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Short Communication

AQUATIC INVERTEBRATE RESTING EGG SENSITIVITY TO GLUTARALDEHYDE AND SODIUM HYPOCHLORITE

DAVID F. RAIKOW,*†‡ PETER F. LANDRUM,‡ and DAVID F. REID†‡
†National Oceanic and Atmospheric Administration, National Center for Research on Aquatic Invasive Species, 2205 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA
‡Great Lakes Environmental Research Laboratory, 2205 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA

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Abstract—Ballast tank treatment technologies are currently in development to reduce the risk of acquiring or transporting viable aquatic organisms that could be introduced to ecosystems and become invasive. Aquatic invertebrate resting eggs represent a challenge to such technologies because of morphological and biochemical adaptations to stress that also protect eggs from artificial stressors. To evaluate the potential efficacy of chemical biocides for ballast tank treatment, the present study examined the acute toxicity of glutaraldehyde and sodium hypochlorite on resting eggs of the freshwater cladoceran *Daphnia mendotae* and marine brine shrimp (*Artemia* sp.). Glutaraldehyde was toxic to resting eggs of *Artemia* sp., as indicated by a lethal concentration to 90% of organisms (LC90) of 95% confidence interval (226 ± 10 mg/L). *Daphnia mendotae*, in contrast, displayed erratic responses to glutaraldehyde. Sodium hypochlorite was similarly toxic to resting eggs of *Artemia* sp. and *D. mendotae*, which displayed LC90s of 86.5 ± 3.0 and 78.3 ± 1.6 mg/L, respectively. Burial in sediment protected resting eggs from toxicants. The present results corroborate those from previous investigations of resting egg sensitivity to artificial stressors, supporting the conclusions that resting eggs are less sensitive than other life stages to artificial stressors and that chemical biocide concentrations effective against other life stages may be ineffective against resting stages.

Keywords—Resting egg Biological invasion Ballast Glutaraldehyde Sodium hypochlorite

INTRODUCTION

Biological invasion by aquatic invasive species (AIS) occurs globally in both freshwater and marine environments [1]. Because such invasions cause major ecological damage and huge economic costs [2–5], the need to manage invasion vectors to reduce invasion rates is widely recognized. Transport and release of ballast water and residual material, including sediment, has been identified as an important AIS introduction vector [6]. Subsequently, best-management practices have been altered to reduce the volume of coastal ballast water imported to some areas, including the Great Lakes (USA) [7,8]. In addition, numerous treatment technologies designed to disinfect ballast water are currently being evaluated for potential deployment on ships. An ideal ballast tank treatment should be effective not only against the broad spectrum of organisms known to occur in ballast water [9] but also against a variety of organism life stages as well. Zooplankton, for example, are known to produce dormant life stages called resting eggs or diapausing cysts [10], which can be found in ballast water and residual material [11]. Resting eggs are particularly challenging for ballast tank treatment technologies, because morphological and biochemical adaptations that impart protection from natural catastrophic events also protect eggs to varying degrees from artificial stressors [12], including the chemical biocide SeaKleen® (Menadione; Garnett, Watkinsville, GA, USA) [13].

Glutaraldehyde and sodium hypochlorite are among the chemical biocides currently being considered for use in ballast water treatment. Both are used as disinfectants against viruses and bacteria in numerous medical and commercial applications. The toxicities of glutaraldehyde and sodium hypochlorite have been evaluated against adult zooplankters and benthic invertebrates and, to a lesser extent, the resting eggs of brine shrimp (*Artemia* sp.; against glutaraldehyde) [14] and the freshwater cladoceran *Daphnia magna* (against sodium hypochlorite) [15]. The toxicities of glutaraldehyde and sodium hypochlorite for adult forms also have been shown to decrease with increasing amounts of sediment in test chambers [14,16]. The present study was conducted for the purpose of evaluating the efficacy of glutaraldehyde and sodium hypochlorite as potential ballast water treatments for the management of AIS vectors. The objective was to evaluate the acute toxicity of glutaraldehyde and sodium hypochlorite to zooplankton resting eggs. Glutaraldehyde and sodium hypochlorite were expected to be toxic to resting eggs. Burial in sediment was expected to reduce organism sensitivity to biocides.

