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Efficacy of 'saltwater flushing' in protecting the Great Lakes from biological invasions by invertebrate eggs in ships' ballast sediment

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SUMMARY

1. Mid-ocean exchange and saltwater flushing were implemented as management practices to reduce the likelihood of new biological invasions in the Laurentian Great Lakes associated with ships' ballast water and sediments. Despite this, there has been no formal assessment of the efficacy of these procedures. Here, we conduct a comparative analysis of community composition of dormant taxa transported by ballast sediment before and after regulations came into effect in 2006.
2. Ballast sediment samples were collected from 17 ships during the post-regulation interval of 2007 and 2008. Invertebrate eggs were counted, hatched and species identified in the laboratory. Results were compared to similar samples collected from 39 ships between 2000 and 2002, prior to implementation of saltwater flushing regulations.
3. The estimated amount of residual ballast sediment transported by vessels was significantly lower during the post-regulation period, ranging from <1 to 45 tonnes per ship, with an average of 5 tonnes. Mean density and number of dormant viable eggs per ship declined 91 and 81%, respectively.
4. Community composition also changed through time, with Rotifera accounting for 78% of taxa transported prior to regulation, whereas Cladocera and Copepoda each accounted for 38% of abundance post-regulation. Although the number of non-indigenous species (NIS) declined 73% per ship after 2006, the reduction was not statistically significant; however, the number of freshwater NIS – which pose the greatest risk of invasion for the Great Lakes – was significantly lowered.
5. Our comparative analysis suggests that ballast management regulations enacted in 2006 markedly reduced the probability of introduction of NIS *via* dormant eggs carried in ballast sediments.

Keywords: ballast sediment, biological invasions, dormant eggs, non-indigenous species, saltwater flushing

Introduction

The introduction of non-indigenous species (NIS) into habitats outside their native range is increasing in frequency worldwide (Mack *et al*., 2000; Wonham & Carlton, 2005; Ricciardi, 2006). The shipping industry has played a major role in the spread of NIS globally. Ships' ballast water and associated sediments are a leading mechanism for NIS introductions into marine ecosystems (Carlton, 1985; Ruiz & Carlton, 2003; Molnar *et al*., 2008) and are particularly important for the Laurentian Great Lakes (Holeck *et al*., 2004; Ricciardi, 2006). Sediment has been implicated as a vector for natural and human-assisted zooplankton

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dispersal (Koste & Shiel, 1989; Hairston *et al*., 1999; Bailey *et al*., 2003, 2005). Ballast sediments may contain very large numbers of active invertebrates as well as their viable dormant stages (Bailey *et al*., 2005; Duggan *et al*., 2005).

To decrease ballast-mediated invasions to the Great Lakes, two different ballast water regulations have been enacted. First, mid-ocean exchange was recommended in 1989 and made mandatory in 1993 to reduce the number of propagules in ballast water (Canadian Coast Guard, 1989; United States Coast Guard, 1993). This regulation was augmented to incorporate management of residual ballast water and accumulated sediments through mandatory saltwater flushing, beginning in 2006 (Government of Canada, 2006; SLSDC, 2008). Mid-ocean exchange involves replacement of water in filled ballast tanks with ocean water, while saltwater flushing involves rinsing ballast tanks containing only residual ballast water and sediments through the uptake and subsequent discharge of several tonnes of ocean water. To assure compliance with the regulations, nearly every ship entering the Great Lakes is inspected by Canadian and/or American agencies (GLBWWG, 2009). In theory, mid-ocean exchange and saltwater flushing should reduce abundance (i.e. propagule pressure) and species richness (i.e. colonisation pressure) by purging coastal water, sediments and taxa from tanks and, for low salinity taxa, by killing remaining individuals with osmotic stress (MacIsaac, Robbins & Lewis, 2002; Lockwood, Cassey & Blackburn, 2009).

Multiple studies have assessed the efficacy of mid-ocean exchange and/or saltwater flushing on active stages of biota (Locke *et al*., 1993; Rigby & Hallegraeff, 1994; Wonham *et al*., 2001; Choi *et al*., 2005; Gray *et al*., 2007; Humphrey, 2008), while only one has examined efficacy of the procedures on dormant eggs of invertebrate species resident in ballast sediment (Gray & MacIsaac, 2010). Gray & MacIsaac (2010) reported only partial effectiveness of mid-ocean exchange at rendering dormant stages non-viable after performing *in situ* tests with ballast sediment. Further, laboratory tests have demonstrated that diapausing eggs are resistant to short-term saltwater exposure (Bailey *et al*., 2004; Gray *et al*., 2005; Bailey, Nandakumar & MacIsaac, 2006). The above studies, however, had small sample sizes and did not evaluate potential cumulative effects of ongoing ballast management, warranting a more substantial analysis of the effect of the current ballast water regulations on dormant stages in sediments.

