles in the controls were gaped, active, and exhibited bright staining. Mortality de-

terminations, assessed by visual examination and vital staining, yielded identical results. In all cases, juveniles classed as gaped and dead exhibited no staining.

Visual inspection of juveniles revealed that 50% of the young *V. iris* subjected to the 24-μg Cu/L treatment were affected (ungaped), and results of vital staining corresponded closely; 45% stained lightly. Complete inhibition of gaping was observed in treatments containing 59 μg Cu/L, and vital staining showed 85% of these juveniles to be affected. At 75 μg Cu/L, all juveniles were classed as affected both by visual inspection and vital staining.

Mortality determinations among juveniles of *A. grandis*, based on vital staining, corresponded to the results of visual inspection. The amber coloration of the valves, however, masked any partial staining of internal anatomy that might have occurred, thus preventing quantification of sublethal responses. Because the coloration of newly excysted juveniles approximates that of mature glochidia (larvae), vital staining may be of limited use for assessing sublethal effects among taxa with colored glochidial valves. Most species within the subfamily Anodontinae possess glochidia with amber-colored valves, but at least seven species exhibit little or no coloration (Hoggarth, M. A., personal communication). Glochidia of species in the subfamilies Ambleminae and Lampsilinae possess uncolored glochidial valves; accordingly, vital staining may be used to determine both mortality and sublethal effects.

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