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Aquatic invasive species vector management: challenges and practical solutions on the eve of new global shipping regulations

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By

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Aquatic invasive species vector management: challenges and practical solutions on the eve of new global shipping regulations

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DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is a result of joint research under the supervision of Dr. H. J. MacIsaac, who contributed to all chapters by providing the opportunities, equipment and facilities necessary to complete the research along with intellectual guidance. In Chapter two, Dr. E. M. Paolucci co-wrote and was responsible for supervising sampling for microplankton and bacterial groups during the execution of the five on-board trials for the larger experiment, Dr. A. Potapov and Dr. M. Lewis carried out the coding in Minitab for evaluation of synergy effect. In Chapter three, Dr. M. L. Johansson contributed coding for the virtual sampling model in R, Dr. Xiao and Lewis tested probability density functions in Minitab with my empirical dataset. In Chapter four, Dr. K. G. Drouillard contributed by providing facilities and guidance and Dr. N. Ismail adapted and optimized the method of TTHMs extraction. In all chapters, the primary contributions, main ideas, field work, experimental designs and data analysis were coordinated and/or performed by the author, Marco R. Hernandez Castañeda. The contribution of co-authors was as described above and through the revision of manuscript drafts.

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I certify that, with the above qualification, this thesis, and the research to which it refers is the product of my own work.

II. Declaration of Previous Publication

This thesis includes two original paper that had been previously published in a peer-reviewed journal, as follows:

Thesis Chapter	Publication title/full citation	Publication Status
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ABSTRACT

A new world standard for ballast water management (IMO-D2) will be enforced commencing September 2017. This thesis aims to achieve required final population abundances for target organisms. In chapter 2, I tested synergy effects with two ballast water treatments (chlorination and ballast water exchange). Chapter 3 evaluated the number and volume of samples required to achieve defined error rates. Chapter 4 estimated potential production and exposure to disinfection by-products that may occur when chlorine-treating ballast water. Shipboard trials were carried out en route from Canada to Brazil with sampling carried out using a multiport ballast-tank sampling installation designed for these experiments, followed by statistical modeling and simulation for accuracy determination. Bench experiments for by-product formation were carried out with water samples collected from the same origin ports and a ballast tank to mimic water salinity and natural organic matter content. By-products were analyzed over time to determine potential exposure of vessel personnel. Combined treatment performed equal or better than each treatment alone. Synergistic effects were found for *Escherichia coli* resulting in greatest reductions when treatments were combined. Antagonistic effects (i.e. less than additive) were detected for phytoplankton and coliform bacteria, possibly due to replenishment of individuals after ballast water exchange. Synergistic effects could not be assessed for zooplankton due to complete elimination of viable individuals in all chlorine treatments. Multiport sampling reduced variability from within-tank aggregation. As volume and replicate number increased, error rates decreased. The best tradeoff for accuracy, precision and practicality was

obtained using $1m^3$ ballast samples. Concerns for potential exposure to chemical treatment by-products for vessel personnel were justified, as single-pulse dosing can lead to significant production of harmful trihalomethane by-products, particularly in brackish ballast water with greater natural organic content, but also for marine and freshwater ballast supplemented with organic content. Freshwater chemical by-product levels were lowest for all treatments examined.

Meeting performance-based ballast water effluent standards starting in 2017 will be challenging. My thesis demonstrates that sample sizes for effluent compliance testing should be substantial ($1\ m^3$), and that combinations of treatments may offer the greatest opportunities for reducing target organism abundances to values below permissible thresholds.

DEDICATION

To my Mom, family, Santha and the Noël-Karamatos family my sincere gratitude.

A La Yola quien inicio todo cuando me mando a la primaria a Texcoco

en lugar de la del pueblo, todo gran viaje inicia con el primer paso.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Biological invasions

Biological invasion is largely a human-mediated process that allows the arrival of a non-indigenous species (NIS) to a novel environment beyond their historical geographic distribution range. NIS may be intentionally or unintentionally transported along a pathway via a vector, surpassing the natural barrier that prevented natural dispersal. Some NIS are able to overcome a series of barriers (Geography, Captivity or Cultivation, Survival/Reproduction, and Dispersal) to enter a succession of stages (Transport, Introduction, Establishment, Spread; Elton, 1958; Blackburn *et al*., 2011; Lockwood *et al*., 2013). The Spread stage of invasion occurs when the NIS becomes established in the new location, from which it may or may not spread. Some established NIS cause damage to the environment, health, or economic activity and thereby acquire the name 'invasive' (see Blackburn *et al*., 2011). Not all NIS are invasive, and it is very important for management policy to understand this fact.

Biological invasions are a leading factor diminishing global biodiversity second only to habitat destruction (Walker and Steffen, 1997). This impact is often the result of multiple or very strong interactions that the invader has with native species in the invaded region. Damage ranges from large-scale disruption of food webs - as with zebra mussels (*Dreissena polymorpha*) in Lake Erie - to elimination of native species via predation, parasitism or competition - as with the accidental introduction of brown tree snake (*Boiga irregularis*) in Guam resulting in the loss of 11 native birds species (Wiles *et al*, 2003). Economic damage includes the cost of actions necessary to eradicate, suppress or control the invader, and the value lost from a commodity when

local species or ecosystem services are adversely affected by the invader (Lovell *et al*., 2006).

1.2 Propagule Pressure and Colonization Pressure

As part of the theoretical framework of invasive species establishment success, propagule pressure encompasses three elements including: Size: how many individuals of a species are released per event; Number: how many introduction events occurred; and Condition: the physiological state in which propagules arrive (Colautti *et al*., 2006, Lockwood *et al*., 2009; Simberloff, 2009). Propagule pressure is a strong predictor of invasion success for many NIS and has been proposed as a null hypothesis (Colautti *et al*., 2006, Lockwood *et al*., 2009; Simberloff, 2009). Reducing propagule pressure is an efficient management option because it reduces probability of a species establishing by enhancing demographic constraints (i.e. Allee effects). Reducing the number of introduction events reduces the likelihood of environmental matching between donor and recipient regions (Lockwood *et al*., 2009; Simberloff 2009).

A second component of many biological invasions is the simultaneous introduction of multiple species, particularly with strong vectors like ballast water (MacIsaac and Johansson 2017). The number of species introduced is often called colonization pressure (Lockwood *et al*., 2009). Introducing more than one species increases risk because there is a greater likelihood that at least one species will have its environmental matching requirements met, akin placing bets to all games in one weekend. From a management perspective, preventing the arrival of new NIS is important since it is cheaper and more effective than trying to eradicate, control or suppress an established invader (Leung *et al*., 2002).

Reducing propagule pressure and colonization pressure with management actions that do not interfere with economic activity is an ideal scenario. Identifying vectors and pathways is crucial to reducing propagule pressure and colonization pressure. Ballast water has been one of the largest – perhaps the largest – vector of introduction to aquatic ecosystems worldwide (Carlton, 1985; Grigorovich *et al*., 2003). Tremendous efforts have been made to reduce the strength of ballast water as a vector, with a desire to reduce both propagule pressure and colonization pressure (Ruiz and Reid, 2007; Briski *et al*., 2010).

1.3 Shipping and global trade around world

Transport of goods and merchandise is a major activity of globalized societies; in the latest report from United Nations Conference on Trade and Development (UNCTAD, 2015) the estimated total cargo transported via shipping for the year 2014 was 9.84 billion tonnes, which corresponds with ~ 80% of total world merchandise trade. Shipping of commodities is traditionally linked to the five major bulks (FMB): iron ore, coal, grain, bauxite/alumina and phosphate rock; these bulk goods collectively represented 3.11 billion tonnes, up 5% from the previous year (UNCTAD, 2015). Other shipment categories include: oil and gas, containers, and other dry cargo (UNCTAD, 2015). However, between 1980 and 2014 the total tonnage almost tripled, with container vessels increasing ten fold and FMB five fold, while the other two types of cargo only managed to double (UNCTAD, 2015). Shipping activity level has an impact on the amount of ballast water transported among ports. The IMO (2014) estimates three to five billion tonnes of ballast is transferred annually. Ballast water needs may differ according to the type of vessel: general cargo and cruise vessels require between 1500

to 5000m^3, while barges, bulk carriers, container ships, and tankers require >5000m^3 (King *et al*., 2012).

1.4 Ballast water as vector for aquatic species

Ballast water is necessary for the safe operation of a vessel, when cargo is unloaded the water replacing the bulk commodity provides stability, manoeuvrability and buoyancy control (Carlton, 1985). Carlton (1985) presented evidence that despite oftentimes harsh conditions inside ballast tanks, transport of living organisms in ballast water was possible between ports. Ballast water assembles organisms from different taxa with different population densities and different survival rates (Wonham *et al*., 2001). Historical evidence links biological invasions in the Great Lakes to shipping activity (Ricciardi and MacIsaac, 2000; Ricciardi, 2006), and ballast water is considered the strongest vector within the basin for introduction of new NIS (Locke *et al*., 1993; Holeck *et al*., 2004; Ricciardi, 2006; Grey *et al*., 2007). Since 1989, ballast water has been a target for preventative action to stem invasions including voluntary ballast water exchange (BWE) (Locke *et al*., 1993). In 1993 the procedure was made mandatory and enforced by the Unites States Coast Guard. This procedure compels any transoceanic vessel with full ballast tanks entering the seaway to empty its tanks filled with water from the port of origin and to refill with mid-ocean seawater. Mid-ocean seawater is defined as water from at least 200 nautical miles into the ocean and at least 200 meters depth (Annex B Regulation B-4, IMO, 2004). In order to comply with this rule the entering vessel reports latitude and longitude in which BWE was carried out and salinity within tanks must reach at least 30‰. In case emptying and refilling of the ballast tanks cannot be carried out due to ship design, then flushing through volumetrically three times is the

required procedure. Thus, using Blackburn *et al*.'s (2013) framework, ballast water management focuses on reducing transport of organisms to new regions.

1.5 Standards for ballast water

The aforementioned ballast water exchange procedure was extended in 2006 (by Canada) and 2008 (by USA) to vessels with only residual water in their ballast tanks. Despite the 1993 regulation, invasion in the Great Lakes appeared to continue apace. Under a new IMO D-2 performance standard, vessels with ballast water in their tanks must produce evidence that they are in compliance with numerical limits established for each target group. These target groups are, broadly-speaking, macroplankton, microplankton, and bacteria indicators. The limits set in this standard are listed as follows:

< 10 organisms m^{-3} for plankton with > 50μm in minimum diameter;

< 10 cells mL^{-1} for plankton with ≤ 50 and > 10μm in minimum diameter;

< 1 colony forming unit (cfu) $100mL^{-1}$ for toxicogenic *Vibrio cholera* (O1 and O139); < 250 cfu $100mL^{-1}$ for *Escherichia coli*; and < 100 cfu $100ml^{-1}$ for intestinal *Enterococcus*.

1.6 Ballast treatment options

According to Lloyd's (2016), there are 57 different commercial devices for shipboard ballast water treatment in different stages of approval from IMO. Devices are grouped into broad categories based upon the process used for disinfection: Solid/liquid separation; filtration, hydrocyclone, and coagulation; Chemical disinfection; chlorination, electrochlorination, ozonation, chlorine dioxide, and paracetic acid/hydrogen peroxide; Chemical disinfection (non-oxidising biocides); menadione and vitamin K; Physical

disinfection; ultraviolet (UV) radiation, deoxygenation, cavitation, and heat. All these devices have inherent advantages and disadvantages. Filtration and hydrocyclone will be less effective if the densities of organisms are high as they may eventually clog. Coagulation requires the addition of materials that will eventually accumulate in the system and need to be discarded on a regular basis. Active substances such as chlorine and ozone remain active in solution and need to be neutralized. UV systems required clear waters to be effective. Deoxygenation requires up to four days to neutralize larger organism in addition to sealed tanks. Cavitation is not very efficient by itself and needs to be used in conjunction with another treatment. Heat may be useful as a cooling system for the engine room, but needs time to reach a temperature that effectively reduces live densities in the ballast water. In all cases the intake flow at which the treatment is effective is often the deciding factor for a vessel owner or manufacturer to decide which device they will install. Biologically, there are differences in how effectively these treatments diminish viable population of target groups for IMO D-2. Tsolaki and Diamadopoulos (2010) reported in a meta-analysis for the treatments that had been submitted for approval that 95% of larger macroplankton was removed in a combination of filtration and cyclone and that was the most frequently used option for treatment onboard as it uses the pumps already installed in vessels. However, flow is limited to ~200 m^3 h^{-1}. Their study also reported that UV could remove 100% of the organism tested if flow >1.6 m^3 h^{-1}; this clearly limits the type of vessel that may opt for UV treatments alone. Chlorine and ozone were reported as the most effective, with >95% of organisms removed. Also, chlorine and ozone were tested against the greatest diversity of organisms according to their dataset. Tsolaki and Diamadopoulos (2010)

concluded that although their study showed interesting results, most treatments were still in the experimental stage with tests carried out in mesocosms, sometimes with cultivated organisms. Grob and Pollet (2016) presented a series of scenarios in which they argued that ballast water treatment could be ignoring the regrowth of bacteria and phytoplankton that may occur within as little as 18 hours and four days after treatment, respectively. While a series of assumptions must be true for those scenarios to occur, this argument underestimates the effectiveness of treatments like UV light or active substances which have residual effects over time. In any case, survival of organisms via resting stages (egg, cyst, or seed) and/or resistance to the treatment of choice could potentially trigger regrowth if conditions in the ballast tank allowed.

1.7 Disinfection by-products

Studies on freshwater supply facilities have revealed the production of chlorinated by-products during the chlorine-based disinfection process owing to contact with natural organic matter (Boorman *et al*., 1999). These by-products are harmful to human health with carcinogenic effects (Richardson *et al*., 2000). The most important group of chlorinated by-products is the trihalomethanes (THMs), as they are formed quickly and easily from a single substitution of hydrogen for chlorine and/or bromine onto the methane molecule (Trussell and Umphres, 1978). Other by-products may be formed, including haloacetic acids and haloacetonitrils; however, these compounds require a longer times to form as it requires a stepwise substitution process and the presence of other complex precursor molecules (amino acids and acetate; Singer, 1999). For that reason the World Health Organization and other local agencies targeted THMs as an overall indicator of by-products presence in the water (WHO, 2005).

Sufficient information exists to limit and control THMs production in freshwater supply systems, however that knowledge and the techniques that have been developed are not easily transferable to ballast water treatments because three major conditions are typically not met: i) removal of organic matter as a pre-treatment; ii) controlled residence time after chlorine dosing; and iii) absence of a homogenous or at least similar source of water. While removal of organic matter is a common practice and the most important for controlling by-products in any utility company that uses active substances as means of disinfection (Chang et al., 2001), in ballast tanks there is no space or time available to allow implementation of this strategy. Even residence time can change as schedules are modified in order to accommodate climatic conditions that modify trip length or destination owing to market demands. Finally, vessels don't choose what type of ballast will fill their tanks, as each port has its own unique water; thus loaded ballast could be fresh, brackish or marine, with different levels of organic matter.

1.8 Objectives

This dissertation addresses three important questions arising with the recent change of international law (i.e. IMO D-2 performance standards) that will be enforced worldwide in September 2017. The second chapter evaluates the gain of additive and synergistic effects of a ballast water chlorinated treatment plus the currently-used ballast water exchange; this standard has been largely effective in preventing the transport of species in transoceanic voyages between freshwater origin and destination ports (Bailey *et al*., 2011). Adding another barrier to the old BWE standard is better than discarding the advances achieved with ballast water management when pursuing a new performance standard.

The third chapter focuses on how to empirically validate the abundance level for larger organisms in ballast water, as required for the IMO D-2 standard. Several studies have dealt with the smaller-sized organisms in which volumes and replicates are easy to collect and manage. This study examines the macroplankton group which is more problematic due to the large volume required to accurately represent the real abundance of organism larger than 50 μm in diameter in a ballast tank. This experiment provides empirical data from a real-time onboard experiment and explores the notion of simplified sampling by assessing the importance of sample number and sample volume while maintaining a 0.05 error rate for both false positives and false negatives. Exploring an alternative sampling design that facilitates access to a tank for direct sampling and used empirical data to evaluate the minimum sample volume and replicate number required to obtain accurate estimations of animal abundances.

The fourth chapter describes bench experiments designed to evaluate the potential production of harmful by-products that results when using chlorine as an active substance to treat ballast water. The main focus was to estimate THMs concentrations in treated ballast water, when the content of organic matter and the type of water varies according to port of origin. While THMs have been evaluated in freshwater supply systems and occasionally in marine waters (Allonier *et al*., 1999; Stack *et al*., 2000; Chowdhury, *et al*., 2008), here it was tested a mixed model of chlorine single-pulse-dose, type of water (fresh, brackish and marine) and varying of organic matter concentrations. This experiment presented very realistic scenarios likely to occur in vessels that opt for chlorine in single dose to treat water, as opposed to novel

technology devices that cannot satisfy treatment demands of >5000m^3 over a short period of time.

The last (fifth) chapter presents the major findings of this dissertation and explores future challenges related to ballast water management under the regulations to be enforced later in 2017. There are clear implications in how the vector of NIS transfer will change, though there exists a need to produce valid empirical data to guide treatments under normal working conditions onboard operating vessels.

CHAPTER 2: HYBRID SYSTEM INCREASES EFFICIENCY OF BALLAST WATER TREATMENT

2.1 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 *et al*., 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 *et al*., 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 vessels over the past fifteen years, although its efficiency varies widely (48 to > 99%) depending on starting inocula, effectiveness of ballast purging, and other factors (e.g. Drake *et al*., 2002; Bailey *et al*., 2011).

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

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

2.2 Materials and methods

Experiments were conducted on the bulk carrier Federal Venture during five trials between Canada and Brazil from April 2012 to March 2013 (Fig. 2.1). 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 measured as practical salinity units (PSU); St.-Onge *et al*., 2004), Trois Rivières and Bécancour are freshwater ports on the Saint Lawrence River (see Fig. 2.1).

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. 2.2). 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 O2 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 pore size glass-fibre filters, and stored at -20°C until weighed. For initial and final total organic carbon (TOC) measures, triplicate unfiltered water samples of 0.5–1 L (from the 20 L containers, below) were filtered through a 0.75-µm pore-size Whatman GF/F glass microfibre, and kept at 4°C for TOC analysis using a Shimadzu TOC-VCSH analyser. Initial measures of TOC were used to estimate trihalomethanes (THMs; a by-product of chlorine reactions with organic matter present and a known health hazard to humans) using a simplified version of Hutton's model (Hutton and Chung 1994) in which:

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

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

Safety and technical issues during the discharge process restricted collection of samples and measurement of chlorine from the main deck, consequently we estimated the initial chlorine concentration based on the volume of chlorine delivered and volume of water pumped into tanks. Once the discharge process was concluded, total chlorine concentration was determined using an ExTech Instruments-CL200 meter, on ballast water pumped from the ballast tank using same system used to collect final samples (see below). Whenever safety and weather permitted, we continued sampling for determination of total chorine.

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

In each of the first two trials, two tanks from each starboard and port side were used for control and chlorine treatments, respectively, and ballast water exchange was not applied to these tanks. The remaining three tanks on each side were used for BWE and BWE + chlorine treatments, respectively (Fig. 2.2), where midocean ballast water exchange was conducted in compliance with International Maritime Organization (IMO) procedures. During BWE the vessel was stopped and allowed to drift (< 28 km). Geographic coordinates of ballast water exchange varied for each trial (Fig. 2.1). In order to balance the total number of replicate tanks per treatment, during trials three and four, two tanks that previously served as BWE and BWE + chlorine treatments were re-assigned to control and chlorine treatments, respectively (see Fig. 2.2). The

arrangement of treatments in the fifth trial was the same as in the first two, except that chlorine was reduced to 10 mg L^{-1} in an attempt to reduce its very strong effect (see results). In total, after five trials, we had 12 control tanks, 12 chlorine-only, 13 BWE-only, and 13 for hybrid treatment.

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

Final sampling was conducted about three days after the second dose of chlorine (i.e. following ballast water exchange) was applied. It was impossible to collect water via the ship's ballast pumps in the engine room, thus all final samples were collected directly from three different levels (top, middle and bottom) in each ballast tank according to Murphy *et al*. (2002). An aliquot of ballast water was pumped from each level using a pneumatic, diaphragm pump (< 35 L $minute^{-1}$; Flowmeters Seametrics). Macroplankton samples were collected using different plankton nets for chlorinated and non-chlorinated treatments. Equal volumes of 333 L were pumped from the top, middle and bottom (total 1 m^3) of each tank. In order to clear water remaining in collection tubing, more than 300 L of ballast water was pumped out between aliquot collections. The sampling device had two outlets with valves and flowmeters; while one was used to collect the macroplankton sample, the other was used to collect unfiltered water from

the same level. These samples of unfiltered water were integrated into a single 20 L sample, which was immediately analysed for microplankton abundance. To avoid contamination of the four treatments, different connecting pipes were attached to the pumps in each treatment. Similarly different pneumatic pumps were used for both port and starboard sides.

