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SENSITIVITY OF AQUATIC INVERTEBRATE RESTING EGGS TO SEAKLEEN® (MENADIONE): A TEST OF POTENTIAL BALLAST TANK TREATMENT OPTIONS

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Abstract—The introduction of aquatic species in resting life stages by the release of ballast water is a less well-known but potentially important invasive species vector. Best-management practices designed to minimize transport of ballast water cannot eliminate this threat, because residual water and sediment are retained in ballast tanks after draining. To evaluate the potential efficacy of chemical treatment of residual material in ship ballast tanks, the present study examined the acute toxicity of the proposed biocide SeaKleen® (menadione; Garnett, Watkinsville, GA, USA) on resting eggs of *Brachionus plicatilis* (a marine rotifer), a freshwater copepod, *Daphnia mendotae* (a freshwater cladoceran), and *Artemia* sp. (a marine brine shrimp). SeaKleen was toxic to resting eggs of all taxa. *Daphnia mendotae* resting eggs encased in protective ephippia were the least sensitive, as indicated by a 24-h lethal concentration of toxicant to 90% of organisms of 8.7 mg/L (95% confidence interval, ± 0.1 mg/L). SeaKleen induced teratogenic effects in *D. mendotae* and *Artemia* sp. Exposure to sunlight quickly degraded SeaKleen, which lost all toxicity after 72 h outdoors. SeaKleen increased in toxicity slightly after 72 h in darkness. Burial of *D. mendotae* ephippia in natural lake sediment reduced SeaKleen toxicity by a factor of 20. Reduced toxicity in the presence of sediment raises serious doubts as to the potential for this, or any, chemical biocide to kill aquatic invertebrate resting stages buried in sediment retained in ship ballast tanks.

Keywords—Resting egg Biological invasion Ballast SeaKleen® Menadione

INTRODUCTION

Biological invasions are the introduction, establishment, and spread of organisms to habitats previously unoccupied by those organisms, usually by anthropogenic means [1]. When a nonindigenous species becomes widespread and negatively affects the ecology of a recipient ecosystem and/or the economy of a region, a value judgment typically is made by stakeholders, and the species is labeled ''invasive'' [2]. Large, well-documented, negative ecological [3] and economic [4] effects of invasive species have instigated a need to control the spread and proliferation of biological invasions globally. This effort requires not only an understanding of invasion biology but also an active managerial response. To that end, key vectors of exotic species introduction have been identified and potential control methods proposed [5]. A current leading invasion vector to freshwater and marine ecosystems globally is the ballast of large oceanic ships [6].

Used to maintain trim and balance structural stress caused by the loading and off-loading of cargo, ballast in modern ships consists of water pumped from the immediate surroundings into tanks integrated into the hull. Required to maintain safe ship operation, retention of ballast can result in its transportation across the globe. Such ballast water can contain large populations of viable organisms [7,8]. Zebra mussels, for example, generally are believed to have been introduced to the Great Lakes (USA) in the form of pelagic veligers (larvae suspended in the water column) released from ballast water [9]. Ballast water also may contain sediments that can settle and collect in the ship's ballast tanks. Current tank-draining mechanisms cannot empty tanks fully, resulting in the retention of residual sediment and water.

Ballast water as a biological invasion vector instigated changes in best-management practices and policies concerning ships entering the North American Great Lakes. For example, regulations now require incoming vessels with ballast water to carry out a ballast water exchange beyond 200 miles and in water more than 2,000 m deep, such that the minimum salinity in the exchanged tanks is greater than 30 ppt; to use an alternative, environmentally sound method approved by the U.S. Coast Guard before the voyage; or to retain the ballast water on board the vessel [10]. International Maritime Organization guidelines direct ships to avoid taking ballast water at night or in muddy water to avoid the acquisition of organisms when possible [11]. The majority of ships entering the St. Lawrence Seaway (Canada) declare ''no ballast on board'' and are exempt from seawater exchange requirements [12]. Residual sediment in ships declaring no ballast on board, however, is known to harbor organisms, including resting eggs that could be resuspended during reballasting activities [13]. Therefore, such management practices are insufficient to prevent the transport of viable and, thus, potentially nonindigenous and invasive species.

Active methods for controlling the presence of viable organisms in ballast tanks are currently in development. Approaches are varied and include filtration, separation of suspended material via hydrocyclones, deoxygenation, heating, electrolysis, application of biocides, or a combination of methods [6]. An ideal chemical biocide would be toxic to target organisms, safe for ship crews to handle, and rapidly degradable when released into the environment.

The life cycles of many aquatic organisms include stages

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that are highly tolerant of adverse environmental conditions and, thus, are amenable to transport in ballast tanks. Cladocerans, for example, can reproduce asexually under favorable environmental conditions but switch to sexual reproduction and produce eggs that are highly resistant to adverse environmental conditions [14]. Also called resting eggs, diapausing eggs, or cysts, these stages typically sink to the sediment, lie dormant, and later hatch and reconstitute the pelagic community [14]. Resting eggs can remain viable for decades, a property that creates a ''seed bank'' in sediment [15]. Moreover, freshwater invertebrate resting eggs exposed to saltwater can survive and hatch when later exposed to freshwater, illustrating their ability to endure adverse conditions such as those that might be encountered during open-ocean ballast water exchange [16,17].

Although cladoceran zooplankters of the genus *Daphnia* in the adult stage are standard bioassay test organisms, the use of resting stages in toxicity studies is uncommon [18]. Neonates derived from the hatching of *Brachinous plicatilis* cysts have been used in acute toxicity bioassays [19]. Resting egg production in the rotifer *Brachionus calyciflorus* has been used as an endpoint in acute toxicity tests, which suggests that resting egg production is highly sensitive to pentachlorophenol and copper [20]. The viability of eggs produced by females exposed to toxicants, however, was not examined [20]. The acute toxicity of glutaraldehyde, sodium hypochlorite, and SeaKleen® (menadione; Garnett, Watkinsville, GA, USA) on the adult stages of several aquatic invertebrates as well as the acute toxicity of glutaraldehyde and sodium hypochlorite on the resting stage of the brine shrimp *Artemia* sp. have been evaluated under standard conditions and in the presence of sediment, which decreased toxicant effectiveness [21,22].

