Hybrid system increases efficiency of ballast water treatment

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Summary

1. Ballast water has been a principal pathway of nonindigenous 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.

Introduction

The use of ballast water in vessels improves vessel stability, manoeuvrability and buoyancy, but is a dominant pathway for the introduction of nonindigenous species (NIS) (e.g. Carlton 1985). The enormous volume of trans-shipped 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 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

76 vessels over the past fifteen years, although its efficiency varies widely (48 to
77 >99%) depending on starting inocula, effectiveness of ballast purging, and other
78 factors (e.g. Drake *et al.* 2002; Bailey *et al.* 2011).
79 As a consequence of this wide variation and a desire for a more uniform
80 and lower maximum total abundance of viable organisms, the IMO has proposed
81 the D-2 performance standard (hereafter IMO D-2 standard; IMO 2004). This
82 standard includes numerical limits for the maximum permissible discharge
83 abundance of five biological indicator groups including intestinal enterococci,
84 *Escherichia coli* (Migula 1895) and *Vibrio cholerae* (Pacini 1854) serotypes O1
85 and O139 bacteria, microplankton – minimum dimension between <50 and ≥10
86 µm, and macroplankton – minimum dimension ≥50 µm. It also includes the
87 promotion of new treatment methodologies for ballast water, which if combined
88 with BWE could improve efficiency owing to synergistic or additive interactions
89 between the two (Briski *et al.* 2013). Each IMO D-2 standard considers the sum of
90 viable organisms within that group, and aims to reduce propagule size to a
91 threshold below which released NIS are unlikely to establish a viable population
92 owing to demographic constraints.
93 Here we explore the efficacy of single and multiple treatment options in
94 experiments conducted aboard an operating commercial bulk carrier. We
95 specifically sought to determine whether a combined hybrid system involving BWE
96 and treatment would provide greater protection than either treatment alone using
97 IMO D-2 groups of bacteria, microplankton and macroplankton as indicators.
98 **Materials and methods**
99 Experiments were conducted on the bulk carrier Federal Venture during five
100 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 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)

126 measures, triplicates unfiltered water samples of 0.5–1 L (from the 20-L
127 containers, below) were filtered through a 0.75-µm pore-size Whatman GF/F glass
128 microfibre, and kept at 4°C for TOC analysis using a Shimadzu TOC-VCSH
129 analyser. Initial measures of TOC were used to estimate trihalomethanes (THMs;
130 a by-product of chlorine reactions with organic matter present and a known health
131 hazard to humans) using a simplified version of Hutton's model (Hutton & Chung
132 1994) in which:

133 $$THM=0.00309\times(TOC\times0.462)\times(Cl_2)^{0.409}\times(t)^{0.265}\times(T)^{1.07}\times(pH-2.6)^{0.695}$$

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

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

143 Initial biological sampling was carried out in port as ballasting was initiated,
144 but at the engine room before the water was dosed with chlorine. These initial
145 samples (for bacteria, microplankton and macroplankton) were collected directly
146 from water bled off the starboard ballast pump discharge gauge in the engine
147 room. One 1-m^3 water sample was filtered using a 35-µm mesh size net for
148 macroplankton. Three additional aliquots of unfiltered port water were collected at
149 different times during the ballasting process, though we avoided the initial and final
150 20 minutes in order to collect representative samples (First *et al.* 2013), and then

151 integrated the samples into a single 20-L sample. Sample volume was monitored
152 using a Hydrobios flowmeter. During this process, as well as during ballast water
153 exchange, the two ballast pumps received water from the same intake pipe, and
154 pumped water at the same time into tanks on each side of the vessel.
155 Consequently, each sample collected from the starboard ballast pump was
156 considered representative of the paired starboard and port tanks.
157 In each of the first two trials, two tanks from each starboard and port side
158 were used for control and chlorine treatments, respectively, and ballast water
159 exchange was not applied to these tanks. The remaining three tanks on each side
160 were used for BWE and BWE+chlorine treatments, respectively (Fig. 2), where
161 mid-ocean ballast water exchange was conducted in compliance with International
162 Maritime Organization (IMO) procedures. During BWE the vessel was stopped
163 and allowed to drift (<28 km). Geographic coordinates of ballast water exchange
164 varied for each trial (Fig. 1). In order to balance the total number of replicate tanks
165 per treatment, during trials three and four, two tanks that previously served as
166 BWE and BWE + chlorine treatments were re-assigned to control and chlorine
167 treatments, respectively (see Fig. 2). The arrangement of treatments in the fifth
168 trial was the same as in the first two, except that chlorine was reduced to 10 mg L^{-1}
169 in an attempt to reduce its very strong effect (see results). In total, after five trials,
170 we had 12 control tanks, 12 chlorine-only, 13 BWE-only, and 13 for hybrid
171 treatment.
172 Ballast water exchange on the Federal Venture was based on the flow-
173 through principle, thus each event requires flushing the tank three times to comply
174 with IMO guidelines. Chlorine was dosed throughout the ballast water exchange
175 procedure to ensure the desired concentration was maintained. In order to analyse

