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Combining ballast water exchange and treatment to maximize prevention of species introductions to freshwater ecosystems

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ABSTRACT

The most effective way to manage species transfers is to prevent their introduction *via* vector regulation. Soon, international ships will be required to meet numeric ballast discharge standards using ballast water treatment (BWT) systems, and ballast water exchange (BWE), currently required by several countries, will be phased out. However, there are concerns that BWT systems may not function reliably in fresh and/or turbid water. A land-based evaluation of simulated 'BWE plus BWT' *versus* 'BWT alone' demonstrated potential benefits of combining BWE with BWT for protection of freshwater ecosystems. We conducted ship-based testing to compare the efficacy of 'BWE plus BWT' *versus* 'BWT alone' on voyages starting with freshwater ballast. We tested the hypotheses that there is an additional effect of 'BWE plus BWT' compared to 'BWT alone' on the reduction of plankton, and that taxa remaining after 'BWE plus BWT' will be marine (low risk for establishment at freshwater recipient ports). Our study found that BWE has significant additional effect on the reduction of plankton, and this effect increases with initial abundance. As per expectations, 'BWT alone' tanks contained higher risk freshwater or euryhaline taxa at discharge, while 'BWE plus BWT' tanks contained mostly lower risk marine taxa unlikely to survive in recipient freshwater ecosystems.

INTRODUCTION

Shipping has been recognized as a primary vector for spread of aquatic species globally.[1-5] To prevent arrival of species by ships' ballast water, Canada, the USA and numerous other countries have implemented regulations requiring transoceanic ships to conduct mid-ocean ballast water exchange (BWE) of tanks that will be discharged into their fresh or marine coastal waters.[5-9] In theory, BWE should expel higher risk coastal species into the ocean, replacing them with oceanic species that would have a lower survival probability along the coast. Though observed efficacy of BWE is mixed for marine ecosystems,[10-13] the strategy is quite protective of freshwater ecosystems due to osmotic shock.[14-17]

In the near future when the International Convention for the Control and Management of Ships' Ballast Water and Sediments will enter into force, all commercial ships trading internationally will be required to meet numeric ballast water discharge standards unless granted an exemption based on risk assessment, excepting emergency situations at sea.[18-19] It should be noted that this convention does not focus on nonindigenous species, but addresses transfers of all harmful aquatic organisms irrespective of their origin.[20]

Numerous commercial ballast water treatment (BWT) systems that use technologies such as filtration, ultraviolet radiation (UV) or chlorination have been developed[5] and BWE will be phased out of use.[21-22] The risk of ballast water treated by BWT systems is expected to be lower than that managed by BWE due to lowered propagule pressure; however, there are concerns that BWT systems may not function reliably in fresh and/or turbid water, that the proposed performance standards are not

stringent enough, and that BWT systems may fail for mechanical or operational reasons.[23-25] Therefore, the government of Canada proposed combining BWE with BWT systems to manage ballast water of ships arriving to freshwater ecosystems in an effort to reap the positive benefits of both management strategies.[26] This combined method addresses two factors of the invasion process - reducing propagule pressure through BWT and reducing environmental tolerance through BWE - and is expected to be more effective than either individual method focusing on only a single component. A land-based evaluation of simulated 'BWE plus BWT' *versus* 'BWT alone' demonstrated potential benefits of combining BWE with BWT;[25] however, a ship-based evaluation was recommended to confirm efficacy and practicality of the combined strategy under real environmental and operational conditions at true size scale.

In this study, we conducted ship-based testing to compare the efficacy of 'BWE plus BWT' *versus* 'BWT alone' for living organisms ≥ 50 μm in minimum dimension (hereafter zooplankton) and living organisms ≥ 10 and < 50 μm in minimum dimension (hereafter phytoplankton). We tested the hypotheses that: (1) there is an additional effect of BWE on top of 'BWT alone' on the reduction of plankton; and (2) taxa present in ballast after 'BWE plus BWT' will be low-risk marine species likely unable to survive in freshwater ecosystems.

METHODS

Experimental design

Between March 2013 and August 2014, we conducted three trials on three individual ships operating along two routes: two trials between Hamburg (Germany,

freshwater) through the Bay of Biscay to the Strait of Gibraltar and one trial between Moerdijk (the Netherlands, freshwater) through the Irminger Basin to Deception Bay (Canada) (Table 1). Each ship had already installed a type-approved BWT system utilizing filtration and electrochlorination, filtration and ultraviolet radiation, or ozonation without filtration (Table 1). The ships were chosen opportunistically as those which already had installed a type-approved BWT system, and which operate on a route permitting uptake of ballast water at a freshwater port followed by BWE, according to the IMO requirements for water depth and distance from the nearest land.[18] Each trial consisted of two different experimental treatments: 1) 'BWT alone' – tank(s) filled at initial freshwater port and treated with a BWT system; and 2) 'BWE plus BWT' – tank(s) filled at initial freshwater port, discharged and refilled in the Atlantic ocean (more than 50 nautical miles from the nearest land and in waters of > 200 metres depth), with a BWT system used to treat both the initial port water and the exchanged ocean water. During the first two trials, experimental treatments were run in parallel (two different tanks were used, each for one experimental treatment; Table 1), while operational limitations on the third voyage resulted in the 'BWT alone' tank being discharged five days before the 'BWE plus BWT' tank (two tanks were used per treatment; the same two tanks were used in time series for both treatments - first for 'BWT alone' then for 'BWE plus BWT' treatment; Table 1). The details on the tanks used, their location on the ships, and capacity are provided in Table 1. Trials lasted between six and 16 days (Table 1).

