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BIODIVERSITY RESEARCH

Does saltwater flushing reduce viability of diapausing eggs in ship ballast sediment?

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ABSTRACT

Flushing of ballast tanks with seawater has been proposed to reduce the risk of invasion associated with residual ballast in 'no ballast on board' ships. The efficacy of this procedure, however, has not been determined. Using diapausing eggs isolated from ballast sediments — as well as from Lake Erie sediment — this study investigated the impact of salinity (0, 8 and 35‰) and temperature (10, 20 and 30 °C) on the cumulative abundance and species richness of hatched zooplankton taxa. The rate and amount of hatching varied dramatically between sediments and across salinity–temperature regimes. Although exposure to saline water inhibited emergence of freshwater taxa during the exposure phase of all trials, mixed results were evident after diapausing eggs were returned to freshwater. The efficacy of salinity as a ballast treatment method was temperature dependent, although the direction of the effect was case-specific. Exposure of eggs to saline water was less effective at 10 and 30 °C than at 20 °C. Although flushing ballast tanks with open ocean water is expected to significantly reduce the number of active invertebrates living in residual ballast water (a potentially larger source of invaders), our results indicate that the most effective treatment conditions for reduction of diapausing egg viability is 8‰ salinity at 20 °C.

Keywords

Ballast water exchange, biological invasions, freshwater invertebrates, NOBOB, resting stages, zooplankton.

INTRODUCTION

Dormant stages of aquatic invertebrates have reportedly evolved as a mechanism for both dispersal and survival, allowing populations to endure conditions intolerable for active life stages. Documented from 67 invertebrate taxa, dormant stages have been reported to withstand factors such as extreme temperature, anoxia, desiccation, and passage through animal digestive tracts (see Cáceres, 1997). Recent work has also demonstrated that many freshwater taxa can survive exposure to saltwater as diapausing eggs when buried in sediments (Gray *et al.*, 2005). In an earlier study, however, Bailey *et al.* (2004) indicated that diapausing eggs of the cladoceran *Daphnia longiremis* Sars and the rotifer *Brachionus calyciflorus* Pallas could be rendered non-viable by exposure to saltwater. Discrepancies in these findings may result from different experimental methodologies, including exposure regimes conducted at different temperatures, as temperature is known to have a species-specific interactive effect on salinity tolerance through changes to metabolic rate (Lee & Bell, 1999).

The effect of temperature and salinity on hatch rate or on viability of diapausing eggs has been investigated only briefly, with a few studies conducting incubation trials at various salinities or temperatures independently (May, 1987; Bailey *et al.*, 2004; Gray *et al.*, 2005; Vandekerkhove *et al.*, 2005). As ballast water exchange (BWE) with saline water has been proposed as a tool for the prevention of freshwater biological invasions via transoceanic shipping, the topic of salinity tolerance and temperature effects must be investigated more thoroughly.

The invasion of numerous species in the Great Lakes and their resultant economic and environmental problems resulted in the enactment of Canadian voluntary BWE procedures for the Great Lakes in 1989. Similar procedures effectively requiring ships to exchange freshwater ballast with open ocean water, taken in waters at least 2000 m deep and 200 nautical miles from shore, were later implemented by the US Coast Guard in 1993. Accordingly, up-bound ships in the St. Lawrence Seaway are only permitted to discharge ballast water in the system if it has a salinity of at least 30‰ (US Coast Guard, 1993). Despite this regulation, reports of new biological invasions via ballast water in the Great Lakes continue (Holeck *et al.*, 2004). This may be due, in part, to the large number of vessels transiting the system that are not subject to the current legislation.