176 the biological composition of marine water pumped into the tanks during ballast
177 water exchange, ‘middle’ samples were collected using the same methodology as
178 per initial sampling in the engine room.
179 Final sampling was conducted about three days after the second dose of
180 chlorine (i.e. following ballast water exchange) was applied. It was impossible to
181 collect water via the ship’s ballast pumps in the engine room, thus all final samples
182 were collected directly from three different levels (top, middle and bottom) in each
183 ballast tank according to Murphy, Ritz & Hewitt (2002). An aliquot of ballast water
184 was pumped from each level using a pneumatic, diaphragm pump (<35 L minute^{-1};
185 Flowmeters Seametrics). Macroplankton samples were collected using different
186 plankton nets for chlorinated and non-chlorinated treatments. Equal volumes of
187 333 L were pumped from the top, middle and bottom (total 1 m^3) of each tank. In
188 order to clear water remaining in collection tubing, more than 300 L of ballast
189 water was pumped out between aliquot collections. The sampling device had two
190 outlets with valves and flowmeters; while one was used to collect the
191 macroplankton sample, the other was used to collect unfiltered water from the
192 same level. These samples of unfiltered water were integrated into a single 20-L
193 sample, which was immediately analysed for microplankton abundance. To avoid
194 contamination of the four treatments, different connecting pipes were attached to
195 the pumps in each treatment. Similarly different pneumatic pumps were used for
196 both port and starboard sides.
197 Triplicate, unfiltered water samples for bacterial analysis were collected
198 directly from the sampling pipe using sterilized 100-mL plastic jars during initial,
199 middle and final sampling. For bacterial analyses, middle samples also included
200 the control and chlorine treatments, which were collected one 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 were 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 2X. 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 hours, 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±0.5ºC and increased the incubation time from 24 to 48 hours for Enterolert kits. For Colilert we used the recommended incubation time but increased incubation temperature from 35 to 36±0.5ºC. These changes allow grow 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 lab for analysis. These samples were processed using a *V. cholerae* (Gene CTX) Real Time *PCR* kit (Liferiver^{TM}), 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) 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 minutes 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 100X under an inverted epifluorescent microscope (Carl Zeiss Axio Vert A1 FL-LED) equipped with an Illuminator LED for transmitted light, and LED Module 470nm. Chlorophyll *a* concentration was determined by *in vivo* fluorescence using a handheld Aquafluor fluorometer (model 8000-010; Turner Designs, Sunnyvale, California).

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:

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

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:

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

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

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

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

where μ_{BWE}, μ_{Cl} and μ_{Cl+BWE} are mean values for different treatments, $\acute{a}_{Cl}$, and $\hat{a}_{BWE}$ are called “effects” for chlorine and BWE treatments, respectively, and $\tilde{a}_{CL+BWE}$ 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: $\tilde{a}_{Cl+BWE} = 0$ or $\mu_{Control} + \mu_{Cl+BWE} - \mu_{BWE} - \mu_{Cl} = 0$; synergistic interaction: H_a: $\tilde{a}_{Cl+BWE}<0$, since $\mu < 0$; or antagonistic interaction: H_a: $\tilde{a}_{Cl+BWE}>0$. 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.

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 ($F_{1, 32}$ = 23.53, P < 0.001; 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,

299 during trials two and four the initial and final values were similar, and only in trial
300 three there was a general increase in final oxygen values, mostly due to low initial
301 values. In general, TSS concentration was higher in control tanks, and lower in
302 tanks with chlorine, BWE, and especially in the hybrid treatment.

303 Initial salinity of the water pumped to ballast tanks was variable between
304 trials at Port Alfred, whereas Trois Rivières and Bécancour had values close to
305 zero due to their location on the Saint Lawrence River. Final salinity values in
306 control and chlorine treatments for all trials were similar to those recorded during
307 initial sampling (Fig. 3). Final salinity was much higher in ballast tanks that
308 involved BWE, reaching the mandatory value of 30 PSU ($F_{3,32}$ = 8.37, P < 0.001;
309 Fig. 3).

310 Our estimated initial chlorine doses for trials one to five averaged between
311 10.0 and 21.8 mg L^{-1} for tanks that were dosed, while all non-dosed tanks were <
312 0.4 mg L^{-1} (Fig. 4). Chlorine concentration decreased rapidly in dosed tanks during
313 the first four days, though decay rate varied from tank to tank during the first four
314 trials (Fig. 4). Measured chlorine decay was very swift during the final trial,
315 dropping to ~0.5 mg L^{-1} within hours of dosing (Fig. 4). Calculated THM
316 concentration ranged between 0.56 and 5.19 µg L^{-1}, with higher values associated
317 with high TOC concentrations in initial ballast water (Table 1).