Sample collection and enumeration of live/dead organisms

Ballast water was sampled each time water was loaded into ballast tanks and during final ballast water discharge. Samples were collected over the whole time that ballast was pumped in (or out) of experimental tanks, resulting in sample volumes between 751 and 1648 L (Table S1). To minimize impacts of organism survival during sample collection and holding time, each sample was collected as two or three sequential subsamples corresponding to the first and second half, or to the beginning, middle, and end of the ballasting process (Table S1).[5,27-28] We aimed for three sequential subsamples, however, due to the smaller tank size on the first voyage and corresponding very short ballasting duration, three subsamples were collected only on uptake in Hamburg while two sequential subsamples (i.e., equivalent to the first and second half) were collected during the remainder of the first voyage. All samples were taken from bent elbow pitot tubes installed for scientific sample collection along straight sections of the ballast piping.[29] Sampled ballast water was pressure-fed by the ships' ballast system through hoses and PVC tubing equipped with a flow meter into a conical plankton net with 50 µm (in diagonal) mesh within a wetted sample tub. The sample collected inside the plankton net was retained for zooplankton analysis. For phytoplankton, a composite sample totalling to ~ 5 L was taken by collecting ~ 0.5 L of water every one to five minutes during each sampling sequence. Salinity and temperature were measured at two to five minute intervals during the sampling process using a calibrated YSI instrument.

Enumeration of live organisms for both taxonomic groups was conducted on board. Zooplankton samples were further concentrated on 50 µm (in diagonal) mesh to 100 or 200 mL volume, of which multiple 2 mL subsamples totalling to 6 to 12 mL were

analysed, depending on available time and sample complexity. A larger subsample volume could not be processed without exceeding the recommended maximum holding time of 6 hours between completing sample collection and completing sample processing.[27-28] The number of fully intact and live individuals of zooplankton in the subsample was determined using a dissecting microscope and standard movement/response to stimuli techniques.[30] The counts were recorded according to broad taxonomic groups, such as Copepoda, Cladocera, Rotifera, Bivalvia, Gastropoda, etc. Representative individuals alive in final discharge samples were isolated and preserved separately in > 95% ethanol for later molecular identification.

For phytoplankton analysis, one 400 mL subsample was removed from each well-mixed 5 L composite sample, concentrated to 100 mL on 10 µm (in diagonal) mesh and a 5 mL subsample stained using FDA (fluorescein diacetate) as a selective indicator of enzymatic activity. The subsample was processed on board immediately after collection using bright field and epifluorescence microscopy (Zeiss Axiovert A1).[31-32] Phytoplankton were not identified to any taxonomic level on board the ship. After staining, the remaining concentrated sample was preserved with Lugol's iodine solution for later morphological identification. On the first trial, phytoplankton were not enumerated on board during the uptake of ballast in the freshwater port (i.e., Hamburg) due to equipment failure.

Laboratory enumeration and taxonomic identification

After the shipboard trials were completed, preserved samples of zooplankton were examined under a dissecting microscope in entirety; representative individuals of

different taxonomic groups were measured and imaged, and twenty individuals from every taxonomic group per sample separated for taxonomic identification. Zooplankton were identified solely by molecular tools in the lab since gross morphological identification was already completed on the ship. DNA was extracted from each whole individual using DNeasy Blood and Tissue Kit (Qiagen Inc., ON, Canada). Fragments of the mitochondrial genes COI and 16S were amplified using the universal COI primers LCO1490 and HCO2190,[33] and 16S primers S1 and S2.[34] PCR reactions followed the protocol from previous studies,[35] and PCR products were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA). Recovered DNA sequences were blasted against those in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the nucleotide blast. The scores resulting in at least 98% similarity to the closest match for COI and 99% for 16S were deemed species level identifications. Freshwater, brackish and/or marine natural habitats of identified species were assigned based on scientific literature review.

Preserved samples of phytoplankton were mixed by overturning by hand more than 20 times, and a volume of 50 mL per sample put in a settling column for 24 hours.[36] Phytoplankton were enumerated and identified morphologically using a Nikon AZ100 inverted microscope. There was no molecular identification of phytoplankton. Identifications were based on literature references.[37-41] Only intact cells with clearly visible cell content were assessed. Freshwater, brackish and/or marine natural habitats of identified species were assigned based on review of scientific literature and taxonomic websites.