Menadione is the parent template of polyisoprenoid-substituted naphthalenediones or the vitamin K group. Synthetic menadione is used as a dietary supplement for livestock, including catfish [23]. As a potential biocide, menadione is marketed under the name SeaKleen. Dinoflagellate (*Glenodinium* sp.) cysts were most resistant, experiencing 100% mortality at 2.0 ppm, in acute toxicity examinations of SeaKleen among a variety of aquatic organisms, including algae, adult copepods, and zebra mussel (*Dreissena polymorpha*) veligers [24]. SeaKleen also disrupted chloroplast development in *Glenodinium foliaceum* cysts at 2.0 ppm [24]. Concentrations of SeaKleen dropped 8.5% after 72 h in the dark and 53% after 72 h of full exposure to sunlight [23].

The purpose of the present study was to directly examine the sensitivity of zooplankton resting eggs to SeaKleen and, thereby, to evaluate the potential efficacy of biocide application to ballast tanks for invasive species vector management. Efficacy of the biocide to resting eggs buried in sediment and of the biocide under light and dark conditions also was examined. Study organisms included the resting stages of one species each of marine rotifer (*B. plicatilis*), freshwater copepod, freshwater cladoceran (*Daphnia mendotae*), and marine brine shrimp (*Artemia* sp.). Sensitivity of organisms in the resting stage was expected to be less than that of adult stages. Burial in the sediment was expected to reduce organism sensitivity, and exposure to light was expected to reduce biocide toxicity. To our knowledge, the present study is the first to examine the acute toxicity of SeaKleen on a variety of aquatic invertebrate resting stages.

MATERIALS AND METHODS

Chemicals

SeaKleen was obtained in the form of a powder in two lots: lot 042602, obtained in 2002; and lot 041504, obtained in 2004. A stock solution of 100 mg/L in water was created on each day of testing and was used immediately. Any solution remaining after the experiments were initiated was discarded. Filtered (Whatman 934-AH, Sanford, ME, USA) Huron River water (Ann Arbor, MI, USA) was used for stock solutions and serial dilutions in tests of freshwater organisms (total hardness, 275 mg/L as $CaCO_3$; conductivity, 913 μS/cm; pH 8.5) [22]. Artificial seawater made using purified (nanopure) water and Instant Ocean® (Aquarium Systems, Mentor, OH, USA) was used for tests of marine organisms. Dilution was evaluated by measuring absorbance of light at 264 nm using a spectrophotometer. Lot 042602 was used initially in tests of *Artemia* sp. On acquisition of the new lot, a bioassay with *Artemia* sp. was run for comparison. Subsequent tests, including tests of other organisms, were run with lot 041504.

Test organisms

Daphnia mendotae and copepod resting eggs were obtained through collection of lake sediment as part of collections made from sites in the western basin of Lake Erie (USA) and Lake Michigan offshore of Muskegon and Muskegon Lake (MI, USA). Sediment also was obtained from the ballast tanks of ships of opportunity. Sediment was stored at 4°C for at least eight weeks before use [13]. Organic material, including resting eggs, was extracted from bulk sediment using sugar flotation [13,15] except for some experiments in which ephippia were simply sieved out of sediments using a 250-μm, stainless-steel sieve.

Artemia cysts were obtained from a local pet store, sorted by placing them in a scintillation vial filled with seawater, and shaken. Cysts were then allowed to separate into those that settled and those that floated. Cysts that settled could be collected more easily with a Pasteur pipette and deposited into a Petri dish with seawater. Individual eggs were then collected using a Pasteur pipette under a dissecting microscope with illumination from below. Dried *B. plicatilis* rotifer cysts were obtained from Florida Aqua Farms (Dade City, FL, USA).

Other organisms were evaluated for potential use, including wetted *Daphnia magna* ephippia, dried *Daphnia pulex* ephippia, and dried *B. calyciflorus* cysts. In addition, cultures of *Daphnia pulicaria*, *Bosmina longirostris*, *D. mendotae*, *Diaphanosoma* sp., and *Ceriodaphnia* sp. were initiated in an attempt to culture resting eggs. Zooplankton cultures were maintained in environmental chambers at 20°C with a 16:8-h light:dark photoperiod and fed live algal cultures of *Nanochloropsis* sp. Cultures were cultivated to produce large population sizes, and a variety of methods were then used to attempt induction of resting egg production, including crowding, reduction of temperature, starvation, and darkness. Resting eggs harvested from *D. pulicaria* cultures were stored at 4°C for at least eight weeks before use.

Test procedures

The hatching rates and temporal hatching patterns of potential test organisms were evaluated before their use in bioassays. Resting eggs were allowed to hatch under conditions optimized for each species [25]. *Daphnia mendotae* ephippia and tests of sediment were run at 20°C, *B. plicatilis* at 25°C,

and *Artemia* sp. at 27°C. All bioassays were run under a 16:8-h light:dark photoperiod except for the light-exposure experiment.

Standard bioassay protocols [26] were adapted for use with invertebrate resting eggs. A range-finding experiment was used to establish a range of five toxicant concentrations for bioassays. Test subjects were exposed to the toxicant SeaKleen at concentrations of 0 (control), 2, 4, 6, 8, and 10 mg/L. Toxicant concentration was modified to the range of 0.5 to 5.0 mg/L for the second run of experiments involving copepods and *B. plicatilis*. Five replicates were run at each concentration, for a total of 30 units per experiment, except for copepods, for which limited sediment supply necessitated three replicates per concentration. Trials of *Artemia* sp., *D. mendotae*, and *B. plicatilis* used 50 eggs per unit. A standardized mass of well-mixed bulk sediment was used per experimental unit in trials using copepods and tests of *D. mendotae* burial in sediment. Resting eggs were exposed to toxicants for 24 h, including the 16:8-h light:dark photoperiod, then transferred to clean medium.

