Diapausing zooplankton eggs remain viable despite exposure to open-ocean ballast water exchange: evidence from in situ exposure experiments

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Abstract: To reduce the transfer of nonindigenous species, regulations require transoceanic ships to exchange ballast with ocean water before discharging into the Great Lakes. Although ballast water exchange (BWE) is effective for live freshwater animals, laboratory experiments provide mixed results with regards to its impact on diapausing zooplankton eggs. We conducted an in situ test of the effectiveness of BWE for treating diapausing eggs in ballast sediments. Incubation chambers containing ballast sediment were placed in ballast tanks of cargo vessels transiting from North America to Europe. Each vessel had paired ballast tanks, one of which remained filled with Great Lakes water (control), while the second was exchanged with mid-ocean water. Laboratory viability tests were then conducted to compare viability of eggs recovered from sediments placed in both treatments, as well as identical sediments that remained at the laboratory in cold storage. No significant differences in egg viability were detected between treatments, but more species hatched from sediment that remained in cold storage. Results indicate that physical conditions in ballast tanks may affect egg viability, but saltwater exposure does not eliminate the risk of species introductions via diapausing eggs. Strategies that minimize sediment accumulation in ballast tanks can reduce the risk of species introductions via diapausing eggs.

Résumé : Afin de réduire les transferts d'espèces non indigènes, la règlementation exige que les navires transocéaniques remplacent leur eau de ballastage avec de l'eau de mer avant de vider leurs ballasts dans les Grands Lacs. Alors que le remplacement de l'eau de ballastage (BWE) est efficace dans le cas des animaux d'eau douce vivants, les expériences de laboratoire fournissent des résultats ambigus sur son impact sur les œufs de diapause du zooplancton. Nous avons procédé à des tests in situ de l'efficacité du BWE pour le traitement des œufs en diapause dans les sédiments des ballasts. Nous avons placé des chambres d'incubation contenant des sédiments provenant des ballasts dans les réservoirs de ballastage de cargos qui transitent d'Amérique du Nord vers l'Europe. Chaque navire possédait une paire de réservoirs, l'un qui restait rempli d'eau des Grands Lacs (témoin) et l'autre dont l'eau était remplacée par de l'eau du milieu de l'océan. Des tests de viabilité en laboratoire ont permis de comparer la viabilité des œufs récupérés dans les sédiments dans chacune des conditions expérimentales, ainsi que celle d'œufs provenant de sédiments identiques gardés en laboratoire en entreposage frigorifique. Aucune différence significative n'a pu être décelée entre les divers traitements, bien qu'un plus grand nombre d'espèces ait éclos des sédiments gardés en entreposage frigorifique. Nos résultats indiquent que les conditions physiques dans les ballasts peuvent affecter la viabilité des œufs, mais que l'exposition à l'eau salée n'élimine pas le risque d'introductions au moyen d'œufs en diapause. Les stratégies qui réduisent au minimum l'accumulation de sédiments dans les ballasts peuvent diminuer le risque d'introductions d'espèces au moyen des œufs en diapause.

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Introduction

The global movement of ballast water by cargo vessels has allowed nonindigenous species (NIS) to establish in many coastal and freshwater ecosystems (Mills et al. 1993; Ruiz et al. 2000; Gollasch 2006). Samples of water and sediment collected from ballast tanks of cargo vessels revealed live zooplankton and benthic invertebrates, as well as a diversity of viable diapausing zooplankton eggs (Bailey et al. 2005*a*; Duggan et al. 2006; Drake and Lodge 2007). Diapausing eggs present in ballast sediments could pose an invasion risk if they are discharged during ballast operations or if they hatch during the course of a voyage and are introduced when the vessel subsequently deballasts (Bailey et al. 2005*b*, 2007).

To decrease the risk of ballast-mediated invasions to the North American Great Lakes, voluntary (1989) and then mandatory (1993) ballast water exchange (BWE) regulations were enacted for ships entering the system carrying fresh or brackish ballast (US Coast Guard 1993). These regulations

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effectively require inbound transoceanic vessels to perform BWE while on the open ocean if their ballast is to be discharged while operating on the Great Lakes (Locke et al. 1993; US Coast Guard 1993). The goal of performing BWE is to flush species out of ballast tanks and kill retained ones through exposure to incoming high salinity seawater (Locke et al. 1993).

Despite the implementation of BWE regulations, new invaders have continued to be reported in the Great Lakes (Ricciardi 2006). This has prompted investigators to consider the role of "empty" ballast tanks that contain unpumpable residual water and sediments. Until recently, ballast tanks containing only residual water and sediment were exempt from ballast exchange regulations. However, live zooplankton, zoobenthos, and diapausing invertebrate eggs have been identified in ballast residuals (Bailey et al. 2005*a*; Duggan et al. 2005). Animals present in residual water and sediments could be released into the Great Lakes if a vessel took on ballast while offloading cargo and then subsequently discharged it to load cargo before leaving the system (Duggan et al. 2005). Canadian regulations extended BWE requirements in 2006 to vessels with unpumpable ballast tank residuals (Government of Canada 2006), while complementary regulations brought into effect in 2008 by the Saint Lawrence Seaway Development Corporation applied the same rules to US-bound vessels.