Statistical analysis

We tested whether there is an additional effect of 'BWE plus BWT' on the reduction of plankton abundance compared to that of 'BWT alone'. To test this hypothesis, we used abundance estimates of both zooplankton and phytoplankton samples collected after treatments (i.e., 'BWE plus BWT' and 'BWT alone') at three sequential time-segments from each of three ship trials (subjects). This allowed samples collected during the same time-segments within each ship to be statistically paired. We computed the proportions (i.e., abundances in 'BWT alone'/abundances in 'BWE plus BWT') for each pair of samples within each ship trial as the dependent variable, and used the $\log_{10}$ transformation to meet the assumption of normality, which we denote by y hereafter. We used the ($\log_{10}$) abundance of 'BWT alone' samples as the independent variable, which we denote by T hereafter. To test the above hypothesis, we tested if y (i.e., difference in log densities between 'BWT alone' and 'BWE plus BWT') increases with increasing T (i.e., densities after 'BWT alone'), such that $y > 0$ (i.e., the difference is positive), using linear mixed effect models, incorporating random effects due to ships (*Ships*), and fixed effects due to sequential time-segments (*Time*) and plankton type (*ZorP*) nested within fixed effects of T. The resulting three alternative models that we analysed using the Linear Mixed Effect Model procedure in SPSS version 22[42] are given in Table 2 with detailed descriptions.

Note that, as we selected three ships from a larger population of ships, here, we would more naturally treat the variable "Ship" as a random effect. That is, we would regard the effects of ship as a random sample of the effects of all the ships in the full population of ships. We would treat explanatory variables T, T(*ZorP*), and T(*Time*) as

fixed effects, assuming there is no randomness in their choice, rather that they are fixed or specific, or the average responses for all subjects. Our choice of linear mixed effect models is because they allow incorporating both fixed and random effects into linear models (a regression type with a hierarchical structure), such that, random effects can account for individual differences in response to an effect, while fixed effect estimate the population level coefficients. Although, we tested numerous other models with different structures and combinations of variables, incorporating non-linear effects also, here, we present only these three alternative nested models as other ones did not improve the goodness of fitness drastically, compared to these three, based on Akaike Information Criteria (AIC).

In these models (Table 2), the response variable *y* was unitless, and the predictor variable *T* was in two different scales, m^{-3} and mL^{-1}, corresponding to factors *Z* (zooplankton) and *P* (phytoplankton), respectively. This scaling was used because the management regulations of the two types of organisms are implemented in these two scales.[18] Therefore, the models quantify scale-free effects on the response variable as a function of the predictor variable, given in two different management scales, corresponding to plankton type. In all these models, we incorporated *Time*-segment as a repeated measure (*RM*) (or a repeated effect), with repeated covariance type - scaled identity, and random effects covariance type - variance components. Using each model with and without incorporation of random effects yielded a total of six alternative models. We used the maximum likelihood estimator in the Mixed Effect Model methodology in SPSS for model parameterization, and AIC for model comparison.

Additionally, we tested the significance of the difference in abundances of plankton type (zooplankton and phytoplankton) between freshwater ports and the ocean to see if treatment of ocean water would require less effort than treatment of fresh water by BWT systems. To test this hypothesis, we transformed the abundance data by $\log_{10}$ to meet the normality assumption, and used paired differences between the ocean and freshwater port samples. For this, we used the Markov Chain Monte Carlo (MCMC) simulation procedure in Poptools (Ver. 3.2): First, we randomly resampled freshwater port abundance data (i.e., the 3 repeated samples) within each ship, and randomly paired them with the ocean abundance data (i.e., the 3 repeated samples) of the same ship, and calculated the average difference in $\log_{10}$ abundances between freshwater ports and ocean intakes across all ships. We repeated this resampling scheme 100 times yielding 100 test values (i.e., the average differences). Then, from each simulated 100 resamples above, we generated another 1000 resamples by randomly mixing both the ocean and freshwater port abundance data (of the 3 repeated samples) within each ship. This yielded the theoretical distribution (i.e., the dependent values) of the average differences of $\log_{10}$ abundances for the case where there is no systematic difference in abundances due to ocean and freshwater port intakes, which is the case if the null hypothesis were true. The *p*-value for the hypothesis, that "there is a difference in abundance of taxa between freshwater port and the ocean intakes", is given by the proportion of simulated resamples (i.e., 10^5) that yielded dependent values greater than the test values. We did this hypothesis test for phytoplankton and zooplankton separately, and also for both taken together.

RESULTS

Community composition of initial freshwater ballast water

Live zooplankton and phytoplankton abundances determined on board in samples collected during ballast uptake in Hamburg/Moerdijk ranged from 1198 to 49,907 individuals per m^3 and from 261 to 1145 cells per mL, respectively (Table S1). Copepoda and Rotifera were dominant zooplankton taxa at source ports ranging between 30% and 76%, and 16% and 68% abundance, respectively (Table S2). Across all trials, laboratory identification of preserved samples revealed at least two Bivalvia, six Cladocera, twelve Copepoda, one Nematoda, six Rotifera, and one Trematoda species (Table S3). All zooplankton species are considered freshwater or euryhaline species, except one Copepoda (*Clausocalanus pergens*) which is previously reported only as a marine species (Table S3). Since species-level identifications for uptake samples were conducted on composite preserved samples, we cannot be certain that the specimen was alive at the time of collection. Laboratory identification of preserved phytoplankton taxa indicated that Bacillariophyceae and Dinophyceae were dominant taxa ranging from 14% to 92%, and 4% and 82% abundance, respectively (Table S4). Chlorophyceae ranged from 1% to 25% (Table S4). Across all trials, at least five Chlorophyceae, two Chrysophyceae, seven Dinophyceae, 33 Bacillariophyceae, one Cyanophyceae, and one Dictyochophyceae species were identified (Table S5). Salinity of water pumped into the tanks ranged from 0.3 – 0.5 ppt (Table S1), but interestingly at least two Dinophyceae, eleven Bacillariophyceae, and one Dictyochophyceae species are to our knowledge marine species, unable to survive in freshwater habitats (Table

S5). Again, we cannot be certain that the individuals of these species were alive at the time of collection (see discussion).