318 *Biota*

319 We observed large differences among trials with respect to initial densities
320 for each biological indicator group (significant block effect; Table 2). Treatment
321 differences in biological conditions were typically minor at the beginning and often
322 very pronounced at the end of a trial, highlighting strong treatment effects (Fig. 5).
323 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 hybrid

349 treatment (Fig. 3 and 5). The chlorine-only treatment was also very effective at
350 reducing macroplankton abundance, though mean abundance exceeded 100 ind.
351 m^{-3} (Fig. 5). Three chlorine trials (third, fourth and fifth) had no viable zooplankton
352 when the experiments ended. Chlorine was the only treatment that affected
353 effective growth rate of macroplankton (P <0.0001, Table 2).
354 While the final absolute abundance of each of the three bacteria indicators
355 was higher when chlorine was dosed at 10 (fifth trial) versus 20 mg L^{-1} (first four
356 trials), only *E. coli* was significantly reduced at the higher dose (Table 2). Similarly,
357 lower microplankton density was observed with the higher dose of chlorine (P
358 =0.0359; Table 2). Chlorine dose had little effect on final viable macroplankton
359 abundance (P =0.1577; Table 2).
360 The effective growth and final abundances of bacteria and microplankton
361 were also affected by an interaction between BWE and chlorination (Figs. 5 and 6;
362 Table 2). This interaction was synergistic for enterococci and *E. coli* (P =0.0321
363 and 0.0228, respectively) but not for coliforms (P =0.2120, Table 2), indicating
364 stronger than additive reductions in abundance for the first two groups.
365 Conversely, microplankton exhibited an antagonistic (i.e. less than additive)
366 interaction (Table 2), signifying that the effect of the hybrid treatment was less
367 than the sum of individual treatments. The hybrid treatment resulted in the lowest
368 final densities for each of these groups. Chlorophyll *a* concentration behaved
369 similarly to microplankton, with each affected by BWE and chlorine application,
370 though the interaction between treatments was not significant (Tables 2, 3). Mean
371 viable macroplankton abundance was much lower in the hybrid than in other
372 treatments (Fig. 5). Even so, the effective growth rate was not affected by an
373 interaction between treatments (Fig. 6; Table 2). Mean final abundance was also

374 slightly above the proposed permissible IMO D-2 performance limit (Fig. 5).
375 Density of macroplankton in BWE-only treatments was often higher than controls,
376 and well in excess of IMO D-2 proposed limits.

377 **Discussion**

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

394 Our experiments were conducted under realistic scenarios on board an
395 operating vessel that was outfitted to allow collection of samples from major
396 sections of ballast tanks, thereby incorporating vertical variation in distributions of
397 biota (Murphy, Ritz & Hewitt 2002; First *et al.* 2013). Reductions in abundance of
398 bacteria, microplankton and macroplankton in untreated (control) ballast water in

399 relation to voyage length are consistent with previous studies (Drake *et al.* 2002;
400 Tomaru *et al.* 2010). Final densities of bacterial indicator taxa in control tanks were
401 very close to or exceeded those prescribed by IMO D-2 limits. Moreover, in some
402 of the trials final densities for bacteria were higher than middle and initial
403 concentrations (Fig. 5), which was probably related to the gradual temperature
404 increase and favourable oxygen conditions as the vessel moved through
405 progressively warmer water, or to increased dissolved organic matter released by
406 decomposition of phytoplankton and zooplankton inside ballast tanks (Tomaru *et*
407 *al.* 2010).

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

417 The higher bacteria and macroplankton densities after BWE relative to
418 controls (Figs 5 & 6), accord with earlier studies conducted in marine
419 environments and highlight the fact that BWE cannot by itself serve as an effective
420 ballast water treatment (e.g. Drake *et al.* 2002; Briski *et al.* 2012 and 2013). Unlike
421 patterns observed in vessels operating between freshwater ports (Bailey *et al.*
422 2011), our final densities were influenced by replenishment of new live marine
423 organisms during the exchange from freshwater to seawater, and consequently

424 macroplankton density exceeded the proposed IMO D-2 standard (Fig. 5). BWE
425 was, however, effective at suppressing abundance of microplankton (Table 2),
426 consistent with other studies (e.g. Drake *et al.* 2002; Taylor *et al.* 2007).
427 The effectiveness of chlorine as a biocide for bacterial and microplankton
428 populations is very well established (Gregg & Hallegraeff 2007; Maranda *et al.*
429 2013), with high efficiency at concentrations ranging from 4 to 50 mg L^{-1}. Our
430 results support this effectiveness, particularly at the higher dose (20 mg L^{-1}; Figs 5
431 & 6). However, the application of chlorine (20 or 10 mg L^{-1}) resulted in consistent
432 achievement of proposed IMO D-2 standards only for bacterial indicators, whereas
433 results for microplankton varied between trials (Fig. 5). This differential was
434 previously observed by Gregg and Hallegraeff (2007), who found complete
435 bacterial inhibition at 15 mg L^{-1}, while more than 25 mg L^{-1} was required to
436 eliminate vegetative cells and cysts of dinoflagellates. Our results demonstrated
437 that a dose of 20 mg L^{-1} yielded significantly higher efficiency than 10 mg L^{-1} with
438 respect to decreasing microplankton density.
439 Many devices under development for ballast water treatment use
440 chlorination either directly applied or via electrochlorination. These devices rely on
441 a timed exposure of a constant dose (Lloyd's 2011), whereas we utilized a pulse
442 that delivered a high initial dose that over time was reduced as chlorine oxidized
443 organic matter. Our aim was to keep the chlorine concentration above 2 mg L^{-1}
444 and therefore effective as a biocide over a long period of time. In our trials
445 macroplankton were very sensitive to chlorine; mean final densities were lowered
446 almost an order of magnitude relative to controls (Fig. 5), and in three of the trials
447 the final abundance was zero. These results mirror those of Maranda *et al.* (2013)
448 despite their use of a constant dose.

