Received: 22 January 2019 | Accepted: 4 October 2019

DOI: 10.1111/1365-2664.13528

RESEARCH ARTICLE

Journal of Applied Ecology | BRITISH ECOLOGICAL SOCIETY

Can chlorination of ballast water reduce biological invasions?

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 31622021; Natural Sciences and Engineering Research Council of Canada (NSERC) Canadian Aquatic Invasive Species Network; Alexander von Humboldt Sofja Kovalevskaja Award; China Postdoctoral Science Foundation, Grant/Award Number: 2017M611020; NSERC Discovery and Canada Research Chair grant

Handling Editor: Qiang He

Abstract

1. Ballast water has been identified as a leading vector for introduction of non-indigenous species. Recently, the International Maritime Organization implemented management standards—D-2—where all large, commercial ships trading internationally are required to adopt an approved treatment system using technologies such as ultraviolet radiation or chlorination. However, current management regulations are based only on the total abundance of viable taxa transported (i.e. total propagule pressure), largely ignoring species richness (i.e. colonization pressure).
2. To determine the efficacy of chlorine treatment in reducing invasion risks and changes in transported biological communities inside ballast tanks, we used DNA metabarcoding-based approaches to estimate colonization pressure (here, the number of species/operational taxonomic units [OTUs] introduced) and relative propagule pressure (relative abundance of each species/OTU) of zooplankton communities in control and chlorine treated tanks during four transatlantic voyages.
3. Our study demonstrated that transport itself did not significantly reduce colonization pressure of zooplankton species, nor did chlorine treatment. Chlorine treatment altered community structure by reducing relative propagule pressure of some taxa such as Mollusca and Rotifera, while increasing relative propagule pressure of some Oligohymenophorea and Copepoda species.
4. *Synthesis and applications.* Chlorine treatment may not reduce invasion risks as much as previously thought. Reduction in total propagule pressure does not mean reduction in abundance of all species equally. While some taxa might experience drastically reduced abundance, others might not change at all or increase due to hatching from dormant stages initiated by chlorine exposure. Therefore, management strategies should consider changes in total propagule pressure and colonization pressure when forecasting risk of new invasions. We therefore recommend adopting new approaches, such as DNA metabarcoding-based methods, to assess the whole biodiversity discharged from ballast water. As species responses to chlorine treatment are variable and affected by concentration, we

also recommend a combination of different technologies to reduce introduction risks of aquatic organisms.

KEYWORDS

ballast water treatment, chlorine, colonization pressure, invasive species, non-indigenous species, relative propagule pressure, zooplankton

1 | INTRODUCTION

Human-mediated introduction and spread of non-indigenous species (NIS) can significantly alter ecosystem processes and species' geographical distributions, contributing to global homogenization of taxa (McGeoch et al., 2010; Molnar, Gamboa, Revenga, & Spalding, 2008). Moreover, the magnitude of this threat is rising with an unprecedented increase in transport networks and human trade and travel (Seebens, Gastner, Blasius, & Courchamp, 2013). The shipping industry transports more than 90% of the world's commodities (Hulme, 2009; International Maritime Organization [IMO], 2006), and ships' ballast water is a leading vector for spread of aquatic NIS (Hulme, 2009). To prevent new introductions of NIS by the shipping industry, a series of international conventions have been devised and implemented, with the most recent establishing discharge standards and regulations for the management and control of ship' ballast water and sediments. On 8 September 2017, the IMO enacted its most stringent management standard—D-2—requiring all commercial ships trading internationally to meet numeric ballast water discharge standards unless granted an exemption based on risk assessment (IMO, 2004). According to the regulation D-2 performance standard, ships conducting ballast water management shall discharge less than 10 viable organisms per m^3 whose minimal diameter is ≥50 μm, less than 10 viable organisms per ml with size between ≥10 and <50 μm, and indicator microbes shall not exceed the specified concentrations (IMO, 2004). Consequently, most ships are now required to install an onboard treatment system to treat ballast water to achieve the new abundance-based performance standard.

Numerous technologies such as filtration, ultraviolet (UV) radiation and chlorination have been applied to ballast water treatment systems and have been approved by IMO. Chlorine treatment, which is used either directly or indirectly via in situ electro-chlorination, is the most widely adopted approach, accounting for more than one third of all ballast water treatment systems installed on ships (Lloyd's Register, 2012). Previous studies reported that chlorine treatment was effective at reducing abundance of transported taxa in ballast water of transoceanic ships, namely enterococci, *Escherichia coli* and other coliform bacteria, as well as phytoplankton and zooplankton, with the latter removed at levels exceeding 96% (Briski et al., 2015; Paolucci, Hernandez, Potapov, Lewis, & MacIsaac, 2015; Paolucci, Ron, & MacIsaac, 2017; Vianna da Silva & da Costa Fernandes, 2004). However, efficiency of chlorine treatment may vary according to residual chlorine concentration, reaction time and environmental factors such as water temperature and pH, as well as inherent variation in vulnerability of various taxa probably to applied chlorine concentrations (Lloyd's Register, 2012; Tsolaki & Diamadopoulos, 2010). Current management regulations are based only on the total abundance of taxa transported (i.e. total propagule pressure; Briski et al., 2012; Lockwood, Cassey, & Blackburn, 2009; IMO, 2004), while species richness (i.e. colonization pressure; Lockwood et al., 2009) is largely overlooked (Paolucci et al., 2017). Therefore, the effect of ballast water treatment on species richness and abundance of individual species remains largely unexplored and needed.

Empirical and statistical evidence indicates that both propagule pressure and colonization pressure influence NIS communities in recipient communities (Briski et al., 2012; Briski, Chan, MacIsaac, & Bailey, 2014; Lockwood et al., 2009). High propagule pressure can reduce or eliminate environmental and/or demographic stochasticity, avoid Allee effects and improve the chance of population establishment in a new region (Blackburn, Lockwood, & Cassey, 2015), while high colonization pressure increases the probability of establishment of at least some species due to higher chance that some of them are pre-adapted to recipient environments (Karatayev, Burlakova, Padilla, Mastitsky, & Olenin, 2009; Lee, 2002; Lockwood et al., 2009). Lockwood et al. (2009) simulation model suggests that mean propagule pressure (i.e. the average abundance of species available in ballast water) increases linearly as the proportion of the initial inoculum transported increases, while colonization pressure increases asymptotically. This finding holds important implications for management since it suggests that mean propagule pressure will always decrease with reduced inoculum, while decline in colonization pressure will depend on the starting point on the curve and the severity of inoculum reduction. Propagule pressure also has parallels to genetic diversity of introduced populations wherein larger propagule pressure typically incorporates more genetic diversity of the source population, possibly enhancing adaptation capacity of introduced populations (Bock et al., 2015; Dlugosch & Parker, 2008). However, transport of ballast water typically has profound effects on both propagule and colonization pressure due to hostile conditions inside ballast tanks, resulting in significant changes in community structure (Briski et al., 2012; Briski, Chan, et al., 2014). More importantly, a recent study suggested that a strong reduction in propagule pressure might not be stochastic

but selective, preserving additive genetic variance important for adaptation to novel environments, and that this selection process may result in a greater likelihood of some populations establishing than predicted by propagule pressure considerations alone (Briski et al., 2018). Consequently, examining changes in community structure and reduction in colonization pressure due to ballast water treatment are of tremendous importance for risk assessment and management, particularly if propagule pressure attenuation is selective.

