

EFFECTS OF PROPOSED PHYSICAL BALLAST TANK TREATMENTS ON AQUATIC INVERTEBRATE RESTING EGGS

DAVID F. RAIKOW,*†‡ DAVID F. REID,†‡ ERNEST R. BLATCHLEY III,§ GREGORY JACOBS,|| and PETER F. LANDRUM‡

†National Oceanic and Atmospheric Administration (NOAA) National Center for Research on Aquatic Invasive Species, 2205 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA

‡NOAA Great Lakes Environmental Research Laboratory, 2205 Commonwealth Boulevard, Ann Arbor, Michigan 48105, USA

§Purdue University, School of Civil Engineering, 550 Stadium Mall Drive, West Lafayette, Indiana 47907, USA

||University of Michigan, School of Natural Resources and Environment, Dana Building, 440 Church Street, Ann Arbor, Michigan 48109, USA

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Abstract—Adaptations in aquatic invertebrate resting eggs that confer protection from natural catastrophic events also could confer protection from treatments applied to ballast water for biological invasion vector management. To evaluate the potential efficacy of physical ballast water treatment methods, the present study examined the acute toxicity of heat (flash and holding methods), ultraviolet (UV) radiation (254 nm), and deoxygenation (acute and chronic) on resting eggs of the freshwater cladoceran *Daphnia mendotae* and the marine brine shrimp *Artemia* sp. Both *D. mendotae* and *Artemia* sp. were similarly sensitive to flash exposures of heat (100% mortality at 70°C), but *D. mendotae* were much more sensitive to prolonged exposures. Exposure to 4,000 mJ/cm² of UV radiation resulted in mortality rates of 59% in *Artemia* sp. and 91% in *D. mendotae*. Deoxygenation to an oxygen concentration of 1 mg/L was maximally toxic to both species. Deoxygenation suppressed hatching of *D. mendotae* resting eggs at oxygen concentrations of less than 5.5 mg/L and of *Artemia* sp. resting eggs at concentrations of less than 1 mg/L. Results suggest that UV radiation and deoxygenation are not viable treatment methods with respect to invertebrate resting eggs because of the impracticality of producing sufficient UV doses and the suppression of hatching at low oxygen concentrations. Results also suggest that the treatment temperatures required to kill resting eggs are much higher than those reported to be effective against other invertebrate life stages and species. The results, however, do not preclude the effectiveness of these treatments against other organisms or life stages. Nevertheless, if ballast tank treatment systems employing the tested methods are intended to include mitigation of viable resting eggs, then physical removal of large resting eggs and ephippia via filtration would be a necessary initial step.

Keywords—Resting egg Biological invasion Heat Ultraviolet radiation Deoxygenation

INTRODUCTION

Continuing invasions of the North American Great Lakes by aquatic invasive species (AIS) are widely known to occur largely through the activities of international commerce, including the aquarium trade, the live-food trade, and the transfer and release of ballast water in large, transoceanic ships [1,2]. The ballast water vector is of particular concern in the Great Lakes, in part because of recent invertebrate invasions attributable to the ballast tank vector, including the zebra mussel (*Dreissena polymorpha*), the quagga mussel (*Dreissena bugensis*), and the predatory zooplankters *Cercopagis pengoi* and *Bythotrephes longimanus* [3,4]. In addition to being ecologically devastating, such invasions can produce negative effects on national economies. Zebra mussels, for example, can cost infested power plants more than \$3,000,000 annually [5], contributing to an estimated total annual U.S. cost from zebra mussels of \$1,000,000,000 [6]. Subsequently, the zebra mussel invasion of the Great Lakes also precipitated the Nonindigenous Aquatic Nuisance Prevention Species and Control Act, brought invasion biology to the attention of the U.S. public,

and continues to alter the ecology of the North American Great Lakes [3,4]. Hence, AIS are pertinent to U.S. federal policy concerning international commerce.

Recognition of the importance of the ballast water AIS vector prompted changes in regulations governing ship activity. Ships are now required to exchange ballast water before entering the St. Lawrence Seaway (Canada) or to provide a declaration of “no ballast on board” [7,8] (<http://globallast.imo.org/resolution.htm>, <http://www.access.gpo.gov/cgi-bin/cfrassemble.cgi?title=200433>). In addition, numerous ballast tank treatment technologies have been proposed to actively mitigate the ballast tank vector, and research is currently underway to develop ballast water monitoring and treatment technologies for implementation onboard ships. The National Research Council identified and ranked several possible treatment technologies, including the following: Filtration, biocides, and heat (promising); ozonation, electrification, and pulse plasma (possible but unsuitable for immediate application); ultraviolet (UV) irradiation, application of sound, application of magnetic fields, and deoxygenation (inappropriate because of unproven effectiveness); and biological control and antifouling coatings (too problematic) [1].

Treatment of water entering or already inside ballast tanks to mitigate biological invasions is difficult because of the potential presence of a wide variety of organisms [9]. Moreover, many species can be entrained in ballast tanks at different life-cycle stages or, potentially, produce different life stages en

* 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, Ohio 45268. This is NOAA Great Lakes Environmental Research Laboratory contribution 1407.

route. Invertebrate resting eggs, for example, can pose a particular challenge to ballast tank treatment technologies. Many aquatic invertebrates, including rotifers, cladocerans, copepods, and shrimp, produce a dormant stage in the form of resting eggs (also called diapausing cysts), which in nature typically settle out of the water column and become incorporated in sediments [10]. Morphological adaptations, such as thick egg walls or chorions, and secondary tissues, such as ephippia, allow the resting eggs of many species and, thus, populations to overwinter or survive adverse environmental conditions, including desiccation [11,12]. Therefore, resting eggs are of concern, because the adaptations that allow them to survive natural catastrophic events also might confer protection from ballast tank treatment technologies, especially when resting eggs are incorporated into residual sediment commonly found in ballast tanks declaring "no ballast on board" [12].