MATERIALS AND METHODS

Artificial seawater was used for tests of marine organisms and made using purified (Nanopure) water and Instant Ocean® (Aquarium Systems, Mentor, OH, USA). Filtered (934-AH; Whatman, Sanford, ME, USA) water from the Huron River (Ann Arbor, MI, USA) was used for tests of freshwater organisms (total hardness, 100 mg/L as $CaCO_3$; conductivity, 913 μs/cm; pH 8.5) [12]. Glutaraldehyde and sodium hypochlorite were obtained from Fisher Scientific (Pittsburgh, PA, USA). Stock solutions of glutaraldehyde were created using 50% solution and stored under refrigeration for up to one month before use. Glutaraldehyde concentrations were checked by colorimetric analysis of diluted solutions, because

* To whom correspondence may be addressed (raikow.david@epamail.epa.gov). The current address of D.F. Raikow is U.S. Environmental Protection Agency, National Exposure Research Laboratory, 26 W. Martin Luther King Jr. Drive, Cincinnati, OH 45268.

concentrations used in experiments were too high to be directly measured. Because of rapid loss of chlorination observed over the course of minutes (not reported), sodium hypochlorite concentrations in solutions used for bioassays were not measured. Instead, nominal sodium hypochlorite serial dilutions were created quickly at the same time as toxicant distribution to test chambers using capped, volumetric flasks under indirect illumination to minimize toxicant volitalization and degradation.

Grades A and B Great Salt Lake (UT, USA) *Artemia* sp. cysts were obtained from Florida Aqua Farms (Dade City, FL, USA). *Artemia* sp. cysts were placed in a scintillation vial filled with artificial seawater, shaken, and allowed to separate into those that settled and those that floated. Cysts that settled were collected with a Pasteur pipette, deposited into Petri dishes containing artificial seawater, and distributed to test chambers using a Pasteur pipette under a dissecting microscope with illumination from below. *Daphnia mendotae* resting eggs were extracted from sediment collected from Muskegon Lake (MI, USA) by sieving with a 250-μm, stainless-steel sieve. The sediment used to obtain *D. mendotae* resting eggs was collected from the same location as samples used to test sensitivity to SeaKleen [13] and physical stressors [12]. Sediment was stored at 4°C for at least eight weeks before use to allow resting eggs to experience a refractory period.

Standard bioassay protocols [17] were adapted for use with invertebrate resting eggs [12,13]. All bioassays of *Artemia* sp. and *D. mendotae* used 50 eggs in each of five replicates for every dose run during three to four experiments under a 16:8-h light:dark photoperiod. *Artemia* sp. bioassays were run at 27°C and *D. mendotae* at 20°C. Control treatments contained water with no toxicant. Test chambers were evaluated and emergent neonates collected once every 24 h for 3 d in the case of *Artemia* sp. and once every 7 d in the case of *D. mendotae*. Neonates were discarded. Mortality rates were calculated by subtracting the number of stocked eggs by the number of successful hatches that occurred over the course of the entire postexposure period. Eggs that did not hatch by the end of the entire postexposure period were considered to be dead and were discarded. A successful hatching was defined as a mobile neonate fully disengaged from the egg shell (e.g., the chorion in *Artemia* sp.) or associated structures (e.g., the ephippium in *D. mendotae*) [12,13]. Teratogenic effects were monitored by noting anomalous morphology in immobile *Artemia* sp. and *D. mendotae* neonates fully disengaged from the egg shell or associated structures [12,13]. *Artemia* sp. that were not counted as successfully hatched included those that stopped development at the umbrella stage; those that were separated from the shell, normal in appearance, and immobile; and those that were separated from the shell, abnormal in appearance, and immobile [12,13,18].

