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Invasion risk associated with invertebrates and their dormant stages in ships entering Canadian ports

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Invasion risk associated with invertebrates and their dormant stages in ships entering Canadian ports

by

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Declaration of Co-Authorship / Previous Publication

I. Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is result of joint research undertaken under the supervision of Dr. Hugh MacIsaac (University of Windsor) as follows: Chapter 2 contains material from a manuscript entitled "Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs" that has been accepted to *Biological Invasions*. This manuscript is co-authored by Briski E., Cristescu M., Bailey S. & MacIsaac H.J. Chapter 3 contains material from the manuscript entitled "Degradation of invertebrate dormant eggs collected in ballast sediment of ships" that has been submitted to *Diversity and Distributions*. This manuscript is co-authored by Briski E., Ghabooli S., Bailey S. & MacIsaac H.J. Chapter 4 contains material from the paper entitled "Efficacy of 'saltwater flushing' in protecting the Great Lakes from biological invasions by invertebrate eggs in ships' ballast sediment" published in *Freshwater Biology*. This manuscript is co-authored by Briski E., Bailey S., Cristescu M. & MacIsaac H.J. Chapter 5 contains material from the manuscript entitled "Regional variation in colonization and propagule pressure of invertebrates and their dormant eggs transported by ships' ballast sediments" that has been submitted to *Limnology and Oceanography*. This manuscript is co-authored by Briski E., Bailey S. & MacIsaac H.J. In all chapters, the main ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through provision of guidance with field and lab work. Chapters published, or accepted for publication, in the above journals have been modified slightly for consistency.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers in my thesis, and have obtained written permission from each of the co-authors to include the above material(s) in my dissertation. I certify that, with the above qualification, this dissertation, and the research to which it refers, is the product of my own work.

II. Declaration of Previous Publication

This thesis includes four original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Dissertation Chapter	Publication title/full citation	Publication status
Chapter 2	Briski E., Cristescu M., Bailey S. & MacIsaac H.J. (2011) Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs. *Biological Invasions* doi: 10.1007/s10530-010-9892-7	In press
Chapter 3	Briski E., Ghabooli S., Bailey S. & MacIsaac H.J. Degradation of invertebrate dormant eggs collected in ballast sediment of ships. In revision at *Diversity and Distribution*.	Submitted
Chapter 4	Briski, E., Bailey S., Cristescu M. & MacIsaac H.J. (2010) Efficacy of 'saltwater flushing' in protecting the Great Lakes from biological invasions by invertebrate eggs in ships' ballast sediment. *Freshwater Biology* 55: 2414-2424.	Published
Chapter 5	Briski E., Bailey S. & MacIsaac H.J. Regional variation in colonization and propagule pressure of invertebrates and their dormant eggs transported by ships' ballast sediments. In review at *Limnology and Oceanography*.	Submitted

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Abstract

The shipping industry has been a leading mechanism for introducing nonindigenous species (NIS) into aquatic ecosystems. Two different ballast water regulations, mid-ocean exchange (MOE) and saltwater flushing, were implemented as management practices to reduce the likelihood of new biological invasions in the Pacific, Great Lakes and Atlantic regions of Canada. There has, however, been no formal assessment of the efficacy of these regulations on invertebrates and their dormant eggs transported in the ballast sediment of ships, and current invasion risk posed by these taxa to different regions of Canada. To determine the potential risk of invasion associated with this vector after the implementation of ballast water regulations, I collected sediment samples from 135 ships entering the Pacific, Great Lakes and Atlantic ports and measured density and diversity of invertebrates as well as viability of their dormant eggs. To accurately identify dormant eggs I tested the application of DNA barcoding using mitochondrial cytochrome *c* oxidase subunit I and 16S rDNA. Further, I explored survival of invertebrate dormant eggs in collected ballast sediment over a one year period to determine if they accumulate inside of ballast tanks. Subsequently, to test efficacy of saltwater flushing, I compared the results of samples I collected in the Great Lakes with the results of similar samples collected prior to the implementation of saltwater flushing regulations, and to test efficacy of MOE I compared voyages with MOE *vs.* voyages without MOE. Finally, I compared vector strength in different regions in Canada. Overall results show DNA barcoding to be a rapid and accurate approach to identification of invertebrate

dormant eggs, and the results indicate possible accumulation of dormant eggs of onychopods and bryozoans inside ballast tanks. This comparative analysis suggests that vector strength varies among different regions in Canada with the Atlantic region being under the highest risk. The two ballast management regulations differently influence the probability of introductions of NIS *via* dormant eggs. Finally, the amount of sediment is the single, most important factor for management of invertebrates and their dormant eggs in ballast sediment and should be treated beyond current ballast management regulations.

For my parents Dragica and Tihomil

and

my husband Mirko

Mojim roditeljima Dragici i Tihomilu

i

suprugu Mirku

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Chapter 1: General introduction

1.1 NIS and their impacts

Human population growth has dramatically increased our collective environmental 'footprint', particularly over the last century (Senbel *et al.*, 2003; MacIsaac, 2005). One of the greatest stressors associated with this 'footprint' is the introduction of nonindigenous species (NIS; also called alien, nonnative, invasive, exotic or introduced species) (Sala *et al.*, 2000; Brönmark & Hansson, 2002). The introduction of species into habitats outside of their native ranges is increasing (Wonham & Carlton, 2005; Ricciardi, 2006) and represents an enormous problem due to the unexpected and unwanted impacts these species might cause (Williamson & Fitter, 1996; MacIsaac, 1996, 1999; Ricciardi, 2001; Leppäkoski *et al.*, 2002; Ricciardi & Atkinson, 2004).

Biodiversity, defined as the variety and variability among living organisms and the ecological complexes in which they occur, is affected by NIS (Sala *et al.*, 2000; Bax *et al.*, 2001). NIS may act as predators, parasites, pathogens or competitors with native species, and their introduction can result in significant habitat changes in ecosystems (Simberloff, 2002). Zebra mussels, *Dreissena polymorpha*, for example, have had considerable impact on the water quality and ecology after establishing in the Laurentian Great Lakes (MacIsaac, 1999). By filtering suspended sediment, zooplankton and phytoplankton from the water column, zebra mussels enhanced water clearance, allowing submerged aquatic plants to increase enormously. These changes altered native fish and

invertebrate communities and probably enhanced the establishment of new NIS (MacIsaac, 1996; Ricciardi, 2001). Some NIS are currently very important components of the Great Lakes food web (Benoit *et al.*, 2002; Vanderploeg *et al.*, 2002). The diatom *Thalassiosira baltica,* for example, is one of the most abundant algal species in the Great Lakes (Edlund *et al.*, 2000), and *Cercopagis pengoi* is a dominant zooplankton species (Laxson *et al.*, 2003). In addition to ecological impacts, NIS may have a tremendous impact on the economy. They damage agriculture, forestry, coastal and marine fisheries and aquaculture (Colautti *et al.*, 2006a). In Canada, the possible damage and control costs for just the 16 NIS considered by Colautti *et al.* (2006a) ranged between $13.2 billion and $34.8 billion CDN per year, while in the United States NIS cause losses amounting to approximately $120 billion USD annually (Pimentel *el al.*, 2005). Biological invasions are also evident in the transmission of human pathogens *Vibrio cholerae*, *Cryptosporidium* sp. and *Giardia* sp. (Ruiz *et al.*, 2000a; Drake *et al.*, 2001). As a consequence of all of the aforementioned impacts of NIS, it is critical to prevent their future transport and spread of new NIS (Simberloff, 2009).

1.2 Conceptual model of invasion and propagule pressure

Invasions can be viewed as a series of transition filters that species must pass through to become established in a new area (Fig. 1.1). The first two filters occur when propagules in the native region are taken into a transport vector (e.g. ballast water), transferred by the vector to another region, and released into a new area (e.g. Kolar & Lodge, 2001; Colautti & MacIsaac, 2004). The third filter

acts on NIS after they arrive to the new area. Some NIS may become widespread in the new area, some may become locally abundant, and some may do both. Collectively, this model consists of three factors: introduction effort (or propagule pressure); physiological tolerance to physical and chemical conditions in new area; and integration into biological community. Most species fail at one of the stages, as they are not able to survive the effects and influences of one or more of the three factors. Considering that number of propagules that survive each stage of the invasion process tends to decline, it would seem that large inocula would favour invasion success. Indeed, the propagule pressure model suggests that the success of an invader is linked to the number of inoculation events, the number of propagules released per event, and the health status of released propagules (Colautti *et al.*, 2006b). This model has been invoked as an explanation of invasion success in aquatic, marine and other ecosystems (Williamson & Fitter, 1996; Kolar & Lodge, 2001; Rouget & Richardson, 2003; Martínez-Ghersa & Ghersa, 2006). Ricciardi & MacIsaac (2000) proposed that NIS invasions by Ponto-Caspian species in the Great Lakes were consistent with the 'propagule pressure' concept even though most vessels do not arrive directly from this part of the world.

1.3 Accurate identification of NIS

An essential but very difficult aspect of measuring propagule pressure is correct identification of the NIS (Bax *et al.*, 2001). Accurate identification is an important element of invasion biology, vital for determining that a species is

indeed a NIS rather than a locally rare or even endangered species (Bax *et al.*, 2001). Incomplete systematic, biogeographic and/or historical data repeatedly result in an inability to categorize study species as native or nonindigenous (Carlton, 2009), while wrong or insufficient species identifications could result in misdirecting resources against false positives, or worse, inaction against false negatives. For centuries species were identified by traditional morphological methods. This approach, however, has some disadvantages, particularly in the case of microorganisms, algae, invertebrates, and their dormant eggs (Bailey *et al.*, 2005; Duggan *et al.*, 2005, 2006; Darling & Blum, 2007; Briski *et al.*, 2010). Currently, molecular identification of species is broadly accepted among scientists (Hebert *et al.*, 2003). DNA identification has been applied to a wide variety of taxa including Copepoda (Bucklin *et al.*, 1999, 2003), Aves (Hebert *et al.*, 2004), Pisces (Ward *et al.*, 2005) and Mammalia (Hajibabaei *et al.*, 2007). However, these methods are in various stages of development (Darling & Blum, 2007), and significant methodological and technological hurdles must be overcome before more reliable applications can be achieved.

1.4 Ships as a vector for NIS

Aquatic NIS are often transported intentionally as aquaculture species and deliberate government-sponsored stocks (Duggan *et al.*, 2006; Rixon *et al.*, 2005), or they may be introduced unintentionally. Major unintentional vectors of aquatic NIS include the external surfaces of ships or the ballast water and ballast sediment inside ships (Carlton, 1985; Carlton, 1989; Carlton & Geller, 1993; Ruiz

et al., 2000b; Leppäkoski *et al.*, 2002; Hayes & Sliwa, 2003; Bailey *et al.*, 2003; Bailey *et al.*, 2005; Sylvester & MacIsaac, 2010). In historical times, solid material (e.g. sand, gravel or stones) was used as ballast (Lindroth, 1958). Beginning in the 19th century, solid ballast was replaced by water. Ballast water is utilized when ships are not fully loaded to control the trim and increase stability. The water can be marine, brackish or fresh water, taken on board in ports, waterways or the open ocean (Carlton, 1985; Carlton, 1987). Species in water and suspended sediments are pumped into ballast tanks. Sediments suspended in the water often settle to the bottom of the tanks depending on the time the water is held in the tanks and on conditions on the ocean (Gollasch & Leppäkoski, 1999). Even if a ship is fully loaded with cargo, it will contain residual ballast water and sediment (Bailey *et al.*, 2003, 2005). It has long been known that ships introduce algae (e.g. Ostenfeld, 1908) and vessels provide habitat for a large variety of species (MacIsaac *et al.*, 2002). In his influential book, Charles Elton (1958) inferred that ships' ballast could be an important vector for NIS. The first scientific studies of ships' ballast water took place almost 20 years later (Medcof, 1975). Major studies on ballast water were subsequently conducted by Carlton (1985, 1987), Hallegraeff & Bolch (1991) and Subba Rao *et al.* (1994), among others. Today, we know that the introduction of NIS by ships is a global issue, and that ships' ballast water is a leading vector in those introductions into marine ecosystems (Carlton, 1985; Ruiz & Carlton, 2003) and the single most significant vector for introductions into the Great Lakes (Holeck *et al.*, 2004; Bailey *et al.*, 2005; Duggan *et al.*, 2005; Ricciardi, 2006; Kelly *et al.*, 2009).

1.5 Mid-ocean exchange and saltwater flushing

With ships' ballast water and sediments being the most important vectors for NIS introductions into aquatic ecosystems (Carlton, 1985; Ruiz & Carlton, 2003; Ricciardi, 2006; Molnar *et al.,* 2008), Canada and the USA have enacted two different ballast water regulations. First, mid-ocean exchange (MOE) was optional in 1989 and became mandatory in 1993 to reduce the number of propagules in ballast water transported to the Great Lakes (Canadian Coast Guard, 1989; United States Coast Guard, 1993). This regulation was extended to the Pacific and Atlantic coasts of Canada, and was further enhanced by including the management of residual ballast water and accumulated sediments through mandatory saltwater flushing, beginning in 2006 (Government of Canada, 2006; SLSDC, 2008). MOE is the replacement of water in filled ballast tanks with ocean water, while saltwater flushing is rinsing ballast tanks containing only residual ballast water and sediments through the uptake and subsequent discharge of several tonnes of ocean water. In theory, MOE and saltwater flushing should reduce abundance and species richness by purging species from the tanks, or particularly in the case of freshwater species, by killing them with highly saline water. The effect of MOE and/or saltwater flushing on the active stages of biota (Locke *et al.,* 1993; Rigby & Hallegraeff, 1994; Wonham *et al.,* 2001; Choi *et al.,* 2005; Gray *et al.,* 2007) as well as on the dormant eggs of invertebrates (Gray & MacIsaac, 2010, Briski *et al.,* 2010) has been assessed by multiple studies with varying results.

1.6 Dissertation objectives

This dissertation describes the first comparative examination of invasion risk posed by invertebrates and their dormant eggs in sediment of ballast tanks of ships operating in three different regions of Canada. This study identifies the best method for species identification of invertebrates and their dormant eggs, explores the survival of eggs in sediment through time, and tests the efficacy of ballast management activities, MOE and saltwater flushing, on the active and dormant eggs of invertebrates.

In chapter 2, I test the application of DNA barcoding using mitochondrial cytochrome *c* oxidase subunit I and 16S rDNA to identify species from dormant eggs collected in the ballast sediment of ships. The accuracy of DNA barcoding identification is tested by comparing results from the molecular markers against each other, and by comparing barcoding results with traditional morphological identification of individuals hatched from dormant eggs. Further, I explore two public genetic databases to determine the broader applicability of DNA barcodes. In this manner, I could determine if DNA barcoding is suitable for identification of invertebrate NIS, particularly those in dormant eggs.

In chapter 3, I explore the survival of invertebrate dormant eggs in collected ballast sediment over a one-year period to determine if there is a possibility of their accumulation inside ballast tanks. The degradation rate of dormant eggs is assessed by enumerating dormant eggs every two months and by conducting viability hatching experiments every six months. This chapter

provides unique evidence about the survivorship of dormant eggs of different taxa in ships' ballast sediment and provides insight into their potential accumulation.

In chapter 4, I test the effect of mandated saltwater flushing on the density and diversity of invertebrate dormant eggs in ballast sediment of transoceanic and coastal vessels arriving to the Great Lakes. I conduct a random survey of ship sediments in 2007 and 2008, and compare my results with those prior to the implementation of saltwater flushing regulations (Bailey *et al.,* 2005). This comparative analysis provides a unique opportunity to test the effect of saltwater flushing enacted in 2006 on dormant eggs carried in ballast sediments.

In chapter 5, I conduct comprehensive sampling of 135 ships arriving in three different regions of Canada to assess colonization and propagule pressure posed by invertebrates and their dormant eggs transported by transoceanic and coastal ships. By sampling ships which followed particular pathways: transoceanic voyages with MOE, coastal voyages with MOE, and coastal voyages without MOE, I am able to compare the vector strength in different regions, the invasion risk of transoceanic *vs.* coastal voyages, and the effect of MOE and duration of voyage on richness and abundance of the species inside tanks.

Finally, in chapter 6 I briefly summarize the novel contributions made by this study, and discuss the importance of ballast sediment as a vector for the introduction of invertebrate NIS.

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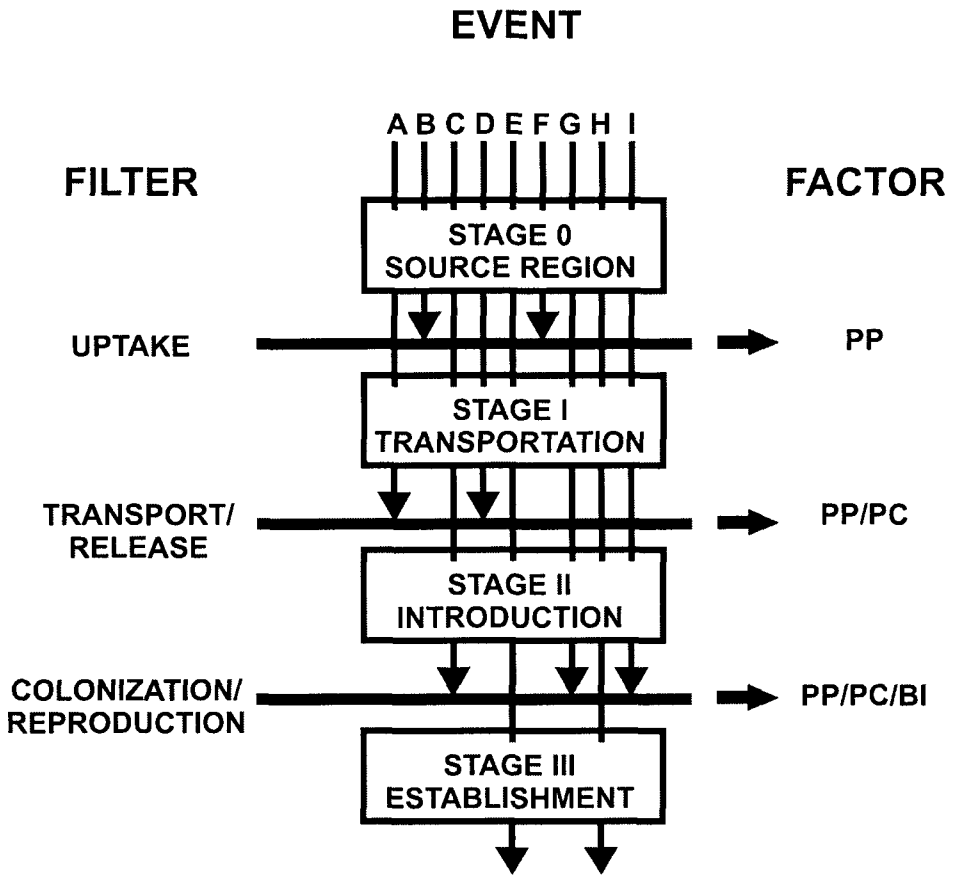

Figure 1.1 Schematic diagram representing sequence of invasion stages. Potential nonindigenous species (NIS), A through I, begin as propagules in a source region (stage 0), and pass through a series of filters that may preclude transition to subsequent stage. Factors affecting stage transition are: propagule pressure (PP), physico-chemical requirements of potential NIS (PC) and biotic interactions (BC). Adapted from Rahel (2002) and Colautti & MacIsaac (2004).

Chapter 2: Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs*

2.1 Introduction

Aquatic nonindigenous species (NIS) are often transported fouled on ships' external surfaces or in ballast water and sediments carried by ships (Carlton & Geller, 1993; Ruiz *et al.*, 2000; Leppäkoski *et al.*, 2002; Bailey *et al.*, 2005; Sylvester & MacIsaac, 2010). In order to most effectively utilize limited resources, managers must be able to quantify the risk of NIS introductions associated with different invasion vectors such as ballast water and sediment. One of the best indicators of invasion risk is "propagule pressure", the frequency and density with which NIS are introduced to new habitats (Colautti *et al.*, 2006; Hayes & Barry, 2008; Lockwood *et al.*, 2009). An essential but very difficult aspect of measuring propagule pressure is correct identification of the NIS associated with each vector (Bax *et al.*, 2001).

Accurate identification is a principal component of invasion biology, essential for determining that a species is indeed a NIS rather than a locally rare or even endangered species (Bax *et al.*, 2001). Effective management of NIS is generally hindered by insufficient information and resources (Byers *et al.*, 2002; Simberloff *et al.*, 2005; Lodge *et al.*, 2006). Limited systematic, biogeographic and/or historical data often results in an inability to categorize study species as

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native or nonindigneous (Carlton, 2009), while inaccurate or insufficient species identifications could result in misdirected resources against false positives, or worse, inaction against false negatives. As false negatives often lead to late detection of NIS, they can lead to difficulty in eradication and/or stopping further spread, as well as concurrent increases in operational costs (Bax *et al.*, 2001; Simberloff, 2009). The success of both prevention and rapid response efforts critically depends on a rapid, accurate and reliable approach to species identifications.

Examination of invertebrate species transported in residual ballast water and sediment of ships has been an active area of recent research (Bailey *et al.*, 2005; Duggan *et al.*, 2005, 2006; Briski *et al.*, 2010). Most of these studies have utilized traditional taxonomic methods to identify individuals collected as active adults or sub-adults, or for diapausing eggs, to identify individuals hatched in the laboratory (Bailey *et al.*, 2003, 2005; Duggan *et al.*, 2006). This approach, however, has some disadvantages. Diapausing eggs will not hatch, even when conditions are favourable, until diapause is broken, and some viable eggs may never hatch in the laboratory as conditions required to induce hatching are complex and vary among taxa (Schwartz & Hebert, 1987). Second, traditional taxonomic keys are often effective only for a particular life stage or sex, and juvenile stages, especially nauplii of Copepoda, often cannot be identified. Third, phenotypic plasticity in the character used for species recognition can lead to incorrect identification, and morphologically cryptic species are common in many taxa (Knowlton, 1993; Jarman & Elliott, 2000). Fourth, traditional taxonomic

identification often demands a very high level of expertise and can be very time consuming, with misidentifications or high uncertainty being a common result (Giangrande, 2003).

Molecular identification of species through the analysis of a small fragment of the genome represents a more promising approach for species identification, and is already broadly accepted among scientists. DNA identification has been applied to a wide variety of taxa including Copepoda (Bucklin *et al.*, 1999, 2003), Lepidoptera (Brown *et al.*, 1999; Janzen *et al.*, 2005), Culicidae (Shouche & Patole, 2000), Araneae (Barrett & Hebert, 2005), Scirtothrips (Rugman-Jones *et al.*, 2006), Aves (Hebert *et al.*, 2004), Pisces (Ward *et al.*, 2005; Ivanova *et al.*, 2007) and Mammalia (Hajibabaei *et al.*, 2007; Imaizumi *et al.*, 2007). The approach consists of amplification and sequencing of a specified 'barcode region', followed by comparison of the recovered sequence(s) to available genetic databases to determine species identity (Hebert *et al.*, 2003). The advantages of DNA barcoding are that it allows for identification of species when morphological identification may offer only estimates of higher taxonomic levels or no estimate at all (Darling & Blum, 2007), it recognizes cryptogenic species (Bickford *et al.*, 2007; Geller *et al.*, 2010), it is rapid and cost-effective (Hebert *et al.*, 2003; Wong & Hanner, 2008).

However, use of barcodes to identify species is not without drawbacks. The utility of barcodes can be limited by overlap of genetic variation between closely related species (Meyer & Paulay, 2005; Monaghan *et al.*, 2005), and by the lack of reference sequences in existing genetic databases (Darling & Blum,

2007). The former problem is more challenging as an insufficient 'barcoding gap', which describes the extent of separation between intraspecific variation and interspecific divergence in the selected molecular marker, can prohibit confident species-level identification (Meyer & Paulay, 2005). Mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rDNA (16S) have been shown to be broadly applicable for use as DNA barcode regions in animals because the evolution of these genes is rapid enough to discriminate to the species level (Hebert *et al.*, 2004; Ward *et al.*, 2005; Hajibabaei *et al.*, 2007; Imaizumi *et al.*, 2007; Ivanova *et al.*, 2007) and because of the availability of robust, universal primers (Folmer *et al.*, 1994; Lopez *et al.*, 1997; Zhang & Hewitt, 1997). COI is suitable for distinguishing not only closely related species but also phylogeographic groups within species (Gómez *et al.*, 2000, 2007; Hebert *et al.*, 2003).

