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Salinity tolerance of Great Lakes invaders

SANDRA ELLIS AND HUGH J. MACISAAC
Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada

SUMMARY

1. The Laurentian Great Lakes are among the most invaded freshwater ecosystems in the world. Historically, the major vector for the introduction of non-indigenous species (NIS) has been the release of contaminated ballast water via transoceanic ships. Despite regulations implemented in 1993, requiring vessels carrying fresh ballast water to exchange this water with saline ocean water, new reports of invasions have continued.

2. NIS often have a wide environmental tolerance allowing them to adapt to and invade a variety of habitats. It has been hypothesized that NIS with broad salinity tolerance may be able to survive ballast water exchange (BWE) and continue to pose an invasion risk to the Great Lakes.

3. We tested the short-term salinity tolerance of eight recent invaders to the Great Lakes, specifically three cladocera (*Bosmina coregoni, Bythotrephes longimanus, Cercopagis pengoi)*, two molluscs (*Dreissena polymorpha, Dreissena rostriformis bugensis)*, and one species each of the families Gammaridae, Mysidae and Gobidae (*Echinogammarus ischnus, Hemimysis anomala, Neogobius melanostomus*) to determine if they could have survived salinities associated with BWE.

4. Overall, short-term exposure to highly saline water dramatically reduced survival of all species. Two different methods of BWE tested, simultaneous and sequential, were equally effective in reducing survival. Species that survived the longest in highly saline water either possess behavioural characteristics that reduce exposure to adverse environments (valve closure; both *Dreissena* species) or are reported to have some degree of salinity tolerance in their native region (*Echinogammarus*). Given that exposure in our trials lasted a maximum of 48 h, and that species in ballast tanks would typically be exposed to saline water for *c.* 5 days, it appears that BWE is an effective method to reduce the survival of these NIS. These results provide impetus for tightening policy and monitoring of BWE, in particular for ships entering the Great Lakes from freshwater ports.

Keywords: ballast water exchange, biological invasions, Great Lakes, non-indigenous species, salinity tolerance

Introduction

International vessels carrying ballast water have been implicated as a major vector for the introduction of non-indigenous species (NIS) to aquatic ecosystems globally, and in particular to the Laurentian Great Lakes (Holeck *et al*., 2004; Ricciardi, 2006). NIS are one of the greatest threats to native biodiversity in freshwater ecosystems; they also cause substantial economic losses due to damage and control measures (Richter *et al*., 1997; Sala *et al*., 2000; Pimentel, Zuniga & Morrison, 2005; Colautti *et al*., 2006a).

In 1993 it became mandatory for ships that discharge fresh or coastal ballast water into the Great Lakes to first exchange that water on the open ocean, to reduce the risk of further species introductions (United States Coast Guard, 1993). Ballast water exchange (BWE) can be either sequential (i.e. empty-refill) or simultaneous (i.e. flow through) depending

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Correspondence: Sandra Ellis, Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Ave, Windsor, ON, Canada, N9B 3P4.
E-mail: sellis@uwindsor.ca

on ship design. During sequential BWE, fresh or coastal water is emptied from the tank, after which open ocean water is pumped in to replace it. During simultaneous BWE, water in the tank is discharged while concurrently ocean water is pumped in to the tank. Current regulations require a minimum of 95% volumetric exchange for BWE, which theoretically requires either one or three complete volume exchanges for sequential or simultaneous exchange, respectively, assuming perfect mixing of water within tanks (Fig. 1).

BWE has two effects which can reduce the abundance of freshwater species. First, assuming a homogeneous distribution, BWE should purge planktonic individuals in proportion to the volume of water exchanged. In the case of simultaneous BWE, it is assumed that the tanks have been optimally designed to allow comprehensive mixing. Usually, however, some 'unpumpable' water remains in the tanks (Bailey *et al.*, 2003). Secondly, for any freshwater individuals that remain within the tank, exposure to full sea water (salinity *c.* 34‰) should inflict mortality owing to physiological stress. Sequential BWE results in a much more rapid accumulation of saline water in a tank than simultaneous BWE (Fig. 1). This has potentially strong implications for viability of species remaining in a tank during exchange, as physiological adjustment would be more rapid during sequential exchange.

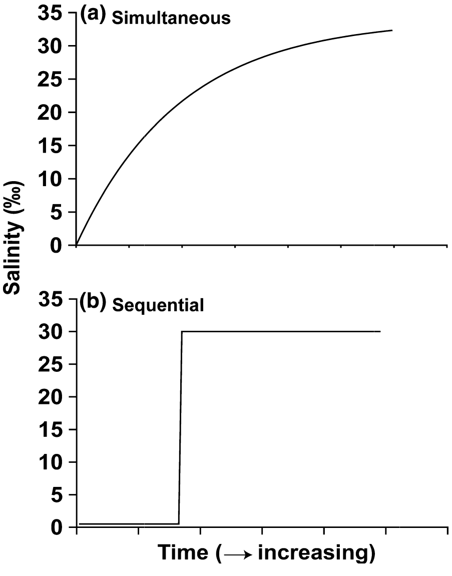

Fig. 1 Conceptual models describing rate of change in salinity in (a) simultaneous and (b) sequential methods of ballast water exchange. For simultaneous exchange the relationship between salinity increases gradually over time (see Rigby & Hallegraeff, 1994).

