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Journal of Applied Ecology
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Journal of Applied Ecology 2015, **52,** 348–357 doi: 10.1111/1365-2664.12397

Hybrid system increases efficiency of ballast water treatment

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Summary

1. Ballast water has been a principal pathway of non-indigenous species introduction to global ports for much of the 20th century. In an effort to reduce the scale of this pathway, and recognizing forthcoming global regulations that will supplant ballast water exchange (BWE) with ballast water treatment (BWT), we explore whether a combined hybrid treatment of BWE and chlorination (Cl) exceeds individual effects of either BWE or chlorination alone in reducing densities of bacteria, microplankton and macroplankton.

2. Five full-scale trials were conducted on an operational bulk carrier travelling between Canada and Brazil.

3. The hybrid treatment generally had the lowest final densities among all treatments for putative enterococci, *Escherichia coli* and coliform bacteria, as well as microplankton and macroplankton, with the former two being synergistically lower than individual treatments alone. Microplankton abundance in the hybrid treatment was significantly but antagonistically reduced relative to individual treatments alone. Macroplankton final density was lowest in the hybrid treatment, though the interaction between treatments was not significant.

4. *Synthesis and applications*. In most cases, the combined hybrid treatment of ballast water exchange (BWE) and chlorination reduced population densities of indicator organisms in ballast water below those proposed by the International Maritime Organization's D-2 performance standards. BWE alone was often ineffective at reducing bacterial and macroplankton densities. Even when performance standards are implemented globally, continued use of BWE could further reduce risk of invasions to freshwater ecosystems that receive ballast water from foreign sources by accentuating the decline in propagule pressure and enhancing demographic constraints for putative invaders.

Key-words: additive, alien species, ballast water treatment, chlorine, IMO-D2, non-indigenous, synergistic

Introduction

The use of ballast water in vessels improves vessel stability, manoeuvrability and buoyancy, but is a dominant pathway for the introduction of non-indigenous species (NIS) (e.g. Carlton 1985). The enormous volume of transshipped ballast water may introduce a large number (i.e. high colonization pressure) and wide abundance (species' propagule pressures) of NIS (Lockwood, Cassey & Blackburn 2009). High colonization pressure favours invasion as it increases the probability that at least one released species will tolerate ambient conditions and possess a minimum required inoculum (Lockwood, Cassey & Blackburn 2009). Propagule pressure has three components: propagule size (number of individuals of one species released in an event), propagule number (number of release events), and health (vitality at the moment of introduction) (Simberloff 2009). Propagule size is critical, as it will influence the existence or severity of demographic constraints, whereas propagule number affects mainly environmental

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and, to a lesser extent, demographic stochasticity (Simberloff 2009).

Adoption of the International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO 2004) included the D-1 procedure requiring at least 95% volumetric exchange of ballast water (BWE) for ocean water at least 1000 m deep and 200 nautical miles from shore. BWE reduces the number of species transported in ballast tanks primarily by physical removal of entrained organisms, while killing remaining ones through osmotic shock (Santagata *et al*. 2008). The procedure has become routine on commercial vessels over the past fifteen years, although its efficiency varies widely (48 to >99%) depending on starting inocula, effectiveness of ballast purging and other factors (e.g. Drake *et al*. 2002; Bailey *et al*. 2011).

As a consequence of this wide variation and a desire for a more uniform and lower maximum total abundance of viable organisms, the IMO has proposed the D-2 performance standard (hereafter IMO D-2 standard; IMO 2004). This standard includes numerical limits for the maximum permissible discharge abundance of five biological indicator groups including intestinal enterococci, *Escherichia coli* (Migula 1895) and *Vibrio cholerae* (Pacini 1854) serotypes O1 and O139 bacteria, microplankton – minimum dimension between <50 and ≥10 μm and macroplankton – minimum dimension ≥50 μm. It also includes the promotion of new treatment methodologies for ballast water, which if combined with BWE could improve efficiency owing to synergistic or additive interactions between the two (Briski *et al*. 2013). Each IMO D-2 standard considers the sum of viable organisms within that group, and aims to reduce propagule size to a threshold below which released NIS are unlikely to establish a viable population owing to demographic constraints.

Here, we explore the efficacy of single and multiple treatment options in experiments conducted aboard an operating commercial bulk carrier. We specifically sought to determine whether a combined hybrid system involving BWE and treatment would provide greater protection than either treatment alone using IMO D-2 groups of bacteria, microplankton and macroplankton as indicators.

Materials and methods

Experiments were conducted on the bulk carrier Federal Venture during five trials between Canada and Brazil from April 2012 and March 2013 (Fig. 1). On the first, third and fifth trials, the vessel departed from Port Alfred, Quebec, whereas on the second and fourth trials, it departed from Trois Rivières and Bécancour, Quebec, respectively. While Port Alfred is a brackish port located on the Saguenay River (salinity range 0–30 PSU; St.-Onge *et al*. 2004), Trois Rivières and Bécancour are freshwater ports on the Saint Lawrence River (see Fig. 1).

Ten ballast tanks were used for the experiments, five matched pairs in starboard and port positions, with individual capacities ranging between 1016 and 1287 tonnes (=m^3; Fig. 2). In every trial, initial ballast water was drawn from the Saguenay or Saint

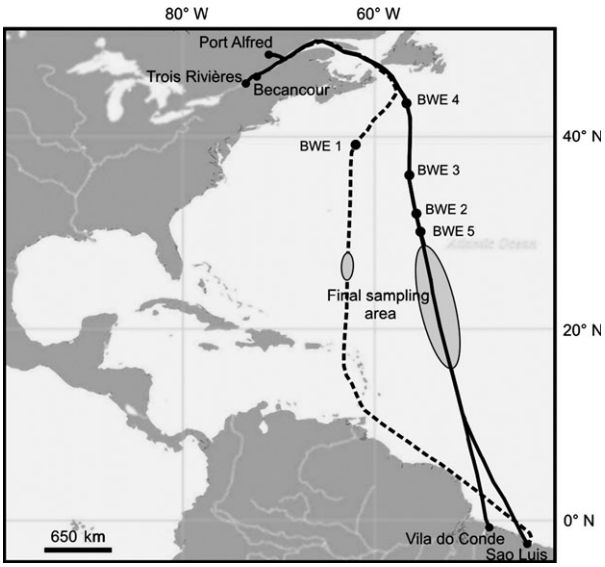

Fig. 1. Routes followed during the five trials (dashed line for the first trial, solid line for trials two through five) between Canada and Brazil. BWE one through five indicate the position of ballast water exchange for the trials one through five, respectively, and the solid line circle indicates area where final sampling was conducted.