Test chambers and procedures were the same as those used to examine the sensitivity of resting eggs to SeaKleen [13]. Test chambers consisted of a pair of nested, 100-ml, polypropylene beakers. The inner beaker was modified by having the bottom cut out and covered with 37-μm Nitex mesh (Terko, Briarcliff Manor, NJ, USA). The outer beaker was not modified. The modified inner beaker filled with water when lowered into the outer beaker that was partially filled with water. Test organisms were sorted and deposited into the inner beaker nested with the outer beaker containing clean medium. Once the appropriate number of test subjects was distributed, the inner beaker was lifted out of the clean medium, allowing it to drain. The exterior bottom surface of the inner beaker was then set on a laboratory tissue for a few seconds to absorb residual medium and then lowered into another outer beaker filled with toxicant. Test chambers were then placed into environmental chambers set to conditions required by the particular species and checked at 23 h. Neonates were then removed under a dissecting microscope. At the end of the 24-h exposure period, the inner beaker was lifted out of the outer beaker, allowing the toxicant to drain from unhatched test subjects. The inner beaker containing wet, unhatched test subjects was then quickly blotted on a laboratory tissue to absorb residual toxicant, and the inner beaker was lowered into a new beaker filled with clean medium and returned to the environmental chambers. No further manipulation of beakers occurred.

Effects of sediment on the toxicity of glutaraldehyde and sodium hypochlorite were tested following procedures used with SeaKleen [13]. Bulk sediment was deposited to a depth of 1 cm (~15 ml) into 100-ml polypropylene beakers. Toxicant was introduced to test chambers by pouring water (~85 ml) onto a floating Styrofoam disk, which prevented any direct disturbance of the sediment. After 24 h, the sediment was sieved, and extracted ephippia were placed in clean medium and allowed to hatch.

Data analysis for all bioassays consisted of computing the 24-h lethal concentration for 90% of tested organisms (LC90) and the 95% confidence interval using Systat® (Systat Software, San Jose, CA, USA) to calculate a logistic regression (LOGIT). Abbott's correction was used on all data [19]. Unhatched eggs were classed as dead, and mobile organisms detached from the shell or ephippium were classed as alive [12,13].

RESULTS

Control hatching rates varied between species. *Artemia* sp. hatched at a rate of 52 to 72% (mean, 63%; standard deviation, 7.4%). *Daphnia mendotae* hatched at a rate of 29 to 44% (mean, 36%; standard deviation, 4.9%). Glutaraldehyde killed resting eggs of *Artemia* sp. and *D. mendotae* (Fig. 1A and B). Sensitivity to glutaraldehyde varied between species (Table 1). *Artemia* sp. displayed a LC90 of 226 ± 10 mg/L. *Daphnia mendotae*, in contrast, displayed erratic responses to glutaraldehyde. Because of the highly variable sensitivity of *D. mendotae* to glutaraldehyde at concentrations that were highly toxic to *Artemia* sp. (<300 mg/L), very high doses (≤2,000 mg/L) were tested. High mortality was not, however, observed at high doses. Because of the highly variable response, the logistic curve fitted to these results was not robust; hence, no lethal concentrations are reported. Sodium hypochlorite killed resting eggs of *Artemia* sp. and *D. mendotae* (Fig. 1C and D). Sensitivity to sodium hypochlorite was similar between species (Table 1), with *Artemia* sp. displaying a LC90 of 86.5 ± 3.0 mg/L and *D. mendotae* a LC90 of 78.3 ± 1.6 mg/L. Responses to sodium hypochlorite were well estimated by logistic regression. No toxicity was observed in *D. mendotae* resting eggs buried in sediment when exposed to glutaraldehyde or sodium hypochlorite at concentrations up to 2,000 mg/L.