Community composition of exchanged oceanic ballast water

Live zooplankton and phytoplankton abundances determined on board in samples collected during BWE in the Bay of Biscay/Irminger Basin ranged from 791 to 4527 individuals per m^3 and from 10 to 2983 cells per mL, respectively (Table S1). Nearly all live zooplankton taxa observed on board the ships were Copepoda (99%; Table S2). Laboratory identification of preserved samples revealed at least 15 Copepoda, two Decapoda, one Gastropoda, and two Thecostraca species across trials - all are considered marine or euryhaline species (Table S3). Laboratory identification of preserved phytoplankton indicated that Bacillariophyceae were dominant taxa in all trials ranging from 93% to 100% (Table S4). In all trials together, at least three Chlorophyceae, six Dinophyceae, 24 Bacillariophyceae, three Ciliophora, one Dictyochophyceae, and two additional species were identified – all are considered marine or euryhaline taxa (Table S5). Salinity of water pumped into the tanks during BWE ranged from 33.5 – 35.3 ppt (Table S1). Statistical comparison of abundance of taxa between freshwater ports and the ocean determined significantly lower abundance of taxa in the ocean: $p = 0.001$ for pooled data, $p = 0.006$ for zooplankton and $p = 0.02$ for phytoplankton.

Community composition at final ballast water discharge

Live zooplankton abundances in samples collected during discharge of 'BWT alone' tanks ranged from 0 to 11,092 individuals per m^3; those of live phytoplankton ranged from 2 to 174 cells per mL (Table S1). Copepoda represented 99% of live taxa observed on board the ships (Table S2). Laboratory identification revealed at least one Amphipoda, four Cladocera, six Copepoda, and one Nematoda species across trials, all of which are expected to thrive in freshwater habitats (Table S3). Laboratory identification of preserved phytoplankton taxa indicated that Bacillariophyceae dominated the first and third trials (98% and 100%, respectively), while Chlorophyceae were most abundant in the second trial (88%; Table S4). Most species observed are previously reported from freshwater habitats, however, in addition to the seven 'marine' species observed during initial uptake, at least five new 'marine' species were detected that to our knowledge are unable to survive in freshwater habitats (four Dinophyceae and one Ciliophora species; Table S5). Again, since species identification was conducted on preserved samples, there might be alternative explanations for the observations.

In the case of 'BWE plus BWT' tanks, live zooplankton abundances in samples collected during discharge ranged from 0 to 124 individuals per m^3; those of live phytoplankton ranged from 0 to 1662 cells per mL (Table S1). Copepoda represented 100% of live taxa in the first two trials, while in the third trial 86% were other taxa (Table S2). Laboratory identification revealed at least two Bivalvia, four Cladocera, ten Copepoda, one Gastropoda, one Nematoda, and one Rotifera species (Table S3). All zooplankton observed alive at the time of sampling are considered marine or euryhaline (Table S3). Laboratory identification of preserved phytoplankton showed that

Bacillariophyceae were dominant in all trials ranging from 57% to 88% abundance, followed by Chlorophyceae ranging from 11% to 23% abundance (Table S4). All phytoplankton identified are considered marine or euryhaline species (Table S5). Salinity of ballast water discharged ranged from 0.3 – 3.8 ppt and 29.7 – 32.9 ppt for 'BWT alone' and 'BWE plus BWT' tanks, respectively (Table S1).

Efficacy of 'BWT alone' *versus* 'BWE plus BWT'

All three fixed effect models (Table 3) yielded significant relationships (gradient > 0) between $\log_{10}$ (abundances in 'BWT alone'/abundances in 'BWE plus BWT') and $\log_{10}$ ('BWT alone') with $p < 0.001$. The predictive $\log_{10}$ ('BWT alone'), nested with plankton type (*ZorP*), yielded a significantly positive gradient of 1.06 for factor *Z*, and 0.87 for factor *P* ($p < 0.001$). The incorporation of nested effects to model gradient was also significant ($p < 0.001$, $F = 18.7$, df = 16,2). Similarly, predictive $\log_{10}$ ('BWT alone'), nested with factor *Time*, yielded significantly positive gradients 0.94, 0.95, and 0.74 ($p < 0.01$), and the incorporation of nested effects to model-gradient was also significant ($p < 0.001$, $F = 12.8$, df = 16,3). Random effects due to type of plankton (*ZorP*) and *Time* were redundant, as they did not improve their respective fixed effect models, so that they are not presented here (Table 3). The AIC values suggested that the simplest model, given by $y \sim T + c + \varepsilon$, was the best predictive model ($p < 0.001$, $F = 35.3$, df = 16,1), demonstrating that regardless of the plankton type or sequential subsample time factor, BWE has an additional effect on the reduction of plankton abundance with $R^2 = 0.69$ (Table 3). The effect of reduction in abundance increases with increasing plankton

abundance in ‘BWT alone’ tanks; a positive effect was not apparent at very low abundances (Fig. 1).