To determine changes in transported biological communities in ballast water, we used DNA metabarcoding-based methods to estimate colonization pressure (i.e. number of operational taxonomic units—OTUs) and relative propagule pressure (i.e. relative abundance of each OTU) of zooplankton communities in control and chlorine-treated tanks during four transatlantic voyages. DNA metabarcoding-based methods were used here as traditional approaches such as morphological identification are prone to incomplete detection and/or uncertain taxonomic identification, in particular for immature individuals (Darling & Frederick, 2018). We focused on zooplankton communities mainly because a recent study showed that zooplankton species still occurred in the communities arriving to new habitats after being transported in ballast tanks (Briski, Chan, et al., 2014). We tested the null hypotheses that there are no changes in transported communities between: (a) initial and final control samples; (b) initial control and final chlorine treated samples; and (c) final control and final chlorine-treated samples.

2 | MATERIALS AND METHODS

2.1 | Experimental design and sample collection

Four experimental voyages were conducted on an operational bulk carrier Federal Venture, provided by Fednav Ltd, travelling from Canada to Brazil between July 2012 and March 2013. The first and third voyages started from Trois Rivières and Bécancour, Quebec, Canada, respectively (freshwater ports), whereas the second and fourth voyages started from Port Alfred (brackish port), Quebec, Canada. We used four ballast tanks (i.e. two control and two chlorine tanks) during the first and fourth voyages and six (i.e. three control and three chlorine tanks) during the second and third voyages for the tests. The ballast capacities ranged between 1,016 and 1,287 t (= m^3). Industrial bleach (sodium hypochlorite 12%, equivalent to 12.0% W/V available Cl_2, Univar Canada) was added into the chlorine-treated tanks during the ballasting, resulting in an initial dose of 20 mg/L for the first three voyages and 10 mg/L for the fourth voyage. Chlorine was directly delivered to the bottom of chlorine-treated tanks, and comprehensively mixed with ballast water using a peristaltic pump. To prevent contamination, chlorine tanks were located at the port side of the vessel while control tanks were at the starboard side, with different pumps used to access each. Zooplankton samples were collected at the beginning of the voyage and prior to ballast water exchange, hereafter referred as different treatments: initial control, final control and final chlorine treatment. The length between the initial and final sample collections lasted eight, nine, 19 and 15 days for the first, second, third and fourth voyage, respectively. Details of zooplankton sample collection were described previously by Paolucci et al. (2015) and Ghabooli et al. (2016). Briefly, at each sampling, 333 L water was pumped from the top, middle and bottom of each tank contributing to a total sample volume of 1 m^3, which was filtered through a 35-μm plankton net that captured plankton organisms while filtering away environmental DNA (eDNA; most abundant below 0.2 μm; Turner et al., 2014). Different nets were used for collection of final control and final chlorine samples. Filtered samples were preserved in 95% ethanol and stored at 4°C on board the vessel and later processed in the laboratory. In total, 30 zooplankton samples were collected during the four voyages, including 10 initial samples, 10 final control samples and 10 final chlorine samples (i.e. six samples [i.e. two initial control, two final control and two final chlorine samples] from the first and fourth voyages; and nine samples [i.e. three initial control, three final control and three final chlorine samples] from the second and third voyages, respectively). Environmental conditions inside tanks were measured during initial and final sampling using an Orion 130A and Orion 230A meters for salinity and pH, respectively, and Orion 810A for temperature (WT) and dissolved oxygen (DO). Chlorophyll *a* (Chl *a*) was determined in vivo using a handheld Aquafluor fluorometer (model 8000-010; Turner Designs). Triplicate, total suspended solid (TSS) samples were filtered on board the vessel using pre-weighed 0.7 μm glass-fibre filters and stored at −20°C until weighed (Paolucci et al., 2015).

2.2 | DNA extraction and sequencing

Ethanol-preserved samples were well shaken to randomize the distribution of zooplankton, followed by removing 1.5 ml of subsamples for DNA extraction. In order to reduce the potential for PCR inhibition, prior to DNA extraction, all samples were thoroughly washed with distilled water using 35-μm mesh that removed any interference with downstream PCR and sequencing (e.g. chlorine, ethanol, and eDNA if there was any). Total genomic DNA was extracted from each subsample using the DNeasy Blood and Tissue Kit (Qiagen Inc.). The kit we used is designed for animal tissues and cells, blood, and bacteria, and it is not suited for dormant stages, thus DNA extractions from dormant stages should be done by other methods and they still represent a challenge (Briski, Cristescu, Bailey, & MacIsaac, 2011; Montero-Pau, Gómez, & Muñoz, 2008). The concentration and quality of extracted DNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The hypervariable V4 region of nuclear small subunit ribosomal DNA (nSSU rDNA) was amplified using a specifically designed primer pair for zooplankton communities (Uni18S: AGGGCAAKYCTGGTGCCAGC; Uni18SR: GRCGGTATCTRATCGYCTT; Zhan et al., 2013). This primer pair has the capacity to amplify and differentiate a wide range of zooplankton taxa (Zhan, Bailey, Heath, & MacIsaac, 2014). PCR amplifications were carried out in 25 μl reactions with six replicates for each sample, using a unique 8-nucleotide-tagged primer set (Parameswaran et al., 2007;

Zhan et al., 2014). PCR mixture contained approximately 50 ng of genomic DNA, 1× PCR buffer, 2 mM of Mg^{2+}, 0.2 mM of each dNTP, 0.4 μM of each primer and 2 U of *Taq* DNA polymerase (Takara Inc.). PCR amplification programme consisted of an initial denaturation step at 95°C for 5 min, followed by 25 amplification cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s and a final extension of 72°C for 10 min. To reduce cross-contamination between samples, eight-tube strip tubes with individually attached lids were used instead of 96-well plates. All six PCR replicates were set up in a dedicated pre-PCR laboratory to avoid contamination then combined and purified using a Qiaquick purification kit (Qiagen, Inc). All samples, except two final controls of the fourth voyage, were pooled with equal molarity into one library using the TruSeq™ DNA sample preparation kit (Illumina™) for Illumina sequencing. Subsequently, the library was sequenced through a paired-end 300-bp sequence read run on an Illumina MiSeq platform. The two controls of the fourth voyage were not processed further due to low-quality and quantity of the DNA extracts.

2.3 | Sequence processing

Raw sequences were demultiplexed and quality-filtered using the UPARSE algorithm (Edgar, 2013). Reads containing errors in primers and tags were discarded using the python-based scripts provided in UPARSE. These scripts also trimmed out the primer and tag sequences. Subsequently, sequences were quality-filtered using a quality score of Q30 and a maximum expected error threshold of 1.0 and then trimmed to 206 bp. The cleaned reads were de-replicated and then clustered into similarity-based OTUs based on a 97% similarity threshold using the UPARSE-OTU algorithm. OTUs were classified taxonomically by BLAST searching against the NCBI database using the pipeline Seed (Větrovský & Baldrian, 2013) with the parameters of *E* value $<10^{-80}$, minimum query coverage >80% and similarity >85% (Zhan et al., 2014). Unassigned sequences and sequences assigned to vertebrates and algae were removed prior to further analysis; the primers used in our study can amplify some vertebrates and algae, however, they cannot amplify whole communities of these taxa.