Despite the regulations implemented in 1993, new invaders continue to be discovered in the Great Lakes (Ricciardi, 2006). Most recently, a Ponto-Caspian mysid *Hemimysis anomala* Sars has been discovered in Lakes Michigan, Erie and Ontario (Pothoven *et al.*, 2007). While some new discoveries may result from time lags between introduction, discovery and reporting, this is unlikely to account for all of the new invaders in the system (Costello, Drake & Lodge, 2007; Ricciardi & MacIsaac, 2008). Another possibility is that BWE is less than 100% effective in eliminating all viable organisms from ballast tanks (see Locke *et al.*, 1993; Bailey *et al.*, 2003; Duggan *et al.*, 2005; Gray *et al.*, 2007). For example, Gray *et al.* (2007) determined experimentally that BWE was highly effective in purging freshwater species, but that some live organisms were present in most ships after water had been exchanged. Finally, it is also possible that some of the invasions recently reported result from introductions by other vectors (Bailey *et al.*, 2005; Duggan *et al.*, 2005; Ricciardi & MacIsaac, 2008).

Ricciardi (2006) evaluated common characteristics of the NIS introduced after 1993 and established that many are euryhaline, benthic invertebrates. A wide salinity tolerance has been presumed to be a factor potentially limiting the efficacy of BWE (Ricciardi & Rasmussen, 1998; Ricciardi & MacIsaac, 2000; MacIsaac, Robbins & Lewis, 2002; Reid & Orlova, 2002; Ricciardi, 2006). While several studies have examined the possibility of lag effects and other ship-mediated vectors, few have explored the ability of freshwater invaders to survive the increases in salinity characteristic of BWE. Gray *et al.*'s (2007) BWE study was conducted on vessels moving from the Great Lakes to European ports. Most recent invaders to the Great Lakes are European, and many are Ponto-Caspian (Holeck *et al.*, 2004; Ricciardi, 2006). Owing to long-term changes in climate, a number of freshwater Ponto-Caspian species have evolved some degree of salinity tolerance (Reid & Orlova, 2002). It is not clear whether this tolerance has played any role in the recent invasion history of the Great Lakes.

In this paper, we explore the ability of recent Great Lakes' invaders to survive high salinities characteristic of BWE. These species have a history of invasion in the Great Lakes, as well as elsewhere, and are reported to have a range of salinity tolerances (Table 1). We were particularly interested in exploring the possibility that some of these invasions could have been prevented had regulations brought into place in 1993 been implemented decades earlier. We also address whether species exhibit differences in salinity tolerance with respect to the type of ballast water exchange (simultaneous and sequential) conducted at sea.

Methods

The effect of simulated BWE on survival was assessed using adults and one juvenile form of eight NIS recently established in the Great Lakes. Collectively, the species selected represent some of the most invasive (widespread and high impact) taxa that have ever invaded the lakes. Many of the species that have invaded the Great Lakes since 1985 are crustaceans, and tests were conducted using these taxa (*Bosmina coregoni* DeMelo & Hebert, *Bythotrephes longimanus* Leydig, *Cercopagis pengoi* Ostroumov, *Echinogammarus ischnus* Stebbing, *Hemimysis anomala*). Crustaceans occur globally across a wide range of salinities (Dorgelo, 1976). We also conducted analyses on the molluscs *Dreissena polymorpha* Pallas and *Dreissena rostriformis bugensis* Therriault and the perciform fish *Neogobius melanostomus* Pallas. Species were selected based upon the date of their discovery in the Great Lakes; five species (*B. coregoni*, *B. longimanus*, *D. r. bugensis*, *D. polymorpha*, *N. melanostomus*) were discovered before BWE regulations were in place, while the other three species (*C. pengoi*, *E. ischnus*, *H. anomala*) were discovered after implementation of regulations. It was assumed that all animals used have no prior history of exposure to high salinity. Animals with a prior history of salinity exposure may be able to adapt to high salinities more readily than those with no prior exposure (Kinne, 1966).

Field collection

Animals were collected from the field for use in laboratory experiments. Collection methods varied based on differences in morphology and life history of the target animal. For example, the amphipod *E. ischnus* was collected in March 2006 from the banks of the Detroit River using a 500 μm mesh kick net. The planktonic species *B. coregoni*, *B. longimanus* and *C. pengoi* were collected in August 2006 using vertical

Table 1 List of species used in ballast water exchange experiments including their native and invasive North American distributions and reported salinity tolerance

Species	Native distribution	North American non-indigenous distribution	Reported salinity tolerance
Bosmina coregoni	Eurasia	Great Lakes, Lake Champlain	0–7.5‰ (Ackefors, 1971)
Bythotrephes longimanus	Eurasia	Great Lakes, inland lakes of the Canadian Shield	0.04–8‰ (Grigorovich *et al*., 1998)
Cercopagis pengoi	Caspian Sea, Aral Sea, Black Sea, Azov Sea	Lake Ontario, Lake Michigan, Lake Erie, Finger Lakes of New York, Muskegon Lake	0–14‰ (MacIsaac *et al*., 1999)
Dreissena rostriformis bugensis	Dnieper River, Bug River	Great Lakes, Mississippi River, Lake Mead, Lake Havasu, Lake Mohave	0–4‰ (Mills *et al*., 1996)
Dreissena polymorpha	Black Sea, Azov Sea, Caspian Sea	Great Lakes, Mississippi River and tributaries, Illinois River, Hudson River, Missouri River, Lake Mead, Inland lakes in Eastern North America	0–13‰ (Orlova *et al*., 1998)
Echinogammarus ischnus	Black Sea, Caspian Sea	Great Lakes, St. Lawrence River	0–23‰ (based on distribution reported in Jażdżewski, 1980)
Hemimysis anomala	Black Sea, Caspian Sea, Azov Sea, Don, Danube, Dnieper and Dniester rivers	Lake Michigan, Lake Ontario, Lake Erie	0.1–18‰ (Pothoven *et al*., 2007)
Neogobius melanostomus	Eurasia including Black Sea, Caspian Sea, Azov Sea and tributaries	Great Lakes and some tributaries of the Great Lakes	0–40.5‰ (Moskal'kova, 1996)