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Table 1 Description of experimental sediments. Place collected refers to ship sampling location, except for the Lake Erie site. Egg density lists sample mean ± standard error for 40 g sediment

Abbreviation	Type	Date collected	Place collected	Pore water salinity (‰)	Egg density	% organic carbon	% oil content
A	Ballast	09/06/03	Toronto Harbour	35	170 ± 4	12.4	0.03
B	Ballast	10/24/02	Burns Harbor	2	188 ± 7	n/a	n/a
C	Ballast	11/19/02	Cleveland Harbor	21	154 ± 11	12.2	0.06
D	Ballast	08/13/02	Windsor Harbour	8	133 ± 13	22.7	2.73
E	Natural	12/04/02	Western Lake Erie	0	269 ± 27	3.7	0.03

According to Colautti *et al.* (2003), the majority (> 90%) of transoceanic vessels operating on the Great Lakes during 1994–2000 declared 'no ballast on board' (NOBOB) status. NOBOB vessels may be an underlying factor contributing to the apparent increase in ballast-mediated invaders because they are not currently subjected to BWE regulations. Although ballast tanks of NOBOB vessels are typically considered empty, they carry residual ballast water and sediment that may harbour a diverse assemblage of live invertebrates and their resting stages (approximately 2.0×10^7 live invertebrates and 9.8×10^6 resting stages per ship, respectively; Bailey *et al.*, 2005a; Duggan *et al.*, 2005). Although live animals in residual ballast are currently considered the largest threat for invasion, the risk posed by resting stages is non-zero (Bailey *et al.*, 2005a,b; Duggan *et al.*, 2005). Since parthenogenetic invertebrates hatched from diapausing eggs are expected to reproduce quickly and to be in better health than are active individuals having just endured transoceanic transport, preventative measures to curtail future invasions via NOBOB vessels should address both active and diapausing life stages.

Flushing of NOBOB tanks with seawater has been proposed as a means to reduce the risk of invasion associated with NOBOB vessels, in the same manner as required for BWE of ballasted vessels but using smaller volumes of water (Locke *et al.*, 1991; Duggan *et al.*, 2005). While this procedure will likely reduce the abundance and viability of active life stages inhabiting residual ballast water and the sediment–water interface, its efficacy has not been tested. Considering the importance of these studies for management of aquatic invasions, an investigation of the impact of various salinities (0, 8 and 35‰) and temperatures (10, 20 and 30 °C) on the hatching success of diapausing eggs of zooplankton was warranted. Using diapausing eggs isolated from both ballast and Lake Erie sediments, we test the hypothesis that the efficacy of saline water as a method to reduce egg viability (hatching abundance) varies in relation to the temperature experienced during exposure.

METHODS

Sediment collection

Sediments were collected from ballast tanks of four transoceanic ships that declared NOBOB status while transiting the Great Lakes. Sediments were collected as ships offloaded cargo on the Great Lakes, as shown in Table 1. Details of collection procedures are described in Bailey *et al.* (2003). Ballast sediments were brought back to the laboratory and stored in the dark at 4 °C until the onset of experimentation. As ballast sediments may have been exposed to varying salinity regimes before experimentation, sediment was also collected from Lake Erie to ensure that at least one trial would be conducted with eggs previously exposed solely to freshwater. Lake Erie sediment was collected using a ponar grab and was also stored in the dark at 4 °C. Sediments were stored for several months to give diapausing eggs a refractory period prior to experimentation (Schwartz & Hebert, 1987). Diapausing egg density, pore water salinity and organic carbon and oil/grease content were characterized for each sediment. Mean egg density was enumerated from four to eleven 40 g replicates after separation of eggs from sediment using a sugar flotation protocol (described below). Analysis of variance (ANOVA) was used to determine if egg densities of the five sediments differed significantly. Pore water salinity was measured with an optical refractometer after separation from a small quantity of sediment by centrifugation (ca. 10 g at $3300 \times g$ for 15 min). Organic carbon content was measured using loss-on-ignition methods, whereas oil/grease content was measured from dichloromethane–hexane extractable residues (K. Drouillard, Great Lakes Institute for Environmental Research, Windsor, Ontario).