2.4 | Statistical analysis

We estimated Pielou's evenness and Shannon–Wiener (*H*) alpha diversity indices using the relative abundance of each OTU by the VEGAN package in R (Oksanen et al., 2015). Relative abundance for phyla was calculated by dividing the number of sequence reads per phylum with the total number of sequence reads in the treatment, while relative abundance of certain OTU was calculated by dividing the number of sequence reads per OTU with the total number of sequence reads in the treatment. We acknowledge that DNA metabarcoding-based approaches are semi-quantitative, and urge caution when using these approaches to make quantitative inferences. However, as previous studies found a general trend that low-abundance species usually correspond to low-abundance sequence reads and high correspondence in community composition estimated from DNA metabarcoding and microscopy (Abad et al., 2016; Sun et al., 2015), we used relative abundance (i.e. relative propagule pressure) to determine changes in community composition. Differences in species richness indices among treatments (i.e. initial control, final control and final chlorine) were assessed using one-way ANOVA implemented in SPSS v.20 (SPSS Inc). Additionally, we conducted one more ANOVA to compare the total number of observed OTUs among the treatments (i.e. initial control, final control and final chlorine). The difference in the number of OTUs for each taxonomic group among treatments (i.e. initial control, final control and final chlorine) was examined by the Mann–Whitney *U* test.

In order to ensure that zooplankton taxa were well recovered from studied samples, we constructed rarefaction curves (Figure S1) with the 'rarecurve' function in the VEGAN package in R (Oksanen et al., 2015). Variation in zooplankton community composition among samples was compared by nonmetric multidimensional scaling ordination (NMDS) based on the distance matrices among samples using the Bray–Curtis index. The abundance of taxa was square-root-transformed before statistical analyses. The significance of separation between NMDS communities was assessed using analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA). Subsequently, the OTUs contributing to the dissimilarity in community structure between sampling time (i.e. initial and final control samples) and chlorine effect (i.e. final control and final chlorine treatments) were identified using similarity percentage (SIMPER) analysis.

To compare the chlorine effects on zooplankton communities, the relative abundance of the OTUs that contributed to the majority of the discrepancy between final control and final chlorine treatments of each tank was visualized using a heat map created using the GPLOTS package in R software (Warnes, Bolker, Bonebakker, & Gentleman, 2016). In addition, the difference in environmental conditions among samples was compared by the NMDS analysis based on the Euclidean distance of variables (WT, pH, DO, Chl *a*, TSS, salinity and Cl^-) of each sample. ANOSIM, SIMPER and NMDS tests were performed using PRIMER 5.0 (Clarke & Gorley, 2001). PERMANOVA analysis was performed in PAST 3.22 (Hammer, Harper, & Ryan, 2001).

According to the analysis of similarity in our study (see the Section 3), metazoan OTUs contributed to the major dissimilarity between the final control and final chlorine samples, therefore a further analysis focused on the efficacy of chlorine treatment on Metazoa. In order to distinguish the effect of chlorine from environmental factors on metazoan community structure, we conducted the linear ordination method of redundancy analysis (RDA) using the "rda" function in the R package VEGAN (Oksanen et al., 2015).

3 | RESULTS

3.1 | Zooplankton community composition of ballast water

A total of 1,819,122 raw sequence reads were obtained after high-throughput sequencing for 28 samples. After quality trimming,

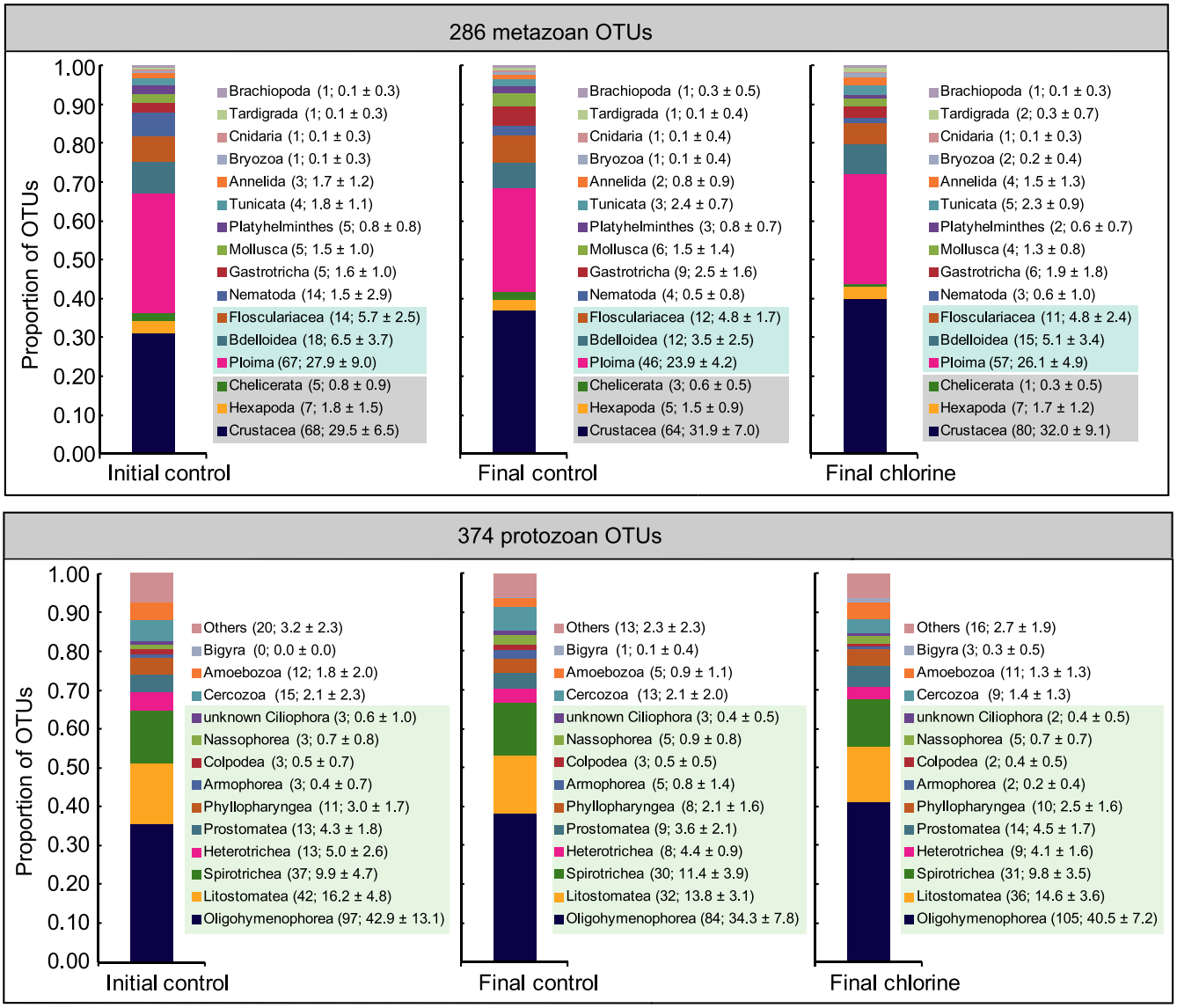

FIGURE 1 Phylum composition of zooplankton communities for both Metazoa (upper figure) and Protozoa (lower figure) from initial control, final control and final chlorine treatment samples. The most abundant phyla (Arthropoda—grey box, Rotifera—blue box, and Ciliophora—green box) were presented at lower taxonomic level as well. Numbers in brackets represent the total number, mean and SD of operational taxonomic units (OTUs) for corresponding taxonomic groups, respectively