Test chambers were evaluated once every 24 h, and emergent neonates were collected each day for 3 d in the case of *Artemia* sp., for 7 d in the case of *D. mendotae* and copepods, and for 14 d in the case of *B. plicatilis*. Several bioassays of *Artemia* sp. and *D. mendotae* were allowed to run for three weeks to test for a delayed-hatching effect. Mortality rates were calculated by subtracting the number of stocked eggs by the number of successful hatches. 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 *Daphnia* sp.). Anomalous morphology in immobile *Artemia* sp. and *D. mendotae* neonates fully disengaged from the egg shell or associated structures was noted and interpreted as an indication of teratogenic effects. Thus, *Artemia* sp. that stopped development at the umbrella stage, were separated from the shell and normal in appearance but immobile, or were separated from the shell and abnormal in appearance and immobile were not counted as successfully hatched [25].

Test chambers were constructed for the present study to control the duration of exposure to the toxicant. For *D. mendotae*, copepods, and *Artemia* sp., test chambers consisted of a pair of nested, 100-ml polypropylene beakers. The inner beaker was modified by having the bottom cut out a few millimeters from the edge. This resulted in a shelf and circular opening. The shelf was scored using a Dremel tool (Racine, WI, USA) to allow sealant to adhere. The opening was then covered with 37-μm Nitex mesh (Terko, Briarcliff Manor, NJ, USA) and glued in place to the inside of the beaker using aquarium silicone that was allowed to cure for several days. The outer beaker was not modified.

When lowered into the outer beaker, which was partially filled with water, the modified inner beaker also filled. Test chambers were initially set up containing clean medium. Test organisms were sorted and deposited into the inner beaker. Once the appropriate numbers of test subjects were 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 lowered into another outer beaker filled with the toxicant. Next, test chambers were 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 with illumination from below, from complete test chambers (i.e., the inner beaker seated within the outer beaker, with toxicant). 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. Finally, the inner beaker was lowered into a new beaker filled with clean medium and returned to the environmental chambers.

The removal of swimming *Artemia* organisms just before full 24-h exposure precluded the potential for death to occur between 23 and 24 h. We chose this procedure because our primary question was the effect of SeaKleen on the resting stage. Alternative methods would have risked killing swimming *Artemia* neonates or allowed resting egg toxicant exposure to exceed 24 h. We decided that full manipulation of test chambers at 24 h could be lethal to swimming *Artemia* organisms and unduly confound results, necessitating the removal of *Artemia* neonates just before 24 h.

Test chambers for *B. plicatilis* consisted of modified, 25-mm polystyrene culture dishes. A notch was cut out of the side of the dish using a Dremel tool. The notch was covered in 37-μm Nitex mesh held in place with silicone. The dish was then placed in an inverted culture dish top and filled with clean medium. Next, *B. plicatilis* cysts were distributed to test chambers. A laboratory tissue was then used to wick the medium from the dish by placing the tissue against the outside of the mesh. Toxicant was added to the dish, and chambers were checked for hatching after 23 h as above. After a total exposure of 24 h, the toxicant was wicked away and replaced with clean medium.

Light-exposure experiment

The effect of exposure to sunlight on SeaKleen effectiveness was evaluated with an experiment using *Artemia* sp. A stock solution of SeaKleen was prepared and a bioassay run under normal conditions to evaluate baseline toxicity. A sample of the stock solution was placed in a sealed glass Erlenmeyer flask, left outdoors, and allowed to be exposed to full sunlight. Initial levels of natural ultraviolet light to which the flask was exposed were measured hourly for 5 h with a model 1700 radiometer and SUD-005 detector with WB-320 filter (International Light, Newburyport, MA, USA). Another sample of the stock solution in an identical flask was placed in an environmental chamber at 20°C and a 0:24-h light:dark photoperiod (i.e., darkness). Bioassays with *Artemia* sp. using toxicant derived from the solutions exposed to sunlight and darkness were run after 24 and 72 h.

Sediment experiment

The effect of sediment on the toxicity of SeaKleen also was evaluated. Bioassays exposing bulk sediment, as opposed to extracted eggs, were run with *D. mendotae*. Sediment was deposited to a depth of 1 cm (15 ml) in 100-ml polypropylene beakers. A styrofoam disk was placed on top of the sediment. Toxicant was then poured onto the disk, which proceeded to float, allowing the beaker to be filled without disturbing the sediment. After 24 h, the sediment was sieved, and extracted ephippia were placed in clean medium and allowed to hatch for 7 d.