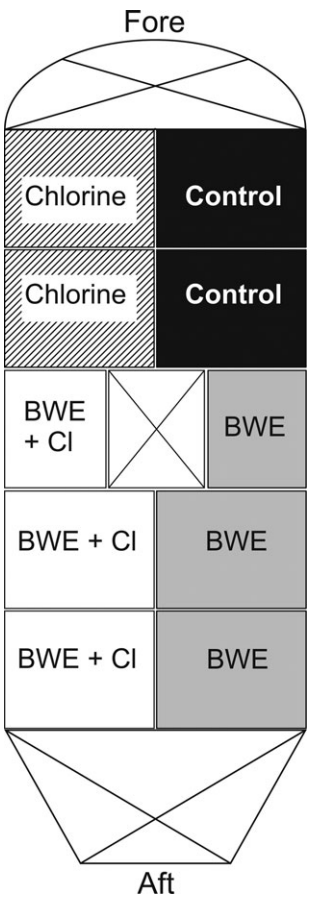

Fig. 2. Ballast tank schematic showing distribution of treatments during the trials one, two and five. Replication varied in trials three and four, with three chlorine, three control, two BWE + chlorine and two BWE tanks per trip.

Lawrence rivers using two pumps, one each on port and starboard sides. Tanks receiving chlorine were located on the port side of the vessel to prevent contamination of non-chlorinated tanks. Chlorine treatment tanks were dosed with industrial bleach (sodium hypochlorite 12%, equivalent to 12·0% W/V available Cl_2, Univar Canada) using a peristaltic pump, resulting in an initial dose of 20 mg L^{-1} (first four trials) or 10 mg L^{-1} (final trial; see below). Chlorine was directly delivered to the bottom of each ballast tank, 1 m from the intake pipe's bell mouth, thus ensuring comprehensive mixing with inflowing ballast water.

Physical and chemical conditions were measured *in situ* at the same time that biological samples were collected on the ballast water pumped to/from ballast tanks during initial and final sampling. Initial measures were carried out at the engine room before the water received the dose of chlorine. Samples were assessed using an Orion A230 meter for pH, Orion 130A meter for salinity and Orion A810 meter for dissolved O_2 and temperature. Triplicate, total suspended solid (TSS) samples were collected during initial and final sampling of each trial, filtered on board the vessel using pre-weighed 0·7 μm glass-fibre filters and stored at −20°C until weighed. For initial and final total organic carbon (TOC) measures, triplicate, unfiltered water samples of 0·5–1 L (from the 20-L containers, below) were filtered through a 0·75-μm pore-size Whatman GF/F glass microfibre, and kept at 4°C for TOC analysis using a Shimadzu TOC-VCSH analyser. Initial measures of TOC were used to estimate trihalomethanes (THMs; a by-product of chlorine reactions with organic matter present and a known health hazard to humans) using a simplified version of Hutton's model (Hutton & Chung 1994) in which:

$$\mathrm{THM} = 0{\cdot}00309 \times (\mathrm{TOC} \times 0{\cdot}462) \times (\mathrm{Cl_2})^{0{\cdot}409} \times (t)^{0{\cdot}265} \times (\mathrm{T})^{1{\cdot}07} \times (\mathrm{pH} - 2{\cdot}6)^{0{\cdot}695}$$

where TOC is total organic carbon in mg L^{-1}, Cl_2 is available chlorine (mg L^{-1}), t is time in hours, and T is temperature (°C).

Safety and technical issues during the discharge process restricted collection of samples and measurement of chlorine from the main deck; consequently, we estimated the initial chlorine concentration based on the volume of chlorine delivered and volume of water pumped into tanks. Once the discharge process was concluded, total chlorine concentration was determined using an ExTech Instruments-CL200 meter, on ballast water pumped from the ballast tank using same system used to collect final samples.

Initial biological sampling was carried out in port as ballasting was initiated, but at the engine room before the water was dosed with chlorine. These initial samples (for bacteria, microplankton and macroplankton) were collected directly from water bled off the starboard ballast pump discharge gauge in the engine room. One 1-m^3 water sample was filtered using a 35-μm mesh-size net for macroplankton. Three additional aliquots of unfiltered port water were collected at different times during the ballasting process, though we avoided the initial and final 20 min in order to collect representative samples (First *et al.* 2013), and then integrated the samples into a single 20-L sample. Sample volume was monitored using a Hydrobios flowmeter. During this process, as well as during ballast water exchange, the two ballast pumps received water from the same intake pipe and pumped water at the same time into tanks on each side of the vessel. Consequently, each sample collected from the starboard ballast pump was considered representative of the paired starboard and port tanks.

In each of the first two trials, two tanks from each starboard and port side were used for control and chlorine treatments, respectively, and ballast water exchange was not applied to these tanks. The remaining three tanks on each side were used for BWE and BWE + chlorine treatments, respectively (Fig. 2), where mid-ocean ballast water exchange was conducted in compliance with International Maritime Organization (IMO) procedures. During BWE, the vessel was stopped and allowed to drift (<28 km). Geographic coordinates of ballast water exchange varied for each trial (Fig. 1). In order to balance the total number of replicate tanks per treatment, during trials three and four, two tanks that previously served as BWE and BWE + chlorine treatments were re-assigned to control and chlorine treatments, respectively (see Fig. 2). The arrangement of treatments in the fifth trial was the same as in the first two, except that chlorine was reduced to 10 mg L^{-1} in an attempt to reduce its very strong effect (see Results). In total, after five trials, we had 12 control tanks, 12 chlorine-only treatment, 13 BWE-only treatment and 13 for hybrid treatment.

Ballast water exchange on the Federal Venture was based on the flow-through principle; thus, each event requires flushing the tank three times to comply with IMO guidelines. Chlorine was dosed throughout the ballast water exchange procedure to ensure the desired concentration was maintained. In order to analyse the biological composition of marine water pumped into the tanks during ballast water exchange, 'middle' samples were collected using the same methodology as per initial sampling in the engine room.

Final sampling was conducted about 3 days after the second dose of chlorine (i.e. following ballast water exchange) was applied. It was impossible to collect water via the ship's ballast pumps in the engine room; thus, all final samples were collected directly from three different levels (top, middle and bottom) in each ballast tank according to Murphy, Ritz & Hewitt (2002). An aliquot of ballast water was pumped from each level using a pneumatic, diaphragm pump (<35 L min^{-1}; Flowmeters Seametrics). Macroplankton samples were collected using different plankton nets for chlorinated and non-chlorinated treatments. Equal volumes of 333 L were pumped from the top, middle and bottom (total 1 m^3) of each tank. In order to clear water remaining in collection tubing, more than 300 L of ballast water was pumped out between aliquot collections. The sampling device had two outlets with valves and flowmeters; while one was used to collect the macroplankton sample, the other was used to collect unfiltered water from the same level. These samples of unfiltered water were integrated into a single 20-L sample, which was immediately analysed for microplankton abundance. To avoid contamination of the four treatments, different connecting pipes were attached to the pumps in each treatment. Similarly, different pneumatic pumps were used for both port and starboard sides.