Lethal temperatures have been determined for a variety of organisms and nonresting juvenile stages relevant to ballast water, including bacteria, phytoplankton, macroalga (spores), mollusks (adults and larvae), and starfish (adults and larvae) [13,14]. Resting eggs also have been tested for sensitivity to heat, including those of the rotifer *Brachionus*, various species of the brine shrimp *Artemia*, and the dinoflagellates *Alexandrium catenella* and *Gymnodinium catenatum* [14–16]. Sensitivity of adult zooplankton (copepods and chaetognaths) during a 30-h onboard test of a ballast water heating system produced 100% mortality after water was heated to greater than 38°C [14]. Laboratory studies commonly employ holding methods, in which exposure time is long (minutes to days), as opposed to brief flash exposures (lasting only seconds). The use of holding methods reflects the exploitation of increased lethality with increased exposure time to create less costly treatment systems that limit the temperatures to which the water must be heated. This has led to the recommendation that heating to between 40 and 45°C may be a cost-effective method of ballast water treatment [14,15].

Ultraviolet radiation causes damage to nucleic acids and proteins, including accumulation of damage to DNA that prevents cellular replication [17]. Examinations of the effects of UV radiation on aquatic invertebrates include studies of increasing exposure to solar radiation as a result of reductions in atmospheric ozone and dissolved organic carbon concentrations [17]. Ultraviolet radiation is commonly used to disinfect water, surfaces, and air by impairing the ability of organisms to replicate or reproduce [18]. The goal of disinfection is to prevent infection in humans, which is analogous to the goal of preventing invasions in ecosystems; hence, impairment of an organism's reproductive ability could be useful in preventing invasions. Technologies incorporating UV radiation into ballast tank treatment systems often use UV radiation as a secondary treatment following filtration, hydrocyclonic removal of organisms and debris, and/or application of chemicals, thus preventing an evaluation of the effectiveness of UV radiation alone. Direct evaluation of UV radiation with respect to ballast tank treatment technologies commonly targets bacteria and phytoplankton, reflecting common applications for disinfecting drinking water [19]. Some tests have targeted aquatic invertebrates at juvenile nonresting life stages; for example, UV radiation at 254 nm delivered at a dose of 400 mJ/cm² has produced 100% mortality of *D. bugensis* veligers [20]. Molecular damage compounded by high rates of mitosis during

embryonic development could infer sensitivity to UV radiation in resting eggs.

Aquatic invertebrates can tolerate moderate hypoxia (O₂, <1–2 mg/L) temporarily through anaerobiosis (i.e., the use of anaerobic metabolic energy pathways), but prolonged exposure is toxic [21,22]. Artificially induced hypoxic conditions were effective in killing pelagic invertebrates [23]. Adult zooplankton and the pelagic larval stages of copepods (nauplii), barnacles (nauplii), *D. polymorpha* (veligers), polychaetes (larvae), shrimp (larvae), and crabs (zoea), for example, were reportedly killed within 3 d by deoxygenation induced through gas extraction by a vacuum [23]. The tubeworm *Ficopomatus enigmaticus* (larvae), the green crab *Carcinus maenas* (larvae), and the zebra mussel *D. polymorpha* (veligers) experienced significant mortality following exposure to hypoxic conditions after 2 or 3 d [24], whereas the starfish *Coscinasteris calamaria* (larvae) and macroalga *Undaria pinnatifida* (spores) experienced 99.9% mortality within 30 min [25], as induced through displacement of dissolved oxygen by purging with nitrogen. In contrast to pelagic larvae, copepod eggs have been shown to not hatch under hypoxic conditions lasting for several days and to hatch on subsequent exposure to normoxic conditions [26,27].

The objective of the present study was to characterize the sensitivity of zooplankton resting eggs to heat, UV radiation, and deoxygenation to evaluate the potential efficacy of proposed ballast tank treatment methods for AIS vector management. Study organisms included a freshwater cladoceran (*Daphnia mendotae*) and a marine brine shrimp (*Artemia* sp.). Heat was expected to be lethal to resting eggs, including greater sensitivity by *D. mendotae* compared to *Artemia* sp. Ultraviolet radiation was not expected to be lethal to resting eggs. Hypoxia was expected to suppress hatching but not kill eggs, which were expected to hatch when subsequently exposed to normoxic conditions.

MATERIALS AND METHODS

Test organisms and procedures

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 CaCO₃; conductivity, 913 µS/cm; pH 8.5). Artificial seawater was made using purified (nanopure) water and Instant Ocean® (Aquarium Systems, Mentor, OH, USA) and used for tests of marine organisms. *Daphnia mendotae* resting eggs were obtained through collection of lake sediment from Muskegon Lake (MI, USA). The sediment used to obtain *D. mendotae* resting eggs was the same sample used to test sensitivity to SeaKleen® (Garnett, Watkinsville, GA, USA) [28]. Sediment often is stored at 4°C for at least eight weeks before use in resting egg studies [12], but the sediment used in the present study was stored, and subsamples used, for up to 19 months. *Daphnia mendotae* resting eggs were extracted from bulk sediment by sieving ephippia using a 250-µm, stainless-steel sieve. A mix of grade A and grade B Great Salt Lake (UT, USA) *Artemia* sp. cysts was obtained from Florida Aqua Farms (Dade City, FL, USA). *Artemia* sp. cysts were sorted by placing them in a scintillation vial filled with artificial seawater, which was then shaken. Cysts were allowed to separate into those that settled and those that floated. Cysts that settled were used because of greater ease in the collection of eggs with a Pasteur pipette and deposition into Petri dishes containing artificial seawater. Individual cysts were then collected using a

Pasteur pipette under a dissecting microscope with illumination from below.