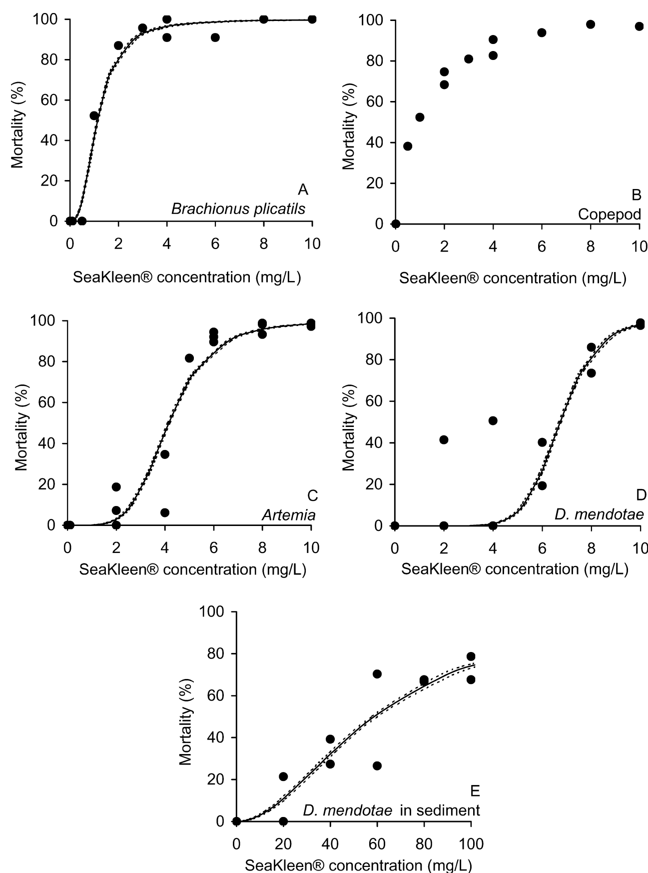

Fig. 1. Dose–response of aquatic invertebrate resting eggs to SeaKleen® (menadione; Garnett, Watkinsville, GA, USA). (**A**) Marine rotifer *Brachionus plicatilis*. (**B**) Freshwater copepod. (**C**) Marine brine shrimp *Artemia* sp. (**D**) Freshwater cladoceran *Daphnia mendotae*. (**E**) *Daphnia mendotae* ephippia (resting eggs) buried in natural lake sediment. Curves are logistic regressions with 95% confidence intervals based on data pooled from two experiments except for *Artemia* sp., which is based on three experiments. Response of copepods (**B**) was best described by $y = -0.1977x^2 + 0.4601x + 1.731$ ($r^2 = 0.99$) in log-log space, but data are presented in normal space to allow visual comparison.

Data analysis

Data analysis for all bioassays consisted of computing the 24-h lethal concentration of toxicant to 50% (LC50) or 90% (LC90) of organisms ± 95% confidence interval using logistic regression. Because of low hatching rates of test organisms in control treatments, Abbott's correction was applied to raw data before analysis [27]. For trials in which standardized masses of well-mixed sediment were used, data were corrected based on the mean number of hatched organisms in control treatments. At least two runs of each experiment were used to calculate lethal concentrations. A Pearson correlation was used to evaluate the relationship between incubation temperature and the LC90.

RESULTS

Test subjects

Lake sediment samples yielded useful densities of resting eggs. Sediment from Muskegon Lake contained a high density of *D. mendotae* ephippia. One sample of sediment from the western basin of Lake Erie displayed a high density of copepod resting eggs and supplied enough material for two bioassays. Because these copepods were collected during experiments as first-instar larval nauplii, identification was not possible. An attempt to culture these nauplii was not successful.

Several organisms evaluated for use were not suitable. Commercially obtained *D. magna* and *D. pulex* ephippia were not suitable because of high cost. Commercially obtained *B. calyciflorus* did not hatch. Zooplankton cultures resulted in inadequate production of resting eggs.

Hatching rates and timing varied between species. *Daphnia mendotae* hatched at a rate of 20% in the control treatments. *Brachinous plicatilis* hatched at a rate of 10% in the control treatments. *Artemia* sp. displayed the highest hatching rate at 60 to 80%. Hatching rate of copepods was unknown, because eggs were not picked from residual extracted organic matter before experiments. *Brachinous plicatilis* and *D. mendotae* hatched later than 24 h in the control treatment and, thus, displayed delayed mortality at 48 h. Up to 7% of copepods in replicates of one experiment hatched within 24 h. Up to 80% of hatching by *Artemia* sp. occurred within 24 h in the 0 (control), 2, and 4 mg/L treatments. Thus, some neonates were exposed to the toxicant after hatching for an unknown period of time before being collected.

SeaKleen toxicity

SeaKleen killed representatives of all organisms tested (Fig. 1). Sensitivity to SeaKleen based on estimated LC90s varied between organisms (Table 1). *Brachinous plicatilis* cysts were most sensitive, displaying a LC90 of 2.6 ± 0.1 mg/L. *Daphnia mendotae* resting eggs in ephippia were the least sensitive,

Table 1. Lethal concentrations of SeaKleen® (Garnett, Watkinsville, GA, USA) to 50% (LC50) and 90% (LC90) of organisms in the resting life stage (±95% confidence interval)

Organism	Experiment	No. of experiments	Temperature (°C)	LC90	LC50
Brachionus plicatilis[a]	Standard	2	25	2.6 (±0.1)	1.1 (±0.1)
Copepod[b]	Standard	2	20	4.9 (±0.2)	0.8 (±0.1)
Artemia sp.[a]	Standard	3	27	6.6 (±0.1)	4.2 (±0.1)
Daphnia mendotae[b]	Standard	2	20	8.7 (±0.1)	6.7 (±0.1)
Artemia sp.	Baseline	1	27	11.9 (±0.5)	6.2 (±0.1)
Artemia sp.	24-h light	1	27	12.7 (±0.4)	9.6 (±0.1)
Artemia sp.	72-h light	1	27	—	—
Artemia sp.	24-h dark	1	27	8.9 (±0.4)	3.8 (±0.1)
Artemia sp.	72-h dark	1	27	5.9 (±0.2)	2.6 (±0.1)
Daphnia mendotae	Sediment	2	27	180 (±10)	59 (±1)

[a] Marine.
[b] Freshwater.

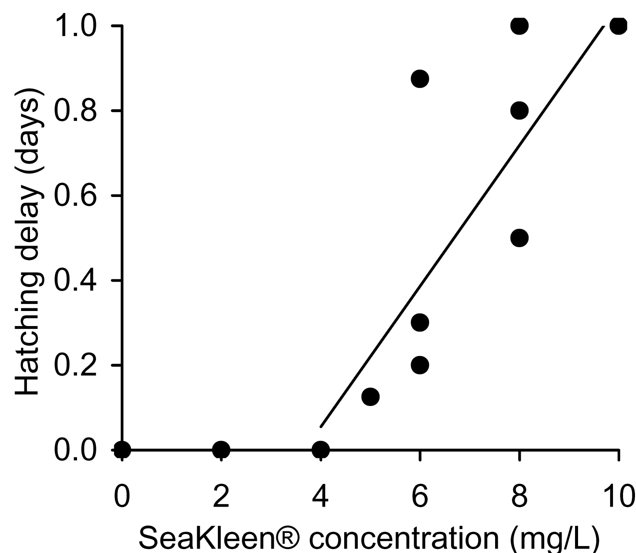

Fig. 2. Delay in hatching of mobile *Artemia* neonates exposed to SeaKleen® (menadione; Garnett, Watkinsville, GA, USA) while in the resting life stage. Data are median hatching delay, averaged across replicates, for three experiments. Linear regression is applied only to data for 4 to 10 mg/L ($y = 0.1659x - 0.6082$, $r^2 = 0.78$, $p < 0.01$).