Triplicate, unfiltered water samples for bacterial analysis were collected directly from the sampling pipe using sterilized 100-mL plastic jars during initial, middle and final sampling. For bacterial analyses, middle samples also included the control and chlorine treatments, which were collected 1 day prior to ballast water exchange. When necessary, bacteria samples were serially diluted using sterile deionized water, and sodium thiosulfate was added to neutralize chlorine. All samples containing marine water, such as those from the BWE treatment, were diluted tenfold using freshwater sterile deionized water before analysing bacterial populations. The number of colony-forming units (cfu) of the three bacterial indicator groups was assessed using US

EPA approved standard methods (Colilert and Enterolert Idexx kits, Idexx Laboratories Inc.). Each sample was mixed with a single test pack, poured and sealed into a Quanti-Tray/2000 using an Idexx Sealer 2×. Negative controls were performed using sterile deionized water every time samples were diluted. A comparator provided by Idexx was used to indicate a positive result via colour change or fluorescence. Protocols were modified from manufacturer recommendations following consultation with Idexx Laboratories personnel; specifically, Colilert and Enterolert trays were incubated for 24 and 48 h, respectively, at 36 ± 0·5°C, following which the number of positive cells were counted and used to estimate the most probable number of colony-forming units per 100 mL using an Idexx MPN table (http://www.idexx.com). We reduced incubation temperature due to space constraints on board the vessel from 41 ± 0·5 to 36 ± 0·5°C and increased the incubation time from 24 to 48 h for Enterolert kits. For Colilert, we used the recommended incubation time but increased incubation temperature from 35 to 36 ± 0·5°C. These changes allow the growth of heterotrophic bacteria in general, but may produce false positives for enterococci bacteria, and consequently overestimate abundance of this group, and, less likely, produce false negatives in Colilert testing. Given these non-standard incubation settings, results for enterococci, coliforms and *E. coli* should be considered putative for those bacterial IMO standards.

During bacterial sampling, an extra 100 mL sample was collected per tank for *Vibrio cholerae* analysis, either from the engine room for the initial and middle samples or from ballast water in ballast tanks for the final samples. Water was filtered through a 22-μm filter at the end of a syringe, following which the filter was washed with 10 mL of potassium buffer solution (Huq *et al.* 2012), frozen and transported to the laboratory for analysis. These samples were processed using a *V. cholerae* (Gene CTX) Real Time *PCR* kit (Liferiver™, Shanghai ZJ Bio-Tech Co, Shanghai, China), with an Applied-Biosystem 7500 Real Time PCR System to selectively identify the presence/absence of pathogenic strains (O1 and O139). Positive, internal (supplied in the kit) and negative controls were run in parallel to samples.

Three random, 500 mL subsamples were collected for microplankton (≥10 μm and <50 μm) analysis from each initial, middle and final sample by homogenizing the 20-L containers within five hours of collection. Fluorescein diacetate (F1303, Molecular Probes; Invitrogen, Carlsbad, CA, USA) and 5-chloromethylfluorescein diacetate, which react only on live cells with metabolic activity, were used to stain unfixed samples (Steinberg, Lemieux & Drake 2011). After staining 1 mL of each subsample and incubating it for 20 min at 25°C, replicates were loaded using a micropipette into 1-mL Sedgewick-Rafter counting chambers etched with 1-mm^2 grids. Fluorescent cells were then observed and counted at 100× under an inverted epifluorescent microscope (Carl Zeiss Axio Vert A1 FL-LED) equipped with an Illuminator LED for transmitted light, and LED Module 470 nm. Chlorophyll *a* concentration was determined by *in vivo* fluorescence using a handheld Aquafluor fluorometer (model 8000-010; Turner Designs, Sunnyvale, CA, USA). This meter was calibrated in the laboratory with a chlorophyll *a* solution of known concentration, which also was used to build a curve concentration–fluorescence values. This curve was adjusted using chlorophyll samples collected on board in each trip by filtering 0·5–1·0 L from the 20-L containers and kept at −20°C until analysed in the laboratory.

Live abundances of macroplankton were estimated by concentrating the 1-m^3 filtered sample into a Hydro-bios dilution bottle with a volume of 250 mL. Three subsamples of 1 mL for trial two and 5 mL in subsequent trials were measured using Hensen-Stempel pipettes. Each subsample was placed in a counting chamber for zooplankton (Hydro-Bios) and observed under a stereoscope (Leica model S8APO) to count live individuals.

The abundance of all taxonomic groups, in addition to chlorophyll *a* concentration, were transformed to satisfy statistical requirements using a log(x + ε) function, where x was the initial or final density of live organisms and ε is 0·1 of the last significant digit in N measurements (0·001 for chlorophyll and 0·1 for others). Additionally, the effective growth rate (r) was calculated as follows:

$$r = \log((N_{\text{final}} + \varepsilon)/(N_{\text{initial}} + \varepsilon) + 1)$$

where N_{final} and N_{initial} are final and initial densities, respectively.

Effective growth rate of each biological indicator was analysed using the following general linear model where we assumed r is a random variable with mean μ:

$$\mu_{\text{Control}} = \mu$$

$$\mu_{\text{BWE}} = \mu + \hat{a}_{\text{BWE}}$$

$$\mu_{\text{Cl}} = \mu + \acute{a}_{\text{Cl}}$$

$$\mu_{\text{Cl+BWE}} = \mu + \acute{a}_{\text{Cl}} + \hat{a}_{\text{BWE}} + \tilde{a}_{\text{Cl+BWE}}$$

where μ_{BWE}, μ_{Cl} and $\mu_{\text{Cl + BWE}}$ are mean values for different treatments, $\acute{a}_{\text{Cl}}$ and $\hat{a}_{\text{BWE}}$ are called 'effects' for chlorine and BWE treatments, respectively, and $\tilde{a}_{\text{CL + BWE}}$ is the interaction. We tested whether there was no interaction between BWE and chlorine treatment effects. Then, the null hypothesis was that there was no interaction: H_0: $\tilde{a}_{\text{Cl+BWE}} = 0$ or $\mu_{\text{Control}} + \mu_{\text{Cl+BWE}} - \mu_{\text{BWE}} - \mu_{\text{Cl}} = 0$; synergistic interaction: H_a: $\tilde{a}_{\text{Cl + BWE}} < 0$, since $\mu < 0$; or antagonistic interaction: H_a: $\tilde{a}_{\text{Cl + BWE}} > 0$. Statistical differences in r values between treatments and interaction effects were analyzed using a block design ANOVA, using trial number as a blocking factor. Our model incorporated two levels for BWE (yes or no), and three levels for chlorine (0, 20 or 10 mg L^{-1}) to assess the effect of these variables for all biological groups. We also tested for differences in environmental variables between sampling time (Initial or final sampling) and among treatments (control, BWE, chlorine, or hybrid) using 2-way ANOVA with Statistica version 7.0 (StatSoft Inc., Tulsa, OK, USA).