Here, we test the effect of the 2006 regulations, which mandated saltwater flushing, on the density and diversity of invertebrate dormant stages in ballast sediment of transoceanic and coastal vessels arriving to the Great Lakes. We conducted a random survey of ship sediments in 2007 and 2008, and compared our results with those of a survey conducted between 2000 and 2002 (Bailey *et al*., 2005). As both studies collected samples after mid-ocean exchange became mandatory, the results reflect only the influence of saltwater flushing regulations; however, for simplicity, in this paper, we refer to pre-regulation (2000–2002) and post-regulation time periods (2007–2008). We tested the hypotheses that post-regulation ships carry less residual sediment, contain a lower abundance of dormant eggs and have lower egg viability than did pre-regulation ships.

Methods

Sediment collection, dormant stage counts and hatching

Ballast sediment was collected opportunistically from 19 ballast tanks on 17 ships, which originated from European, South American and Atlantic ports in the USA, arriving to the Great Lakes during 2007 and 2008. Approximately 6 kg of sediment was collected from each ballast tank for laboratory analysis of the density, diversity and viability of invertebrate dormant eggs. Methodology was consistent with that of Bailey *et al*. (2003, 2005), allowing for comparison of results pre- and post-regulation. We note that Bailey *et al*. (2005) sampled ships that carried only residual ballast water at the time of entry to the Great Lakes, while this study sampled ships with full ballast tanks that were discharged after entry to the Great Lakes (Table 1). Following Bailey *et al*. (2005), results from multiple tanks sampled from a single ship at a single sampling event were averaged, while independent trips into the Great Lakes by a single vessel were considered independent samples since new ballast had been held in tanks between sampling intervals. Personal observations of sediment depth and per cent cover inside ballast tanks, combined with architectural diagrams of ships' tanks, were used to estimate the amount of residual sediment carried by each ship. We obtained data about each ship's ballast history,

Table 1 Differences in methodology between the pre-regulation and the post-regulation periods

	This study (the post-regulation period)	Bailey *et al.* (2005) (the pre-regulation period)
Sampling		
Tank status	Ballast water discharged after entering the Great Lakes	Ballast water discharged outside the Great Lakes
Total number of tanks sampled	19	69
Total number of ships sampled	17	39
Number of ships where two tanks were sampled	2	26
Number of ships where three tanks were sampled	0	2
Egg density counts		
Number of tanks counted	19	69
Hatching experiments		
Maximum diversity experiments	19 tanks; four replicates; 0‰, 15 and 30‰; at 20 °C	Five tanks; four replicates; 0 and 8‰; at 10 °C and 20 °C and 50 tank; one replicate; 0‰; at 20 °C
Whole sediment experiments	19 tanks; four replicates; 0, 15 and 30‰; at 20 °C	19 tanks; four replicates; 0‰; at 20 °C and 10 tanks; four replicates; 8, 16 and 32‰; at 20 °C
Identification of taxa		
Molecular markers COI and 16S applied to eggs	Yes	No
Morphological identification applied to hatched animals	Yes	Yes

including total ballast capacity and previous dates and locations of ballast uptake and discharge, from ships' crews and mandatory reporting forms submitted to Transport Canada.

Upon return to the laboratory, sediment was homogenised by thorough mixing. Four 40-g subsamples were taken from each tank sediment sample for egg density counts. Subsamples were preserved in 95% ethanol, followed by washing through a 45-μm sieve to remove fine sediment. Eggs were separated from the remaining sediment using the colloidal silica Ludox® HS 40 (Burgess, 2001). Dormant stages were enumerated under a dissecting microscope, and the average density of eggs from the four subsamples was extrapolated to the number of dormant propagules per ship.