filtering and demultiplexing, 227,406 clean reads were obtained, representing a total of 801 OTUs. When unassigned sequences and those assigned to algae or vertebrates were removed, 660 OTUs remained for downstream analyses of zooplankton communities (Figure 1). Across all samples, the number of observed OTUs varied between 105 and 266 (Table 1). Approximately 43.3% (286 OTUs) of assigned OTUs belonged to Metazoa, with the majority being Arthropoda (117 OTUs; 40.9%), including 99 Crustacea, 11 Hexapoda and seven of Chelicerata, followed by Rotifera (110 OTUs; 38.5%) that consisted of 72, 22 and 16 OTUs of Ploima, Bdelloidea and Flosculariaceae, respectively (Figure S2). More than half of the OTUs were assigned to Protozoa (374 OTUs; 56.7%), with the largest group belonging to Ciliophora (297 OTUs; 79.4%), followed by Cercozoa (26 OTUs; 7.0%) and Amoebozoa (15 OTUs; 4.0%).

3.2 | Effect of "Transport" versus "Chlorine Treatment"

In general, the number of observed OTUs and two diversity indices (Pielou's evenness and Shannon–Wiener diversity index) decreased slightly but not significantly in both final control and final chlorine samples when compared to the initial samples (one-way ANOVA; $p > .05$ for all pairs; Figure S3). We also did not detect any difference in the number of observed OTUs nor diversity indices between final control and final chlorine treatment samples (Mann–Whitney U test; $p > .05$ for all pairs; Figure 1; Figure S3). Consequently, neither "Transport" nor "Chlorine Treatment" significantly reduced colonization pressure of transported communities.

The distribution of reads across treatments by taxonomic groups (i.e. relative propagule pressure) demonstrated considerable

TABLE 1 Number of operational taxonomic units (OTUs) and sequences for each sample

Ballast tank	Ballast source	Sampling period	Treatment	No. all OTUs	No. metazoan OTUs	No. sequences
T2-1	Trois Rivières, Quebec	Initial	Control	153	75	3,418
T2-2	Trois Rivières, Quebec	Initial	Control	187	104	14,503
T3-1	Port Alfred, Quebec	Initial	Control	230	107	23,584
T3-2	Port Alfred, Quebec	Initial	Control	133	63	1,463
T3-3	Port Alfred, Quebec	Initial	Control	149	77	2,244
T4-1	Bécancour, Quebec	Initial	Control	266	118	12,570
T4-2	Bécancour, Quebec	Initial	Control	162	61	3,504
T4-3	Bécancour, Quebec	Initial	Control	175	76	4,646
T5-1	Port Alfred, Quebec	Initial	Control	162	80	3,787
T5-2	Port Alfred, Quebec	Initial	Control	105	50	1,069
T2-4	Trois Rivières, Quebec	Final	Control	144	69	16,478
T2-6	Trois Rivières, Quebec	Final	Control	161	85	8,271
T3-5	Port Alfred, Quebec	Final	Control	155	63	2,800
T3-7	Port Alfred, Quebec	Final	Control	184	71	5,490
T3-9	Port Alfred, Quebec	Final	Control	110	59	1,505
T4-5	Bécancour, Quebec	Final	Control	152	84	3,984
T4-7	Bécancour, Quebec	Final	Control	139	73	4,941
T4-9	Bécancour, Quebec	Final	Control	176	93	5,398
T2-3	Trois Rivières, Quebec	Final	Chlorine	175	92	15,882
T2-5	Trois Rivières, Quebec	Final	Chlorine	129	58	3,779
T3-4	Port Alfred, Quebec	Final	Chlorine	167	67	2,993
T3-6	Port Alfred, Quebec	Final	Chlorine	151	84	7,173
T3-8	Port Alfred, Quebec	Final	Chlorine	160	71	3,109
T4-4	Bécancour, Quebec	Final	Chlorine	137	65	2,360
T4-6	Bécancour, Quebec	Final	Chlorine	200	109	6,675
T4-8	Bécancour, Quebec	Final	Chlorine	174	94	9,420
T5-3	Port Alfred, Quebec	Final	Chlorine	141	63	5,271
T5-5	Port Alfred, Quebec	Final	Chlorine	189	78	3,912

structural difference both in metazoan and protozoan communities (Figure 2; proportional abundance for each sample is provided in Figures S4 and S5). In the case of Metazoa, Arthropoda had the highest relative propagule pressure across all treatments, with 18.1% increase in final control and 28.5% increase in final chlorine samples compared to the initial control. The second highest relative propagule pressure belonged to Rotifera both in initial control and final chlorine, but in final control, Mollusca was particularly pronounced (12.5% vs. 1.6% and 0.1% for initial control and final chlorine; Figure 2a). Relative abundance of the 20 most abundant OTUs was influenced by both 'Transport' and 'Chlorine Treatment' (Figure 2). In the case of Protozoa, the pattern of relative propagule pressure also varied by treatment. Initial control was dominated by Oligohymenophorea (58.0%) and Litostomatea (18.7%), whereas the final control and final chlorine had relatively few Litostomatea (5.8% for final control and 7.9% for final chlorine) but were dominated by Oligohymenophorea (68.8% for final control and 63.8% for final chlorine) and Phyllopharyngea (15.1% for final control and 20.5% for final chlorine; Figure 2b). Among the 20 most abundant OTUs, relative propagule pressure varied between initial control and both final control and final chlorine, while a certain degree of similarity was detected between final control and final chlorine.

When all samples were collectively analysed using nonmetric multidimensional scaling analysis of OTUs, only minor differences were observed among treatments (ANOSIM global R = −0.041, p = .766; PERMANOVA, F = 0.812, p = .715), and considerable overlap was evident in zooplankton communities of different treatments except for the freshwater initial samples that were clustered into a distinct group (Figure 3a). When freshwater samples were analysed separately, the composition of zooplankton communities changed significantly between initial and final samples (ANOSIM global R = .324, p = .014; Figure 3b; PERMANOVA, F = 2.050, p = .010; Table S6), while there was no clear segregation between final control and chlorine samples (Figure 3b). With brackish samples, zooplankton communities again did not differ significantly between treatments (ANOSIM global R = −.031, p = .532; Figure 3c; PERMANOVA,