DISCUSSION

Our study found that BWE used in combination with BWT provides a significant additional reduction of plankton abundance, and this effect increases with greater abundance (after treatment) in ‘BWT alone’ tanks. As per expectations, ‘BWT alone’ tanks filled in freshwater ports contained mainly freshwater or euryhaline taxa at discharge, while ‘BWE plus BWT’ tanks contained mainly marine taxa that primarily originated from the BWE area, and would likely not survive if discharged into freshwater ecosystems. Due to the almost exclusively marine composition of live zooplankton taxa after BWE, the ‘BWE plus BWT’ strategy notably reduces introduction risk of zooplankton through environmental mismatching. The environmental mismatching effect is less clear for phytoplankton, since many marine and euryhaline species were observed in the initial ballast water uptake sample of the freshwater ports though it is unknown if they were alive. Notably, there were no freshwater phytoplankton species observed in discharge samples of the ‘BWE plus BWT’ experiments. A recent study examining BWE plus chlorination *versus* BWE or chlorination alone found similar results, with the hybrid treatment generally having lowest densities of plankton and microbes at discharge, although they did not assess the risk of the species composition resulting from the different management strategies.[43]

When BWE was first introduced, it was presumed that incoming ocean taxa would be both lower in density and have a lower survival probability along the coast

than those taken up at coastal ports.[44] Empirical studies conducted since then have indicated that both abundance and species richness of plankton may increase immediately after BWE,[10,45-46] but that longer voyages result in lower abundance and species richness of zooplankton and diatoms, and lower species richness of dinoflagellates due to mortality.[2,46-49] During our trials, plankton abundance was consistently lower in the ocean than in coastal freshwater ports. As a result, BWE used in combination with BWT might result in additional benefit by lowering the 'challenge' faced by the BWT systems.

While we are expecting that BWT systems will greatly reduce transport and introduction of aquatic species into new habitats, our study demonstrates that taxa such as Copepoda, Gastropoda and Nematoda may survive BWT applications. In particular, Copepoda were recorded alive after all three trials. As transport vectors change through time, the associated species assemblage will also change, such as when the replacement of solid ballast with ballast water in the late nineteenth century led to a change in ship-mediated introductions from insects, plants, and earthworms to aquatic taxa.[5,50] Previous studies testing BWT systems similarly observed that smaller, soft-body zooplankton and/or zooplankton with small juvenile stages such as Rotifera, Copepoda, or Mollusca selectively survived treatment.[32,51] With the application of BWT systems in the future, under both 'BWT alone' and 'BWE plus BWT' scenarios, we may observe a reduction in the rate of establishment of new species, with selection towards Copepoda as forthcoming aquatic non-indigenous taxa. Similar taxonomic shifts may be expected in phytoplankton as well.

The zooplankton taxonomic composition in the two freshwater ports used as starting points for our trials was composed of freshwater or euryhaline species, with only one marine species recorded; interestingly, beside freshwater or euryhaline species of phytoplankton identified, at least 14 phytoplankton species found in the ballast water at uptake are considered marine. Our phytoplankton species identifications were completed several months after the trials on Lugol's solution preserved samples, therefore, it is not clear if the marine species recovered were alive during the trials. Possibly, these species were present as contaminants in the ballast pipework of the ships, or might have been recently discharged into the ports by other ships but due to mismatch in environmental conditions were in a state of dying or already dead. Furthermore, the port of Hamburg is located in an inner estuary with tidal amplitude of more than 2 m, thus marine species could possibly occur as a result of tidal water influx. The long term viability of those individuals in freshwater would again be questionable. On the other hand, a more intriguing explanation might be that some, or even all of those species, were alive and established in the freshwater port ecosystems. Some marine species discovered in our study have already been reported in the estuarine Elbe River and the freshwater Port of Hamburg.[52] Invasions of freshwater habitats by marine and brackish species have become increasingly common in recent years.[53-54] Most biodiversity studies are conducted in protected areas and habitats less impacted by human activities, so consequently, our knowledge on biodiversity in ports - invasion hubs - is often poor.

This study is the first research conducted on operational ships fitted with type approved BWT systems to test BWT in combination with BWE as a ballast water

management method, as well as its efficacy compared with BWT systems alone. While our purpose was not to confirm compliance with any ballast water discharge standard, we observed that efficacy among the three different BWT systems was quite mixed. There are several sources of error which can affect the accuracy of numeric organism counts obtained during our testing, including sample collection method, sample size, and conditions encountered on board ships (e.g., vibration, ship rolling and pitching). As a result, our counts should be viewed as an 'estimate' of plankton density, perhaps accurate only within one order of magnitude. With this in mind, it appears that the BWT systems more effectively managed zooplankton on the first two voyages than on the third voyage. Conversely, BWT appeared least effective for phytoplankton on the second voyage. In general, our past experience indicates that most BWT systems utilize a two-stage process to separately manage zooplankton (e.g., filtration) and phytoplankton (e.g., chlorination or UV). As the BWT system on the third voyage utilized only a single stage treatment process (i.e., ozone), the variability in zooplankton densities at discharge across voyages might be attributed to the absence of a filter. The higher density of phytoplankton observed on the second voyage is possibly explained by the delayed metabolic reaction to ultraviolet radiation as measured by FDA staining.[55] The efficacy of BWT systems might also be affected by environmental factors such as temperature, turbidity, or ionic composition (salinity) of the water; due to the small sample size in our study, we were not able to test for the effect of environmental factors.