Standard bioassay protocols [29] were adapted for use with invertebrate resting eggs [28]. All bioassays of *Artemia* sp. and *D. mendotae* used 50 eggs per replicate and were run under a 16:8-h light:dark photoperiod. Heat and deoxygenation bioassays were run with five replicates per treatment level, and UV radiation bioassays were run with three replicates. *Daphnia mendotae* bioassays were run at 20°C and *Artemia* sp. bioassays at 27°C. Test chambers were evaluated once every 24 h, and emergent neonates were collected each day for 3 d in the case of *Artemia* sp. and for 7 d in the case of *D. mendotae*, except as noted in deoxygenation bioassays. Neonates were discarded. Mortality and effect rates were calculated by subtracting the number of stocked eggs by the number of successful hatches that occurred during the course of the entire postexposure period. Eggs that did not hatch by the end of the postexposure period were considered to be dead and were discarded. Dormancy was calculated by subtracting the number of stocked eggs by the number of successful hatches recorded at 24 h for heat and UV-radiation bioassays or, for deoxygenation bioassays, at the end of the exposure period if longer than 24 h. 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*) [28]. 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 [28]. *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 and normal in appearance but immobile, and those that were separated from the shell but abnormal in appearance and immobile [28,30].

Heat

To accomplish flash heat exposures, resting eggs were deposited into 25-ml glass beakers filled with 2 ml of room-temperature (20°C) water. Other beakers with 15 ml of water were then heated and maintained in a heat bath. Heated water (15 ml) within an individual heated beaker was added to an individual beaker containing eggs. Beakers with eggs and heated water were then immediately transferred to a cold bath (10°C) and quickly cooled to room temperature (20°C). On reaching room temperature, the beakers were removed from the cold bath and transferred to an environmental chamber. Hatching was then monitored as described above.

For holding heat exposures, resting eggs were distributed to 25-ml glass beakers filled with 2 ml of room-temperature water. Beakers were then placed in a heat bath set to 40, 45, or 50°C to bracket proposed holding temperature exposures for use in ballast water treatment. Following various exposure times, at intervals of 5, 10, or 20 min for up to 120 min, replicates of each treatment level were removed, placed in a cold bath and quickly cooled to room temperature, filled to 15 ml with room-temperature water, and placed in environmental chambers. Hatching was then monitored as described above. Both methods of heat exposure produced exposure times much shorter than the 24-h exposure periods used in other experiments.

UV radiation

Resting eggs were distributed to glass Petri dishes filled with water (depth, 1 cm). Replicate Petri dishes were placed

under a custom-built beam collimator (E. Blatchley, Purdue University, IN, USA). The collimator held UV bulbs above test subjects and directed the resulting light as a beam downward so that the light hit the test subjects at an angle perpendicular to the water surface (i.e., directly) and shielded investigators from direct exposure to UV radiation. The collimator held two low-pressure mercury-quartz bulbs (Enaqua, Vista, CA, USA). Incident light intensity at 254 nm (in the UV-C range) was measured with a 400A Radiometer (International Light, Newburyport, MA, USA). Incident intensity at a position 2 cm below the collimator stabilized at approximately 0.6 mW/cm² after 10 min, so the collimator was turned on and run for 10 min before measurement of light intensity to allow bulb output to stabilize for each experiment. Dosage levels were achieved by varying the time that test subjects were exposed to UV radiation under the collimator. Ultraviolet-radiation doses were quantified in units of energy (J). A dose of 1,000 mJ/cm², for example, required an exposure time of approximately 28 min. A correction for attenuation of light through 1 cm of artificial seawater was applied to dosage calculations for *Artemia* sp., because those resting eggs sank to the bottom of the dish, but was not applied to *D. mendotae*, because those resting eggs floated. Following exposure to UV radiation, Petri dishes were transferred to environmental chambers. Hatching was then monitored as described above. Methods of UV-radiation exposure produced exposure times much shorter than the 24-h exposure periods used in other experiments.

Deoxygenation

Deoxygenation was achieved by displacing atmospheric oxygen with nitrogen and sparging water with nitrogen. A microscope and necessary materials were placed within a medium-sized Atmosbag (an inflatable polyethylene chamber with built-in gloves; Aldrich, Milwaukee, WI, USA). The chamber was sealed and the air pumped out. Compressed, high-purity nitrogen was then released continuously into the chamber. Subsequent procedures were carried out within the chamber to sort resting eggs under low-oxygen conditions. Oxygen saturation of the atmosphere within the chamber and water prepared while in the chamber were monitored using a YSI 550A dissolved oxygen instrument (YSI Environmental, Yellow Springs, OH, USA) placed entirely in the chamber. On reaching the desired low-oxygen atmosphere, the oxygen saturation of water in an Erlenmeyer flask was measured. The water was then sparged with nitrogen, if necessary, to match the saturation of the atmosphere as closely as possible. Next, resting eggs were distributed to glass beakers filled with 15 ml of deoxygenated water. Beakers were then sealed in an airtight standard or large GasPak EZ container (Becton Dickinson and Company, Franklin Lanes, NJ, USA). The Atmosbag was then opened, and the GasPak was removed and placed in an environmental chamber. At the end of the exposure period, the GasPak was opened, the oxygen saturation of the water in the beakers immediately measured, and hatching recorded. Beakers were then returned to the incubator and exposed to ambient atmospheres for the remainder of the experiment. Hatching was subsequently monitored as described above. Controls were prepared while exposed to ambient atmospheres and placed in water with normal oxygen saturations (~95%).

Artemia sp. were exposed to deoxygenation for 24 h and *D. mendotae* for 72 h to compare between test species the effect of deoxygenation during the entire typical period of

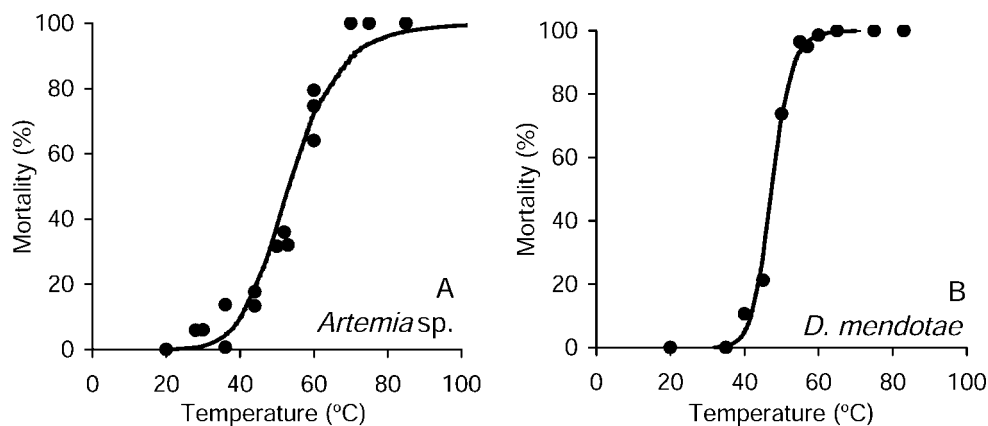


Fig. 1. Dose–response of aquatic invertebrate resting eggs to heat (flash exposure). (A) Marine brine shrimp (*Artemia* sp.). (B) Freshwater cladoceran (*Daphnia mendotae*). Curves are logistic regressions with 95% confidence intervals based on data pooled from multiple experiments.