449 Regardless of the chlorine and initial organism concentrations, when
450 chlorine was combined with BWE the final bacterial, microplankton and
451 macroplankton densities were the lowest recorded (Fig. 3 and Table 1). Briski *et*
452 *al.* (2013) also demonstrated potential benefits of combining BWE with ballast
453 water treatment (UV radiation), which resulted in a strong reduction of all groups.
454 At least two non-exclusive mechanisms may explain the significant
455 synergistic interaction observed with bacterial populations. First, higher killing
456 efficiency of chlorine may result from osmotic shock associated with BWE (Briski
457 *et al.* 2013). Secondly, lower organic matter concentration of open ocean water
458 relative to freshwater may better facilitate biocide action (Dychdala 1968).
459 The hybrid treatment resulted in a significant antagonistic interaction for
460 microplankton, with the final density higher than would be expected if the two
461 treatments were additive (Fig. 5). A likely reason for this lower efficiency is the
462 higher resistance to chlorine of some microplankton, such as cyst-forming
463 dinoflagellates (Gregg & Hallegraeff 2007). Despite this undesirable antagonistic
464 interaction effect, the hybrid treatment was the only one in which final
465 microplankton density was consistently below the prescribed IMO D-2 limit.
466 The interaction term between treatments was not significant for
467 macroplankton due mostly to the effectiveness of the chlorine-only treatment. We
468 acknowledge that there exists extensive variability in our data for this group (Fig.
469 5). The hybrid treatment was still the most effective, reducing final densities by
470 almost an order of magnitude versus chlorine alone, and more than an order of
471 magnitude versus ballast water exchange alone (Fig. 5).
472 The IMO D-2 performance standard refers to live organisms without regard
473 to origin or, in most cases, taxonomy. Our studies confirm that combining BWE

474 with chlorination offers enhanced efficiency with respect to reducing propagule
475 pressure better than any either treatment alone for a variety of aquatic groups.
476 Although, it remains unclear exactly how low propagule pressure must be to
477 prevent an invasion, it is a key factor in reducing overall invasion risk (Lockwood,
478 Cassey & Blackburn 2005). Nevertheless, any treatment that reduces propagule
479 pressure, such as the hybrid management that combines treatment and BWE,
480 should also reduce overall invasion risk. Middle ocean ballast exchange may
481 provide an additional benefit for freshwater habitats (e.g. Great Lakes) that receive
482 foreign ballast because freshwater organisms in original ballast are replaced by
483 oceanic taxa that are unlikely to survive environmental conditions upon discharge
484 into a freshwater port (Briski *et al.* 2013).

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

494 The ecotoxicity of chlorination, which generates byproducts including
495 trihalomethanes (THMs) in substantially larger quantity than occur naturally, must
496 be monitored to ensure compliance with existing law. Although our estimates
497 express the maximum possible amount of THMs generated, the actual amount
498 produced could be lower. Nevertheless, any commercial treatment system that

499 utilizes chlorine as a biocide must be cognizant and monitor production of THMs
500 as well as residual chlorine in discharged ballast water.
501
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510
511 **Data accessibility**
512 Data are available from the Dryad Digital Repository:
513 http://dx.doi.org/10.5061/dryad.rm83s
514
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597 reducing propagule pressure: a model for ballast-water exchange. *Frontiers*
598 *in Ecology and the Environment,* **3,** 473-478.

599 Table 1. Formation of trihalomethanes (THMs; μg L^{-1}) estimated using the Hutton

600 model (Hutton & Chung, 1994) and total organic carbon (TOC; μg L^{-1}) (in brackets) in

601 ballast water at the port of origin

602

	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

603

604 Table 2. Effect of Ballast Water Exchange (yes or no) and chlorine (0, 20 or 10
605 mg L^{-1}) on indicator group abundances. ANOVA models also considered trial
606 number (Trial #) as a blocking factor. Effect size represents the percentage of the
607 final treatment (BWE, chlorine and hybrid) as a function of the control. Error
608 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	