Separation of diapausing eggs from sediments

Diapausing eggs of invertebrates were separated from sediments by sugar flotation (see Bailey *et al.*, 2003). This involved sieving 40 g well-mixed sediment through 45 μm mesh and transferring the retained matter into 50 mL plastic centrifuge tubes with concentrated sucrose solution for centrifugation at $27 \times g$ for 5 min. The supernatant, containing diapausing eggs and additional organic matter, was subsequently decanted onto clean 45 μm mesh and washed thoroughly using sterile pond water to remove sucrose solution. The supernatant of each 40 g replicate was randomly assigned to one of nine treatment regimes for the first 10 days of the experiment (using all possible combinations of 0, 8 and 35‰ at 10, 20 and 30 °C, respectively). Artificial pond water was used for 0‰ trials (recipe given in Hebert & Crease, 1980); whereas seawater collected from transoceanic ships entering the Great Lakes was filtered (using 2.5 μm Whatman filter

paper, Whatman Inc., Florham Park, NJ, USA) and diluted (using sterile artificial pond water) for 8 and 35‰ trials. Experimental dishes were immediately incubated in environmental chambers under 16: 8 h light:dark at 10, 20 or 30 °C. Each dish was examined under a dissection microscope at regular intervals (typically every 24 h). During observation, all hatched individuals were removed by pipette and identified using standard taxonomic keys. Quadruplicate samples were run for the 0‰, 20 °C treatment for all experiments, whereas triplicate samples were run for all other salinity–temperature regimes.

In order to determine if saltwater exposure impacted viability of diapausing eggs, or simply restricted hatching under unfavourable conditions, we replaced all hatching media with artificial pond water (0‰) after an exposure period of 10 days. This was carried out by carefully washing each replicates' contents with pond water over clean 45 μm mesh and transferring all contents into new experimental dishes containing 40 mL fresh pond water. These dishes were immediately put back into an environmental chamber at 20 °C with a 16: 8 h light:dark cycle to encourage maximum hatching abundance. As in the initial 10 days of the experiment, each replicate was checked regularly and all hatched organisms were removed and identified. The 10-day exposure period is considered typical for the length of time an operational vessel could provide for ballast treatment during a transoceanic voyage (Gray *et al.*, 2005).

Statistical analysis

Three-way multivariate analysis of variance (MANOVA) was used to discern the effect of temperature, salinity and sediment type on the abundance and species richness of hatched taxa for all trials (SYSTAT 8.0, SPSS Inc., Chicago, IL, USA). Both dependent variables were standardized for differences in initial egg density prior to analysis. Subsequently, as the effect of temperature and salinity was dependent on sediment type, two-way MANOVAS were conducted to explore the effect of salinity and temperature on abundance and richness data for each sediment trial. The experiment-wise error rate was adjusted for multiple tests using the Dunn–Šidák method, to give a significance level of $P = 0.01$ (Sokal & Rohlf, 1995).

We recognize that the experimental design outlined above, although relevant biologically, makes statistical analysis and interpretation difficult by introducing a confounding effect of temperature change at day 10 for the trials initially incubated at 10 and 30 °C. Therefore, we conducted a second, separate analysis using only data for trials run exclusively at 20 °C. This one-way MANOVA allowed us to remove the confounding effect of temperature for a subset of the trials conducted at different salinities.

For all analyses, data collected for taxa incapable of completing their life cycle in freshwater were excluded, as these species pose little risk of invasion to the Great Lakes. Analyses were conducted separately on cumulative abundance and richness values recorded through days 10 (exposure period) and 20 (complete trial). Finally, both total abundance and species richness were log- or square-root-transformed to improve normality before analysis.

RESULTS

Experimental sediments varied in egg density, pore water salinity and in organic carbon and oil/grease content (Table 1). The density of diapausing eggs was statistically different between sediments (ANOVA, $F_{4,28} = 16.080$, $P < 0.001$), with the Lake Erie sediment containing more eggs than did each ballast sediment (Bonferroni post hoc test, $P < 0.001$). Diapausing egg density tended to increase with the organic carbon content of the sediment (Table 1); egg density did not appear to be related to either pore water salinity or oil/grease content, although the sediment with the highest grease content did exhibit the lowest egg density.