Here, I use the COI and 16S genes to test the utility of DNA barcodes as a tool for species-level identification of diapausing eggs of aquatic invertebrates found in ships' ballast sediment. For simplicity, the term 'diapausing egg' is used in a broad sense in this paper and includes eggs, statoblasts, and other types of diapausing and non-diapausing dormant (or resting) stages. While the focus of this assessment was diapausing eggs in the strictest sense, I acknowledge that additional dormant stages were not excluded from analysis. The study is based on DNA extraction, PCR amplification using universal COI and 16S primers, and assignment of species identity by comparing resulting sequences with reference databases: GenBank and the Barcode of Life Database (BOLD). I then assess: i) the accuracy of the DNA barcode identifications by comparing DNA barcode

results generated by two molecular markers, and by comparing DNA barcode results to morphological identification; ii) the efficacy of DNA barcoding by comparing the number of species identifications obtained *via* molecular identification of diapausing eggs *versus* traditional morphological identification of animals hatched from diapausing eggs; and iii) the utility of DNA barcoding by examining the availability of sequences of invasive invertebrate species in existing reference genetic databases.

2.2 Materials and methods

Sample collection and extraction of eggs from sediment

Ballast sediments were collected opportunistically from 13 transoceanic ships arriving to Great Lakes ports (Hamilton, Windsor, Sarnia, Toledo and Detroit) and five transoceanic ships arriving to Sept-Îles, Quebec between June 2007 and September 2008. Approximately 6 kg of sediment was collected from a single tank of each ship. Sediment was homogenized before removal of four 40 g subsamples from each sample. Eggs were separated from sediment using a sugar flotation method (Hairston, 1996). Sediment was sieved through a 45 µm sieve, with the retained material washed into centrifuge tubes using a 1:1 mixture (weight:volume) of sucrose and water and centrifuged at approximately 650 rpm (7.7 m s^{-2}) for 5 min. The supernatant was then decanted into a 45 µm mesh sieve and rinsed with water. Diapausing eggs were classified into groups based on size and morphology using a dissecting microscope before DNA extraction and hatching experiments (Fig. 2.1). Every type of egg was photographed (Fig.

2.2). A maximum of 15 eggs per group were isolated for DNA extraction. Eggs that appeared completely intact were preferentially selected over those that appeared degraded, when possible; when less than 15 eggs per group were available, all eggs were used regardless of quality (Fig. 2.2).

Species-level identifications using DNA barcoding

Selected eggs were rinsed thoroughly in double-distilled H_2O several times to remove external debris before DNA extraction. DNA was extracted directly from diapausing eggs using the HotSHOT method (Montero-Pau *et al.*, 2008). Individual diapausing eggs were transferred to 200 µL reaction tubes containing 15 µL of alkaline lysis buffer (NaOH 25mM, disodium EDTA 0.2mM, pH 8.0). Once in the buffer, the egg was gently crushed against the side of the tube using a sterile needle under a dissecting microscope. Samples were incubated at 95°C for 30 min and placed on ice for 3 min. Finally, 15 µL of neutralizing buffer (Tris-HCL 40 mM, pH 5.0) was added to each tube. DNA was quantified using a Nanovue spectrophotometer (GE Healthcare UK Limited). Fragments of the mitochondrial genes COI and 16S were amplified using the universal COI primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2190 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994), and universal 16S primers S1 (5'-CGC CTG TTT ATC AAA AAC AT-3') and S2 (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Palumbi, 1996). PCR reactions were performed in a total volume of 25 µL using 5 µL of DNA extract, 1x PCR buffer, 0.13 mM trehalose, 0.1 µM of each primer, 2.5 mM $MgCl_2$, 0.14 mM

dNTPs and 0.4 U Top*Taq* DNA polymerase (Qiagen, Canada). The thermal profile consisted of a 1 minute initial cycle at 94°C, followed by 5 cycles of 94°C (40 sec), 45°C (40 sec) and 72°C (1 min), 35 cycles of 94°C (40 sec), 50°C (40 sec) and 72°C (1 min), and a final extension of 72°C for 5 minutes. I did not attempt to concentrate DNA extracts resulting in unsuccessful PCR or to amplify smaller fragments from within the barcode region using primers other than universal Folmer *et al.* (1994) or Palumbi (1996) primers.

PCR products from eggs were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA) and DNA sequences were blasted against GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the nucleotide blast (default parameters). In addition, COI sequences were compared to the BOLD (http://www.barcodinglife.org), using the identification engine BOLD-IDS, with the option 'All Barcode Records on BOLD'. Based on a maximum 4% intraspecific variation in the COI gene reported for Copepoda by Bucklin *et al.* (2003) and for Cladocera and Amphipoda by Costa *et al.* (2007), a score resulting in at least 96% similarity to the closest match was deemed a species level identification. As 16S evolves approximately 2 times slower than COI (Adamowicz *et al.*, 2009), 98% similarity was used for discriminations for species level using 16S. Matches lower than 96 and 98% for COI and 16S, respectively, were not assigned to any particular taxonomic level.

Species-level identifications using traditional taxonomy

More than 5100 diapausing eggs were incubated to conduct traditional morphological identification on hatched individuals (Fig. 2.1). Sediments were stored in the dark at 4°C for at least 4 weeks to break diapause of dormant eggs before hatching experiments commenced (Grice & Marcus, 1981; Schwartz & Hebert, 1987; Dahms, 1995). Subsequently, diapausing eggs were isolated from 40 g replicate subsamples of sediment using the sugar flotation method described above. Isolated eggs were immediately placed into vials containing 15 mL of sterile synthetic pond water (0 parts per thousand salinity (‰); Hebert & Crease, 1980) or sterile seawater medium (15 or 30‰) under a light:dark cycle of 16:8 h at 20°C, using a stratified random design. The seawater medium was prepared from natural seawater ballast collected from a vessel transiting the Great Lakes, filtered through 2.5 µm Whatman paper filter, and diluted with the sterile, synthetic pond water. Each vial contained between 6 and 81 eggs, depending on the density of eggs in sediment samples.

Three different salinities were used in an attempt to match unknown species to optimum fresh-, brackish- or saltwater habitat to maximize hatching success. Controls containing only hatching media were kept in each treatment group to monitor for the introduction of organisms from the environment. Following Bailey *et al.* (2005), vials were checked for emergence every 24 h for the first 10 days and every 48 h for a subsequent 10 days, with media renewed every 5 days. Hatched individuals were removed to separate vials and identified morphologically in the laboratory; taxonomic experts were consulted when

identifications were uncertain. Hatching percentage was calculated by dividing the total number of animals hatched by the total number of eggs isolated for hatching and multiplied by 100.

Confirmation of species identifications and efficacy of DNA barcoding

A DNA barcoding identification was considered correct when a second methodology gave the same result (i.e., when both molecular markers (COI and 16S), or when morphological identification and one molecular marker, identified eggs from the same group as the same species). To assess efficacy of DNA barcoding I compared the total number of species identified and the variety of taxa identified by DNA barcoding to that of traditional morphological identification of animals hatched from diapausing eggs. Additionally, financial costs and time spent to conduct DNA barcoding *versus* morphological methods were estimated.

GenBank sequence availability

To determine the broader applicability of DNA barcoding for species level identification of diapausing eggs transported by ship sediments, I searched two primary public genetic databases to determine the number of species with available gene sequences in comparison to the number of described species. Rotifera, Bryozoa, Branchiopoda and Copepoda species were investigated for availability of any type of sequence, and specifically for COI and 16S genes, in GenBank (http://www.ncbi.nlm.nih.gov/), and for the COI gene in the BOLD (http://www.barcodinglife.org) on 23 February 2010. In addition, I examined

sequence availability of 34 established NIS of Bryozoa, Branchiopoda and Copepoda reported from the Northeast Pacific Ocean (Wonham & Carlton, 2005), Laurentian Great Lakes (Ricciardi, 2006), and East Coast of Canada (A. Locke, unpublished data) to gain a better understanding of NIS sequence availability on a broader scale. Finally, sequence availability for 55 invasive animal species on the Global Invasive Species Database's "100 of the World's Worst Invasive Alien Species" list (http://www.issg.org/database/welcome/) were examined to determine if notorious animal invaders were better represented than NIS in general.

2.3 Results

Species-level identifications using DNA barcoding

I isolated 289 diapausing eggs from 18 ballast tanks for DNA barcoding. Of the 289 eggs isolated, DNA was successfully extracted from 96 eggs (33%). Extraction from the remaining 193 eggs resulted in < 1 ng/μL of DNA (as quantified using a Nanovue spectrophotometer), and unsuccessful PCR amplification for both COI and 16S DNA fragments. I obtained 139 successful PCR products and 139 sequences using the two sets of universal primers, including 66 COI and 73 16S sequences (Fig. 2.1). Of the 96 diapausing eggs for which I obtained barcodes, I was able to identify 61 eggs to species level and a further 35 to possible family/order level. Species level identifications were obtained for ten Branchiopoda (44 eggs), one Rotifera (5 eggs), three Bryozoa (6 eggs), four Copepoda (5 eggs) and one Ascidia (1 egg)(Fig. 2.1, Appendix 2.1).

DNA barcoding of diapausing eggs was most successful for species level identification of Branchiopoda belonging to the families Podonidae and Daphniidae. I was able to identify all four Podonidae species and five out of six Daphniidae species. Further, one *Diaphanosoma* was identified to species-level and one to possible genus, while *Moina* and *Bosmina* were poorly represented, resulting in no species identifications. All three Bryozoa species were identified, as was one out of three Rotifera species. Copepoda was the least represented group in the genetic databases; of nine possible species, only four were identified (Appendix 2.1).

Six of the 19 species identified by molecular methods are nonindigenous to the Great Lakes region (i.e. *Daphnia magna, Podon intermedius, Pleopis polyphemoides, Cercopagis pengoi, Acartia tonsa* and *Botryllus schlosseri*), while three are nonindigenous to the east coast region (i.e. *D. magna, Calanus euxinus* and *Plumatella emarginata*).

Species-level identifications using traditional taxonomy

Hatching trials were conducted on 5106 diapausing eggs, of which 161 eggs (3%) were successfully hatched. There was no introduction of organisms from the environment into the negative controls. Hatched taxa included Branchiopoda, Copepoda and Rotifera. Morphological species level identification was successful for nine Branchiopoda (109 individuals) and one Rotifera species (19 individuals), but no Copepoda (Appendix 2.1). Juvenile naupliar stages of many species of Copepoda are morphologically indistinguishable (Kiesling *et al.*,

2002), thus even taxonomic experts could not identify hatched individuals. Three of the 10 species identified morphologically were nonindigenous to the Great Lakes region (i.e. *D. magna, P. intermedius* and *P. polyphemoides*), and one was nonindigenous to the east coast region (i.e. *D. magna*).

Confirmation of species identifications and efficacy of DNA barcoding

Of 61 species level identifications by DNA barcoding, 48 were confirmed by a second method: 30 confirmed by both morphological identification and another marker, 14 confirmed only by morphological identification and four confirmed only by another marker (Table 2.1). Thirteen additional sequences resulted in a species level identification, but were not confirmed by a second method. Forty-four sequences had identification matches between 79 and 95%, resulting in identification only to the possible family/order level (Fig. 2.1, Appendix 2.1). One 16S sequence had an identification match of 99%, but still resulted in only genus level identification as the GenBank reference sequence was only identified to the genus level itself (Appendix 2.1). Species level identifications were entirely consistent between methodologies.

Comparison of DNA barcoding and morphological methods revealed that DNA barcoding resolved a greater number of species. While nine Branchiopoda (*D. mendotae, D. parvula, D. magna, D. pulex, D. galeata, Diaphanosoma brachyurum, P. intermedius, P. polyphemoides* and *Evadne normanni*) and one Rotifera (*Brachionus calyciflorus*) were identified by both methods, nine species could be identified only by DNA barcodes: four Copepoda (*Leptodiaptomus*

siciloides, A. tonsa, Eurytemora affinis and *C. euxius*), one Branchiopoda (*C. pengoi*), three Bryozoa (*P. emarginata, P. reticulata* and *P. casmiana*) and one Ascidia (*B. schlosseri*) (Fig. 2.3, Appendix 2.1). The success of DNA barcoding identification (19 species) was nearly double that of traditional morphological methods (10 species) (Figs. 2.1 and 2.3). Further, estimated costs (supplies and labour) and time spent on molecular identification using both markers (unsuccessful tries included) were approximately $1800 (CND) and 72 h, respectively, *versus* approximately $2600 (CND) and 300 h for morphological identification.

GenBank sequence availability

My inspection of two public databases revealed the availability of COI and/or 16S sequences for 102, 176, 488 and 416 species for Rotifera, Bryozoa, Branchiopoda and Copepoda, respectively (Table 2.2, consulted 23 Feb 2010). This represents ~ 5, 3.5, 54 and 3.5% of described Rotifera, Bryozoa, Branchiopoda and Copepoda species, respectively (Ruppert *et al.*, 2004; BOLD (http://www.barcodinglife.org), consulted 23 Feb 2010) (Table 2.2). However, searching for COI and 16S sequences of NIS of Bryozoa, Branchiopoda and Copepoda established in the Northeast Pacific Ocean, the Laurentian Great Lakes, and East Coast of Canada resolved available sequences for 7 (44%), 7 (44%) and 2 (100%) species, respectively (Table 2.3). Of the 55 worst invasive animals reported in the Global Invasive Species Database, 52 (94%) had COI and/or 16S sequences available.

2.4 Discussion

Results from this study indicate that DNA barcoding resolved nearly double the number of species identified by traditional morphological taxonomy (19 *vs.* 10), and was suitable for a wide range of taxa, including Branchiopoda, Copepoda, Rotifera, Bryozoa and Ascidia. Branchiopoda and Copepoda were respectively the best and worst represented groups in genetic databases. Nevertheless, notorious invaders were well represented, making high priority NIS detectable. Of the 96 diapausing eggs for which I obtained barcodes, I was able to identify 64% to species level and a further 36% to possible family/order level.

Correct identification of species is essential to invasion biology, yet identification of morphologically cryptic species and those which are present as diapausing eggs remains a major challenge. Challenges associated with morphological identification of sub-adult stages render molecular genetic analyses particularly advantageous (Hebert *et al.*, 2003), though accuracy of the method for many taxonomic groups has yet to be demonstrated. For example, Schubart *et al.* (2008) reported the same COI sequence for two genera of freshwater crabs, while Bucklin *et al.* (2003) and Costa *et al.* (2007) reported a 'barcoding gap' for species of Copepoda, Cladocera and Amphipoda. I tested DNA barcoding accuracy for taxa of interest by direct comparison of DNA barcoding results using two gene markers to each other, and by comparison of DNA barcoding and morphological identification results. I found no disagreement among the three methodologies. DNA barcoding using mitochondrial COI and16S genes provides a rapid, accurate method for identification of species from

diapausing eggs, and overcomes several problems posed by traditional morphological identification. Even though morphological identification showed the same accuracy as molecular, I estimated that DNA barcoding method is at least 4x times faster and 30% cheaper than morphological identification (not including initial equipment costs). Furthermore, notorious invaders such as *C. pengoi* and *B. schlosseri* were identified only by the molecular method. I found DNA barcoding was suitable for a range of taxa, including Branchiopoda, Copepoda, Rotifera, Bryozoa and Ascidia.

By using both markers for each individual egg, and two public databases - GenBank and BOLD - I obtained more sequences and increased the chance of a sequence match, thereby providing higher confidence in identifications. Branchiopoda were the best resolved taxa, possibly due to the fact that about 54% of the species in this group are represented in public genetic databases. In addition, the genus *Daphnia* - which represents 13% of Branchiopoda taxa (Forró *et al.*, 2008) - are used as model organisms for genomics (http://wfleabase.org/) and evolutionary studies (e.g. Hebert *et al.*, 2002). Conversely, only 3.5% of Copepoda have been entered into genetic databases, limiting the current utility of molecular identification methodologies for a group notorious for difficult taxonomic identification (Rombouts *et al.*, 2009). Given morphologically indistinguishable immature stages in this order (Kiesling *et al.*, 2002), augmentation of sequence databases may prove particularly useful for identification of Copepoda in the future.

Unbalanced representation of taxa in sequence databases has been observed by other scientists. Puillandre *et al.* (2009) reported that sequence availability for marine gastropods is low, with only 4% of taxa identified to species level. Conversely, Wong & Hanner (2008) obtained 99% species identifications for market seafood. Though DNA databases are undergoing continual and rapid expansion, sequence availability for poorly studied taxa remains low compared to commercially important or otherwise better studied taxa. Despite this current limitation, identification of invasive species such as *C. pengoi* and *B. schlosseri* was successful. Sequences exist for 94% of the world's 55 worst invasive animals in the two explored genetic databases. As studies of population genetics of NIS are a major source for sequences, species with small, spatially restricted populations, and those that cause no discernible economical or ecological problems, are rarely studied. Because control and eradication of NIS is usually only possible at the earliest stages of invasion (Bax *et al.*, 2001), DNA barcoding may be especially useful for management of notorious invaders which are already well represented in genetic databases. Presently, barcoding technology is being used to assay for presence of silver (*Hypophthalmichthys molitrix*) and bighead (*H. nobilis*) carp in waterways adjacent to Chicago, Illinois, as part of an early detection program to prevent spread to Lake Michigan.

Unsuccessful DNA extractions from 67% of eggs in my study were most likely due to the condition of eggs, as eggs that were visibly degraded never hatched. Previous testing of the HotSHOT method (Montero-Pau *et al.*, 2008) for DNA extraction success on freshly cultured *Daphnia pulex* eggs from Dr. Melania

Cristescu's lab resulted in 96% success (E. Briski, unpublished data) with similar observations reported by Montero-Pau *et al.* (2008) for diapausing eggs of Rotifera, Cladocera, Anostraca and Notostraca, indicating that the methodology was robust. Considering that diapausing eggs in this study were collected in ships' ballast tanks, degradation of eggs is common and faster than degradation of dormant eggs in natural habitats (E. Briski, unpublished data). As universal COI and 16S primers amplify products of similar size, they can be considered a positive control for each other (Ivanova *et al.*, 2007). Failed amplification of one primer can be attributed to primer mismatch. In cases where both markers failed, there is a distinct possibility that the DNA template was degraded (Ivanova *et al.*, 2007). This phenomenon provides possible insight into the viability of diapausing eggs. In my case, 3% of the eggs hatched, while DNA was successfully extracted from 33% of eggs. While the percentage of eggs hatched in the laboratory likely underestimates the true viability of the eggs, the percentage of successful DNA extractions may overestimate viability. The physiology of dormant eggs is very complex, and hatch success depends on the degree of diapause termination, energy content of the eggs, number of non-viable embryos and environmental factors (Carvalho & Wolf, 1989; Lavens & Sorgeloos, 1996; Gilbert, 2004; Pauwels *et al.*, 2007; Briski *et al.*, 2008). As a result, many eggs will not hatch in the laboratory even under favourable conditions (Schwartz & Hebert, 1987; Bailey *et al.*, 2003). In contrast, successful DNA extraction may overestimate viability if extraction methods are sensitive enough to amplify degraded DNA of non-viable eggs. Thus, the true viability of diapausing eggs recovered from sediments may

be somewhere between the number of hatched individuals and the number of eggs from which DNA was successfully extracted. This information could prove useful to risk assessments, as diapausing eggs that are not viable do not constitute an invasion risk.

Aside from the fact that I found two public sequence databases underpopulated, DNA barcoding still yielded greater taxonomic identification capability than traditional morphological methods. The problem of underpopulated sequence databases is least acute for problematic NIS, which tend to be well represented in existing databases. As sequencing technology improves and DNA barcoding becomes more commonplace, I expect that DNA barcoding for species identifications will become routine for an ever increasing number of taxonomic groups.

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Table 2.1 List of taxa identified by DNA barcoding using mitochondrial genes COI and 16S. Species level identifications were considered accurate if the two genes gave the same results and/or were verified by morphological identification.

Primary marker	Secondary confirmation	Branchiopoda	Copepoda	Rotifera	Ascidia	Bryozoa	Total
	16S and morphological	25	0	5	0	0	30
COI	Morphological	2	0	0	0	0	2
	16S	3	1	0	0	0	4
	No extra confirmation	2	4	0	1	0	7
16S	Morphological	12	0	0	0	0	12
	No extra confirmation	0	0	0	0	6	6
	Total	44	5	5	1	6	61

Table 2.2 Number of described species of Rotifera, Bryozoa, Branchiopoda and Copepoda compared to the number of species for which gene sequences are available in two public databases: GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD (http://www.barcodinglife.org). Comparison was conducted 23 Feb 2010.

Taxa	Number of described species	GenBank			BOLD	Total number of distinct species from GenBank and BOLD (COI + 16S)
		Number of species (all sequences)	Number of species (COI sequence)	Number of species (16S sequence)	Number of species (COI sequence)	
Rotifera	~ 2,000[1]	205	78	33	34	102
Bryozoa	~ 5,000[1]	239	76	103	20	176
Branchiopoda	~ 900[2]	582	364	230	374	488
Copepoda	~ 12,000[1]	598	256	123	296	416

[1] Ruppert *et al.* 2004

[2] BOLD (http://www.barcodinglife.org); consulted 23 Feb 2010.

Table 2.3 Gene sequence availability for 34 aquatic NIS recorded in the North Pacific Ocean, the Laurentian Great Lakes and the East Coast of Canada and the 55 worst animal invaders globally in two public databases: GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD (http://www.barcodinglife.org); consulted 23 Feb 2010.

Region	Number of NIS	GenBank			BOLD	Total number of distinct species from GenBank and BOLD (COI + 16S)
		Number of species (all sequences)	Number of species (COI sequence)	Number of species (16S sequence)	Number of species (COI sequence)	
Northeast Pacific Ocean[1]	16	7	7	3	6	7
Laurentian Great Lakes[2]	16	7	5	6	6	7
East Coast of Canada[3]	2	2	2	1	2	2
Total	34	16	14	10	14	16
55 worst animal invaders[4]	55	53	46	39	48	52

[1] Wonham & Carlton (2005)

[2] Ricciardi (2006)

[3] A. Locke (unpublished data)

[4] "100 of the World's Worst Invasive Alien Species" reported on Global Invasive Species Database (http://www.issg.org/database/welcome/)

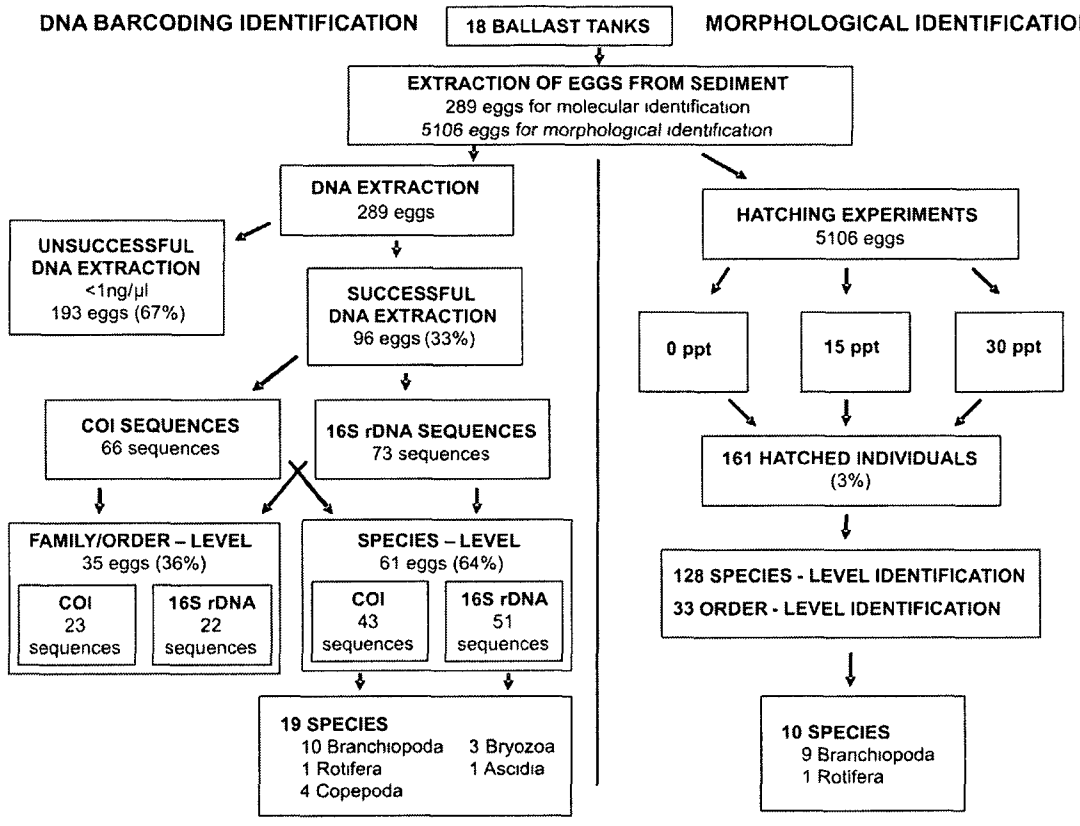

Figure 2.1 Schematic representation of DNA barcoding and morphological identification methods used for diapausing egg.