DISCUSSION

Sensitivity of *Artemia* sp. resting eggs to glutaraldehyde was greater than, but of the same magnitude as, the reported LC90 of 550 mg/L for the epibenthic amphipod *Hyalella azteca* to glutaraldehyde [16]. *Artemia* sp. sensitivity to glutaraldehyde was less than the reported sensitivity of other or-

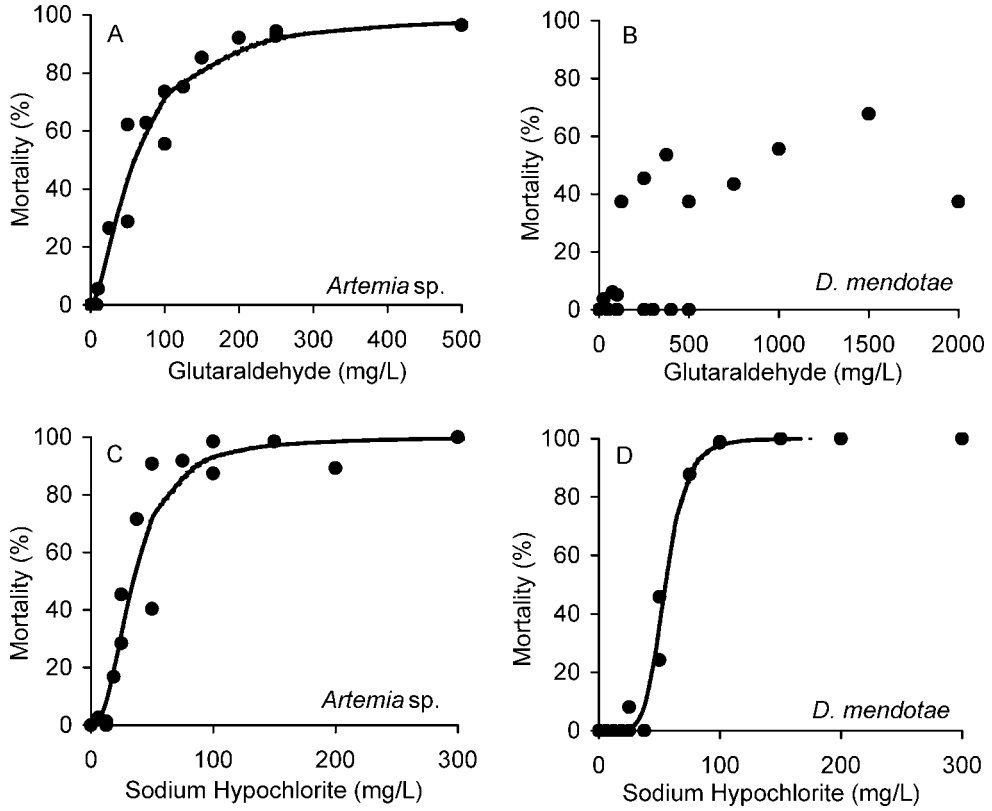

Fig. 1. Dose–response relationship of aquatic invertebrate resting eggs to chemical biocides. Data are results from several experiments. (**A**) Response of marine brine shrimp (*Artemia* sp.) to glutaraldehyde. (**B**) Response of freshwater cladoceran *Daphnia mendotae* to glutaraldehyde. (**C**) Response of *Artemia* sp. to sodium hypochlorite. (**D**) Response of *D. mendotae* to sodium hypochlorite.

ganisms, including the benthic oligochaete *Lumbriculus variegatus* (LC90, 12.9 mg/L), the cladoceran *Ceriodaphnia dubia* as adults (LC90, 12 mg/L), and the cladoceran *Daphnia magna* as adults (LC90, 102 mg/L) [16]. Hence, application of glutaraldehyde to levels sufficient to kill *H. azteca*—levels that also are highly effective against adult pelagic zooplankters—should be highly effective against resting eggs of *Artemia* sp.