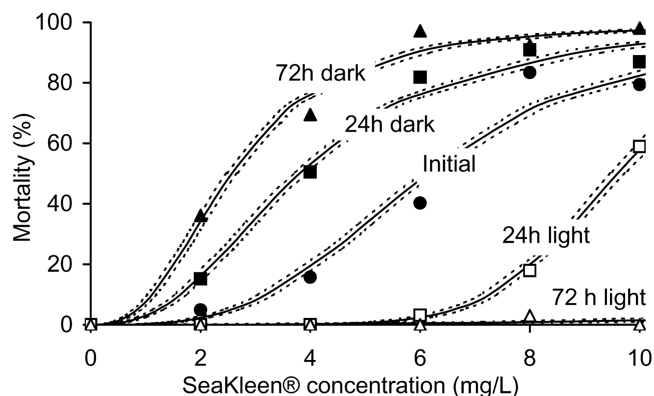

Fig. 4. Dose–response of *Artemia* resting eggs to SeaKleen® (menadione; Garnett, Watkinsville, GA, USA) exposed to natural light or darkness.

displaying a LC90 of 8.7 ± 0.1 mg/L. Incubation temperature and LC90s between taxa were negatively correlated, but this relationship was not statistically significant ($r = -0.333$, $p = 0.67$, $n = 4$). Toxicity of the newer lot (i.e., lot 041504) of SeaKleen on *Artemia* sp. compared favorably to that of the older lot, displaying a LC90 of 6.1 ± 0.2 mg/L. Based on this result, subsequent tests were run with the newer lot. This lot displayed unexpectedly lower toxicity (higher LC90), however, in the baseline (initial) bioassay of the light-exposure experiment.

The most varied effects of SeaKleen were observed in *Artemia* sp. SeaKleen delayed hatching of *Artemia* sp. up to 1 d relative to controls at concentrations of greater than 4 mg/L (Fig. 2). No hatching delays in treatments with SeaKleen were observed in other species.

The frequency of hatched, normal in appearance, but immobile *Artemia* neonates observed over the course of complete experiments peaked at midlevel concentrations (Fig. 3A). Although immobile neonates were observed on each day of collection, the majority appeared at 48 h. Because the survey of organism condition was visual and superficial, it was not possible to distinguish (at 24 h) between *Artemia* neonates that died as a result of teratogenic effects not visible externally and those that died as a result of exposure while swimming following normal development. Thus, the rapid hatching of *Artemia* eggs confounded strict interpretation of results for this species. Counting *Artemia* neonates that were superficially normal but immobile at 24 h as successfully hatched, thus assuming they died as a result of exposure while swimming, lowered calculated toxicity (raised LC90) marginally, producing a LC90 of 7.0 ± 0.1 mg/L and LC50 of 4.5 ± 0.1 mg/L.

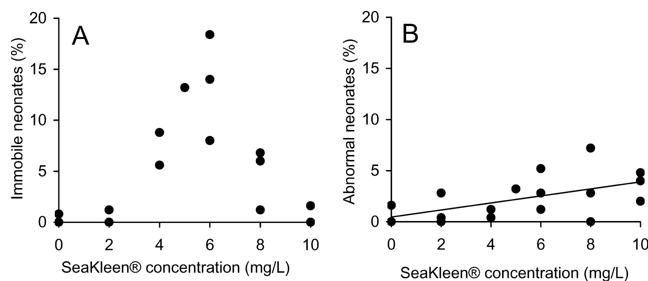

Fig. 3. Occurrence of immobile and abnormally developed *Artemia* neonates exposed to SeaKleen® (menadione; Garnett, Watkinsville, GA, USA). (**A**) Percentage of hatched immobile neonates normal in external appearance observed over the course of complete experiments. (**B**) Percentage of hatched immobile neonates abnormal in external appearance ($y = 0.3436x + 0.4627$, $r^2 = 0.332$, $p < 0.02$).

SeaKleen clearly affected the embryonic development of *D. mendotae* and *Artemia* sp. Abnormal development in both species presented as a lack of appendages and a relatively undifferentiated body. The frequency of *Artemia* neonates that separated from the egg shell but were immobile, having developed improperly, increased with SeaKleen concentration (Fig. 3B). Others stopped developing at the umbrella stage, at which an embryo is visible emerging from the shell. Most affected *Artemia* eggs simply did not hatch, especially at higher concentrations.