A recent study by Gray et al. (2007) demonstrated that open-ocean BWE could be highly effective for removing live freshwater zooplankton from ballast tanks. Moreover, in situ hatching experiments suggest that BWE decreases the risk of NIS introductions via diapausing eggs by causing mortality of previously hatched animals (Gray et al. 2007). However, it is unlikely that BWE completely eliminates the risk of NIS introductions through diapausing eggs for the following three reasons. First, laboratory studies suggest that diapausing eggs are resistant to short-term saltwater exposure (Bailey et al. 2004, 2006; Gray et al. 2005). Second, accumulations of eggs in residual sediments suggest that they are not easily flushed from ballast tanks (Bailey et al. 2003, 2005*a*). Third, although BWE may eliminate individuals that hatch during the course of a transoceanic voyage, multiport operations in freshwater systems such as the Great Lakes may provide opportunities for in situ hatching and reproduction before ballast is discharged (Bailey et al. 2005*b*).

Although laboratory studies have been conducted to simulate BWE, it is difficult to replicate the conditions experienced in ballast tanks during a transoceanic voyage. As a result, the impact of BWE on diapausing eggs has not been tested under field conditions in which physical variables such as temperature and oxygen vary throughout the course of a voyage (Gray et al. 2007). Recent laboratory studies have suggested that physical variables such as temperature may interact with salinity to determine post-BWE egg viability (Bailey et al. 2006). Therefore, field studies that incorporate realistic variation in physical variables are necessary to gain a true measure of the influence of BWE on diapausing eggs. Furthermore, laboratory experiments have made it difficult to determine if ambient conditions in ballast tanks, independent of BWE, have any affect on diapausing egg viability. If inhospitable conditions in ballast tanks lower diapausing egg viability, this could presumably decrease the risk of species invasions through this vector.

To provide a realistic test of the influence of open-ocean BWE on diapausing egg viability, we conducted controlled exposure experiments aboard six transoceanic vessels traveling between North America and Europe. Our experiments were designed to evaluate the impact of BWE on diapausing eggs, as well as to determine if ambient conditions in ballast tanks have any discernable impact on egg viability.

Materials and methods

This study is a companion to Gray et al. (2007), which assessed the impact of BWE on live zooplankton and zoobenthos. Experiments for Gray et al. (2007) and the current study were conducted aboard six vessels traveling from the Great Lakes to European ports of call between October 2004 and September 2006 (Table 1). Paired ballast tanks were utilized on each voyage, one of which was designated as a control that remained filled with Great Lakes' water, while the other was exchanged with saltwater at mid-ocean. The exchange tank was randomly selected at the outset of the study and underwent BWE during the transatlantic voyage. Prior to ship departure, Great Lakes' water from the port-of-origin was added to fill each tank. BWE was conducted >200 nautical miles from shore in water >2000 m in depth utilizing the sequential (empty–refill) method of BWE. Voyages ranged from 13 to 17 days depending on the travel distance, weather conditions, and port delays.

In situ exposure

Incubation chambers designed to hold ballast sediment containing diapausing eggs were constructed out of PVC piping components (Fig. 1; see Bailey et al. 2005*b*; Gray et al. 2007). The chambers were constructed using 15 cm (inside diameter) pipe caps with threaded, sealable lids. Each chamber was bolted to a rectangular PVC platform and boltholes were sealed with silicone. Three chambers were attached to each rectangular platform (Fig. 1). To allow for the exchange of water between the inside of the chamber and the ballast tank, 12 holes of 2.5–4.0 cm diameter were drilled through the lid (four holes) and halfway up the wall (eight holes) of each chamber. Nitex mesh (60 μm) was affixed to the exterior surface of each chamber body and interior surface of each top to completely cover all holes and was secured with PVC cement and 18 cm diameter hose clamps. To eliminate glue residues, chambers were submersed in water for seven days in the laboratory before use.

Ballast sediments used for experimentation were collected from six transoceanic ships operating on the Great Lakes between 2003 and 2005 and were stored in a cold room at 4 °C prior to use. For each of our six shipboard experiments, we selected only one of these six sediments (i.e., sediments collected from separate ships were not homogenized together). Prior to running shipboard experiments, the sediment that we selected was homogenized, and 300 g aliquots were distributed into sterile sample containers. Sample containers were then randomly assigned to one of the three treatments: (*i*) exposure to saltwater during BWE in the exchanged ballast tank; (*ii*) exposure only to fresh water in the control ballast tank (not exchanged); or (*iii*) remaining at the

Table 1. Information on vessels used for this study, including departure and destination ports, vessel type, dates of voyage, and date of ballast water exchange (BWE).