Triplicate, unfiltered water samples for bacterial analysis were collected directly from the sampling pipe using sterilized 100 mL plastic jars during initial, middle and final sampling. For bacterial analyses, middle samples also included the control and chlorine treatments, which were collected 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 fresh 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 growth of heterotrophic bacteria in general, but may produce false positives for enterococci bacteria, and consequently overestimate abundance of this group, and, less likely, produce false negatives in Colilert testing. Given these non-standard incubation settings, results for enterococci, coliforms, and *E. coli* should be considered putative for those bacterial IMO standards.

During bacterial sampling, an extra 100 mL sample was collected per tank for *Vibrio cholerae* analysis, either from the engine room for the initial and middle samples or from ballast water in ballast tanks for the final samples. Water was filtered through a 2.2 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 (LiferiverTM), 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

and 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. This solution was also used to build a curve for 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 $\log(x + \varepsilon)$ 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: Ha: $\tilde{a}_{Cl+BWE} > 0$. 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.

2.3 Results

2.3.1 Environmental conditions

While initial temperature of ballast water varied between trials, all treatments within a trial had similar initial conditions (Fig. 2.3). Temperature tended to increase in all trials as time progressed ($F_{1, 32}$ = 23.53, P < 0.001; Fig. 2.3), particularly in those that received BWE (Fig. 2.3). 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. 2.3). Control ballast declined slightly in pH over the course of the experiments (Fig. 2.3).

Oxygen and TSS concentrations exhibited variation between tanks at both initial and final sampling (Fig. 2.3). During trials one and five, oxygen concentration decreased in treatments with BWE as compared to those without it. However, during trials two and four the initial and final values were similar, and only in trial three there was a general increase in final oxygen values, mostly due to low initial values. In general, TSS concentrations were higher in control tanks, and lower in tanks with chlorine, BWE, and especially in the hybrid treatment.

Initial salinity of the water pumped to ballast tanks was variable between trials at Port Alfred, whereas Trois Rivières and Bécancour had values close to zero due to their location on the Saint Lawrence River. Final salinity values in control and chlorine treatments for all trials were similar to those recorded during initial sampling (Fig. 2.3). Final salinity was much higher in ballast tanks that involved BWE, reaching the mandatory value of 30 PSU ($F_{3,32}$ = 8.37, P < 0.001; Fig. 2.3).

Our estimated initial chlorine doses for trials one to five averaged between 10.0 and 21.8 mg L^{-1} for tanks that were dosed, while all non-dosed tanks were < 0.4 mg L^{-1} (Fig. 2.4). Chlorine concentration decreased rapidly in dosed tanks during the first four days, though decay rate varied from tank to tank during the first four trials (Fig. 2.4). Measured chlorine decay was very swift during the final trial, dropping to ~0.5 mg L^{-1} within hours of dosing (Fig. 2.4). Calculated THM concentration ranged between 0.56 and 5.19 µg L^{-1}, with higher values associated with high TOC concentrations in initial ballast water (Table 2.1).

2.3.2 Biota

We observed large differences among trials with respect to initial densities for each biological indicator group (significant block effect; Table 2.2). Treatment differences in biological conditions were typically minor at the beginning and often very pronounced at the end of a trial, highlighting strong treatment effects (Fig. 2.5). For all biological indicators (enterococci, coliforms, *E. coli*, microplankton, and macroplankton), the BWE plus chlorination treatment had the lowest final mean density, often followed closely by the chlorine-only treatment (Table 2.2 and Fig. 2.5).

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. 2.5). The overall effect of BWE was significant only for microplankton and chlorophyll *a*

concentration (Table 2.2 and Fig. 2.5). Surprisingly, BWE resulted in higher mean final abundances of enterococci and *E. coli* relative to controls, although differences were minor and not significant (P >0.05) owing to pronounced variation within treatments and trials. 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. 2.6). Our macroplankton samples from oceanic water during BWE (labelled “Middle” in Fig. 2.5) 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. 2.5). Chlorine had a strong suppressive effect on IMO indicator groups as well as coliform bacteria and chlorophyll a concentration (two way ANOVA tests, $P = 0.0001$; Table 2.2), though often not as strong as in the hybrid treatment (Fig. 2.3 and 2.5). The chlorine-only treatment was also very effective at reducing macroplankton abundance, though mean abundance exceeded 100 ind. m^{-3} (Fig. 2.5). Three chlorine trials (third, fourth and fifth) had no viable zooplankton when the experiments ended. Chlorine was the only treatment that affected effective growth rate of macroplankton ($P < 0.0001$, Table 2.2).

While the final absolute abundance of each of the three bacteria indicators was higher when chlorine was dosed at 10 (fifth trial) versus 20 mg L^{-1} (first four trials), only *E. coli* was significantly reduced at the higher dose (Table 2.2). Similarly, lower

microplankton density was observed with the higher dose of chlorine ($P < 0.05$; Table 2.2). Chlorine dose had little effect on final viable macroplankton abundance ($P > 0.1$; Table 2.2).

The effective growth rate and final abundances of bacteria and microplankton were also affected by an interaction between BWE and chlorination (Figs. 2.5 and 2.6; Table 2.2). This interaction was synergistic for enterococci and *E. coli* ($P = 0.03$ and 0.02, respectively) but not for coliforms ($P = 0.21$, Table 2.2), indicating stronger than additive reductions in abundance for the first two groups. Conversely, microplankton exhibited an antagonistic (i.e. less than additive) interaction (Table 2.2), signifying that the effect of the hybrid treatment was less than the sum of individual treatments. The hybrid treatment resulted in the lowest final densities for each of these groups. Chlorophyll *a* concentration behaved similarly to microplankton, with each affected by BWE and chlorine application, though the interaction between treatments was not significant (Tables 2.2 and 2.3). Mean viable macroplankton abundance was much lower in the hybrid than in other treatments (Fig. 2.5). Even so, the effective growth rate was not affected by an interaction between treatments (Fig. 2.6; Table 2.2). Mean final abundance was also slightly above the proposed permissible IMO D-2 performance limit (Fig. 2.5). Density of macroplankton in BWE-only treatments was often higher than controls, and well in excess of IMO D-2 limits.

2.4 Discussion

Ballast water has been a key pathway for global spread of aquatic nonindigenous species during the 20th century (Carlton, 1985). Management of ballast water has evolved over the past three decades, from a virtual laissez-faire approach to global

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

Our experiments were conducted under realistic scenarios on board an operating vessel that was outfitted to allow collection of samples from major sections of ballast tanks, thereby incorporating vertical variation in distributions of biota (Murphy *et al.*, 2002; First *et al*., 2013). Reductions in abundance of bacteria, microplankton and macroplankton in untreated (control) ballast water in relation to voyage length are consistent with previous studies (Drake *et al*., 2002; Tomaru *et al*., 2010). Final densities of bacterial indicator taxa in control tanks were very close to or exceeded those prescribed by IMO D-2 limits. Moreover, in some of the trials, final densities for bacteria were higher than middle and initial concentrations (Fig. 2.5), which was probably related to the gradual temperature increase and favourable oxygen conditions as the vessel moved through progressively warmer water, or to increased dissolved

organic matter released by decomposition of phytoplankton and zooplankton inside ballast tanks (Tomaru *et al*., 2010).

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

The higher bacteria and macroplankton densities after BWE relative to controls (Figs. 2.5 and 2.6), accord with earlier studies conducted in marine environments and highlight the fact that BWE cannot by itself serve as an effective ballast water treatment (e.g. Drake *et al*., 2002; Briski *et al*., 2012 and 2013). Unlike patterns observed in vessels operating between freshwater ports (Bailey *et al*., 2011), our final densities were influenced by replenishment of new live marine organisms during the exchange from fresh to sea water, and consequently macroplankton density exceeded the IMO D-2 standard (Fig. 2.5). BWE was, however, effective at suppressing abundance of microplankton (Table 2.2), consistent with other studies (e.g. Drake *et al*., 2002; Taylor *et al*., 2007).

The effectiveness of chlorine as a biocide for bacterial and microplankton populations is very well established (Gregg and Hallegraeff 2007; Maranda *et al*., 2013), with high efficiency at concentrations ranging from 4 to 50 mg L^{-1}. Our results support

this effectiveness, particularly at the higher dose (20 mg L^{-1}; Figs. 2.5 and 2.6). However, the application of chlorine (20 or 10 mg L^{-1}) resulted in consistent achievement of proposed IMO D-2 standards only for bacterial indicators, whereas results for microplankton varied among trials (Fig. 2.5). This difference was previously observed by Gregg and Hallegraeff (2007), who found complete bacterial inhibition at 15 mg L^{-1}, while more than 25 mg L^{-1} was required to eliminate vegetative cells and cysts of dinoflagellates. Our results demonstrated that a dose of 20 mg L^{-1} yielded significantly higher efficiency than 10 mg L^{-1} with respect to decreasing microplankton density.

Many devices under development for ballast water treatment use chlorination either directly applied or via electrochlorination. These devices rely on a timed exposure of a constant dose (Lloyd's, 2011), whereas we utilized a pulse that delivered a high initial dose that over time was reduced as chlorine oxidized organic matter. Our aim was to keep the chlorine concentration above 2 mg L^{-1} and therefore effective as a biocide over a long period of time. In our trials macroplankton were very sensitive to chlorine; mean final densities were lowered almost an order of magnitude relative to controls (Fig. 2.5), and in three of the trials the final abundance was zero. These results mirror those of Maranda *et al*. (2013) despite their use of a constant dose.

Regardless of the chlorine and initial organism concentrations, when chlorine was combined with BWE the final bacterial, microplankton and macroplankton densities were the lowest recorded (Fig. 2.3 and Table 2.1). Briski *et al*. (2013) also demonstrated potential benefits of combining BWE with ballast water treatment (UV radiation), which resulted in a strong reduction of all groups.

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

The hybrid treatment resulted in a significant antagonistic interaction for microplankton, with the final density higher than would be expected if the two treatments were additive (Fig. 2.5). A likely reason for this lower efficiency is the higher resistance to chlorine of some microplankton, such as cyst-forming dinoflagellates (Gregg and Hallegraeff, 2007). Despite this undesirable antagonistic interaction effect, the hybrid treatment was the only one in which final microplankton density was consistently below the prescribed IMO D-2 limit.

The interaction term between treatments was not significant for macroplankton due mostly to the effectiveness of the chlorine-only treatment. We acknowledge that there exists extensive variability in our data for this group (Fig. 2.5). The hybrid treatment was still the most effective, reducing final densities by almost an order of magnitude versus chlorine alone, and more than an order of magnitude versus ballast water exchange alone (Fig. 2.5).

The IMO D-2 performance standard refers to live organisms without regard to origin or, in most cases, taxonomy. Our studies confirm that combining BWE with chlorination offers enhanced efficiency with respect to reducing propagule pressure better than any either treatment alone for a variety of aquatic groups. Although, it remains unclear exactly how low propagule pressure must be to prevent an invasion, it

is a key factor in reducing overall invasion risk (Lockwood *et al*., 2005). Nevertheless, any treatment that reduces propagule pressure, such as the hybrid management that combines treatment and BWE, should also reduce overall invasion risk. Middle ocean ballast exchange may provide an additional benefit for freshwater habitats (e.g. Great Lakes) that receive foreign ballast because freshwater organisms in original ballast are replaced by oceanic taxa that are unlikely to survive environmental conditions upon discharge into a freshwater port (Briski *et al*., 2013).

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

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

Table 2.1. Formation of trihalomethanes (THMs; μg L^{-1}) estimated using the Hutton model (Hutton and Chung, 1994) and total organic carbon (TOC; mg L^{-1}) (in brackets) in ballast water at the port of origin.

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
THM (mean±SD)	1.19 ± 0.95	4.25 ± 0.77	0.93 ± 0.52	5.19 ± 6.10	0.56 ± 0.35
TOC (mean±SD)	2.95 ± 2.33	4.35 ± 0.93	2.18 ± 0.78	9.74 ± 11.84	4.73 ± 2.39

Table 2.2. Effect of Ballast Water Exchange (yes or no) and chlorine (0, 20 or 10 mg L^{-1}) on indicator group abundances. ANOVA models also considered trial number (Trial #) as a blocking factor. Effect size represents the percentage of the final treatment (BWE, chlorine and hybrid) as a function of the control. Error degree of freedom (d.f.): 31 for macroplankton and 40 for the other groups.

Source	d.f.	F	*P*	Coefficients	Effect size (%)
***enterococci* bacteria**					
Trial #	4	7.53	<0.001		
BWE	1	0.00	>0.9	1.14	334.80
Chlorine	1	146.94	<0.001	-5.31	7.59
BWE*Chlorine	1	4.93	<0.03	-1.52	0.19
10 vs. 20 ppm	1	0.07	>0.8	2.10	
Coliform bacteria					
Trial #	4	14.02	<0.001		
BWE	1	0.78	>0.3	0.15	46.20
Chlorine	1	454.57	<0.001	-7.91	0.01
BWE*Chlorine	1	1.61	>0.2	-0.87	0.00
10 vs. 20 ppm	1	0.19	>0.6	0.86	
***E. coli* bacteria**					
Trial #	4	23.80	<0.001		
BWE	1	2.77	>0.1	1.60	874.70
Chlorine	1	93.51	<0.001	-2.58	0.64
BWE*Chlorine	1	5.61	>0.02	-1.65	0.00
10 vs. 20 ppm	1	3.83	>0.05	-1.10	
Microplankton					
Trial #	4	3.93	>0.008		
BWE	1	10.60	>0.002	-2.19	6.03
Chlorine	1	37.66	<0.001	-3.96	0.48
BWE*Chlorine	1	4.02	>0.05	1.96	0.29
10 vs. 20 ppm	1	4.72	0.0359	2.99	
Chlorophyll (algae)					
Trial #	4	3.09	>0.02		
BWE	1	13.52	<0.001	-0.48	56.54
Chlorine	1	8.74	>0.005	-0.52	69.93
BWE*Chlorine	1	0.11	>0.7	0.14	43.85
10 vs. 20 ppm	1	0.22	>0.6	0.88	
Macroplankton					
Trial #	3	2.61	>0.06		
BWE	1	0.51	>0.4	-1.00	21.33
Chlorine	1	52.96	<0.001	-5.23	11.33
BWE*Chlorine	1	0.33	>0.5	0.66	1.26
10 vs. 20 ppm	1	2.10	>0.1	-3.18	

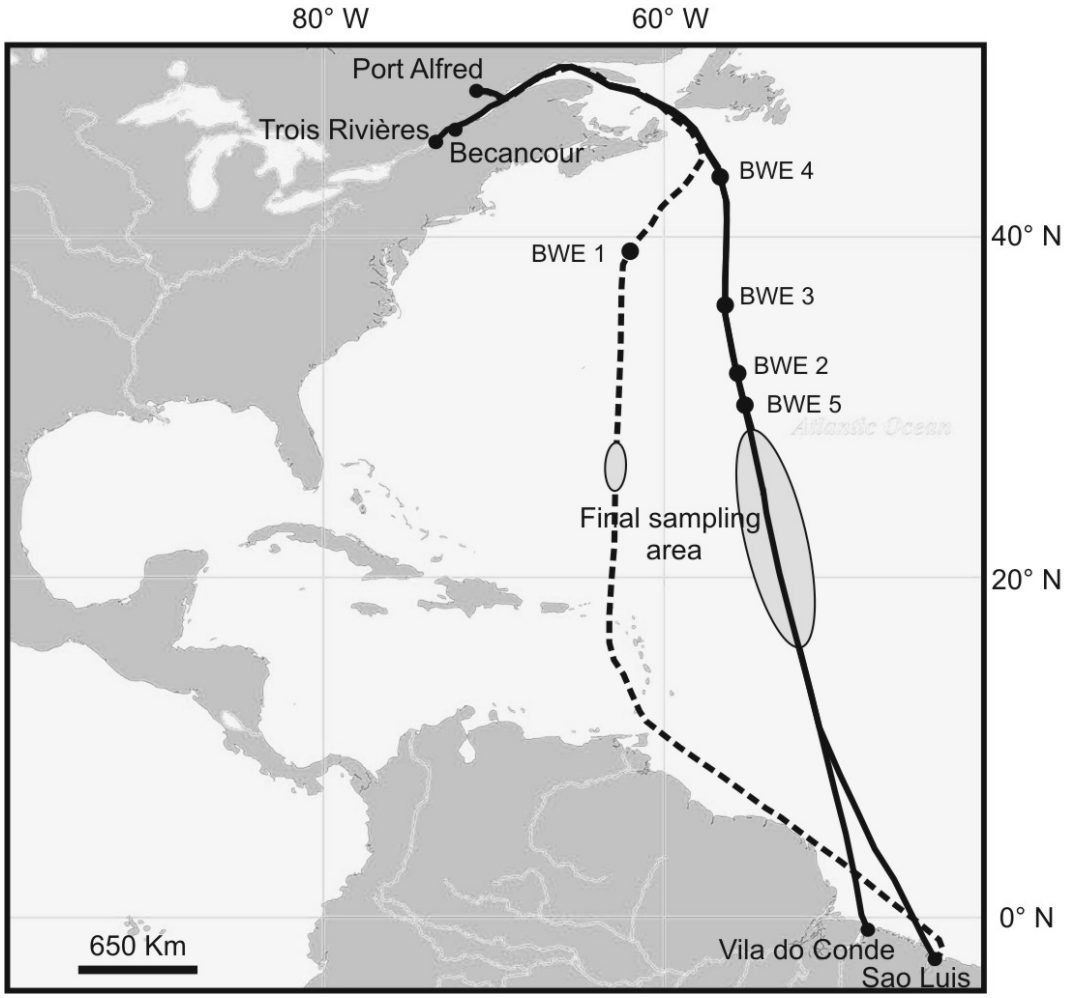

Figure 2.1. Routes followed during the five trials (dashed line for the first trial, solid line for trials two through five) between Canada and Brazil. BWE one through five indicate the position of ballast water exchange for the trials one through five, respectively, and the solid line circle indicates area where final sampling was conducted.

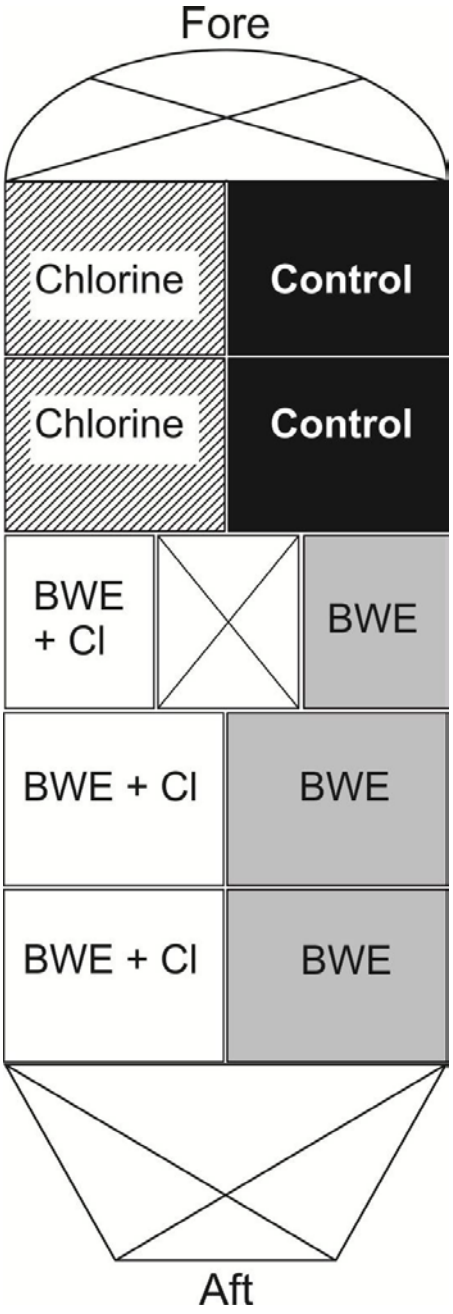

Figure 2.2. Ballast tank schematic showing distribution of treatments during the trials one, two and five. Replication varied in trials three and four, with three chlorine, three control, two BWE+chlorine, and two BWE tanks per trip.