All remaining sediment was stored in the dark at 4 °C for at least 4 weeks to break the diapause cycle of dormant stages before hatching experiments commenced (Schwartz & Hebert, 1987; Dahms, 1995). Two types of hatching experiments were conducted on all 19 tank sediments following the methodology of Bailey *et al.* (2005). First, 'maximum diversity experiments' isolated eggs from sediments prior to hatching to determine the number of viable species. Second, to represent more realistic hatching conditions inside ballast tanks, 'whole sediment experiments' were conducted, which did not separate eggs from sediments (Table 1). All experiments were conducted using a light : dark cycle of 16 : 8 h.

For maximum diversity experiments, diapausing eggs were isolated from 40-g sediment subsamples using a sugar flotation method (Hairston, 1996; Bailey *et al.*, 2003, 2005). Extracted eggs were placed into vials containing sterile synthetic pond water [0 parts per thousand (‰) salinity; Hebert & Crease, 1980] or a sterile seawater medium with salinity of 15 or 30‰. The seawater medium was prepared using mid-ocean ballast water collected from a vessel transiting the Great Lakes, filtered through 2.5-μm Whatman paper filter and diluted to 15 or 30‰ with the sterile, synthetic pond water. Four replicates were placed into each of the 0, 15 and 30‰ treatments at 20 °C (Table 1). Whole sediment experiments were

conducted by placing 40-g sediment subsamples directly into 500-mL glass vessels. Four replicates were placed into each of the 0, 15 or 30‰ treatments, with 150 mL of media added to each vessel before incubation at 20 °C.

Three different salinities were used in both types of hatching experiments in an attempt to match unknown species to a fresh-, brackish- or salt water habitat to promote maximum hatching (Table 1). The list of species generated from hatching experiments was used to estimate egg viability as well as the effect of saltwater flushing on freshwater, brackish and saltwater taxa. Hatching percentage (H%) was calculated by dividing the total number of animals hatched by the total number of eggs isolated for hatching, and multiplying by 100.

We use the number of hatched eggs as a proxy measure of egg viability, although we acknowledge that some eggs that did not hatch may have been viable but did not receive appropriate hatching cues. All NIS hatched in the 0‰ treatment were considered high-risk taxa with potential to establish populations under environmental conditions of the Great Lakes, unless an established population of the species already exists. The freshwater species *Daphnia magna* was not considered a high-risk NIS, however, as the species almost certainly has been introduced into the Great Lakes multiple times by both shipping (Bailey *et al.*, 2003, 2005; Duggan *et al.*, 2005) and natural (Louette & De Meester, 2005) vectors but has not established a self-sustaining population; biotic or abiotic factors may preclude invasion by this species (Lauridsen & Lodge, 1996).

Identification of dormant eggs was conducted directly using molecular methods, as well as through traditional morphological taxonomy of hatched individuals (Table 1). DNA was extracted directly from diapausing eggs using a HotSHOT method (Montero-Pau, Gómez & Muñoz, 2008). Fragments of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) and 16S rDNA gene were amplified from each egg using the universal primers LCO1490 and HCO2190 (Folmer *et al.*, 1994) and S1 and S2 (Palumbi, 1996), respectively. PCR were performed in a total volume of 25 μL using 5 μL of DNA extract, 1× PCR buffer, 12.5 μL of 10% trehalose, 0.1 μM of each primer, 2.5 mM $MgCl_2$, 0.14 mM dNTPs and 0.4 U *Taq* DNA polymerase. The thermal profile consisted of a 1-min initial cycle at 94 °C, followed by five cycles of 94 °C (40 s), 45 °C (40 s) and 72 °C (1 min), 35 cycles of 94 °C (40 s), 50 °C (40 s) and 72 °C (1 min) and a final extension of 72 °C for 5 min. PCR products were sequenced by an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

Although our post-regulation methodology followed that used during the pre-regulation period (Bailey *et al.*, 2005), there are some differences (Table 1). We conducted both types of hatching experiments on all 19 tank samples in a fully replicated fashion using three growth media at a single temperature (0, 15 and 30‰, at 20 °C). In contrast, Bailey *et al.* (2005) conducted experiments using four growth media at two temperatures (0, 8, 16 and 32‰ at 10 and 20 °C), but were unable to fully replicate all experiments because of large sample size (69 tanks). Furthermore, Bailey *et al.* (2005) used only traditional morphological taxonomy to identify hatched individuals to species level. Morphological identification of dormant eggs can be difficult, even to the class level, and almost 10% of unhatched eggs were reported by Bailey *et al.* (2005) as 'indeterminate' taxon (Table 2). While the molecular methods used in this study were not able to identify all eggs to species level, the number of species identifications was double that of morphological methods (E. Briski, unpubl. data).