Results

ENVIRONMENTAL CONDITIONS

While initial temperature of ballast water varied between trials, all treatments had similar initial conditions (Fig. 3). Temperature tended to increase in all trials as time progressed ($F_{1,\ 32} = 23{\cdot}53$, $P < 0{\cdot}001$; Fig. 3), particularly in those that received BWE (Fig. 3). Similarly, most of the variation in final pH values also was associated with BWE, which increased from 7–7·5 to ~8 over the duration of the experiments (Fig. 3). Freshwater ballast declined slightly in pH over the course of the experiments (Fig. 3).

Oxygen and TSS concentrations exhibited variation between tanks at both initial and final sampling (Fig. 3). During trials one and five, oxygen concentration decreased in treatments with BWE as compared to those without it. However, during trials two and four, the initial and final values were similar, and only in trial three, there was a general increase in final oxygen values, mostly due to low initial values. In general, TSS concentration was higher in control tanks, and lower in tanks with chlorine, BWE and especially in the hybrid treatment.

Initial salinity of the water pumped to ballast tanks was variable between trials at Port Alfred, whereas Trois Rivières and Bécancour had values close to zero due to their location on the Saint Lawrence River. Final salinity values in control and chlorine treatments for all trials were similar to those recorded during initial sampling (Fig. 3). Final salinity was much higher in ballast tanks that involved BWE, reaching the mandatory value of 30 PSU ($F_{3,32} = 8{\cdot}37$, $P < 0{\cdot}001$; Fig. 3).

Our estimated initial chlorine doses for trials one to five averaged between 10·0 and 21·8 mg L^{-1} for tanks that were

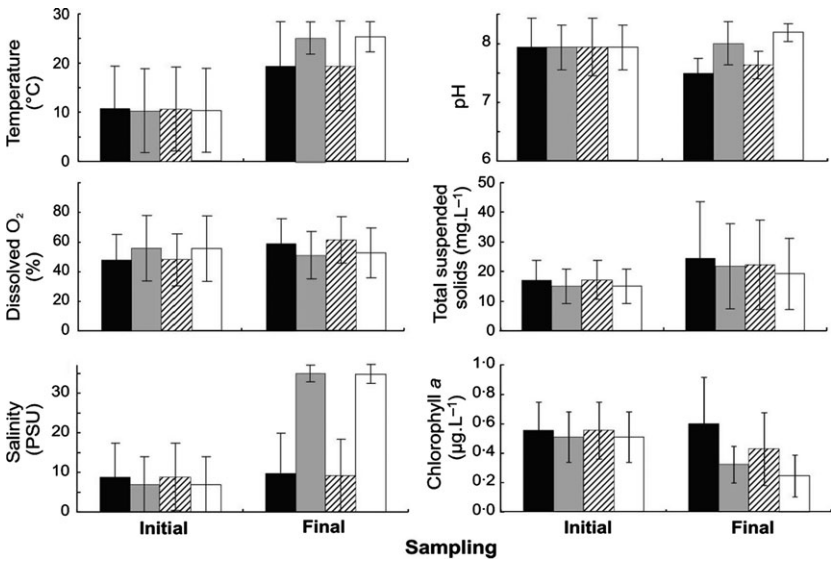

Fig. 3. Initial and final mean (±SD) values for environmental variables for control (black bars), BWE (grey bars), chlorine (diagonal striped bars) and hybrid treatments (white bars).

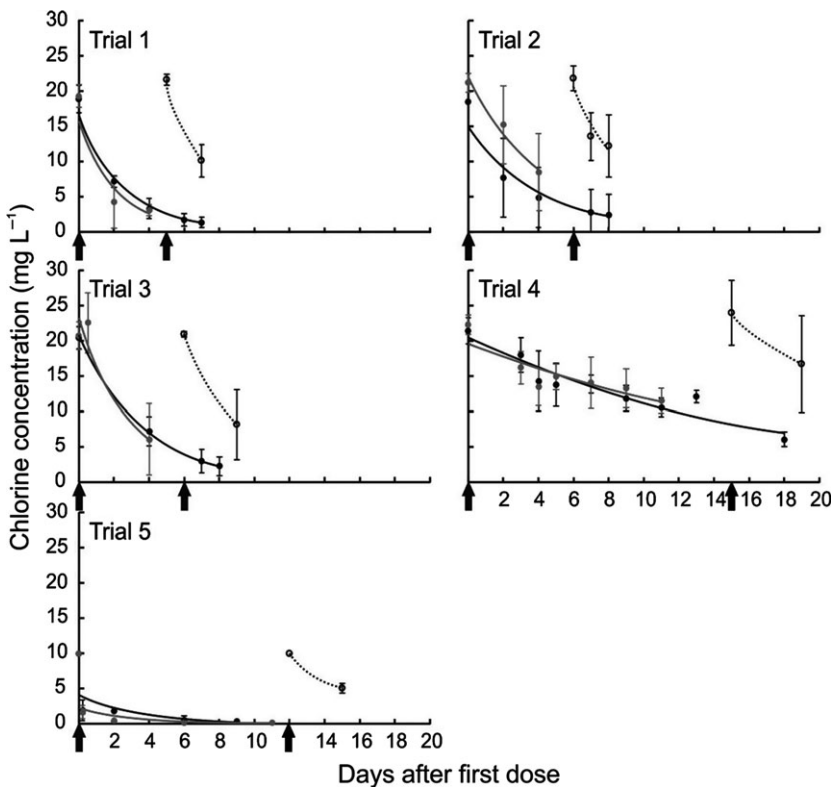

Fig. 4. Mean (±SD; dots and vertical lines) and modelled (solid lines) chlorine concentration (mg L^{-1}) in ballast tanks during trials one to five. The onset of chlorination is indicated by vertical arrows below the *x*-axis. Dashed lines represent chlorine concentration for the ballast tanks that received a second dose of chlorine during the BWE (hybrid treatment).