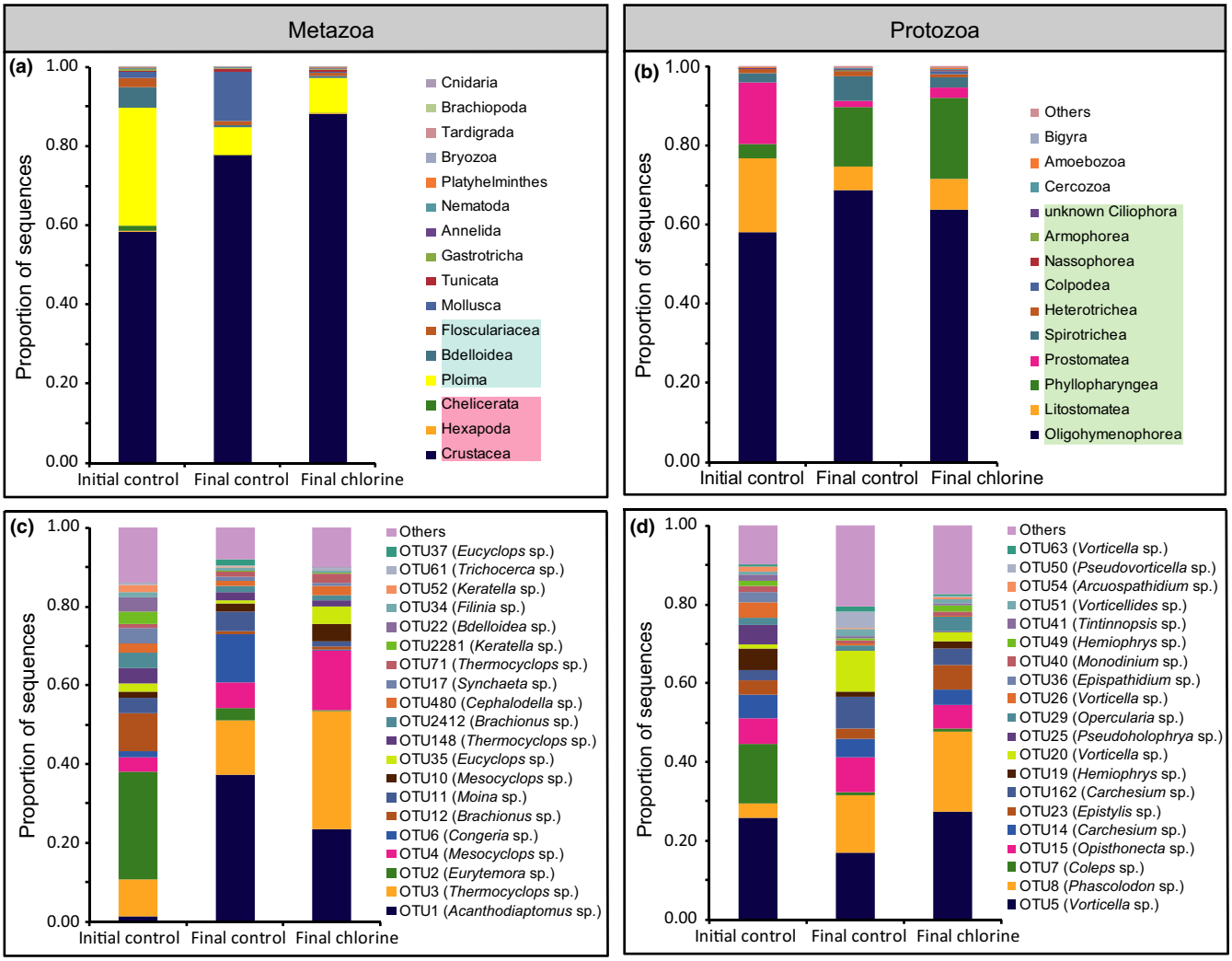

FIGURE 2 Relative abundances of each metazoan (a) and protozoan phylum (b) and the 20 most abundant metazoan (c) and protozoan OTUs (d) for each sample. The most abundant phyla (Arthropoda—pink box, Rotifera—blue box, and Ciliophora—green box) were presented at the lower taxonomic level

F = 0.623, p = .907), although there was some degree of segregation between them. NMDS analysis suggested that freshwater communities were more prone to change than marine ones during transoceanic transfers in ballast water. Our similarity percentage analysis determined 56.1%, 56.5% and 45.2% dissimilarity between initial control and final control, initial control and final chlorine, and final control and final chlorine treatments in freshwater samples, respectively.

3.3 | Effect of chlorine on metazoa

Among the OTUs that contributed to 40% of the discrepancy between final control and final chlorine treatment communities, 12.5% were Protozoa (i.e. 10 OTUs) and 27.5% Metazoa (i.e. 18 OTUs). Therefore, we explored changes of Metazoa in more detail. The heat map demonstrated that relative propagule pressure of Metazoa was lower in the low (10 mg/L) than in the higher chlorine concentration (20 mg/L; Figure 4). Among the top 10 abundant OTUs, relative propagule pressure of six OTUs decreased in the final chlorine treatment samples relative to the final control samples (Figure 5). The relative propagule pressure of OTU6 (*Congeria* sp.) and OTU11 (*Moina* sp.) were higher in final control samples than in initial control samples, while it decreased dramatically in chlorine treatment samples (Figure 5).

To identify the environmental and biological variables responsible for the observed changes in metazoan communities, we used seven environmental (i.e. WT, pH, DO, Chl *a*, TSS, salinity and Cl^-) and one biological (i.e. Protozoa) variable to build a parsimonious RDA model (F = 1.668, p = .0004 for all canonical axes; Figure 6). The composition of metazoan communities varied both between freshwater and brackish ports, and among treatments with distinct environmental conditions (Figure 6 and Figure S7). RDA demonstrated that the first three canonical axes were significant (F = 4.7, p = .0001, F = 3.8, p = .0002 and F = 2.5, p = .0008 respectively), with 26.5% of overall variability of metazoan community explained by the first two