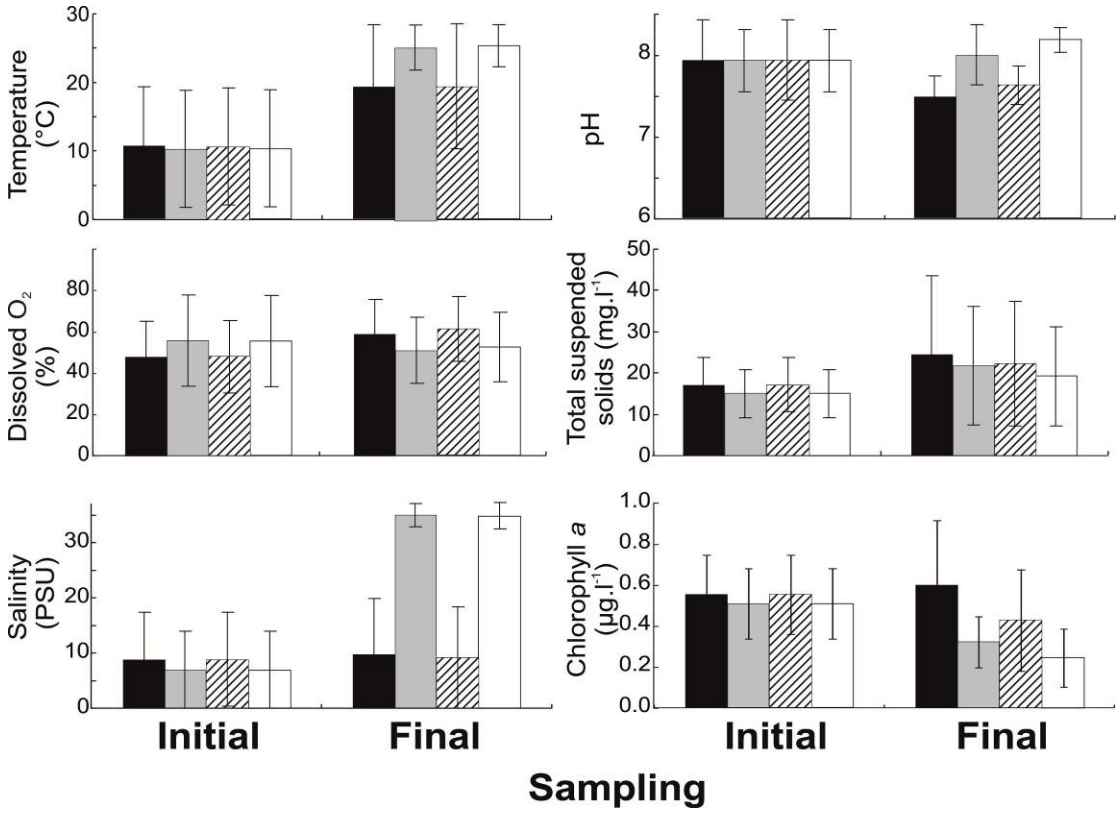

Figure 2.3. Initial and final mean (±SD) values for environmental variables for control (black bars), BWE (grey bars), chlorine (diagonal striped bars), and hybrid treatments (white bars).

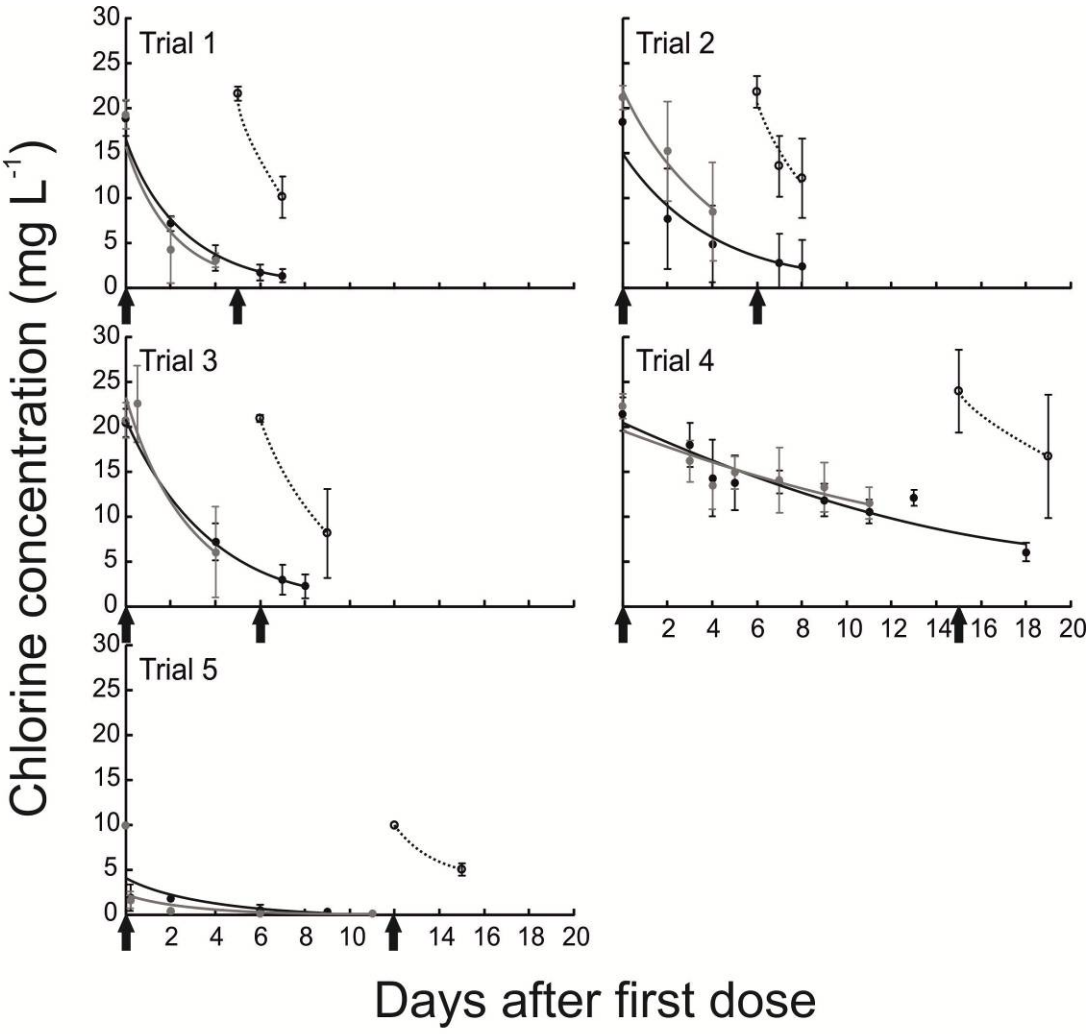

Figure 2.4. Mean (±SD; dots and vertical lines) and modelled (solid lines) chlorine concentration (mg L^{-1}) in ballast tanks during trials one to five. The onset of chlorination is indicated by vertical arrows below the x-axis. Dashed lines represent chlorine concentration for the ballast tanks that received a second dose of chlorine during the BWE (Hybrid treatment).

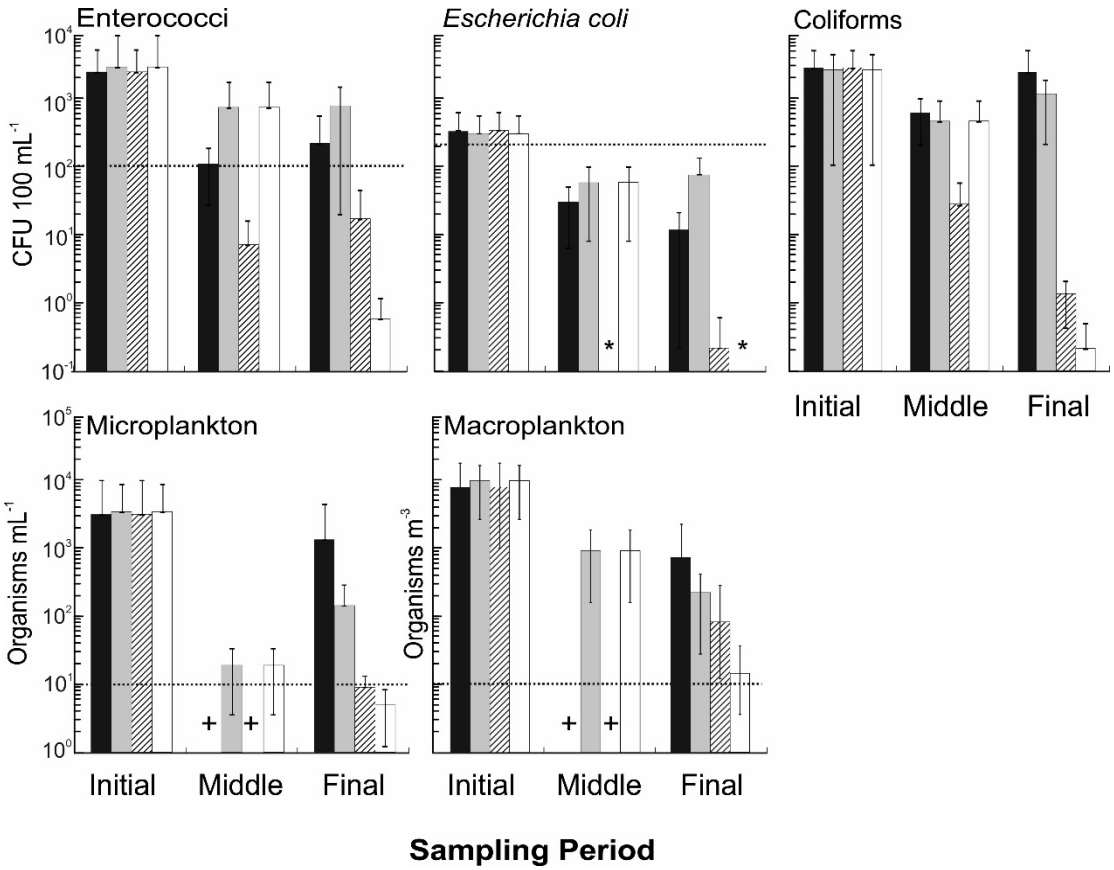

Figure 2.5. Changes in densities (log- transformed initial, middle and final mean values ±SD) of putative enterococci, coliforms, *E. coli*, viable microplankton (≥10 µm and <50 µm) and viable macroplankton (≥50 µm) in all four treatments. Black, grey, diagonally striped, and white bars are control, BWE, chlorine, and hybrid treatments, respectively. Dotted lines indicate the IMO D-2 performance standard maximum limit for each group. * = 0; + = No sample. CFU = colony forming units.

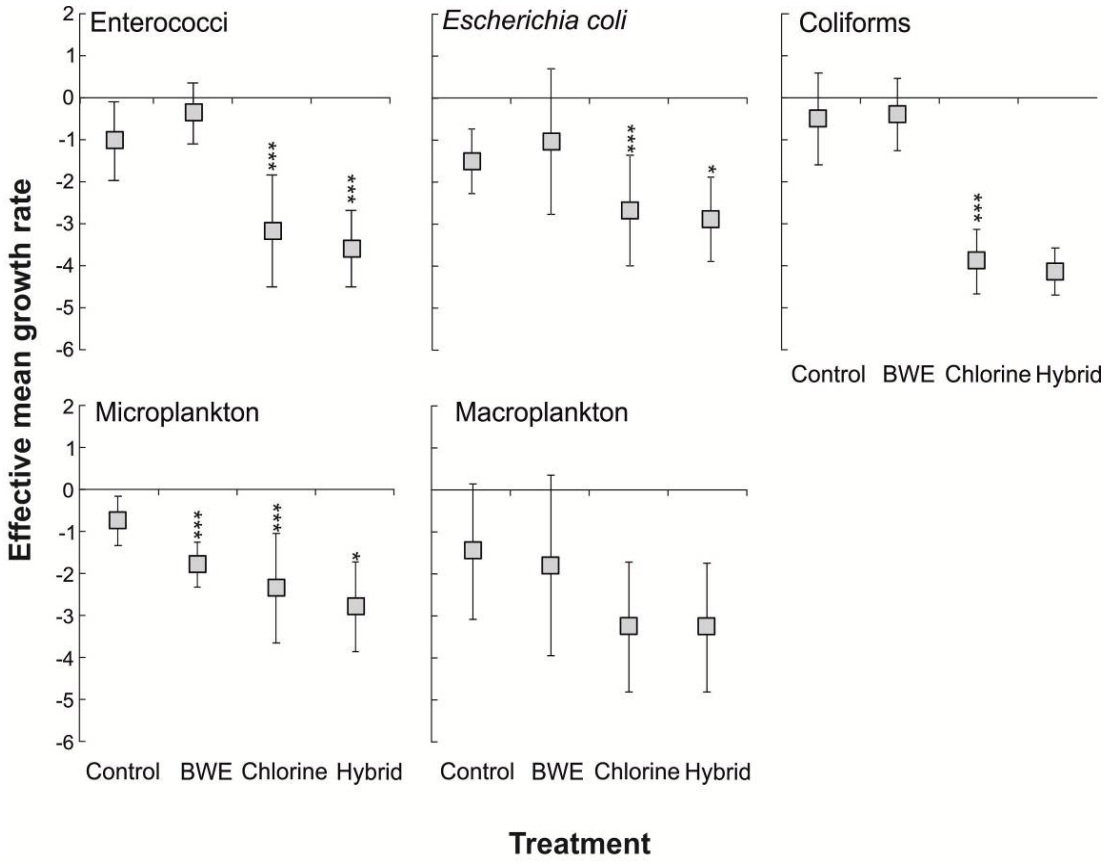

Figure 2.6. Effective mean growth rate ± SD (r; grey squares) for the five biological indicators in control, BWE, chlorine, and hybrid treatments. Upper asterisk indicates significant treatment effects with P ≤ 0.0001 (***) and 0.05 (*) based on two-way ANOVA.

CHAPTER 3: MODELING SAMPLING STRATEGIES FOR DETERMINATION OF ZOOPLANKTON ABUNDANCE IN BALLAST WATER

3.1 Introduction

Ballast water is one of the world's largest vectors for non-indigenous species (NIS) transfer (Molnar *et al*., 2008). Efforts to control this vector in the Great Lakes began in 1989 with voluntary mid-ocean ballast water exchange (BWE) for vessels entering with filled ballast-water tanks, which was followed by mandatory regulations in 1993. Regulations were extended to vessels with 'empty' ballast-water tanks in 2006 and 2008 in Canada and the USA, respectively. Ballast water management (BWM) has become a standard procedure worldwide, and is overseen by the International Maritime Organization (IMO). Current IMO best management practises request vessels with full ballast tanks conduct exchange on the open ocean to ensure that 95% of the ballast volume has been exchanged, to achieve an in-tank salinity of at least 30‰ (IMO, 2008a). While this procedure is effective in preventing the movement of NIS between freshwater ports that are connected by transoceanic routes (Bailey *et al*., 2011), it is less effective when both origin and destination ports are marine (Wonham *et al*., 2001). In 2004 the IMO proposed new performance standards (IMO D-2) (IMO, 2004). This agreement sets numerical limits on the density of two plankton size groups (< 10 viable organisms m^{-3} for minimum dimension > 50 μm and < 10 viable cells mL^{-1} for organisms between 10 and 50 μm) as well as for three bacteria indicators (IMO, 2004). The IMO D-2 convention was ratified in 2016 and will be implemented in 2017 (IMO, 2004).

Many companies and research groups are testing technology and processes to ensure compliance with IMO D-2 standards. Initial steps for approval include testing of devices by an independent third party at verification facilities designed to provide bench-scale estimations, usually referred to as land-based testing. Verification centers also must replicate treatment trials as part of the bench-scale evaluation. Sampling strategies and sampling effort are intended to be easily replicable (IMO, 2008b). Model ballast tanks must be ≥ 200 m^3. For shipboard sampling, control and treated samples need to be collected in triplicate, that uptake and final densities be determined for control tanks, and that viable organism density be assessed before discharge of treated ballast water (IMO, 2008c). However, current guidelines provide no guidance on sample volumes or how they are collected.

Current technology has been tested primarily using land-based tests, though a subset have also used shipboard testing (Gollasch and David, 2010). However, no clear method exists for sampling onboard vessels, particularly for sampling directly from ballast tanks. Thus, an imbalance exists in the prescribed sampling process for land-based versus shipboard testing. Onboard sampling poses a major challenge as the IMO D-2 standard requires very low densities of zooplankton, and estimating density of live organisms requires large sample volumes, even under the best case (and unrealistic) scenario that organisms are randomly distributed (Lee II *et al*., 2010; Miller *et al*., 2011; Frazier *et al*., 2013). Moreover, random dispersion of zooplankton in ballast tanks cannot be assumed, as organisms may aggregate and thus exhibit a patchy distribution (Murphy *et al*., 2002; First *et al*., 2013).

Given that access to tanks is often limited, one important question researchers seek to answer is the relationship between sampling method and sample representativeness (Gollasch and David, 2011). Zooplankton sampling in ballast tanks may be done using plankton nets via hatches (Briski *et al*., 2013; Simard *et al*., 2011) or, less commonly, by pumping a known volume from the tank into a plankton net (McCollin et al., *2008*; Veldhuis *et al*., 2009; Gollasch and David, 2010). Sampling a ballast tank is complicated as access is limited while in port and very difficult while en route (Wright and Mackey, 2008). Samples must be representative of the entire population, easy to replicate, and unbiased. Another consideration is inherent stochasticity associated with low population densities, with concerns regarding both accuracy and precision (Lemieux *et al*., 2008). In addition, the sampling strategy must allow inferences to be made regarding densities of viable zooplankton in treated water.

Another important element is to determine the minimum water volume adequate for representative sampling (Gollasch and David, 2011). Several studies have addressed the effects of low organism density and sample volume on estimating the true density of zooplankton, using both Poisson and negative binomial distributions (Lee *et al*., 2010; Miller *et al*., 2011; Frazier *et al*., 2013; Costa *et al*., 2015). The validity of this theoretical approach has not yet been affirmed empirically. The Poisson distribution is suitable under the assumption of a centralized outflow that can be sampled entirely or in equal time intervals (First *et al*., 2013). A key challenge is access to the entire water column of a tank. Net tows likely introduce bias as only the upper portion of the tank is typically sampled.

In this study, we tested different sampling volumes using three in-tank sampling points to sample the full depth of a ballast tank on a working cargo vessel. Our goal was to identify the sampling efforts that will provide accurate density estimations of zooplankton at the very low abundances that the IMO D-2 standard requires for compliance. We also designed a simple model to contrast common distributions that have been examined theoretically to provide a sample volume that managers can utilize to verify compliance with the IMO D-2 standard.

3.2 Methods

Ballast samples were collected during voyages by the Federal Venture, between 2012 and 2013 [see Paolucci *et al*., 2015]. The vessel transited from three ports (Saguenay, Trois Rivières, and Bécancour) in Quebec, Canada to two ports (Vila do Conde and Sao Luis) in Brazil. A single trial was conducted during each voyage where samples were taken and analyzed. Samples were collected from the largest ballast tank (Tank 2) on the starboard side, with 25 mm diameter inlet pipes (Alfagomma 266GL Water S&D PVC Standard Duty) installed at three depths (4.5, 14.5 and 16.0 m below top deck level) to account for vertical variation in organism distribution (Fig. 3.1). We selected those depths based on the geometry of the tank: 4.5 m is the middle section of the attached wing tank, 14.5 m is the highest open space in the double-bottom tank, and 16.0 m is just above the baffle line in the deepest portion of the tank. Each inlet pipe contributed one third of the total sample volume. To assess sampling effort, triplicate samples totalling 0.10, 0.25, 0.50, 1.00 or 3.00 m^3 were collected. Samples were collected two days after ballast-water exchange was performed in the North Atlantic region using a pneumatic, self-priming diaphragm pump. Ballast water was

transferred from the tank to the forepeak of the vessel where it was filtered through a 35 µm plankton net. Water volume sampled was measured with a Seametrics flowmeter (WMP-Series Plastic-Bodied Magmeter). In-line valves were used to keep water flow rate to 40 L minute^{-1} in order to avoid mortality due to strong currents. Samples were then fixed in 95% ethanol for microscope counting. We assumed that all intact individuals encountered when processing under the microscope were alive at the time of capture. Each sample was counted entirely to assess population density. The order in which sample volumes were collected was randomized using a random number generator in Excel (Microsoft Inc.).