Effect of light on SeaKleen toxicity

The toxicity of SeaKleen diminished after exposure to sunlight (Fig. 4). During the first 5 h, the sample was exposed to an average of 2.8 μW/cm (standard deviation, ±0.7 μW/cm). The toxicity of SeaKleen in the initial (baseline) test conducted under normal conditions, as indicated by a LC90 of 11.9 ± 0.5 mg/L, was lower than expected based on the results of previous tests. After 24 h under natural light conditions, the LC90 fell to 12.7 ± 0.4 mg/L, although this estimate was less robust because of truncation of the response curve. After 72 h under natural light conditions, SeaKleen was not toxic; that is, the slope of response curve was not significantly different from zero ($F = 0.49$, $p = 0.56$). Surprisingly, the toxicity of SeaKleen increased relative to the baseline test after 24 and 72 h in the dark, reaching LC90s of 8.9 ± 0.4 and 5.9 ± 0.2 mg/L, respectively. This effect was corroborated by spectrophotometry. Peaks clearly visible in the initial sample between 240 and 280 nm diminished over time in the sample exposed to sunlight and, by 219 h, were not present (Fig. 5A). Concurrently, a new peak developed at 210 nm, which could be degradation products. The initial peaks, in contrast, increased over 24 and 72 h in the dark (Fig. 5B). By 219 h, the increased peaks diminished. That a peak also developed at 210 nm during time in the dark may indicate that increases in peaks between 220 and 280 nm were caused by the formation of degradation

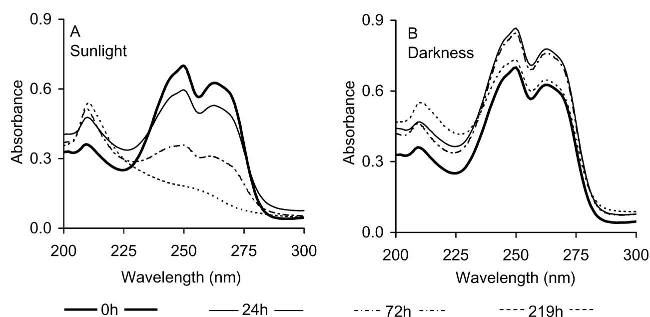

Fig. 5. Absorbance of light by SeaKleen® (menadione; Garnett, Watkinsville, GA, USA) exposed to natural light or darkness. (**A**) Effect of exposure to sunlight outdoors during normal diel cycles. (**B**) Effect of exposure to darkness in environmental chambers. Baseline (0-h) response is represented by the same curve in both panels.

compounds that also absorb light at those wavelengths and are sensitive to degradation by ultraviolet light.

Effect of sediment on SeaKleen toxicity

The toxicity of SeaKleen for buried *D. mendotae* ephippia was much less than that for aqueous exposed ephippia, with a LC90 of 180 ± 11 mg/L (Fig. 1E). Use of the styrofoam disk successfully allowed toxicant to be introduced into test chambers without disturbing sediment. No hatching occurred within 24 h, and hatching generally was delayed 1 d relative to ephippia not buried in sediments.

DISCUSSION

SeaKleen displayed characteristics desired for a potential disinfectant of ballast tanks. The compound was toxic to freshwater and marine aquatic invertebrate resting eggs and degraded quickly on exposure to sunlight. Reduced toxicity in the presence of sediment, however, raises serious doubts as to the potential for this, or any, chemical biocide to kill aquatic invertebrate resting stages buried in the retained sediment of ship ballast tanks.

Use of resting eggs in laboratory experiments

Of the variety of potential test subjects evaluated for the present study, few proved to be appropriate for experimental use. Ideal test subjects would have the following characteristics: Available in large quantities; inexpensive; high hatching rate; easy to handle and store; hatch quickly, but later than 24 h; easy to see and catch under a microscope; and not mixed with extraneous material. None of the test subjects used in the present study were ideal, but some were much better than others. Commercially obtained cladoceran ephippia were simply too expensive and displayed low hatching rates. Low or variable hatching rates are not a problem for establishing cultures because of asexual reproduction and rapid turnover rates of adults. Bioassays, however, require faster and more precise hatching. Cladoceran culturing may be too risky an endeavor to rely on for a resting egg supply in the quantities needed for bioassays. *Brachinous plicatilis* cysts are relatively inexpensive and available in vast quantities but are more difficult to handle in a quantitative fashion, because they are very small and translucent, requiring a micropipette and clear visual field for collection.

Sediment provides a potentially excellent source of resting eggs and may allow opportunistic testing of unusual taxa, as illustrated by copepods in the present study. Sediment samples, however, are highly variable in resting egg density (unpublished data). Collection of sediment is no guarantee of obtaining test subjects in sufficient quantities, and hatching rates can be variable. The sediment that yielded high densities of cladoceran resting eggs was obtained from a region known to have high *D. mendotae* resting egg densities [28]. Sediment also is not ideal because of the presence of extraneous material. Sugar flotation or sieving can remove much of this material, and this can be sufficient processing for use in bioassays.

Artemia organisms proved to be the most useful test subject. *Artemia* cysts were inexpensive, plentiful, and packaged as pure cultures with no extraneous material. Cysts were quickly and easily sorted in great numbers. Hatching rates were high. Storage was achieved on a dry shelf for long time periods. Neonates were opaque, relatively large, and did not respond to the intrusion of pipettes into chambers (as opposed to *D. mendotae*, which quickly swam away from pipette tips). In addition, teratogenic effects were easily observed.

The major drawback of *Artemia* cysts was hatching within 24 h. The presence of hatched and superficially normal in appearance but immobile neonates after 24 h was minor, but it did erode interpretation of the effects of SeaKleen on hatching in the present study. Nevertheless, we recommend *Artemia* cysts as a standard test organism for evaluation of toxicity on aquatic invertebrate resting life stages.

SeaKleen toxicity

SeaKleen was toxic to the resting stages of a marine rotifer, a freshwater copepod, a freshwater cladoceran, and a marine brine shrimp. The LC90s for these organisms were greater than 100% lethal concentrations of up to 2 mg/L for a variety of pelagic organisms, including bacteria, algae, zebra mussel (*D. polymorpha*) larvae, and adult copepods [23,24]. This result was not surprising, because resting eggs are adapted to withstand adverse environmental conditions through a variety of physical characters. Incubation temperature did not explain observed patterns of LC90s between taxa.