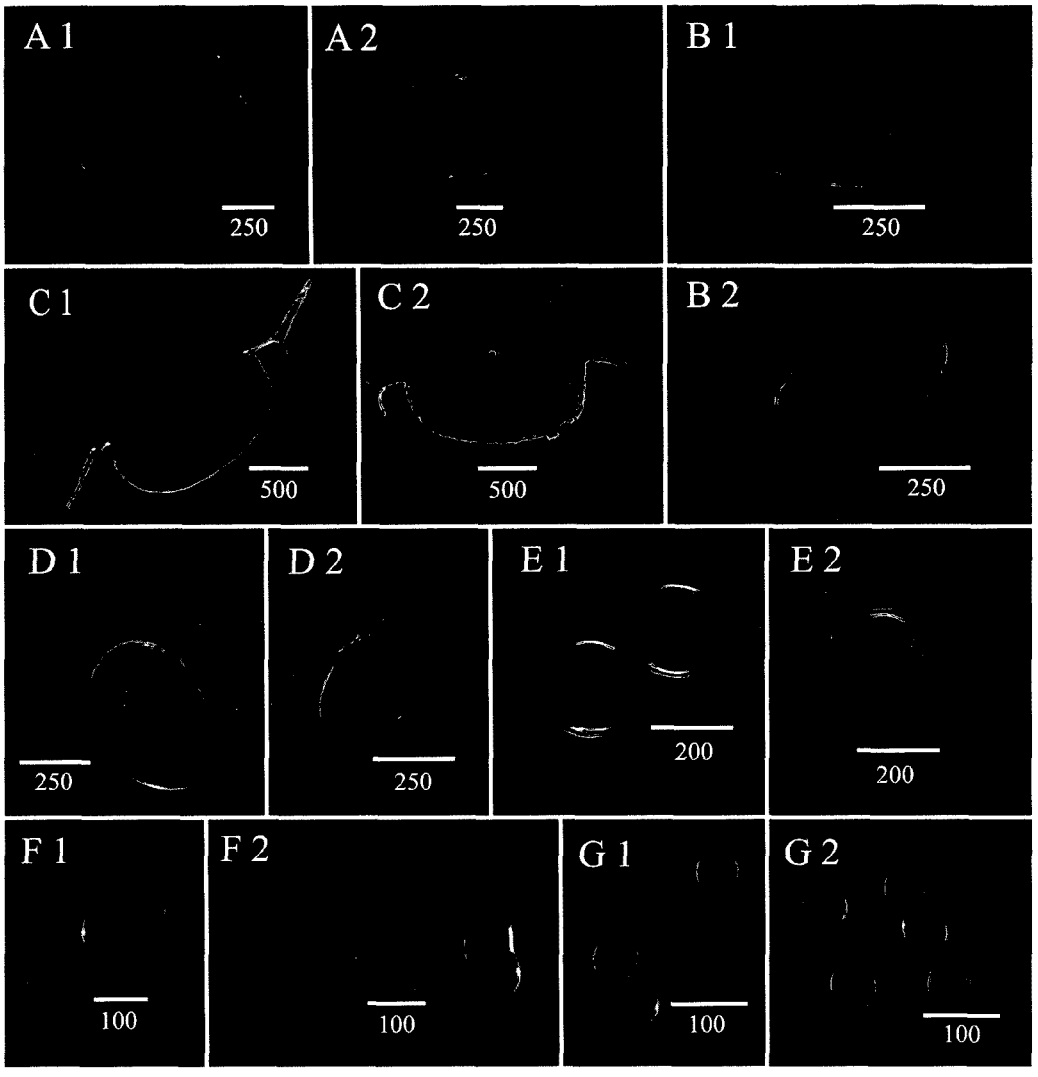

Figure 2.2 Resting egg morphotypes with successful (1) and unsuccessful (2) DNA extraction. Branchiopoda: (A1) *Daphnia mendotae*, (A2) *Daphnia* sp., (C1) *Daphnia magna,* (C2) *Daphnia* sp., (D1) *Daphnia magna,* (D2) Branchiopoda, (E1) *Podon intermedius,* (E2) Branchiopoda. Bryozoa: (B1) *Plumatella emarginata*, (B2) *Plumatella* sp. Rotifera: (F1) *Brachionus calyciflorus*, (F2) *Brachionus* spp. Copepoda: (G1) *Leptodiaptomus siciloides*, (G2) various Copepoda. Scale bars (μm) are included on each image.

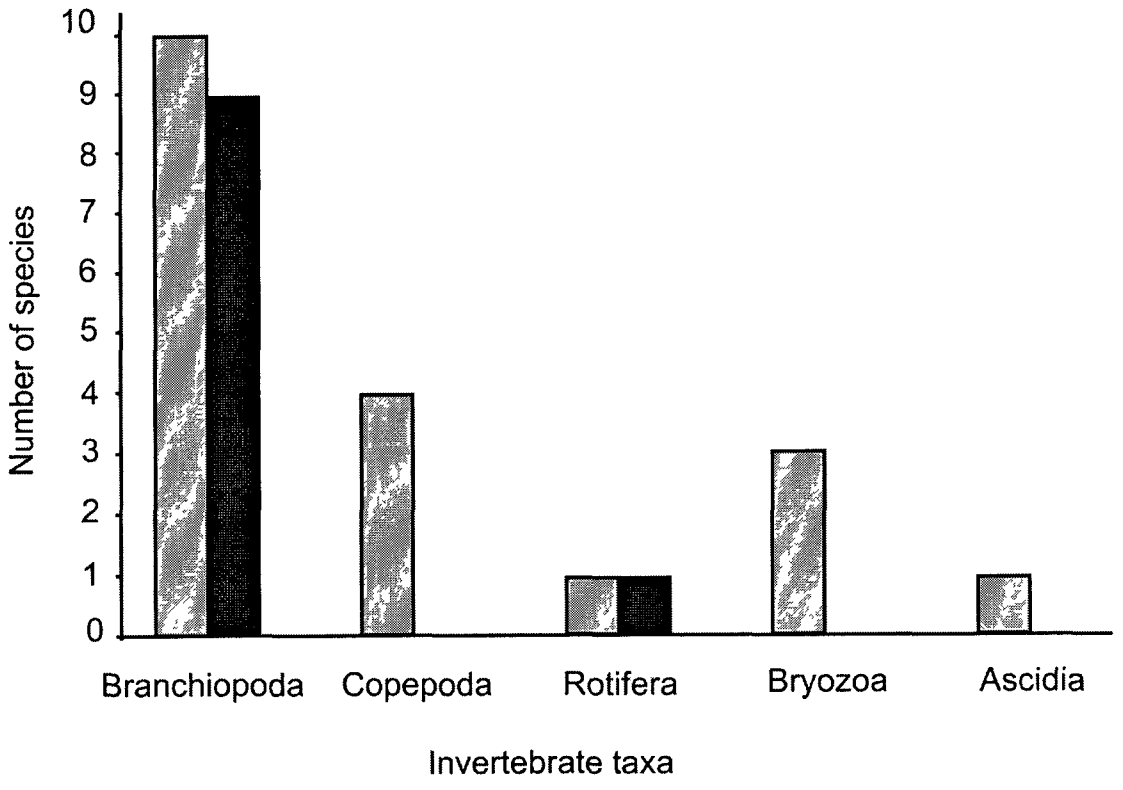

Figure 2.3 Number of species identified using DNA barcodes from diapausing eggs (gray bars), and morphological identification of hatched animals (black bar).

Chapter 3: Degradation of invertebrate dormant eggs collected in ballast sediment of ships

3.1 Introduction

Humans have traded and transported species for centuries. In recent decades, however, increases in transport networks and demand for commodities have unintentionally accelerated the introduction of nonindigenous species (NIS) beyond their native ranges in both terrestrial and aquatic environments worldwide (Ruiz & Carlton, 2003; Wonham & Carlton, 2005; Ricciardi, 2006; Hulme, 2009). As commercial vessels transport over 90% of the world's trade (Hulme, 2009; IMO, 2010), it is not surprising that they are a leading mechanism for the spread of NIS globally (Holeck *et al.*, 2004; Ricciardi, 2006; Molnar *et al.*, 2008).

Ballast water, typically utilized to control the trim and stability of a vessel when not fully loaded with cargo, can contain significant amounts of suspended sediment that later settles to the tank bottom (Carlton, 1985; Gollasch & Leppäkoski, 1999). Ballast sediments can contain large numbers of active invertebrates as well as their viable dormant stages (e.g., dormant eggs, diapausing eggs, resting eggs, cysts, statoblasts) that could pose an invasion risk if they are discharged along with ballast water, or if they hatch during a voyage and are subsequently discharged (Bailey *et al.*, 2005a, 2007; Duggan *et al.*, 2005; Drake & Lodge, 2007; Briski *et al.*, 2010; Kipp *et al.*, 2010). For simplicity, the term 'dormant egg' is used in a broad sense in this paper and includes eggs, statoblasts, and other types of diapausing or resting stages. A unique feature of

dormant eggs is that they are very resistant to harsh environments (Cáceres, 1997; Schröder, 2005) and can remain viable in nature from decades to several centuries (Hairston *et al.*, 1995, 1999; Hairston, 1996). The accumulation of dormant eggs is a natural process in lakes, with most viable eggs found in the upper layers of sediment (Brendonck & De Meester, 2003). The dense nature of ballast sediment, combined with its tendency to accumulate in tanks, suggests that dormant eggs may not be easily expelled from tanks during ballasting operations (Bailey *et al.,* 2005b), leading to a continuous increase in the abundance of dormant eggs inside tanks.

While a large number of studies have addressed viability and senescence of dormant eggs of invertebrates in natural habitat (Hairston *et al.*, 1995, 1999; Hairston, 1996; Cáceres, 1997; Brendonck & De Meester, 2003; Schröder, 2005; De Stasio, 2007) and several studies have explored the abundance, species richness and viability of dormant eggs inside ballast tanks on single occasions (Bailey *et al.,* 2005a; Duggan *et al.,* 2005; Drake & Lodge, 2007; Kipp *et al.,* 2010), the accumulation and viability of dormant eggs over time in ballast tanks have not been studied previously. To evaluate this phenomenon, one would ideally sample the same ballast tank repeatedly over time; however, sampling costs and logistical constraints associated with sampling commercial vessels (i.e. tight shipping schedules and unpredictable changes in shipping operations) make this task almost impossible. Therefore, I explore long-term survivorship of dormant eggs collected from ballast sediment in the lab, as a conservative means to estimate survival and viability. Using sediment samples containing high

densities of dormant eggs, I explored the abundance and viability of dormant eggs over time. I tested the hypotheses that the abundance and viability of dormant eggs will not change over the twelve month time period.

3.2 Materials and methods

Nineteen ballast samples, collected as part of a larger study, were selected for examination because they contained high densities of dormant eggs. All selected sediments contained at least 40 dormant eggs of at least one species per 40 g of sediment. In addition, one sample containing a lower density of dormant eggs was included as it contained species of rotifers and copepods not found in the other samples. Approximately 6 kg of sediment was collected from each ship and thoroughly homogenized before being stored in dark conditions at 4°C (see Briski *et al.,* 2010). While I acknowledge that conditions inside ballast tanks are less stable than in the lab, often with pronounced variability in temperature, salinity, and concentration of different gasses over time (Reid *et al.*, 2007; Sutherland *et al.,* 2009), my methodology will provide a conservative assessment of the degradation rate within ballast tanks since variable conditions are expected to increase rates of degradation (Keller & Pitblado, 1984; Onbé, 1985; Hairston *et al.,* 1999; Brendonck & De Meester, 2003; Gray & MacIsaac, 2010; Briski *et al.,* 2010).

To examine the rate of visible degradation, dormant eggs were enumerated every two months, for twelve months. Enumeration was first conducted one week after samples were collected. At each time point, four 40 g

subsamples were removed from each sediment sample and washed through a 45 µm sieve to remove fine sediment. Dormant eggs were separated from the remaining sediment using the colloidal silica Ludox® HS 40 (Burgess, 2001) and enumerated under a dissecting microscope. For each sediment sample, all extracted dormant eggs were counted and grouped based on size and morphology, and a maximum of 20 dormant eggs per morphological group were taken for molecular identification following the methods of Briski *et al.* (2010, 2011).

Further, to assess viability of dormant eggs over time, I conducted hatching experiments one, six, and twelve months after collection of sediment. At each time point, dormant eggs were isolated from 40 g sediment subsamples using a sugar flotation method (Hairston, 1996; Bailey *et al.*, 2003; Briski *et al.*, 2010). Extracted dormant eggs were placed in vials containing sterile synthetic pond water (0 parts per thousand (‰) salinity; Hebert & Crease (1980)) or a sterile seawater medium with salinity of 15 or 30‰ (see Briski *et al.* (2010)). The seawater medium was prepared using ballast water of open ocean origin, filtered through 2.5 µm Whatman paper filter and diluted to 15 or 30‰ with the sterile, synthetic pond water. Different salinities were used in an attempt to provide unknown species with optimum fresh-, brackish- or saltwater habitat and to maximize hatching success. Four replicates were placed in each of the 0, 15 and 30‰ treatments at 20°C. Owing to the large sample size, I was able to conduct hatching experiments at only one temperature; therefore I selected 20°C as the optimal temperature based on the results of Bailey *et al.* (2005). To monitor for

the introduction of organisms from the environment, controls containing only hatching media were kept in each treatment group. Hatching percentage (H%) was calculated by dividing the total number of individuals hatched by the total number of dormant eggs isolated for hatching, multiplied by 100.

Statistical Analysis

After enumerating and identifying the dormant eggs throughout the year, I tested for differences in the onset and rate of visible degradation between dormant eggs within species on different ships, between species which belonged to the same taxonomic group (i.e. copepods, rotifers and anomopods), and between different taxonomic groups. To test for differences within species I constructed a degradation curve for each species for each sediment sample, using data from all four replicates, described by the equation:

$$y=100/1+e^{-Z(t-Q)} \quad (1)$$

where t is time, Z is the rate of degradation and Q is the onset of degradation. The model was expanded to compare the rate and the onset of degradation between two curves using the equation:

$$y=100/1+e^{-(Z_1+Z_2)(t-Q_1-Q_2)} \quad (2)$$

where Z_1 and Z_2 are the rates of degradation for the first and second curves, and Q_1 and Q_2 are the points of onset of degradation for the first and second curves, respectively. All possible combinations of curve pairs were compared statistically by the Fit Nonlinear Model using Generalized Least Squares (S-Plus® 6.1, 2002, Insightful Corp., Seattle, Washington, USA). Further, to test for differences between species which belonged to the same taxonomic group, degradation curves were constructed as explained above, using data for each species combined across ships. Again, statistical comparisons were conducted using all possible combinations of species pairs within each taxonomic group using the nonlinear model. Finally, to test for differences between different taxonomic groups, data for each taxonomic group was combined across all ships for comparison as above. Significant levels for statistical comparisons of estimated parameters Z_1 and Z_2, and Q_1 and Q_2, were adjusted for multiple pairwise comparisons by Bonferroni-type correction to guard against inflating the Type I error rate. The family-wise error rate of 0.05 was used. All tests were performed using the computer program S-Plus 6.1 (S-Plus® 6.1, 2002, Insightful Corp., Seattle, Washington, USA). Dormant eggs of two taxonomic groups (i.e. bryozoans and onychopods) did not visibly degrade at all, and were excluded from statistical analyses.

Given that viability hatching assessments were conducted by repeated sediment subsampling over time, one-way analysis of variance with repeated measures (ANOVA) and post-hoc Bonferroni tests were used to test for differences in absolute numbers of eggs hatched among three hatching

experiments (i.e. three time points). The tests were performed separately for rotifers, copepods and anomopods (SPSS 11.5.0, SPSS Inc., 1989 - 2002; Chicago, Illinois, USA). A logarithmic transformation was applied to all datasets to meet assumptions of parametric tests. Greenhouse-Geisser corrections were used when sphericity was violated. A significance level of 95% was used for within subject effect statistical analyses, while Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise comparisons.

3.3 Results

The visible degradation and viability of dormant eggs of 18 taxa were examined over a 12 month period. Examined taxa included three rotifers, eight copepods, three anomopods, two onychopods and two bryozoans (Table 3.1). Ninety-eight percent of dormant eggs of rotifers, with a mean density of 11 eggs per 40 g of sediment at T_0, completely degraded in six months (Table 3.1; Figs. 3.1 and 3.2), with all eggs degraded within ten months. Dormant eggs of copepods, with a mean density of 149 eggs per 40 g of sediment at T_0, degraded to a mean density of 36 eggs per 40 g of sediment at T_6, and finally to a mean density of 5 eggs per 40 g at T_{12}. On average 75% of dormant eggs of copepods degraded in the first six months, while an additional 22% degraded thereafter (Table 3.1; Figs. 3.1 and 3.2). Degradation rate of anomopod dormant eggs was significantly slower than that of rotifer and copepod eggs ($P < 0.05$, Tables 3.1 and 3.2, Fig. 3.2), with only 20% of eggs degrading within the first six months and

35% degrading through the entire year. Dormant eggs of onychopods and bryozoans, with respective mean densities of 12 and 6 eggs per 40 g of sediment at T_0, did not visibly degrade at all during the experiment.

Eight species (*Brachionus plicatilis*, *Brachionus* sp., *Eurytemora affinis*, *Calanus euxinus*, an unidentified calanoid copepod, *Daphnia magna, Evadne nordmanni* and *Plumatella emarginata*) occurred in multiple sediment samples (Table 3.1). Thirty-one paired statistical comparisons of degradation curves within species across ships exhibited significant differences in neither the onset nor the rate of degradation ($P > 0.05$). Further, three, 28 and three statistical comparisons were applied to pairs of species within rotifer, copepod and anomopod taxonomic groups, respectively. Degradation curves for species within rotifer and anomopod groupings did not differ significantly from each other, though variability was observed among some species within the copepod group ($P < 0.05$, Table 3.2, Fig. 3.1). Eggs of *E. affinis* began to degrade significantly later than for all other copepod species, and *E. affinis* was the only species having eggs degrade significantly slower than eggs of *Acartia pacifica*. Other copepod species pairs exhibited significant differences only for the onset of the degradation ($P < 0.05$, Table 3.2, Fig. 3.1) or not at all. Further, it is interesting to point out that dormant eggs of freshwater copepods (i.e. *Diacyclops thomasi, Leptodiaptomus siciloides* and *E. affinis*) started to degrade later than marine species (i.e. *A. pacifica,* an unidentified harpacticoid copepod hatched only at 30‰ and *C. euxinus*)(Fig. 3.1). When all copepod data were entered in the model, the constructed curve was significantly different from that of rotifers (Table

3.2, Fig. 3.2). However, when curves of copepod species were individually compared to those of rotifers, curves for *A. pacifica*, the unidentified harpacticoid copepod, *C. euxinus* and *D. thomasi* were not significantly different from those of the rotifers. Eggs of anomopods degraded very slowly, with the onset and degradation rate significantly different from both rotifers and copepods: after twelve months, < 50% of anomopod eggs degraded (Table 3.2, Fig. 3.2).

B. plicatilis was the only rotifer hatched in viability experiments, with a mean hatch rate of 64% (Table 3.3). Six months later, the same species hatched from only one sample. While the total number of hatched individuals was lower than in T_1, eggs that remained were nearly all viable (95%). The number of rotifers hatched at T_1 was significantly higher than at T_6 and T_{12} (ANOVA; $P < 0.05$; Table 3.4). Five taxa of copepods hatched from eight samples at T_1, with hatch rate varying from 6 to 54% (Table 3.3). Six months later, only two taxa hatched from five samples, with a 100% success rate for both taxa. While the absolute number of individuals of the two taxa (unidentified calanoid and harpacticoid copepods) hatched at T_6 were higher than T_1, the difference was not statistically significant (ANOVA; $P > 0.05$; Tables 3.3 and 3.4). After twelve months, copepods were the only taxonomic group that hatched, though only from two samples. The absolute number of copepods hatched in T_{12} was significantly lower than in T_1 and T_6 (ANOVA; $P < 0.05$; Tables 3.3 and 3.4). In the anomopod taxonomic group, *Moina* sp. hatched from only one sample at T_1 (13%, Table 3.3). No other anomopod species hatched at T_1. *D. magna* hatched after six months, even though it did not hatch at T_1 (Table 3.3). There was no significant

difference in the number of individuals hatched between time points for the anomopods ($P > 0.05$; Table 3.4). Dormant eggs of onychopods and bryozoans did not hatch in any of the three hatching trials.

3.4 Discussion

Comparison to natural habitats

A large number of studies have addressed questions regarding production, timing, and viability of dormant eggs of invertebrates (Hairston *et al.*, 1995, 1999; Hairston, 1996; Cáceres, 1997; Brendonck & De Meester, 2003; Schröder, 2005) but few have addressed dormant egg senescence in natural habitats (Hairston *et al.*, 1995; De Stasio, 2007) and no studies have explored survivorship, viability, and senescence of dormant eggs transported by vessels. This study highlighted key differences in survival of eggs retained in ballast sediment - differences that could profoundly influence spread potential of different taxonomic groups *via* ships' ballast sediment.

While dormant eggs of rotifers and copepods have been reported as viable for decades or even centuries (Marcus *et al.,* 1994; Hairston *et al.,* 1995; Hairston, 1996; Hairston & Kearns, 1996; Cáceres, 1997), most research indicates loss of viability within 12 – 24 months (Chittapun *et al.,* 2005; De Stasio, 2007). Viability of dormant rotifer eggs is reduced by exposure to severe drought (Schröder, 2005), bacterial infections (De Stasio, 2007) or high amounts of organic matter (Hagiwara *et al.,* 1997). Similarly, environmental pollutants including heavy metals, acidic water, low oxygen, elevated hydrogen sulphide,

high temperature, and parasitism may reduce viability of dormant eggs of copepods (Keller & Pitblado, 1984; Kerfoot *et al.,* 1999; Brendonck & De Meester, 2003). Such adverse conditions are often found in ballast tanks (Wagner *et al.,* 1996; Reid *et al.*, 2007; Ago *et al.,* 2008; Sutherland *et al.,* 2009; E. Briski, personal observation), thus it is not surprising that dormant rotifer and copepod eggs degraded rapidly in this study. It is surprising, however, that the dormant rotifer and copepod eggs examined in this study degraded in half the time reported in nature.

The viability of dormant eggs of cladocerans could be reduced by exposure to the same conditions as mentioned above for rotifers and copepods (Keller & Pitblado, 1984; Onbé, 1985; Hairston *et al.,* 1999). In this study, cladocerans exhibited two patterns of dormant egg degradation: anomopods degraded at a very slow rate, while onychopods did not degrade at all. While anomopod degradation rate was similar to that reported in nature by Hairston *et al.* (1999), the lack of degradation of dormant onychopod eggs was surprising as Onbé (1985) reported degradation of onychopod dormant eggs with exposure to environmental pollutants such as those listed above. The last taxonomic group explored in my study were bryozoans, whose dormant stages (i.e. statoblasts) are reported to be resistant to extreme temperature, drought, heavy metals, toxins, freezing, and desiccation (Bushnell & Rao, 1974; Kipp *et al.,* 2010) and can remain viable from 2 to 4 years (Cáceres, 1997; Wood, 2005). My findings for bryozoans were consistent with those from natural habitats.