Table 1. Formation of trihalomethanes (THMs; μg L^{-1}) estimated using the Hutton model (Hutton & Chung 1994) and total organic carbon (TOC; μg L^{-1}) (in brackets) in ballast water at the port of origin

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
THM (mean ± SD)	1·19 ± 0·95	4·25 ± 0·77	0·93 ± 0·52	5·19 ± 6·10	0·56 ± 0·35
TOC (mean ± SD)	2·95 ± 2·33	4·35 ± 0·93	2·18 ± 0·78	9·74 ± 11·84	4·73 ± 2·39

dosed, while all non-dosed tanks were <0·4 mg L^{-1} (Fig. 4). Chlorine concentration decreased rapidly in dosed tanks during the first 4 days, though decay rate varied from tank to tank during the first four trials (Fig. 4). Measured chlorine decay was very swift during the final trial, dropping to ~0·5 mg L^{-1} within hours of dosing (Fig. 4). Calculated THM concentration ranged between 0·56 and 5·19 μg L^{-1}, with higher values associated with high TOC concentrations in initial ballast water (Table 1).

Table 2. Effect of ballast water exchange (yes or no) and chlorine (0, 20 or 10 mg L^{-1}) on indicator group abundances. ANOVA models also considered trial number (Trial #) as a blocking factor. Effect size represents the percentage of the final treatment (BWE, chlorine and hybrid) as a function of the control. Error degree of freedom (d.f.): 31 for macroplankton and 40 for the other groups

Source	d.f.	*F*	*P*	Coefficients	Effect size (%)
Enterococci bacteria					
Trial #	4	7·53	0·0002		
BWE	1	0·00	0·9947	1·14	334·80
Chlorine	1	146·94	0·0001	−5·31	7·59
BWE*Chlorine	1	4·93	0·0321	−1·52	0·19
10 vs. 20 ppm	1	0·07	0·7908	2·10	
Coliform bacteria					
Trial #	4	14·02	0·0001		
BWE	1	0·78	0·3830	0·15	46·20
Chlorine	1	454·57	0·0001	−7·91	0·01
BWE*Chlorine	1	1·61	0·2120	−0·87	0·00
10 vs. 20 ppm	1	0·19	0·665	0·86	
E. coli bacteria					
Trial #	4	23·80	0·0001		
BWE	1	2·77	0·1040	1·60	874·70
Chlorine	1	93·51	0·0001	−2·58	0·64
BWE*Chlorine	1	5·61	0·0228	−1·65	0·00
10 vs. 20 ppm	1	3·83	0·0573	−1·10	
Microplankton					
Trial #	4	3·93	0·0088		
BWE	1	10·60	0·0023	−2·19	6·03
Chlorine	1	37·66	0·0001	−3·96	0·48
BWE*Chlorine	1	4·02	0·0518	1·96	0·29
10 vs. 20 ppm	1	4·72	0·0359	2·99	
Chlorophyll (algae)					
Trial #	4	3·09	0·0261		
BWE	1	13·52	0·0007	−0·48	56·54
Chlorine	1	8·74	0·0052	−0·52	69·93
BWE*Chlorine	1	0·11	0·7427	0·14	43·85
10 vs. 20 ppm	1	0·22	0·6400	0·88	
Macroplankton					
Trial #	3	2·61	0·0691		
BWE	1	0·51	0·4791	−1·00	21·33
Chlorine	1	52·96	0·0001	−5·23	11·33
BWE*Chlorine	1	0·33	0·5710	0·66	1·26
10 vs. 20 ppm	1	2·10	0·1577	−3·18	

BIOTA

We observed large differences among trials with respect to initial densities for each biological indicator group (significant block effect; Table 2). Treatment differences in biological conditions were typically minor at the beginning and often very pronounced at the end of a trial, highlighting strong treatment effects (Fig. 5). For all biological indicators (enterococci, coliforms, *E. coli*, microplankton and macroplankton), the BWE plus chlorination treatment had the lowest final mean density, often followed closely by the chlorine-only treatment (Table 2 and Fig. 5).

In most cases, we observed a trend of decreasing abundance over time for all biological indicators, except for *E. coli* in the first and third trials of the BWE treatment, coliforms in the first trial and enterococci in the third trial. Toxigenic *Vibrio cholerae* O1 or O139 were not detected in any samples.

The control treatment had the highest final abundance of coliforms, microplankton and macroplankton, followed by the BWE treatment (Fig. 5). The overall effect of BWE was significant only for microplankton and chlorophyll *a* concentration (Table 2 and Fig. 5). Surprisingly, BWE resulted in higher mean final abundances of enterococci and *E. coli* relative to controls, although differences were minor ($P > 0·05$) owing to pronounced variation within treatments and trails. Variation was especially pronounced for *E. coli* and enterococci in the third trial, and for *E. coli* and coliforms in the first trial. Similar results were obtained for relative growth rates of these indicator taxa (Fig. 6). Our macroplankton samples from oceanic water during BWE (labelled 'Middle' in Fig. 5) demonstrated entrainment of a new community, which almost certainly influenced final abundances. Macroplankton final densities never exceeded 500 ind. m^{-3} and were lowest in the fourth trial, which also happened to be the longest.

In general, the chlorine-only and hybrid treatments had the lowest final abundance values and thus highest efficiency among all treatments for enterococci, coliforms, *E. coli*, microplankton and macroplankton (Fig. 5). Chlorine had a strong suppressive effect on IMO indicator groups as well as coliform bacteria and chlorophyll *a* concentration (two-way ANOVA tests, $P = 0·0001$ and 0·0052, respectively; Table 2), though were often not as strong as in the