Statistical analysis

We tested for differences in the cumulative mean density of diapausing eggs, the mean density of viable (hatched) eggs and the mean density of eggs of NIS in 40 g subsamples between the pre- and post-regulation sampling periods using *t*-tests and Mann–Whitney *U*-tests (SPSS 11.5.0; SPSS Inc., 1989–2002, Chicago, IL, USA). A logarithmic transformation was applied to both datasets to meet assumptions of parametric tests. When a normal distribution was not achieved, or if results of a Levene's test for homogeneity of variances was significant, the non-parametric Mann–Whitney *U*-test was used (Table 3). After analysis of 40-g sediment subsamples was completed, further tests were conducted on extrapolated total egg abundance, total number of viable eggs and abundance of eggs of NIS by multiplying the average of four 40-g subsamples from each ship by the amount of sediment carried by that ship. The extrapolated data were again compared to Bailey

Table 2 Per cent occurrence and abundance of dormant stages collected before (39 vessels) and after (17 vessels) implementation of Canadian ballast water management regulations in 2006. Dormant stages are arranged phylogenetically by taxon. Pre-regulation data modified from Bailey *et al.* (2005)

	Pre-regulation period		Post-regulation period	
Taxon	% Occurrence	% Abundance	% Occurrence	% Abundance
Rotifera	**100**	**77.9**	**95**	**19.9**
Asplanchna spp.	66.7	1.0	0	0
Brachionus spp.	97.4	76.2	95	19.9
Conochilus spp.	5.1	<1	0	0
Filinia spp.	48.2	<1	0	0
Synchaeta spp.	5.1	<1	0	0
Bryozoa	**61.5**	**<1**	**63.2**	**3.9**
Cladocera	**76.9**	**9.3**	**89.5**	**37.8**
Bosmina spp.	51.3	<1	10.5	1.3
Chydoridae	5.1	<1	0	0
Daphnia spp.	46.2	7.9	89.5	19.5
Diaphanosoma spp.	2.6	<1	1	<1
Moina spp.	25.6	<1	15	12.8
Onychopoda	1	<1	21	3.3
Copepoda	**76.9**	**2.6**	**100**	**37.8**
Indeterminate*	**100**	**9.8**	**26.3**	**<1**

*Indeterminate represent eggs that were not identified to any taxonomic level.

Table 3 Significance levels for statistical comparisons of experimental data between the pre-regulation and the post-regulation periods. Significant *P*-values are presented in bold. A significance level of 95% was used for all statistical analyses

Experiment type	Treatment compared	Leven's test for equality of variances (*P*)	*t*-test (*P*)	Mann–Whitney *U* test (*P*)
Egg counts	Number of eggs in 40-g subsample	0.925	**0.019**	
	Amount of sediment per ship	0.330	**0.001**	
	Number of eggs per ship	0.916	**<0.001**	
Maximum diversity	Number of hatched eggs from 40-g subsample	**0.001**		**<0.001**
	Number of eggs of NIS in 40-g subsample	0.400	0.173	
	Number of high risk eggs of NIS in 40-g subsample	**<0.001**		**<0.001**
	Number of hatched eggs per ship	**0.037**		**0.009**
	Number of eggs of NIS per ship	**0.007**		0.814
	Number of high risk eggs of NIS per ship	**<0.001**		**<0.001**
Whole sediment	Number of eggs hatched from 40 g subsample	**<0.001**		**0.007**
	Number of hatched eggs per ship	**<0.001**		**<0.001**

NIS, non-indigenous species.

et al.'s (2005) samples using *t*-tests and Mann–Whitney *U*-tests (SPSS 11.5.0, SPSS Inc.) (Table 3). A significance level of 95% was used for all statistical analyses. Power analyses estimating the number of ship samples required to differentiate significant differences in abundance of NIS between the two time periods were calculated using JMP 7.0.2 (2007 SAS Institute Inc., Cary, NC, USA).