Species hatched during these experiments belonged to three taxa: Rotifera, Cladocera and Copepoda (see Appendices S1–S5 in Supplementary Material). The abundance of all individuals hatched was not correlated with initial egg density, nor with pore water salinity, organic carbon or oil/grease content of the sediment (Pearson's correlation, $P > 0.05$). Brachionid rotifers were typically the most abundant taxon, although the cladoceran *Moina micrura* Kurz dominated ballast sediment D. The majority of species hatched were freshwater taxa, with the highest diversity recorded from the Lake Erie sediment (54 distinct species). The lowest number of species emerged from ballast sediment D (8 species), with remaining ballast sediments A, B and C containing 25, 35 and 19 species, respectively. A number of the freshwater taxa could tolerate brackish water, as indicated by their ability to fully develop and hatch in 8‰ water during the exposure period. These species included *Brachionus budapestinensis* Daday, *Brachionus calyciflorus*, *Hexarthra mira* (Hudson), *Daphnia ambigua* Scourfield and copepod nauplii (see Appendices S1–S5). The only marine species to emerge was the rotifer *Synchaeta baltica* Ehrenberg, although several euryhaline species also hatched (*Brachionus urceolaris* O.F. Müller, *Synchaeta kitina* Rousselet, *Synchaeta pectinata* Ehrenberg, *Synchaeta stylata* Wierzejski and copepod nauplii; Appendices S1–S5).

The rate and cumulative number of individuals hatched varied dramatically between sediments and across salinity–temperature regimes. Exposure to saline water during the initial 10 day exposure period inhibited emergence from diapausing eggs. The effect of salinity on both abundance and species richness at day 10 was significant for all five sediments (Figs 1 & 2; Table 2). During this time, hatching occurred predominantly in the 0‰ trials, irrespective of temperature. Temperature did appear, however, to influence the rate of emergence in the 0‰ treatment, with the slowest hatch rate typically occurring at 10 °C (Fig. 3). Although the 20 °C and 30 °C treatments appeared to have similar hatch rates, the cumulative number of individuals hatched was generally lower at 30 °C (Fig. 1). The effect of temperature on abundance and species richness, however, was not statistically significant for all sediments (Table 2).

The incidence of hatching continued after samples were transferred to 0‰, 20 °C at day 10, most notably for those treatments that were transferred from brackish or saltwater media (Fig. 3). Mixed results were observed at the termination of these experiments, however, as salinity exposure significantly affected abundance and species richness in only three of five trials at day 20 (Table 3).

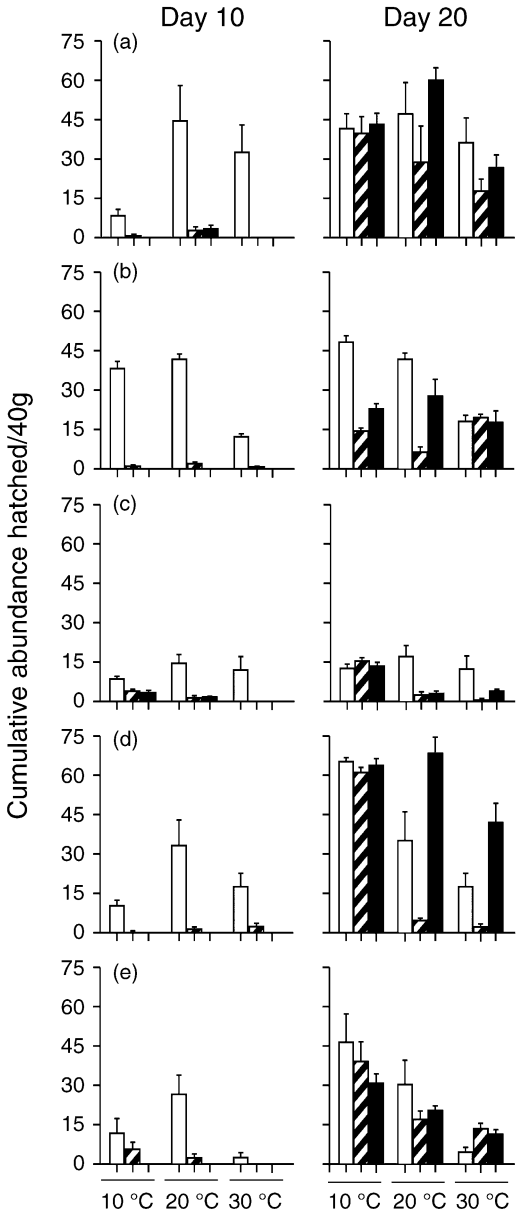

Figure 1 Mean (+ SE) cumulative abundance of individuals hatched under different salinity and temperature treatments, by sediment, at days 10 (left panel) and 20 (right panel). Salinity treatments are 0‰ (open bars), 8‰ (striped bars) and 35‰ (solid bars).