Dormant eggs in ships' ballast tanks

In this study I tracked the survivorship and viability of dormant eggs collected from ballast tanks. I acknowledge two major limitations in this study. First, I do not know the history of eggs in different ballast sediments, including their age, source or conditions to which they were exposed. Secondly, environmental conditions inside ballast tanks are less stable than storage in the laboratory; yet, my study still provides insight into the survivorship and viability of dormant eggs.

Mid-ocean exchange (MOE) and saltwater flushing are procedures mandated by the Canadian and American governments to prevent introduction to the Great Lakes and the Pacific and Atlantic coasts of North America of new NIS by ships' ballast water and sediment. These procedures influence the abundance and viability of dormant eggs of invertebrates in ballast tanks (Gray & MacIsaac, 2010; Briski *et al.,* 2010). As MOE involves the replacement of water in filled ballast tanks with that from open ocean, and saltwater flushing involves rinsing ballast residuals with smaller volumes of ocean water, these activities increase the variability of environmental conditions inside ballast tanks. Reid *et al.* (2007) measured changes in oxygen concentration from 10 to 1 mg/L and then back to 10 mg/L, and salinities from 0 to 35‰ and then back to 0‰ every several days. Saltwater flushing also reduces the volume of accumulated sediments in ballast tanks, leaving dormant eggs less protected (Briski *et al.,* 2010). Dehydration of dormant eggs could occur more frequently if they are not deeply buried in the sediment (Sutherland *et al.,* 2009). On the other hand, if a tank contains higher

amounts of sediment, only dormant eggs in the top layers might be expected to hatch. Some eggs may be buried and not exposed to hatching stimuli, resulting in a lower hatch rate and lower invasion risk than my lab results suggest. The variable and often adverse environment of ballast tanks would lead to a higher and more rapid mortality rate for dormant eggs as compared to natural habitats.

Degradation rates

While I found degradation rates among different species of rotifers and anomopods fairly consistent, I caution that sample size may be a confounding factor since differences in degradation rate were observed for copepods, the group with largest sample size. Inconsistencies in the onset of degradation in dormant eggs of different copepod species could not be accounted for by variation among ships, as I did not find differences in the onset or rate of degradation within species sampled from different ships in thirteen species pairs comparisons. One reason for the high mortality rate and inconsistent onset of degradation of eggs of different copepod species in my samples could be that some species produce mostly subitaneous rather than dormant eggs (Marcus, 1996). It is very difficult to distinguish between dormant eggs and subitaneous eggs of copepods without using an electron microscope (Dharani & Altaff, 2004). The observed differences may be a result of evolutionary history since the onset of degradation for eggs of freshwater species was later than that of marine taxa, possibly resulting in higher invasion potential for freshwater copepods and

making freshwater ecosystems more vulnerable to copepod invasions than marine habitats.

Dormant egg viability

Although onychopod and bryozoan dormant eggs did not hatch in my viability experiments, I cannot conclude that the eggs are not viable due to the small number of eggs set in my hatching trials. The physiology of dormant eggs is very complex, and hatching success depends on the degree of diapause termination, energy content of the eggs and hatching conditions (Schwartz & Hebert, 1987; Lavens & Sorgeloos, 1996; Bailey *et al.,* 2003; Sopanen, 2008), with low hatching success a common result (Hairston *et al.,* 1999; Bailey *et al.,* 2003; Simm & Ojaveer, 2006). Successful hatching of onychopod species was reported by Bailey *et al.* (2005a), Simm & Ojaveer (2006) and Sopanen (2008) after incubation at 10°C, while Sopanen (2008) stated that 18°C exceeds limits for development and hatching of *Cercopagis pengoi*. Considering that my hatching trials were set at 20°C, that there was no visible degradation of eggs, that DNA extraction was equally successful at the beginning and end of the experimental year, and that low numbers of eggs were set for hatching in my hatching trials, I argue that the onychopod eggs were viable and pose an invasion risk. Similarly, demonstrated resistance by bryozoan eggs to all kinds of extreme environmental conditions (Bushnell & Rao, 1974; Wood, 2005; Kipp *et al.,* 2010) and extremely low hatching success in the laboratory (S.A. Bailey, unpublished

data) leads me to conclude that the bryozoan eggs were likely viable, and also pose an invasion risk.

Ballast sediment as a vector of invertebrate invasions

The rapid degradation rate observed across species of rotifers and copepods, even though samples were collected from different tanks and at different times, suggests that eggs of these taxa entered the tanks not long before samples were collected. The low number of ships carrying a high abundance of dormant eggs (Bailey *et al.,* 2005a; Duggan *et al.,* 2005; Briski *et al.*, 2010) supports the conclusion that dormant eggs of rotifers and copepods do not continually accumulate inside ballast tanks. Rare observations of high densities of rotifer and copepod dormant eggs in ballast sediments could be associated with the uptake of ballast water during seasonal peaks of dormant egg production in particular locations. This finding suggests that rotifers and copepods are less likely to be transported as dormant eggs in ships' ballast sediment than as active planktonic adults in ballast water (see also Bailey *et al.,* 2005b; Duggan *et al.,* 2005). In contrast, dormant eggs of anomopods, onychopods and bryozoans may accumulate in ballast sediments because of their slow, or nil, degradation rates.

While the number of species documented as successful invaders is similar among all five taxonomic groups examined here, the geographical extent and impact of past invasions by onychopods has been much more pronounced than for the other taxonomic groups (Wonham & Carlton, 2005; Ricciardi, 2006;

Molnar *et al.*, 2008; Kipp *et al.,* 2010; A. Locke, unpublished data). For example, *Cercopagis pengoi* has invaded Eastern European waterways and reservoirs, the Baltic Sea, the Laurentian Great Lakes, and the Finger Lakes in North America, causing very prominent ecological and economic impacts (Leppäkoski & Olenin, 2000; Therriault *et al.,* 2002; Vanderploeg *et al.,* 2002). Notorious species like *C. pengoi* and *Bythotrephes longimanus*, while not encountered during this study, are previously reported from ballast tanks (Briski *et al.*, 2010; E. Briski, unpublished data) and results here indicate that dormant eggs in ballast sediments are a viable vector for onychopod invasions.

Conclusions

Ballast-borne dormant eggs of rotifers and copepods degrade at twice the rate observed in nature. Even though their dormant eggs may not accumulate in ballast tanks, dormant eggs of copepods remain viable and represent an invasion risk for at least six months; dormant eggs of freshwater copepods appear to represent a higher invasion risk than do marine species. In contrast, dormant eggs of anomopods, onychopods and bryozoans do not appear to degrade in ballast sediments, for at least one year, resulting in higher invasion potential. Further work to quantify risk of onychopod invasions by this vector is warranted.

3.5 References

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Table 3.1 Mean and standard error (S.E.) of dormant egg density, by species, counted initially (T_0), after six months (T_6) and one year later (T_{12}). Note the increase in abundance for a few cases at T_6 and T_{12} is the result of random sampling.

Higher taxa level	Species level	# of samples T_0	Mean density of eggs T_0	S.E T_0	# of samples T_6	Mean density of eggs T_6	S.E. T_6	# of samples T_{12}	Mean density of eggs T_{12}	S.E. T_{12}
Rotifer	*Brachionus plicatilis*	6	12.5	1.7	4	2.1	0.4	0	0	0
Rotifer	*Brachionus calyciflorus*	1	11.2	0.3	1	1.7	1	0	0	0
Rotifer	*Brachionus* sp.	2	9.7	2.6	0	0	0	0	0	0
Copepod	*Eurytemora affinis*	5	347.4	66.3	5	113.9	21.4	5	10	3.5
Copepod	*Calanus euxinus*	3	169.9	14.5	3	29.1	2.4	3	7.7	1.1
Copepod	*Leptodiaptomus siciloides*	1	5	0.7	1	1	0	0	0	0
Copepod	*Acartia pacifica*	1	116.7	8.2	1	13.7	1.2	0	0	0
Copepod	Calanoid copepod	5	402.4	64.3	5	108.3	22.2	5	18.2	5.3

Copepod	*Diacyclops thomasi*	1	32.2	0.7	1	5.2	0.2	0	0	0
Copepod	Harpacticoid copepod	1	69.5	7.7	1	11.5	1.5	1	1.7	1.1
Copepod	Unidentified copepod	1	46.2	8.3	1	6.7	0.2	1	0.2	0.2
Anomopod	*Daphnia magna*	3	15.1	1.4	3	12.9	1.2	3	11.6	1.1
Anomopod	*Ceriodaphnia dubia*	1	46.7	4.9	1	34.5	3.9	1	29.2	1.2
Anomopod	*Moina* sp.	1	70.2	4.3	1	58	2.9	1	44.5	3.5
Onychopod	*Evadne nordmanni*	6	14.3	2.2	6	14.7	2.2	6	14.9	2.0
Onychopod	*Pleopis polyphemoides*	1	9.7	0.2	1	10	0.4	1	10.5	0.6
Bryozoan	*Plumatella emarginata*	12	4.2	0.5	12	4.3	0.5	12	4.3	0.5
Bryozoan	Unidentified bryozoan	1	7.7	0.4	1	7	0.4	1	7.5	0.6

Table 3.2 Significance levels for statistical comparisons of parameters between pairs of fitted curves, which showed significant difference in the onset or rate of degradation, or both. The *t*-test incorporated in the Fit Nonlinear Model using Generalized Least Squares was used to test for significant differences between estimated parameters Z_1 and Z_2, and Q_1 and Q_2. Significant *P*-values are presented in bold. Bonferroni-type protection to guard against inflating the Type I error rate and family-wise error rate of 0.05 were used for pairwise statistical comparisons. Results which were not significantly different are not shown.

Level of comparison	Pairs compared	The onset of degradation (*P*-value)	The rate of degradation (*P*-value)
Within copepods			
	Eurytemora affinis – *Calanus euxinus*	**< 0.001**	0.1063
	Eurytemora affinis – harpacticoid copepod	**< 0.001**	0.2320
	Eurytemora affinis – *Diacyclops thomasi*	**< 0.001**	0.1771
	Eurytemora affinis – *Acartia pacifica*	**< 0.001**	**< 0.001**
	Eurytemora affinis – *Leptodiaptomus siciloides*	**< 0.001**	0.585

	Harpacticoid copepod – *Leptodiaptomus siciloides*	**< 0.001**	0.1499
	Acartia pacifica – *Leptodiaptomus siciloides*	**< 0.001**	0.1323
Different taxonomic groups			
	Rotifers - copepods	**< 0.001**	**< 0.001**
	Rotifers - anomopods	**< 0.001**	**< 0.001**
	Copepods - anomopods	**< 0.001**	**< 0.001**

Table 3.3 Mean (± standard error) number of dormant eggs hatched, by species, at one month (T_1), six months (T_6) and twelve months (T_{12}) after collection of the sediment. The number of samples each particular species hatched from at T_1, T_6 and T_{12}, and hatching percentages (H%), are included. * denotes cases where more dormant eggs hatched after six months than after one month.

Higher taxa level	Species level	# of samples T_1	Mean # of viable eggs T_1 (S.E.)	H% T_1	# of samples T_6	Mean # of viable eggs T_6 (S.E.)	H% T_6	# of samples T_{12}	Mean # of viable eggs T_{12} (S.E.)	H% T_{12}
Rotifer	*Brachionus plicatilis*	3	8 (5.7)	64	1	2 (1.2)	95	0	0	0
Copepod	*Eurytemora affinis*	1	22.4 (19.2)	6.4	0	0	0	0	0	0
Copepod	*Calanus euxinus*	2	92.6 (47.4)	54.5	0	0	0	0	0	0
Copepod	Calanoid copepod	3	81.2 (50.3)	20.1	4	109 *(81.9)	100	1	9.8 (7.2)	53.8
Copepod	Harpacticoid copepod	1	31(19.1)	44.6	1	11.5* (29.1)	100	1	2 (4.7)	100
Copepod	Unidentified copepod	1	10.6 (3.2)	22.9	0	0	0	0	0	0

Anomopod	*Daphnia magna*	0	0	0	1	0.1* (0.1)	0.7	0	0	0
Anomopod	*Moina* sp.	1	9 (9)	12.8	0	0	0	0	0	0

Table 3.4 Statistical comparisons of hatching data among three time points tested by repeated measures ANOVA and Bonferroni tests. Significant *P*-values are presented in bold.

Taxa	Within subject effects (*P*)	Pairwise comparisons (*P*)		
		$T_1 - T_6$	$T_1 - T_{12}$	$T_6 - T_{12}$
Rotifers	**0.036**	**0.009**	**0.011**	0.992
Copepods	**0.002**	0.247	**0.002**	**0.014**
Anomopods	0.392	1	0.992	0.992

Within subject *P*-values for anomopods are with Greenhouse-Geisser corrections.

Figure 3.1 Degradation rates for dormant eggs of rotifer (A), copepod (B) and anomopod (C) species. The curves were constructed using data from all four replicates from all ships where a particular species was found.

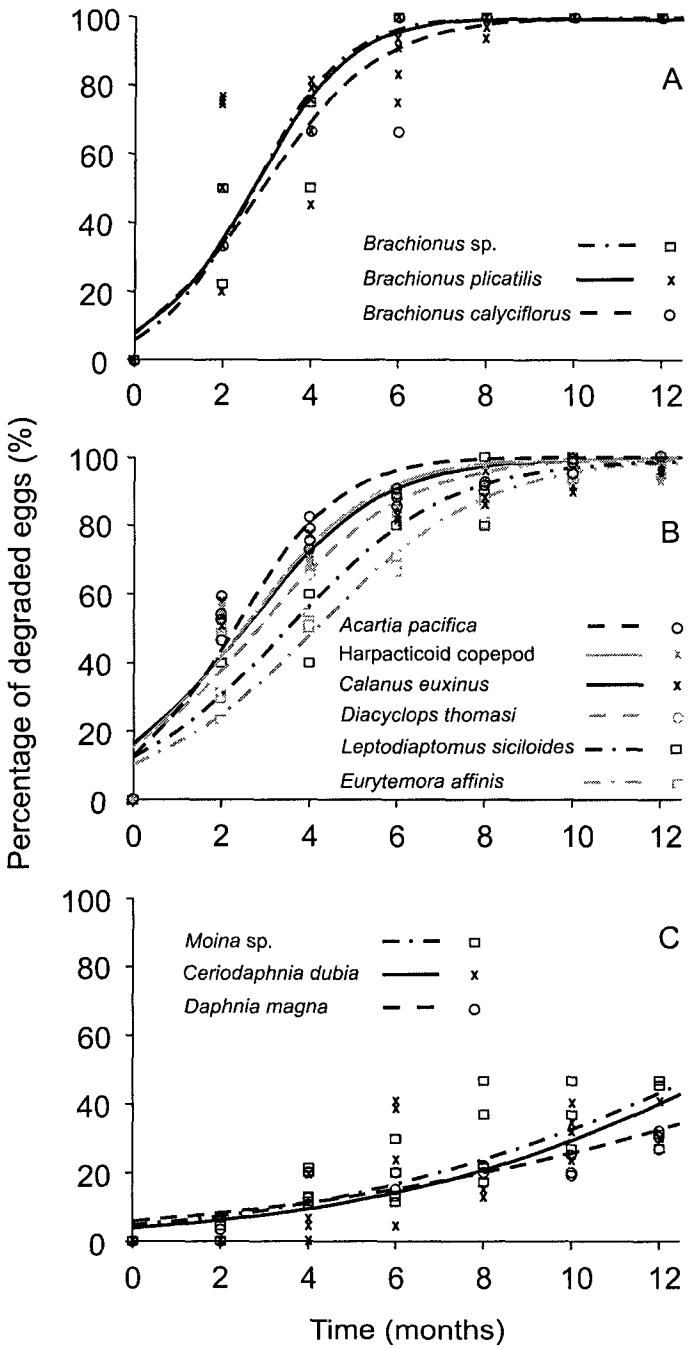
Percentage of degraded eggs (%)
Time (months)
A
Brachionus sp.
Brachionus plicatilis
Brachionus calyciflorus
B
Acartia pacifica
Harpacticoid copepod
Calanus euxinus
Diacyclops thomasi
Leptodiaptomus siciloides
Eurytemora affinis
C
Moina sp.
Ceriodaphnia dubia
Daphnia magna

Figure 3.1

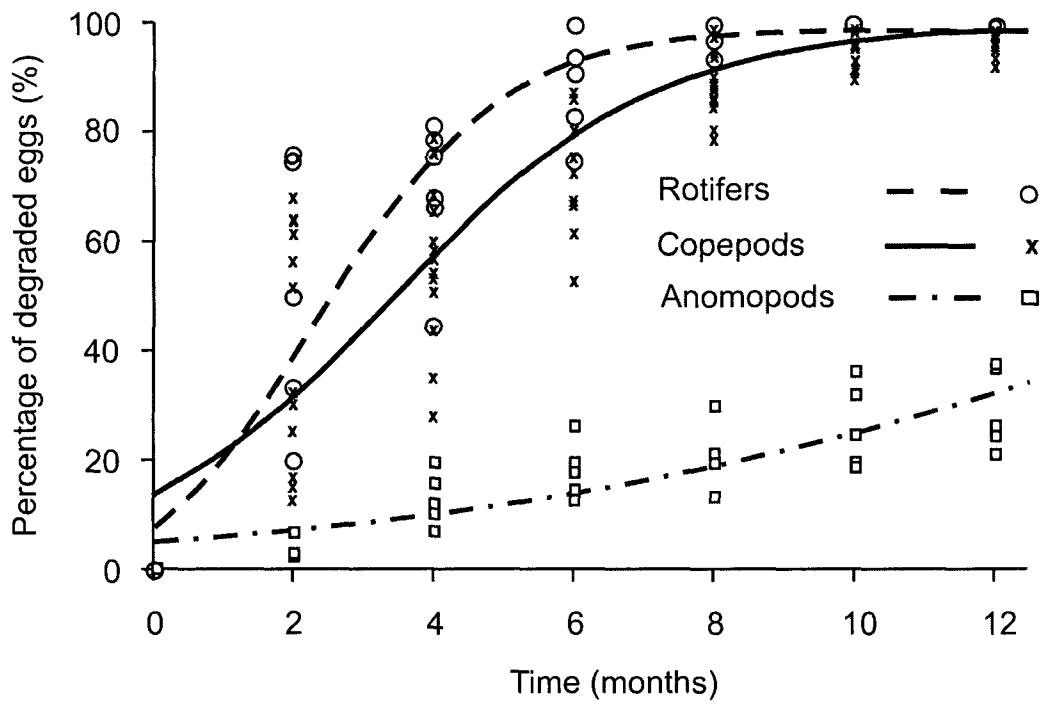

Figure 3.2 Degradation rates for dormant eggs of rotifer, copepod and anomopod taxonomic groups. Curves were constructed using data from all four replicates from all ships where a particular taxonomic group was found. Degradation rates of onychopods and bryozoans, having poor fit to the model, are not shown.

Chapter 4: Efficacy of 'saltwater flushing' in protecting the Great Lakes from biological invasions by invertebrate eggs in ships' ballast sediment**

4.1 Introduction

The introduction of nonindigenous species (NIS) into habitats outside their native range is increasing in frequency worldwide (Mack *et al.,* 2000; Wonham & Carlton, 2005; Ricciardi, 2006). The shipping industry has played a major role in the spread of NIS globally. Ships' ballast water and associated sediments are a leading mechanism for NIS introductions into marine ecosystems (Carlton, 1985; Ruiz & Carlton, 2003; Molnar *et al.*, 2008), and are particularly important for the Laurentian Great Lakes (Holeck *et al.,* 2004; Ricciardi, 2006). Sediment has been implicated as a vector for natural and human-assisted zooplankton dispersal (Koste & Shiel, 1989; Hairston *et al.,* 1999; Bailey *et al.,* 2003, 2005). Ballast sediments may contain very large numbers of active invertebrates as well as their viable dormant stages (Bailey *et al.,* 2005; Duggan *et al.,* 2005).

To decrease ballast-mediated invasions to the Great Lakes, two different ballast water regulations have been enacted. First, mid-ocean exchange was recommended in 1989 and made mandatory in 1993 to reduce the number of propagules in ballast water (Canadian Coast Guard, 1989; United States Coast Guard, 1993). This regulation was augmented to incorporate management of residual ballast water and accumulated sediments through mandatory saltwater

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flushing, beginning in 2006 (Government of Canada, 2006; SLSDC, 2008). Mid-ocean exchange involves replacement of water in filled ballast tanks with ocean water, while saltwater flushing involves rinsing ballast tanks containing only residual ballast water and sediments through the uptake and subsequent discharge of several tonnes of ocean water. To assure compliance with the regulations, nearly every ship entering the Great Lakes is inspected by Canadian and/or American agencies (GLBWWG, 2009). In theory, mid-ocean exchange and saltwater flushing should reduce abundance (i.e. propagule pressure) and species richness (i.e. colonization pressure) by purging coastal water, sediments and taxa from tanks and, for low salinity taxa, by killing remaining individuals with osmotic stress (MacIsaac *et al.,* 2002; Lockwood *et al.,* 2009).

Multiple studies have assessed the efficacy of mid-ocean exchange and/or saltwater flushing on active stages of biota (Locke *et al.,* 1993; Rigby & Hallegraeff, 1994; Wonham *et al.,* 2001; Choi *et al.,* 2005; Gray *et al.,* 2007; Humphrey, 2008), while only one has examined efficacy of the procedures on dormant eggs of invertebrate species resident in ballast sediment (Gray & MacIsaac, 2010). Gray & MacIsaac (2010) reported only partial effectiveness of mid-ocean exchange at rendering dormant stages non-viable after performing *in-situ* tests with ballast sediment. Further, laboratory tests have demonstrated that diapausing eggs are resistant to short-term saltwater exposure (Bailey *et al.,* 2004, 2006; Gray *et al.,* 2005). The above studies, however, had small sample sizes and did not evaluate potential cumulative effects of ongoing ballast

management, warranting a more substantial analysis of the effect of the current ballast water regulations on dormant stages in sediments.

Here I test the effect of the 2006 regulations, which mandated saltwater flushing, on the density and diversity of invertebrate dormant stages in ballast sediment of transoceanic and coastal vessels arriving to the Great Lakes. I conducted a random survey of ship sediments in 2007 and 2008, and compared my results with those of a survey conducted between 2000 and 2002 (Bailey *et al.,* 2005). As both studies collected samples after mid-ocean exchange became mandatory, the results reflect only the influence of saltwater flushing regulations; however, for simplicity, in this paper I refer to pre-regulation (2000 - 2002) and post-regulation time periods (2007 – 2008). I tested the hypotheses that post-regulation ships carry less residual sediment, contain a lower abundance of dormant eggs and have lower egg viability than did pre-regulation ships.

4.2 Materials and methods

Sediment collection, dormant stage counts and hatching

Ballast sediment was collected opportunistically from 19 ballast tanks on 17 ships, which originated from European, South American and Atlantic ports in the U.S.A., arriving to the Great Lakes during 2007 and 2008. Approximately 6 kg of sediment was collected from each ballast tank for laboratory analysis of the density, diversity and viability of invertebrate dormant eggs. Methodology was consistent with that of Bailey *et al.* (2003, 2005), allowing for comparison of results pre- and post-regulation. I note that Bailey *et al.* (2005) sampled ships that

carried only residual ballast water at the time of entry to the Great Lakes, while this study sampled ships with full ballast tanks that were discharged after entry to the Great Lakes (Table 4.1). Following Bailey *et al.* (2005), results from multiple tanks sampled from a single ship at a single sampling event were averaged, while independent trips into the Great Lakes by a single vessel were considered independent samples since new ballast had been held in tanks between sampling intervals. Personal observations of sediment depth and percent cover inside ballast tanks, combined with architectural diagrams of ships' tanks, were used to estimate the amount of residual sediment carried by each ship. I obtained data about each ship's ballast history, including total ballast capacity and previous dates and locations of ballast uptake and discharge, from ships' crews and mandatory reporting forms submitted to Transport Canada.