and horizontal plankton net tows (253, 950 and 500 μm mesh respectively) from Lake Erie, Peninsula Lake (Ontario) and Lake Ontario, respectively. All other collections were made in 2007. Similar to the cladocerans, *H. anomala* was sampled using horizontal plankton tows (500 μm mesh) in a channel connecting Muskegon Lake and Lake Michigan. Adults of both dreissenid species were collected in May from Lake St. Clair using a sled-dredge. Mussel veliger larvae were collected from the same location in June using vertical plankton tows (53 μm mesh). Finally, *N. melanostomus* was collected using a seine net with a mesh size of 5 mm from the Detroit River. Once collected, all species were placed in 10 or 20 L buckets filled with site water and brought back to the laboratory.

Laboratory protocol

Samples were brought back to the laboratory and, if sample temperature was different from 20 °C (±1 °C), they were placed in a walk-in environmental chamber and the temperature raised or lowered by 2 °C/h until 20 °C was reached. Then, samples were sorted, using a dissecting microscope when required, and healthy adults or veligers were placed (10 per replicate) in filtered [GF/F Whatman filter, 0.7 μm pore size (Whatman International Ltd., Maidstone, England)] water from the collection site. All animals, except *N. melanostomus*, were placed in small glass jars (130 mL), with 100 mL of filtered site water. *Neogobius melanostomus* is much larger than the other species, and was consequently placed in aquaria (38 L, 51 cm × 5 cm × 30 cm) filled with 16 L of filtered (5 μm pore size) site water. Sorting and transfer techniques varied by species, primarily due to differences in size, and were selected to minimize the stress on the animal (Table 2). Replicates containing *B. coregoni* were sprinkled with *c.* 1.00 mg of cetyl alcohol (1-hexadecanol) in order to prevent animals from becoming trapped in the surface film. Previous experiments indicate that cetyl alcohol, used in low concentration, increases survival of *Bosmina* (S. Ellis, pers. obs.). This surfactant is widely used to keep *Daphnia* out of the surface tension and is highly hydrophobic making toxic effects unlikely (Desmarais, 1997). Animals were left for a maximum of 24 h in an environmental chamber at 20 °C and a 16 : 8 light : dark regime before experimental treatments began, and were not fed during this interval. Animals which appeared healthy after a 24-h period were selected for use in experiments.

Ballast water exchange experiments

Simultaneous and sequential BWE treatments were simulated in laboratory experiments. When possible experiments were conducted twice based on abundance and availability of animals. In the simultaneous BWE treatment, animals were introduced to water of gradually increasing salinity, beginning at 4‰ and ending at 30‰. In the sequential BWE treatment, animals were immediately exposed to water with a salinity of 30‰. Currently, Canadian law requires that salinity within exchanged tanks reach a minimum of 30‰ (Canada Shipping Act, 2006). The third treatment was a control in which animals were placed in ambient salinity, filtered (0.7 μm pore size) site water. Salt water for ballast water treatments was produced by mixing filtered (0.7 μm pores size) site water with Instant Ocean® (Spectrum Brands Inc., Atlanta, GA, U.S.A.), a synthetically manufactured aquarium salt. Instant Ocean® is a widely used aquarium salt having

Table 2 Transfer techniques used while sorting animals during the course of experimental trials and end point mortality for each species

Species	Transfer technique	End point mortality
Bosmina coregoni	Pipette	Postabdominal claw ceases movement and heart ceases beating
Bythotrephes longimanus	Pipette	Pleopods cease beating
Cercopagis pengoi	Pipette	Pleopods cease beating
Dreissena polymorpha	Forceps, by hand	Mantle drawn away from interior shell; no resistance when shell opened
Dreissena rostriformis bugensis	Forceps, by hand	Mantle drawn away from interior shell; no resistance when shell opened
Dreissena veliger larvae	Pipette	Cessation of movement of internal organs
Echinogammarus ischnus	Scoop	Pleopods cease beating
Neogobius melanostomus	Aquarium net	Decreased and laboured respiration, erratic swimming behaviour
Hemimysis anomala	Scoop	Pleopods cease beating

ionic and trace element concentrations similar to natural seawater (Arnold *et al.*, 2007). Salinity was determined by a handheld refractometer.

Each treatment consisted of four replicates containing 10 individuals per 100 mL of water, except for *C. pengoi* and *N. melanostomus*. Negative intraspecific interactions in experiments with *C. pengoi* required that trials be conducted with only five individuals in 100 mL of water, though replicate number was increased to eight. *Neogobius melanostomus* was housed in aquaria, with 10 individuals in 16 L of water. All replicates were covered loosely with cellophane to reduce evaporation, which may alter salinity. Replicates were not aerated, except in the case of *N. melanostomus*.