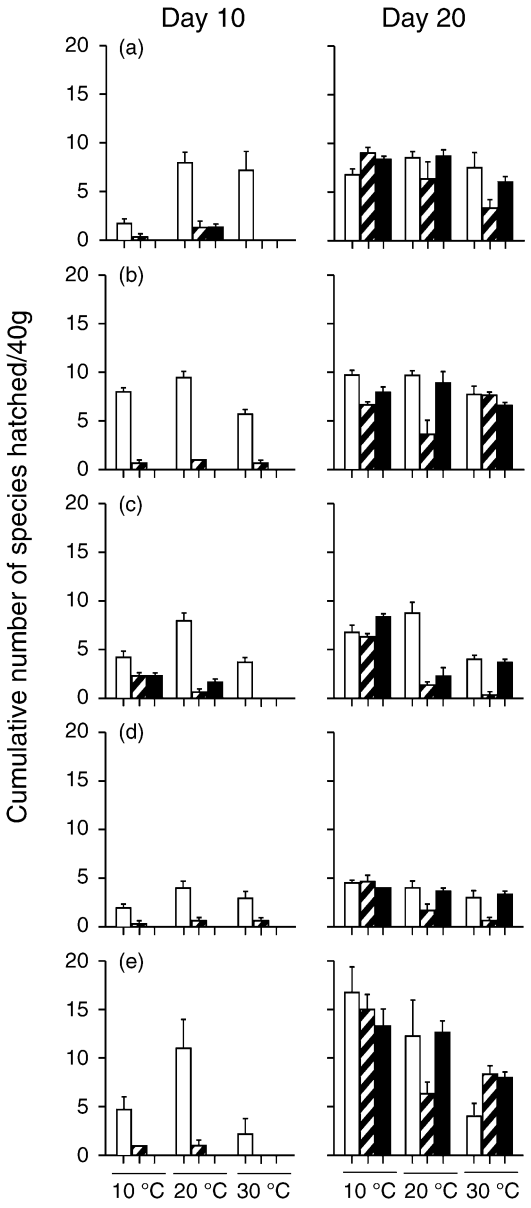

Figure 2 Mean (+ SE) cumulative number of species hatched under different salinity and temperature treatments, by sediment, at days 10 (left panel) and 20 (right panel). Salinity treatments are 0‰ (open bars), 8‰ (striped bars) and 35‰ (solid bars).

For these three sediments (B, C, D), cumulative abundance and species richness tended to decrease following exposure to saline water, particularly in replicates previously exposed to the 8‰ medium (Figs 1 & 2). Generalizations as to the effect of salinity are difficult, however, owing to interactive effects between salinity and temperature for sediments A through D, although enhanced hatch rates were observed for eggs previously exposed to 35‰ in two trials (Fig. 1b,d). Analysis of trials conducted only at 20 °C provided no additional clarification on the effect of salinity when confounding temperature effects were removed (Table 4).

DISCUSSION

The inter- and intraspecific variation in hatch rates observed here is not surprising. Pre-incubation conditions, maternal effects and intraspecific genetic variation are all known to influence rates of diapause termination in response to abiotic cues (see review by Gyllström & Hansson, 2004). The ability of a wide variety of taxa to withstand exposure to saline water as unprotected diapausing eggs, however, is unexpected and has potentially important implications for long-term population dynamics in habitats periodically inundated with saline water (see Bailey *et al.*, 2004).