Upon return to the laboratory, sediment was homogenized by thorough mixing. Four 40 g subsamples were taken from each tank sediment sample for egg density counts. Subsamples were preserved in 95% ethanol, followed by washing through a 45 µm sieve to remove fine sediment. Eggs were separated from the remaining sediment using the colloidal silica Ludox® HS 40 (Burgess, 2001). Dormant stages were enumerated under a dissecting microscope, and the average density of eggs from the four subsamples was extrapolated to the number of dormant propagules per ship.

All remaining sediment was stored in the dark at 4°C for at least four weeks to break the diapause cycle of dormant stages before hatching experiments commenced (Schwartz & Hebert, 1987; Dahms, 1995). Two types of

hatching experiments were conducted on all 19 tank sediments following the methodology of Bailey *et al.* (2005). Firstly, 'maximum diversity experiments' isolated eggs from sediments prior to hatching to determine the number of viable species. Secondly, to represent more realistic hatching conditions inside ballast tanks, 'whole sediment experiments' were conducted, which did not separate eggs from sediments (Table 4.1). All experiments were conducted using a light:dark cycle of 16:8 hours.

For maximum diversity experiments, diapausing eggs were isolated from 40 g sediment subsamples using a sugar flotation method (Hairston, 1996; Bailey *et al.*, 2003, 2005). Extracted eggs were placed into vials containing sterile synthetic pond water (0 parts per thousand (‰) salinity; Hebert & Crease, 1980) or a sterile seawater medium with salinity of 15 or 30‰. The seawater medium was prepared using mid-ocean ballast water collected from a vessel transiting the Great Lakes, filtered through 2.5 µm Whatman paper filter and diluted to 15 or 30‰ with the sterile, synthetic pond water. Four replicates were placed into each of the 0, 15 and 30‰ treatments at 20°C (Table 4.1). Whole sediment experiments were conducted by placing 40 g sediment subsamples directly into 500 mL glass vessels. Four replicates were placed into each of the 0, 15 or 30‰ treatments, with 150 mL of media added to each vessel before incubation at 20°C.

Three different salinities were used in both types of hatching experiments in an attempt to match unknown species to a fresh-, brackish- or saltwater habitat to promote maximum hatching (Table 4.1). The list of species generated from

hatching experiments was used to estimate egg viability as well as the effect of saltwater flushing on freshwater, brackish and saltwater taxa. Hatching percentage (H%) was calculated by dividing the total number of animals hatched by the total number of eggs isolated for hatching, and multiplying by 100.

I use the number of hatched eggs as a proxy measure of egg viability, although I acknowledge that some eggs that did not hatch may have been viable but did not receive appropriate hatching cues. All NIS hatched in the 0‰ treatment were considered high risk taxa with potential to establish populations under environmental conditions of the Great Lakes, unless an established population of the species already exists. The freshwater species *Daphnia magna* was not considered a high risk NIS, however, as the species almost certainly has been introduced to the Great Lakes multiple times by both shipping (Bailey *et al.,* 2003, 2005; Duggan *et al.,* 2005) and natural (Louette & De Meester, 2005) vectors but has not established a self-sustaining population; biotic or abiotic factors may preclude invasion by this species (Lauridsen & Lodge, 1996).

Identification of dormant eggs was conducted directly using molecular methods, as well as through traditional morphological taxonomy of hatched individuals (Table 4.1). DNA was extracted directly from diapausing eggs using a HotSHOT method (Montero-Pau *et al.,* 2008). Fragments of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) and 16S rDNA gene were amplified from each egg using the universal primers LCO1490 and HCO2190 (Folmer *et al.,* 1994) and S1 and S2 (Palumbi, 1996), respectively. PCR reactions were performed in a total volume of 25 µL using 5 µL of DNA extract, 1x PCR buffer,

12.5 µL of 10% trehalose, 0.1 µM of each primer, 2.5 mM $MgCl_2$, 0.14 mM dNTPs and 0.4 U *Taq* DNA polymerase. The thermal profile consisted of a 1 min initial cycle at 94°C, followed by 5 cycles of 94°C (40 sec), 45°C (40 sec) and 72°C (1 min), 35 cycles of 94°C (40 sec), 50°C (40 sec) and 72°C (1 min) and a final extension of 72°C for 5 min. PCR products were sequenced by an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA).

Although my post-regulation methodology followed that used during the pre-regulation period (Bailey *et al.,* 2005), there are some differences (Table 4.1). I conducted both types of hatching experiments on all 19 tank samples in a fully replicated fashion using three growth media at a single temperature (0, 15 and 30‰, at 20°C). In contrast, Bailey *et al.* (2005) conducted experiments using four growth media at two temperatures (0, 8, 16 and 32‰ at 10°C and 20°C), but were unable to fully replicate all experiments due to large sample size (69 tanks). Furthermore, Bailey *et al.* (2005) used only traditional morphological taxonomy to identify hatched individuals to species level. Morphological identification of dormant eggs can be difficult, even to the class level, and almost 10% of unhatched eggs were reported by Bailey *et al.* (2005) as 'indeterminate' taxon (Table 4.2). While the molecular methods used in this study were not able to identify all eggs to species level, the number of species identifications was double that of morphological methods (E. Briski, unpublished data).

Statistical analysis

I tested for differences in the cumulative mean density of diapausing eggs, the mean density of viable (hatched) eggs and the mean density of eggs of NIS in 40 g subsamples between the pre- and post-regulation sampling periods using *t*-tests and Mann-Whitney U-tests (SPSS 11.5.0, SPSS Inc., 1989 - 2002). A logarithmic transformation was applied to both datasets to meet assumptions of parametric tests. When a normal distribution was not achieved, or if results of a Levene's test for homogeneity of variances were significant, the non-parametric Mann-Whitney U-test was used (Table 4.3). After analysis of 40 g sediment subsamples was completed, further tests were conducted on extrapolated total egg abundance, total number of viable eggs and abundance of eggs of NIS by multiplying the average of four 40 g subsamples from each ship by the amount of sediment carried by that ship. The extrapolated data were again compared to Bailey *et al.*'s (2005) samples using *t*-tests and Mann-Whitney U-tests (SPSS 11.5.0, SPSS Inc.) (Table 4.3). A significance level of 95% was used for all statistical analyses. Power analyses estimating the number of ship samples required to differentiate significant differences in abundance of NIS between the two time periods were calculated using JMP 7.0.2 (2007 SAS Institute Inc).

To estimate species richness of the larger vessel population based on findings from my sampled vessels, I calculated Chao-1, an estimator of species richness based on the number of rare species in a sample (Chao, 1984; Chao & Shen, 2003). I compared Chao-1 species richness estimates for the pre- and post-regulation periods to examine the efficacy of ballast water flushing. Sample-

based species rarefaction curves were generated for both sampling periods to determine if a significant difference existed given my small sample size. Confidence intervals (95%) were generated to test for significant differences between the two sampling periods (Chao & Shen, 2006; Gotelli & Entsminger, 2006). Chao-1 estimates were calculated using SPADE software (Chao & Shen, 2006), while rarefaction curves were generated with 5000 random iterations using ECOSIM (Gotelli & Entsminger, 2006). Species richness comparisons were conducted for both total richness and richness of NIS.

4.3 Results

The estimated amount of residual sediment per vessel ranged from < 1 to 45 tonnes, with the mean tonnage (5 tonnes) being significantly lower than in the pre-regulation period (14 tonnes per vessel; $P < 0.05$, Table 4.3). Similarly, total density of dormant eggs during the post-regulation period, which ranged from 1 to 80 eggs per 40 g sediment with mean density 20.3 eggs per 40 g, was significantly lower than in pre-regulation samples (143.5 eggs per 40 g; Fig. 4.1; Table 4.3). The density of viable eggs, which ranged from 0 to 17 eggs per 40 g (mean density of 1.9 eggs per 40 g; 6.2% hatching success), was also significantly lower than in pre-regulation period (24.4 eggs per 40 g; 24.8%; $P < 0.05$; Table 4.3, Fig. 4.1). While the mean density of viable eggs of NIS post-regulation (1.3 eggs per 40g sediment; 1.6% hatch rate) did not decrease significantly (4.7 eggs per 40 g; < 1% hatch rate for pre-regulation period), there

was a significant difference if only eggs of high risk NIS are considered (Fig. 4.1; Table 4.3).

The identification of diapausing eggs using molecular markers COI and 16S resulted in 17 distinct taxa (Appendix 4.1). Only eight of the 13 distinct morphological groups of dormant stages recorded by Bailey *et al.* (2005) were also recorded in this study. While Bailey *et al.* (2005) found that community composition of dormant eggs was dominated by Rotifera (77.9%), I found that dormant eggs of Cladocera and Copepoda were most abundant, each representing 37.8% of total abundance. Seven NIS were identified, including five Cladocera, one Copepoda and one Ascidia (Table 4.4). Four of the NIS were freshwater taxa (*D. magna, D. galeata, Cercopagis pengoi* and *Acartia tonsa*), although only one is considered high risk (*A. tonsa*; Table 4.4).

Maximum diversity experiments confirmed the viability of 10 taxa, with species richness ranging from 0 to 5 viable taxa per 40 g sediment (median 2; no eggs were hatched from eight tanks). Cladocera were the most species-rich group, representing 80% of all taxa hatched. The remaining species belonged to Rotifera and Copepoda (see Appendix 4.1). Forty percent of ships sampled in the post-regulation period carried viable dormant stages of NIS (0 - 2 eggs per 40 g sediment), although only one taxon (*D. magna*) hatched from only one of the 19 tanks during whole sediment experiments. The estimated species richness of the post-regulation vessel population was 27.2 species including 8.3 NIS, which was significantly lower than estimates for the pre-regulation period (126 and 33

species estimated for total richness and NIS richness, respectively, for the pre-regulation period) (Fig. 4.2).

Extrapolation of post-regulation subsample results to whole ships resulted in mean and median abundances of 3.5×10^6 and 9.0×10^4 eggs ship^{-1}, respectively. The mean and median numbers of viable eggs per ship were estimated at 1.9×10^6 and 1.3×10^5 eggs ship^{-1}, respectively. Finally, the mean and median abundances of dormant eggs of NIS were estimated as 1.8×10^5 and 0 eggs ship^{-1}, respectively. The total abundance of dormant eggs, number of eggs hatched and number of eggs of high risk NIS per ship were each significantly lower in the post- *versus* pre-regulation period ($P < 0.05$; Table4.3). The estimated average total ballast capacity of ships in my study was 14532 m^{-3}, and assuming that all eggs from sediment would hatch, it could result in average of 130 individuals m^{-3} of which 12.3 individuals m^{-3} are NIS. However, considering hatching results from whole sediment experiments, the average number of invertebrates released from eggs may be as low as 0.014 individuals m^{-3}.

4.4 Discussion

Results from this study indicate that the ballast management regulations implemented in 2006 have markedly reduced the probability of introduction of invertebrates to the Great Lakes *via* dormant eggs. Sediment accumulation has been significantly reduced and with it the abundance of dormant stages of invertebrate species. On average, ships in the post-regulation period carried a potential inoculum one order of magnitude lower than those sampled in the pre-

regulation period. Egg viability was lower and fewer eggs of perceived high risk NIS were present during the post-regulation period. Estimated species richness for the vessel population was also much lower in the post-regulation period (27 *vs.* 126 species).

The addition of saltwater flushing to the ballast water management regime reduced the tonnage of accumulated sediments in ballast tanks threefold. Physical removal of sediments likely contributed to the observed reduction in egg number, as eggs are likely discharged along with sediments. Less sediment accumulation in tanks could also impact viability of retained eggs if they are more exposed to saltwater exposure during mid-ocean exchange and/or saltwater flushing. Bailey *et al.* (2004, 2006) determined that saltwater exposure was significantly more detrimental to viability of eggs extracted from sediments than for those retained within sediment. Furthermore, Reid *et al.* (2007) reported a reduction in oxygen concentration in ballast tank water owing to decaying organic matter. Reduced sediment accumulation in tanks may also expose a larger proportion of retained eggs to unfavourable oxygen concentration at the sediment-water interface. Reduction in sediment accumulation could also negatively impact egg viability *via* desiccation. Dry sediments have been documented for ships arriving to the west coast of Canada and to the Great Lakes (Sutherland *et al.,* 2009; S.A. Bailey, unpublished data), and long periods of desiccation or repeated hydration-dehydration cycles can negatively impact viability of dormant eggs (Lavens & Sorgeloos, 1987; Hagiwara *et al.,* 1997). Furthermore, egg viability may be reduced if small amounts of sediment facilitate

gradual rather than rapid changes in abiotic conditions. For example, exposure to brackish water (8‰) has a greater effect on viability of dormant eggs of freshwater species like *Bosmina leideri* De Melo and Hebert, 1994 and *Daphnia longiremis* (Sars, 1861) than does exposure to ocean water (32‰; Bailey *et al.,* 2004). Similarly, egg viability is reduced more by exposure to low levels of oxygen than to complete anoxia (Lutz *et al.,* 1994). Under extreme abiotic conditions, such as complete anoxia or high salinity, dormant eggs remain inactive, while sub-optimal conditions can initiate termination of diapause (Clegg & Trotman, 2002; García-Roger *et al.,* 2005; Pauwels *et al.,* 2007). Eggs that begin to develop under less-than-optimal conditions may allocate extra energy to adjust their metabolism to environmental conditions such that energy reserves are depleted before development is complete and emergence occurs (Van Stappen, 1996; Bailey *et al.,* 2004). Whatever the mechanism, the results of this study indicate that egg viability – as determined by hatching success – was significantly reduced in vessels entering the Great Lakes following implementation of ballast regulations for residual sediment and water.

Current ballast water management activities seemingly exert differential impacts on different taxa, as evidenced by the change in dormant egg community dominance from Rotifera to Cladocera and Copepoda. Dormant eggs of Rotifera are best preserved in constant salinity with low amounts of organic matter (Hagiwara *et al.,* 1997), while Cladocera have a hard ephippial structure around eggs that enhances resistance to desiccation and rapid abiotic changes (Altermatt *et al.,* 2009). It is unclear why abundance of Copepoda would increase

compared to other taxa, but it is possible that a portion of Copepoda eggs were classified as 'indeterminate' in the pre-regulation period (see Methods; Table 4.2) since species identifications did not include molecular analysis in the earlier study. Other possible reasons for the change in taxonomic composition between pre- and post-regulation periods may be that ships sampled in the latter period had followed different geographic pathways than ships in the former period, or because of different ballasting/deballasting patterns (i.e. ships sampled in the pre-regulation period were entering the Great Lakes with empty tanks and had been sampled before ballasting, while those sampled in the post-regulation period were discharging ballast water into the Great Lakes just before samples were collected,Table 4.1).

While I observed a significant decrease in the total abundance, viability and species richness of dormant eggs, egg abundance of NIS did not decrease in the post-regulation period. This observation is almost certainly due to insufficient sample size. Using power analysis, I estimated that up to 171 ship samples would be required to confirm a significant difference in NIS egg abundance between the two studies. However, egg abundance of high risk taxa dropped from an average of 78% during the pre-regulation period to an average of 1% post-regulation. Only four NIS capable of tolerating fresh water were recorded during this study. Cladocera *D. magna* and *C. pengoi* were recorded in two ships, while *D. galeata* and Copepoda *A. tonsa* were observed in one vessel each. Following my earlier line of reasoning, *D. magna* appears to be a low risk for successful establishment in the Great Lakes. *C. pengoi* and *D. galeata* represent the next highest risk for

introduction based on propagule pressure, but both have already established in the system (Taylor & Hebert, 1993; MacIsaac *et al.*, 1999). Indeed, presence of viable eggs of these species in ballast sediments highlights the possibility that these species were vectored to the lakes in ballast sediment rather than ballast water. As a result, *A. tonsa* is the only species recorded during this study which presents a relatively high risk for invasion for the Great Lakes *via* retained eggs in treated ballast sediments.

Preventing species introductions *via* dormant invertebrate eggs is a particularly challenging task because ballast sediments are not easily flushed from tanks and because dormant eggs are resistant to a wide array of adverse environmental conditions and treatment strategies (Bailey *et al.,* 2005, 2006; Gray *et al.,* 2006). My results from whole sediment experiments indicate that the current propagule pressure posed by dormant eggs hatched into ballast water, estimated at 0.014 hatched individuals m^{-3}, would make an insignificant contribution to the median number of propagules typically carried in exchanged ballast water (2672.9 ind. m^{-3}; S.A. Bailey, unpublished data). As the proposed international ballast water discharge standard applicable to invertebrate zooplankton stipulates that treated ballast water must contain less than 10 viable individuals m^{-3} (IMO, 2004), my results suggest that the risk of introductions *via in situ* hatching is adequately managed through saltwater flushing. In the worst case scenario, if all eggs in the sediment were to hatch and become available for discharge, or if all eggs in the sediment would be discharged directly, the estimated abundance of viable individuals in filled ballast tanks would increase by

130 individuals m^{-3}, which would be noncompliant with the proposed international ballast water discharge standard.

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Table 4.1 Differences in methodology between the pre-regulation and the post-regulation periods.

		This study (the post-regulation period)	Bailey *et al.* (2005) (the pre-regulation period)
Sampling			
	Tank status	Ballast water discharged after entering the Great Lakes	Ballast water discharged outside the Great Lakes
	Total number of tanks sampled	19	69
	Total number of ships sampled	17	39
	Number of ships where two tanks were sampled	2	26
	Number of ships where three tanks were sampled	0	2
Egg density counts			
	Number of tanks counted (Ludox® HS 40 method; 40 g; four replicates)	19	69

Hatching experiments		
Maximum diversity experiments (sugar flotation method)	19 tanks; four replicates; 0, 15 and 30‰; at 20°C	five tanks; four replicates; 0 and 8‰; at 10°C and 20°C and 50 tank; one replicate; 0‰; at 20°C.
Whole sediment experiments	19 tanks; four replicates; 0, 15 and 30‰; at 20°C	19 tanks; four replicates; 0, at 20°C and 10 tanks; four replicates; 8, 16 and 32‰; at 20°C.
Identification of taxa		
Molecular markers COI and 16S applied to eggs	Yes	No
Morphological identification applied to hatched animals	Yes	Yes

Table 4.2 Percent occurrence and abundance of dormant stages collected before (39 vessels) and after (17 vessels) implementation of Canadian ballast water management regulations in 2006. Dormant stages are arranged phylogenetically by taxon. Pre-regulation data modified from Bailey *et al.* (2005).

Taxon	Pre-regulation period		Post-regulation period	
	% occurrence	% abundance	% occurrence	% abundance
Rotifera	**100**	**77.9**	**95**	**19.9**
Asplanchna spp.	66.7	1.0	0	0
Brachionus spp.	97.4	76.2	95	19.9
Conochilus spp.	5.1	< 1	0	0
Filinia spp.	48.2	< 1	0	0
Synchaeta spp.	5.1	< 1	0	0

Bryozoa	**61.5**	**< 1**	**63.2**	**3.9**
Cladocera	**76.9**	**9.3**	**89.5**	**37.8**
Bosmina spp.	51.3	< 1	10.5	1.3
Chydoridae	5.1	< 1	0	0
Daphnia spp.	46.2	7.9	89.5	19.5
Diaphanosoma spp.	2.6	< 1	1	<1
Moina spp.	25.6	< 1	15	12.8
Onychopoda	1	< 1	21	3.3
Copepoda	**76.9**	**2.6**	**100**	**37.8**
Indeterminate*	**100**	**9.8**	**26.3**	**< 1**

*Indeterminate represents eggs which were not identified to any taxonomic level.

Table 4.3 Significance levels for statistical comparisons of experimental data between the pre-regulation and the post-regulation periods. Significant *P*-values are presented in bold. A significance level of 95% was used for all statistical analyses.

Experiment type	Treatment compared	Leven's test for equality of variances (*P*)	*t*-test (*P*)	Mann-Whitney U test (*P*)
Egg counts				
	Number of eggs in 40 g subsample	0.925	**0.019**	
	Amount of sediment per ship	0.330	**0.001**	
	Number of eggs per ship	0.916	**< 0.001**	
Maximum diversity				
	Number of hatched eggs from 40 g subsample	**0.001**		**< 0.001**
	Number of eggs of NIS in 40 g subsample	0.400	0.173	

	Number of high risk eggs of NIS in 40 g subsample	**< 0.001**	**< 0.001**
	Number of hatched eggs per ship	**0.037**	**0.009**
	Number of eggs of NIS per ship	**0.007**	0.814
	Number of high risk eggs of NIS per ship	**< 0.001**	**< 0.001**
Whole sediment			
	Number of eggs hatched from 40 g subsample	**< 0.001**	**0.007**
	Number of hatched eggs per ship	**< 0.001**	**< 0.001**

Table 4.4 Nonindigenous species transported as dormant eggs in residual ballast sediment to the Great Lakes. Species are listed in order of decreasing frequency and abundance of resting eggs, and ability to tolerate freshwater habitats. Occurrence identifies the number of ships that the species was collected from. Abundance is the cumulative mean number of eggs identified from 40 g sediment for all ships in which each species was found. Species hatched in 0‰ medium during laboratory experiments were considered an environmental match for the Great Lakes. Further, species identified by molecular markers that did not hatch were assigned habitat match based on literature research (*Cercopagis pengoi* and *Botrillus schlosseri*). Pre-regulation data are modified from Bailey *et al.* (2005).

Species Name	Pre-regulation period		Post-regulation period		Habitat Match
	Occurrence	Abundance	Occurrence	Abundance	
Daphnia magna Straus, 1820	4	6	2	20.5	Y
Filinia passa (Muller, 1786)	4	3.5			Y
Brachionus leydigi Cohn, 1862	4	3			Y

Filinia cornuta (Weisse, 1847)	3	3			Y
Asplanchna girodi (De Geurne, 1888)	2	1			Y
Cephalodella sterea (Gosse, 1887)	1	4.75			Y
Cercopagis pengoi (Ostroumov, 1891)*			2	0.75	Y
Bosmina maritima (Muller, 1867)	1	2			Y
Diaphanosoma orghidani Negrea, 1982	1	1.25			Y
Daphnia galeata Sars, 1864			1	2	Y
Brachionus forficula Wierzejski, 1891	1	1			Y
Brachionus nilsoni Ahlstrom, 1940	1	1			Y
Conochilus coenobasis (Skorikov, 1914)	1	0.5			Y

Diaphanosoma mongolianum Ueno, 1938	1	0.5			Y
Cephalodella cf. stenroosi Wulfert, 1937	1	0.3			Y
Alona rustica Scott, 1895	1	0.25			Y
Brachionus bennini Leissling, 1924	1	0.25			Y
Brachionus diversicornis (Daday, 1883)	1	0.25			Y
Diaphanosoma sarsi Richar, 1894	1	0.25			Y
Hexarthra intermedia Wiszniewski, 1929	1	0.25			Y
Moina affinis Birge, 1893	1	N/A			Y
Acartia tonsa Dana, 1849			1	0.25	Y
Synchaeta baltica Ehrenberg, 1834	1	2.75			N

Synchaeta bacillifera Smirnov, 1933	1	2.25			N
Evadne nordmanni Lovén, 1836	1	0.5			N
Pleopis polyphemoides (Leuckart, 1859)	1	N/A	1	0.5	N
Podon intermedius Lilljeborg, 1853			1	0.5	N
Botrillus schlosseri (Pallas, 1766)**			1	0.25	N

* *Cercopagis pengoi* did not hatch in my experiments, though it is established in the Great Lakes.

** *Botrillus schlosseri* did not hatch in my experiments; habitat matching designation based on Lambert (2005).

Figure 4.1 Mean (± standard error of mean) and median (horizontal line in bar) density of invertebrate dormant eggs (total), of viable invertebrate eggs (hatched), of viable eggs of nonindigenous species (NIS) and of eggs of high risk NIS in ballast sediment samples from pre-regulation (grey bars) and post-regulation sampling periods (black bar). The asterisk denotes a significant difference between paired bars.