In contrast, *D. mendotae* resting eggs displayed erratic responses to glutaraldehyde that precluded comparison to the sensitivity of other organisms. Although increased bacterial activity is associated with more rapid degradation of glutaraldehyde [20], exposure durations in the present study were much shorter than previous experimental durations examining glutaraldehyde degradation. Thus, it is not likely that potential differences in bacterial activity between freshwater and saline bioassays in the present study explain the observed differences in organism sensitivity to glutaraldehyde. Neither are differences in oxidizing properties between glutaraldehyde, which is nonoxidizing, and sodium hypochlorite, which is oxidizing, likely to explain the observed differences. Deactivation of glutaraldehyde, however, could have occurred through a Schiff base reaction. Variation in ephippia integrity also might explain, in part, variation in sensitivity—that is, damaged or partially opened ephippia could have allowed increased exposure of eggs to the toxicant. The presence of ephippia, however, did not protect cladoceran resting eggs from sodium hypochlorite, nor did ephippia prevent SeaKleen from being toxic to resting eggs [13]. Cladoceran eggs not encased in ephippia lack a tough outer shell, such as the chorion in *Artemia* sp., so loose cladoceran eggs or other zooplankton eggs not encased in ephippia, such as those of copepods, may be more sensitive to glutaraldehyde.

Sensitivity of *Artemia* sp. resting eggs to sodium hypochlorite was consistent with the reported LC90 of 53 mg/L for *Artemia* sp. resting eggs [14], having fallen within the reported 95% confidence interval (28–147 mg/L). Sensitivity of *Artemia* sp. resting eggs observed in the present study, as indicated by lethal concentration to 99% of organisms (LC99, 234 ± 15 mg/L) was of the same magnitude as that reported for

Table 1. Lethal concentrations (mg/L) to 90% (LC90) and 50% (LC50) of organisms in the resting life stage

Toxicant	Organism	No. of experiments	LC90[a] (mg/L)	LC50[a] (mg/L)
Glutaraldehyde	*Artemia* sp.[b]	4	226 ± 10	59.3 ± 1.5
	Daphnia mendotae[c]	4	—	—
Sodium hypochlorite	*Artemia* sp.	3	86.5 ± 3.0	34.6 ± 0.7
	Daphnia mendotae	3	78.3 ± 1.6	55.0 ± 0.6

[a] Values in parentheses are ± 95% confidence interval.
[b] Marine, incubated at 27°C.
[c] Freshwater, incubated at 20°C.

Artemia salina resting eggs (LC99, 486 ppm [15]). Exposure methods used to obtain previously reported values differed from those used in the present study, which could explain differences between the present and previous studies. Resting eggs of *D. mendotae* encased in ephippia displaying a LC99 of 115 ± 4 mg/L in the present study were slightly less sensitive to sodium hypochlorite compared with those of *D. magna* encased in ephippia (reported LC99, 76.3 ppm [15]). Results of resting egg sensitivity trials clearly demonstrate that resting eggs are much less sensitive to sodium hypochlorite compared to adult *D. magna* (LC90, 0.7 mg/L), *H. azteca* (LC90, 4.7 mg/L) , and *L. variegatus* (LC90, 1.0 mg/L) [16]. Zebra mussel (*Dreissena polymorpha*) adults, however, are less sensitive than zooplankton resting eggs (LC90, 130 mg/L) [16].

Results from sediment experiments were consistent with those of previous experiments that tested the effect of sediment on aquatic invertebrate sensitivity to chemical biocides. Previous experiments have shown that *D. mendotae* resting egg sensitivity to SeaKleen, for example, decreases 10-fold when eggs are buried in sediment [13]. In the presence of sediment, *L. variegatus*, which burrows into sediment, is much less sensitive to glutaraldehyde and sodium hypochlorite than *H. azteca*, which does not burrow [14,16]. The likely presence of amines in sediment could explain deactivation of glutaraldehyde, again through a Schiff base interaction. Hence, the conclusions reached concerning resting egg response to potential ballast water treatment technologies in previous studies are further supported by the present study—namely, burial in sediment confers protection from chemical biocides; resting eggs are less sensitive than adult life stages to artificial stressors, including chemical biocides; and toxicant concentrations effective against other organisms or life stages may be ineffective against resting eggs [12,13,16].

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