Table 2 Results of two-way MANOVA assessing the effect of salinity and temperature on the cumulative abundance and species richness of freshwater taxa hatched through day 10, by sediment. Experiment-wise error rate adjusted for multiple tests using Dunn–Šidák method (**P = 0.001, *P = 0.01, ^{ns} = not significant)

	Sediment				
Variable	A	B	C	D	E
Salinity					
Univariate *F*-tests (d.f.)					
Total abundance (2,21)	33.075**	517.333**	66.108**	87.661**	20.668**
Species richness (2,21)	44.696**	453.020**	118.669**	48.389**	23.099**
Multivariate test					
Wilks' lambda	0.190**	0.016**	0.081**	0.105**	0.225**
Temperature					
Univariate *F*-tests (d.f.)					
Total abundance (2,21)	5.789*	14.120**	5.940*	2.578^{ns}	6.349*
Species richness (2,21)	10.328**	6.154*	19.656**	1.857^{ns}	4.158^{ns}
Multivariate test					
Wilks' lambda	0.428*	0.408**	0.323**	0.786^{ns}	0.507*
Interaction					
Univariate *F*-tests (d.f.)					
Total abundance (4,21)	0.824^{ns}	6.916**	1.779^{ns}	1.328^{ns}	2.815^{ns}
Species richness (4,21)	3.386^{ns}	3.684^{ns}	5.097*	0.918^{ns}	2.477^{ns}
Multivariate test					
Wilks' lambda	0.441^{ns}	0.367*	0.325*	0.753^{ns}	0.520^{ns}

Table 3 Results of two-way MANOVA assessing the effect of salinity and temperature on the cumulative abundance and species richness of freshwater taxa hatched through day 20, by sediment. Experiment-wise error rate adjusted for multiple tests using Dunn–Šidák method (**P = 0.001, *P = 0.01, ^{ns} = not significant)

	Sediment				
Variable	A	B	C	D	E
Salinity					
Univariate *F*-tests (d.f.)					
Total abundance (2,21)	2.075^{ns}	33.007**	18.029**	36.514**	0.499^{ns}
Species richness (2,21)	2.251^{ns}	11.423**	46.556**	9.126**	0.469^{ns}
Multivariate test					
Wilks' lambda	0.814^{ns}	0.238**	0.149**	0.194**	0.704^{ns}
Temperature					
Univariate *F*-tests (d.f.)					
Total abundance (2,21)	2.019^{ns}	4.061^{ns}	15.424**	33.995**	20.686**
Species richness (2,21)	5.508^{ns}	1.124^{ns}	39.232**	11.723**	11.111**
Multivariate test					
Wilks' lambda	0.550^{ns}	0.696^{ns}	0.200**	0.232**	0.275**
Interaction					
Univariate *F*-tests (d.f.)					
Total abundance (4,21)	0.667^{ns}	12.693**	5.868*	8.606**	2.910^{ns}
Species richness (4,21)	2.621^{ns}	5.439*	14.278**	3.432^{ns}	2.549^{ns}
Multivariate test					
Wilks' lambda	0.324*	0.246**	0.169**	0.350*	0.452^{ns}

While a significant reduction in hatch rate was observed for eggs incubated in brackish and saline water, the effect was not permanent. Once returned to favourable (freshwater) incubation conditions, the effect of salinity appeared to be dependent on temperature, with the direction of the effect being case-specific. Exposure of eggs to saltwater (both 8 and 35‰) generally did not reduce egg viability at 10 °C, as three of the five trials appeared to show no effect of salinity at this temperature. This may be the