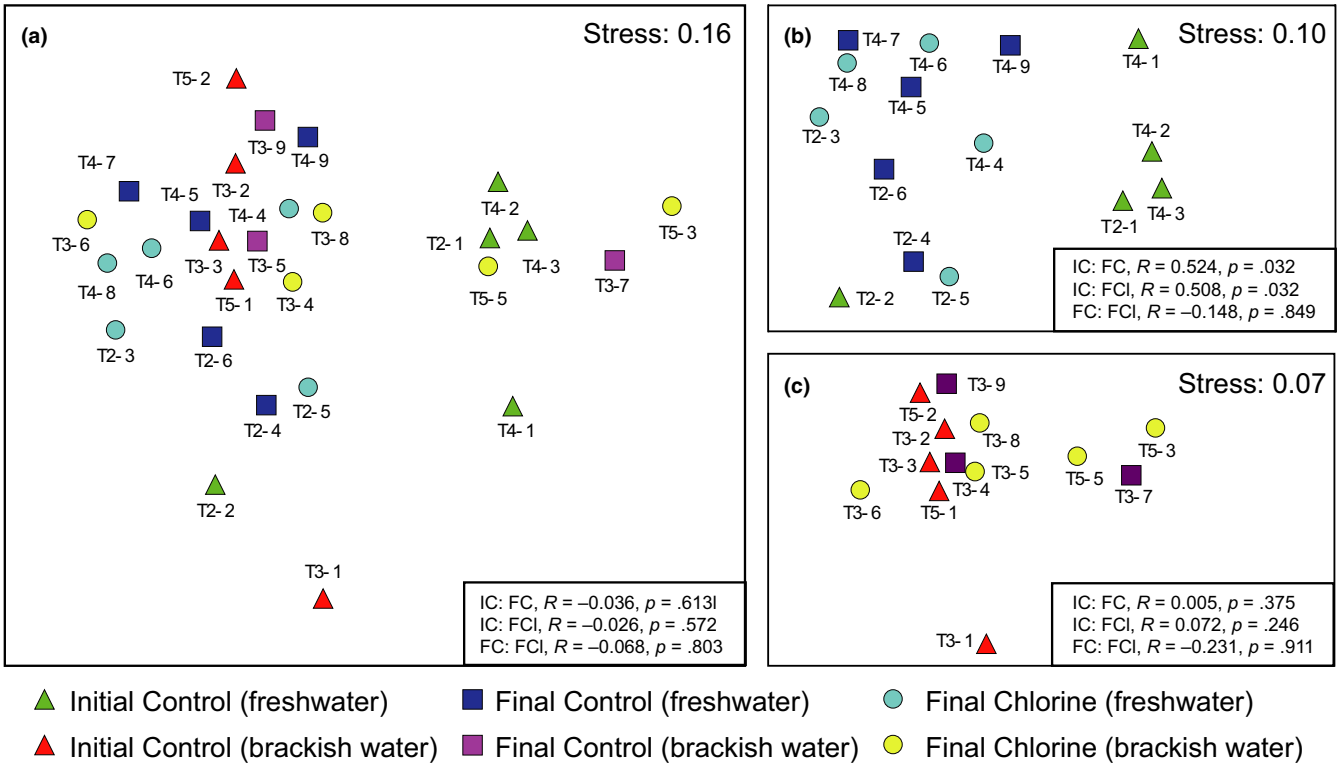

FIGURE 3 The results of nonmetric multidimensional scaling ordination (NMDS) based on the Bray–Curtis similarity of zooplankton communities from all samples (a), and freshwater ports (b) and brackish ports samples (c) separately. IC, FC and FCI denote initial control, final control, and final chlorine treatments, respectively

principal components (RDA1, explained 14.6% and RDA2, 11.9% of variance). RDA1 was mostly explained by Protozoa, and followed by Chl *a* and Chlorine, whereas RDA2 was mostly explained by Chl *a* and TSS. According to the RDA ordination plot (Figure 6), the metazoan communities of the final control samples and final chlorine samples were grouped separately along the chlorine gradient in the freshwater port samples, while this pattern was not found in the brackish samples. In addition, the chlorine treatment had different influence on different taxa. For example, Rotifera and Annelida were generally negatively correlated with chlorine, while Copepoda and Phyllopoda demonstrated the opposite pattern, being more abundant in the chlorine-treatment samples (Figure 6).

4 | DISCUSSION

In this study, we used DNA metabarcoding-based analyses to investigate changes in zooplankton communities in ballast tanks during transoceanic voyages and in application of chlorine treatment to reduce introduction risk of zooplankton. In accordance with previous studies (Briski, Chan, et al., 2014; Briski, Drake, Chan, Bailey, & MacIsaac, 2014; Chan, Briski, Bailey, & MacIsaac, 2014), neither transport itself nor chlorine treatment significantly reduced zooplankton colonization pressure. However, chlorine treatment altered community structure by reducing relative propagule pressure of some taxa such as Mollusca and Rotifera, but increasing relative propagule pressure of some Protozoa (e.g. Oligohymenophorea) and Arthropoda (e.g. Copepoda), particularly in freshwater ballast samples. Our method did not allow us to estimate changes in absolute propagule pressure, but only estimate relative propagule pressure. However, as our study used the same samples as Paolucci et al. (2015), we know that absolute propagule pressure of total zooplankton was reduced in final control (178 microplankton individuals per ml and 1,104 macroplankton individuals per m^3) and final chlorine samples (5 microplankton individuals per mL and 125 macroplankton individuals per m^3) when compared to initial control samples (657 microplankton individuals per ml and 12,613 macroplankton individuals per m^3). Consequently, though absolute propagule pressure of total zooplankton was reduced at the end of voyages, in particular in chlorine-treated tanks (Paolucci et al., 2015), our study determined that colonization pressure was still similar to that at the beginning of the voyage. This finding indicates that if the reduction in absolute propagule pressure was selective (Briski et al., 2018), neither travel nor chlorine treatment necessarily reduced the likelihood of new invasions.

Briski et al. (2018) suggested that a reduction in absolute propagule pressure of a transported species might not reduce invasion risk if the reduction was selective rather than stochastic, with exapted individuals (genotypes) surviving best. In this case, genetic composition of the introduced population would deviate from the original population loaded into the ballast tank, with subsequent random mating of only pre-adapted individuals to new conditions.

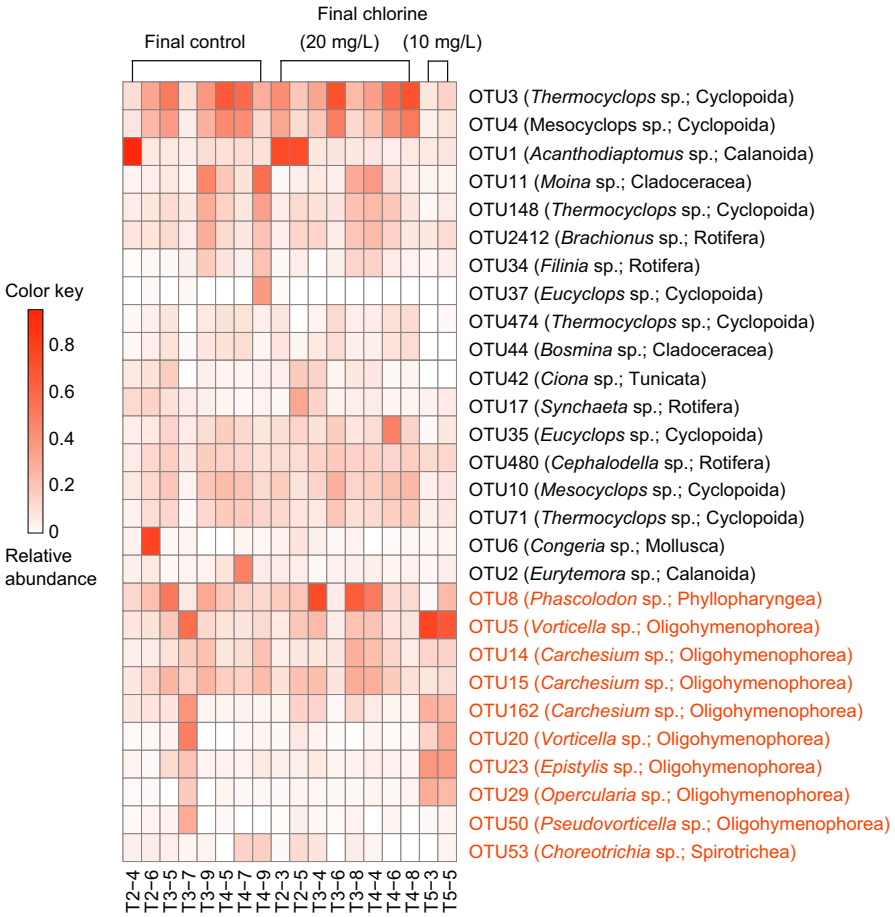

FIGURE 4 Heatmap showing the relative abundance of the operational taxonomic units (OTUs) that contributed to 40% of the discrepancy between the final control and the final chlorine treatments (12.5% Protozoa and 27.5% Metazoa) selected by SIMPER. Red text indicates protozoan, while black metazoan OTUs