Experiments began by placing healthy adult individuals into filtered water of ambient salinity in the control replicates, 4‰ in the simultaneous BWE treatment, and 30‰ in the sequential BWE treatment (Fig. 2). All species except *N. melanostomus* were checked for mortality after 1 h dead animals were preserved in 95% ethanol. During this time, *N. melanostomus* was observed for signs of distress, including decreased and laboured respiratory rates and erratic swimming behaviour. If signs of distress were observed, individuals were anaesthetized using 5 mg L^{-1} clove oil dissolved in water and were then killed by increasing the dose to 100 mg L^{-1} (in accordance with University animal care criteria). End point mortality for other species was determined by observing animals and placing them in filtered freshwater in order to determine if they could recover. These endpoints varied between species due to differences in morphology (Table 2). After 1 h animals that were alive, or showed no signs of distress (in the case of *N. melanostomus*), were transferred to water at 8‰ salinity for the simultaneous treatment, while those in the sequential and control treatments were transferred to water at 30‰ and ambient salinity, respectively. Subsequent to the first transfer in the simultaneous treatment, three other graded transfers were conducted (Fig. 2). Animals in this treatment were transferred every hour to increasing salinity, from 8, 14, 24 and finally to 30‰ (Fig. 2). Sequential and control treatments were also transferred to solutions of constant salinity (30‰ and ambient salinity water, respectively) to ensure all animals received the same amount of handling (Fig. 2). Survival was recorded at each time of transfer and 1 h after the final transfer. Animals were then monitored after 24 and 48 h, at which time all living animals were transferred to ambient salinity water. This transfer to ambient salinity water (<0.2‰) was completed to examine if species could recover from osmotic stress caused by exposure to high salinity. In addition, after BWE is completed and vessels enter the Great Lakes, they will discharge animals from highly saline ballast water into the freshwater of the Great Lakes; transferring species to water of ambient salinity will mimic this process. Species that may be able to survive in high salinities may not be able to adapt to the subsequent exposure to freshwater.

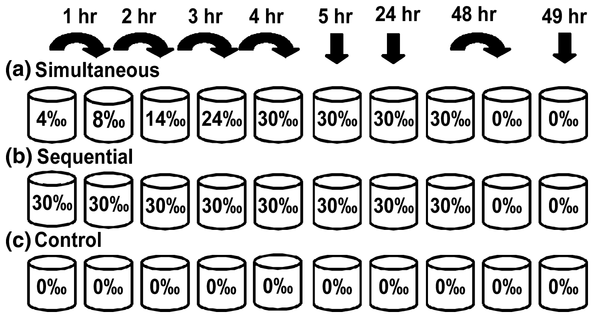

Fig. 2 Transfer sequence used during experimental trials. Time above arrows indicates cumulative experimental time and whether animals were observed and transferred (curved arrow) or only observed (straight arrow).

Due to high mortality in controls during experiments with *C. pengoi* and veliger larvae, after 24 h all replicates for these species were transferred to freshwater for 1 h, after which time survival was assessed and experiments ended. After 1 h in freshwater, survival was observed and all animals were preserved in 95% ethanol.

Tests were conducted in darkened environmental chambers at 20 °C to represent realistic ballast tank conditions during much of the shipping season. However, during observation periods animals were brought into the laboratory for assessment, where light levels were substantially higher than under the experimental conditions of the chambers.

Statistical analysis

Results from ballast water exchange simulations were calculated as proportion of individuals surviving at each separate time point, using time as a discrete variable. The proportion of individuals surviving was calculated as the number of individuals alive at an observation point divided by the number alive at the end of the previous observation point. Additionally, the net proportion of individuals surviving over time was calculated, as this value describes the overall response to treatments across time and is presented in all figures. The net proportion of individuals surviving was calculated as the number of individuals surviving at a specific time point divided by the total number of individuals entering the treatment at the beginning of the experiment.

Analysis of variance (ANOVA) was used to examine differences between the proportions of individuals surviving among all three treatments. Levene's test was used to ensure data met criteria for homogeneity of variance, and a *post-hoc* Tukey test was used to identify specific differences (Levene, 1960; Sokal & Rholf, 1995). Once all individuals in a treatment died, leaving two treatments, differences between the two surviving treatments were analysed using a Student's *t*-test. When data violated the assumption of homoscedasticity, a Welch's test was used.

The aspect of significance from a biological perspective in experiments was 100% mortality. Once a treatment had reached this point it was no longer relevant to compare the response with treatments that still had living individuals. One hundred per cent mortality within a treatment would also indicate that this treatment is effective in eliminating animals and invasion risk, which is the appropriate metric for management of ballast water.

Finally, species comparisons were completed using the same methods of ANOVA, Student's or Welch's *t*-tests. Each time point was examined separately using proportion of individuals surviving.

Results

Time to mortality varied between experimental treatments and species, with the sequential treatment causing mortality more rapidly than the simultaneous treatment in all species (Fig. 3). Four of eight species experienced 100% mortality in both treatments during the initial 5-h observation period (Fig. 3).

The outcome of trials with *B. coregoni*, *B. longimanus* and *C. pengoi* was identical, with 100% mortality after 1 h in the sequential treatment, and after 4 h and a salinity of 24‰ in the simultaneous treatment (Fig. 3). Interestingly, the simultaneous BWE treatment for these three species did not differ significantly (Student's and Welch's *t*-tests, $P > 0.20$) from the control treatment until 3 h had passed [Student's (*B. coregoni*) and Welch's (*B. longimanus, C. pengoi*) *t*-tests, $P < 0.001$] and animals had been exposed to 14‰ for 1 h (Fig. 3).

Hemimysis anomala had a response comparable to the cladocerans, with the sequential treatment causing 100% mortality after 3 h. The simultaneous treatment caused 100% mortality after 5 h and water salinity had increased to 30‰ for 1 h (Fig. 3).