We conducted basic descriptive statistics (mean and standard deviation) for our four trials. Variance was grouped for fall and spring as those samples were not statistically different and mean densities were similar. Our first goal was to determine the best volume for sampling. Since the true density of organisms in the ballast tank was not known, we assumed that the mean density of organisms over all sample volumes in each trial was an accurate estimate of true density. Preliminary analysis of variance (ANOVA) revealed that volume sampled had a large impact on the density of organisms in the tank ($p=0.0056$). We estimated density based on the data points collected from the same volume. We assumed that if we sampled at the same volume repeatedly inside the tank, the density of organisms would follow a given probability distribution function (PDF). We performed the following analysis on each of five PDFs (Poisson, Weibull, Negative binomial, Gamma, and Log-normal) with respect to each volume individually. We estimated the parameters of each PDF by maximum likelihood estimation (MLE). Then, we created random number generators based on the estimated

PDFs to sample more data points (i.e. one thousand data points) for the density of organisms for each volume, and calculated the mean square error (MSE) based on our assumption that the true density was the average of density estimates in all trials for each volume (Walther and Moore, 2005).

Modeling PDF for distribution of zooplankton

Our second goal was to determine how altering the spatial distribution of zooplankton would affect the sampling error rate. Specifically, our objective was to identify the number of samples of a particular volume that would be required to confidently state that a vessel was compliant with the IMO D-2 limit of < 10 viable organisms m^{-3} for zooplankton-sized organisms while keeping the rate of Type I and II errors below 5%. In other words, the cumulative sample number of each individual density (from 1 to 20 organisms m^{-3}) required in each scenario was constrained to no more than a 0.05 error rate for both false positives and false negatives.

We modeled sampling from the ballast tank using a three-dimensional array in R (R Development Core Team, 2016). To simulate sampling from the tank, we defined each cell of the array as 1 L of water and the total volume of the array as approximately equal to the actual capacity of the tank used for our sampling (1,279,400 L in the actual tank, 1,300,000 L in our model 100x100x130 cell array). For each of 1000 replicates, we populated each cell in the array by drawing randomly from two commonly used PDFs (Poisson and Gamma) with mean densities from 1 to 20 organisms m^{-3}. For each PDF, we then sampled between 1 and 30 replicates using sampling points placed at particular heights in the array (to model our field design) but with randomly assigned length and width coordinates. The decision to cut off sampling at 30 replicates was somewhat

arbitrary, but reflects the reality that it is impossible to collect and process large numbers of samples within a reasonable time in order to assess compliance. Thirty represents a number of replicates somewhat above that which would normally be used in field sampling. In each case, we assessed the rate of false positives and false negatives (i.e. we tallied the number of cases where the true mean density was below 10, and the estimate was above 10, or where the true density was above 10 and the estimate was below 10) for all combinations of sample volume and replicate number and determined the minimum replicate number required to achieve error rates less than 5% for each volume.

For the Poisson distribution, we also tested the effect on error rates of having organisms randomly but evenly distributed in the array (Even scenario) at the target density versus organisms preferring the upper wing tank (Uneven scenario: organisms randomly distributed in the 501,400 L upper section at a much higher density [up to ~500X higher density] than the 778,000 L lower region while still achieving the same overall density as the even distribution). In addition, we modeled the effect of sampling only from the upper wing tank, as typically occurs in current working vessels. In an ideal Poisson situation with evenly distributed organisms, there should be no difference between sampling a given volume in a single large replicate versus a number of small replicates. However, because our simulations sampled randomly from a distribution, some variance between replicates occurred.

For the Gamma distribution, we simulated three different distribution shapes to test the effect of variance on our ability to accurately estimate the true density with different sample volumes and replicate numbers. In each simulation, we tested three

levels of dispersion by setting the rate to 0.5, 1.0, and 2.0 to correspond with wide, medium, and narrow distributions, respectively, and then stepwise-adjusted the shape to achieve the desired mean, from 1 to 20 organisms m^{-3}.

3.3 Results

Although the vessel traversed essentially the same route from Canada to Brazil during all four trials, the geographic position of ballast-water exchange and subsequent location of sampling varied slightly from one trial to the next. Mean plankton density ranged from 285 to 1170 organisms m^{-3} (horizontal lines, Fig. 3.2), with a clear seasonal pattern: trial 1 (July) was highest, trial 3 (November) the lowest, and trials 2 and 4 (September and March) were similar and had intermediate densities (Fig. 3.2). From our field sampling, it was also evident that dispersion is larger in smaller volumes and that it is generally low at volumes > 0.50 m^3 (Fig. 3.2).

We observed no significant difference fitting the five distribution functions in our MLE for PDFs (Fig. 3.3), possibly owing to our small empirical dataset (12 data points from each sample volume). We did, however, note that the 1.00m^3 sampling volume exhibited the lowest MSE term relative to other volumes tested (Table 3.1).

When organisms were evenly Poisson distributed in the ballast tank, simulations exhibited a clear relationship between sample volume, replicate number, and our ability to confidently state whether the ballast tank was compliant or not. As mean density of the sample approached the permissible limit of 10 organisms m^{-3}, the total volume of samples required to assess compliance increased (Fig. 3.4, upper panel), and all sampling volumes eventually required >30 samples to assess compliance. Smaller sampling volumes reached our arbitrary limit of 30 replicates earlier than did larger

ones, leading to a larger window where sample sizes were insufficient to confidently assess compliance. For example, in our simulations a single 0.10 m^3 sample (purple line, Fig. 3.4 upper panel) could theoretically be sufficient to identify the sample as compliant (i.e. < 10 organisms m^{-3}) if the true density was below three organisms m^{-3}. However, it would be impossible to confidently assess compliance of a sample with fewer than 30 replicate samples of 0.10 m^3 if true density were between eight and 14 organisms m^{-3}. Overall, increasing the volume of samples improves our ability to confidently assess compliance as the true density approaches the 10 organisms m^{-3} limit (dotted vertical line, Fig. 3.4, upper panel).

In contrast to small volume samples, those of 3.00 m^3 required three or fewer replicate samples to confidently determine compliance when the true density was below eight organisms m^{-3} or above 12 organisms m^{-3} (red line, Fig. 3.4 upper panel), and compliance could be assessed with 11-12 replicates if true density was very close to the maximum permissible limit (i.e. nine or 11 organisms m^{-3}). Intermediate sample sizes could be used to confidently assess compliance when the true density was <7 or >13 organisms m^{-3}, but as sample volume declined, the number of replicates required increased (Fig. 3.4, upper panel). As expected, across the range of densities tested, total sample volume seemed to be the key determinant of our ability to confidently assess compliance when organisms were evenly Poisson distributed. For example, at a true density of seven organisms m^{-3}, compliance could be assessed with a minimum of 24, 9, 5, 3 or 1 sample(s) with corresponding volumes of 0.10, 0.25, 0.50, 1.00, or 3.00 m^3, respectively. This reflects the expectation that, for Poisson-distributed populations, sampling a given volume in one large replicate or multiple small replicates should be

mathematically equivalent. Here, differences likely reflect variation due to random sampling of our model tanks.

When organisms were unevenly distributed and were sampled from the full depth of the ballast tank (all three sampling ports), we saw a very similar pattern though the window of non-confidence (error rate >0.05) moved toward false negatives (Fig. 3.4, lower panel). All volumes except for 0.10 m^3 could be used to assess compliance when the true density of organisms was ≤ 9 organisms m^{-3} (purple line, Fig. 3.4, lower panel); however, when the sample volume was low (e.g. 0.25 m^3), a large (20) number of replicates was required (green line). The number of replicates required to confidently assess compliance dropped progressively from eight to four to two replicates at 0.50, 1.00 and 3.00 m^3 (blue, black , red lines, respectively). The lower total volume required for samples of 1.00 m^3 (4 m^3) versus 3.00 m^3 (6 m^3) suggests that multiple 1.00 m^3 samples might be the most parsimonious sampling scheme given the time required to process samples under the microscope. The major difference between “uneven” and “even” scenarios is that there were more true densities above the compliance limit where we could not confidently assess compliance in the former scenarios. At a density of 13 organisms m^{-3}, we could confidently assess compliance with sample volumes of 1.00 m^3 (black line) and 3.00 m^3 (red line), but both required sampling impractically large volumes of water: 20 m^3 (20 samples) for 1.00 m^3 and 18 m^3 (6 samples) for 3.00 m^3.

In the uneven Poisson scenario, where organisms were concentrated in the top section of the tank and only that region was sampled (Fig. 3.4, lower panel), results were quite different. As organism density in the upper portion of the tank was much

higher than the overall mean density, it was very easy to overestimate mean density; consequently, large sample volumes from tanks with low overall density (i.e. <3 organisms m^{-3}) were required to achieve an acceptable rate of false positives. In contrast, it took relatively small sample volumes (i.e. 1.00 m^3 total from any sample volume/replicate combination) to avoid false negatives, as few samples estimated densities lower than 10 organisms m^{-3}.

Similar to the Poisson results sampled from throughout the tank, all sampling volumes with the Gamma PDF had a window of non-confidence for densities approaching the IMO D-2 standard of 10 organisms m^{-3}. Overall, the relationships between different sample sizes were similar to that seen in the Poisson model, above. In all three dispersion scenarios, larger samples had narrower ranges where we failed to confidently assign compliance with reasonable replicate numbers (i.e. <30 replicates; Fig. 3.5). In the Gamma simulations, the key difference among the three different dispersion scenarios is that as dispersion decreased (rate increased), the range where we could not confidently assign compliance narrowed. This was most apparent in the smallest sample size (0.10 m^3, Fig. 3.5, purple line). In the highest dispersion (rate=0.5) model, we failed to confidently assign compliance for true densities from seven to 15 organisms m^{-3}, while for the intermediate dispersion (rate=1.0) model the range is eight to 14 organisms m^{-3}, and for the more aggregated organisms (rate=2.0) model the range is nine to 12 organisms m^{-3}. The other sample volumes tested exhibited a similar, if less pronounced, pattern. The other major difference was that the number of replicates for a given volume decreased with decreasing statistical dispersion. This was very pronounced in the 3.00 m^3 sample size, which maintained the same narrow range

of non-confidence throughout all three rate scenarios, but required >20 replicates for confidence when dispersion was highest, 10-12 replicates at intermediate dispersion, and 5-6 replicates when dispersion was low (Fig. 3.5, red line). This pattern of a narrowing of the non-confidence range with decreasing dispersion, and a decrease in replicates required for confidence, was consistent across all five sample volumes. Consistent with the Poisson model, the largest sample sizes again returned the narrowest range of non-confidence for tractable sample numbers.

3.4 Discussion

Even at very low densities, sampling volumes of 1.00 and 3.00 m^3 were able to accurately estimate zooplankton density in ballast tanks. However, the improvement in accuracy by adding additional samples was more practical for 1.00 m^3 than for 3.00 m^3 samples. The1.00 m^3 samples had the lowest MSE scores in five out of six PDFs tested (all except Log-normal), and were, therefore, the most accurate of all volumes tested (Table 3.1 and Fig. 3.3).

Sampling across the water column addresses problems inherent in sampling species with patchy distributions, and is required for testing IMO D-2 compliance (IMO, 2008b; Murphy *et al*., 2002). Zooplankton tend to aggregate in natural waters (First *et al*., 2013) and likely do so in ballast tanks as well. Our multiport sampling design allowed us to sample the entire water column, including the double-bottom portion, which is usually inaccessible. Thus, multiple sampling ports provide more accurate estimates of organism density than single ports or if researchers use deck-based plankton nets. Although we used an equal number of ports as Murphy *et al*. (2002), our design allowed us to collect water from the lower portion of the tank, something that

their system was unable to carry out. This portion is also inaccessible to open hatch tow sampling. Our design also made it possible to take as many replicate samples as desired within a short period of time without affecting vessel operations.

The Poisson distribution had the lowest MSE scores in all volumes (Table 3.1). The results we obtained were similar for Gamma distribution in deriving the likelihood of over dispersion due to clumping. The Poisson distribution is commonly used for modeling zooplankton distributions in ballast tanks (Lee II *et al*., 2010; Miller *et al*., 2011; Frazier *et al*., 2013; Costa *et al*., 2015), however, the Gamma distribution also has been used as a Poisson approximation. Gamma distribution estimates abundance distributions (Egen and Lande, 1996) and has been suggested for zooplankton in ballast water (Costa *et al*., 2015). A need exists to build data sets that allow identification of an appropriate PDF based on empirical data. Our attempt with a rather limited data set proved inconclusive.

True zooplankton densities were not known in our trials, thus we relied on a series of assumptions that justified using the mean of all sampling efforts per trial. Under these assumptions, large volume samples had higher precision and lower variability. Trials 1 and 3 also demonstrated that the largest volume (3.00 m^3) estimated density better than smaller ones. However, in Trials 2 and 4 large volumes underestimated densities. While larger volumes - such as 3.00 m^3 - provided- in general- better estimates, they increased work load prohibitively and thus cannot be recommended (see Frazier *et al*., 2013). We observed that 1.00 m^3 samples had the lowest MSE and provided a good estimation with a low rate of false positives when organism abundance was ≤10 individuals m^{-3}, and a low false negative rate when

density ≥10 individuals m^{-3} for the two PDFs evaluated here. The error rate can be improved for estimates based on 1.00 m^3 samples by increasing the number of replicates (Figs. 3.4 and 3.5). Because our sampling technique was already an integration of three equal volumes, even a single replicate enhanced accuracy of the density estimate, and replicates at this volume are manageable.

There exists support for the argument that large volume samples offer better estimations assuming Poisson-based models (e.g. see Lee II *et al*., 2010; Miller *et al*., 2011). However when the dispersion of organisms in the tank is unknown, there is a possibility to overestimate densities and wrongly conclude that vessels are not in compliance with the IMO D-2 standard (see Fig. 3.4). In our 'uneven' Poisson simulations, altering how animals are distributed in the tank modified not only the proportion of false positives and negatives, but the capability to accurately assess organism densities at all tested volumes. We agree with the aforementioned authors that larger volumes (e.g. 7.00 m^3) provide a better estimator of density, though these volumes are impractical for organism enumeration at anything other than, and possibly including, a land-based testing facility. Our three sampling port design provides better opportunities to accurately quantify plankton present at low density. Theoretical minimum sampling volumes under our design slightly differed from those estimated by Frazier *et al.* (2013). We found that it would be theoretically possible to assess compliance with a single 0.1 m^3 sample, if true organism density was <3 individuals m^{-3}, whereas Frazier *et al*. (2013) assert that a minimum of 0.4m^3 would need to be sampled to assess compliance. We argue that the differences between our findings reflect the different mathematical approaches used, rather than any significant disagreement in

sampling recommendations. Differences may also stem from the composite nature of our samples, where every sample consisted of three 1/3 samples, taken from different parts of the ballast tank.

Our descriptive statistics highlighted that dispersion was larger on small sample volumes and decreased as volume increased (Fig. 3.2). Despite the non-significant difference among sampling volumes, we observed that sampling volumes below 0.50 m^3 are much more variable and thus less reliable (Fig. 3.2). Our comparison of MSE scores for all trials and volumes demonstrated that 1.00 m^3 had the smallest MSE and thus the best accuracy.

The two PDFs that we used to simulate sampling allow us to infer that when zooplankton populations are present at low densities, both 1.00 and 3.00 m^3 sample volumes provide good estimates of density with acceptable error rates (<0.05) versus smaller volumes.

Our study is limited by the number of trials and replicates within each sample volume, however it presents realistic working conditions and constraints likely to be encountered on ocean-going vessels. Validation procedures for IMO D-2 standard are in development. At present there exist no clear guidelines on sample volumes or sample number. We suggest 1.00 m^3 as a starting point and encourage collection of additional empirical data and assessment of sampling strategies.

Empirical data highlighted that integrative samples added precision to density estimations by reducing variance, and that large but practicable volumes - such as 1.00 m^3 - benefit from it. MSE scores for 1.00 m^3 were lowest regardless of which PDF was used to fit our data, suggesting that this volume most accurately estimated true density.

Finally, our simulations revealed that increasing the size and number of samples improves confidence in compliance assessments, with the best tradeoff between accuracy, precision, and work load seemingly optimized with 1.00 m^3 samples.

Table 3.1. Mean squared error (MSE*10^{-5}) computed for each probability density function and each volume (m^3). Lower values indicate less dispersion between data points and the distribution curve.

Volume (m^3)	Poisson	Weibull	Negative Binomial	Gamma	Log-normal
0.10	1.30	2.60	2.54	2.54	2.70
0.25	2.01	3.95	4.07	4.08	4.74
0.50	1.67	3.30	4.02	4.10	6.36
1.00	0.79	1.53	1.72	1.78	2.37
3.00	1.41	2.89	3.23	3.23	5.60

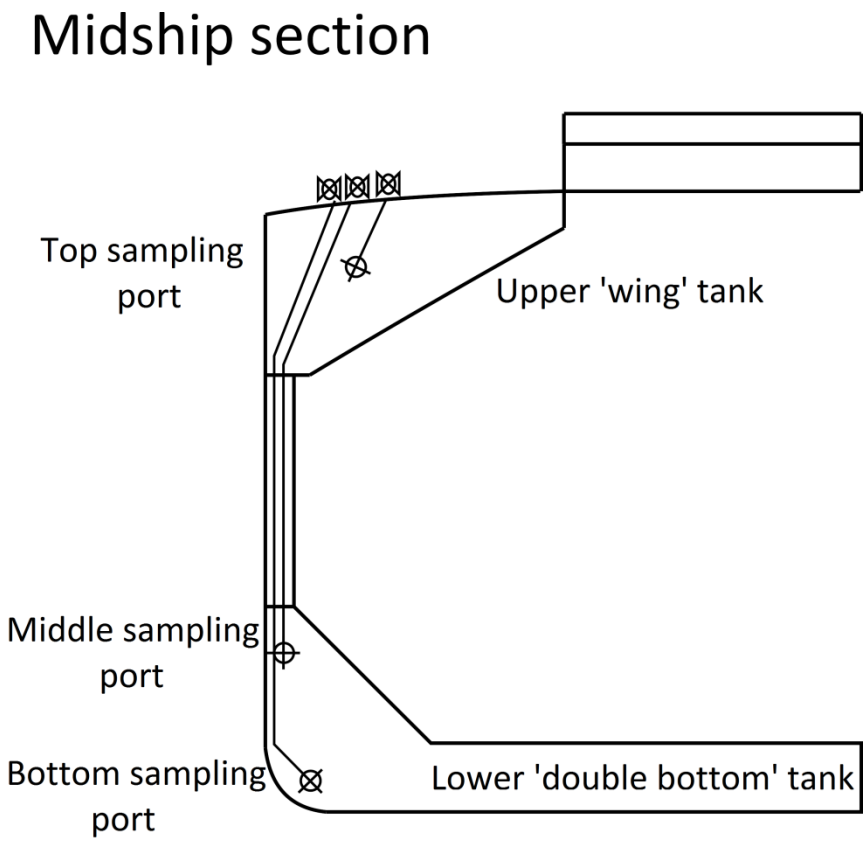

Figure 3.1. Location of sampling ports inside the ballast tank.

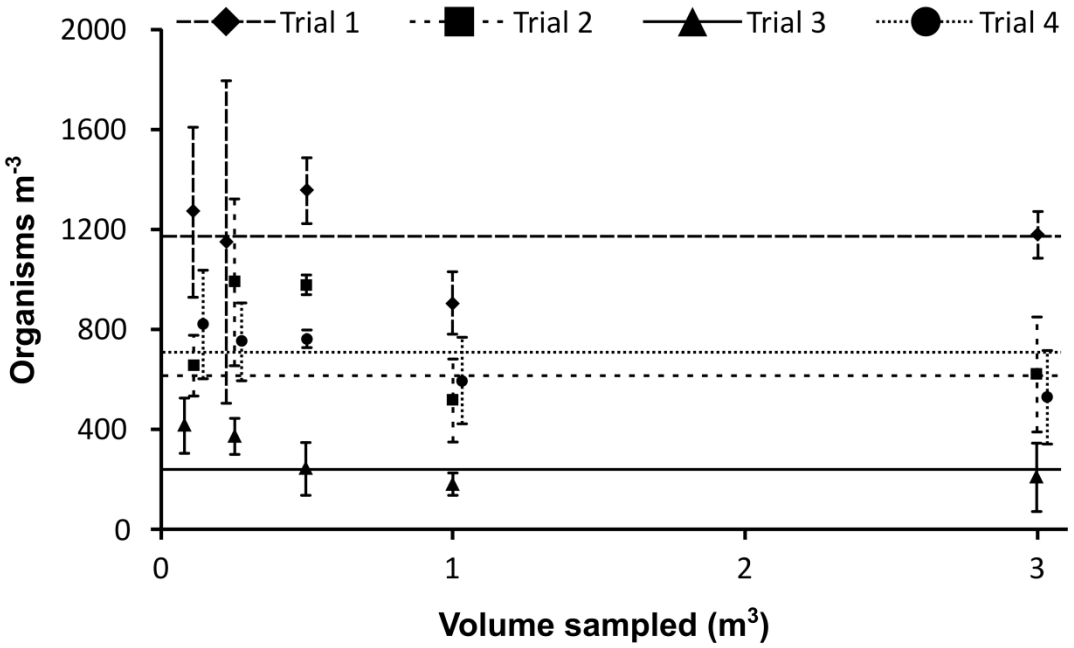

Figure 3.2. Densities estimated from all four trials and five sampling efforts (0.10, 0.25, 0.50, 1.00, and 3.00m^3). Markers (diamonds – Trial 1, squares – Trial 2, triangles – Trial 3, and circles – Trial 4) indicate mean volume (n=3) ± one standard deviation.