To estimate species richness of the larger vessel population based on findings from our sampled vessels, we calculated Chao-1, an estimator of species richness based on the number of rare species in a sample (Chao, 1984; Chao & Shen, 2003). We compared Chao-1 species richness estimates for the pre- and post-regulation periods to examine the efficacy of ballast water flushing. Sample-based species rarefaction curves were generated for both sampling periods to determine whether a significant difference existed given our small sample size. Confidence intervals (95%) were generated to test for significant differences between the two sampling periods (Chao & Shen, 2006; Gotelli & Entsminger, 2006). Chao-1 estimates were calculated using SPADE software (Chao & Shen, 2006), while rarefaction curves were generated with 5000 random iterations using ECOSIM (Gotelli & Entsminger, 2006). Species richness comparisons were conducted for both total richness and richness of NIS.

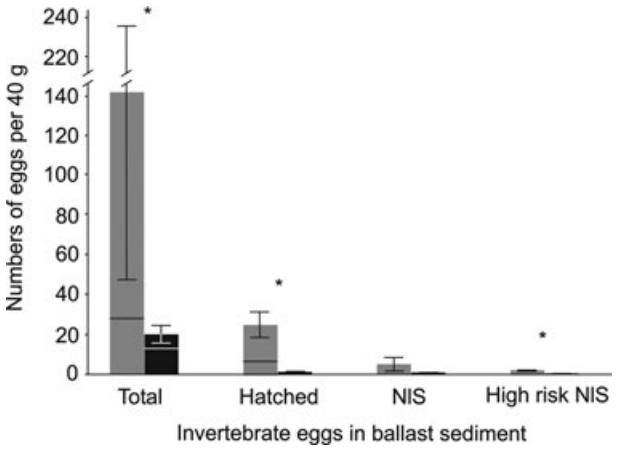

Fig. 1 Mean (±standard error of mean) and median (horizontal line in bar) density of invertebrate dormant eggs (total), of viable invertebrate eggs (hatched), of viable eggs of non-indigenous species (NIS) and of eggs of high-risk NIS in ballast sediment samples from pre-regulation (grey bars) and post-regulation sampling periods (black bar). The asterisk denotes a significant difference between paired bars.

Results

The estimated amount of residual sediment per vessel ranged from <1 to 45 tonnes, with the mean tonnage (5 tonnes) being significantly lower than in the pre-regulation period (14 tonnes per vessel; $P < 0.05$, Table 3). Similarly, total density of dormant eggs during the post-regulation period, which ranged from 1 to 80 eggs per 40-g sediment with mean density 20.3 eggs per 40 g, was significantly lower than in pre-regulation samples (143.5 eggs per 40 g; Fig. 1; Table 3). The density of viable eggs, which ranged from 0 to 17 eggs per 40 g (mean density of 1.9 eggs per 40 g; 6.2% hatching success), was also significantly lower than in pre-regulation period (24.4 eggs per 40 g; 24.8%; $P < 0.05$; Table 3, Fig. 1). While the mean density of viable eggs of NIS post-regulation (1.3 eggs per 40-g sediment; 1.6% hatch rate) did not decrease significantly (4.7 eggs per 40 g; <1% hatch rate for pre-regulation period), there was a significant difference if only eggs of high-risk NIS are considered (Fig. 1; Table 3).

The identification of diapausing eggs using molecular markers COI and 16S resulted in 17 distinct taxa (Appendix S1). Only eight of the 13 distinct morphological groups of dormant stages recorded by Bailey *et al.* (2005) were also recorded in this study. While Bailey *et al.* (2005) found that community composition of dormant eggs was dominated by Rotifera (77.9%), we found that dormant eggs of Cladocera and Copepoda were most abundant, each representing 37.8% of total abundance. Seven NIS were identified, including five Cladocera, one Copepoda and one Ascidia (Table 4). Four of the NIS were freshwater taxa (*D. magna*, *Daphnia galeata*, *Cercopagis pengoi* and *Acartia tonsa*), although only one is considered high risk (*A. tonsa*; Table 4).

Maximum diversity experiments confirmed the viability of 10 taxa, with species richness ranging from 0 to 5 viable taxa per 40-g sediment (median 2; no eggs were hatched from eight tanks). Cladocera were the most species-rich group, representing 80% of all taxa hatched. The remaining species belonged to Rotifera and Copepoda (see Appendix S1). Forty per cent of ships sampled in the post-regulation period carried viable dormant stages of NIS (0–2 eggs per 40 g sediment), although only one taxon (*D. magna*) hatched from only one of the 19 tanks during whole sediment experiments. The estimated species richness of the post-regulation vessel population was 27.2 species including 8.3 NIS, which was significantly lower than estimates for the pre-regulation period (126 and 33 species estimated for total richness and NIS richness, respectively, for the pre-regulation period) (Fig. 2).