The invasion process consists of a series of stages, with successful transition between stages dependent on the abundance of individuals introduced, their tolerance

to environmental conditions in a new habitat, and assimilation into the biological community.[5,56-57] As a result, the combined 'BWE plus BWT' strategy that targets two factors in the invasion process (i.e., propagule pressure and environmental tolerance) proved to be more effective in reducing invasion risk to freshwater recipient systems than 'BWT alone'. However, we noted exceptions to the effect of environmental mismatch during our study, and caution that marine species released into freshwater habitats could potentially adapt to lower salinity.[53-54] Consequently, more studies exploring rapid evolution, species adaptation and phenotypic plasticity during the invasion process would be informative.[58] Furthermore, additional tests to determine precision and accuracy of different ballast water sampling and analysis protocols are needed to quantify sampling and counting error, in order to improve assessments of plankton density in treated ballast water discharges.[27-28]

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Supporting Information Available

The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. Detailed information describing sampling scenarios, treatment systems used, ballast tanks, locations and dates of ballast uptake in freshwater ports and mid-ocean areas and ballast discharge for three ship-based trials conducted. EC, UV, P, S and n/a, denote electrochlorination, ultraviolet radiation, port side of ship, starboard side of ship, and not applicable, respectively.

				Uptake		Uptake		Discharge	
	Sampling scenario	Treatment system	Ballast tank(s) number and capacity (m^3)	Freshwater port	Date	Mid-ocean area	Date	Area	Date
Trial 1	'BWT alone'	filter + EC	6 P (656.8)	Hamburg	15.03.2013	n/a	n/a	Coast of Portugal	20.03.2013
	'BWE plus BWT'	filter + EC	6 S (656.8)	Hamburg	15.03.2013	Bay of Biscay	19.03.2013	Coast of Portugal	20.03.2013
Trial 2	'BWT alone'	filter + UV	4 S (2850.4)	Hamburg	18.11.2013	n/a	n/a	Coast of Portugal	24.11.2013
	'BWE plus BWT'	filter + UV	9 S (1187.7)	Hamburg	18.11.2013	Bay of Biscay	23.11.2013	Coast of Portugal	24.11.2013
Trial 3	'BWT alone'	ozone	1 P (916.3) and 1 S (916.3)	Moerdijk	25.07.2014	n/a	n/a	Irminger Basin	04.08.2014
	'BWE plus BWT'	ozone	1 P (916.3) and 1 S (916.3)	Moerdijk	25.07.2014	Irminger Basin	04.08.2014	Deception Bay	09.08.2014

Table 2. Alternative linear mixed effect models fitted to data, where y~$\log_{10}$ (abundances in 'BWT alone'/abundances in 'BWE plus BWT') is the dependent variable, which is dimensionless, and *T*~$\log_{10}$ (abundances in 'BWT alone') is a covariate. Zooplankton and phytoplankton abundances were measured in management scales (i.e., m^{-3} and mL^{-1}, respectively). Here, *c*, *ε* denote the intercept and residuals, respectively.

Alternative Models	Description
$y \sim T + (1\|Ship) + c + \varepsilon$;	*T* non-nested with plankton type (*ZorP*) as a factor. Fixed Effects: T, c; Random Effects: Ship; Repeated Measures: Time.
$y \sim T\,(ZorP) + (1\|Ship) + c + \varepsilon$	*T*(*ZorP*) denotes the plankton type (*ZorP*: Zooplankton or Phytoplankton) nested within *T* as a factor. Fixed Effects: T(ZorP), c; Random Effects: Ship; Repeated Measures: Time
$y \sim T(Time) + (1\|Ship) + c + \varepsilon$,	*T*(*Time*) denotes the time-segment nested within *T* as a factor. Fixed Effects: T(time), c; Random Effects: Ship; Repeated Measures: Time