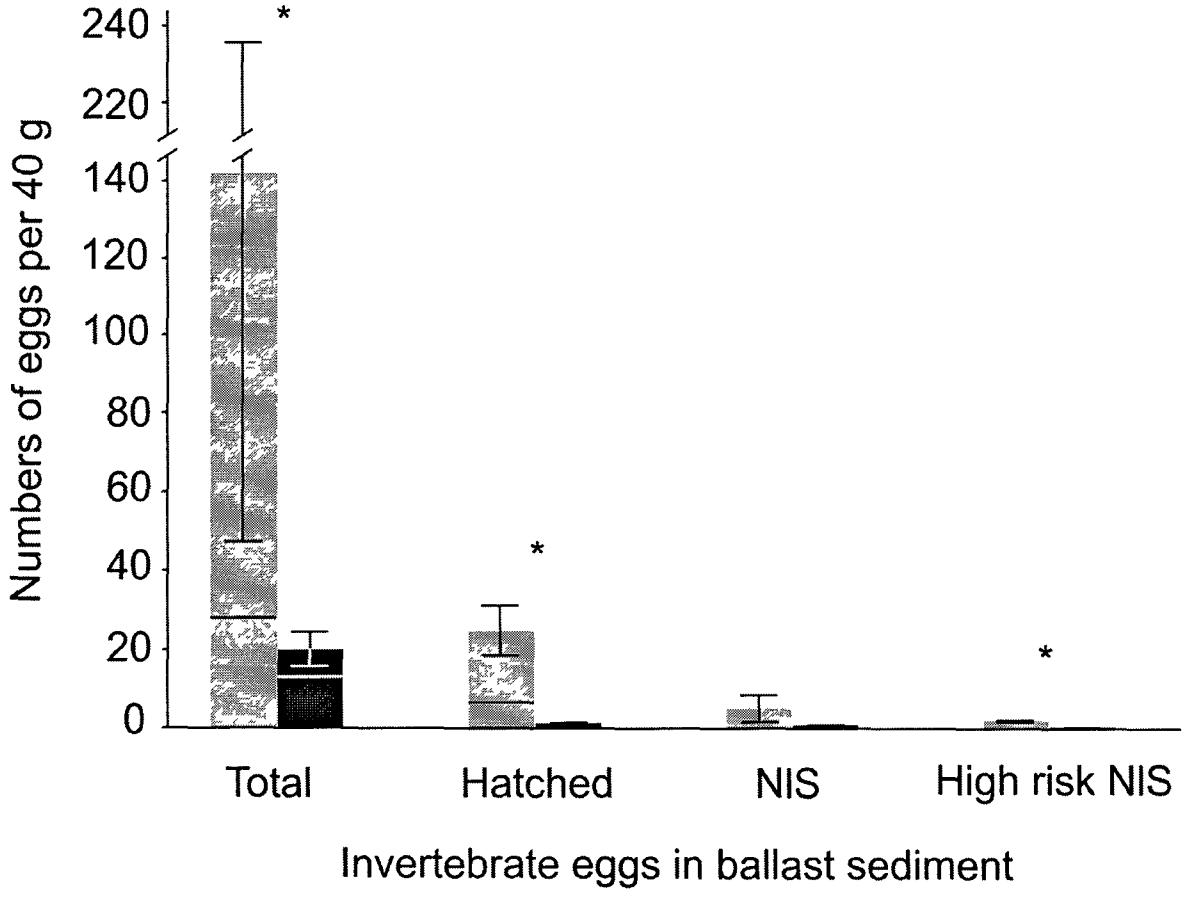
240
220
140
120
100
80
60
40
20
0
Numbers of eggs per 40 g
*
*
*
Total
Hatched
NIS
High risk NIS
Invertebrate eggs in ballast sediment

Figure 4.1

Figure 4.2 Sample-based rarefaction curves from the pre-regulation (grey line, ± 95% C.I.) and the post-regulation (black line, ± 95% C.I.) periods for: (A) all ships sampled and (B) ships containing nonindigenous species (NIS). Also shown are species richness estimates for the vessel population (Chao-1 ± 95% C.I.) for the pre-regulation (grey bar) and the post-regulation period (black bar). Note the difference in scales for each x and y axis.

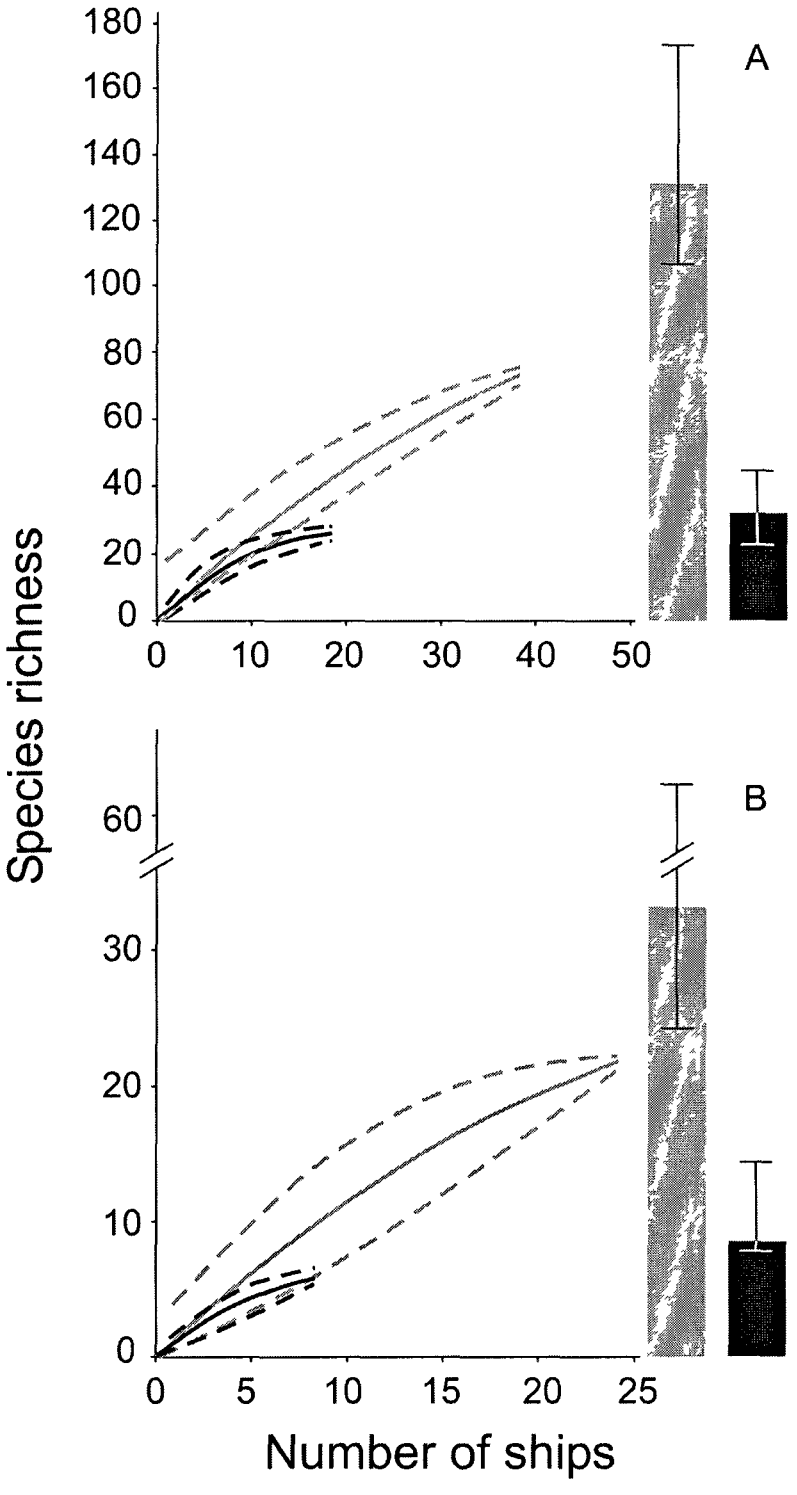
Species richness
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
A
60
30
20
10
0
0
5
10
15
20
25
B
Number of ships

Figure 4.2

Chapter 5: Regional variation in colonization and propagule pressure of invertebrates and their dormant eggs transported by ships' ballast sediments

5.1 Introduction

Global trade and travel have increased greatly in recent years, as have biological invasions concomitantly (Wonham & Carlton, 2005; Ricciardi, 2006; Hulme, 2009). A better understanding of the invasion process has been gained by dividing it into several stages, including introduction, establishment and spread. Filters reduce the number of successful nonindigenous species (NIS) at each successive stage (e.g. Kolar & Lodge, 2001; Colautti & MacIsaac, 2004). Transitions between stages are affected individually or by combinations of factors including propagule pressure (i.e. the frequency and density with which NIS are introduced to new habitats), physico-chemical factors, and by community interactions (see Colautti & MacIsaac, 2004). Cost-effective management of NIS is most successfully conducted at the introduction stage through prevention efforts (Simberloff, 2009a).

With 90% of world trade transported by ships (Hulme, 2009; IMO, 2010), and ships' ballast water and sediments being leading mechanisms for NIS introductions (Carlton, 1985; Ricciardi, 2006; Molnar *et al.*, 2008), ballast water regulations have been enacted by the USA and Canada in an attempt to manage this vector. First, mid-ocean exchange (MOE) was recommended in 1989, and became mandatory in 1993, for vessels entering the Great Lakes with filled

ballast tanks in an effort to reduce the number of propagules transported to the lakes (Canadian Coast Guard, 1989; United States Coast Guard, 1993). Beginning in 2006, this regulation was extended to the Pacific and Atlantic coasts of Canada, and was further improved by including management of residual ballast water and accumulated sediments through mandatory saltwater flushing for vessels carrying only ballast residuals (Government of Canada, 2006; SLSDC, 2008). By purging fresh or coastal water, sediments, and taxa contained therein from tanks, and by exposing taxa remaining in tanks to mid-ocean water, MOE and saltwater flushing should reduce both species richness (i.e. colonization pressure) and abundance (i.e. propagule pressure) inside tanks (MacIsaac *et al.*, 2002; Bailey *et al.*, 2005a; Briski *et al.*, 2010).

Invertebrate species may be present in ballast tanks as active (i.e. metabolically active) or as dormant stages (e.g. dormant eggs, resting eggs, statoblasts and cysts) which are in some type of metabolic and/or developmental depression (Cáceres, 1997; Bailey *et al.*, 2005a; Duggan *et al.*, 2005). The effect of MOE and/or saltwater flushing on active (Wonham *et al.*, 2001; Choi *et al.*, 2005; Gray *et al.*, 2007) and dormant (Gray & MacIsaac, 2010, Briski *et al.*, 2010) stages of invertebrates has been assessed in a number of studies. Humphrey (2008) compared the invasion risk posed by invertebrate active stages arriving to different regions, though no similar studies have been conducted for their dormant eggs. In addition, most ballast water studies have typically focused on transoceanic ships, while neglecting intra-coastal ship traffic (Levings *et al.*, 2004; Rup *et al.*, 2010). This intra-coastal gap neglects the possibility of secondary

transfer of NIS from heavily invaded hub sites, such as San Francisco Bay, to smaller, regional ports that lack international shipping (Wasson *et al.*, 2001). Indeed, it is possible that intra-coastal shipping could represent an important invasion pathway depending on the proportion of the total ship traffic that it represents (e.g. Verling *et al.*, 2005; Rup *et al.*, 2010). Many intra-coastal ship transits are exempt from mandatory flushing/exchange regulations on the presumption that movement of ballast water is occurring only within a single marine ecoregion (Powles *et al.*, 2004) and thus is viewed as non-risky (Government of Canada, 2006; SLSDC, 2008). This assumption may not be valid if NIS established in one part of the region are transferred in ballast water to another location in the ecoregion where the NIS is not present. Such intra-coastal transfers could be particularly effective at introducing NIS since these voyages typically are of relatively short duration, with a relatively high survivorship of NIS in ballast tanks in consequence (Carlton, 1985; Verling *et al.*, 2005).

Ballast sediment has been implicated as a vector for human-assisted zooplankton dispersal (Bailey *et al.*, 2005a; Briski *et al.*, 2010), and may contain very large numbers of invertebrates and their viable dormant eggs (Bailey *et al.*, 2005a; Duggan *et al.*, 2005). In this study, I explore species richness and abundance of invertebrate active and dormant stages vectored by ballast sediment between and within ecoregions, and consider invasion risk for different areas receiving ballast discharges. Ship pathways considered include transoceanic voyages with MOE (TOE), coastal voyages with MOE (CE), and

coastal voyages without MOE (CNE). Specifically, I compare the ballast sediment vector strength for ports in the Pacific, Atlantic and Great Lakes regions of Canada, and invasion risk of transoceanic *versus* coastal voyages. I also examine the influence of voyage length on colonization and propagule pressure of invertebrates and their dormant eggs contained in ballast sediments.

5.2 Materials and methods

Sampling

One hundred forty-two sediment samples were collected between May 2007 and August 2009, inclusive (Appendix 5.1). I sought to sample twenty tanks from each of three different ship pathways in each of the Pacific and Atlantic regions (TOE, CE and CNE) and in the Great Lakes region (TOE). TOE ships were defined as ships coming from any continent except North America to ports in Canada and/or the Great Lakes. The CE pathway included ships arriving from ports in the southern USA, defined as south of Cape Blanco, Oregon on the Pacific coast and south of Cape Cod, Massachusetts on the Atlantic coast. The CNE pathway includes ships arriving from American and Canadian ports north of Cape Blanco on the Pacific coast and Cape Cod on the Atlantic coast (Canadian Government, 2006; Fig. 5.1). In reality, I was able to sample 20 TOE, 19 CE, and 21 CNE tanks in the Pacific region. Comparable numbers in the Atlantic region were 22 TOE, 20 CE and 21 CNE tanks, while in the Great Lakes region I sampled 14 TOE and five CE tanks (Appendix 5.1). In seven cases, two tanks were sampled from the same ship (Appendix 5.1). In all cases, sediment was

collected from different areas inside the ballast tank and homogenized. The only exception to this protocol occurred with six TOE tanks in the Great Lakes region, for which four different areas inside the tanks were sampled and processed separately to determine if spatial differences in biological composition exist within tanks. Whenever possible, 6 kg of sediment was collected per tank. All sampled tanks were inspected for presence of macroinvertebrates such as Cnidaria, Decapoda, Gastropoda, Bivalvia, which were collected when found. Sampling teams also inspected sediment depth and percent cover inside ballast tanks. Ballast water reporting forms, which included total ballast capacity and previous dates and locations of ballast uptake and discharge, were collected from each ship's crew. Information on the amount of sediment per tank, combined with architectural diagrams of ships' tanks, was used to estimate the amount of residual sediment carried per 10000 m^3 ballast capacity for each ship. Ship ballast capacity is directly related to ship size, and as ships operating in different regions varied in size, I used the 10000 m^3 ballast capacity to correct for differences in ship size.

Density counts, viability experiment and identification

Macroinvertebrates were separated from sediment and stored in 95% ethanol until further processed. Sediment was thoroughly homogenized in the laboratory and stored at 4°C. Egg density counts were conducted one week after samples were collected. Four 40 g subsamples were taken from each sediment sample. Eggs were separated from sediment using colloidal silica Ludox® HS 40

(Burgess, 2001). Dormant eggs were enumerated under a dissecting microscope, grouped by size and gross morphology, and a maximum of 20 dormant eggs per morphological group was taken for molecular identification (see Briski *et al.*, 2010, 2011). Identification of dormant eggs was conducted using molecular methods and traditional morphological taxonomy of hatched individuals (see Briski *et al.*, 2010, 2011).

The remaining sediment was stored for four weeks to break the diapause of dormant eggs before hatching experiments were performed (Schwartz & Hebert, 1987; Dahms, 1995). For hatching experiments, dormant eggs were isolated from 40 g sediment subsamples using a sugar flotation method (Hairston, 1996). Extracted dormant eggs were placed in vials containing sterile synthetic pond water (0 parts per thousand (‰) salinity; Hebert & Crease, 1980) or a sterile seawater medium with salinity of 15 or 30‰ (see Briski *et al.*, 2010). Four replicates were placed in each of the 0, 15 and 30‰ treatments at 20°C. Owing to the large sample size, I was able to conduct hatching experiments at only one temperature; therefore I selected 20°C as the optimal temperature based on the results of Bailey *et al.* (2005). I used the number of hatched eggs as a proxy measure of egg viability, although I acknowledge that some eggs that did not hatch may have been viable but did not receive appropriate hatching cues (Schwartz & Hebert, 1987).

Statistical analysis

Estimated sediment tonnage carried per 10000 m^3 of ballast capacity of ships arriving to different regions, following different pathways, were compared using nested ANOVA and post-hoc Bonferroni test (SPSS 11.5.0). Significance levels for statistical comparisons were adjusted for multiple pair-wise comparisons by Bonferroni-type correction to guard against inflating the Type I error rate. The family-wise error rate of 0.05 was used.

Following Bailey *et al.* (2005a), I averaged results from multiple tanks sampled from a single ship at a single sampling event. To determine if different areas inside tanks exhibited biological differences, Sorensen's coefficient of similarity (Krebs, 1999) was calculated for samples (i.e. dormant eggs) taken from four different areas in six TOE tanks in the Great Lakes region. Sorensen's coefficient is based on presence/absence of species in each sample, and ranges from zero to one, with higher values representing greater similarity of samples. I calculated Sorensen's coefficients for 16 randomly drawn pairs of samples from within tanks and compared them to those of 16 randomly drawn pairs of samples between tanks from different ships using a Mann-Whitney U-test since data did not follow a normal distribution and the result of a Levene's test for homogeneity of variances was significant (SPSS 11.5.0). Furthermore, to determine if the density of dormant eggs within tanks varied, I compared egg density counts of four replicated samples taken from six TOE tanks in the Great Lakes region using one-way ANOVA (SPSS 11.5.0). A logarithmic transformation was applied to meet assumptions of parametric tests. Since the spatial analyses determined that

samples within tanks were more similar than samples between tanks, data from replicated samples were averaged and processed as single samples.

I used parametric (Pearson's) correlation analysis to explore the relationship between sediment tonnage inside tanks with density and viability of dormant eggs in 40 g of sediment subsamples, and with density of dormant eggs of NIS in 40 g sediment subsamples. I also used Pearson's correlation to explore the relationship between voyage length (calculated using ballast water reporting forms) and density and viability of dormant eggs in 40 g of sediment. All data were log transformed to meet statistical assumptions.

Variation in density and viability of dormant eggs in 40 g sediment subsamples between regions and between different ship pathways within regions were analyzed using nested ANOVA (SPSS 11.5.0) and post-hoc Bonferroni test (SPSS 11.5.0). Significance levels for statistical comparisons were again adjusted for multiple pair-wise comparisons by Bonferroni-type correction with a family-wise error rate of 0.05.

After completing analyses of 40 g sediment subsamples, additional tests were conducted on extrapolated egg abundance and number of viable eggs by multiplying the average of four 40 g subsamples from each ship by the amount of sediment carried per 10000 m^3 ballast capacity for that ship. Sediment tonnage per region and per ship pathway, and extrapolated egg abundance and viability, were analyzed using two-way multivariate analysis of variance (MANOVA, SPSS 11.5.0). Macroinvertebrates were excluded from all statistical analyses.

Species richness of the larger ship population was estimated by calculating 1st order jackknife, an estimator of species richness based on the number of rare species in a sample (Burnham & Overton, 1979; Heltshe & Forrester, 1983; Chao & Shen, 2003). I compared these species richness estimates for three regions and three ship pathways to determine if region, duration of voyage, or application of MOE were important factors. Sample-based species rarefaction curves were generated and contrasted for all sampling regions and pathways. Confidence intervals (95%) were generated to test for significant differences between sampling regions and ship pathways (Chao & Shen, 2006; Gotelli & Entsminger, 2006). 1st order jackknife estimates were calculated using SPADE software (Chao & Shen, 2006), while rarefaction curves were generated with 5000 random iterations using ECOSIM (Gotelli & Entsminger, 2006).

5.3 Results

Ships sampled in this study serviced both international and domestic Canadian routes, although those arriving to the Atlantic and Great Lakes regions typically operated between Atlantic ports, whereas those arriving to Pacific Canadian ports mainly traveled among Pacific ports. Ships operating in different regions tended to vary in size, with mean ballast capacity of 21,835, 11,726 and 32,381 m^3 for Pacific, Great Lakes and Atlantic regions, respectively. Voyage length also varied across pathways, with mean durations of 19.5, 10.2 and 6.0 days for TOE, CE and CNE pathways, respectively. Significantly more ballast

sediment was found in TOE and CE ships than in CNE ships in the Atlantic region, TOE vessels in the Great Lakes region, and in all ship pathways in the Pacific region (Post-hoc Bonferonni test, $P < 0.05$). Sediment tonnage (per 10000 m^3 ballast capacity) of TOE and CE ships in the Atlantic region averaged 9.8 and 14.3 tonnes, respectively (Table 5.1). Comparable CE ships from the Great Lakes region averaged 16 tonnes of sediment. However, high variation in ballast tonnage among CE ships from the Great Lakes region precluded significant differences between either TOE and CE in the Atlantic, TOE in the Great Lakes, or any ship pathway in the Pacific region. All other ships carried an average of < 4 tonnes of sediment (Table 5.1).

Spatial analyses of four different areas in six TOE tanks in the Great Lakes region illustrated that samples within tanks are more similar than those between tanks. Sorensen's coefficients of similarity for 16 randomly drawn pairs of samples within tanks were significantly (Mann-Whitney U-test, $Z = - 4.423$, $P < 0.05$) higher than those of 16 randomly drawn pairs of samples between tanks from different ships (means of 0.85 and 0.17, respectively). Furthermore, densities of eggs collected from different areas in tanks were not significantly different from each other (ANOVA, $F_{3,20} = 0.042$, $P > 0.05$).

Sediment volume in ballast tanks was correlated with total density of dormant eggs (Pearson's correlation, $r^2 = 0.029$, $P = 0.022$, Fig. 5.2) but not viability ($r^2 = 0.001$, $P = 0.339$) or density of NIS dormant eggs ($r^2 = 0.018$, $P = 0.056$) in 40 g sediment subsamples. Also, there was no relationship between voyage length and total dormant egg density ($r^2 = 0.017$, $P = 0.120$) or viability (r^2

= 0.005, P = 0.403) in 40 g sediment subsamples. However, active macroinvertebrates were found only in ships with voyage length of five days or less.

Mean density of eggs in sediment subsamples ranged from 4.5 to 17.3, 18.4 to 33.6, and 84.0 to 129.1 eggs 40 g^{-1} in the Pacific, Great Lakes and Atlantic regions, respectively (Table 5.1). Dormant egg densities were log-normally distributed and were significantly different between regions, though not between pathways within regions (Nested ANOVA, Table 5.2). Density of dormant eggs in the Pacific region was significantly lower than those in Atlantic and Great Lakes regions (Post-hoc Bonferonni test, $P < 0.05$). In addition, no dormant eggs collected from ships in the Pacific region hatched in the laboratory, while the mean density of viable eggs in the Great Lakes and Atlantic regions ranged from 1.0 to 1.2 and 4.5 to 29.8 eggs 40 g^{-1}, respectively (Table 5.1). Viability of dormant eggs was also significantly different between regions, but non-significant for pathways within regions (Nested ANOVA, Table 5.2). Again, viability of dormant eggs in the Pacific region was significantly lower than that in the Atlantic and Great Lakes regions (Post-hoc Bonferonni test, $P < 0.05$).

After extrapolating results of 40 g subsamples to the sediment tonnage per 10000 m^3 ballast capacity, mean abundance of dormant eggs ranged from 0.13 to 0.29, 3.42 to 8.65, and 7.81 to 28.60 million eggs in the Pacific, Great Lakes and Atlantic regions, respectively (Table 5.1). Dormant egg viability for Atlantic ships was at least an order of magnitude higher than Great Lakes ships (Table 5.1). A multivariate test showed significant influence of region but not of ship

pathway on three dependent variables (sediment tonnage, abundance and viability of dormant eggs) (MANOVA, $p < 0.05$, Table 5.3). While univariate analyses indicated a regional influence on all three dependent variables, ship pathway independently influenced only the amount of sediment in tanks (MANOVA, Table 5.3).

Seventeen, 24 and 29 distinct species, representing 14 taxonomic groups, were identified from the Pacific, Great Lakes and Atlantic region samples, respectively (Appendix 5.2). Corresponding richness estimates for the general vessel populations were 18, 29 and 37 species, respectively (Fig. 5.3). Species richness estimates were significantly higher for the Great Lakes and Atlantic regions than for the Pacific region. In addition, richness estimates were not different for ship pathways within the Pacific and Atlantic region, however, a significant difference was observed between pathways in the Great Lakes region (Fig. 5.3). Still, this difference should be interpreted with caution because of the small sample size for this ship pathway in the Great Lakes region (five ships, Appendix 5.1).