609 **Figure Legends**

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

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

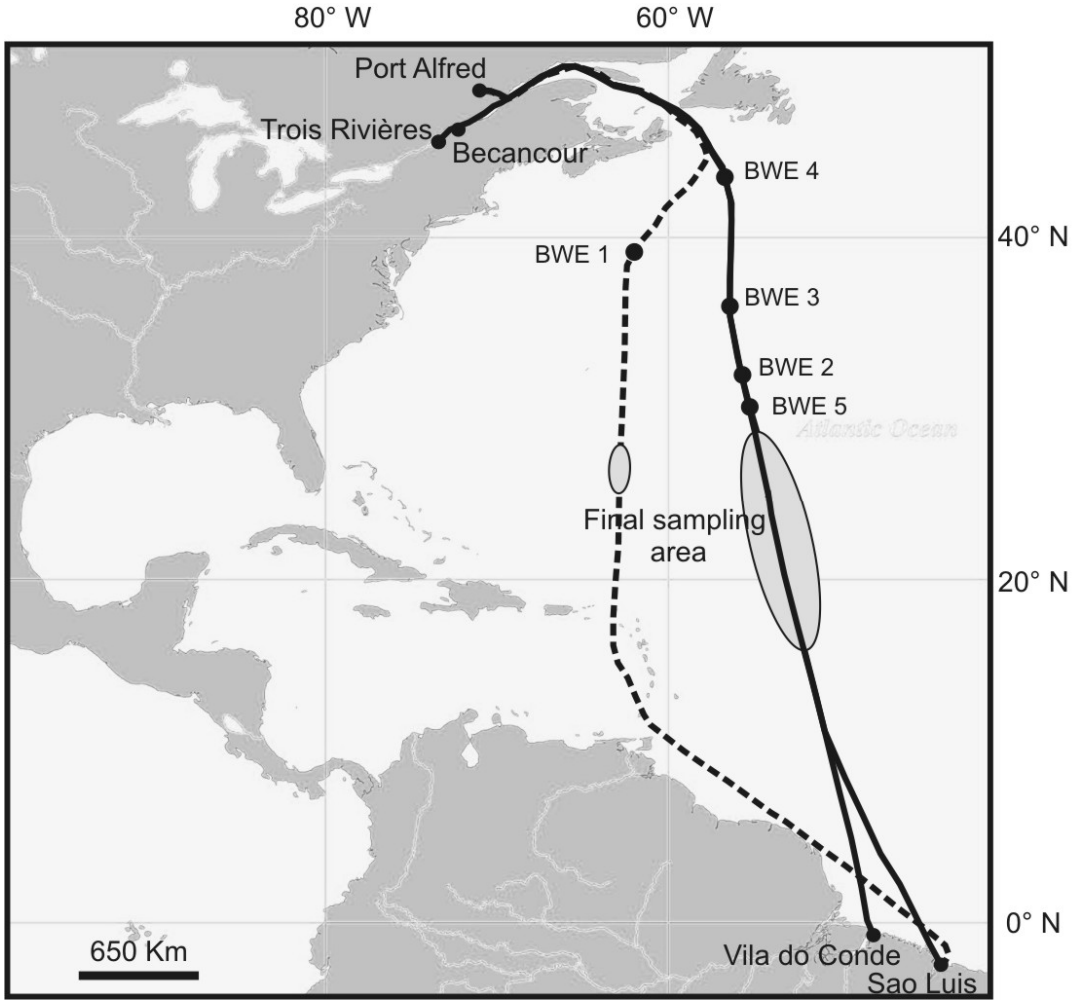
618 Figure 3. Initial and final mean (±SD) values for environmental variables for control
619 (black bars), BWE (grey bars), chlorine (diagonal striped bars), and hybrid
620 treatments (white bars).

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

626 Figure 5. Changes in densities (log-transformed initial, middle and final mean
627 values ±SD) of putative *enterococci*, coliforms, *E. coli*, viable microplankton
628 (≥10 μm and <50 μm) and viable macroplankton (≥50 μm) in all four
629 treatments. Black, grey, diagonally striped, and white bars are control, BWE,
630 chlorine, and hybrid treatments, respectively. Dotted lines indicate the
631 proposed IMO D-2 performance standard maximum limit for each group. * = 0;
632 + = No sample. CFU = colony forming units.

633 Figure 6. Effective mean growth rate ± SD (r; grey squares) for the five biological
634 indicators in control, BWE, chlorine, and hybrid treatments. Upper asterisk
635 indicates significant treatment effects with $P \leq 0.0001$ (***) and 0.05 (*) based
636 on two-way ANOVA.