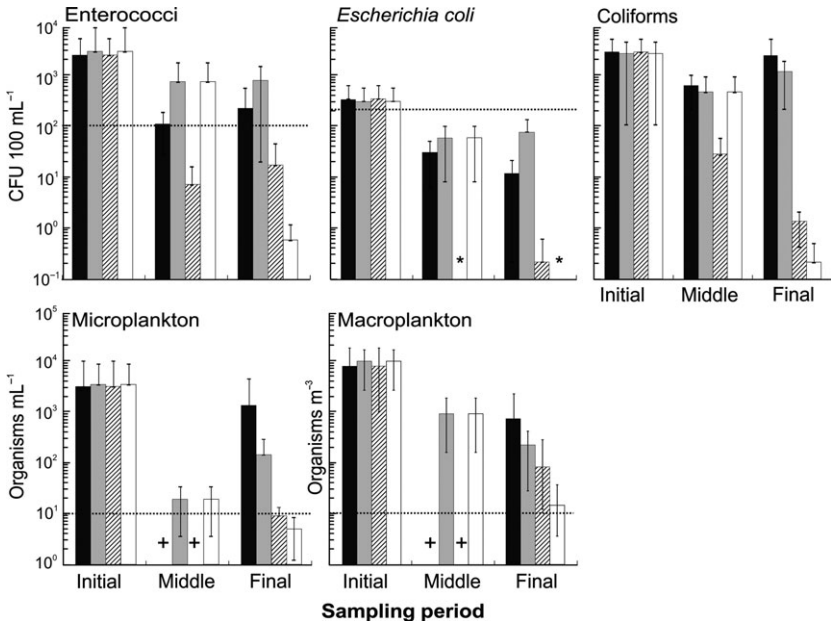

Fig. 5. Changes in densities (log-transformed initial, middle and final mean values ± SD) of putative enterococci, coliforms, *E. coli*, viable microplankton (≥10 μm and <50 μm) and viable macroplankton (≥50 μm) in all four treatments. Black, grey, diagonally striped and white bars are control, BWE, chlorine and hybrid treatments, respectively. Dotted lines indicate the proposed IMO D-2 performance standard maximum limit for each group. * = 0; + = No sample. CFU, colony-forming units.

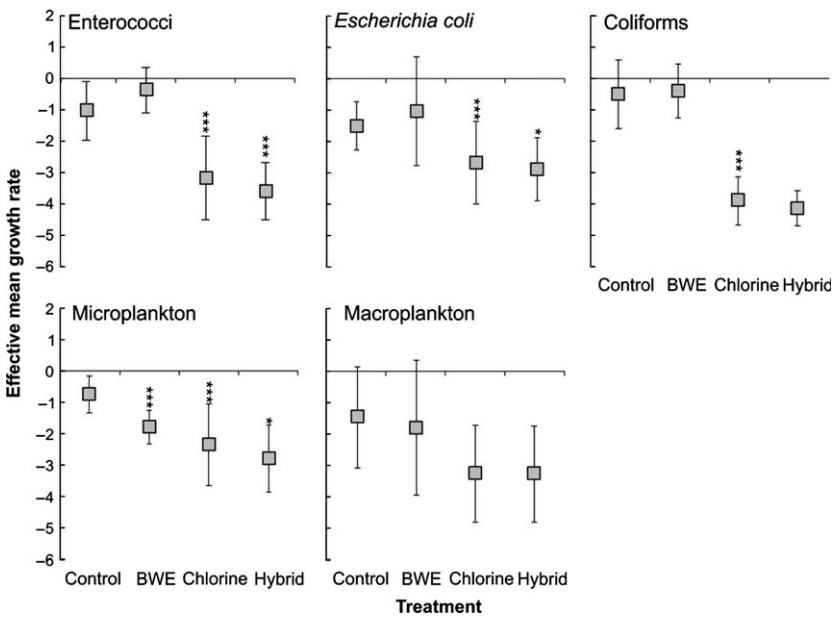

Fig. 6. Effective mean growth rate ± SD (r; grey squares) for the five biological indicators in control, BWE, chlorine and hybrid treatments. Upper asterisk indicates significant treatment effects with $P \leq 0{\cdot}0001$ (***) and 0·05 (*) based on two-way ANOVA.

hybrid treatment (Figs 3 and 5). The chlorine-only treatment was also very effective at reducing macroplankton abundance, though mean abundance exceeded 100 ind. m^{-3} (Fig. 5). Three chlorine trials (third, fourth and fifth) had no viable zooplankton when the experiments ended. Chlorine was the only treatment that affected effective growth rate of macroplankton ($P < 0{\cdot}0001$, Table 2).

While the final absolute abundance of each of the three bacteria indicators was higher when chlorine was dosed at 10 (fifth trial) vs. 20 mg L^{-1} (first four trials), only *E. coli* was significantly reduced at the higher dose (Table 2). Similarly, lower microplankton density was observed with the higher dose of chlorine ($P = 0{\cdot}0359$; Table 2). Chlorine dose had little effect on final viable macroplankton abundance ($P = 0{\cdot}1577$; Table 2).

The effective growth and final abundances of bacteria and microplankton were also affected by an interaction between BWE and chlorination (Figs 5 and 6; Table 2). This interaction was synergistic for enterococci and *E. coli* ($P = 0{\cdot}0321$ and 0·0228, respectively) but not for coliforms ($P = 0{\cdot}2120$, Table 2), indicating stronger than additive reductions in abundance for the first two groups. Conversely, microplankton exhibited an antagonistic (i.e. less than additive) interaction (Table 2), signifying that the effect of the hybrid treatment was less than the sum of individual treatments. The hybrid treatment resulted in the lowest final densities for each of these groups. Chlorophyll *a* concentration behaved similarly to microplankton, with each affected by BWE and chlorine application, though the interaction between treatments was not

significant (Table 2). Mean viable macroplankton abundance was much lower in the hybrid than in other treatments (Fig. 5). Even so, the effective growth rate was not affected by an interaction between treatments (Fig. 6; Table 2). Mean final abundance was also slightly above the proposed permissible IMO D-2 performance limit (Fig. 5). Density of macroplankton in BWE-only treatments was often higher than controls, and well in excess of IMO D-2 proposed limits.

Discussion

Ballast water has been a key pathway for global spread of aquatic non-indigenous species during the 20th century (Carlton 1985). Management of ballast water has evolved over the past three decades, from a virtual laissez-faire approach to global standards via treaties developed by the IMO. Currently, ballast water management typically involves protective guidelines such as not ballasting at night in areas with known invasive species and/or 95% volumetric BWE on the open ocean (IMO D-1 standard). Some countries (e.g. Canada, Norway, Australia, USA) have codified this standard into enforceable domestic regulations. The IMO's proposed performance standards (D-2) will place numerical limits on permissible discharges of viable organisms from ballast water. Our on-board experiments demonstrated the greatest population reductions of organisms subject to D-2 performance standards with the hybrid treatment (BWE + Cl), with a significant synergistic interaction between these treatments for some indicators. These results underscore the potential benefit of combining BWE with treatment technologies to consistently reduce population abundances of aquatic organisms beyond the current and widespread use of ballast water exchange alone.