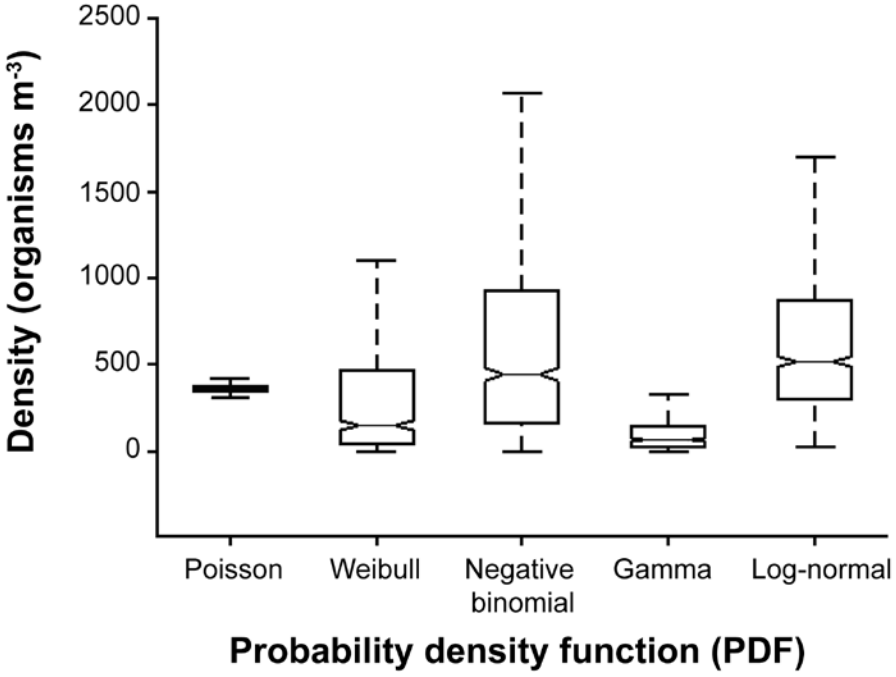

Figure 3.3. Box and whisker plot for maximum likelihood of five probability density function testing 1.00 m^3 sample volumes.

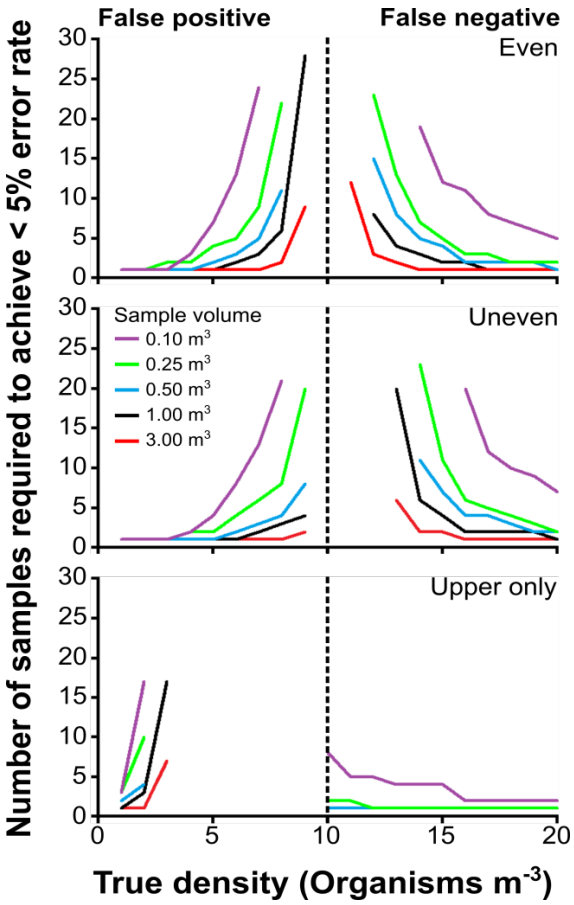

Figure 3.4. Minimum sample numbers required at a given animal density and sample volume to achieve < 5% false positive/false negative rate for Poisson-distributed organisms. False positives are shown to the left of the midline, false negatives to the right. The central gap in each line indicates that the minimum sample number required to achieve <5% false positive/false negative rate exceeds our arbitrary cutoff of 30 replicates at a given volume for those densities of organisms. The upper panel represents a case where organisms are evenly distributed throughout the tank. Middle panel shows the case where organisms favor the upper 1/3 of the tank and sampling is through three sampling ports (as in our field experiment). In the bottom panel, organisms are aggregated in the upper 1/3 of the tank and sampling is restricted to the upper portion of the tank. Each density was simulated 1,000 times for all five volumes.

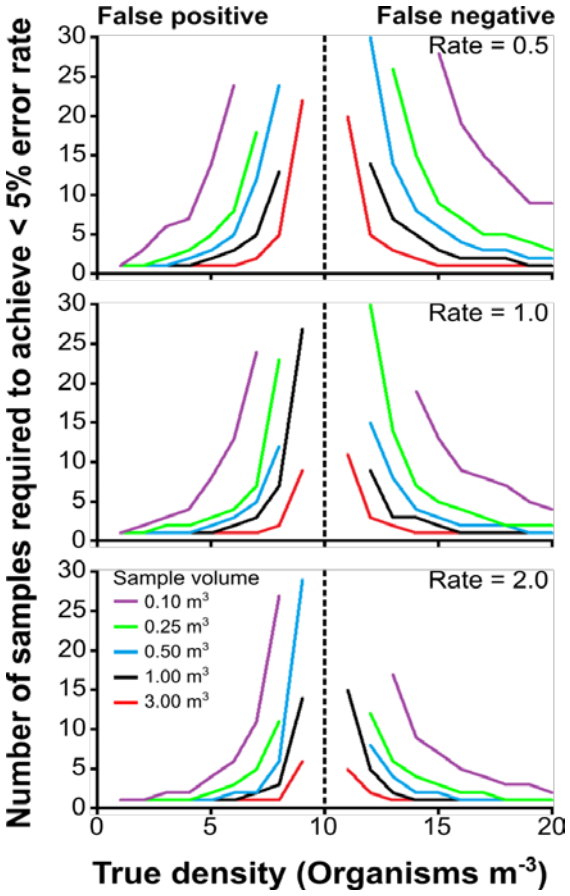

Figure 3.5. Minimum sample numbers required at a given animal density and sample volume to achieve < 5% false positive/false negative rate for Gamma-distributed organisms. False positives are shown to the left of the midline, false negatives to the right. Panels represent high-dispersion (top, rate=0.5), moderate-dispersion (middle, rate=1), and low-dispersion (bottom, rate=2) scenarios. Other details are as per Figure 3.4.

CHAPTER 4: PRODUCTION OF TRIHALOMETHANES IN CHLORINATED BALLAST WATER

4.1 Introduction

Vessels use ballast water to preserve buoyancy and maneuverability (Carlton, 1987); by design ballast tanks hold a volume sufficient to equal the tonnage of dry cargo (IMO, 2008d). It is estimated that three to five billion tons of ballast water are transported every year (Globallast IMO, 2015). New regulations for ballast water management will be globally implemented beginning September 2017. These regulations are designed to reduce the movement of non-indigenous species by setting numerical limits for abundance of two planktonic groups and three health-related bacteria of concern (IMO, 2016). Different alternatives to achieve these limits exist, including use of strong oxidants such as chlorine (e.g. Werschkun *et al*., 2012; Zhang *et al*., 2013). Chlorine may be applied to ballast water either directly or indirectly via in situ electro-chlorination of sea water. Large vessels like bulk carriers and tankers can discharge between 15,000 and 113,000m^3 of treated ballast water in a single event, thereby posing an invasion risk for recipient ports.

Chlorine is the most widely used chemical for disinfection of fresh water, as it eliminates active pathogens. However, chlorine treatment of water is associated with undesirable by-products, some of which have carcinogenic effects (Boorman *et al*., 1999). Trihalomethanes (THMs), which result when three halogen atoms are substituted for hydrogen atoms in the methane molecule, are the most commonly observed by-product of chlorination (Budziak *et al*., 2007).

THMs formation depends on the availability of both chlorine and natural organic matter (NOM). Limiting production of THMs by pre-treatment to reduce NOMs is a common practice in public utilities (Bull *et al*., 1995). This capability does not extend to ballast water, where large volumes of water are loaded and discharged, and little space exists for on-board pre-treatment. Consequently, ballast water treatment has focused on the control of the oxidant dose (Tsolaki *et al*., 2010; Paolucci *et al*., 2015). Salinity of ballast water varies according to the geographic location where it is loaded.

Formation of THMs requires dissolved NOM such as humic substances and/or fulvic acid (Madabhushi, 1999) and halogens dissolved in water. Both humic and fulvic acids constitute the largest portion of dissolved organic carbon (DOC) in waters. In addition to DOC, natural waters contain particulate organic carbon (POC), which represents debris from plants and animals. The availability of NOM and POC in water plays a key role in the quantity of THMs generated; however, there exists a long list of organic compounds that can constitute NOM and POC in water which will vary depending upon its source (Liu *et al*., 2015). The sum of DOC and POC equals total organic carbon (TOC) and is typically used as a proxy for the potential reactive pool for THMs generation (Bruchet *et al*., 1990; Singer, 1999).

THMs are continuously produced if NOM is present and the halogen supply is not exhausted (Stack *et al*., 2000). The most abundant halogen used in ballast treatment is chlorine because it is inexpensive and can be readily added from sources such as sodium hypochlorite (NaClO). If the ballast is fresh water, $CHCl_3$ may constitute the most abundant THM, based upon utility plant experience (Ivahnenko and Zogorski, 2006). However, THMs abundance and composition change in the presence of

bromine. High concentrations of bromine result in brominated THMs even when chlorine is added as the active substance of disinfection (Bull *et al*., 1995). Ballast from brackish and marine waters may produce brominated THMs owing to the higher bromine content of these water sources (Ged and Boyer, 2014). Speciation of THMs occurs when bromine is present in the water, leading to the formation of $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$, with the sum of these plus $CHCl_3$ equalling total THMS (TTHMs; Singer, 1999). The ratio of chlorinated to brominated species can be estimated based on molar ratios of each halogen and then extrapolated using probability models when analysis is limited to final concentration of TTHMs and not initial doses of chlorine and bromine (Chang *et al*., 2001).

Here we evaluate potential TTHMs production in ballast water treated with chlorine at doses recommended for use to reduce target organisms in ballast water (see Paolucci *et al*., 2015), specifically exploring the effects of both water salinity and NOM. Experiments were performed using natural water sources representative of fresh water and brackish waters from shipping ports and marine water derived from a ballast sample. In addition, we augmented samples with humic acids to contrast the effects of NOM concentration on THM generation.

4.2 Materials and Methods

Water samples were collected from two different ports and one vessel according to their salinity as a follow-up to a larger ballast water treatment experiment (see Paolucci *et al*., 2015). Sampled water included fresh water (0.1 practical salinity units; PSU) from Trois Rivières, Québec and brackish water (11.3 PSU) from Port Alfred, Québec. Marine water (34.0 PSU) was collected from a ballast tank of a general cargo

vessel whose water was exchanged in the North Atlantic region (38°08.7' N, 67°23.1' W) according to its ballast water management record. All samples were kept in the dark at 4°C until the day of analysis. Water in amber glass bottles at ambient temperature was used hereafter to mimic light exposure in a ballast tank.

The experimental design was full factorial with two fixed factors: source of water (according to salinity) and organic matter content. Three conditions for organic content were tested: i) natural condition (water as it was collected); ii) removal by filtration (POC and other suspended solids were removed) with a 0.45 µm glass fibre filter; and iii) enrichment with humic acid (HUMICan 100, AgroCare Canada) to increase content of TOC to 25 mg L^{-1}. The resulting 3x3 combinations were prepared in a 500mL sterilized, amber glass bottle with a Teflon lined cap. 500mL were measured with a volumetric flask and dosed (single pulse) to 10 mg L^{-1} Cl^{-1} using commercial pool bleach at 10% weight-to-weight (w/w) solution. All nine treatments (3x3 combinations) were analyzed in triplicate (3 replicates per treatment) across three time intervals (t1≈1 hour, t2≈2 hours, and t3≈24 hours) to assess THMs maximum production. TOC was measured prior to incubation using a Shimadzu TOC-VCSH analyzer (lowest detectable level 1 mg L^{-1}) and chlorine (estimated detection limit 0.1 µg L^{-1}) using a Hach Pocket ColorimeterTM II (Cat. No. 58700-12). Incubation times 1-3 are reported accordingly on the x axis in Fig. 4. 1. Amber bottles were kept in the dark and the temperature in the lab was constant at 20°C.

At sampling, THMs were extracted from water samples using the method of headspace solid-phase microextraction (SPME) followed by analysis by GC-MSD (Stack *et al*., 2000; Zhao *et al*., 2004). A manual SPME device Supelco part # 57318

(SPME fibre 1 cm long, retractable SPME fibre, 75µm film thickness, thin-fused silica optical fibre, coated with thin-film of CAR/PDMS, 24 gauge needle, and SPME holder; Supelco part # 5-7330) fibre was conditioned at 300°C for 5 min before and after each extraction. SPME extraction was performed by transferring 20g of water sample from a given incubation vessel into a precleaned amber VOA vial with screw cap and PTFE/Silicone septum (EPA VO vials, Supelco part # 23189), containing 7.2g of NaCl and spiked with a mix of recovery surrogate standard solution containing p-bromofluorobenzene and d8-Toluene [CPL-PS-4X (concentration: 2 µg mL^{-1}; 10 µL)]. The sample was vortexed for 1 min, following which a SPME needle (protecting fibre) was pierced through the septum and into the vial. Needle depth was adjusted to keep the fibre above the liquid layer in the headspace environment. The SPME extraction initiated after exposing the fibre into the headspace and heating the vial indirectly at 45°C ± 1°C with constant stirring at 300 rpm for 20 min. Our methods differ slightly from those of Stack *et al*. (2000) and Zhao *et al*. (2004), the former because we used moderate stirring for 20 min as opposed to low stirring for a longer period, the latter because we increased temperature. Volatiles were absorbed/adsorbed to the fibre and concentrated, followed by retraction of fibre into the needle. Thermal desorption of THMs from the fibre occurred when the needle was directly introduced to the GC inlet and pushed out the fibre from the needle and introduced to the hot GC inlet.

The Gas Chromatograph with MSD (GC/MSD) instrument (Hewlett Packard 6890/5973) was equipped with a GC capillary column [VF-624ms; 30m x 0.25mm I.D. x 1.4µm film thickness (J&W)]. The inlet was set at 250°C in a splitless mode and carrier gas (UHP) at a flow rate of 0.8 mL min^{-1} with column head pressure 4.8 psi. The MSD

operated in EI SIM mode. Oven temperature was set at 40°C for an initial time of 2.0 min and increased at a rate of 7°C min^{-1} and held at 130°C for 1.0 min. The total analysis time was 15.86 min with equilibration time at 0.5 min. Calibration was carried out with the same procedure replacing the sample water with 20 mL of buffer solution [sodium chloride (360g) in Milli-Q water (1L) fixed at pH 2.0 with ortho-phosphoric acid (85% weight/weight)].

Known concentrations of THMs were loaded into 20 g of water using the THM standard mix (M-501-10X) to generate a calibration curve. Determination of method detection limits (MDLs) for the THMs was based on a signal-to-noise ratio (S/N*5) at low concentration and were between 0.04 to 0.05 µg L-1 for $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$ and $CHBr_3$. Recovery rate for our surrogate was 94.3%. However, it was not used in MDLs determination because it was within acceptable range for volatiles. Additionally Fresh, Brackish and Marine water samples with no dose of chlorine added were processed in the same method as quality control for matrix effects (see last column Table 4.1)

A univariate general linear model was conducted on the production of TTHMs using two fixed factors (water source and TOC content), and a covariate (time after dose). We also tested for an interaction between the fixed factors. Additionally, we performed an independent sample t-test for TOC content between natural and filtered for all sources of water. All statistical analyses were carried out with IBM SPSS Statistics version 23 (IBM Corp., Armonk, OK, USA).

4.3 Results

Filtered and natural TOC concentrations were low in both natural and filtered water for fresh and brackish waters, and slightly higher in marine waters (Table 4.1).

There were no detectable concentrations of TTHMs in samples prior to chlorine addition (Table 4.1). Total THM production varied significantly by water source and by TOC content at the outset of the experiment, and by an interaction of these parameters (Table 4.2). Fresh water produced on average less TTHMs than any other source, while marine water produced an intermediate amount of TTHMs and brackish water the highest amount (Table 4.2, Fig. 4.1).

Enrichment of TOC increased TTHMs production for fresh and marine waters but not for brackish water (Table 4.2; Fig. 4.1). Filtering significantly reduced TOC concentration versus natural conditions ($t = 5.17$, $df = 16$, and $p < 0.001$). Although non-significant, filtered samples yielded the highest production of TTHMs for brackish water (Table 4.2; Fig. 4.2).

TTHMs maximum production was achieved very quickly (i.e. within 1 hour) and was sustained over the 24-hour follow-up measure in all TOC treatments for fresh and marine water (Fig. 4.1, right and left panels). Brackish water almost doubled TTHMs in the first hours after dose for natural and filtered treatments, and sustained the same levels in the enriched treatment (Fig. 4.1, middle panel).

$CHCl_3$ was the major constituent of TTHMs in freshwater, whereas brackish or marine water treatments had a higher ratio of brominated to chlorinated species of TTHMs owing to the very low presence of bromine in fresh water (Table 4.2). Most production in natural or filtered fresh water was by $CHBrCl_2$ (Table 4.2). By contrast, marine and brackish water produced more $CHBr_3$ but under different conditions, with the former being greatest in the enhanced TOC treatments and the latter in natural and filtered treatments (Table 4.2).

4.4 Discussion

The World Health Organization (WHO) limits TTHMs to 200µg L^{-1}; with individual conditions for chloroform ($CHCl_3$) to 200µg L^{-1}, bromoform ($CHBr_3$) to 100µg L^{-1}, dibromochloromethane ($CHClBr_2$) to 100 µg L^{-1}, and bromodichloromethane ($CHCl_2Br$) to 60 µg L^{-1} (Stack *et al*., 2000). We found concentrations of TTHMs in fresh ballast water for natural and filtered treatments were well below the 200 µg L^{-1} limit, and thus compliant with WHO regulations for continental waters (Agus *et al*., 2009; Werschkun *et al*., 2012). It is more likely that a source of fresh ballast water with a maximum TOC content of 16mg L^{-1} would produce similar or lower concentrations of TTHMs with doses of $Cl^{-1} \leq 10$ mg L^{-1}.

Similarly, we expect that marine water will be below permissible limits set by WHO regulations for with TTHMs. However, under conditions of enhanced TOC concentration, we anticipate that production of TTHMs would greatly increase (Table 4.2) and possibly exceed these regulations. Production of TTHMs in enriched marine water was almost 10 times higher than in filtered or natural water (Table 4.2). It is apparent that the largest limiting factor for THMs production in marine ballast water used in this experiment is NOM in the water.