Vessel	Departure port	Destination port	Vessel type	Date of voyage	Date of BWE
1	Hamilton, Ontario	Cartagena, Spain	BC	1 Oct. 2004 – 18 Oct. 2004	13 Oct. 2004
2	Hamilton, Ontario	Hamburg, Germany	CT	23 July 2005 – 9 Aug. 2005	2 Aug. 2005
3	Montreal, Quebec	Rotterdam, Holland	BC	29 Sept. 2005 – 11 Oct. 2005	5 Oct. 2005
4	Hamilton, Ontario	Hamburg, Germany	CT	5 Dec. 2005 – 20 Dec. 2005	12 Dec. 2005
5	Hamilton, Ontario	Hamburg, Germany	CT	25 Apr. 2006 – 9 May 2006	3 May 2006
6	Hamilton, Ontario	Reyðarfjörður, Iceland	BC	1 Sept. 2006 – 14 Sept. 2006	7 Sept. 2006

Note: Vessel type: BC, bulk carrier; CT, chemical tanker. Table is modified from Gray et al. (2007).

Fig. 1. Sediment is placed into polyvinyl chloride (PVC) incubation chambers moored to the bottom of a ballast tank.

laboratory in cold storage at 4 °C. Four experiments (numbered 1, 4, 5, and 6 in Table 1) used four replicates for each treatment. Extra incubator chambers were available for experiments 2 and 3; therefore, the number of replicates for each treatment was increased to five, requiring fifteen 300 g aliquots of sediment.

The density of diapausing eggs in the ballast sediment used for our experiments was doubled to increase the probability of hatching for experiments described in Gray et al. (2007). For each 300 g aliquot of sediment used in a chamber, a 300 g sample from the same sediment had been subjected to a sugar flotation procedure, which isolates but does not harm eggs (Onbe 1978; Bailey et al. 2005*b*). The eggs extracted by sugar flotation were then added to the 300 g aliquot to be used in the incubation chambers (Gray et al. 2007). This artificial increase in egg density serves no purpose for the current study; therefore, we scaled back our hatching data by half to reflect this supplementation. However, it is important to note that sugar flotation may be less than 100% effective at isolating organic matter from sediments. For example, Onbe (1978) calculated a 90% extraction rate for marine cladocerans and copepods using this procedure. As a result, our estimates for the total number of hatched individuals are probably conservative.

The diversity and abundance of diapausing eggs present in the sugar flotation supplemented sediments was characterized prior to starting experiments using a Ludox HS40 protocol (Burgess 2001). The Ludox extraction procedure developed by Burgess (2001) operates by exploiting differences in density between sediments (composed mostly of high-density minerals) and benthic meiofauna. The density of the Ludox solution used for extractions is intermediate between those of the sediments and meiofauna, causing animals to float to the surface after a series of steps involving fluidization and centrifugation of the Ludox–sediment solution (for details, see Burgess 2001). Eggs were isolated from three replicate 40 g aliquots of sediment and were then enumerated under a stereomicroscope at ~32× magnification (Table 2).

Plates with three incubator chambers each were strapped to the bottom of both exchange and control ballast tanks before vessels disembarked for their transatlantic voyage. Incubation chambers were installed in upper-wing ballast tanks in all vessels, except for vessel 3, where they were placed in double-bottom tanks. Prior to filling the ships' ballast tanks with water, 300 g of ballast sediment (as described above) was transferred into each incubation chamber. After sediment had been added to all of the incubation chambers, the tops were screwed on and the ballast tanks were flooded with Great Lakes' water. Nitex mesh affixed to the incubation chambers allowed the sediment inside to be exposed to saltwater during ballast water exchange (the exchanged tank) or to remain in fresh water (the control tank). Diapausing eggs in exchanged ballast tanks were exposed to saltwater between 5 and 8 (average = 6.5) days before the vessels reached their European destination (Table 1). At the end of the voyage in Europe, sediment was collected from the incubation chambers using sterile scoops and spatulas and was shipped back to the laboratory on ice for use in viability experiments.

Laboratory viability experiments

Viability experiments were conducted following the methodology presented in Gray et al. (2005) and were designed to assess the viability of the eggs under conditions similar to those in the Great Lakes. Sediments collected from incubation chambers were stored in the laboratory in cold storage for at least two weeks prior to beginning the viability experiments to provide a refractory period for the eggs (Jo and Marcus 2004). Viability trials for the three treatments (exchanged, control, and left at laboratory) were conducted concurrently to ensure that any differences detected among treatments were not due to variability in the length of the refractory period.