embryonic development as determined under normal conditions. The effect of chronic exposure to deoxygenation was further evaluated with an experiment in which *Artemia* sp. were exposed to deoxygenation for 3, 6, and 10 d. For this experiment, three treatment groups were established at the same time under deoxygenation as described above and were opened sequentially. Oxygen levels and hatching were then recorded.

Data analysis

Data analysis consisted of computing the lethal temperature to 50% (LT₅₀) and 90% (LT₉₀) of organisms for flash heat bioassays, the lethal time at specified temperatures to 50% (LT_{i50}) and 90% (LT_{i90}) of organisms for holding heat bioassays, the lethal dose to 50% (LD₅₀) and 90% (LD₉₀) of organisms for UV-radiation bioassays, the lethal and effect concentrations to 50% (LC₅₀ and EC₅₀, respectively) and 90% (LC₉₀ and EC₉₀, respectively) of organisms for deoxygenation bioassays, and the 95% confidence intervals using logistic regression. Because of drifting of dissolved oxygen values during the deoxygenation experiments, the dissolved oxygen concentration in beakers at the end of the experiment was used to estimate lethal and effect concentrations to produce conservative estimates. Because of low hatching rates of test organisms in control treatments, Abbott's correction was applied to raw data before analysis [31].

RESULTS

Hatching rates varied between species. *Artemia* sp. hatched at a rate of 51 to 84% (mean, 64%; standard deviation, 15%). *Daphnia mendotae* hatched at a rate of 23 to 56% (mean, 42%; standard deviation, 14%).

Heat

The flash heat procedure exposed test organisms to treatment temperatures for no more than 3 to 5 s. Flash and holding heat exposures killed resting eggs of *Artemia* sp. and *D. mendotae* (Figs. 1 and 2). Sensitivity to heat differed between species (Table 1). *Daphnia mendotae* were more sensitive to flash heat exposures, displaying a LT₉₀ of $53.6 \pm 0.4^\circ\text{C}$, compared to *Artemia* sp., which displayed a LT₉₀ of $70.5 \pm 0.7^\circ\text{C}$. This estimation of the LT₉₀, however, is low, because 100% mortality was observed at 70°C and above in both species in the present study. Neither species displayed delayed hatching, nor were there appreciable numbers of immobile or malformed neonates.

In tests of holding heat exposures, water in beakers was quickly heated to within 5°C of the target temperature, but shorter-duration treatment levels may not have reached the target temperature by the end of the exposure period. Subsequently, target temperatures were expressed as ranges (i.e., 35–40, 40–45, and 45–50°C). Hatching of *Artemia* sp. was

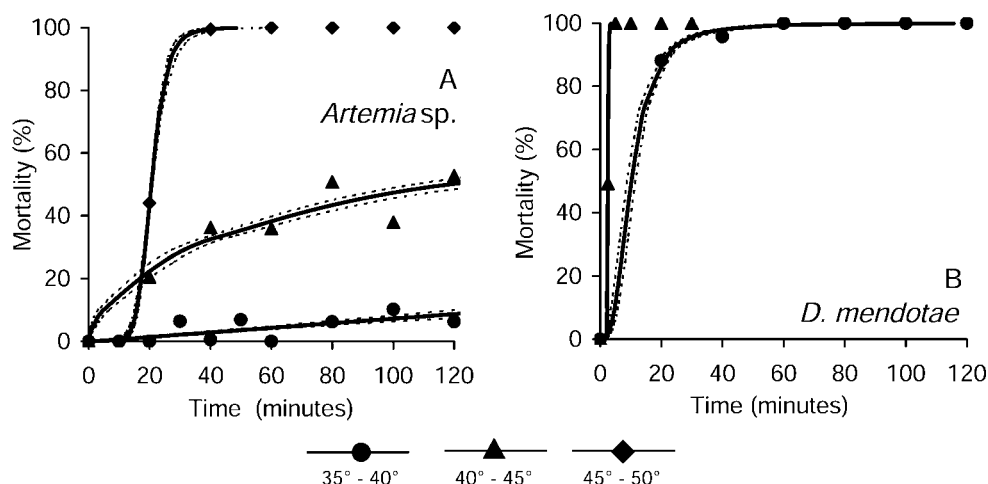


Fig. 2. Dose–response of aquatic invertebrate resting eggs to heat (holding exposure). (A) Marine brine shrimp (*Artemia* sp.). (B) Freshwater cladoceran (*Daphnia mendotae*). Curves are logistic regressions with 95% confidence intervals based on data pooled from multiple experiments.