Three of 17 species identified in the Pacific region were NIS (*Bosmina freyi, Ceriodaphnia dubia* and *Daphnia retrocurva*) but are likely incapable of surviving salinity conditions in the region (Appendix 5.3). In the Great Lakes, five of 24 species identified were NIS (*Daphnia magna, Cercopagis pengoi, Pleopis polyphemoides, Podon intermedius* and *Acartia tonsa*), including two freshwater taxa capable of survival in the Great Lakes (*D. magna* and *A. tonsa*) and one species already established there (*C. pengoi*)(Appendix 5.3). In the Atlantic

region, nine of 29 distinct species were NIS (*Hyalinella punctata, Plumatella emarginata, D. magna, Daphnia galeata, Daphnia cucullata, Calanus euxinus, Carcinus maenas, Littorina littorea* and *Hyotiss numisma*), including four species able to tolerate salinity conditions typical in the Atlantic region (*C. euxinus, C. maenas, L. littorea* and *H. numisma*)(Appendix 5.3).

5.4 Discussion

The risk of aquatic invasions by ballast sediment is not uniform across regions despite implementation of common, national management practices across Canada. I found that ships arriving to the Atlantic region not only carry more ballast sediment than do those arriving to the Pacific and Great Lakes regions, but also transported more species (i.e. colonization pressure) at higher abundance (i.e. propagule pressure) within that sediment. The amount of sediment carried by ships may depend on the geography of ship activities. TOE and CE ships in the Atlantic region typically carried an order of magnitude more ballast sediment than those in the Pacific or Great Lakes regions. As ships arriving to the Atlantic region mostly operate in the South-eastern US, North and Baltic Sea ports, which are shallow and sandy (European Commission, 2004), the high amount of accumulated sediment found in their ballast tanks is not surprising. In contrast, TOE and CE ships arriving to Pacific region mainly operate in the Pacific Ocean where ports are predominantly rocky and deep (Zhou *et al.*, 1993). TOE ships servicing the Great Lakes region contained less sediment than those arriving to the Atlantic region even though they largely

operate between the same ports. Approximately 90% of ships entering the Great Lakes carry cargo (MacIsaac *et al.*, 2002) and manage 'empty' ballast tanks with saltwater flushing rather than MOE, which could more effectively purge accumulated sediments (Briski *et al.*, 2010). Another explanation for differences in the amount of sediment between ships arriving at Atlantic ports and those arriving in the Great Lakes region may be attributed to the greater stringency in the enforcement of regulations in the latter region.

While MOE effectively manages active invertebrates in ballast water (Gray *et al.*, 2007), I did not find any effect on dormant eggs in ballast sediment. This could be attributed to the unique ability of dormant eggs to withstand very harsh environments (Cáceres, 1997), remaining viable inside ballast tanks from several months to longer than a year (E. Briski, unpubl.). Furthermore, ballast tanks with more sediment build-up contained higher densities of dormant eggs, implying that thicker sediment provides more protection for dormant eggs, a possibility that is consistent with reports that saltwater exposure is significantly more detrimental to viability of eggs extracted from sediments than for those retained within sediment (Bailey *et al.*, 2004; Bailey *et al.*, 2006; Briski *et al.*, 2010). Sutherland *et al.* (2009) observed a positive correlation between suspended particulate matter in tanks and invertebrate abundance and species richness, while Briski *et al.* (2010) reported a reduction in ballast sediment and colonization and propagule pressure in ships entering the Great Lakes after implementation of saltwater flushing; thus, the most effective means to reduce propagule pressure associated with ballast sediment is almost certainly physical removal of eggs together with sediment

from tanks, either through manual tank cleaning or saltwater flushing on the open sea. Additional research is required to determine whether MOE is a less effective means to manage sediments, as well as the relative efficacy of both strategies with respect to freshwater *versus* marine taxa.

As with MOE, the length of a voyage did not influence density or viability of dormant eggs. However, this study indicates that active macroinvertebrates survived only in ships undertaking shorter voyages. Reid *et al.* (2007) reported a rapid decline of dissolved oxygen concentration inside ballast tanks, with 90% of initial oxygen content lost in 10 days at temperatures above 20°C. With shorter voyages, water inside tanks is renewed more frequently by ballasting/deballasting activities and/or by ballast management activities, which may enhance survival of active macroinvertebrates.

Several species recorded during this study are NIS of global concern (ISSG, 2010). For example, the fishhook waterflea *Cercopagis pengoi*, found in two Great Lakes ships, is already established in this system (Laxon *et al.,* 2003). *C. pengoi* is a potent predator and may suppress both invertebrate and vertebrate taxa (Laxon *et al.*, 2003). The European green crab *Carcinus maenas*, found in two ships servicing Atlantic Canadian ports, is a generalist predator that is attributed with the decline of other crab and bivalve species (Grosholz & Ruiz, 1995). Although these two NIS are already established in North America, a risk exists of increasing genetic variation with additional introductions of new individuals (Simberloff, 2009b). Increased genetic variation from continued propagule pressure could lead to production of new genotypes that are better

adapted to the local environment or adjacent areas, potentially enhancing further spread (McCauley, 1991). Other problematic NIS recorded in this study include the crab *Rhithropanopeus harrisii,* ascidian *Botryllus schlosseri,* and bivalve *Mytilus galloprovincialis.*

Numerous vectors operate in all biogeographic regions, with the relative importance of each vector changing through time (Wonham & Carlton, 2005; Ricciardi, 2006). The cumulative colonization and propagule pressure has resulted in a diverse community of established NIS globally. This study indicates that the relative importance of the ballast sediment vector also changes regionally within Canada, currently posing the greatest invasion risk for ports in the Atlantic region. With no active animals recorded, and no viable dormant eggs, this study shows very low invasion risk for the Pacific region from this vector. Furthermore, considering fewer and smaller ships arrive to the Great Lakes than to either Pacific or Atlantic Canadian ports (S. Bailey, unpubl.), I also conclude a relatively low risk of sediment-borne invasions for the Great Lakes.

Current ballast water management activities exert differential impacts by region. I have identified the amount of sediment as the single, most important factor for management of invertebrates and their dormant eggs in ballast sediment. As more sediment in tanks represents disproportionately higher propagule and colonization pressure, a precautionary approach to ballasting should be undertaken with the objective to limit or avoid entrainment of sediments by not loading ballast in turbid or shallow water. This approach, however, can be problematic because of tight schedules and working rules of the dock (Reid *et al.*,

2007). Nevertheless, frequent inspection and manual cleaning of ballast tanks could result in important reductions in colonization and propagule pressure of invertebrates and their dormant eggs in ballast sediment. Future ballast management by technological treatment systems must include means to remove sediments, such as filtration. Enhanced sediment management actions may be required for ships particularly prone to sediment accumulation.

5.5 References

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Table 5.1 Mean (standard error (S.E.)) and median dormant egg density and viability per 40 g subsample of sediment and sediment tonnage per 10000 m^3 of ballast capacity, dormant egg density and viability per 10000 m^3 of ballast capacity, in three regions (Pacific, Great Lakes and Atlantic) of North America, from three ship pathways (transoceanic voyages with mid-ocean exchange (TOE), coastal voyages with mid-ocean exchange (CE) and coastal voyages exempt from mid-ocean exchange (CNE)). Note the difference in scale (10^6 *versus* 10^5) for dormant egg density and viability per 10000 m^3 of ballast capacity, respectively.

Region	Ship pathway	Density·40 g^{-1}		Viability·40 g^{-1}		Sediment·10000 m^3 ballast capacity^{-1} (tonnes)		Density·10000 m^3 ballast capacity^{-1} (x 10^6)		Viability·10000 m^3 ballast capacity^{-1} (x 10^5)	
		Mean (S.E.)	Median	Mean (S.E.)	Median	Mean (S.E.)	Median	Mean (S.E.)	Median	Mean (S.E.)	Median
Pacific	TOE	5.2 (1.8)	1	0	0	2.5 (1.1)	0.5	0.3 (0.1)	0.02	0	0
	CE	17.3 (6.8)	4.2	0	0	1.5 (0.7)	0.5	0.2 (0.08)	0	0	0
	CNE	4.5(2.1)	0	0	0	3.6 (1.8)	0.2	0.1 (0.1)	0	0	0
Great Lakes	TOE	18.4 (7.1)	11.0	1.2 (0.5)	0.4	2.5 (1.3)	0.3	3.4 (2.2)	0.03	1.9 (1.3)	0.01
	CE	33.6 (7.5)	40.5	1 (0.6)	0.4	16.0 (12.4)	6.0	8.6 (6.0)	1.9	0.7 (0.6)	0.03
Atlantic	TOE	129(47.6)	52.1	4.5 (2.4)	0	9.8 (2.0)	7.7	28.6 (14.2)	5.5	5.1 (3.2)	0
	CE	84 (43.2)	16.7	4.9 (4.2)	0	14.3 (3.9)	5.2	13.9 (6.0)	2.3	7.4 (4.1)	0
	CNE	100 (27.2)	48.5	29.8 (25.5)	0	2.1 (1.1)	0.9	7.8 (5.1)	0.5	19.5 (13.5)	0

Table 5.2 Results of nested ANOVAs addressing densities and viability of dormant eggs in 40 g sediment samples collected from three different ship pathways (transoceanic ships performing mid-ocean exchange (TOE), coastal ships performing mid-ocean exchange (CE) and coastal ships exempt from mid-ocean exchange (CNE)) in three regions (Pacific, Great Lakes and Atlantic region) of North America. Significant *P*-values are presented in bold.

Variable	Density			Viability		
	df	*F*	*P*	df	*F*	*P*
Intercept	1	179.04	**0.000**	1	24.846	**0.001**
Region	2	19.123	**0.002**	2	10.437	**0.005**
Region (Pathway)	5	1.672	0.146	5	0.738	0.596

Table 5.3 Results of two-way multivariate analysis of variance (MANOVA) addressing the effect of different regions and different pathways of ships on the amount of sediment, abundance of dormant eggs, and their viabilities. Significant *P*-values are presented in bold.

Variable	df	*F*	*P*
Region			
Univariate *F* tests			
Sediment	2	10.428	**< 0.001**
Abundance of dormant eggs per ship	2	36.696	**< 0.001**
Viability of dormant eggs per ship	2	17.099	**< 0.001**
Multivariate test			
Wilks' lambda = 0.576	6	13.950	**< 0.001**
Ship pathway			
Univariate *F* tests			
Sediment	2	5.273	**0.006**
Abundance of dormant eggs per ship	2	0.839	0.434
Viability of dormant eggs per ship	2	0.223	0.800
Multivariate test			
Wilks' lambda = 0.919	6	1.889	0.083
Interaction			
Univariate *F* tests			
Sediment	3	5.656	**0.001**
Abundance of dormant eggs per ship	3	0.953	0.417

Viability of dormant eggs per ship	3	0.145	0.932
Multivariate test			
Wilks' lambda = 0.847	9	2.527	**0.008**

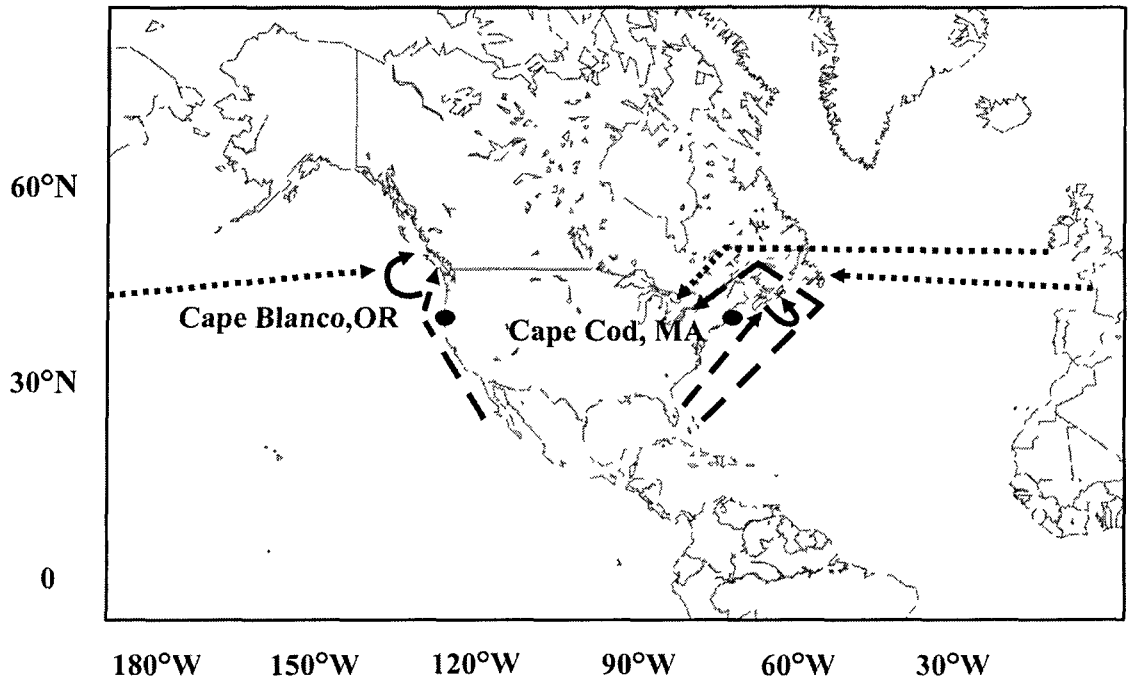

Figure 5.1 Schematic representation of pathways utilized by ships that were sampled in the Pacific, Great Lakes, and Atlantic regions. Dotted lines represent vessels which arrived following transoceanic voyages (TOE) and which conducted mid-ocean exchange (MOE), dashed lines represent vessels on coastal voyages with MOE (CE), and solid lines represent vessels on coastal voyages that were exempt from MOE (CNE). Vessels arriving from south of Cape Blanco, Oregon (OR) or Cape Cod, Massachusetts (MA) are required to conduct ballast water management prior to entry, whereas those arriving from north of these locations are exempt from MOE (Government of Canada, 2006).

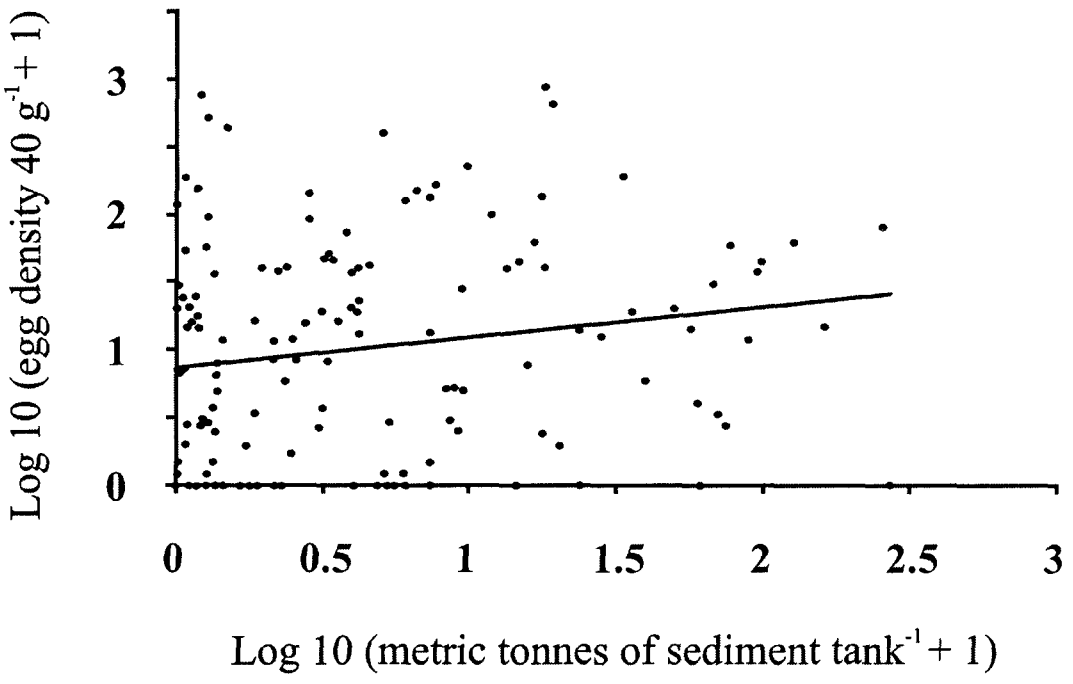

Figure 5.2 Pearson's correlation of the amount of sediment inside tanks and density of dormant eggs in 40 g of sediment. Both data sets are log-transformed.

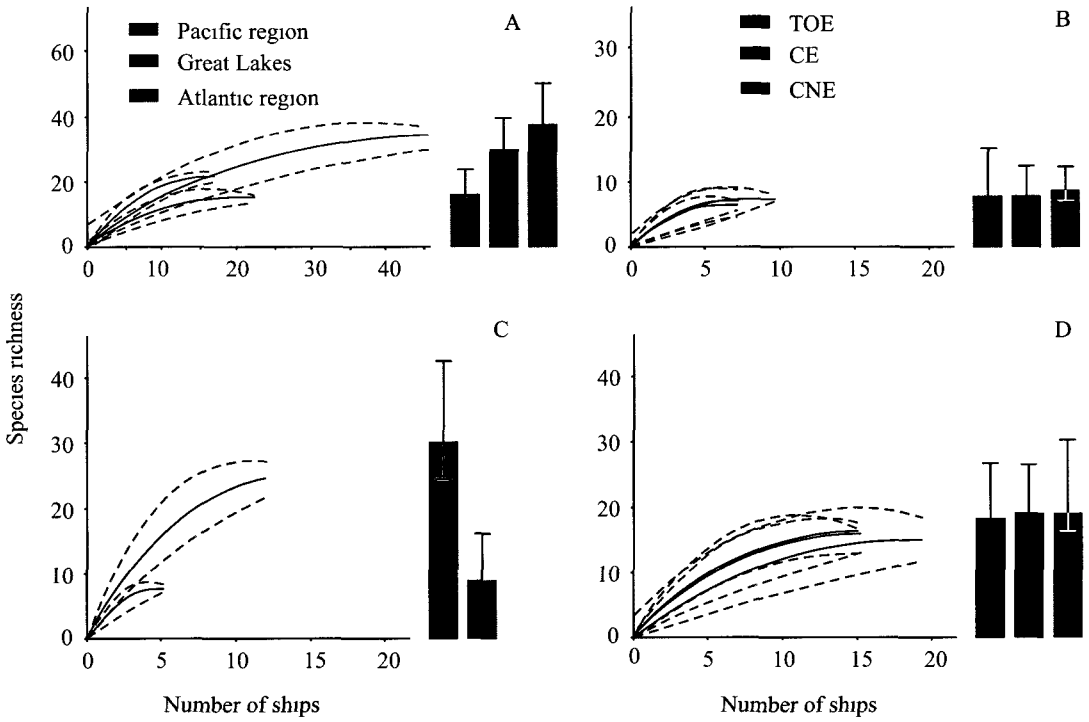

Figure 5.3 Sample-based rarefaction curves (± 95% C.I.) for ships sampled in all regions (A), in the Pacific region (B), in the Great Lakes region (C), and in the Atlantic region (D). Also shown are species richness estimates for the vessel population (1st order jackknife ± 95% C.I.). Note the difference in scales for each x and y axis.

Chapter 6: General discussion

6.1 Invasion risk posed by invertebrates

A common theme underlying this dissertation is that preventing the transport of NIS or reducing the intensity of transported propagules is the first, and perhaps most important, step in dealing with NIS. To most effectively utilize limited resources, managers must be able to quantify the risk of NIS introductions associated with different transport vectors such as aquaculture, aquarium and garden trades, and shipping activities (Bax *et al.,* 2001; Simberloff, 2009). One of the best indicators of invasion risk is "propagule pressure", the frequency and density of NIS introductions into new habitats (Colautti *et al.,* 2006; Hayes & Barry, 2008; Lockwood *et al.*, 2009). Based on two findings: that ships are a leading vector for transportation of NIS in aquatic habitats (Carlton, 1985; Ruiz & Carlton, 2003; Ricciardi, 2006; Molnar *et al.,* 2008) and that ballast sediments may contain large numbers of active invertebrates as well as their viable dormant eggs (Bailey *et al.,* 2005; Duggan *et al.,* 2005), I explored propagule pressure of invertebrates and their dormant eggs transported in ballast sediment of ships arriving at Canadian ports.

An essential but exceedingly difficult aspect of measuring propagule pressure is the correct identification of the NIS associated with each vector (Bax *et al.,* 2001). While traditional taxonomic methods are hampered by difficulties in identifying invertebrates, and particularly their dormant eggs, molecular methods are becoming more readily applicable (Hebert *et al.,* 2003). DNA barcoding, an

approach that employs the sequencing of a small fragment of DNA, typically a short portion of a single gene, to provide a unique identifier has been developed for species identifications (Hebert *et al.,* 2003). However, DNA barcoding has its limitations. For example, the utility of species-specific barcodes may be limited by overlap of genetic variation between closely related species (Meyer & Paulay, 2005; Monaghan *et al.,* 2005), and further by the lack of existing reference sequences in genetic databases (Darling & Blum, 2007). Despite reports of identical COI sequences shared by morphologically distinct invertebrate species, such as two genera of freshwater crabs (Schubart *et al.,* 2008), chapter 2 of this dissertation demonstrated that the use of DNA barcoding is a much more effective approach to identifying potential NIS, and it is faster and cheaper than the traditional taxonomic approach for species identification of invertebrates. By using two markers for each individual egg (i.e. COI and 16S), and two public databases (i.e. GenBank and BOLD), I obtained more sequences and increased the chance of a sequence match, thereby providing high confidence in identifications.

With 90% of world trade transported by ships (Hulme, 2009; IMO, 2010), and ships' ballast water and sediments being leading mechanisms for NIS introductions (Carlton, 1985; Ricciardi, 2006; Molnar *et al.*, 2008), two ballast management regulations, MOE and saltwater flushing, were implemented to reduce the likelihood of new ballast-mediated invasions to Canada (Government of Canada, 2006). As ballast management activities often produce extreme fluctuations in environmental conditions in ballast tanks (i.e. changes in oxygen

concentration from 10 to 1 mg/L and then back to 10 mg/L, and salinities from 0 to 35‰ and then back to 0‰ every several days (Reid *et al.*, 2007)), these activities should theoretically reduce or even completely eliminate the transport of ballast-mediated propagules. The effect of MOE on the active stages of biota (Locke *et al.,* 1993; Rigby & Hallegraeff, 1994; Wonham *et al.,* 2001; Choi *et al.,* 2005; Gray *et al.,* 2007; Humphrey, 2008) as well as on the dormant eggs of invertebrates (Gray & MacIsaac, 2010) has been assessed by multiple studies with varying results. However, there was no assessment of saltwater flushing on either active or dormant stages of invertebrates. Hence, chapter 4 is the first formal assessment of the efficacy of saltwater flushing on dormant eggs. In this chapter, I compared pre- and post-regulation data, and reported a reduction in ballast sediment, and density and viability of invertebrate dormant eggs in sediment of ships entering the Great Lakes after the implementation of the saltwater flushing regulation. While active invertebrates in ballast waters may be managed by MOE (Gray *et al.,* 2007; Humphrey, 2008), chapter 5 of this dissertation showed that invertebrate dormant eggs from ballast sediment still pose a high invasion risk to the Atlantic region in Canada. Ballast management activities may reduce the invasion risk posed by dormant eggs in two ways: by reducing the viability of dormant eggs by exposing them to rapid environmental changes and by physically purging eggs together with suspended sediment from tanks. However, exposure to saltwater during MOE and saltwater flushing seems not to reduce the viability of dormant eggs significantly (Bailey *et al.,* 2004, 2006; Gray & MacIsaac, 2010). Similarly, in chapter 5 of this dissertation, comparative

assessment of 81 tanks, 39 with and 42 without MOE, did not show any reduction in either the propagule or colonization pressure of invertebrate dormant eggs in the coastal areas of Canada. As Sutherland *et al.* (2009) reported positive correlation between suspended particulate matter in tanks and invertebrate abundance and species richness, the main way of reducing propagule pressure inside ballast tanks is almost certainly by physically purging eggs together with sediment from tanks during saltwater flushing. This finding may imply that large differences exist between the efficacies of the two ballast management activities, with saltwater flushing being much more effective in managing propagule pressure of invertebrates' dormant eggs than MOE.