Sediment collected from each incubation chamber was thoroughly mixed using a sterile spatula, and a 40 g subsample was placed in a 500 mL glass vessel. Synthetic pond water (150 mL; Hebert and Crease 1980) was then added to the vessel, and the vessel was agitated gently by hand for

Table 2. Mean diapause egg density per 40 g of ballast sediment used for experiments.

	Mean diapause egg density					
Egg type	Vessel 1	Vessel 2	Vessel 3	Vessel 4	Vessel 5	Vessel 6
Asplanchna	1.4	—	1.0	0.6	—	—
Brachionus	50.4	394.4	44.6	104.6	300.4	240.1
Filinia	4.2	1.0	12.4	6.4	2.6	1.4
Synchaeta	0.6	80.6	131.0	—	114.6	94.8
Unidentified Rotifera	7.0	44.8	32.4	2.6	36.4	27.3
Bosmina	—	2.8	1.6	3.0	4.2	4.3
Daphnia	—	—	10.4	5.0	—	—
Unidentified Cladocera	18.0	4.6	15.2	10.6	8.4	6.8
Copepoda	6.6	11.2	96.6	0.6	7.2	3.3
Total number of eggs	88.2	539.4	345.1	133.3	473.8	378.1
% hatched in experiments	2.4	1.1	11.8	2.1	4.0	1.2

Note: The ballast sediments listed were collected from six separate vessels operating on the Great Lakes. Vessel numbers (1–6) refer to the vessels in Table 1 on which these sediments were used. The percentage of eggs that hatched during experiments was calculated by dividing the mean number of hatched individuals per 40 g of sediment by the total number of eggs found in 40 g of sediment.

approximately 5 s. Sediment was then allowed to settle out of the water (approximately 2 h) before the water overlaying the sediment was carefully decanted and replaced with fresh pond water. This exchange of water was performed to ensure that the salinity of the incubation media (pond water) was not affected by residual salt present from BWE.

After the incubation media was exchanged, the vessels were placed in an environmental chamber at 20 °C with a 16 h light – 8 h dark cycle. Vessels were checked for hatched animals every 48 h by carefully decanting the water through a 30 μm sieve and examining the contents under a stereomicroscope. Decanted water was then replaced in each vessel, and the vessel was returned to the environmental chamber. All vessels were examined for 10–20 days, with the experiment terminated when no hatching was observed on any day after the first 10 days.

To test for differences in egg viability among the three treatments and six ships, a two-factor analysis of variance (ANOVA) was conducted using ship (1–6) as a random factor and treatment (control, exchange, or left at laboratory) as a fixed effect. There were four replicates per treatment for ships 1, 4, 5, and 6, whereas experiments 2 and 3 had five replicates per treatment. Satterthwaite's (1946) denominator synthesis method, as implemented in Statistica 7.0 (StatSoft Inc., Tulsa, Oklahoma), was used to calculate error terms in the mixed-model ANOVA. This method finds the linear combinations of sources of random variation that serve as appropriate error terms and can result in fractional degrees of freedom for the denominator mean square (Satterthwaite 1946). Separate ANOVAs were conducted to determine if the total number of hatched individuals per 40 g of sediment and the species richness (total number of species) of hatched individuals per 40 g of sediment differed among treatments and ships. Data representing the total number of hatched individuals per 40 g of sediment and the species richness of hatched individuals per 40 g of sediment were both log-transformed prior to analyses to meet the assumption of normality (Lilliefor's tests, $p > 0.05$). Both sets of data were also tested for homogeneity of variances among treatments using Levene's test ($p > 0.05$ in both cases). Following the ANOVAs, Tukey's honestly significant differences (HSD) tests were conducted to compare treatment means. In the case of a significant interaction between factors, an interaction plot displaying the cell means from the ANOVA table was plotted and visually examined. All statistical tests were conducted using Statistica 7.0 software (Statsoft Inc.).

To calculate the percentage of eggs that hatched during each experiment the total number of eggs found in 40 g of sediment was divided by the average number of individuals that hatched per 40 g of that sediment.

Results

The ANOVA conducted using the total number of individuals hatched per 40 g of sediment as the dependent variable revealed a ship × treatment interaction (Table 3). A visual assessment of the interaction plot suggests that the interaction can be explained by a larger number of individuals hatching from sediments left at the laboratory, compared with sediments that were placed in ballast tanks, for ships 3 and 6 (Fig. 2). Consistent differences in hatching between control and exchanged treatments were not evident (Fig. 2). In total, 27 rotifer species, three cladoceran species, and unidentified copepod nauplii hatched during laboratory viability experiments (Appendix A, Table A1). Mean hatching ranged from approximately 2.2 individuals per 40 g of sediment for ship 1 to 40.6 individuals per 40 g of sediment for ship 3 (Fig. 3).