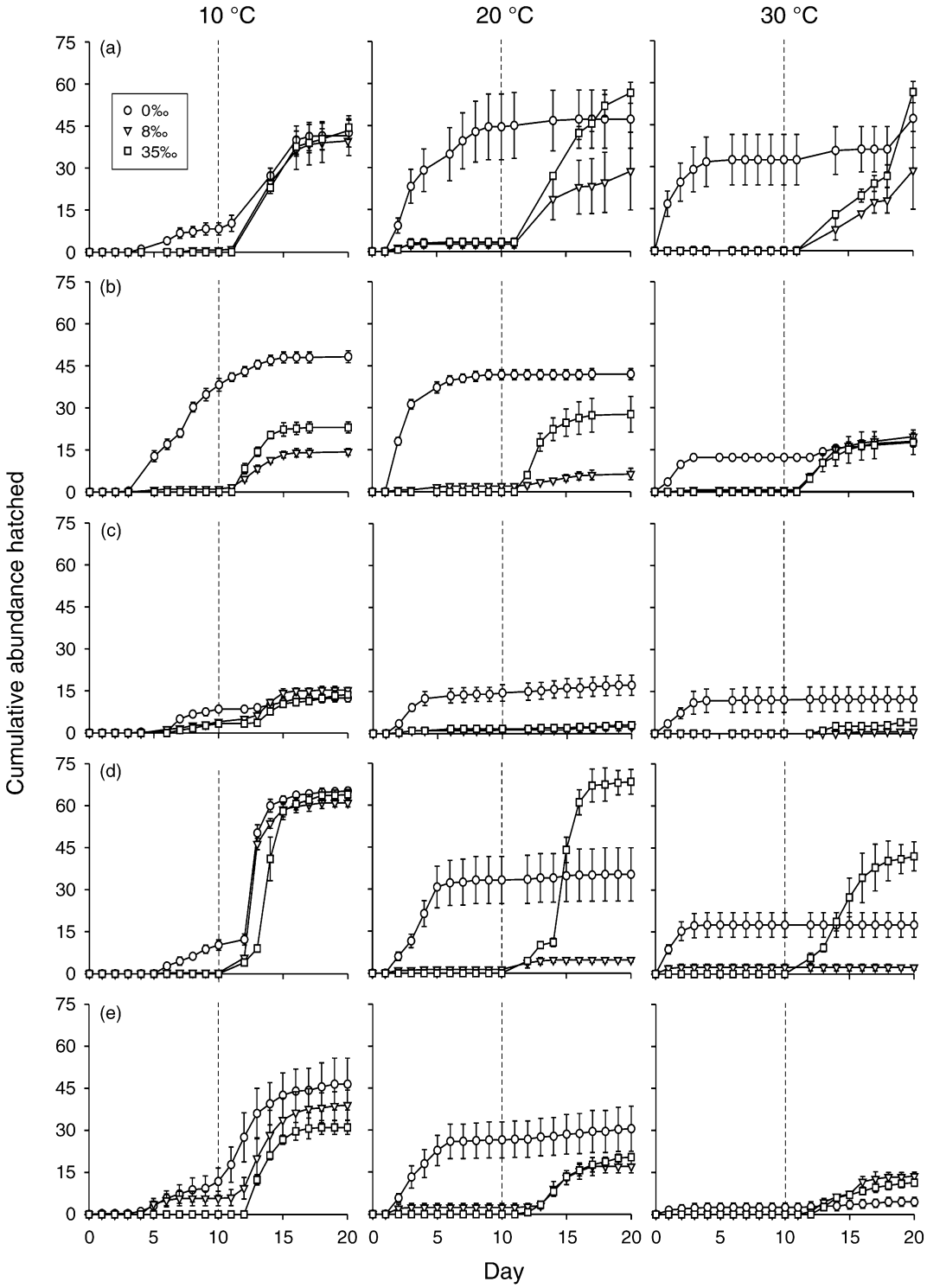

Figure 3 Mean (± SE) cumulative abundance of individuals hatched under salinity and temperature treatments, by sediment (a–e). After 10 days (dashed vertical line) all unhatched eggs in each treatment group were transferred to 0‰ media at 20 °C.

result of a lowered metabolic rate with low temperature, which in turn would reduce the amount of interaction developing eggs would have with their surrounding environment. Treatment of eggs at 30 °C also appears less than favourable, as one trial showed no effect of salinity, whereas two additional trials indicated that the combination of high salinity (35‰) and high temperature may actually promote more eggs to hatch than would 'ideal' conditions of zero salinity exposure. This finding may occur if high salinity prevents diapause termination in resting eggs, essentially isolating the freshwater species' eggs from