637 Figure 1



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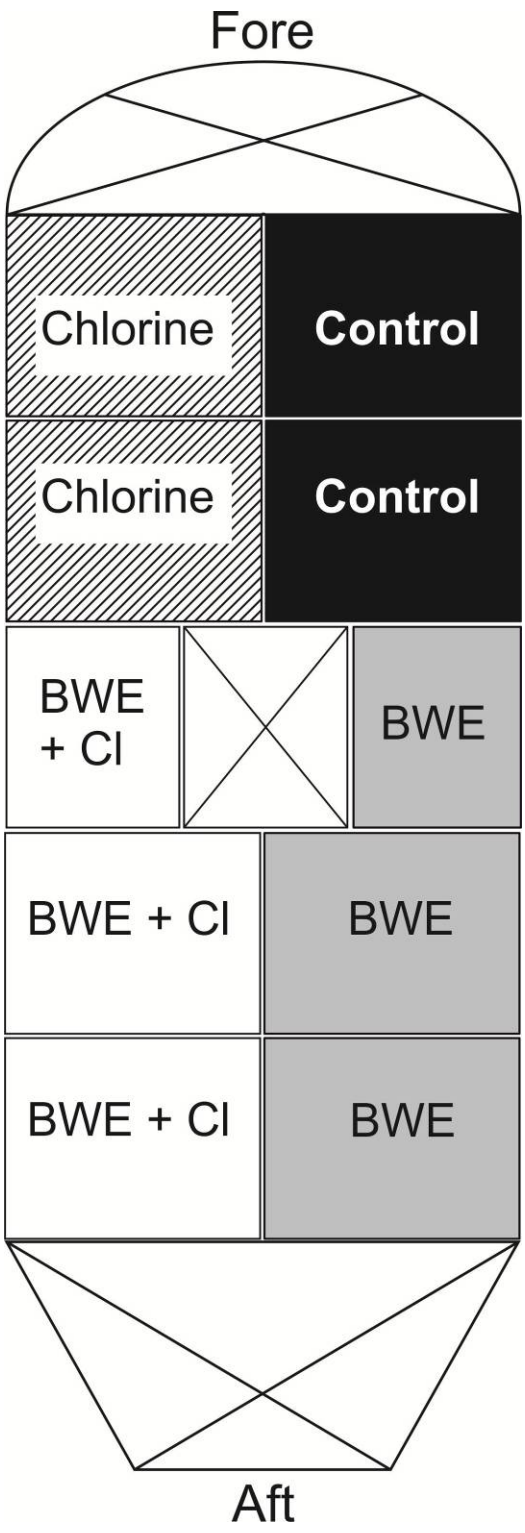
643 Figure 2



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645 Figure 3
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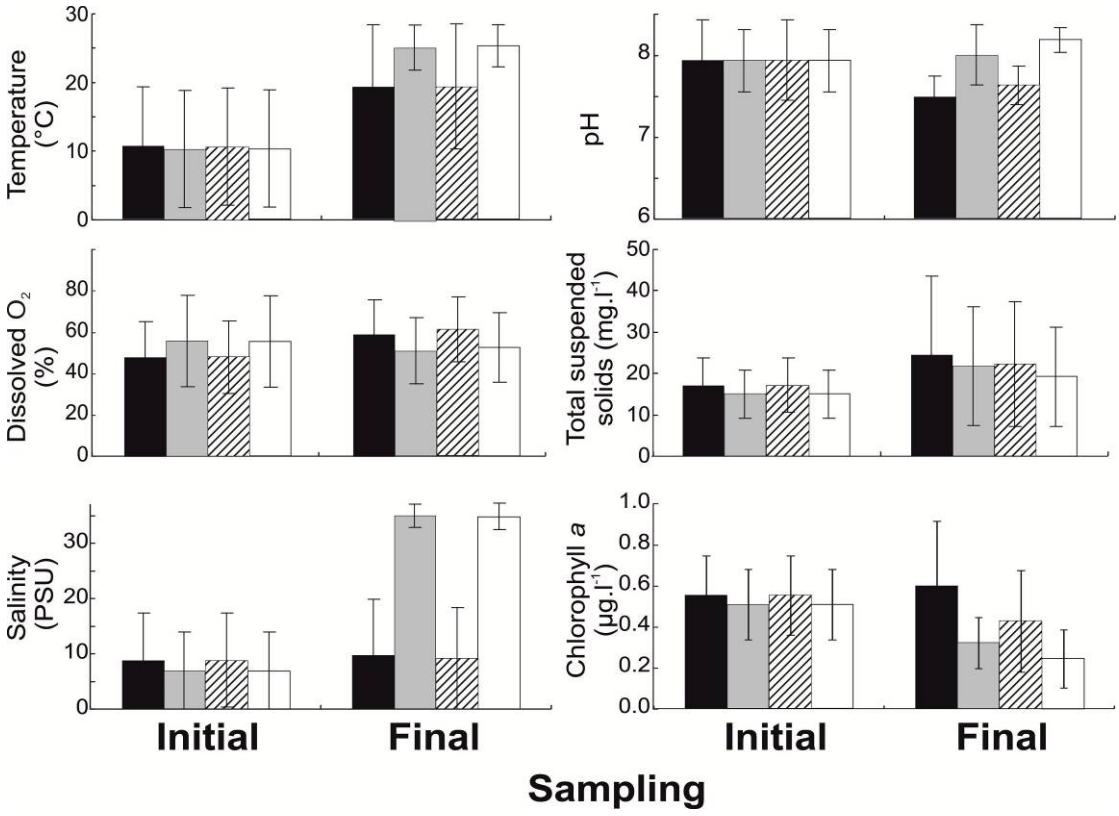