The species richness of hatched individuals per 40 g of sediment varied significantly among the three treatments (Table 3). Sediment left at the laboratory had significantly higher species richness of hatched individuals compared with sediment that was placed in ships' ballast tanks (Tukey's HSD tests, p values < 0.05). In contrast, there was no significant difference in hatched species richness between sediment exposed to BWE versus sediment placed in control ballast tanks (Tukey's HSD tests, p values > 0.05).

The percentage of eggs found in sediment that hatched during experiments, based on egg counts conducted prior to experiments and hatching data from laboratory viability experiments, ranged from 1.1% for sediment used with ship 2 to 11.8% for sediment used with ship 3 (Table 2).

Table 3. Results of a two-factor analysis of variance conducted using abundance and species richness as dependent variables.

Dependent variable	Source	df	Sum of squares	Mean square	Denominator synthesis error df	Denominator synthesis error mean square	*F* value	*p* value
No. of hatched individuals per 40 g of sediment	Ship	5	14.738	2.947	10.000	0.238	12.351	<0.001
	Treatment	2	1.448	0.724	10.028	0.236	3.056	0.092
	Ship × treatment	10	2.386	0.238	60.000	0.039	6.095	<0.001
	Error	60	2.349	0.039				
Species richness of hatched individuals per 40 g of sediment	Ship	5	2.975	0.595	10.000	0.039	14.910	<0.001
	Treatment	2	0.514	0.257	10.116	0.039	6.464	0.015
	Ship × treatment	10	0.399	0.039	60.000	0.026	1.501	0.161
	Error	60	1.594	0.026				

Note: Sediments containing diapausing eggs were exposed to one of three treatments: (*i*) placed in control ballast tanks; (*ii*) placed in exchanged ballast tanks; or (*iii*) left at laboratory in cold storage. Ship numbers correspond with those listed in Table 1. Degrees of freedom, df.

Fig. 2. Interaction plot describing how ship (1–6) and treatment interacted to determine the mean number of individuals hatched per 40 g of sediment: control treatment, continuous line; sediment left at laboratory, dotted line; exchanged treatment, broken line. Error bars represent standard error of the mean.

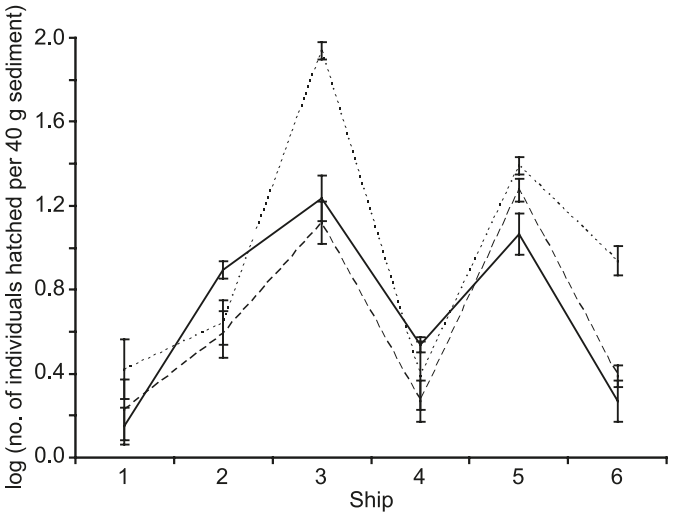
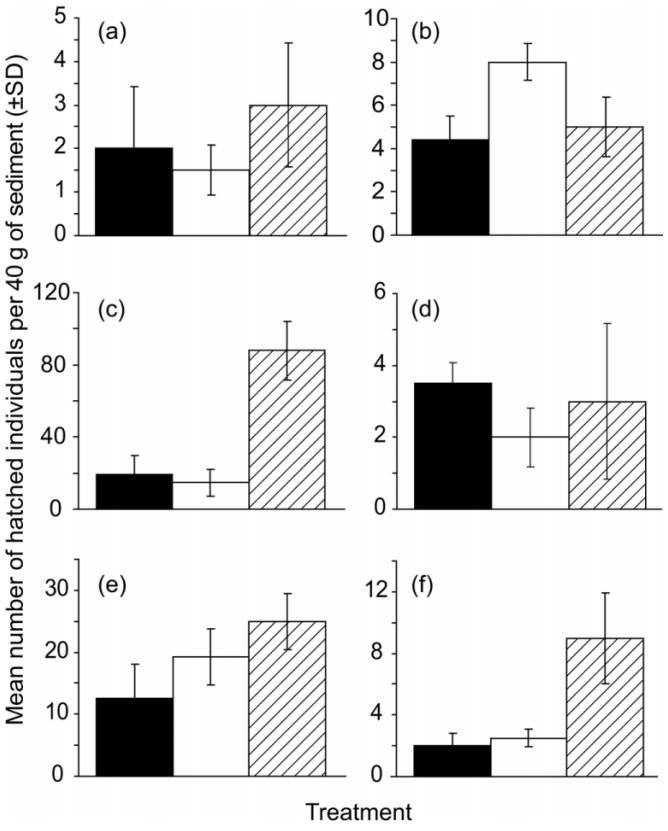


Fig. 3. Mean number of hatched individuals per 40 g of sediment that emerged during laboratory viability trials: exchanged ballast tank treatment, solid bars; control tank treatment, open bars; treatment that was left at the laboratory in cold storage, hatched bars. Each panel represents one of the six experiments: (*a*) ship 1; (*b*) ship 2; (*c*) ship 3; (*d*) ship 4; (*e*) ship 5; (*f*) ship 6.