Echinogammarus ischnus, *D. rostriformis bugensis*, *D. polymorpha* and *N. melanostomus* did not experience high mortality in any treatment before the 5-h. Among these four species, *N. melanostomus* exhibited the most rapid mortality in response to increasing salinity (Fig. 4). The proportion of *N. melanostomus* surviving for 24 h was not significantly different between the two treatments (ANOVA, $P > 0.05$), with 14.4 and 5.0% of individuals surviving this long in the simultaneous and sequential BWE treatments, respectively. However, both exchange treatments differed significantly from the control (ANOVA, Tukey *post-hoc* test, $P < 0.001$) (Fig. 4). After 48 h, animals in both treatments suffered 100% mortality, while those in controls survived until the end of the experiment (Fig. 4). By contrast, *E. ischnus*, *D. r. bugensis* and *D. polymorpha* all had individuals that survived after 48 h in both simultaneous and sequential BWE treatments (Fig. 4).

The proportion of individuals surviving to 48 h in the sequential and simultaneous treatments were not significantly different for *E. ischnus*, with 2.5% and 5% of individuals surviving in the sequential and simultaneous treatments, respectively (Fig. 4) (ANOVA, $P > 0.05$). This trend continued after 1 h's exposure to freshwater with 1.3% of individuals surviving in the sequential treatment and 5% of individuals surviving the simultaneous treatment (Fig. 5).

After 48 h the net proportion of *D. r. bugensis* individuals surviving the sequential and simultaneous BWE treatments did not differ significantly (8.8 and 6.3%, respectively; Fig. 4) (ANOVA, $P > 0.05$). Similarly, after 1 h's exposure to freshwater three (3.8%) and five (6.3%) individual *D. r. bugensis* survived from these treatments, respectively (Fig. 5).

Dreissena polymorpha had the greatest net proportion of individuals that survived in both the simultaneous and sequential treatment after 48 h (Fig. 4). In the simultaneous treatment, survival was the highest for

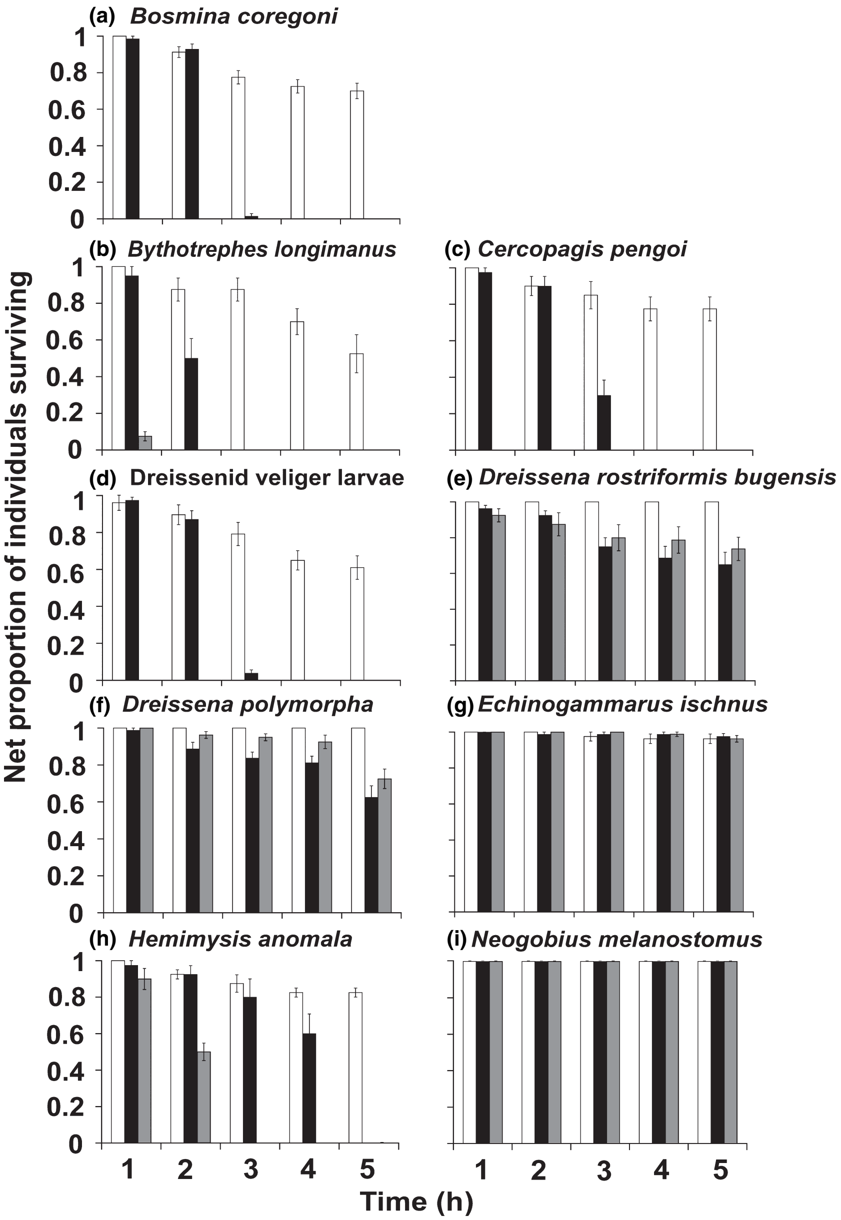

Fig. 3 Net proportion of individuals surviving for the first 5 h exposure (±SE) for all species in control (open bar), simultaneous (solid bar) and sequential (grey bar) exchange treatments.

all species, with a net proportion of 10% surviving to 48 h, while in the sequential treatment 15% survived to 48 h (Fig. 4). Though the simultaneous treatment had the highest survival for any species, this survival rate was not significantly different from that of *E. ischnus* and *D. r. bugensis* (ANOVA, $P > 0.08$). After 1 h in freshwater, all individuals from the sequential BWE treatment died, whereas one individual survived from the simultaneous treatment (Fig. 5).