Brackish water produced less TTHMs under enrichment than under natural or filtered conditions. We propose that an inhibitor may have prevented the oxidation process in water collected at Port Alfred. Further, we propose that some macromolecules may sequester chlorine in the natural condition, because filtering removes suspended particles above 0.45 µm in size. It has been documented that ammonia reduced THMs production during chlorination despite the presence of humic

substances (Amy *et al*., 1984). High production of TTHMs in brackish versus marine or fresh water has been documented in at least five ballast water treatment systems (OceanSaver, CleanBallast, Greenship, TG, OceanGuard) (Werschkun *et al*., 2012). Similar results were also found when the OceanGuard system was tested on land, with $CHBr_3$ accounting for almost 90% of the total 670 μg L^{-1} TTHM produced (Werschkun *et al*., 2012).

Experience with chlorination in marine water as a means to control biofouling used doses from 0.5 to 1.5 μg L^{-1} and resulted in TTHMs concentrations of 2.5 to 18.5 μg L^{-1} (Boudjellaba *et al*., 2016). However, the IMO D-2 performance standard targets two planktonic groups that will require a higher dose to achieve lethality (Gregg *et al*., 2009). Our TTHM production results for marine water were an order of magnitude greater than those of Boudjellaba *et al*. (2016). Other studies that have evaluated differences in TTHM production using nearshore and deep-ocean waters revealed that THM production differed by orders of magnitude, with nearshore water having the highest TTHM production (Fabbricino and Korshin, 2005). Werschkun *et al*., (2012) reported that three commercial ballast water treatment systems (CleanBallast, TG and OceanGuard), tested with marine water and a lower chlorine dose resulted in TTHMs production just below 200 μg L^{-1} over a five day cycle (as required by IMO G9; IMO, 2008e). OceanSaver produced relatively less TTHMs and were similar to our results (Fig. 4.1 left panel). Cowman and Singer (1996) documented a shift of brominated species in disinfection by-products, where hypochlorous acid continuously integrates bromide into THM formation. The free chloride from the completion of this reaction will restart the process again. Marine water produced more TTHMs when enriched

compared with fresh water, it appears that similarly to what Symons *et al*. (1993) found in fresh water in the presence of precursors, DOC and bromide with as little as 3 mg L^{-1} residual free available, chlorine will promote production of brominated species until complete exhaustion of one precursor. In contrast, in fresh water production will stop production when hypochlorous acid no longer can react with organic matter.

Chlorination is an effective alternative to ballast water exchange under the new IMO ballast water standard. Bench-scale experiments like ours allow inferences to be made regarding patterns and trends, though care must be taken when extrapolating to the field. Paim *et al*. (2007) spiked fresh water with humic acids to 23.7 mg L^{-1} and, using a 5 µg L^{-1} chlorine dose, reported a maximum production of $CHCl_3$ of 18 µg L^{-1}. Our experiments with fresh water, conducted under laboratory conditions and 10 µg L^{-1} Cl^{-1} and 23.9 mg L^{-1} TOC, revealed much higher production of TTHMs (Fig. 4.1) during the first hours. This large difference might stem from the higher chlorine dose and its apparent immediate impact on $CHCl_3$ production. It is apparent that filtering will remove the particulate fraction, yet it had little or no net positive effect on subsequent TTHMs production because it is apparent that only the dissolved fraction of organic carbon was involved in reactions that produced TTHMs. Liu *et al*. (2015) observed that only four species of organic carbon (glycolic, alginic, citric, humic acids and urea) enhanced TTHM production. While it will be difficult for crew and port authorities to analyze what species of organic carbon is in the water, the option exists to adjust the dose of chlorine. In addition, it is possible to track free chlorine in real time using electronic sensors in the tanks (Zimmer-Faust *et al*., 2014).

This study assessed the importance of both ballast water source and its organic carbon content to production of TTHMs. This has been overlooked as evaluations are carried out during final discharge several days after dosing (IMO, 2008e). We observed clear differences in TTHM production in brackish, fresh and marine water, which has implications for where ballast water should be loaded and its likely generation of TTHMs. Ballast water loaded in freshwater ports - even if TOC load is high - may pose less risk of TTHM production than that loaded in brackish or marine water. However, many global ports are brackish or marine (Werschkun *et al*., 2014), thus by-product generation of TTHMs may pose a problem, particularly in carbon-enriched marine or filtered brackish water for voyages shorter than five days (see guidelines G8 and G9; IMO 2008d,e). Our results suggest that if treated ballast water is discharged within the first two days there is a risk of releasing sufficient TTHMs to cause environmental harm.

Table 4.1. Mean (SD) total organic carbon (TOC; mg L^{-1}), pH, salinity (PSU) and TTHMs (µg L^{-1}) measured before dosing samples with chlorine.

Water Source	Natural	Filtered	Enriched	pH	Salinity	TTHMs
Fresh	15.6 (0.8)	7.3 (0.0)	23.9 (3.2)	8.2 (0.2)	0.1 (0.0)	0 (0)
Brackish	11.4 (0.4)	8.6 (0.2)	26.1 (2.4)	7.2 (0.2)	11.3 (0.2)	0 (0)
Marine	18.8 (1.8)	11.4 (0.1)	22.1 (2.1)	7.8 (0.1)	34.0 (0.1)	0 (0)

Table 4.2. Mean production (+/- standard error) of THMs by species (μg L^{-1}) in each combination of fixed factors.

Water source	TOC	$CHCl_3$	$CHBrCl_2$	$CHBr_2Cl$	$CHBr_3$	Total THMs
Marine	Enriched	0.9 (0.2)	15.0 (0.3)	32.9 (4.0)	545.6 (37.1)	581.1 (41.1)
Marine	Filtered	1.3 (0.5)	5.7 (0.3)	7.7 (6.2)	75.7 (18.9)	86.6 (33.1)
Marine	Natural	0.6 (0.1)	2.2 (0.1)	3.4 (0.7)	57.4 (15.8)	61.6 (16.5)
Brackish	Enriched	2.7 (0.8)	128.1 (3.1)	73.5 (6.9)	341.8 (15.8)	432.2 (14.8)
Brackish	Filtered	1.6 (0.5)	21.2 (0.2)	34.7 (3.5)	633.4 (94.1)	672.0 (97.1)
Brackish	Natural	0.8 (0.3)	14.7 (0.3)	28.1 (4.3)	575.0 (108.3)	605.5 (112.1)
Fresh	Enriched	169.8 (42.7)	113.0 (3.6)	14.9 (11.8)	43.3 (37.8)	240.5 (38.8)
Fresh	Filtered	31.9 (10.1)	97.6 (2.7)	7.4 (1.5)	25.4 (5.0)	75.6 (12.5)
Fresh	Natural	40.2 (12.4)	119.4 (1.6)	10.0 (2.3)	33.3 (8.7)	96.8 (8.5)

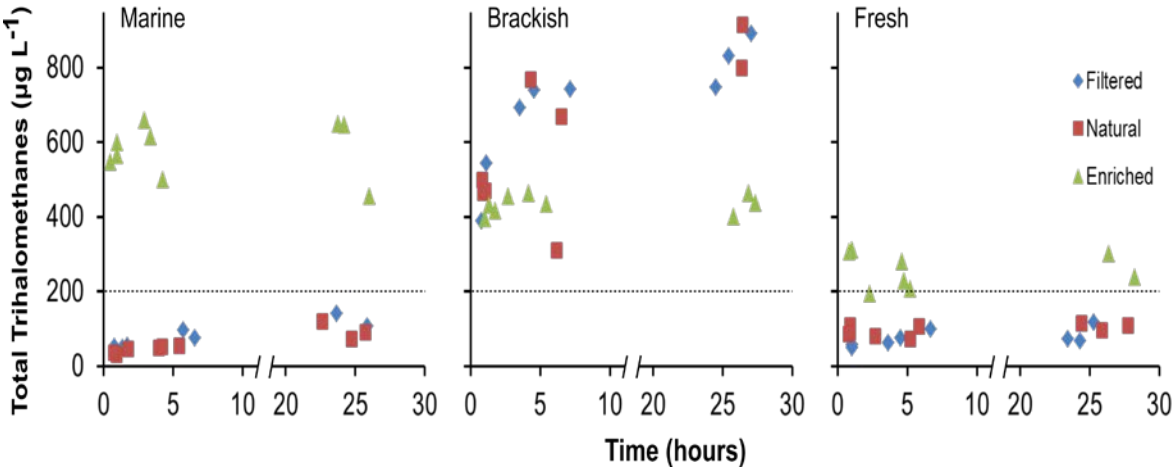

Figure 4.1. Concentration of total trihalomethanes (TTHMs; µg L^{-1}) over time for all replicates analyzed, series corresponds to TOC content and individual panels displayed water type commonly used as ballast. Horizontal dotted lines represent the WHO limit for TTHMs.

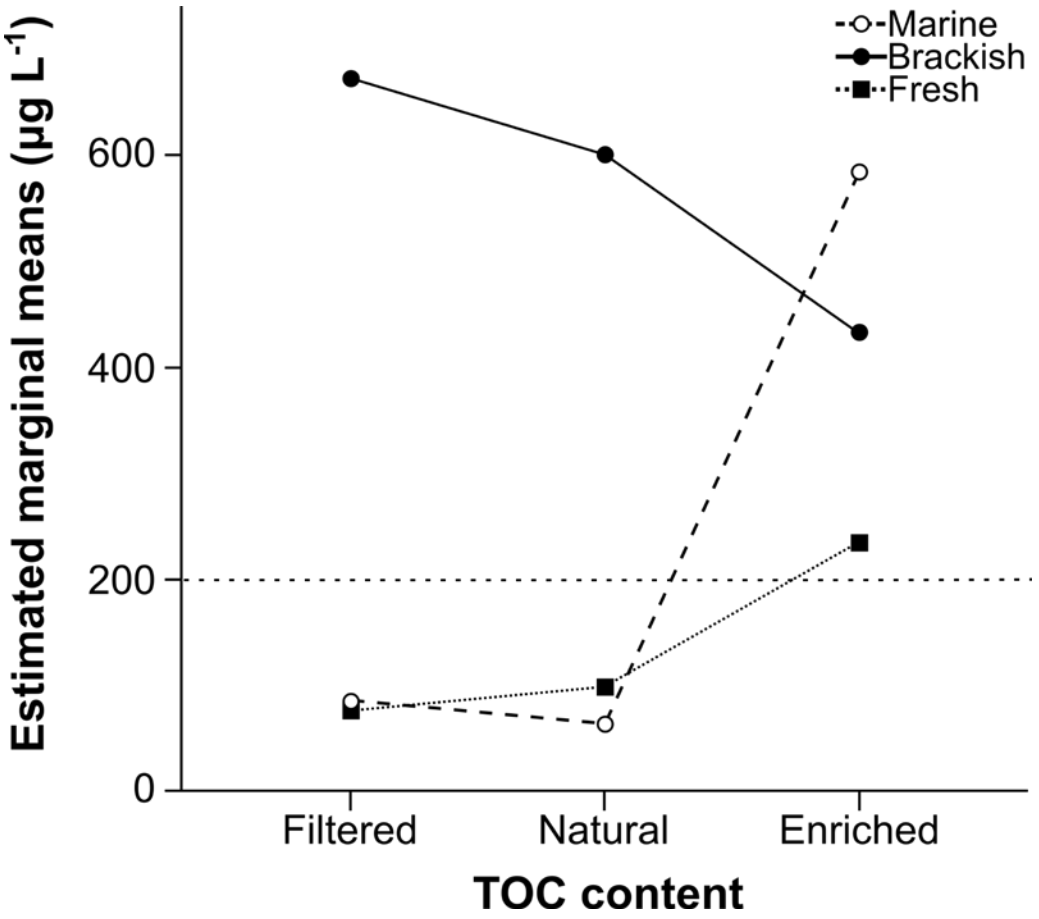

Figure 4.2. Estimated marginal means (µg L^{-1}) from general linear model displaying calculated TTHMs production means from model for all TOC source ballast waters. Horizontal dotted line represents the WHO limit for TTHMs.

CHAPTER 5: CONCLUSIONS

This dissertation addresses a number of critical issues pertaining to ballast water treatment assessment, including sampling volume and replication (for effluent testing), and the occurrence of synergistic effects that may occur when ballast water exchange (BWE) is combined with ballast water treatment (e.g. chlorination) in order to achieve low population densities of target organisms required by new IMO D-2 performance standards. I found that a combined BWE plus ballast water treatment was either equal to or better than each treatment alone in reducing viable populations of target organisms. Thus, combined BWE and chlorine treatment reduces the overall propagule pressure and colonization pressure of the ballast water vector for the transfer of non-indigenous species (NIS) between aquatic environments. Rather than simply turning to ballast water treatment, results from this thesis support the notion that combined BWE and ballast water treatment offers greater protection than treatment alone on transoceanic routes that connect freshwater ports (Bailey *et al*., 2011). It also preserves that protection through chlorination treatment directly into a ballast tank, providing a simple and economical alternative to other treatment methods (Chapter 2). Ballast treatment devices that employ chlorination are most suitable for tankers, barges and bulk carriers, as they require large volumes of ballast water over a short period of time and will benefit from synergistic treatment effects. Collection of data in vessels that continue to perform BWE after mandatory treatment would allow a more robust examination of the utility of combined treatment, and the nature of the treatment interactions for different biological groups.

Sampling for macroplankton presents challenges of practicality, replicability and accuracy. Currently there is a discussion on how and when to sample ballast water for adherence to performance standards (see Gollasch and David 2017); the design used here employs a multiport sampling outlet, which does not require open tank access and allows for easy replication (Chapters 2 and 3). The onboard experiment provided empirical evidence that was complemented with modeling and supports the argument that $1m^3$ is the optimal volume for allowing quick and practical sampling within defined and acceptable error rates. Multiport sampling at different depths in the water column can account for tank design and geometry and is more accurate than other open tank sampling techniques (e.g. net tows). Multiple $1m^3$ samples provide better estimates than sampling larger volumes with fewer replicates regardless of the probability density function used (Chapter 3). This is extremely important from the standpoint of port authorities and ship owners, as they have to meet the new standard and efficiently provide evidence thereof.

Active substances, such as chlorine, produce undesirable by-products (i.e. trihalomethanes, THMs) when applied directly and without a pre-treatment. Ballast water is a generic term that encompasses water loaded from an enormous number of possible sources that varies in both chemical composition and organic matter concentration. Specific chemical qualities of ballast water have strong impacts on how quickly and the total amount of THMs are produced, although organic matter concentration is the limiting reactant. In Chapter 4, I evaluated potential THM production assuming the same conditions that I recommended for reducing viable populations in Chapter 1. Thus I used a fixed dose of 10 mg L^{-1} chlorine with fresh and brackish water

collected from the same ports and ballast tank water that originated in the North Atlantic Ocean.

Sixteen devices that Lloyd's (2016) reported as possessing final approval have not disclosed the full array of by-products present in discharged ballast water (Werschkun *et al*., 2012). Assuming that "procedures for approval of ballast water treatment that make use of active substance (G9)" were followed over the mandatory five day period at port facilities, results from this facilities can only be extrapolated to waters of similar chemical qualities. Current guidelines are very broad and could underestimate the role of chemical composition of ballast water. Similarly, a five day trial test is not representative of all shipping operations. Another equally important aspect is that the highest production of THMs will occur the first 48 hours after dosing, when there is a potential occupational exposure of ships' crews. Ships may move from port to port in shorter periods of time while moving cargo in unscheduled itinerary changes (e.g. in the Baltic or North Seas). There is a potential environmental contamination risk due to chemical content of ballast water and the short time period between ballasting and discharge. My goal here is not to discourage the use of chlorine, but rather to encourage end users to carefully consider these caveats and to put into place contingency plans to address these situations. In particular, consideration needs to be given to close monitoring, regulated dosing, and application of neutralizing agents, to mention a few issues. Port authorities may wish to establish periodic monitoring for active substance residuals and a list of likely by-products in port waters.

As my onboard experiments were conducted under normal operational procedures on an active vessel, they were subjected to multiple factors that varied

between and within trials. These difficult-to-control factors included variation in plankton population abundances, as well as ballast water salinity, temperature, pH, total organic carbon, and intake flow. This variability leads to diverse ballast water assemblages. Due to design, space or even cost, shipping companies may not be able to afford retrofitting and installation of new and very costly ballast water treatments devices (King *et al*., 2012). For this reason, the use of chlorine as broad biocide is a sensible option (17 out of 57 available commercial treatment devices used chlorine as active substance; Lloyds, 2016). In this dissertation, I found that high to moderate doses of chlorine (20 to 10 mg L^{-1}) delivered in a single pulse were effective in reducing viable populations for trips longer than three days.

The Hutton model used in chapter 2, when fed with actual values from experiment in chapter 4, appears to underestimate the production of TTHMs (Table 5.1). It was developed and calibrated for the San Joaquin Valley in California, USA. My findings in chapter 4 indicated that the larger contribution of TTHMs comes from brominated species in brackish and marine water. There is bias towards clean fresh water with this model however; it still fails to estimate CHCl3 by ≈50%. It appears that waters from ports provide not only more TOC in solution but the species of organic carbon that enhance THM production. Trials for the chapter 2 experiment in general have less TOC (Table 2.1) and lower temperatures when compared with the ambient temperature of the lab; both factors are well-known inhibitors of THM production. Additionally our heating and spinning process was designed to maximize TTHM extraction. While I chose the Hutton model for practicality, as one that I could feed with environmental data that could be accurately collected in the field with limited field

equipment, the Hutton model used only environmental samples and then developed the algorithm to estimate THM production. I carried out the evaluation on THMs in the lab knowing that this was a grey area on the general evaluation of chlorinated ballast water treatments.

Chlorine delivery can be simplified and integrated into routine operations of ballasting by a single inlet in the main ballast pipeline at the engine room, thereby reducing price, increasing efficiency, and allowing delivery of very low doses in a homogenous mix. I did not have the opportunity to evaluate low chlorine doses as my experiment was a proof-of-principle only, but other researchers are using low doses sustained over a long time interval (e.g. Maranda *et al*., 2013). However, large vessels that require >5000 m^3 of ballast could not afford a time-consuming treatment and it is not known whether a low dose would be as effective as those tested here.

Changes to regulations always involve a learning curve, however the work described in this dissertation offers a set of methodological improvements to achieve the new performance standard, to validate results while providing data of sufficient quality within margins of acceptable error, and to be prepared for potential challenges when new elements are included in routine operations. Ballast water has been identified as the strongest vector for aquatic invasive species in many fresh water and some marine systems (Carlton, 1985; Ricciardi, 2006). New regulations set to take hold in 2017 represent a major change in management. My data chapters outline three issues that offer a solution for specific aspects of these new regulations. Some of these issues can be addressed formally as new treatment systems are deployed and opportunities for formal tests arise.

Table 5.1. TTHMs (μg L^{-1}) estimated by Hutton model for a chlorine dose of 10 mg L^{-1} using values of temperature (°C) and pH from lab experiment in Chapter 4 for all water sources and total organic carbon (TOC; mg L^{-1}) concentrations

Water source	TOC		Chlorine	Time (hours)	Temperature	pH	TTHMs
Fresh	Filtered	7.3	10	30	24	8.2	6.5
Fresh	Natural	15.6	10	30	24	8.2	14.0
Fresh	Enriched	23.9	10	30	24	8.2	21.4
Brackish	Filtered	8.5	10	30	24	7.2	6.6
Brackish	Natural	11.4	10	30	24	7.2	8.9
Brackish	Enriched	26.1	10	30	24	7.2	20.4
Marine	Filtered	11.4	10	30	24	7.8	9.7
Marine	Natural	18.8	10	30	24	7.8	16.0
Marine	Enriched	22.1	10	30	24	7.8	18.8

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APPENDIX A: ADDITIONAL PUBLICATIONS

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Open Access

Short Communication

Water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) in the Great Lakes: playing with fire?

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Abstract

The Laurentian Great Lakes have been successfully invaded by at least 182 nonindigenous species. Here we report on two new species, water hyacinth *Eichhornia crassipes* and water lettuce *Pistia stratiotes*, that were found at a number of locations in Lake St. Clair and Detroit River during autumn 2010. Both species are commonly sold in the water garden and aquarium trade in southern Ontario and elsewhere. While it is not clear whether these species are established or can establish in the Great Lakes, the historic assumption that neither of these subtropical to tropical plants pose an invasion risk must be questioned in the light of changing environmental conditions associated with climate warming that may render Great Lakes' habitats more suitable for these species and increase the likelihood of their successful establishment.