Table 1. Lethal temperature, lethal time at specified temperatures, lethal dose, lethal concentration and effect concentration of heat, ultraviolet radiation, and deoxygenation during the resting life stage^a

Stressor	Unit	Organism	No. of experiments		
Heat, flash	Lethal temperature (°C)	<i>Daphnia mendotae</i> ^b <i>Artemia</i> sp. ^d	2 3	LT ₉₀	LT ₅₀
				53.6 (±0.4) ^c	47.3 (±0.2)
				70.5 (±0.7) ^c	53.2 (±0.3)
Heat, holding: 35–40°C Heat, holding: 40–45°C Heat, holding: 35–40°C Heat, holding: 40–45°C Heat, holding: 45–50°C	Lethal time heated (min)	<i>Daphnia mendotae</i> <i>Daphnia mendotae</i> <i>Artemia</i> sp. <i>Artemia</i> sp. <i>Artemia</i> sp.	1 1 1 1 1	LTi ₉₀	LTi ₅₀
				22.1 (±1.3)	10.2 (±1.3)
				2.8 ^e	2.5 ^e
				— ^f	— ^f
				2,917 (±1207)	118 (±14)
Ultraviolet radiation	Lethal dose (mJ/cm ²)	<i>Daphnia mendotae</i> <i>Artemia</i> sp.	2 2	LD ₉₀	LD ₅₀
				9,500 (±1,500)	1,000 (±50)
				11,700 (±3,800)	3,900 (±20)
Deoxygenation	Lethal concentration (mg O ₂ /L)	<i>Daphnia mendotae</i> <i>Artemia</i> sp.	2 3	LC ₉₀	LC ₅₀
				0.93(±0.03)	1.5(±0.02)
				— ^f	1.3 (±0.04)
Deoxygenation	Effect concentration (mg O ₂ /L) ^g	<i>Daphnia mendotae</i> <i>Artemia</i> sp.	2 3	EC ₉₀	EC ₅₀
				— ^f	— ^f
				0.96 (±0.03)	1.8 (±0.03)

^a Values in parentheses are the 90% confidence intervals. LT₉₀ = lethal temperature to 90% of organisms; LT₅₀ = lethal temperature to 50% of organisms; LTi₉₀ = lethal time heated at a specified temperature to 90% of organisms; LTi₅₀ = lethal time heated at a specified temperature to 50% of organisms; LD₉₀ = lethal dose to 90% of organisms; LD₅₀ = lethal dose to 50% of organisms; LC₉₀ = lethal concentration to 90% of organisms; LC₅₀ = lethal concentration to 50% of organisms; EC₉₀ = 90% effect concentration; EC₅₀ = 50% effect concentration.

^b Freshwater, incubated at 20°C.

^c 100% mortality observed at 70°C and above.

^d Marine, incubated at 27°C.

^e 95% confidence interval not calculable.

^f Insufficient or poorly fitting data for logistic regression.

^g Effect = dormancy.

not appreciably affected by exposure to water at 35 to 40°C for up to 120 min (Fig. 2A). The insensitivity of *Artemia* sp. at the 35 to 40°C temperature range precluded reliable calculation of lethal times; hence, estimates are not reported. *Artemia* sp. displayed increasing mortality with increasing time at the 40 to 45°C temperature range, but mortality did not exceed 55% at 120 min. At the 40 to 45°C temperature range, *Artemia* sp. displayed a LTi₉₀ of 2,917 ± 1,207 min (48.6 ± 20.1 h), but truncation of the logistic curve made lethal temperature estimations less robust. At the 45 to 50°C temperature range, *Artemia* sp. displayed a LTi₉₀ of 27.0 ± 1.4 min, with 99.5% mortality at 40 min and 100% mortality at 60 min. *Daphnia mendotae* were more sensitive to holding exposures to heat, displaying a LTi₉₀ of 22.1 ± 1.3 min, with

97.7% mortality at 40 min and 100% mortality at 60 min at the 35 to 40°C temperature range (Fig. 2B). At the 40 to 45°C temperature range, *D. mendotae* displayed a LTi₉₀ of 2.8 min, with 100% mortality at 5 min.

UV radiation

Ultraviolet radiation killed representatives of both species (Fig. 3). Sensitivity differed between species (Table 1). *Daphnia mendotae* were more sensitive to UV radiation, displaying a LD₉₀ of 9,500 ± 1,500 mJ/cm², compared to *Artemia* sp., which displayed a LD₉₀ of 11,700 ± 1,400 mJ/cm², as estimated by logistic regression using truncated curves for both species. Toxicity did not exceed 59% in *Artemia* sp. and 91% in *D. mendotae* at 4,000 mJ/cm² (Fig. 3).

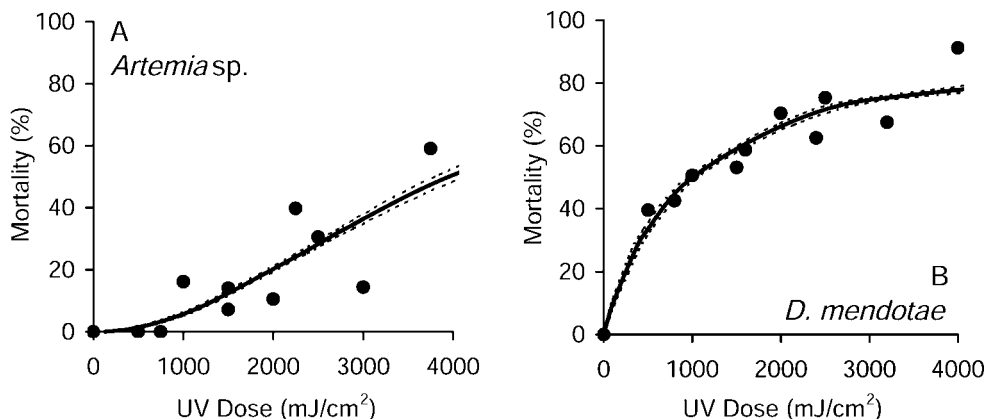


Fig. 3. Dose–response of aquatic invertebrate resting eggs to ultraviolet (UV) radiation (254 nm). (A) Marine brine shrimp (*Artemia* sp.). (B) Freshwater cladoceran (*Daphnia mendotae*). Curves are logistic regressions with 95% confidence intervals based on data pooled from multiple experiments.