In comparison to the prolonged of survival of adults of both dreissenid species, *Dreissena spp.* veliger larvae had more rapid mortality in response to increasing salinity (Fig. 3). Veliger larvae experienced 100% mortality after 3 and 2 h in the simultaneous and sequential BWE treatments, respectively (Fig. 3).

Discussion

In this paper, laboratory trials were conducted to explore the possibility that the salinity tolerance of NIS may have allowed them to colonize the Great Lakes despite current regulations governing ballast water discharge. Overall, there was variation in the salinity tolerance of the species tested, it being greatest for the

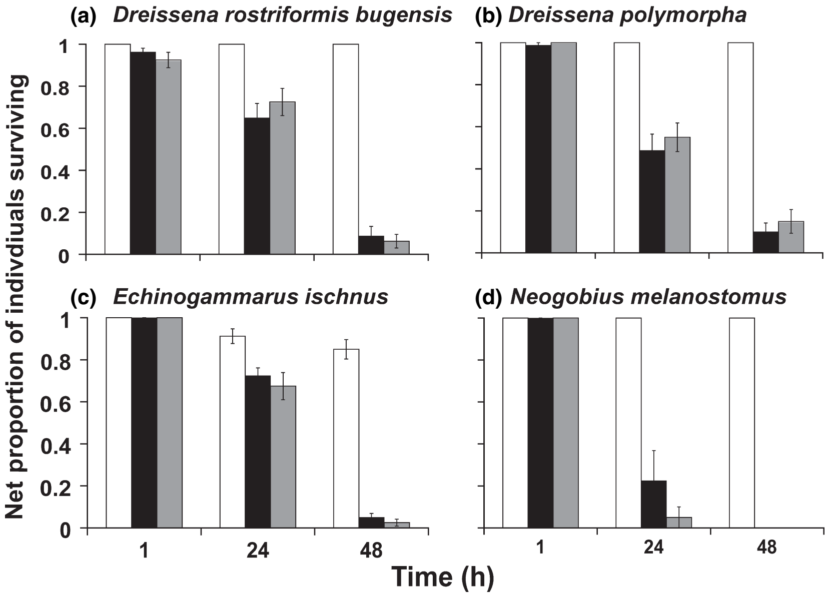

Fig. 4 Net proportion of individuals surviving after 1, 24 and 48 h (±SE) for those species with individuals surviving at least 24 h in either the simultaneous or sequential treatment for control (open bar), simultaneous (solid bar) and sequential (grey bar) exchange treatments.

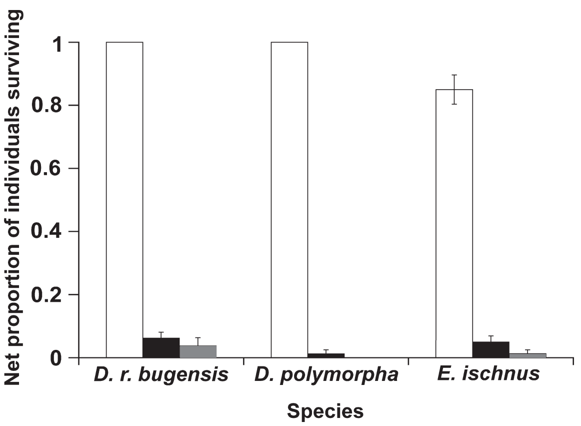

Fig. 5 Net proportion of individuals surviving after 1 h transfer to freshwater (±SE), and a total experimental duration of 49 h, for all species with individuals surviving in either the simultaneous or sequential treatment for control (open bar), simultaneous (solid bar) and sequential (grey bar) treatments.

crustacean *E. ischnus* and molluscs *D. polymorpha* and *D. r. bugensis.* However, the tolerance of even these species to full sea water depended greatly on length of exposure and the rate of salinity increase. Longer exposures were highly effective in dramatically reducing the viability of these species.

The ability of *D. polymorpha*, *D. r. bugensis* and *E. ischnus* to survive in water of 30‰ was unexpected, as none of these species has been reported alive in water even close to this salinity (Table 1). It should be noted, however, that these trials were relatively short-term and may not reflect actual conditions experienced by these species in a ballast tank of a vessel bound for the Great Lakes. Between 1 May and after 1 December, it is legally permissible for vessels to exchange water off the east coast of Canada in a region east of 63°W longitude (in the Laurentian Channel) (Canada Shipping Act, 2006). In most cases, vessels conduct BWE on the open Atlantic Ocean, off of the continental shelf. The time required to enter freshwater ports like Montreal or those on the Great Lakes would typically exceed five days from the time of BWE, far exceeding the length of trials conducted here and the reported tolerance of the species studied (Claudi & Ravishankar, 2006). If ships are closer to North America when completing BWE, such as in the Laurentian channel, it is possible, though improbable, for the most tolerant species tested here to survive until deballasting occurred in freshwater. In 2006, Transport Canada extended the ballast water flushing requirement to no ballast on board (NOBOB) vessels carrying only residual water and sediment to prevent possible introductions from these vessels (Canada Shipping Act, 2006). When salinity was gradually increased to 30‰ over a period of 72 h, all three of these species experienced 100% mortality (S. Ellis, pers. obs). Results of this study indicate that inherent salinity tolerance may allow certain species to survive in exchanged ballast for short periods of time, although it seems unlikely that this would be sufficient to allow introduction of large numbers of propagules. These findings are in broad accord with the experimental study of Gray *et al.* (2007), who observed very low survival of both planktonic and benthic invertebrates in exchanged ballast water.