Key words: nonindigenous, alien, macrophyte, *Eichhornia crassipes*, *Pistia stratiotes*

Introduction

The Laurentian Great Lakes have a long legacy of species introductions. The Great Lakes Aquatic Nonindigenous Species Information System currently lists 182 nonindigenous species (NIS) as established in the Great Lakes (NOAA 2010). Many of the more problematic NIS in the system are invertebrates or fishes, although 55 introduced, wetland or aquatic plant species are currently established in the basin (NOAA 2010). The predominant vector of introduction of NIS to the Great Lakes over the past 60 years has been the discharge of contaminated ballast water, which accounts for at least 55% of established NIS, most of which have been introduced from European sources (e.g. Kelly et al. 2009). Historically, a number of other possible vectors, including connecting channels and the aquarium, human food, and live garden trades, appeared much less important. However, connecting channels have attracted significant attention recently, as bighead *Hypophthalmichthys nobilis* (Richardson, 1845) and silver carp *H. molitrix* (Valenciennes, 1844) are poised to enter the Great Lakes *via* Chicago-area canals that link the Mississippi River and Lake Michigan (see Cooke and Hill 2010). In addition, a variety of fishes and molluscs are sold commercially in the aquarium and water garden trades that potentially could survive if released into the Great Lakes (Rixon et al. 2005; Gertzen et al. 2008).

Aquarium and water garden (i.e. pond) shops in the lower Great Lakes region also sell at least 19 species of macrophytes, including a number of species considered to be highly problematic in some areas where they have been introduced (Rixon et al. 2005). These taxa include *Ceratophyllum demersum* L. 1753, *Egeria densa* Planch. 1857, *Myriophyllum aquaticum* (Vell.) Verdc., *Cabomba caroliniana* Gray 1837, *Pistia stratiotes* L. 1753 and *Eichhornia crassipes* (Mart.) Solms 1883. Rixon et al. (2005) indicated that the former four plants could overwinter in the Great Lakes, and, indeed, *C. demersum* is native to the system. The same study suggested that water hyacinth and water lettuce could not survive Great Lakes' winters.

Commercial sale of invasive NIS may portend subsequent release and establishment in the wild, as Duggan et al. (2006) observed a positive correlation between popularity (i.e. frequency) of fishes sold in aquarium stores and their establishment in the wild. Only one of the aforementioned plants, water lettuce, has been reported in the Great Lakes proper, at a single location in Metzer Marsh in western Lake Erie in 2000, although the species did not persist at that site (USGS 2010; D. Wilcox, pers. comm.). Water lettuce is possibly a South American native (Cordo et al. 1981; USGS 2010) that occurs on all continents except Antarctica (Holm et al. 1977; Dray and Center 2002). The species has been recorded in waterways adjacent to the Laurentian Great Lakes including Bull Creek, adjacent to the Erie Canal, New York, and in the Rideau River, Ontario.

Water hyacinth is a South American native that has attained a very broad global distribution in tropical and semi-tropical countries. Its established distribution in North America is limited mainly to the southeastern United States and California, although non-permanent populations occur farther north in Illinois, Wisconsin, New York and Pennsylvania (USGS 2010).

Introduced aquatic plants can cause myriad changes in invaded ecosystems, including reduced water flow and a dramatic increase light attenuation, with consequent effects on primary and secondary production (e.g. Carpenter and Lodge 1986). In addition, mass accumulations of aquatic plants may strongly interfere with recreational and commercial vessel navigation, fisheries, and human health (e.g. Opande et al. 2004; Hershner and Havens 2008; Villamagna and Murphy 2010).

In this report, we describe the presence of water lettuce and water hyacinth in the lower Great Lakes.

Methods

Following a report from a citizen, Tim Duckett, regarding the suspected presence of water lettuce and water hyacinth in a river flowing into Lake St. Clair, we conducted surveys on 28-29 October 2010 to visually determine occurrence of both species at seven sites and 11 locations in total in major rivers and creeks adjacent to Windsor, Ontario (Table 1; Figure 1). We examined waterway margins on foot, while littoral zones were surveyed by a boat capable of manoeuvring in shallow waters. Each location was surveyed for ca. 2 hours, with longitude, latitude and water temperature recorded. Mature plants were collected in plastic containers, while young leaves were preserved in 95% ethanol and subsequently used for barcoding and species identity confirmation. We also collected 5L of water at each site where these macrophytes occurred, and buckets of surface sediment from Puce River and Turkey Creek to screen for seeds. In the laboratory, sediment was passed through a 0.7 mm sieve; matter retained on the sieve was hand-processed for seeds, which were examined under a microscope at 16x magnification. Water hyacinth reproduces largely via clonal growth, though sexual reproduction also may occur – with consequent production of seeds – albeit at reduced frequency in introduced, temperate populations (Barrett 1980; Zhang et al. 2010).

Images of plants were sent to Ted Center, U.S. Department of Agriculture, for confirmation of identification. In addition, three to four individuals of each species from each location of occurrence were utilized for DNA barcoding. However, many reactions failed owing to poor DNA isolation. Water hyacinth was more difficult to process than water lettuce owing to its rigid cell wall structures. Both plant species were barcoded for molecular identification using two chloroplast gene fragments, RNA polymerase C (rpoC1) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) (Newmaster et al. 2006; Kress and Erickson 2007).

Total genomic DNA was extracted from young leaves according to the proteinase K method (Waters et al. 2000). The primer pairs, rpoC1-2F (GGCAAAGAGGGAAGATTTCG) and rpoC1-4R (CCATAAGCATATCTTGAG TTGG) (Sass et al. 2007), and rbcL-1F (ATGTCACCACAAACAGAAAC) and rbcL-724R (CATGTACCTGCAGTAGC) (Asmussen and Chase 2001) were used to amplify rpoC1 and rbcL genes, respectively. PCR amplifications were performed in a 25 µL reaction volume containing ~50 ng of genomic DNA, 0.5 U of *Taq* polymerase, 1 x PCR buffer, 2 mM of Mg^{2+}, 0.2 µM of dNTPs, and 0.4 µM of each primer. PCR was conducted with an initial denaturing step at 95°C for 5 min, followed by 35 amplification cycles: 95°C for 30 s, 50°C for 30 s, 72°C for 60 s, and a final elongation step at 72°C for 5 min. All PCR products were verified on 1% agarose gel and subsequently purified using Agencourt® CleanSEQ protocol

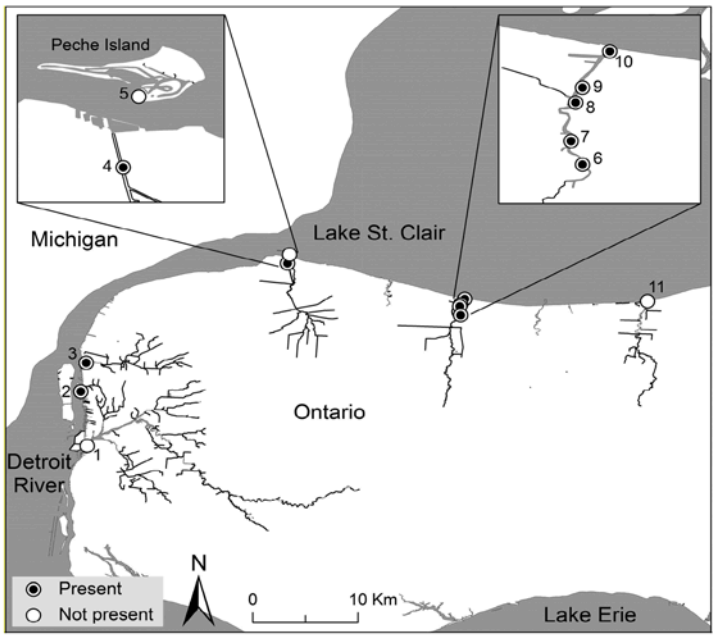

Figure 1. Sampling locations for water hyacinth and water lettuce in Lake Saint Clair and Detroit River in October 2010. Sites where both species were present (filled symbols or absent (open symbols) are indicated.

Table 1. Sampling sites in Ontario where surveys for water hyacinth and water lettuce were conducted on Lake St. Clair and the Detroit River. Site number refers to locations in Figure 1. Both species were either present (P) or absent (A) at each surveyed location. Sites are ordered by presence/absence.

Location	Site	Coordinates	Present
Puce River	6	N 42°18'10" W 82°46'41"	P
	7	N 42°17'53" W 82°46'53"	P
	8	N 42°17'28" W 82°46'58"	P
	9	N 42°17'46" W 82°46'56"	P
	10	N 42°17'17" W 82°46'53"	P
Little River	4	N 42°20'07" W 82°55'46"	P
Turkey Creek	3	N 42°14'41" W 83°06'06"	P
Island View Marina	2	N 42°13'07" W 83°06'23"	P
Peche Island	5	N 42°20'36" W 82°55'40"	A
Ruscom River	11	N 42°18'03" W 82°37'18"	A
Canard River	1	N 42°10'07" W 83°06'03"	A

(Agencourt). Sequencing was performed on purified PCR products using the forward primers for each gene, BigDye Terminator 3.1 sequencing chemistry and an ABI 3130XL automated sequencer. Sequences were annotated using the BLASTN algorithm on NCBI website *http://blast.ncbi.nlm.nih.gov/Blast.cgi*. All sequences were aligned using BioEdit software. Polymorphisms at different locations were assessed using DnaSP software. rbcL and rpoC sequences for both species were deposited in the GenBank barcoding databases (accession numbers HQ702899 - HQ702907 for rbcL gene and HQ702908 - HQ702915 for rpoC1 gene).

Results and discussion

Air and water temperatures on collection dates ranged between 8-10°C and 10-11°C, respectively. Water lettuce (Figure 2A) and water hyacinth (Figure 2B) were found at four of the

seven surveyed sites in rivers connected to Lake St. Clair and in the Detroit River, and the species always co-occurred (Table 1; Figure 1). These species have been observed in the Puce River for the last two and three years, respectively (Tim Duckett, pers. comm.). Previous studies also have reported co-occurrence of these taxa (e.g. Agami and Reddy 1990). Species identification was confirmed by Ted Center using photographic images and by molecular analysis. In total, eight water lettuce individuals sampled across all seven locations in four rivers, and one water hyacinth individual collected from Puce River, were successfully sequenced for both markers. The BLASTN searches using the rbcL gene confirmed identities of both species with 100% certainty. We observed only one haplotype for water lettuce across all locations in all rivers, suggesting that they may be derived from a single stock. Low observed genetic diversity is consistent with observations for a number of other introduced, nonindigenous plants (see Zhang et al. 2010).

Recovered plants appeared as moderately to very healthy, with all water hyacinth and most water lettuce exhibiting no signs of dieback (ie. chlorosis or wilt; Figure 2). Plant abundance was lower (<10 plants) at Turkey Creek, Harbour View Marina, and Little River than at other sites where the species were found (Figure 1). Both species were also found stranded on the beach in Lake St. Clair, adjacent to the Puce River, and water lettuce was observed floating into the lake from the river mouth.

A central question with the discovery of these plants in or adjacent to the Great Lakes is whether they can establish, or are established, in the basin. Observations of both species in the Puce River over multiple years would seemingly require production and subsequent germination of viable seeds, survival by some colonies during winter freezing followed by clonal growth, or repeated stocking events in different years, possibly at multiple sites. Recolonization of water hyacinth *via* seeds seems unlikely given the absence of genetic diversity in introduced populations, and low seed production in temperate areas (Barrett 1980; Spencer Barrett, pers. comm.). In addition, we recovered no seeds of either species from sediment collected from areas with extant populations of these species, although lack of comprehensive sampling precludes a conclusion that they are not produced as only ca. 3.8kg of sediment was collected, sieved and examined.

Figure 2. Images of water lettuce (Figure 1A) and water hyacinth (Figure 1B) collected from the Puce River, Ontario adjacent to Lake Saint Clair.

Presence of a refugium from freezing conditions during winter also seems unlikely in the areas surveyed as there are no major thermal effluent inputs in the area where plants were recovered. Water lettuce is highly vulnerable to low temperatures, with populations in south Florida experiencing high winter mortality when temperature approached freezing (Dewald and Lounibos 1990). Populations in a thermally enhanced stream in Slovenia experienced loss of leaves and decline in rosette size during winter, although plants were never exposed to freezing conditions (Šajna et al. 2007). However, the species has been recorded in a number of locations in Europe that experience freezing conditions (see Šajna et al. 2007). Water hyacinth range (Owens and Madsen 1995, and references therein) and growth (Center and Spencer 1981; Rodríguez-Gallego 2004; Wilson et al. 2005) also are influenced by cold temperatures (~5 - 8.1°C). Owens and Madsen (1995) determined that regrowth following exposure to freezing temperatures was typically higher for rooted water hyacinth than for floating

plants. Owens and Madsen (1995) speculated that winter mortality can influence the degree of population regrowth and infestation the following year, particularly along northern range boundaries. Thus, it seems improbable these species overwintered as adult plants in the Great Lakes.

It is possible that the plants may be introduced frequently by citizens seeking to dispose of excessive production from their personal water gardens. Water gardens are very popular in southern Ontario, and both species are sold in commercial trade (Rixon et al. 2005). However, if this vector were responsible, the observed distribution would require repetitive introductions at one and possibly multiple sites.

Even if the plants are not presently established in the basin, their repeated occurrence in surveyed waterways may pose localized navigational problems and precipitate ecological changes. It is possible, and perhaps probable, that additional introduced macrophytes may be established in the Great Lakes but hitherto have escaped formal reporting. We expect that under-reporting would be most likely for submerged species (e.g. *Hydrilla verticillata*, *Cabomba caroliniana*, *Egeria densa*), which may not be detected until their population densities impede navigation. *Cabomba* is present in the Great Lakes watershed and is dispersing slowly in Ontario (Jacobs and MacIsaac 2009).

Climate change and nonindigenous species

Species distributions may change in response to variation in key environmental drivers, notably those associated with climate warming (Baskin 1998; Parmesan 2006). The sale of potentially invasive macrophyte plants by the water garden trade in the Great Lakes basin is a risk factor for unanticipated aquatic introductions. Considering that water hyacinth and water lettuce are amongst the most commonly occurring macrophyte species sold in aquarium shops in southern Ontario (in 30% and 20% of stores, respectively), opportunities clearly exist for intentional or accidental release into the wild (Rixon et al. 2005). Lakes in the northern hemisphere have experienced later ice formation and earlier breakup in conjunction with climate warming (Magnuson et al. 2000). Winter temperature is predicted to be up to 4-6°C warmer in southern Ontario over the coming century (Colombo et al. 2007). If these forecasts are accurate, habitats previously unsuitable for completion of species' life cycles may become increasingly suitable, and establishment of some NIS may be anticipated (Hellmann et al. 2007; Dytham 2009). Such a scenario suggests that a review of species offered for sale by live garden and aquarium trades is warranted, perhaps including formal risk assessment under a scenario of warmer water temperature regimes during critical seasons (Champion et al. 2010; Andreu and Vilà 2010).

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Population attenuation in zooplankton communities during transoceanic transfer in ballast water

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Abstract

Successful biological invasion requires introduction of a viable population of a nonindigenous species (NIS). Rarely have ecologists assessed changes in populations while entrained in invasion pathways. Here, we investigate how zooplankton communities resident in ballast water change during transoceanic voyages. We used next-generation sequencing technology to sequence a nuclear small subunit ribosomal DNA fragment of zooplankton from ballast water during initial, middle, and final segments as a vessel transited between Canada and Brazil. Operational taxonomic unit (OTU) diversity decreased as voyage duration increased, indicating loss of community-based genetic diversity and development of bottlenecks for zooplankton taxa prior to discharge of ballast water. On average, we observed 47, 26, and 24 OTUs in initial, middle, and final samples, respectively. Moreover, a comparison of genetic diversity within taxa indicated likely attenuation of OTUs in final relative to initial samples. Abundance of the most common taxa (copepods) declined in all final relative to initial samples. Some taxa (e.g., Copepoda) were represented by a high number of OTUs throughout the voyage, and thus had a high level of intraspecific genetic variation. It is not clear whether genotypes that were most successful in surviving transit in ballast water will be the most successful upon introduction to novel environments. This study highlights that population bottlenecks may be common prior to introduction of NIS to new ecosystems.

Introduction

Biological invasions are commonplace in many habitats colonized by humans. Successful invasions are contingent upon introduction of sufficient individuals to constitute a viable population, tolerance of ambient conditions, and successful integration into the existing community (Colautti et al. 2006; Blackburn et al. 2011). These requirements must be met across an ordered series of stages from transport, introduction, establishment, and spread (Blackburn et al. 2015). Small population inocula and differences between native and introduced habitats may cause invasions to fail or trigger evolutionary changes in colonizing species (e.g., Phillips et al. 2006; Moran and Alexander 2014; Blackburn et al. 2015). Biological invasions may be viewed as examples of in situ evolution in consequence (Lee 2002; Facon et al. 2006; Barrett 2015; Colautti and Lau 2015).

A number of studies have documented successfully introduced populations with the same or higher levels of genetic diversity than putative source populations (e.g., Roman 2006; Taylor and Keller 2007; Gillis et al. 2009).

Enhanced genetic diversity may result from high propagule pressure (i.e., number of introduced individuals), particularly if it involves admixis from more than one source population (Roman and Darling 2007; Muirhead et al. 2008). In seemingly rare instances, small population size may be beneficial if some of the introduced individuals carry genotypes preadapted to the novel environment (e.g., Lavergne and Molofsky 2007). More typically, however, attenuation of propagules during transportation may result in small population inocula, with population genetic bottlenecks resulting from either losses during transportation or immediately upon introduction (see Roman and Darling 2007). Loss of genetic diversity can be fatal for introduced populations if they are unable to respond to selective pressures in the new region (e.g., Dlugosch and Parker 2008; Dlugosch et al. 2015). Impoverished genetic diversity also may result from postestablishment processes, notably genetic drift and selection in the new environment (e.g., Koskinen et al. 2002; Lee et al. 2007).

Few studies have focused on dynamics that occur while nonindigenous species (NIS) are carried by the invasion pathway (Olenin et al. 2000; Ruiz et al. 2000; Wonham et al. 2001; Briski et al. 2014). This dearth of research is surprising given that principal aquatic invasion pathways such as ships' ballast water and hull fouling each may carry dozens or more species at once (Sylvester et al. 2011; Briski et al. 2013). Wonham et al. (2001) found more than 50% loss of plankton taxa in ballast water of an ocean-going vessel that travelled from Hadera, Israel to Baltimore, USA, during a 16-day voyage, while Briski's et al. (2014) conceptual model of community dynamics during transportation indicates loss of 80–99% of individuals per species depending of taxonomic group during 25 days of transport in ships' ballast tanks. The endpoint for ballast populations that have suffered severe demographic decline could be local extirpation. Examination of community dynamics during transport may help determine whether bottlenecks in NIS populations develop before and/or after introduction.

Detecting species present at very low population density can be highly problematical, although advances in genetic technologies may assist researchers in this endeavor (Jerde et al. 2011; Zhan and MacIsaac 2015). The growing use of next-generation sequencing (NGS) is one such technology that may be employed in biodiversity studies (Hajibabaei et al. 2011; Zhan et al. 2013). For example, Zhan et al. (2013) determined that NGS could detect individual larvae or fragments down to 10^{-5}% biomass contribution in plankton samples, far below traditional microscopical analysis. Here, we use NGS to assess community changes in zooplankton entrained in ballast water of vessels moving from Canada to Brazil. We assess temporal changes in zooplankton community and determine the severity of population attenuation and whether genetic bottlenecks may have resulted in consequence prior to ballast water discharge.

Materials and Methods

We assessed zooplankton community dynamics in a vessel moving from Canada to Brazil during voyages in July, September, and October 2012 (Fig. 1). Two ballast tanks (three tanks for the second voyage) were sampled at the beginning, middle, and prior to the end of the voyage when mandatory ballast water exchange (BWE) occurred. Middle samples were not taken in voyage three due to inclement weather. In total, 19 ballast water samples were collected during the three voyages. Equal volumes of water were pumped from three different depths in each ballast tank and combined to achieve a total sample volume of 1000 L, following which it was processed through a 35-μm plankton net. Filtered samples were transferred to 95% ethanol and stored at cool temperature on the vessel, and later processed in the lab.