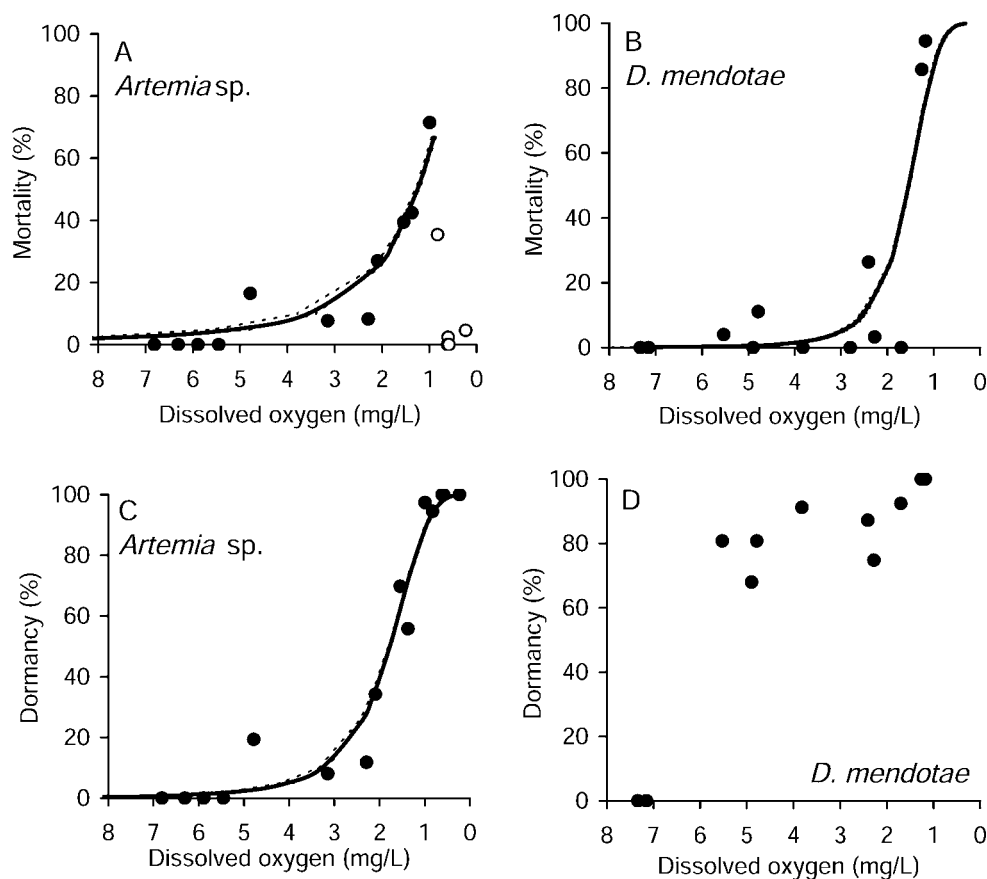


Fig. 4. Dose–response of aquatic invertebrate resting eggs to deoxygenation. (A) Marine brine shrimp (*Artemia* sp.). Open circles are excluded from analysis because of maintenance of dormancy. (B) Freshwater cladoceran (*Daphnia mendotae*). (C) *Artemia* sp. effect response (dormancy) to deoxygenation. (D) *Daphnia mendotae* effect response (dormancy) to deoxygenation. Curves are logistic regressions with 95% confidence intervals based on data pooled from multiple experiments.

Deoxygenation

Deoxygenation killed representatives of both species (Fig. 4A and B). Delayed hatching (dormancy) was observed in both species (Fig. 4C and D) and, using *Artemia* sp., subsequently studied further. *Daphnia mendotae* were more sensitive to deoxygenation, displaying a LC90 of 0.93 ± 0.03 mg/L (O_2 , 11.3%) (Table 1). Because mortality did not exceed 72% at 1 mg/L (O_2 , 14.2%), only a LC50 of 1.3 ± 0.04 mg/L (O_2 , 18.5%) could be calculated for *Artemia* sp. Tests of *Artemia* sp. contained treatment levels that were maintained at oxygen concentrations of less than 1 mg/L. Mortality of *Artemia* sp. increased with decreasing oxygen concentrations until 1 mg/L, whereupon mortality rates decreased. Based on rates of dormancy, mortality data at concentrations of less than 1 mg/L were excluded from the estimation of lethal concentrations. Although the tests run with *D. mendotae* were established with treatments at concentrations of oxygen less than 1 mg/L, oxygen concentrations migrated a bit to levels slightly greater than 1 mg/L.

Daphnia mendotae displayed dormancy patterns that differed from those of *Artemia* sp. The effect rate of dormancy in *Artemia* sp. followed a typical response curve (Fig. 4C), reaching 100% dormancy by 0.5 mg/L (O_2 , 7.1%). In contrast, *D. mendotae* began to display prolonged dormancy at higher oxygen concentrations compared to *Artemia* sp., and this pattern did not follow a logistic response curve (Fig. 4D). Moreover, mortality rates were low for dormant *D. mendotae* resting

eggs until oxygen concentrations approached 1.0 mg/L (O_2 , 12.2%), whereupon mortality rates increased dramatically.

Observed temporal patterns of hatching showed that in control treatments, the majority of neonates hatched within 24 h for *Artemia* sp. and within 72 h for *D. mendotae* [28]. Dormancy (effect) was thus defined as the proportion of eggs not hatched at 24 h for *Artemia* sp. and at 72 h for *D. mendotae*. Because mortality was defined as the proportion of neonates not hatched after 72 h for *Artemia* sp. and after 168 h for *D. mendotae*, eggs not hatched at earlier times in response to deoxygenation had enough time to hatch and be counted after subsequent exposure to normoxia following the exposure period. Dormancy of *Artemia* sp. increased with decreasing oxygen concentrations, displaying a 90% effect dose of 0.96 ± 0.03 mg/L (O_2 , 13.7%). Treatments at or below 0.61 mg/L (O_2 , 8.7%) displayed 100% dormancy and no more than 5% mortality. *Daphnia mendotae* displayed rates of dormancy greater than 68% at less than 5.5 mg/L (O_2 , 66.9%), and data did not fit a logistic regression.