Classical invasion theory suggests that species with broad environmental tolerances would have the best opportunities to invade new areas, given that they would be most likely to find suitable conditions in a new habitat (see Lodge, 1993). With particular regard to salinity tolerance, it is worth noting that the species with the greatest tolerance (*E. ischnus*, *D. polymorpha*, *D. r. bugensis*) in our experiments were not consistent with patterns reported in the literature (Table 1). Species including *C. pengoi, D. polymorpha, E. ischnus, H. anomala* and *N. melanostomus* from saline sections of the Caspian and Aral Seas, are exposed to $CaSO_4$ (rather than NaCl) dominant salinity in their native habitat (Strayer & Smith, 1993). Ocean water is typically dominated by NaCl, as are synthetic sea salts such as the Instant Ocean® used here. Mount *et al.* (1997) showed freshwater species have a lower tolerance to NaCl than to $CaSO_4$ salinity. Different ionic compositions may account for the discrepancies in reported salinity tolerance and that of species tested in solutions of Instant Ocean®. However, it is interesting that among the species tested, *D. polymorpha* has the greatest introduced range in Europe and North America (Table 1).

The salinity tolerances of *Dreissena polymorpha* and *D. r. bugensis* are <13‰ and <4‰, respectively (Mills *et al.*, 1996; Orlova, Khlebovich & Komendantov, 1998). A few individuals of these species survived at 30‰ for 48 h (Fig. 4). This ability to survive for extended periods in unfavourable conditions may be a consequence of life history and physiology. Mussels respond to toxins by closing shell valves to avoid exposure (Rusznak, Mincar & Smolik, 1994). Zebra mussels can remain closed for >48 h, depending on temperature and size (Rusznak *et al.*, 1994). These animals may experience hypoxia while valves are closed, though they periodically open them to assess conditions (Ricciardi, Serrouya & Whoriskey, 1995; Rajagopal *et al.*, 2003). Animals exposed to saline treatments were often in poor condition, with valves gaped. These animals were typically unresponsive to probing and occasionally were floating, indicating that they were highly stressed and near death. A conservative endpoint was selected, based upon previous observation that some mussels could recover in freshwater even if their valves were gaping and they were unresponsive to probing (S. Ellis, pers. obs.). Therefore, survival may have been overestimated based on these experiments. While both *D. polymorpha* and *D. r. bugensis* possess limited ability to adapt to slow and small increases in salinity, these species probably would not survive the period of salinity increase characteristic of BWE, or the duration following BWE required to reach port.

Survival of *Dreissena* veliger larvae was poor, and less than that of adults (Fig. 4). These findings conflict with those of studies which found veliger tolerance was similar to that of adults (Kilgour *et al.*, 1994; McMahon, 1996; Barnard, Frenette & Vincet, 2003). While adult mussels are protected by their impermeable valves, veligers lack this structure and therefore are exposed directly to unfavourable conditions. It is unlikely that dreissenids would be transferred to the Great Lakes via exchanged ballast water as adults. Minchin & Gollasch (2003) proposed that adult dreissenids are more likely to enter the Great Lakes through hull fouling.

Some *E. ischnus* individuals survived extended periods in both treatments (Figs 4 & 5). *Echinogammarus ischnus* experienced some mortality in controls due to cannibalism and therefore results from the treatments may overestimate mortality. However, even after taking mortality in control treatments into consideration, results indicate that simultaneous and sequential exchange both cause substantial mortality, as compared to untreated ballast. This is a euryhaline species with a European distribution that extends from the Black and Caspian Sea, through the canals and inland waters of Europe, to the Baltic and North Seas (Jażdżewski, 1980) (Table 1). A recent *in situ* experiment involving caged *E. ischnus* within ballast tanks revealed no survival following open ocean sequential BWE, although survival was moderate in unexchanged, companion tanks (Gray *et al.*, 2007). This finding suggests that our laboratory experiment may have overestimated survival relative to ballast tank conditions. Within ballast tanks, mortality may be caused by interacting stressors including anoxia, fluctuations in pH and temperature, agitation of water within the tank, inadequate food supply and complete darkness (Gollasch *et al.*, 2000; Olenin *et al.*, 2000). However, laboratory results are based on the assumption of completing mixing within ballast tanks. Recent computational fluid dynamics models of ballast tanks indicate that mixing is incomplete (Wilson *et al.*, 2006); therefore salinity within tanks may not be uniform. If portions of the tank remain brackish, our results may underestimate survival.

Neogobius melanostomus had a surprisingly low ability to survive at high salinities (30‰) when compared to its reported salinity tolerance (Table 1). This may be due to differences in salinity composition or in the acclimation history of animals in the Great Lakes, as compared to those in the Ponto-Caspian region. It has been established that distinct populations of a species can have variable tolerance ranges, potentially indicating that those *N. melanostomus* found in the Aral Sea are from a different population than that which colonized the Great Lakes (Boersma, De Meester & Spaak, 1999; Kamal & Mair, 2005). This experiment indicates that the *N. melanostomus* population sampled for this study would not be able to survive either type of BWE.

The three cladoceran species tested (*B. coregoni*, *B. longimanus*, *C. pengoi*) experienced 100% mortality in <5 h in both simultaneous and sequential BWE treatments (Fig. 3). These species experienced some mortality in controls due to handling. However, the mortality of *C. pengoi* in controls was also due to cannibalism and negative interactions caused by tail spine entanglement. Taking these factors into account, these species still appear to be sensitive to the salinity increases characteristic of BWE, as each have a low reported salinity tolerance (Table 1). However, these species all produce diapausing eggs, which are far more resistant than adults. Holeck *et al.* (2004) examined different shipping vectors that could have brought these species into the Great Lakes. It is not possible to discount entry in low salinity residual ballast water by adults or by resting stages in ballast tanks.