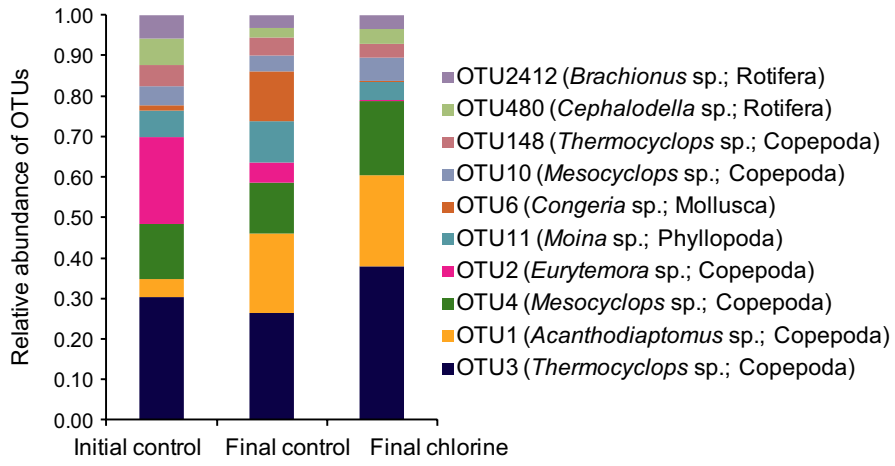

FIGURE 5 The relative abundance of the top 10 abundant operational taxonomic units (OTUs) for initial, final control and final chlorine treatment samples

Consequently, the remaining selected population would have greater mean fitness than the original population and a higher probability of establishment despite low propagule pressure upon introduction (Briski et al., 2018). As Paolucci et al. (2015) reported very strong reductions in absolute propagule pressure, it is reasonable to assume that selection occurred in at least some of the transported species. Therefore, taxa with high relative propagule pressure in our study—such as some Oligohymenophorea and Copepoda species—might be selected and at the same time contain a sufficient number of individuals to overcome demographic stochasticity upon introductions. Consequently, while chlorine treatment may have reduced invasion risk for some species such as Rotifera and Mollusca, it likely increases risk of establishment of exapted species such as Oligohymenophorea and Copepoda.

In contrast to reported efficiency of chlorine treatment on reducing absolute propagule pressure of microplankton and macroplankton using traditional microscopic approaches (Paolucci et al., 2015), our results demonstrated that colonization pressure was not reduced. While some taxa may be eliminated by chlorine treatment, there is also possibility of occurrence of new species due to hatching

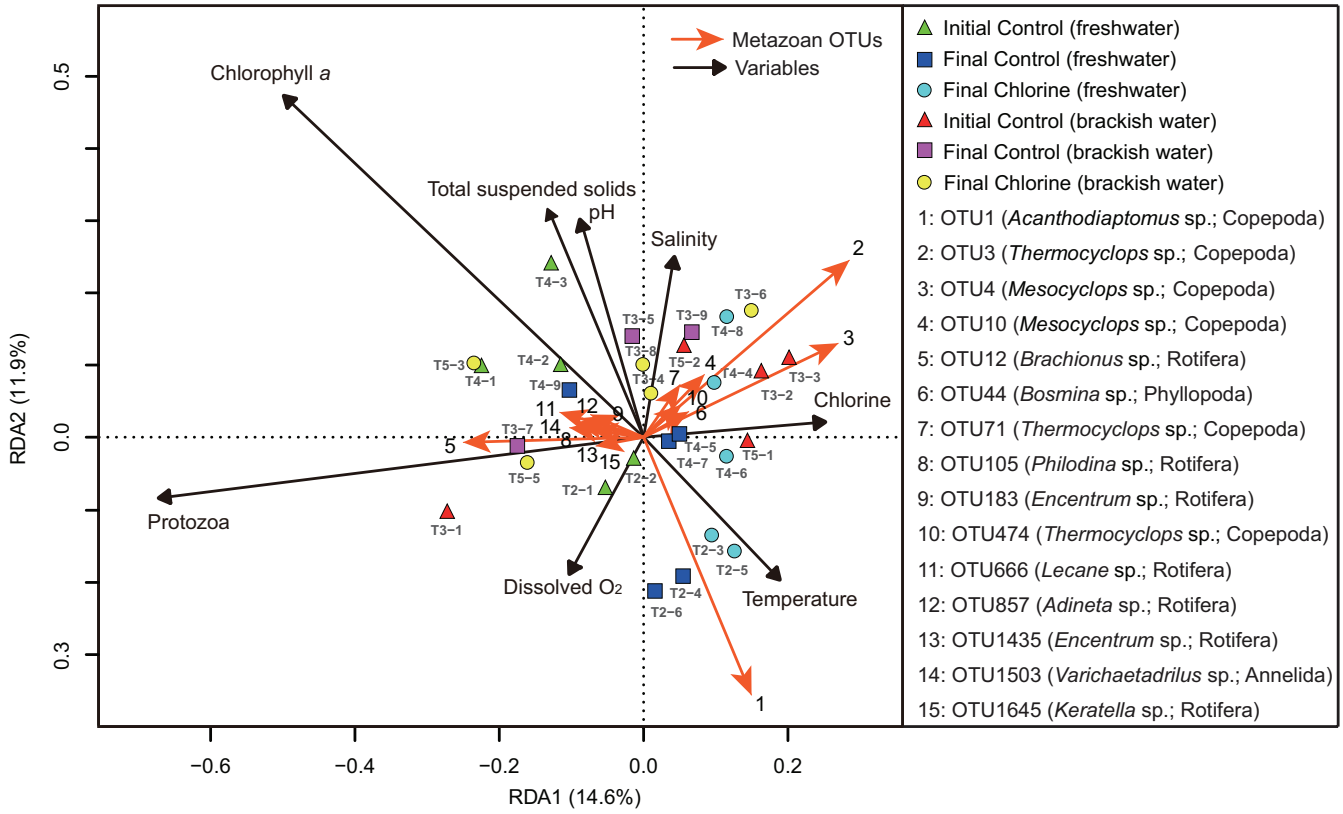

FIGURE 6 The ordination plot based on redundancy analysis of metazoan communities. Arrows in red and black represent metazoan OTUs and environmental and biological variables (Protozoa), respectively. The 15 OTUs most strongly associated with the first two axes are show in the diagram

of dormant stages from ballast tank's sediment (Bailey et al., 2005; Briski, Ghabooli, Bailey, & MacIsaac, 2011; Duggan et al., 2005). The production of dormant, diapausing or resting eggs, cysts or statoblasts is common in many bacterial, fungal, protist, plant and animal species, and dormant stages are commonly found in sediment of ballast tanks (Bailey et al., 2005; Briski, Ghabooli, et al., 2011; Cáceres, 1997; Duggan et al., 2005). Dormancy is a life history strategy involving some types of metabolic and/or developmental depression that organisms produce or enter in during unfavourable environmental conditions (Bailey et al., 2005; Cáceres, 1997; Duggan et al., 2005). While some taxa remain dormant only as long as environmental conditions are unfavourable, others can remain dormant from decades to several centuries (Hairston, 1996; Hairston et al., 1999; Hairston, Van Brunt, Kearns, & Engstrom, 1995). Sodium hypochlorite (NaOCl) has antibacterial activity and a scarification effect on dormant stage coverings, resulting in an increased hatching success (Balompapueng, Munuswamy, Hagiwara, & Kirayama, 1997; Douillet, 1998; Gray, Duggan, & MacIsaac, 2006). Interestingly, our study clearly showed that some Oligohymenophorean OTUs had higher relative abundance at low (10 mg/L) than at high (20 mg/L) NaOCl concentration. This finding accords with several studies that demonstrated that lower concentrations of NaOCl did not decrease but rather increased hatching success of dormant stages (Balompapueng et al., 1997; Douillet, 1998; Gray et al., 2006). According to Gray et al. (2006), exposure of copepod dormant eggs (Crustacea) to a high concentration of NaOCl (e.g. 500 mg/L NaOCl) resulted in reduced hatching. Consequently, while application of chlorine may reduce introduction risk of some taxa, at the same time it may increase risks of taxa being in dormant stages in ballast tanks, such as Crustacea.