Our experiments were conducted under realistic scenarios on board an operating vessel that was outfitted to allow collection of samples from major sections of ballast tanks, thereby incorporating vertical variation in distributions of biota (Murphy, Ritz & Hewitt 2002; First *et al.* 2013). Reductions in abundance of bacteria, microplankton and macroplankton in untreated (control) ballast water in relation to voyage length are consistent with previous studies (Drake *et al.* 2002; Tomaru *et al.* 2010). Final densities of bacterial indicator taxa in control tanks were very close to or exceeded those prescribed by IMO D-2 limits. Moreover, in some of the trials, final densities for bacteria were higher than middle and initial concentrations (Fig. 5), which was probably related to the gradual temperature increase and favourable oxygen conditions as the vessel moved through progressively warmer water, or to increased dissolved organic matter released by decomposition of phytoplankton and zooplankton inside ballast tanks (Tomaru *et al.* 2010).

Microplankton experienced a sharp reduction in abundance in control tanks over time, consistent with other reports of effects of darkened conditions in ballast tanks on photosynthetic biota (Gollasch *et al.* 2000; Drake *et al.* 2002). Nevertheless, final mean values exceeded the IMO's D-2 standard of 10 ind. mL^{-1}. Absent ballast water management, a comparatively large number of individuals of macroplankton could be released at the recipient port in violation of the proposed IMO D-2 performance standard. This problem would be particularly acute on short trips, as final abundance is affected by voyage time and survival rate (Wonham, Lewis & MacIsaac 2005; Chan *et al.* 2014).

The higher bacteria and macroplankton densities after BWE relative to controls (Figs 5 and 6) accord with earlier studies conducted in marine environments and highlight the fact that BWE cannot by itself serve as an effective ballast water treatment (e.g. Drake *et al.* 2002 and Briski *et al.* 2012, 2013). Unlike patterns observed in vessels operating between freshwater ports (Bailey *et al.* 2011), our final densities were influenced by replenishment of new live marine organisms during the exchange from freshwater to seawater, and consequently, macroplankton density exceeded the proposed IMO D-2 standard (Fig. 5). BWE was, however, effective at suppressing abundance of microplankton (Table 2), consistent with other studies (e.g. Drake *et al.* 2002; Taylor *et al.* 2007).

The effectiveness of chlorine as a biocide for bacterial and microplankton populations is very well-established (Gregg & Hallegraeff 2007; Maranda *et al.* 2013), with high efficiency at concentrations ranging from 4 to 50 mg L^{-1}. Our results support this effectiveness, particularly at the higher dose (20 mg L^{-1}; Figs 5 and 6). However, the application of chlorine (20 or 10 mg L^{-1}) resulted in consistent achievement of proposed IMO D-2 standards only for bacterial indicators, whereas results for microplankton varied between trials (Fig. 5). This differential was previously observed by Gregg & Hallegraeff (2007), who found complete bacterial inhibition at 15 mg L^{-1}, while more than 25 mg L^{-1} was required to eliminate vegetative cells and cysts of dinoflagellates. Our results demonstrated that a dose of 20 mg L^{-1} yielded significantly higher efficiency than 10 mg L^{-1} with respect to decreasing microplankton density.

Many devices under development for ballast water treatment use chlorination either directly applied or via electrochlorination. These devices rely on a timed exposure of a constant dose (Lloyd's 2011), whereas we utilized a pulse that delivered a high initial dose that over time was reduced as chlorine oxidized organic matter. Our aim was to keep the chlorine concentration above 2 mg L^{-1} and therefore effective as a biocide over a long period of time. In our trials, macroplankton were very sensitive to chlorine; mean final densities were lowered almost an order of magnitude relative to controls (Fig. 5), and in three of the trials, the final abundance was zero. These results mirror those of Maranda *et al.* (2013) despite their use of a constant dose.

Regardless of the chlorine and initial organism concentrations, when chlorine was combined with BWE the final

bacterial, microplankton and macroplankton densities were the lowest recorded (Fig. 3 and Table 1). Briski *et al.* (2013) also demonstrated potential benefits of combining BWE with ballast water treatment (UV radiation), which resulted in a strong reduction of all groups.

At least two non-exclusive mechanisms may explain the significant synergistic interaction observed with bacterial populations. First, higher killing efficiency of chlorine may result from osmotic shock associated with BWE (Briski *et al.* 2013). Secondly, lower organic matter concentration of open ocean water relative to freshwater may better facilitate biocide action (Dychdala 1968).

The hybrid treatment resulted in a significant antagonistic interaction for microplankton, with the final density higher than would be expected if the two treatments were additive (Fig. 5). A likely reason for this lower efficiency is the higher resistance to chlorine of some microplankton, such as cyst-forming dinoflagellates (Gregg & Hallegraeff 2007). Despite this undesirable antagonistic interaction effect, the hybrid treatment was the only one in which final microplankton density was consistently below the prescribed IMO D-2 limit.

The interaction term between treatments was not significant for macroplankton due mostly to the effectiveness of the chlorine-only treatment. We acknowledge that there exists extensive variability in our data for this group (Fig. 5). The hybrid treatment was still the most effective, reducing final densities by almost an order of magnitude vs. chlorine alone, and more than an order of magnitude vs. ballast water exchange alone (Fig. 5).

The IMO D-2 performance standard refers to live organisms without regard to origin or, in most cases, taxonomy. Our studies confirm that combining BWE with chlorination offers enhanced efficiency with respect to reducing propagule pressure better than any either treatment alone for a variety of aquatic groups. Although, it remains unclear exactly how low propagule pressure must be to prevent an invasion, it is a key factor in reducing overall invasion risk (Lockwood, Cassey & Blackburn 2005). Nevertheless, any treatment that reduces propagule pressure, such as the hybrid management that combines treatment and BWE, should also reduce overall invasion risk. Middle ocean ballast exchange may provide an additional benefit for freshwater habitats (e.g. Great Lakes) that receive foreign ballast because freshwater organisms in original ballast are replaced by oceanic taxa that are unlikely to survive environmental conditions upon discharge into a freshwater port (Briski *et al.* 2013).

The IMO D-2 performance standard seeks to prevent new invasions primarily by reducing propagule pressure below critical thresholds, such that populations are introduced at densities below those requires for establishment. It is not yet clear, however, how the vastly different standards that will apply to microplankton and macroplankton will influence future invasion patterns (Briski *et al.* 2013). It seems plausible that macroplankton may become less frequent invaders and that future invasions could be dominated by microplankton as the proposed standard appears to be far more robust for the former than the latter group.

The ecotoxicity of chlorination, which generates by-products including trihalomethanes (THMs) in substantially larger quantity than occur naturally, must be monitored to ensure compliance with existing law. Although our estimates express the maximum possible amount of THMs generated, the actual amount produced could be lower. Nevertheless, any commercial treatment system that utilizes chlorine as a biocide must be cognizant and monitor production of THMs as well as residual chlorine in discharged ballast water.