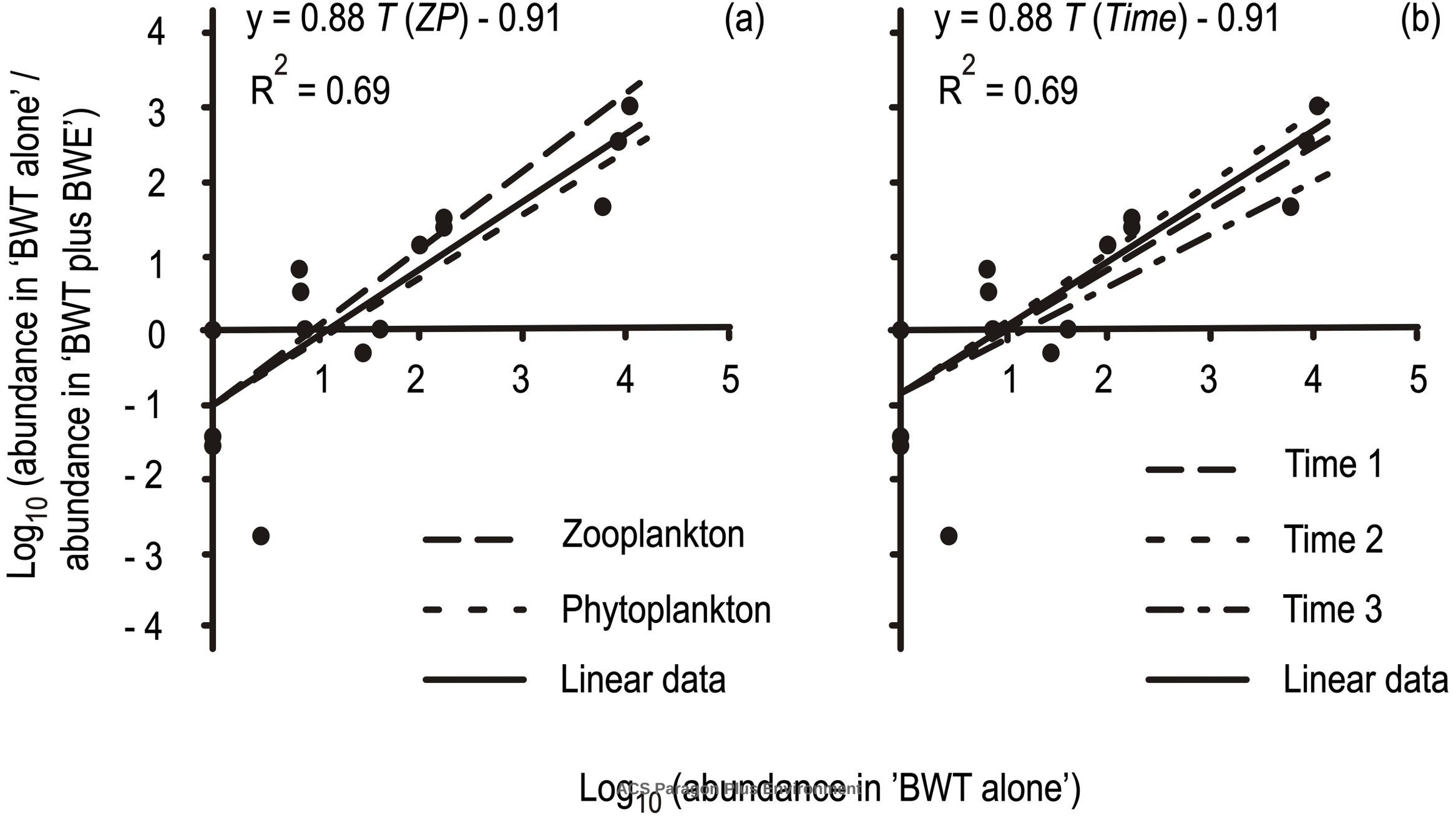
Table 3. Results of alternative linear mixed effect models fitted to data such that $y \sim \log_{10}$ (abundances in 'BWT alone'/abundances in 'BWE plus BWT'), which is dimensionless, and $T \sim \log_{10}$ (abundances in 'BWT alone') as a covariate, with non-nested (model 1), nested with plankton type (*ZorP*) as a factor (model 2), and nested with *Time* as a factor (model 3). *Time* was considered as a repeated measure. Zooplankton and phytoplankton abundances were measured in management scales (i.e., m^{-3} and mL^{-1}, respectively). The results of random effects due to *Ship* and *ZorP* as factors are not presented, as those effects were redundant. Here, *c*, *ε*, FE, and RM denote intercept, residuals, fixed effects, and repeated measures, respectively, while est, var, stde, AIC, Coef, LB, and UB denote estimates, variance, standard error, Akaike Information Criteria, coefficients, lower bound, and upper bound. * denotes significant difference at 95% level.