Discussion

The results of our BWE exposure experiments suggest that diapausing invertebrate eggs may be largely resistant to saltwater exposure. Neither total abundance nor species richness of hatched individuals differed significantly between sediments collected from incubation chambers in the exchanged versus control (not exchanged) ballast tanks. Similar results were presented in Gray et al. (2005), although their experiments simulated BWE in the laboratory rather than performing the saltwater exposure under operational conditions in ballast tanks. In that study, there was no significant difference in abundances of hatched animals or in species richness of animals hatched between sediments that had been exposed to saltwater (32‰) for 10 days versus those that had not been exposed. However, the relationship between saltwater exposure and viability is not straightforward. Bailey et al. (2004) found significant differences in viability when they performed salinity exposure experiments with eggs of the cladocerans *Bosmina liederi* and *Daphnia longiremis* and the rotifer *Brachionus calyciflorus*. Similarly, Bailey et al. (2006) found significant differences in the abundance of hatched animals among treatments exposed to salinities of 0‰, 8‰, and 32‰. However, in both of the aforementioned studies, the eggs had been isolated from sediment with a sugar flotation procedure prior to saltwater exposure, whereas Gray et al. (2005) and the current study conducted whole-sediment incubation experiments. The inability of saltwater to reduce the viability of eggs in the latter studies may have resulted from protection provided by the sediments in which they were contained. If this is the case, then two

contrasting arguments can be made. First, BWE exchange may not be effective under operational conditions when eggs in the ballast tanks are embedded in sediments. Alternatively, eggs closest to the surface of the sediment should pose the highest risk for receiving hatching cues or being ejected from ballast tanks, and these would also be the most likely to receive exposure to saline water during BWE. Regardless, past studies indicate that exposure to saline water does not appear to completely inactivate diapausing eggs, even in the absence of sediment (Bailey et al. 2004, 2006).

We obtained mixed results when assessing the impact of physical conditions in ballast tanks on diapausing egg viability. When comparing the total number of animals that hatched from sediments placed in ballast tanks versus those that were left at the laboratory, we detected a statistical interaction between ship and treatment. A graphical analysis of this interaction suggested that the total number of animals hatched per 40 g of sediment was higher for sediments left at the laboratory for two of the six experiments, indicating that the impact of ballast tank conditions on egg viability may not be consistent. However, species richness of hatched animals was significantly higher for sediments that were left at the laboratory versus those that were placed in ballast tanks. We expected differences in abundance and species richness only for the BWE treatment; however, because species richness of hatched animals appears to be significantly higher for sediments left at the laboratory, we suggest that there may be some kind of "ballast tank effect". The factors responsible for the differences in species richness values are unclear; however, wide temperature variation and decreases in oxygen concentration have been measured in ballast tanks during the course of a ship's voyage (Gray et al. 2007). In addition, previous studies have noted the presence of oil and grease in ballast sediments (Bailey et al. 2006) that could possibly have an impact on ballast tank biota.

Although we were mainly concerned with testing for differences among the three treatments in this study, our ANOVAs also allowed us to explore differences in hatching among the six separate experiments that we conducted (ships 1–6). Our analyses suggested that the species richness of hatched individuals varied significantly among the six experiments and that the number of hatched individuals differed among ships dependent on treatment level (exchange, control, left at laboratory). Variation in the average number of individuals hatched or average species richness of hatched individuals did not appear to be related to the density of eggs in the sediment (Appendix A, Fig. A1*a*), suggesting that simple egg counts were not a good predictor of the number or diversity of potential colonists that could have been introduced via these ballast sediments. Instead, differences in the number and richness of hatched individuals appeared to be driven largely by variation in the percentage of eggs that hatched from each of the sediments during experiments. For example, sediments obtained from ships 3 and 6 had similar egg densities, but the abundance of hatched individuals was higher for the former due to differences in the percentage of eggs that hatched (11.8% vs. 1.2%, respectively). Although we have limited data for comparison with only six experiments, it does not appear that the average percentage of hatched eggs is related to the duration of saltwater exposure (number of days post-BWE; Appendix A, Fig. A1*b*). In addition, we do not believe that differences in hatching among experiments are a result of experimental error, as field and laboratory methods and physical conditions in environmental chambers were consistent across all experiments. Although we cannot determine what factors were responsible for differences among the experiments, we speculate that the past history of the eggs contained in the sediments, including factors such as age of eggs (Hairston and Van Brunt 1994; Hairston et al. 1995), and history of ballast treatments (e.g., multiple BWE exposures) could potentially explain observed differences in egg viability. Regardless of the explanation for these differences, our results suggest that the risk of species introductions is not uniform for all vessels as propagule supplies may vary depending on the viability of eggs present in ballast sediments (see also Bailey et al. 2005*b*).