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654 Figure 4

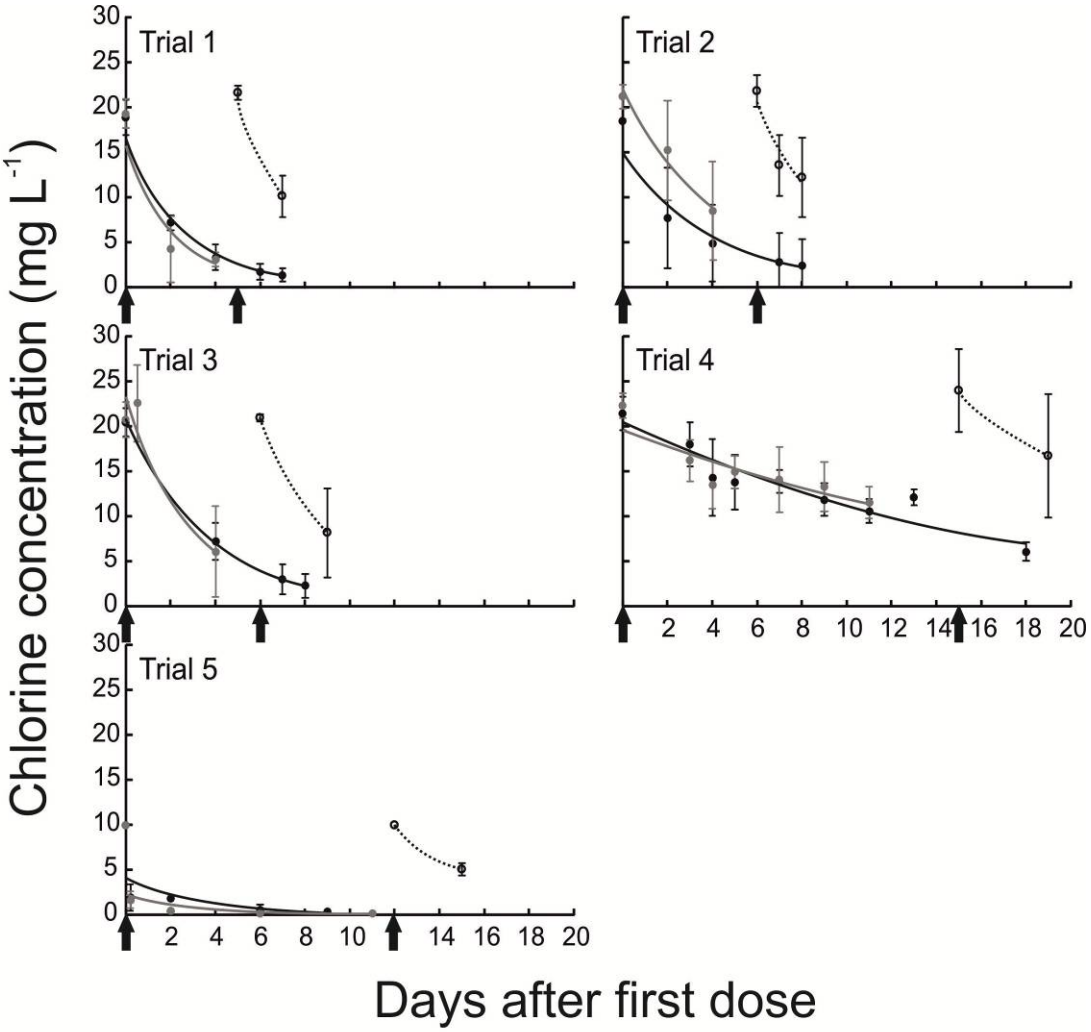

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Figure 5

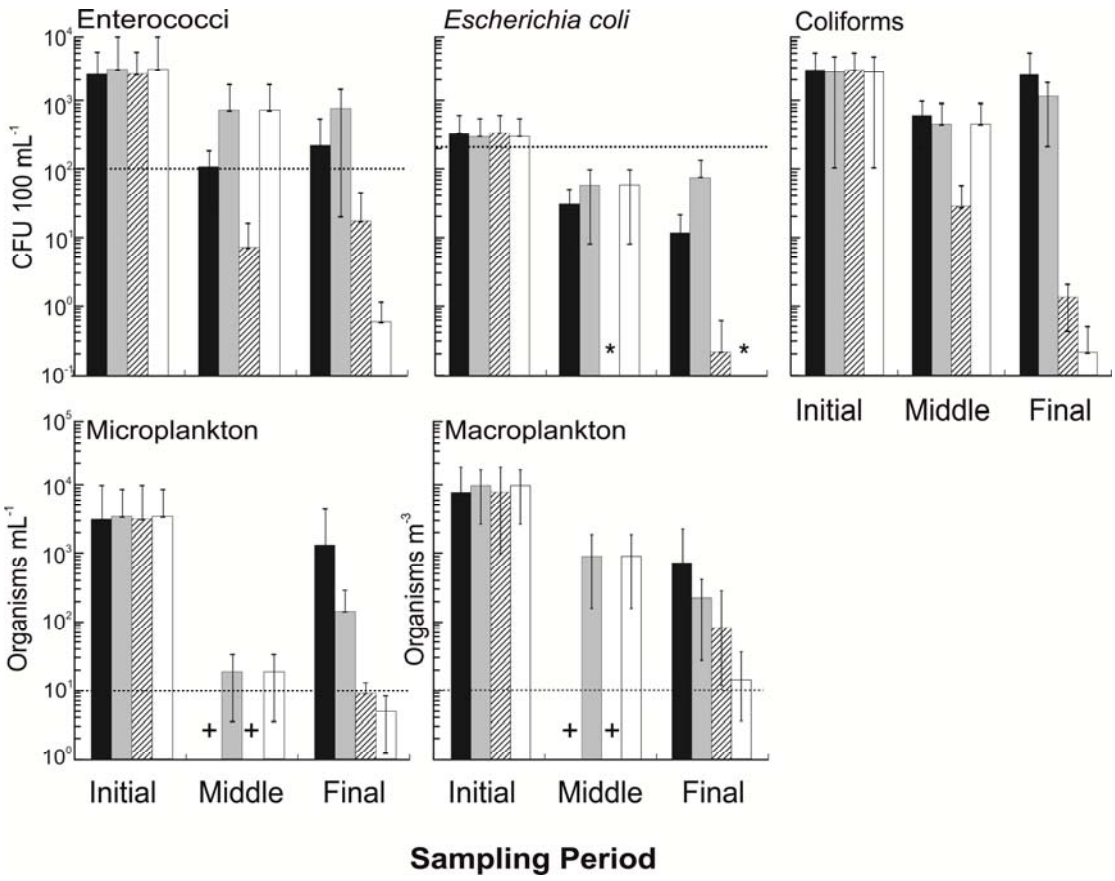
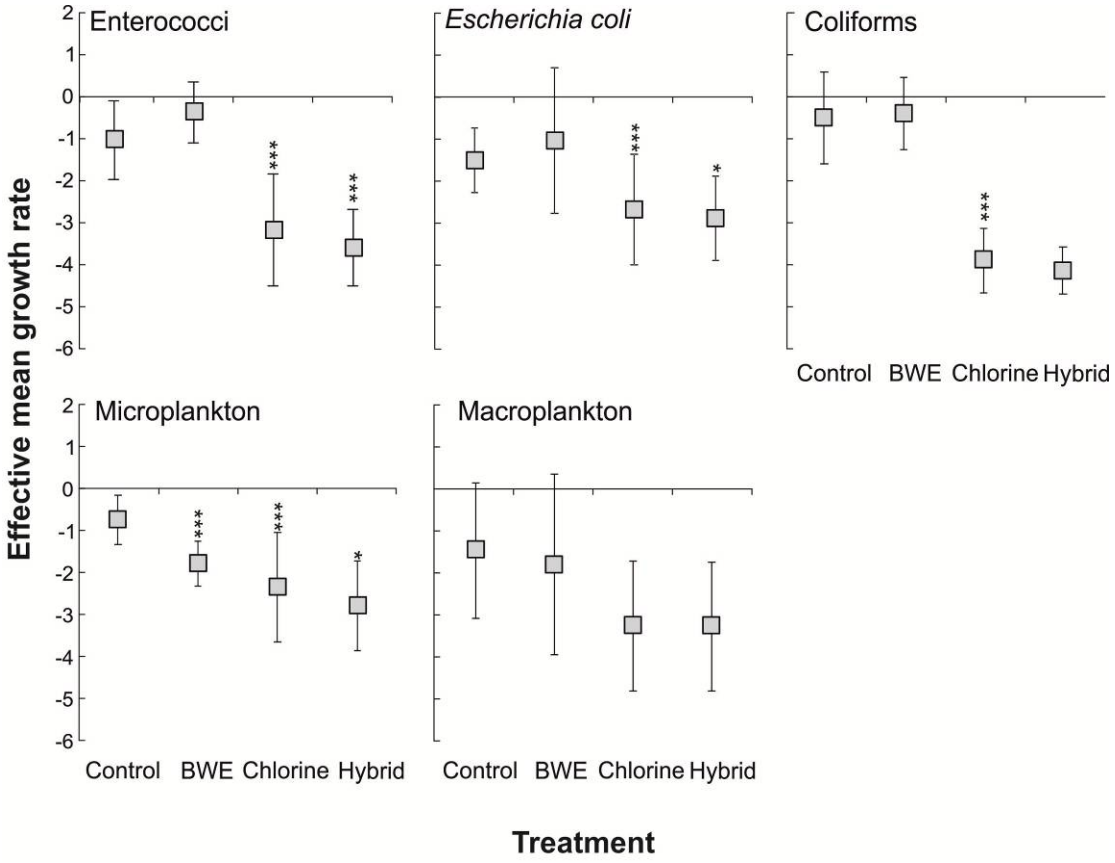

Enterococci
Escherichia coli
Coliforms
CFU 100 mL-1
Microplankton
Macroplankton
Organisms mL-1
Organisms m-3
Initial
Middle
Final
Sampling Period

Figure 6


Enterococci
Escherichia coli
Coliforms
Microplankton
Macroplankton
Effective mean growth rate
Control
BWE
Chlorine
Hybrid
Treatment