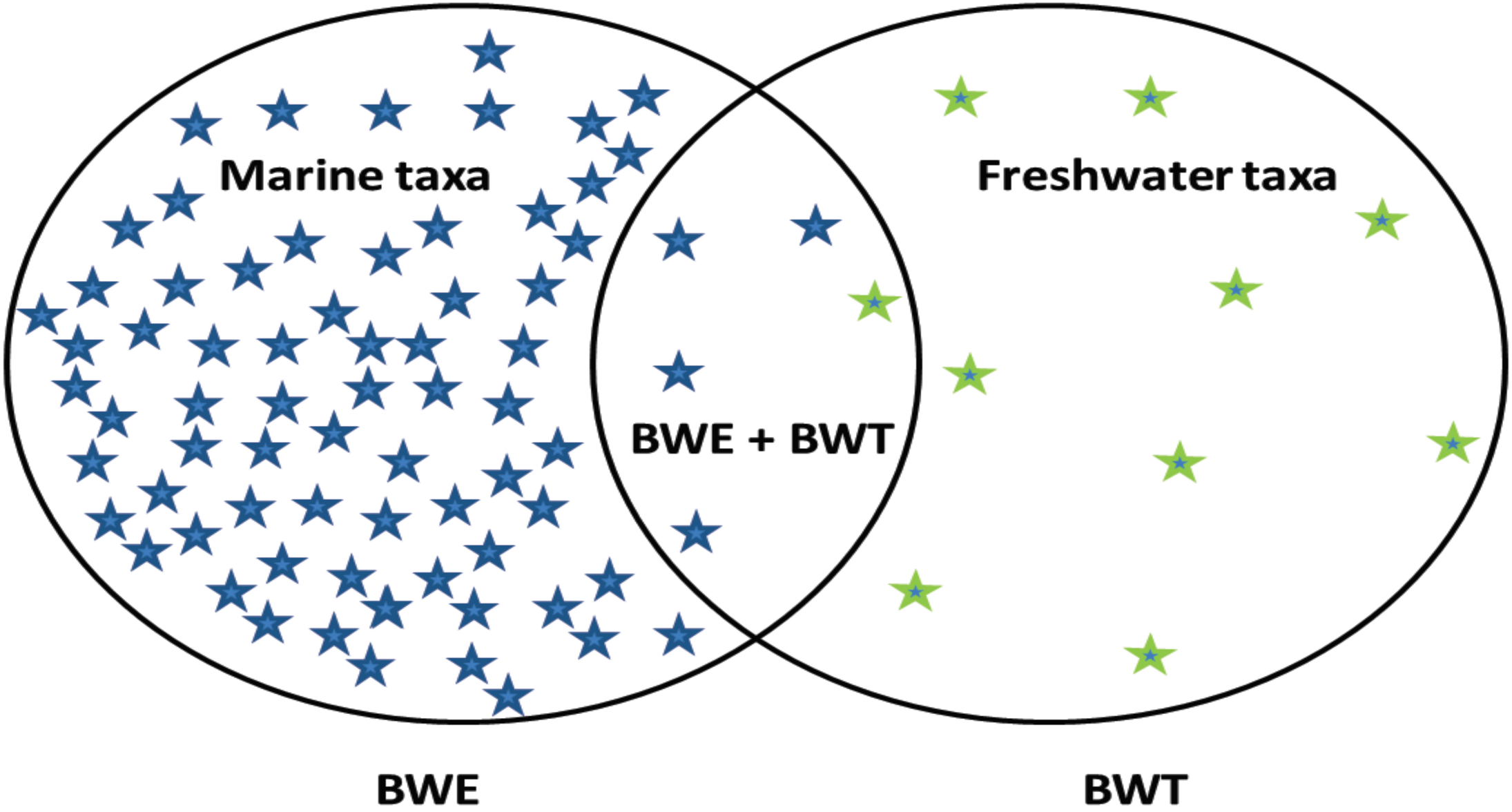
			Fixed effects				Repeated measures		AIC
Alternative Models			Est	*p*-value	*F*(t*), df	95% CI LB and UB	Var	Var stde	
$y \sim T + c + \varepsilon$	FE:	*T*		0.000	35.3, 16,1				44.90
		c		0.009	8.9, 16,1				
	Coef:	*T*	0.88	0.000	*5.9, 16	0.57, 1.20			
		C	-0.91	0.009	*-2.9, 16	-1.56, -0.27			
	RM:	*Time*					0.67	0.24	
$y \sim T(ZorP) + c + \varepsilon$	FE:	*T(ZorP)*		0.000	18.7, 16,2				46.25

:*ZorP*-nested		*c*		0.007	9.7, 16,1				
	Coef:	*T(P)*	0.87	0.000		0.56, 1.18			
		T(Z)	1.06	0.001		0.50, 1.63			
		c	-1.03	0.007		-1.73, -0.33			
	RM:	*Time*					0.64	0.23	
y ~ *T*(*Time*) + *c* + *ε*	FE:	*T(Time)*		0.000	12.8, 16,3				47.96
:*Time*-nested		*c*		0.007	9.5, 16,1				
	Coef:	*T(Time1)*	0.95	0.000	*4.8, 16	053, 1.37			
		T(Time2)	0.94	0.000	*4.8, 16	0.53, 1.36			
		T(Time3)	0.74	0.002	*3.7, 16	0.31, 1.17			
		c	-0.92	0.007	*-3.1, 16	-1.55, -0.29			
	RM:	*Time*					0.63	0.22	

Figure Legends

Fig. 1 Graphical comparison of plankton abundance in 'BWT alone' against 'BWE plus BWT' trials. Solid lines are given by fixed effect model, $y \sim T + c + \varepsilon$, where $y \sim \log_{10}$ (abundances in 'BWT alone'/abundances in 'BWE plus BWT'). On the panel (a) $y \sim T(ZorP) + c + \varepsilon$, where plankton type *ZorP* is nested within $T \sim \log_{10}$ (abundances in 'BWT alone'). Dashed lines indicate the nested fixed effect regression lines given for *Z* and *P*. On the panel (b) $y \sim T(Time) + c + \varepsilon$, where *Time* is nested within *T*. Dashed lines indicate the nested fixed effect regression lines given for *Times* of data collection. *Time* was considered as a repeated measure. Zooplankton and phytoplankton abundances were measured in management scales (m^{-3} and mL^{-1}, respectively).




Marine taxa
Freshwater taxa
BWE + BWT
BWE
BWT