Observed patterns in LC90 values between taxa might be explained, however, by increasing egg size and morphological complexity. *Brachinous plicatilis* and copepods, for example, were the most sensitive resting eggs tested and also the smallest at 60 to 90 and 100 μm in diameter, respectively. Rotifer eggs are surrounded by a membrane described as thick by Wallace and Snell [29]. Whereas such membranes can aid in the resistance of adverse environmental conditions, the small size of eggs may have resulted in faster diffusion of biocide into the egg relative to other larger species. Copepod resting eggs were similarly simple [13]. Artemia eggs were much larger, at 250 μm in diameter in the present study, and protected by a more substantial membrane, the chorion. In contrast to the other taxa examined in the present study, *D. mendotae* eggs were larger and deposited within a chitinous envelope, the ephippium [14]. The ovoid *D. mendotae* eggs used in the present study, for example, measured 270 μm in diameter along the long axis, with the ephippium measuring 750 μm in length. That *D. mendotae* eggs used in the present study were contained within ephippia is important to note, because cladoceran eggs released from ephippia might be more sensitive to biocides. Survival of cladoceran eggs ejected from ephippia passing through fish guts has been observed to be low compared to survival of rotifer and copepod eggs, for example, which is an effect attributed to fragile membranes [30].

Although many resting eggs possess protective characters, organisms in this life stage are at a disadvantage with respect to biocide exposure relative to adults because of required developmental processes. Rapid cellular division and differentiation expose the organism to potential teratogens. *Artemia* organisms normally emerge from the egg but remain attached, in the umbrella stage, until appendage development is complete and it can freely swim away [25]. Thus, the presence of hatched (i.e., free of the shell) neonates with obvious external abnormalities allowed direct observation of teratogenic effects. SeaKleen induced similar effects in *D. mendotae*. Delayed hatching also indicated disrupted development. Thus, at low toxicant concentrations, development was delayed slightly. At midlevel toxicant concentrations, the frequency of hatched and superficially normal but immobile neonates peaked, which may indicate that death occurred at later developmental stages rather than through exposure to the toxicant while swimming. At high toxicant levels, most death occurred before successful hatching but also included the highest frequency of hatched but visibly abnormal neonates.

Effect of light and sediment on SeaKleen toxicity

SeaKleen degraded quickly on exposure to sunlight, as indicated by a reduction in toxicity. It is possible that the test solution exposed to sunlight was heated to such a degree as to cause degradation. Differential effects of heat on SeaKleen also might have contributed to differences in lethal concentrations observed between taxa, because taxa were reared at different temperatures. The relative importance of heat, however, was not directly evaluated in the present study. Moreover, the small sample size available for evaluation of correlation between incubation temperature and LC90 may have masked an effect of heat. The effect of heat on the toxicity of SeaKleen should be evaluated in future studies. Light can be eliminated as a confounding factor in such experiments by storing unused stock solution at test temperatures for 24 h in the dark before use in bioassays, as indicated by the maintenance of toxicity in the dark that was observed in the present study. That toxicity actually changed over time in the dark was surprising, and it hints at chemical transformations. It should be noted, however, that the initial toxicity of the toxicant used in the light-exposure experiment was lower than that in other experiments using the same lot and increased in toxicity to levels observed previously. Nevertheless, the maintenance of toxicity in the dark and quick degradation in sunlight are desirable properties in ballast tank treatment applications.

Burial of resting eggs in sediment reduced toxicant effectiveness. This result was consistent with observed protective effects of sediment on toxicant exposure by benthic aquatic macroinvertebrates in other studies [21,22]. The LC90 of SeaKleen for buried ephippia relative to that experienced by exposed ephippia increased by a factor of 20, a result similar in magnitude to that experienced by adult *Lumbriculus variegatus* (an oligochaete worm) with respect to SeaKleen [22]. The likely mechanism of protection was decreased exposure resulting from low diffusion rates of the toxicant into sediment, coupled with adsorption of the toxicant onto organic detritus. The sediment used for burial experiments in the present study was mixed artificially to promote uniform resting egg dispersion but, nonetheless, was natural in origin. The consistency of this material was quite different from the much denser and dryer mud collected in ship ballast tanks. Thus, we can speculate that the protective effects of dense mud found in ballast tanks will be greater than that observed in the present study.

Efficacy of treating residual sediment in ballast tanks

The present study examined the basic sensitivity of aquatic invertebrate resting eggs to SeaKleen, a biocide proposed for application to ballast tank treatment to prevent new aquatic biological invasions. Aquatic invertebrates in the resting life stage were sensitive to the biocide despite morphological adaptations for survival during adverse environmental conditions. The adaptations of resting eggs, including egg walls and/or chitinous cases, arguably did provide some protection, as indicated by higher LC90s (lower toxicity) compared to those of SeaKleen trials against adult aquatic invertebrates in other studies [24]. However, whereas lethal toxicant concentrations observed for resting eggs in the present study may theoretically be practical to apply in the field, serious doubt as to the efficacy of chemical treatment of residual material in ballast tanks is raised by the results of the sediment burial experiment.

Burial of *D. mendotae* ephippia provided protection from SeaKleen. Lethal concentrations of SeaKleen necessary to affect ephippia buried in up to 1 cm of sediment in the present study are not practical to achieve in the field. Moreover, the sediment inside ballast tanks often is much denser than the sediment used in the present study and deeper that 1 cm (unpublished data). Given such considerations, chemical treatment of residual sediment in ballast tanks for the purpose of killing aquatic invertebrate resting stages may not be effective.

This conclusion cannot be applied to the potential efficacy of chemical treatment of ballast water and targeting of pelagic organisms, including the use of SeaKleen. Chemical treatment of ballast water may still be a viable option for the control of ballast tanks as a biological invasion vector. Moreover, the risk of invasion by introduction of, or hatching en route by, resting eggs relative to the risk of invasion by pelagic organisms may be low and, thus, would make control of pelagic organisms a higher priority [31]. Nevertheless, we still must take action against potential biological invaders, and ballast tanks remain an important pathway. We may have to accept, however, that if residual material is present in ballast tanks, it will remain a potential threat despite efforts at ballast tank treatment.