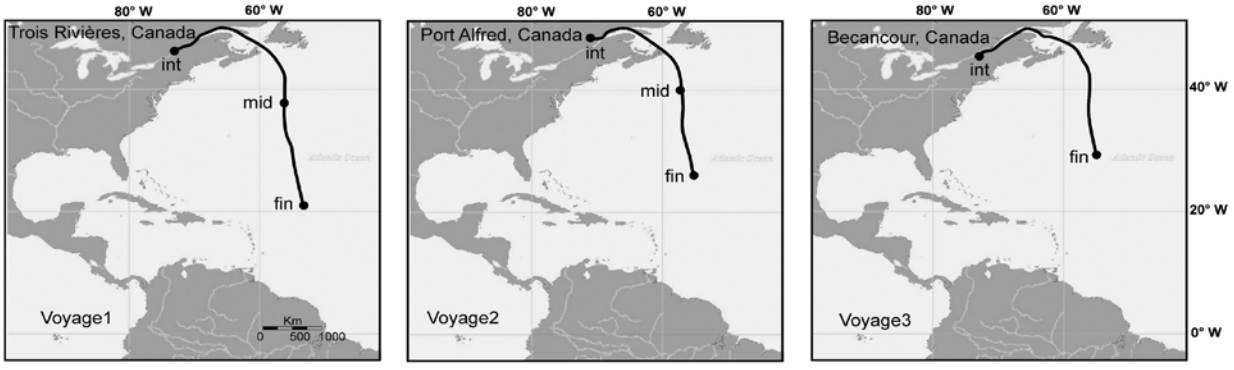

Figure 1. Voyage routes and the sampling locations at the initial (int), middle (mid), and final (fin) point of the experiment.

Zooplankton community genetic composition

Ethanol-preserved samples (~60 mL) were shaken to randomize the distribution of plankton. Two replicates of 1.5 mL were taken from each preserved sample using eppendorf tubes. Tubes were centrifuged at 9279.4 g to remove ethanol. Total genomic DNA was extracted from each sample using DNeasy Blood and Tissue Kit (Qiagen Toronto, ON, Canada). Extracted DNA was PCR-amplified using the primer pair Uni18S (5′-AGGGCAAKYCTGGTGCCAGC-3′)—Uni18SR (5′-GRCGGTATCTR ATCGYCTT-3′) spanning the hypervariable V4 region of nuclear small subunit ribosomal DNA (nSSU rDNA) (Zhan et al. 2014). A 25 μL PCR cocktail contained 100 ng of genomic DNA, 1 × PCR buffer, 2 mmol/L of Mg^{2+}, 0.2 mmol/L of dNTPs, 0.4 μmol/L of each primer, and 2U of *Taq* DNA polymerase (Genscript). PCR cycling parameters consisted of an initial denaturation step at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, and a final elongation step at 72°C for 10 min. Two PCR replicates were prepared for each sample. Samples were prepared for amplicon sequencing on an Ion Torrent Personal Genome Machine (PGM) according to the manufacturer's protocols.

Raw sequences obtained from Ion Torrent PGM were trimmed (e.g., homopolymer ≤8, maximum number of ambiguous nucleotides = 0) using the software Mothur v. 1.31.2 (Schloss et al. 2009). The UPARSE v7.0.1001 pipeline was used to remove chimeric sequences and errors/artifacts with the default settings (Edgar 2013). The resulting sequences were clustered into similarity-based operational taxonomic units (OTUs) at a cutoff value of 3% divergence (Kunin et al. 2010; Edgar 2013). Taxonomic status of OTUs was defined by BLASTn queries against the GenBank database implemented in the pipeline Seed v.1.1.35 (Větrovsky and Baldrian 2013). OTUs with minimum query coverage of 70% and E-value $<10^{-70}$ were used for downstream analyses. High levels of intraspecific genetic divergence and polymorphism increase the chance of error when comparing genetic diversity of different samples (Lee 2000; Brown et al. 2015). Hence, we defined taxa at the family level to avoid uncertainty in defining intraspecific genetic diversity (Fig. S1). Analysis of variance (one-way ANOVA) implemented in SPSS v.20 (SPSS Inc, Chicago, IL) was performed to investigate differences among average number of OTUs/sequences obtained from initial, middle, and final samples using a block design ANOVA and tanks as the blocking factor. Phylogenetic relationships of OTUs were reconstructed using neighbor-joining (NJ) analysis in MEGA v.4 (Tamura et al. 2007).

Zooplankton community abundance

Numerical abundance of zooplankton present in ballast samples was enumerated after taking subsamples for DNA extraction. This was carried out to evaluate the results from genetic analysis. As not all taxa were present in all samples, we focused on the most abundant taxon (i.e., Copepoda). All copepods including nauplii were counted. To estimate OTUs of the larger sampling size (i.e., more tanks) based on findings from our sampled tanks, we calculated Chao-1, an estimator of species richness based on the number of rare species in a sample (Chao 1984; Chao and Shen 2003). Sample-based OTUs rarefaction curves were generated to determine whether a significant difference existed given our small sample size. Chao-1 estimates were calculated using SPADE software (Chao and Shen 2006), while rarefaction curves were generated with 5000 random iterations using ECOSIM (Gotelli and Entsminger 2006).

Results

A total of 3,576,841 sequences were obtained from 19 samples taken from ballast tanks during the three voyages. After filtering and removing low-quality sequences, as well as removing sequences from other groups such as bacteria and algae, 3.10% of sequences were used for downstream analyses of zooplankton community. The number of obtained OTUs varied between 12 and 64 among samples (Table 1).

The number of OTUs decreased from the start to the end of each voyage, suggesting zooplankton die-off in ballast tanks (Fig. 2). The mean number of OTUs recovered from initial samples of all three voyages differed significantly from that found in the middle and final samples (ANOVA, $F = 15.17$, $P = 0.001$) (Fig. 3A), while trip differences (i.e., block effect) were not significant ($F = 0.83$, $P = 0.574$) (Table S2). Conversely, the mean number of sequences obtained from initial, middle, and final samples did not differ significantly (ANOVA, $F = 1.19$, $P = 0.345$), although a significant block effect was observed ($F = 4.80$, $P = 0.015$) (Table S2). These results indicate that differences in OTU depletion rate over time were not due to the number of recovered sequences (Fig. 3B).

Voyage one exhibited the highest loss of OTUs from initial to final samples, declining by 61.4% and 76.0% in tanks 1A and 1B, respectively (Table 1). In voyage two, attenuation was less severe, with losses of 14.2%, 33.3%, and 8.6% for tanks 2A, 2B, and 2C, respectively (Table 1). A small rebound in the number of OTUs was experienced at the end of the trip in tank 2A. There were slightly more OTUs in final samples than those collected at the midpoint of the trip (Table 1). In voyage three, 68.7% and 51.2% of OTUs were lost between initial and

Table 1. Operational taxonomic units (OTUs) and number of copepods recovered from three ballast tanks (A, B, and C) during three Atlantic voyages of a vessel. Each tank was sampled at the beginning, middle, and near the end of the voyage. Days refer to the time since start of the voyage when sampling was conducted.

Tank	Sampling period	Days	No. of OTUs	No. of taxa (Families)	No. of copepods	No. of OTUs (copepods)
1A	Initial	0	57	23	5340	20
	Middle	4	30	10	4179	13
	Final	8	22	10	1050	10
1B	Initial	0	50	17	1,1804	17
	Middle	3	28	12	1,1231	11
	Final	7	12	7	2140	9
2A	Initial	0	35	18	4058	15
	Middle	3	18	10	3005	10
	Final	7	30	17	1431	12
2B	Initial	0	39	18	2500	18
	Middle	3	26	14	2221	17
	Final	7	26	12	896	16
2C	Initial	0	46	16	3421	24
	Middle	3	30	15	2483	16
	Final	7	42	23	1762	27
3A	Initial	0	64	34	1503	28
	Final	12	20	12	25	5
3B	Initial	0	41	25	1048	15
	Final	14	20	15	17	8

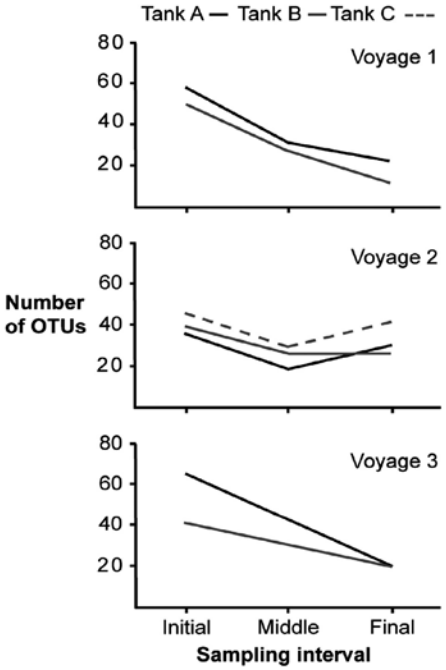

Figure 2. Number of OTUs (total counts) recovered from initial, middle, and final samples. Three different ballast tanks were sampled: A (black line), B (gray line), and C (dashed line). Voyage 3 was sampled only at beginning and end.

final samples in tanks 3A and 3B, respectively (Table 1). The initial sample collected from tank 3A contained the highest number of OTUs (64) and recovered taxa (34 taxa) (Fig. 2, Table 1), while the final sample of tank 1B exhibited the lowest number of OTUs (12) and recovered only seven taxa (Fig. 2, Table 1). Some major groups such as copepods, molluscs, and protozoans appeared in all samples (Table 2). However, bryozoans, cnidarians, gastrotriches, nematodes, platyhelminthes, poriferans, and rotifers were present in only some samples (Table 2).

In voyage one, only 12 of the initial 27 taxa were present in final samples (Fig. S2). Copepods had the highest number of OTUs recovered in final samples of this voyage, representing six taxa (Fig. S2). Another six taxa were recovered (one bryozoa, two mollusca, and three protozoa) in final samples. Tetrahymenidae (Phylum: Ciliophora) was the only taxon represented by two OTUs and a single sequence in final samples of tank 1A and was not detected in previous samples of the voyage. We recovered 36 taxa from samples of voyage two, only four of which were not recovered from final samples, while 12 taxa (five copepoda, one mollusca, one cnidaria, and five protozoa) had a higher number of OTUs relative to initial samples (Fig. S3). The overall number of OTUs declined or remained the same in all major groups in this voyage, except for cnidarians which contained more OTUs in final (4) than initial samples (3) (Fig. S3). In total, 38 taxa were obtained from initial samples of voyage three, 18 of which were not present in final samples. The number of OTUs declined over time in all groups, with protozoa and copepods containing the highest number of OTUs in final samples relative to other groups (Fig. S4).

Similar to the number of OTUs, the abundance of copepods declined from the start to the end of each voyage (Fig. 4). The initial sample collected from tank 1B contained the highest number of copepods ($n = 11804$), while final sample of tank 3B had the lowest ($n = 17$) (Table 1). The highest and lowest number of copepod OTUs ($n = 28$, $n = 5$) was recovered from initial and final sample of tank 3A, respectively (Table 1). The mean number of copepods and their OTUs recovered from initial samples of all three voyages differed significantly from that found in the final samples (ANOVA, $F = 5.02$, $P = 0.020$; $F = 4.09$, $P = 0.036$, respectively) (Fig. 4).

Discussion

In current study, we assessed changes in zooplankton communities in ballast water during the course of three Atlantic voyages. Our findings indicate attenuation of broad zooplankton groups during each of the voyages

(Figs S2–S4, Table 1). We also demonstrate that genetic diversity is lost prior to an introduction event, although results were taxon-specific as some species were detected for the first time toward the end of the voyage. Consistent with Wonham et al. (2001), we found that zooplankton species represented by OTUs and copepod abundance were reduced preintroduction and that not all taxa survive to the end of the voyage (Figs S2–S4, Table 1). Copepods, mollusks (veliger larvae), and protozoans were dominant among groups whose genetic diversity did not decline during voyages.

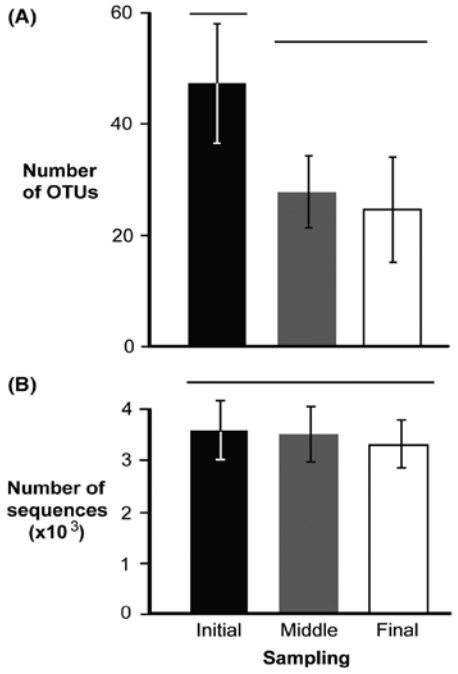

Figure 3. Average (±SD) number of OTUs (A) and average (±SD) number of sequences (B) obtained from all initial (black bar), middle (gray bar), and final (white bar) samples. Groups that are significantly different are not joined by the same line above the bars.

The total number of OTUs decreased along each voyage, and initial samples contained taxa that were not recovered at the end of voyage (Table 2, Figs S2–S4). Thus, our findings suggest the development of a genetic bottleneck and loss of potential genetic diversity prior to introduction. The loss of diversity is generally perceived as a significant barrier to successful establishment that must be overcome at the initial stage of an invasion (Blackburn et al. 2011). However, our results suggest that the same barrier may also occur within species.

Voyage one samples exhibited the highest loss of OTUs (76% for tank 1B) from initial to final samples (Table 1). This high loss of OTUs relative to other voyages may be due to enhanced fluctuations in temperature and salinity during the sampling period (Table S1). Temperature decreased by 5.3°C from initial samples to middle samples and then increased by 7.2°C between middle and final samples. During the same voyage, mean salinity increased in middle samples (3.1 ppt) relative to initial ones (0.1 ppt) but then decreased to final samples (0.3 ppt) (Table S1). Such fluctuations in environmental characteristics could trigger physiological shock in some taxa with adverse effects on genetic diversity in zooplankton (e.g., Cervetto et al. 1999; Zajaczkowski and Legezynska 2001).

In contrast, voyage two exhibited the lowest loss in OTU number, ranging from 8.6% to 33.3% relative to initial samples. Environmental temperature increased by 15.9°C from initial to final sample periods, while salinity decreased after initial sampling and remained relatively constant thereafter (Table S1). We observed a high loss of OTUs (>50%) for both tanks during voyage three (Table 1). This voyage was the longest trip (12 and 14 days before taking final sample for tanks 3A and 3B, respectively), which lasted for 7 days before final sampling was conducted (Table 1). Temperature of ballast

Table 2. Number of OTUs recovered from ballast tanks (A, B, and C) for three Atlantic voyages after BLASTn query against GenBank nucleotide database. Numbers indicate results for 18S marker obtained from Ion Torrent Personal Genomic Machine at the initial (int), middle (mid), and final (fin) day of the voyage. Refer Table 1 for number of days between initial, middle, and final samples.

Tank	Bryozoa	Cnidaria	Copepoda	Gastrotricha	Mollusca	Nematoda	Platyhelminthes	Porifera	Protozoa	Rotifera
No. of OTUs per group (int/mid/fin)										
1A	1/1/1		20/13/10	2/0/0	18/11/8		1/0/0	1/0/0	14/5/3	
1B	1/1/0	1/1/0	18/11/9	1/0/0	21/8/2	1/0/0	1/0/0		6/7/1	
2A	2/0/1	0/1/1	15/10/12	1/0/0	3/3/3	1/0/1	1/0/0		10/4/11	2/0/1
2B	1/0/0	1/1/1	18/17/16		4/3/3	1/0/0	1/1/0		12/3/6	1/1/0
2C	1/1/0	0/0/2	24/16/27	2/0/0	5/3/3	1/0/1			12/8/8	1/1/1
No. of OTUs per group (int/fin)										
3A	1/0	1/0	28/5	2/1	4/1	2/0	2/0		20/13	4/0
3B	1/1		15/8	2/1	3/2	2/1	2/0		11/5	5/2

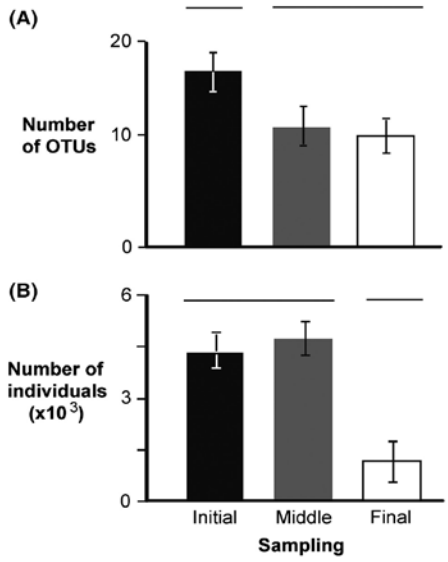

Figure 4. Average (±SD) number of OTUs (A) and average (±SD) number of individuals (B) for copepods obtained from all initial (black bar), middle (gray bar), and final (white bar) samples. Groups that are significantly different are not joined by the same line above the bars.

water decreased by 5.1°C and salinity increased during voyage three (Table S1). Based on the above, environmental factors in ballast tanks during each voyage appear to influence the rate at which OTUs were lost or, more rarely, gained. The appearance of some taxa or an increase in their OTU number in final samples could be the result of random sampling errors (Olenin et al. 2000) or population growth (Gray and MacIsaac 2010) during the voyage, perhaps from hatching of dormant stages (Briski et al. 2010, 2011).

The total number of copepods decreased along all voyages. Voyage three—the longest trip—exhibited the highest loss of individuals at about 98%. In voyage one, more than 80% of copepods were lost in final samples. However, voyage two exhibited the lowest loss in number of copepods. A conceptual model developed by Briski et al. (2014) suggests that factors such as the length of transport and taxon-specific survival could affect the magnitude of change in zooplankton community of ballast tanks.

A number of studies have investigated common errors associated with Ion Torrent PGM data, including erroneous insertions/deletions (i.e., indels) (Loman et al. 2012; Quail et al. 2012). Indels introduced by inaccurate flow calls appear at a rate of 1.38% in PGM data (e.g., Bragg et al. 2013). There exist a growing number of algorithms to minimize these errors for downstream analyses (Yeo et al. 2012; Flynn et al. 2015). However, much improvement is required to increase the efficiency of these methods. Effects of such errors are more pronounced when NGS data are used for polymorphism studies (Bragg et al. 2013). We used the UPARSE pipeline (Edgar 2013), which promises to produce the most accurate number of OTUs. In this method, OTUs are produced with ≤1% incorrect bases versus >3% generated by other methods (e.g., Mothur, QIIME) which tend to overestimate OTU number (Edgar 2013). Even though the UPARSE method might not represent the exact number of OTUs present in each sample, it appears to be among the most reliable methods currently available for such analyses (Edgar 2013; Flynn et al. 2015).

Results from BLAST may not be fully accurate in part due to a lack of online sequence references for particular taxonomic groups (Briski et al. 2016). Moreover, studies have shown that some groups of zooplankton—such as copepods and rotifers—form species complexes that are poorly defined taxonomically (e.g., Lee 2000; Gomez et al. 2002). We acknowledge that the number of sequences might not directly correspond to the number of propagules in ballast water (Weber and Pawlowski 2013; Flynn et al. 2015), as multiple divergent amplicons can be produced from a single individual or closely related taxa might be joined into one OTU. Therefore, our results are based upon genetic composition of the zooplankton community in the ballast water and do not fully correspond to the actual abundance of species. However, results from the abundance of copepods were in agreement with the genetic composition of zooplankton found in our ballast tanks.

In conclusion, this study highlights the possible creation of population bottlenecks prior to introduction of NIS to a novel environment, with about 50% of copepods lost prior to discharge of ballast water. It appears that population loss caused the attenuation of OTUs in final samples. Therefore, our findings highlight that events that occur prior to introduction may influence genetic diversity of newly introduced populations, which, in turn, could affect subsequent establishment success.

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Conflict of Interest

None declared.

Data Accessibility

OTUs and their matching accession numbers for each sample: Dryad doi: 10.5061/dryad.77sr0.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Protocol for analysis of 19 ballast water samples collected during three Atlantic voyages.
Figure S2. Neighbor-joining tree for all OTUs recovered from voyage one.
Figure S3. Neighbor-joining tree for all OTUs recovered from voyage two.
Figure S4. Neighbor-joining tree for all OTUs recovered from voyage three.
Figure S5. Sample-based rarefaction curves from the initial (red lines), middle (green), and final (blue) sampling and 95% confidence intervals (dashed lines).
Table S1. Environmental characteristics (temperature, pH, dissolved oxygen (D.O.), and salinity) of three ballast water (A, B, and C) samples obtained at the initial (int), middle (mid), and final (fin) day during three voyages of a vessel transiting between Canada and Brazil.
Table S2. Standard ANOVA table for randomized block design based on the number of OTUs/sequences recovered from all voyages.

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