It is important to note that the history of the six different ballast sediments that we collected for use in our experiments is not known. Therefore, we do not know if these sediments were previously exposed to BWE prior to the dates on which we made our collections. If the sediments were previously exposed to saline water, then it is possible that diapausing eggs sensitive to salinity changes may have been eliminated, leaving only resistant eggs. This would presumably have led to less pronounced differences in hatching during our laboratory viability trials when comparing species richness and abundance of individuals between the BWE and control treatments. However, simulated BWE experiments conducted by Gray et al. (2005) using sediments collected from natural habitats also failed to find significant differences in species richness or abundance of hatched individuals when comparing exchanged versus control treatments. This suggests that even with the use of a naïve egg bank, salinity exposure has little effect on eggs contained in sediment. As an alternative to using ballast sediments, we could have collected sediments from natural habitats for use in our emergence chambers. However, we chose not to do this for two reasons: (*i*) we felt that it would be unethical to risk the introduction of NIS into European waters if animals escaped from our emergence chambers; and (*ii*) we wanted to provide maximum realism for our experiments, so the use of ballast sediment seemed to be the best choice. In addition to the unknown history of ballast sediments used for this study, a further caveat is that we measured viability based on the number and richness of animals that hatched under our experimental conditions. These experimental conditions, including photoperiod, temperature, and composition of the growth medium, were designed to mimic conditions in the Great Lakes. Because cues for dormancy termination vary widely among and within species (Gyllström 2004; Vandekerkhove et al. 2005), our experimental conditions may not have provided the appropriate cues for hatching, even if some unhatched eggs were viable. The distinction between hatching and actual egg viability is probably not important for comparisons among our three treatments, but it could mean that our estimates for total hatching and species richness are conservative.

Past studies have suggested that the risk of introduction of NIS to freshwater ports via diapausing eggs is probably low as only a small proportion appear to hatch in situ and it is

unlikely that eggs embedded in compacted sediment will be ejected during ballasting activities (Bailey et al. 2005*a*, 2005*b*; Gray et al. 2005). However, numerical models incorporating reproduction of hatched individuals identify species introductions via diapausing eggs as a plausible invasion threat for the Great Lakes, despite low hatching rates (Wonham et al. 2005). In addition, some researchers have speculated that diapausing eggs may facilitate transport and introduction of NIS as 16 of the 19 crustaceans that have successfully invaded the Great Lakes are known or suspected to produce a dormant life history stage (Bailey et al. 2007). Now that BWE regulations have been extended to include vessels carrying both full ballast tanks and residual-only ballast, the arrival of large populations of live invertebrates is highly unlikely due to high mortality in flushed (exchanged) water (Gray et al. 2007). The principal remaining internal risk may thus be related to presence of resting stages. That many of these diapausing eggs appear to be largely resistant to saltwater exposure when embedded in sediments leads us to speculate that the relative importance of this subvector for introductions of NIS may increase relative to the overlying ballast water. Even this risk may be substantially lower today than prior to implementation of regulations requiring ballast water flushing for NOBOB (no ballast on board) tanks. For example, NOBOB tanks have significantly less sediment accumulation (and presumably fewer resting eggs) following implementation of tank-flushing regulations relative to the period before this procedure was mandated (E. Briski, University of Windsor, Great Lakes Institute for Environmental Research, Windsor, Ontario, personal communication).

Future ballast water treatment options should reduce not only active forms of zooplankton and other groups, but also their diapausing eggs. Chemical and physical treatments should be explored; however, the presence of large quantities of accumulated sediment in ballast tanks makes treatment difficult (Gray et al. 2006; Raikow et al. 2007). Therefore, we suggest that the best approach may be to explore ballast tank designs, ballast tank maintenance procedures, and filtration technologies that prevent the accumulation of sediment.

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Appendix A

Table A1. List of species that emerged during laboratory incubation experiments.