Table 4 Results of one-way MANOVA assessing the effect of salinity on the cumulative abundance and species richness of freshwater taxa hatched in the 20 °C trials through day 20, by sediment. Experiment-wise error rate adjusted for multiple tests using Dunn–Šidák method (**$P = 0.001$, *$P = 0.01$, ^{ns} = not significant)

	Sediment				
Variable	A	B	C	D	E
Salinity					
Univariate *F*-tests (d.f.)					
Total abundance (2,7)	1.402^{ns}	24.596**	11.005*	23.388**	0.426^{ns}
Species richness (2,7)	1.462^{ns}	8.643^{ns}	29.502**	4.763^{ns}	1.401^{ns}
Multivariate test					
Wilks' lambda	0.554^{ns}	0.062**	0.104*	0.101*	0.322^{ns}

the saline environment. In contrast, eggs not exposed to saline water can initiate development, but as 30 °C appears to be above the tolerable temperature limits of most species, eggs are rendered non-viable before full emergence. This trend may have implications for any ballast treatment method looking to combine chemical and heat treatments. In particular, it may be feasible to use waste heat from the ship's main engine to heat ballast water as it is loaded to 20 or 30 °C (see Rigby *et al.*, 1999).

Ultimately, it appears that the most effective temperature for reduction of egg viability associated with saltwater exposure is 20 °C, although the results are less than ideal. Exposure to 8‰ water at this temperature resulted in a significant reduction in the cumulative number of resting eggs hatched in all five cases; however, the same result was true for only three of the five trials conducted at 35‰. In the other two trials, the number of individuals hatched after exposure to 35‰ water at 20 °C was actually greater or equal to that of no salinity exposure. Just as a transfer to 15–20 °C after storage of eggs at low temperature (e.g. 4 °C) can stimulate hatching (e.g. Yurista, 1997), these results indicate that the return of eggs to 0‰ water after exposure to 35‰ may act as a cue to terminate diapause and initiate development for hatching. Our results support earlier work indicating that 8‰ water is the most effective salinity for reduction of viability of diapausing eggs of freshwater species (Bailey *et al.*, 2004). Our results, however, indicate that the efficacy of saltwater treatment will be dependent on water temperature and will certainly not render 100% of eggs non-viable. Furthermore, exposure to salinity had no effect on hatching of eggs isolated from Lake Erie sediments, which, ironically, were the only ones that presumably lacked a recent history of exposure to saline water.

Although sediments comprise one-fifth of residual ballast by volume, the number of eggs buried in sediments is typically 1200 times greater than that in residual waters (S.A. Bailey and C.D.A. van Overdijk, University of Windsor, unpublished data). Previous studies examining chemical disinfectants for ballast treatment indicated that the presence of sediments can reduce biocide efficacy (Sano *et al.*, 2003, 2004). Thus, the results observed in this study should be considered as a 'best case' scenario, with the expectation that efficacy may decline under operational conditions. This effect would be largely offset, however, by the reduction in hatch rates for sediment-bound eggs as opposed to isolated ones (Bailey *et al.*, 2005b).

Flushing of tanks of NOBOB vessels with saline water (> 30‰) should be an effective management tool for freshwater taxa living in residual ballast water owing to osmotic stress associated with saltwater exposure for individuals remaining in the tanks. Furthermore, active animals residing in the residual water or sediment–water interface will have a greater chance for discharge from tanks with flushing than would active or dormant taxa buried in sediments (see Duggan *et al.*, 2005). Flushing of tanks may also affect viability of resting eggs in sediment, although the effect will vary according to water salinity and temperature and the species-specific complement of resting eggs in ballast sediments.

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SUPPLEMENTARY MATERIAL

The following Supplementary Material is available from www.blackwell-synergy.com/loi/ddi

Appendix S1 List of species emerged from diapausing eggs isolated from sediment A

Appendix S2 List of species emerged from diapausing eggs isolated from sediment B

Appendix S3 List of species emerged from diapausing eggs isolated from sediment C

Appendix S4 List of species emerged from diapausing eggs isolated from sediment D

Appendix S5 List of species emerged from diapausing eggs isolated from sediment E