The most recently discovered NIS, *Hemimysis anomala*, responded to salinity exposure in a manner similar to that of the cladocerans, with both simultaneous and sequential treatments resulting in 100% mortality in <5 h, despite having a higher reported salinity tolerance (Table 1). Native to the Ponto-Caspian region, *H. anomala* was discovered in Lake Michigan in water temperatures of 6.4–8 °C (Pothoven *et al.*, 2007). In this study, *H. anomala* was collected at a temperature of 5.6 °C in April 2007, and the population reached peak abundance soon afterwards (S. Pothoven, pers. comm.). When returned to the laboratory, animals were acclimated to a temperature of 20 °C, which is the maximum reported temperature at which they survive (Pothoven *et al.*, 2007). The relationship between temperature and salinity is well documented, with salinity tolerance of crustaceans inversely related to temperature (Dorgelo, 1976). The mortality of *H. anomala* in controls was low (i.e. 30% after 48 h), indicating that temperature alone did not cause mortality in BWE treatments. The interaction between salinity and temperature may have caused individuals to die more quickly than if the temperature had been lower (Dorgelo, 1976). However, the reported salinity tolerance of 18‰ suggests that, even if temperature was lower, animals would not have been able to survive for the duration of the experiment in either the sequential or simultaneous BWE treatments.

These results may be used to infer transport mechanisms for recent NIS, to assess whether ballast water exchange could have prevented introductions had such regulations been in place before 1993, and to demonstrate the efficacy of BWE upon the survival of known NIS. Five of the eight species (*B. coregoni*, *B. longimanus*, *D. polymorpha*, *D. r. bugensis*, *N. melanostomus*) studied were discovered in the Great Lakes before BWE regulations were mandatory. *Bosmina coregoni* and *B. longimanus* were reportedly established in the Great Lakes in 1966 and 1982, respectively, and results indicate that their introductions could have been prevented had regulations been in place for all vessels, unless entry was by diapausing stages (Wells, 1970; Johannsson, Mills & O'Gorman, 1991). *Dreissena polymorpha*, discovered in Lake St. Clair in 1988, would probably not have survived BWE and, even if it did, there would have been few founders of an invasion (Hebert, Muncaster & Mackie, 1989). Finally, *D. r. bugensis* and *N. melanostomus* were found in 1989 and 1990, respectively, and probably entered via ships that did not conduct BWE at sea (Jude, Reider & Smith, 1992; Mills *et al.*, 1993).

The remaining three species (*E. ischnus*, *C. pengoi*, *H. anomala*) were found following the implementation of BWE regulations. *Echinogammarus ischnus* was discovered in the Detroit River in 1995 (later backdated to 1994 or 1993), and probably could not have entered in exchanged ballast water (Witt, Hebert & Morton, 1997; van Overdijk *et al.*, 2003). Similarly, *C. pengoi* and *H. anomala*, both reported well after ballast regulations were implemented, do not appear capable of surviving the high salinities achieved after a tank has completed a full ballast exchange (MacIsaac *et al.*, 1999; Pothoven *et al.*, 2007). It is unlikely that these species experienced an extensive time lag between introduction and discovery, as both are conspicuous (Ricciardi & MacIsaac, 2008). These species may have entered through ballast water which did not reach the required salinity of 30‰ or, in the case of the *C. pengoi*, as resting stages in ballast sediment or through an untreated NOBOB vessel. NOBOBs are vessels which enter the Great Lakes carrying cargo and therefore have no declarable ballast on board. However, ballast tanks have been found to carry residual sediment and water that contains both viable individuals and resting stages (Bailey *et al.*, 2005). When cargo is discharged, ballast water is loaded and mixing of ballast water and residuals occur. This mix of ballast water and residuals, containing potential invaders, can subsequently be discharged at the next port of call, when cargo is loaded and ballast discharged (Duggan *et al.*, 2005).

The theory of 'propagule pressure' relates the probability of a successful biological invasion to the number of individuals introduced per event and the frequency of introductions (Williamson, 1996; Colautti, Grigorovich & MacIsaac, 2006b). As propagule pressure increases, so does the likelihood of a successful invasion (Colautti *et al.*, 2006b). Reducing the number of individuals within ballast tanks should decrease propagule pressure and the likelihood of successful invasion. In our studies, survival sometimes depended on which type of ballast exchange had occurred, though as time progressed all species converged on 100% mortality. When salinity was gradually increased to 30‰ over 72 h, all species experienced 100% mortality (S. Ellis, pers. obs). This suggests that propagule pressure for all of these species would be very low following BWE and, therefore, the probability of establishment would be low. While it is impossible to turn back the clock to prevent these species from colonizing the Great Lakes, ballast treatments (including BWE) that reduce effective propagule pressure should help slow global spread by shipping.

Simultaneous and sequential BWE were equally effective in reducing the viability of animals for all species studied. While sequential BWE appeared to reduce survival more quickly than simultaneous BWE exchange, for the majority of species these differences were small and not significant.

Ballast water exchange is an effective management tool for preventing the introduction of freshwater species into the Great Lakes. Flushing residual freshwater within ballast tanks with saline ocean water should similarly be effective, as long as exposure period is protracted and the species are of freshwater origin. Future studies should focus on the ability of estuarine species to survive highly saline conditions characteristic of BWE.

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