Our high-throughput sequencing approach allowed a more comprehensive analysis of changes in zooplankton communities than previous studies based on microscopic approaches, thus providing a thorough assessment of the efficacy of chlorine treatment. Overcoming the main limitations of morphological identification, the high-throughput sequencing approach greatly increased resolution at low taxonomic levels (e.g. genus- and species-level), especially for organisms that are difficult to identify, such as early developmental stages (e.g. larvae, eggs and juveniles), morphologically indistinguishable species, and taxa present in low abundance which are not detectable using the traditional microscopy approach (Gollasch et al., 2002; Rey, Basurko, & Rodríguez-Ezpeleta, 2018; Xiong, Li, & Zhan, 2016; Zhan & MacIsaac, 2015). Although our results of high-throughput sequencing data are promising, DNA metabarcoding-based analyses have inherent drawbacks including distinguishing between living/dead organisms (Barnes et al., 2014; Thomsen et al., 2012; Zaiko et al., 2015) and the possibility of 'tag jumping' (Schnell, Bohmann, & Gilbert, 2015; Xiong et al., 2016). We acknowledge that the potential influence of dead organisms on our results could not be fully excluded and this requires further investigation. However, a previous study reported that the fraction of observed

dead organisms was consistently low across all ballast water samples from vessels in six to 38-day voyages (Carney et al., 2017), and our former study based on microscopic examination of plankton samples collected for this study also confirmed the low ratio of dead animals (Paolucci et al., 2015). As our voyages lasted from eight to 19 days and we revealed the occurrence of new taxa after chlorine treatment, we believe that dead organisms did not affect the main conclusions of our study (i.e. occurrence of new taxa and species abundance). We cannot entirely rule out the possibility that our results were inflated due to the presence of eDNA. However, such an influence should be very limited, as captured plankton samples were filtered through 35-μm nets and then thoroughly washed with distilled water to remove chemical residuals as well as eDNA (see Section 2). Studies have demonstrated that such treatments effectively reduce the influence of residual eDNA in samples (e.g. Turner et al., 2014). We also acknowledge that 'tag jumping' (Schnell et al., 2015) might happen during library preparation. According to Schnell et al. (2015), 'tag jumping' was nearly inevitable with high-throughput sequencing technologies, and may on average account for up to 2.4% of sequences and contribute to false positives. As we used strategies recommended by Xiong et al. (2016) to avoid possible errors caused by 'tag jumping', as well as the evidence of significant community structure among samples (Figures 1–3), we assert that 'tag jumping' could affect the variation among samples to a limited degree. Furthermore, while barcoding databases are increasing very rapidly (Briski, Ghabooli, Bailey, & MacIsaac, 2016), substantial variation in coverage of different taxonomic groups hinders matching to field-based sequences. Nevertheless, high-resolution DNA metabarcoding-based approaches are an important step toward improving the resolution and efficacy of NIS surveillance and risk assessment (Darling & Frederick, 2018; Johansson et al., 2017; Rey et al., 2018; Zaiko et al., 2015). We advocate that combing DNA with RNA analysis, which is capable of differentiating living and dead portions of communities, will improve DNA metabarcoding-based tools for NIS risk assessment and management.

Both transport and chlorine treatment altered zooplankton communities inside ballast tanks. While introduction risks in general might be reduced by a reduction in absolute propagule pressure, as well as in relative propagule pressure of Mollusca and Rotifera, the risk of other groups (e.g. Copepoda and Oligohymenophorea) might be increased. This may be particularly true if the reduction in propagule pressure was selective, and remaining taxa were exapted to environmental conditions encountered both during transport and in recipient habitat (Briski et al., 2018). Furthermore, chlorine treatment could potentially increase colonization pressure of some groups by triggering hatching of dormant stages from ballast sediments. As the increase in relative propagule pressure of some taxa was more pronounced in freshwater than brackish ballast, and the fact that dormancy is more common in freshwater than marine taxa (Cáceres, 1997; de Stasio, 2007), our study suggests that freshwater habitats might be under greater invasion risk than marine or brackish habitats if treatment occurs by chlorination only. Additionally, chlorination systems that depend on chlorine sourced from loaded ballast water (i.e. marine water) might be less effective if the source water is fresh, as electrochlorination creates reactive chlorine compounds by passing an electric current through saline ballast water (Evoqua Water Technologies, 2019; Vorkapić, Radonja, & Zec, 2018). Additional studies should be conducted to determine establishment success of populations inoculated at low propagule pressure and possible selection during transport. Finally, it is important to note that the IMO performance standard pertaining to zooplankton-sized organisms considers only total propagule pressure, when in fact all invasions occur at the level of individual species. True risk reduction can occur only if individual species sustain reductions in population abundance and fitness. It remains to be determined how well total propagule pressure equates to individual species' propagule pressures when applied to ballast water treatment systems.

ACKNOWLEDGEMENTS

Financial support was provided by: the National Natural Science Foundation of China (31622011) to A.Z.; Alexander von Humboldt Sofja Kovalevskaja Award to E.B.; China Postdoctoral Science Foundation (2017M611020) to Y.L.; Natural Sciences and Engineering Research Council of Canada (NSERC) Canadian Aquatic Invasive Species Network; and NSERC Discovery and Canada Research Chair grants to H.J.M. We are grateful to Fednav Limited`s staff, captains and crew of the Federal Venture, and colleagues Samir Quershi, William Gaspar, Leila Ron, Colin Van Overdijk and Amanda Eryaud. Special thanks to Zhiqiang Xia for assistance in sample preparation.

AUTHORS' CONTRIBUTIONS

Y.L., A.Z., H.J.M. and E.B. conceived the study; Y.L., M.R.H. and E.P. collected the data; Y.L., A.Z. and E.B. conducted data analyses; all the authors contributed to the writing of the manuscript and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

OTUs and their matching accession numbers for each sample are available on PANGAEA https://doi.pangaea.de/10.1594/PANGAEA.900272 (Lin et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Lin Y, Zhan A, Hernandez MR, Paolucci E, MacIsaac HJ, Briski E. Can chlorination of ballast water reduce biological invasions? *J Appl Ecol*. 2019;00:1–13. https://doi.org/10.1111/1365-2664.13528