Experiment	Group	Species	Exchanged tank	Control tank	Left at lab
1	Rotifera	*Brachionus angularis*	0	×	×
		Brachionus bidentata	×	0	0
		Brachionus budapestinensis	0	×	0
		Brachionus calyciflorus	0	×	×
		Cephalodella gibba	0	0	×
		Filinia longiseta	×	0	0
		Synchaeta kitina	0	0	×
		Keratella valga	0	×	×
		Polyarthra minor	0	0	×
	Cladocera	*Daphnia* sp.	0	×	0
		Diaphanosoma birgei	×	×	0
	Copepoda	Unidentified nauplii	×	0	0
2	Rotifera	*Brachionus angularis*	×	×	0
		Brachionus bidentata	0	×	0
		Brachionus budapestinensis	×	×	×
		Brachionus calyciflorus	×	×	×
		Brachionus urceolaris	×	0	0
		Filinia longiseta	×	0	0
		Keratella cochlearis	0	0	×
		Polyarthra major	×	×	0
		Pompholyx sulcata	×	×	×
		Synchaeta kitina	0	0	×
		Trichocerca pusilla	×	×	×
	Copepoda	Unidentified nauplii	×	0	0
3	Rotifera	*Ascomorpha* sp.	×	0	0

Table A1 (*continued*).

Experiment	Group	Species	Exchanged tank	Control tank	Left at lab
		Asplanchna sp.	0	×	×
		Brachionus angularis	×	×	×
		Brachionus bidentata	×	×	×
		Brachionus budapestinensis	×	0	×
		Brachionus calyciflorus	×	×	×
		Brachionus caudatus	×	0	×
		Brachionus havanaensis	×	×	0
		Brachionus quadridentatus	×	0	0
		Brachionus urceolaris	0	0	×
		Filinia longiseta	×	×	×
		Hexarthra mira	0	0	×
		Keratella cochlearis	0	0	×
		Keratella quadrata	×	×	×
		Polyarthra dolichoptera	×	×	×
		Proales sp.	×	×	×
		Synchaeta grandis	×	×	×
		Synchaeta kitina	×	0	×
		Synchaeta lakowitziana	0	0	×
		Trichocerca pusilla	×	×	×
	Cladocera	*Diaphanosoma brachyurum*	×	×	×
		Bosmina longirostris	0	0	×
	Copepoda	Unidentified nauplii	×	×	0
4	Rotifera	*Brachionus bidentata*	×	×	0
		Brachionus calyciflorus	×	×	×
		Filinia longiseta	×	×	0
		Polyarthra minor	0	0	×
		Trichocerca pusilla	×	×	0
		Brachionus budapestinensis	×	×	0
	Cladocera	*Diaphanosoma brachyurum*	0	0	×
5	Rotifera	*Asplanchna brightwelli*	0	×	0
		Brachionus angularis	×	×	×
		Brachionus bidentata	×	0	×
		Brachionus budapestinensis	×	×	×
		Brachionus calyciflorus	×	×	×
		Brachionus caudatus	0	0	×
		Brachionus rubens	0	0	×
		Brachionus urceolaris	0	×	0
		Collotheca pelagica	×	0	0
		Filinia longiseta	×	×	×
		Keratella testudo	×	×	×
		Keratella valga	×	×	×
		Polyarthra dolichoptera	×	×	×
		Pompholyx sulcata	0	×	0
		Synchaeta kitina	0	0	×
		Synchaeta sp.	×	×	0
		Trichocerca pusilla	×	×	0
		Trichocerca sp.	0	0	×
	Cladocera	*Diaphanosoma* sp.	×	0	×
	Copepoda	Unidentified nauplii	×	0	×

Table A1 (*concluded*).

Experiment	Group	Species	Exchanged tank	Control tank	Left at lab
6	Rotifera	*Asplanchna brightwelli*	×	×	×
		Brachionus angularis	×	×	×
		Brachionus bidentata	0	0	×
		Brachionus budapestinensis	0	0	×
		Brachionus calyciflorus	0	0	×
		Diaphanasoma sp.	0	0	×
		Keratella testudo	0	0	×
		Keratella valga	0	0	×
		Polyarthra dolichoptera	0	0	×
		Pompholyx sulcata	0	0	×
		Synchaeta sp.	0	0	×
		Trichocerca pusilla	0	0	×

Note: Columns for each of the three sediment treatments (exchanged tank, control tank, and left at laboratory) are included. Experiment number corresponds to the vessel numbers listed in Table 1. Species hatched from this sediment, ×; species that did not hatch, 0.

Fig. A1. (*a*) Mean diapausing egg density per 40 g of sediment versus mean (± standard error, SE) number of hatched individuals (diamonds) and number of species (solid squares) that hatched during experiments from 40 g aliquots of sediment, and (*b*) days of saltwater exposure versus percentage of eggs that hatched in each experiment. Each data point represents one of the six experiments (vessels 1–6). The number of days of saltwater exposure refers to the number of days that sediments were exposed to saltwater before samples were collected from ballast tanks. The percentage of hatched eggs was calculated by dividing the average number of individuals hatched per 40 g of sediment by the average number of eggs counted in 40 g of that sediment.