Oxygen levels migrated during the course of the chronic deoxygenation experiment. All treatments began with an initial oxygen level of less than 0.15 mg/L (O_2 , 2.1% O_2) (Fig. 5A). Oxygen levels increased during the course of the experiment, with the 10-d treatment reaching an average oxygen concentration of 1.22 mg/L (O_2 , 17.4%). Temporal hatching patterns observed in the control treatment were typical of control treatments in all experiments, in which most *Artemia* sp. hatching

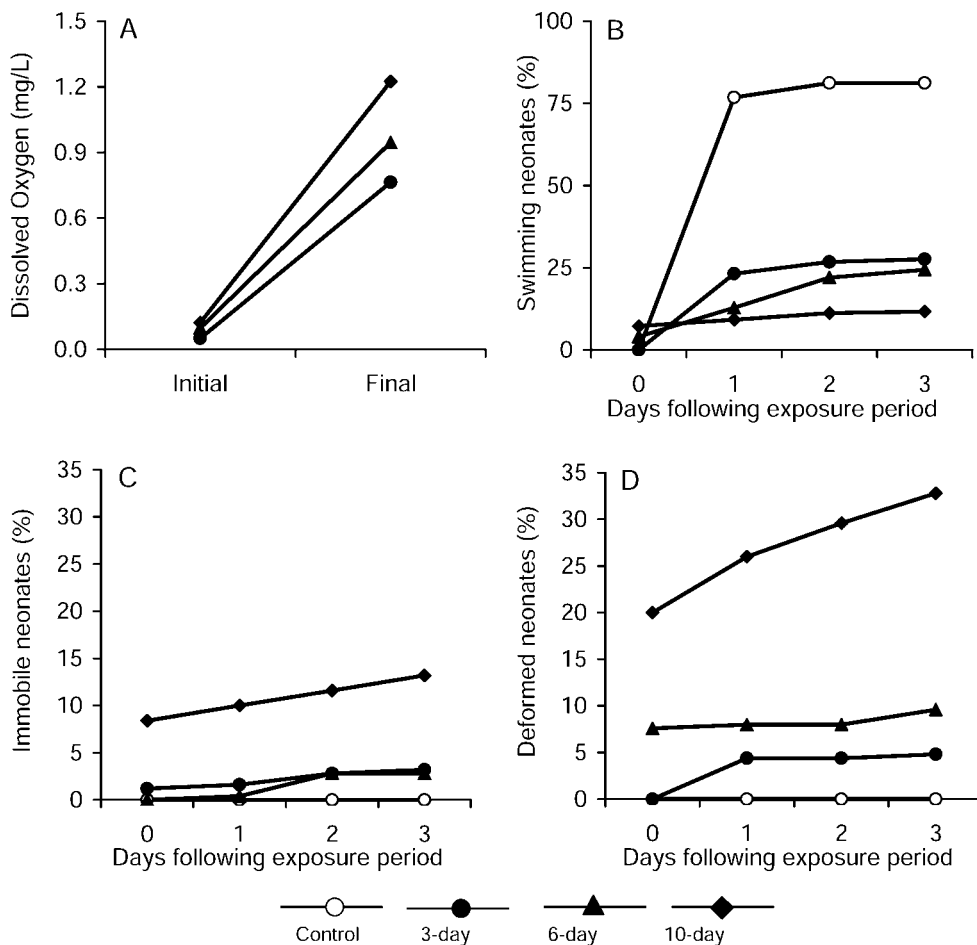


Fig. 5. Response of aquatic invertebrate resting eggs to chronic deoxygenation exposure. (A) Drift of dissolved oxygen concentrations during exposure period. (B) Occurrence of hatching following exposure period. (C) Occurrence of immobile *Artemia* sp. neonates following exposure period. (D) Occurrence of abnormally developed *Artemia* sp. neonates following exposure period.

occurred within 24 h (Fig. 5B). The 3-d exposure to low oxygen concentration also displayed the typical temporal pattern, but only reached a hatching rate of 28%, or one-third the hatching rate of the control. The 10-d exposure to low oxygen concentration deviated from typical hatching patterns. Swimming neonates were observed in the 6- and 10-d treatments immediately following the exposure period, indicating that hatching occurred under deoxygenation. Immobile neonates were observed with increasing frequency at longer exposure times (Fig. 5C), including after exposure to normal atmospheres, indicating improper embryonic development caused by deoxygenation during the exposure period (Fig. 5C). Further evidence of improper embryonic development under deoxygenation was observed, with an increasing frequency of deformed neonates with increasing exposure time (Fig. 5D).

DISCUSSION

Heat, UV radiation, and deoxygenation were toxic to freshwater and marine aquatic invertebrate resting eggs. Toxicity varied greatly between stressors, however, as a result of both morphological and biochemical adaptations to stress. These results demonstrate the relatively high resistance of aquatic invertebrate resting life stages to potential ballast tank treatment methods.

Both holding and flash heat exposures revealed differential heat tolerance of resting eggs between test species. Greater sensitivity to heat exposure by *D. mendotae* was not surprising

given optimum culturing temperatures for *Daphnia magna*, another freshwater cladoceran, of 18 to 22°C [30]. Moreover, the maximum average surface temperature of Lake Michigan (USA), into which the location of egg collection (Muskegon Lake, MI, USA) drains, has not exceeded 25°C in the last five years (the time period from which data are available; <http://coastwatch.glerl.noaa.gov/>). Hence, increased sensitivity to heat by *D. mendotae* is likely the result of adaptation to colder habitats than those to which *Artemia* sp. has adapted. Tolerance of heat (at 40° for comparison) during prolonged holding exposures by *D. mendotae* resting eggs was greater than tolerances reported for *C. calamaria* (starfish larvae), *U. pinnatifida* (macroalga spores), and *G. catenatum* (dinoflagellate cysts) and similar to tolerances reported for *A. catenella* (dinoflagellate cysts) and *Crassostrea gigas* (oyster larvae) [13–16].

Greater observed tolerance of *Artemia* sp. to heat was consistent with known optimal culturing temperatures of 26 to 31°C for Great Salt Lake *Artemia* sp. cysts, which is not the highest optimal temperature range when compared to globally distributed *Artemia* sp. populations [32]. In addition, *Artemia* sp. cysts are more tolerant of heat compared with *Artemia* sp. neonates [33]. Heat tolerance in *Artemia* sp. cysts can be attributed to the production of heat shock proteins, which repair thermal damage to cells, allowing acclimation to higher temperatures [34]. Moreover, greater sensitivity to heat by *Artemia* sp. collected from cold, high-altitude lakes, among other glob-

ally distributed populations, as well as evolution of heat tolerance within introduced populations demonstrate the ability of *Artemia* sp. to adapt a wide range of temperatures [35]. Tolerance of heat by *Artemia* sp. resting eggs was much greater than tolerances for the species reported above [13–16].