Acknowledgements

We thank staff of Fednav Limited including Georges Robichon, John Stubbs, Mark Harney, Rajendra Singh and Roy Avijit. We also are grateful to captains and crew of the Federal Venture, and colleagues Samir Quershi, William Gaspar, Leila Ron, Colin Van Overdijk and Amanda Eryaud. This study was supported by Fednav Ltd. and the NSERC Canadian Aquatic Invasive Species Network. M.L. and H.J.M. were supported by NSERC Discovery Grants and Canada Research Chairs. We are grateful to the reviewers and Shelley Arnott for helpful comments on the manuscript.

Data accessibility

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.rm83s (Paolucci *et al.* 2015).

References

Bailey, S.A., Deneau, M.G., Jean, L., Wiley, C.J., Leung, B. & MacIsaac, H.J. (2011) Evaluating efficacy of an environmental policy to prevent biological invasions. *Environmental Science & Technology*, **45**, 2554–2561.

Briski, E., Bailey, S.A., Casas-Monroy, O., DiBacco, C., Kaczmarska, I., Levings, C. *et al.* (2012) Relationship between propagule pressure and colonization pressure in invasion ecology: a test with ships' ballast. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 2990–2997.

Briski, E., Allinger, L.E., Balcer, M., Cangelosi, A., Fanberg, L., Markee, T.P. *et al.* (2013) Multidimensional approach to invasive species prevention. *Environmental Science & Technology*, **47**, 1216–1221.

Carlton, J.T. (1985) Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. *Oceanography and Marine Biology: An Annual Review*, **23**, 313–372.

Chan, F.T., Briski, E., Bailey, S.A. & MacIsaac, H.J. (2014) Richness–abundance relationships for zooplankton in ballast water: temperate versus Arctic comparisons. *ICES Journal of Marine Science* **71**, 1876–1884.

Drake, L.A., Ruiz, G.M., Galil, B.S., Mullady, T.L., Friedman, D.O. & Dobbs, F.C. (2002) Microbial ecology of ballast water during a transoceanic voyage and the effects of open-ocean exchange. *Marine Ecology Progress Series*, **233**, 13–20.

Dychdala, G.R. (1968) Chlorine and chlorine compounds. *Disinfection, Sterilization, and Preservation* (eds C.A. Lawrence, S.S. Block & G.F. Reddish), pp. 135–158. Lea & Febiger Lippincott, Philadelphia.

First, M.R., Robbins-Wamsley, S.H., Riley, S.C., Moser, C.S., Smith, G.E., Tamburri, M.N. & Drake, L.A. (2013) Stratification of living organisms in ballast tanks: how do organism concentrations vary as ballast water Is discharged? *Environmental Science & Technology*, **47**, 4442–4448.

Gollasch, S., Lenz, J., Dammer, M. & Andres, H.G. (2000) Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. *Journal of Plankton Research*, **22**, 923–937.

Gregg, M.D. & Hallegraeff, G.M. (2007) Efficacy of three commercially available ballast water biocides against vegetative microalgae, dinoflagellate cysts and bacteria. *Harmful Algae*, **6**, 567–584.

Huq, A., Haley, B.J., Taviani, E., Chen, A., Hasan, N.A. & Colwell, R.R. (2012) *Detection, Isolation, and Identification of Vibrio Cholerae From the Environment*. *Current Protocols in Microbiology*, doi: 10.1002/9780471729259.mc06a05s26.

Hutton, P. & Chung, F. (1994) Correlating trihalomethane data. *Journal of Environmental Engineering*, **120**, 219–241.

IMO (2004) *International convention for the control and management of ships ballast water and sediments*. As Adopted by Consensus at a Diplomatic Conference at IMO (International Maritime Organization), London, England, February 13, 2004.

Lloyd's Register (2011) Ballast water treatment technology current status June 2011.

Lockwood, J.L., Cassey, P. & Blackburn, T. (2005) The role of propagule pressure in explaining species invasions. *Trends in Ecology & Evolution*, **20**, 223–228.

Lockwood, J.L., Cassey, P. & Blackburn, T.M. (2009) The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. *Diversity and Distributions*, **15**, 904–910.

Maranda, L., Cox, A.M., Campbell, R.G. & Smith, D.C. (2013) Chlorine dioxide as a treatment for ballast water to control invasive species: shipboard testing. *Marine Pollution Bulletin*, **75**, 76–89.

Murphy, K.R., Ritz, D. & Hewitt, C.L. (2002) Heterogeneous zooplankton distribution in a ship's ballast tanks. *Journal of Plankton Research*, **24**, 729–734.

Paolucci, E.M., Hernandez, M.R., Potapov, A., Lewis, M.A. & MacIsaac, H.J. (2015) Data from: Hybrid system increases efficiency of ballast water treatment. *Dryad Digital Repository*, http://dx.doi.org/10.5061/dryad.rm83s.

Santagata, S., Gasiūnaite, Z., Verling, E., Cordell, J., Eason, K., Cohen, J. *et al*. (2008) Effect of osmotic shock as a management strategy to reduce transfers of non-indigenous species among low-salinity ports by ships. *Aquatic Invasions*, **3**, 61–76.

Simberloff, D. (2009) The role of propagule pressure in biological invasions. *Annual Review of Ecology Evolution and Systematics*, **40**, 81–102.

Steinberg, M.K., Lemieux, E.J. & Drake, L.A. (2011) Determining the viability of marine protists using a combination of vital, fluorescent stains. *Marine Biology*, **158**, 1431–1437.

St.-Onge, G., Mulder, T., Piper, D.J.W., Hillaire-Marcel, C. & Stoner, J.S. (2004) Earthquake and flood-induced turbidites in the Saguenay Fjord (Québec): a Holocene paleoseismicity record. *Quaternary Science Reviews*, **23**, 283–294.

Taylor, M.D., MacKenzie, L.M., Dodgshun, T.J., Hopkins, G.A., De Zwart, E.J. & Hunt, C.D. (2007) Trans-Pacific shipboard trials on planktonic communities as indicators of open ocean ballast water exchange. *Marine Ecology Progress Series*, **350**, 41–54.

Tomaru, A., Kawachi, M., Demura, M. & Fukuyo, Y. (2010) Denaturing gradient gel electrophoresis shows that bacterial communities change with mid-ocean ballast water exchange. *Marine Pollution Bulletin*, **60**, 299–302.

Wonham, M.J., Lewis, M.A. & MacIsaac, H.J. (2005) Minimizing invasion risk by reducing propagule pressure: a model for ballast-water exchange. *Frontiers in Ecology and the Environment*, **3**, 473–478.

Received 30 August 2014; accepted 21 January 2015
Handling Editor: Shelley Arnott