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REFERENCES

1. Elton CS. 2000. *The Ecology of Invasions by Animals and Plants with a Foreword by Daniel Simberloff.* University of Chicago Press, Chicago, IL, USA.
2. Coulatti RI, MacIssac HJ. 2004. A neutral terminology to describe 'invasive' species. *Divers Distrib* 10:135–141.
3. Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA. 2000. Biotic invasion: Causes, epidemiology, global consequences, and control. *Ecol Appl* 10:689–710.
4. Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52:273–288.
5. Ruiz GM, Carlton JT, eds. 2003. *Invasive Species: Vectors and Management Strategies.* Island, Washington, DC.
6. National Research Council. 1996. *Stemming the Tide*. National Academy, Washington, DC.

7. Carlton JT, Geller JB. 1993. Ecological roulette: The global transport of nonindigenous marine organisms. *Science* 261:78–82.
8. Ruiz GM, Rawlings TK, Dobbs FC, Drake LA, Mullady T, Huq A, Colwell RR. 2000. Global spread of microorganisms by ships. *Nature* 408:49–50.
9. McMahon RF. 1996. The physiological ecology of the zebra mussel, *Dreissena polymorpha*, in North America and Europe. *Am Zool* 36:339–363.
10. Code of Federal Regulations. 2004. Ballast water management for control of nonindigenous species in the Great Lakes and Hudson River. CFR 33, Part 151, Subpart C. U.S. Government Printing Office, Washington, DC.
11. International Maritime Organization. 1997. Guidelines for the control and management of ships' ballast water to minimize the transfer of harmful aquatic organisms and pathogens. Resolution A.868(20). London, UK.
12. Colautti R, Niimi AJ, van Overdijk CDA, Mills EL, Holeck K, MacIsaac HJ. 2003. Spatial and temporal analysis of transoceanic shipping vectors to the Great Lakes. In Ruiz GM, Carlton JT, eds, *Invasive Species: Vectors and Management Strategies*. Island, Washington, DC, pp 227–246.
13. Bailey SA, Duggan IC, van Overdijk CDA. 2003. Viability of invertebrate eggs collected from residual ballast sediment. *Limnol Oceanogr* 48:1701–1710.
14. Dodson SI, Frey DG. 2001. Cladocera and other Branchiopoda. In Thorp JH, Covich AP, eds, *Ecology and Classification of North American Freshwater Invertebrates*. Academic, San Diego, CA, USA, pp 849–913.
15. Hairston NG, Van Brunt RA, Kearns CM, Engstrom DR. 1995. Age and survivorship of diapausing eggs in a sediment egg bank. *Ecology* 76:1706–1711.
16. Bailey SA, Duggan IC, van Overdijk CDA, Johengen TH, Reid DF, MacIsaac HJ. 2004. Salinity tolerance of diapausing eggs of freshwater zooplankton. *Freshw Biol* 49:286–295.
17. Gray DK, Bailey SA, Duggan IC, MacIsaac HJ. 2005. Viability of invertebrate diapausing eggs exposed to saltwater: Implications for Great Lakes' ship ballast management. *Biol Invasions* 7:531–539.
18. Persoone G, Janssen CR. 1993. Freshwater invertebrate toxicity tests. In Calow P, ed, *Handbook of Ecotoxicology,* Vol 1. Blackwell Scientific, Cambridge, MA, USA, pp 51–65.
19. Snell TW, Persoone G. 1989. Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatilis*. *Aquat Toxicol* 14:65–80.
20. Preston BL, Snell TW. 2001. Full life-cycle toxicity assessment using rotifer resting egg production: Implications for ecological risk assessment. *Environ Pollut* 114:399–406.
21. Sano LL, Moll RA, Krueger AM, Landrum PF. 2003. Assessing the potential efficacy of glutaraldehyde for biocide treatment of unballasted transoceanic vessels. *J Gt Lakes Res* 29:545–557.
22. Sano LL, Mapili MA, Krueger AM, Garcia E, Gossiaux D, Phillips K, Landrum PF. 2004. Comparative efficacy of potential chemical disinfectants for treating unballasted vessels. *J Gt Lakes Res* 30:201–216.
23. Culter SJ, Cutler HG, Glinski J, Wright D, Dawson R, Lauren D. 2004. SeaKleen, a potential product for controlling aquatic pests in ships' ballast water. *Proceedings*, Second International Ballast Water Treatment R&D Symposium, London, UK, July 21–23, 2001, pp 164–174.
24. Wright DA, Dawson R. 2003. SeaKleen®—A potential natural biocide for ballast water treatment. *Proceedings*, First International Ballast Water Treatment R&D Symposium, London, UK, March 26–27, 2001, pp 73–75.
25. Hoff FH, Snell TW. 2004. *Plankton Culture Manual*, 6th ed. Florida Aqua Farms, Dade City, FL, USA.
26. American Society for Testing and Materials. 2003. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E 729-96. In *Annual Book of ASTM Standards,* Section 11, Vol 11.05. Philadelphia, PA, pp 1–22.
27. SPSS. 2000. *Systat*, Ver 10. Chicago, IL, USA.
28. Kerfoot WC, Budd JW, Eadie BJ, Vanderploeg HA, Agy M. 2004. Winter storms: Sequential trap records record ephippial production, resuspension, and sediment interactions. *Limnol Oceanogr* 49:1365–1381.
29. Wallace RL, Snell TW. 2001. Phylum Rotifera. In Thorp JH, Covich AP, eds, *Ecology and Classification of North American Freshwater Invertebrates.* Academic, San Diego, CA, USA, pp 195–254.
30. Saint-Jean L, Pagano M. 1995. Egg mortality through predation in egg-carrying zooplankters, studies on *Heterobranchus longifilis* larvae fed on copepods, cladocerans, and rotifers. *J Plankton Res* 17:1501–1512.
31. Bailey SA. 2005. Sediments as a dispersal vector of aquatic invertebrates: An estimation of propagule pressure associated with 'no ballast on board' vessels. PhD thesis. University of Windsor, Windsor, ON, Canada.