Ultraviolet radiation was toxic to resting eggs only at high doses. Response curves were truncated, but the results clearly indicated that high toxicity rates would only occur at much higher doses. Although we hypothesized that resting eggs might be sensitive to UV radiation as a result of cellular division during embryonic development, these results were not surprising. Both *Artemia* sp. and *D. mendotae* possess tissues that shield embryos from UV radiation (the chorion and the ephippium, respectively). Decapsulated (having the egg “shell” or chorion removed) *Artemia* sp. cysts, for example, have been shown to display 100% mortality at 910 mJ/cm², whereas intact cysts display approximately 0% mortality [36]. Observed responses of intact *Artemia* sp. cysts in the present study, in which 0% mortality at 750 mJ/cm² and 16% mortality at 1,000 mJ/cm² were observed, were in agreement with previously reported results [36].

Prolonged dormancy under deoxygenation and increasing rates of abnormally developed neonates with increasing chronic exposure time to deoxygenation suggest that midlevel oxygen concentrations terminate diapause, allowing development to commence, which then proceeds improperly, causing death. This interpretation also is supported by the increasing rate of immobile neonates hatched under normoxia following increasingly long chronic exposures to hypoxia. Oxygen concentration has been reported to be a cue for termination of diapause in the copepod *Diaptomus stagnalis*, and other copepod eggs maintain dormancy at extremely low oxygen levels (O₂, <0.03 mg/L) [10,26]. Results of the present study suggest that oxygen concentration is a cue for diapause termination in both *D. mendotae* and *Artemia* sp.

Implications for ballast tank treatment

Greater tolerance to heat by *Artemia* sp. creates difficulties for heat-based ballast tank treatment methods. Based on the results of the present study, a flash heat exposure at 65°C would kill 100% of *D. mendotae* resting eggs and could reasonably be expected to be lethal to all *Daphnia* cysts, if not also to the resting stages of all cladocerans and copepods, because of similar morphology, and to all rotifers, because of smaller size. In contrast, a flash heat exposure of 70°C or higher would be required to kill 100% of *Artemia* sp. resting eggs. A holding heat regimen of 40 to 45°C for less than 5 min would be sufficient to kill resting eggs of *D. mendotae* and, by extension, the resting eggs of similar organisms. Such a regimen would not, however, be sufficient to kill *Artemia* sp. resting eggs. Instead, the holding heat exposure would have to be raised to between 45 and 50°C for 45 to 60 min. This interpretation is consistent with other tests of heat regimens applied to Great Salt Lake *Artemia* sp. resting eggs, in which 100% mortality occurred at 50°C when applied for 60 min [33]. A regimen of 50° for 60 min, however, was not 100% lethal to San Francisco Bay (CA, USA) *Artemia* sp. resting eggs introduced into Vietnamese ponds as well as to other *Artemia* sp., with the exception of *Artemia* sp. obtained from a Tibetan Lake [35]. Given that *Artemia* sp., including American strains, have become invasive [37], the results of the present study suggest that proposed ballast water heating regimens of 40 to 45°C [14,15], which are insufficient to kill marine bacteria [14], may

be inadequate to kill all planktonic life stages potentially entrained in ballast water.

Biological weighting functions, or the relative importance of wavelengths to the induction of detrimental effects, have been shown to increase with decreasing wavelength across ranges meant to represent the potential for harm caused by solar radiation, typically 280 to 320 nm (UV-B range). Thus, it is reasonable to expect that application of radiation in the UV-C range (e.g., 254 nm), as studied here, affects at least some organisms potentially present in ballast water. In addition, because the presence of UV-A and visible light supports amelioration of UV-induced molecular damage through photoenzymatic repair [38], the lack of such radiation inside ballast tanks would likely enhance the effects of UV radiation.

Ultraviolet radiation was not, however, found to be an effective biocidal treatment against invertebrate resting eggs, because inadequate toxicity was observed at extremely high doses, suggesting that adequate mortality could be achieved at doses that would be prohibitively expensive to induce onboard ships. Granted, the reproductive capacity of test organisms could have been compromised, and sterilization would be as effective as death in AIS vector management by preventing introduced eggs from becoming reproducing populations despite maturation. Hence, should UV radiation be seriously considered as a ballast tank treatment method, effects on reproductive capacity of zooplankton should be studied.

Because hypoxia may suppress embryonic development, the greatest lethal effect of deoxygenation applied to ballast tanks would be achieved at oxygen levels near 1.0 mg/L. *Artemia* sp., however, displayed greater tolerance to deoxygenation, only reaching approximately 71% mortality at 1.0 mg/L. The pattern of resting egg mortality response to deoxygenation is similar to that observed for salinity, where resting eggs exposed to high salinity remained dormant and those exposed to midlevel salinity hatched and died [39].

The results of the present study indicate that UV radiation and deoxygenation are inadequate ballast tank treatment methods with respect to invertebrate resting eggs. Heat is highly effective, but the required temperatures and exposure times could be too costly. These methods, however, may still be useful in the overall treatment of ballast water when coupled with other methods. Thus, a first step of physical removal by filtration may be the best option to deal with resting eggs. Hydrocyclonic separation of material may not be able to separate resting eggs because of low density compared to mineral grains. In addition to preventing ephippia and large eggs from entering tanks, filtration also could remove sediment, preventing the accumulation of residuals that are known to protect resting eggs and other life stages from treatment methods [28]. Small or loose eggs separated from ephippia that pass through the physical separation step can be expected to be more sensitive to subsequent treatments. A second step of deoxygenation could suppress diapause termination, allowing resting eggs to survive transport. A second step of UV radiation may not be lethal, although sterilization is theoretically possible. A second step of heat could be highly effective if sufficient temperatures and exposure durations are achieved but may be too costly to implement. Thus, a two-step process of physical separation followed by chemical application would appear to be the most viable option for ballast tank treatment with respect to resting eggs, but only if residual sediment is removed before application.

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