Extrapolation of post-regulation subsample results to whole ships resulted in mean and median abundances of 3.5×10^6 and 9.0×10^4 eggs ship^{-1}, respectively. The mean and median numbers of viable eggs per ship were estimated at 1.9×10^6 and 1.3×10^5 eggs ship^{-1}, respectively. Finally, the mean and median abundances of dormant eggs of NIS were estimated as 1.8×10^5 and 0 eggs ship^{-1}, respectively. The total abundance of dormant eggs, number of eggs hatched and number of eggs of high risk NIS per ship were each significantly lower in the post- versus pre-regulation period ($P < 0.05$; Table 3). The estimated average total ballast capacity of ships in our study was 14532 m^{-3}, and assuming that all eggs from sediment would hatch, it could result in average of 130 individuals m^{-3} of which 12.3 individuals m^{-3} are NIS. However, considering hatching results from whole sediment experiments, the average number of invertebrates released from eggs may be as low as 0.014 individuals m^{-3}.

Discussion

Results from this study indicate that the ballast management regulations implemented in 2006 have markedly reduced the probability of introduction of

Table 4 Non-indigenous species transported as dormant eggs in residual ballast sediment to the Great Lakes. Species are listed in order of decreasing frequency and abundance of resting eggs, and ability to tolerate freshwater habitats. Occurrence identifies the number of ships that the species was collected from. Abundance is the cumulative mean number of eggs identified from 40-g sediment for all ships in which each species was found. Species hatched in 0‰ medium during laboratory experiments were considered an environmental match for the Great Lakes. Further, species identified by molecular markers that did not hatch were assigned habitat match based on literature research (*Cercopagis pengoi* and *Botrillus schlosseri*). Pre-regulation data are modified from Bailey *et al.* (2005)

	Pre-regulation period		Post-regulation period		
Species name	Occurrence	Abundance	Occurrence	Abundance	Habitat match
Daphnia magna (Straus, 1820)	4	6	2	20.5	Y
Filinia passa (Muller, 1786)	4	3.5			Y
Brachionus leydigi (Cohn, 1862)	4	3			Y
Filinia cornuta (Weisse, 1847)	3	3			Y
Asplanchna girodi (De Geurne, 1888)	2	1			Y
Cephalodella sterea (Gosse, 1887)	1	4.75			Y
C. pengoi (Ostroumov, 1891)*			2	0.75	Y
Bosmina maritima (Muller, 1867)	1	2			Y
Diaphanosoma orghidani (Negrea, 1982)	1	1.25			Y
Daphnia galeata (Sars, 1864)			1	2	Y
Brachionus forficula (Wierzejski, 1891)	1	1			Y
Brachionus nilsoni (Ahlstrom, 1940)	1	1			Y
Conochilus coenobasis (Skorikov, 1914)	1	0.5			Y
Diaphanosoma mongolianum (Ueno, 1938)	1	0.5			Y
Cephalodella cf. stenroosi (Wulfert, 1937)	1	0.3			Y
Alona rustica (Scott, 1895)	1	0.25			Y
Brachionus bennini (Leissling, 1924)	1	0.25			Y
Brachionus diversicornis (Daday, 1883)	1	0.25			Y
Diaphanosoma sarsi (Richar, 1894)	1	0.25			Y
Hexarthra intermedia (Wiszniewski, 1929)	1	0.25			Y
Moina affinis (Birge, 1893)	1	N/A			Y
Acartia tonsa (Dana, 1849)			1	0.25	Y
Synchaeta baltica (Ehrenberg, 1834)	1	2.75			N
Synchaeta bacillifera (Smirnov, 1933)	1	2.25			N
Evadne nordmanni (Lovén, 1836)	1	0.5			N
Pleopis polyphemoides (Leuckart, 1859)	1	N/A	1	0.5	N
Podon intermedius (Lilljeborg, 1853)			1	0.5	N
B. schlosseri (Pallas, 1766)†			1	0.25	N

*C. *pengoi* did not hatch in our experiments, although it is established in the Great Lakes.
†*B. schlosseri* did not hatch in our experiments; habitat matching designation based on Lambert (2005).

invertebrates to the Great Lakes via dormant eggs. Sediment accumulation has been significantly reduced, and with it the abundance of dormant stages of invertebrate species. On average, ships in the post-regulation period carried a potential inoculum one order of magnitude lower than those sampled in the pre-regulation period. Egg viability was lower, and fewer eggs of perceived high-risk NIS were present during the post-regulation period. Estimated species richness for the vessel population was also much lower in the post-regulation period (27 versus 126 species).

The addition of saltwater flushing to the ballast water management regime reduced the tonnage of accumulated sediments in ballast tanks threefold. Physical removal of sediments probably contributed to the observed reduction in egg number, as eggs are probably discharged along with sediments. Less sediment accumulation in tanks could also impact viability of retained eggs if they are more exposed to saltwater exposure during mid-ocean exchange and/or saltwater flushing. Bailey *et al.* (2004, 2006) determined that saltwater exposure was significantly more detrimental to viability of eggs extracted from sediments than for those retained within sediment. Furthermore, Reid *et al.* (2007) reported a reduction in oxygen concentration in ballast tank water owing to decaying organic matter. Reduced sediment accumulation in tanks may also expose a larger proportion of retained eggs to unfavourable oxygen concentration at the sediment–water interface. Reduction in sediment

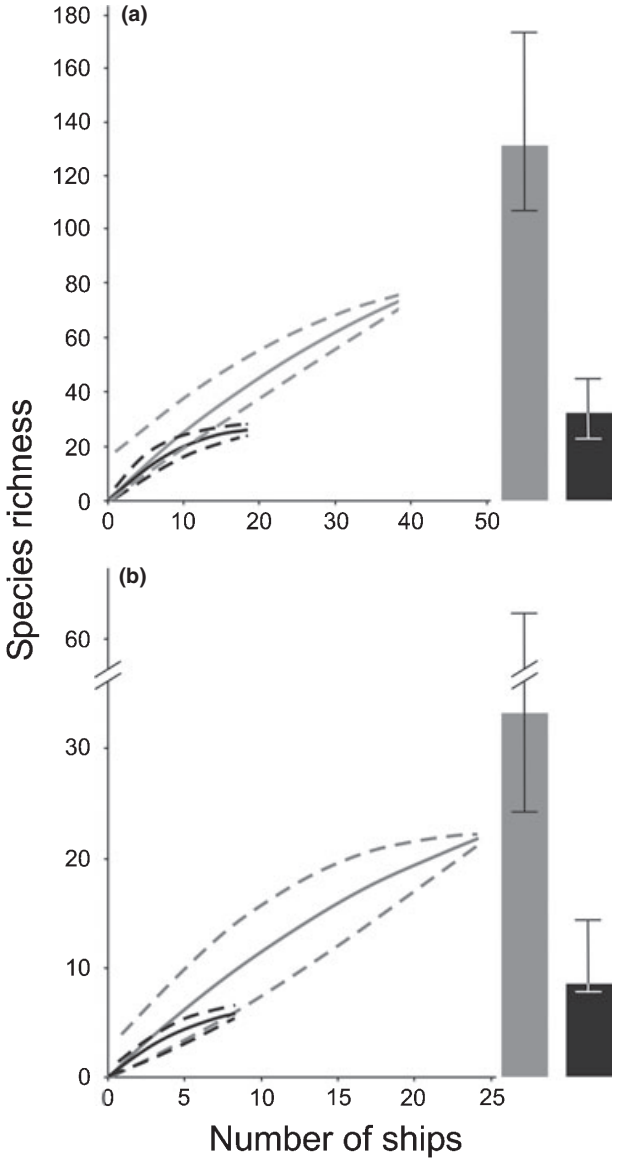

Fig. 2 Sample-based rarefaction curves from the pre-regulation (grey line, ±95% C.I.) and the post-regulation (black line, ±95% C.I.) periods for: (a) all ships sampled and (b) ships containing non-indigenous species. Also shown are species richness estimates for the vessel population (Chao-1 ± 95% C.I.) for the pre-regulation (grey bar) and the post-regulation period (black bar). Note the difference in scales for each *x* and *y*-axis.

accumulation could also negatively impact egg viability *via* desiccation. Dry sediments have been documented for ships arriving to the west coast of Canada and to the Great Lakes (Sutherland, Levings & Wiley, 2009; S. A. Bailey, unpubl. data), and long periods of desiccation or repeated hydration–dehydration cycles can negatively impact viability of dormant eggs (Lavens & Sorgeloos, 1987; Hagiwara *et al.*, 1997). Furthermore, egg viability may be reduced if small amounts of sediments facilitate gradual rather than rapid changes in abiotic conditions. For example, exposure to brackish water (8‰) has a greater effect on viability of dormant eggs of freshwater species like *Bosmina leideri* De Melo and Hebert, 1994 and *Daphnia longiremis* (Sars, 1861) than does exposure to ocean water (32‰; Bailey *et al.*, 2004). Similarly, egg viability is reduced more by exposure to low levels of oxygen than to complete anoxia (Lutz, Marcus & Chanton, 1994). Under extreme abiotic conditions, such as complete anoxia or high salinity, dormant eggs remain inactive, while sub-optimal conditions can initiate termination of diapause (Clegg & Trotman, 2002; García-Roger, Carmona & Serra, 2005; Pauwels *et al.*, 2007). Eggs that begin to develop under less-than-optimal conditions may allocate extra energy to adjust their metabolism to environmental conditions such that energy reserves are depleted before development is complete and emergence occurs (Van Stappen, 1996; Bailey *et al.*, 2004). Whatever the mechanism, the results of this study indicate that egg viability – as determined by hatching success – was significantly reduced in vessels entering the Great Lakes following implementation of ballast regulations for residual sediment and water.

Current ballast water management activities seemingly exert differential impacts on different taxa, as evidenced by the change in dormant egg community dominance from Rotifera to Cladocera and Copepoda. Dormant eggs of Rotifera are best preserved in constant salinity with low amounts of organic matter (Hagiwara *et al.*, 1997), while Cladocera have a hard ephippial structure around eggs that enhances resistance to desiccation and rapid abiotic changes (Altermatt, Pajunen & Ebert, 2009). It is unclear why abundance of Copepoda would increase compared to other taxa, but it is possible that a portion of Copepoda eggs were classified as 'indeterminate' in the pre-regulation period (see Methods; Table 2), since species identifications did not include molecular analysis in the earlier study.

While we observed a significant decrease in the total abundance, viability and species richness of dormant eggs, egg abundance of NIS did not decrease in the post-regulation period. This observation is almost certainly as a result of insufficient sample size. Using power analysis, we estimated that up to 171 ship samples would be required to confirm a

significant difference in NIS egg abundance between the two studies. However, egg abundance of high-risk taxa dropped from an average of 78% during the pre-regulation period to an average of 1% post-regulation. Only four NIS capable of tolerating fresh water were recorded during this study. Cladocera *D. magna* and *C. pengoi* were recorded in two ships, while *D. galeata* and Copepoda *A. tonsa* were observed in one vessel each. Following our earlier line of reasoning, *D. magna* appears to be a low risk for successful establishment in the Great Lakes. *Cercopagis pengoi* and *D. galeata* represent the next highest risk for introduction based on propagule pressure, but both have already established in the system (Taylor & Hebert, 1993; MacIsaac *et al*., 1999). Indeed, presence of viable eggs of these species in ballast sediments highlights the possibility that these species were vectored to the lakes in ballast sediment rather than ballast water. As a result, *A. tonsa* is the only species recorded during this study which presents a relatively high risk for invasion for the Great Lakes via retained eggs in treated ballast sediments.

Preventing species introductions *via* dormant invertebrate eggs is a particularly challenging task, because ballast sediments are not easily flushed from tanks and because dormant eggs are resistant to a wide array of adverse environmental conditions and treatment strategies (Bailey *et al*., 2005, 2006; Gray, Duggan & MacIsaac, 2006). Our results from whole sediment experiments indicate that the current propagule pressure posed by dormant eggs hatched into ballast water, estimated at 0.014 hatched individuals m^{-3}, would make an insignificant contribution to the median number of propagules typically carried in exchanged ballast water (2672.9 ind. m^{-3}; S. A. Bailey, unpubl. data). As the proposed international ballast water discharge standard applicable to invertebrate zooplankton stipulates that treated ballast water must contain less than 10 viable individuals m^{-3} (IMO, 2004), our results suggest that the risk of introductions *via in situ* hatching is adequately managed through saltwater flushing. In the worst case scenario, if all eggs in the sediment were to hatch and become available for discharge, or if all eggs in the sediment would be discharged directly, the estimated abundance of viable individuals in filled ballast tanks would increase by 130 individuals m^{-3}, which would be non-compliant with the proposed international ballast water discharge standard.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of invertebrate taxa identified during pre-regulation and post-regulation studies.

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