Despite implementation of common, national management practices across Canada (Government of Canada, 2006), chapter 5 of this dissertation indicated that invertebrates and their dormant eggs present in ballast sediment of transoceanic and coastal ships entering Canadian Pacific, Great Lakes and Atlantic ports posed different invasion risks to these different regions of Canada, with the Atlantic region being under the greatest risk. Ships carrying more sediment in their tanks also transported more species (i.e. colonization pressure) at higher abundance (i.e. propagule pressure). The amount of sediment carried by ships may depend on the geography of ship activities (Zhou *et al.*, 1993; European Commission, 2004), the managament regulation applied (i.e. MOE *vs.* saltwater flushing), but it may also be attributed to varying degrees of stringency in the enforcement of regulations in different regions.

Furthermore, in chapter 5, comparative analysis of the amounts of sediment in ballast tanks and the density of dormant eggs in ballast sediment supported the hypothesis that ballast sediment protects dormant eggs. Dormancy is a frequently encountered life stage of invertebrates which ensures survival through periods when active individuals are not able to survive (Cáceres, 1997; Schröder, 2005). At the end of a growing season, or when the environment becomes hostile, most invertebrates produce dormant eggs which typically sink to the bottom of a pond, lake or sea, slowly become covered by sediment, and remain protected until the next growing season or until favourable conditions are restored (Brendonck & De Meester, 2003). Depending on the taxa, dormant eggs are often able to withstand extreme conditions in terms of temperature, oxygen, salinity or drought (Cáceres, 1997; Brendonck & De Meester, 2003; Schröder, 2005).

There are two possible ways for dormant eggs to enter ballast tanks. They may be pumped into tanks together with suspended sediment during the ballasting operation, and/or they may be produced inside ballast tanks by active adults that were pumped into tanks. However dormant eggs enter tanks, if they are not buried in sediment, they may experience adverse environmental conditions (Reid *et al.,* 2007). Some of these conditions are induced by chance (e.g. high levels of H_2S and ammonium and the depletion of oxygen resulting from microbial and/or algal activities) and some are induced by humans (e.g. rapid changes in salinities and temperatures caused by ballast activities) (Reid *et al.,* 2007). As dormant eggs were reported to be able to survive diverse

environmental conditions (Cáceres, 1997; Brendonck & De Meester, 2003; Schröder, 2005), in chapter 3 I followed the degradation of dormant eggs of invertebrates collected in ballast tanks and discovered that, depending on the diverse survival and longevity of dormant eggs of different taxa, there are two patterns of invasion risk posed by invertebrates' dormant eggs. More than 75% of dormant eggs of rotifers and copepods degraded within six months, thus these taxa are unlikely to accumulate inside tanks. Rare observations of high densities of rotifer and copepod dormant eggs in ballast sediments (Bailey *et al.,* 2005; Duggan *et al.,* 2005; this dissertation) could be associated with the uptake of ballast water during seasonal peaks of dormant egg production in particular locations, implying that their dormant eggs pose high invasion risks only occasionally during ideal reproductive seasons. In contrast, the slow degradation rate of anomopod dormant eggs and the lack of degradation of onychopod and bryozoan dormant eggs could result in accumulation inside tanks and consequently a high invasion risk throughout the year.

6.2 Main conclusions and future recommendations

This dissertation demonstrated that DNA barcoding is a rapid and accurate approach to identifying invertebrates and their dormant eggs. There is a possibility for dormant eggs of anomopod, onychopods and bryozoans to accumulate inside ballast tanks. This comparative analysis suggested that vector strength varies in different regions, and the two ballast management regulations differently influence the probability of introductions of NIS *via* dormant eggs.

Ships carrying high amounts of sediment inside their ballast tanks have a high potential for subsequent invasion by invertebrates.

Frequent inspections and cleaning of ballast tanks could result in significant reductions in both colonization and propagule pressure posed by invertebrates and their dormant eggs. The key conclusion of my dissertation is that ships' ballast sediment should be treated beyond current ballast management activities. Ongoing research by scientists working on bacteria, viruses and algae in ships' subvectors (i.e. ballast water, ballast sediment and hull fouling) likewise suggest a need for additional treatment of ships entering Canadian ports (R. Rivkin, J. Lawrence, S. Roy, I. Kaczmarska, C. DiBacco, personal communications).

6.3 References

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Appendices

Chapter 2

Appendix 2.1 List of species identified by morphological methods and DNA barcodes. The maximum identification match (%) of my sequences with those found on BOLD and GenBank is also listed. Match scores ≥ 96 or ≥ 98%, for COI and 16S, respectively, were considered species level identifications (highlighted in bold), while < 96 or < 98%, for COI and 16S, respectively, were considered possible family/order level identifications. GenBank accession numbers for sequences from this study are also provided. # = number of morphologically identified individuals. GL = Great Lakes, EC = east coast of North America.

Ship ID	Morphological identification of animals[1]		DNA barcoding identification of eggs[2]						GenBank accession number	
			BOLD		GenBank					
	Organism	#	Organism	COI %	Organism	COI %	Organism	16S %	COI	16S
GL2	*Daphnia mendotae*	3	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475272	GQ343261
			Daphnia mendotae	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475273	GQ343262
			Daphnia mendotae	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475274	GQ343263
	Daphnia parvula	6					*Daphnia parvula*	**99**		GQ343264
							Daphnia parvula	**99**		GQ343265
							Daphnia parvula	**99**		GQ343266

							Daphnia parvula	**100**		GQ343267
							Daphnia parvula	**99**		GQ343268
							Daphnia parvula	**100**		GQ343269
GL3	*Brachionus calyciflorus*	7	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**100**	GQ475275	GQ343270
			Brachionus calyciflorus	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**98**	GQ475276	GQ343273
	Diaphanosoma brachyurum	3					*Diaphanosoma brachyurum*	**99**		GQ343272
							Diaphanosoma brachyurum	94		
	Daphnia parvula	6					*Daphnia parvula*	**100**		GQ343271
GL4	*Daphnia magna*	45	*Daphnia magna*	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457315	GQ343274
							Moina sp.	95		
							Moina sp.	95		
							Moina sp.	95		
							Moina sp.	95		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	85		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	85		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	83		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	83		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	84		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	84		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	84		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	85		
			Moina cf micrura[3]	86	*Moina cf micrura*[3]	86	*Bosmina longirostris*[3]	85		
			Moina cf micrura	86	*Moina cf micrura*	86				
			Moina cf micrura	86	*Moina cf micrura*	86				
			Moina cf micrura	86	*Moina cf micrura*	85				
			Moina cf micrura	86	*Moina cf micrura*	86				
GL5	*Daphnia pulex*	3	*Daphnia pulex*	**99**	*Daphnia pulex*	**99**	*Daphnia pulex*	**99**	GQ475277	GQ343275
GL8	*Podon intermedius*	1	*Podon intermedius*	**99**	*Podon intermedius*	**99**	*Podon intermedius*	**99**	GQ475278	GQ343276

							Plumatella emarginata	**99**		GQ343277
	Calanoida copepod nauplii	6	*Leptodiaptomus siciloides*	**98**	*Leptodiaptomus siciloides*	**98**			GQ466409	
GL10	*Pleopis polyphemoides*	1	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**100**	GQ475279	GQ343278
	Daphnia pulex	9	*Daphnia pulex*	**99**	*Daphnia pulex*	**100**	*Daphnia pulex*	**99**	GQ466410	GQ343279
			Cercopagis pengoi	**100**	*Cercopagis pengoi*	**100**	*Cercopagis pengoi*	**99**	GQ466411	GQ343280
			Cercopagis pengoi	**98**	*Cercopagis pengoi*	**98**	*Cercopagis pengoi*	**99**	GQ466412	GQ343281
							Bosmina fatalis	**86**		
							Bosmina fatalis	**86**		
							Moina cf micrura	**88**		
							Moina cf micrura	**88**		
GL13	*Daphnia magna*	20	*Daphnia magna*	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457316	GQ343282
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457317	GQ343283
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457318	GQ343284
			Daphnia magna	**100**	*Daphnia magna*	**100**	*Daphnia magna*	**98**	GQ457319	GQ343285
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457320	GQ343286
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457321	GQ343287
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457322	GQ343288
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457323	GQ343289
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457324	GQ343290
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457325	GQ343291
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457326	GQ343292
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457327	GQ343293
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457328	GQ343294
			Daphnia magna	**100**	*Daphnia magna*	**99**			GQ457329	
			Daphnia magna	**100**	*Daphnia magna*	**99**			GQ457330	
	Brachionus calyciflorus	4	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**98**	GQ466413	GQ466404
							Brachionus rubens	86		
							Brachionus rubens	87		
GL14							*Plumatella reticulata*	**98**		GQ343295
							Plumatella emarginata	**99**		GQ343296

							Plumatella casmiana	**100**		GQ343297
			Botryllus schlosseri	**99**	*Botryllus schlosseri*	**99**			GQ457331	
GL15	*Brachionus calyciflorus*	2	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	GQ466414	GQ466405
	Calanoida copepod nauplii	4	*Eurytemora affinis*[4]	86	*Boeckella meteoris*[4]	84				
GL16			*Diaphanosoma brachyurum*[4]	**100**	*Diaphanosoma* sp.[4]	91			GQ924683	
			Diaphanosoma brachyurum[4]	**100**	*Diaphanosoma* sp.[4]	91			GQ924684	
GL17	*Daphnia galeata*	7	*Daphnia galeata*	**100**	*Daphnia galeata*	**99**	*Daphnia galeata*	**99**	GQ457332	GQ466407
							Daphnia sp.	**99**		GQ343298
	Brachionus calyciflorus	6	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**99**	GQ466415	GQ466406
			Brachionus calyciflorus	91	*Brachionus calyciflorus*	91				
GL21			*Cercopagis pengoi*	**100**	*Cercopagis pengoi*	**99**	*Cercopagis pengoi*	**99**	GQ466416	GQ343299
GL22	Calanoida copepod nauplii	8	*Acartia tonsa*	**99**	*Acartia tonsa*	**100**	*Acartia tonsa*	**98**	GQ466417	GQ466408
			Paraeuchaeta rubra[4]	83	*Boeckella meteoris*[4]	83				
			Boeckella poopoensis[4]	83	*Boeckella meteoris*[4]	83				
EC1	Calanoida copepod nauplii	2	Calanoida[4]	89	*Acartia hudsonica*[4]	79				
			Eurytemora affinis[4]	82	*Acartia tonsa*[4]	81				
							Plumatella emarginata	**98**		GQ343300
							Plumatella emarginata	**98**		GQ343301
EC3	*Daphnia magna*	2	*Daphnia magna*	**98**	*Daphnia magna*	**96**	*Daphnia magna*	**98**	GQ457333	GQ343302
EC12	Calanoida copepod nauplii	6	*Eurytemora affinis*[4]	**99**	*Boeckella poppei*[4]	82			GQ924685	

			Eurytemora affinis[4]	**98**	*Boeckella poppei*[4]	82			GQ924686	
			Eurytemora affinis[4]	89	*Paracalanus parvus*[4]	79				
			Eurytemora affinis[4]	88	*Clausocalanus parapergens*[4]	79				
			Eurytemora affinis[4]	89	*Clausocalanus parapergens*[4]	79				
EC18	Calanoida copepod nauplii	7	*Calanus euxinus*	**99**	*Calanus euxinus*	**98**			GQ457334	
			Austrochiltonia sp.[4]	89	*Acartia tonsa*[4]	81				
EC37	*Evadne normanni*	2					*Evadne normanni*	**99**		GQ343303
							Evadne normanni	**99**		GQ343304
							Evadne normanni	**100**		GQ343305
							Evadne normanni	**100**		GQ343306
	Pleopis polyphemoides	1	*Pleopis polyphemoides*	**99**	*Pleopis polyphemoides*	**99**	*Pleopis polyphemoides*	**100**	GQ475280	GQ343307
			Pleopis polyphemoides	**99**	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**100**	GQ475281	GQ343308

[1] Morphological identification of animals was done from hatched eggs, which were the same distinct group based on size and morphology as those used for DNA barcoding.

[2] The same egg is used for both markers (COI and 16S).

[3] Divergence from closest match exceeded 4 or 2% for COI and 16S, respectively, and different markers revealed different closest matches (16S: *Bosmina longirostris* and COI: *Moina cf micrura*).

[4] Divergence from closest match exceeded 4%, and different public database (BOLD and GenBank) revealed different closest matches.

Chapter 4

Appendix 4.1 List of invertebrate taxa identified during pre-regulation and post-regulation studies. Occurrence lists number of ships that the species was collected on, from a possible 17. Abundance lists the range (median) number of individuals identified from 40 g sediment for all ships. Pre-regulation data are modified from Bailey *et al.* (2005).

Taxon	Pre-regulation period		Post-regulation period	
	Occurrence	Abundance	Occurrence	Abundance
Gastrotricha				
Chaetonotidae, unidentified	1	3		
Rotifera				
Ascomorpha ecaudis Perty, 1850	1	0.25		
Ascomorpha saltans Bartsch, 1870	1	0.25		
Ascomorpha sp.	1	1		
Asplanchna brightwelli Gosse, 1850	4	0.25-1 (0.75)		
Asplanchna girodi (De Geurne, 1888)	2	0.5 (0.5)		

Asplanchna priodonta Gosse, 1850	3	1-11.5 (3)		
Brachionus angularis Gosse, 1851	21	0.25-21.8 (4)		
Brachionus bennini Leissling, 1924	1	0.25		
Brachionus budapestinensis Daday, 1885	15	0.75-341.5 (3)		
Brachionus calyciflorus Pallas, 1766	25	0.63-77.77 (3)	4	0.25-1.75 (0.5)
Brachionus caudatus Barrois and Dadai, 1894	2	0.5-2 (1.25)		
Brachionus diversicornis (Daday, 1883)	1	0.25		
Brachionus forficula Wierzejski, 1891	1	1		
Brachionus havanaënsis Rousselet, 1911	2	1 (1)		
Brachionus leydigi Cohn, 1862	4	0.25-1 (0.78)		
Brachionus nilsoni Ahlstrom, 1940	1	1		
Brachionus quadridentatus Hermann, 1783	4	0.5-12.25 (1.25)		
Brachionus urceolaris Muller, 1773	9	0.25-78 (1)		
Brachionus sp.			2*	0.25-0.5 (0.37)

Cephalodella catellina (Muller, 1786)	2	0.25 (0.25)
Cephalodella forficula (Ehrenberg, 1831)	1	0.25
Cephalodella cf. stenroosi Wulfert, 1937	1	0.3
Cephalodella sterea (Gosse, 1887)	1	4.75
Cephalodella cf. theodora Koch-Althaus, 1961	1	0.25
Cephalodella sp.	1	1
Conochilus coenobasis (Skorikov, 1914)	1	0.5
Conochilus dossuarius Hudson, 1885	1	1
Conochilus hippocrepis (Schrank, 1830)	2	1 (1)
Conochilus cf. natans (Seligo, 1900)	1	0.25
Conochilus unicornis Rousselet, 1892	1	0.8
Dicranophoridae, unidentified	1	83
Euchlanis cf. dilatata Ehrenberg, 1832	2	0.25-1 (0.63)
Filinia brachiata Rousselet, 1901	1	0.25

Filinia cornuta (Weisse, 1847)	3	0.5-1 (0.5)
Filinia longiseta (Ehrenberg, 1834)	6	0.25-4 (1)
Filinia passa (Muller, 1786)	4	0.25-1 (0.75)
Filinia terminalis (Plate, 1886)	5	0.38-2.5 (1)
Floscularidae, unidentified	1	0.25
Hexarthra intermedia Wiszniewski, 1929	1	0.25
Hexarthra mira (Hudson,1871)	3	0.25-1 (1)
Keratella cochlearis Gosse, 1851	3	0.25-1 (1)
Keratella quadrata (Muller, 1786)	5	0.25-4 (0.5)
Keratella tropica (Apstein 1907)	1	2
Keratella sp.	1	1
Lacinularia sp.	1	0.25
Lecane closterocerca (Schmarda, 1853)	2	0.3-0.5 (0.4)
Lecane flexilis (Gosse, 1886)	1	0.25

Lindia truncata (Jennings, 1894)	1	0.5
Ploesoma truncatum (Levander, 1894)	3	0.25-2 (2)
Polyarthra dolichoptera Idelson, 1925	9	0.5-5 (1)
Polyarthra vulgaris Carlin, 1943	6	0.25-21 (2)
Polyarthra spp.	2	0.25-1 (0.63)
Pompholyx sulcata Hudson, 1885	4	0.25-7 (3.5)
Synchaeta bacillifera Smirnov, 1933	1	2.25
Synchaeta baltica Ehrenberg, 1834	1	2.75
Synchaeta kitina Rousselet, 1902	1	0.25
Synchaeta oblonga Ehrenberg, 1832	1	0.25
Synchaeta stylata Wierzejski, 1893	4	0.25-1 (0.28)
Synchaeta tremula (Muller, 1786)	2	1-3.5 (1)
Synchaeta sp.	1	0.25
Trichocerca multicrinis (Kellicott, 1897)	1	39

Trichocerca pusilla (Jennings, 1903)	7	1-17.63 (1.25)		
Trichocerca rattus (Muller, 1776)	1	1		
Trichocerca similis (Wierzejski, 1893)	1	0.25		
Monogonont, unidentified	2	1 (1)		
Bryozoa				
Plumatella casmiana Oka, 1907	2	0.25-1 (0.63)	1*	0.25
Plumatella emarginata Allman, 1844			2*	0.25 (0.25)
Plumatella reticulata Wood, 1988			1*	0.25
Plumatella sp.	1	0.25		
Anomopoda				
Alona rectangula Sars, 1861	1	0.5		
Alona rustica Scott, 1895	1	0.25		
Bosmina liederi De Melo and Hebert, 1994	3	1-6 (1)		
Bosmina maritima (Muller, 1867)	1	2		

Bosmina spp.	2	1 (1)	1*	0.5
Ceriodaphnia quadrangula (Muller, 1785)	1	0.25		
Ceriodaphnia sp.	2	1 (1)		
Daphnia longiremis Sars, 1861	2	1 (1)		
Daphnia magna Straus, 1820	4	0.5-2 (1)	2	0.25-11.25 (4.37)
Daphnia retrocurva Forbes, 1882	1	2		
Daphnia mendotae Birge, 1918			1	0.75
Daphnia parvula Fordyce, 1901			2	0.25-1.5 (1.5)
Daphnia pulex (De Geer, 1778)			2	0.25-2.25 (0.5)
Daphnia galeata Sars, 1864			1	2
Daphnia sp.			1*	0.25
Disparalona leei (Chien, 1970)	1	0.25		
Moina micrura Kurz, 1874	2	1-47.88 (24.44)		
Moina sp.	1	1	2*	0.5-1 (0.75)

Anomopoda, unidentified			1*	3.25
Ctenopoda				
Diaphanosoma birgei Korinek, 1981	2	0.75-6 (3.38)		
Diaphanosoma brachyurum (Lievin, 1848)	1	0.25	2	0.25-0.75 (0.5)
Diaphanosoma mongolianum Ueno, 1938	1	0.5		
Diaphanosoma orghidani Negrea, 1982	1	1.25		
Diaphanosoma sarsi Richar, 1894	1	0.25		
Diaphanosoma spp.	6	1 (1)	1*	0.25
Onychopoda				
Evadne nordmanni Loven, 1836	1	0.5		
Cercopagis pengoi (Ostroumov, 1891)			2*	0.25-0.5 (0.37)
Pleopis polyphemoides (Leuckart, 1859)			1	0.25
Podon intermedius Lilljeborg, 1853			1	0.25
Copepoda				

Acanthocyclops robustus (Sars, 1863)	1	0.8		
Cyclopoida, unidentified	3	0.25-1.25 (0.25)		
Nitocra lacustris (Shmankevich, 1875)	1	1		
Leptodiaptomus siciloides (Lilljeborg in Guerne and Richard, 1889)			1*	0.25
Acartia tonsa Dana, 1849			1*	0.25
Copepoda, unidentified	14	0.25-20 (3)	2	0.25-2 (1)
Ascidia				
Botryllus schlosseri (Pallas, 1766)			1*	0.25

* taxa that did not hatch in post-regulation hatching experiments.

Chapter 5

Appendix 5.1 Sampling scheme for three regions: Pacific, Great Lakes and Atlantic, and three ship pathways: transoceanic exchanged ships (TOE), coastal exchanged ships (CE), and coastal not exchanged ships (CNE).

	Pacific region				Great Lakes region			Atlantic region			
	Total	TOE	CE	CNE	Total	TOE	CE	Total	TOE	CE	CNE
# of ships sampled	60	20	19	21	17	13	4	58	22	20	16
# of tanks sampled	60	20	19	21	19	14	5	63	22	20	21
# of ships with two tanks sampled					2	1	1	5			5
# of tanks with four spatial samples sampled					6	6					

Appendix 5.2 List of invertebrate taxa identified in this study and arranged taxonomically. Egg taxa were identified directly from dormant eggs using mitochondrial markers COI and 16S following Briski *et al.* (2010, 2011), and morphologically after eggs hatched. Live animals were identified using mitochondrial markers COI and 16S and morphologically. Occurrence lists number of ships that the species was collected on, from a possible 135. Abundance lists the range (median) number of egg identified from 40 g sediment or range (median) number of active macroinvertebrates per tank (*).

Taxon	Pacific region		Great Lakes		Atlantic region	
	Occurrence	Abundance	Occurrence	Abundance	Occurrence	Abundance
Porifera						
Ephydatia fluviatilis	1[1]	0.25				
Cnidaria						
Actinaria, unidentified					1[3]	1*
Rotifera						
Asplanchna sp.	7[3]	0.25-1 (0.25)			5[1,2]	0.25-14.5 (1.75)

Brachionus calyciflorus	2[1]	0.25-3.25 (1.75)	8[1,2]	0.25-43.25 (11.75)		
Brachionus plicatilis					18[1,2,3]	0.25-25.5 (4.87)
Brachionus sp.			2[1,2]	0.25-0.5 (0.37)		
Synchaeta sp.	6[1,2]	0.25-7.25 (2.25)			13[1,2,3]	0.25-6.75 (0.25)
Bryozoa						
Hyalinella punctata					1[2]	4
Plumatella casmiana			2[1]	0.25 (0.25)		
Plumatella emarginata			3[1,2]	0.25-2.5 (0.25)	28[1,2,3]	0.25-12.25 (1.5)
Plumatella reticulata			1[1]	0.25		
Pectinatella sp.	8[1,2,3]	0.25-4 (0.25)				
Anomopoda						
Bosmina freyi	3[2]	0.25-8.5 (6.25)				
Bosmina sp.			1[1]	0.25	6[2]	0.25-8.5 (0.62)
Ceriodaphnia dubia	3[2]	3-46.8 (4.255)				

Daphnia magna			2[1,2]	0.25-51.5 (25.87)	11[1,2,3]	0.25-21 (2.25)
Daphnia retrocurva	5[1]	0.25-2.25 (0.25)				
Daphnia mendotae			1[1]	1.75		
Daphnia parvula			2[1]	0.25-1.5 (0.87)		
Daphnia pulex			2[1,2]	0.25-2.25 (1.25)		
Daphnia galeata			1[1]	2	2[2]	1.5-4 (2.8)
Daphnia cucullata					1[1]	1.8
Daphnia sp.	7[1,2]	0.25-17.5 (5.75)	1[1]	0.25		
Ilyocryptus sp.					1[2]	3
Moina sp.			2[1]	0.5-1 (0.75)	1[2]	1
Anomopoda, unidentified			1[1]	70.25		
Ctenopoda						
Diaphanosoma brachyurum			2[1]	0.25-0.75 (0.5)		
Diaphanosoma sp.			1[1]	0.25	3[1]	1-2 (1.75)

Onychopoda						
Evadne nordmanni					19[1,3]	0.25-136.5 (9.75)
Cercopagis pengoi			2[1]	0.25-0.5 (0.37)		
Pleopis polyphemoides	9[3]	0.25-7 (0.75)	1[1]	0.25	2[2]	2-9.75 (5.88)
Podon intermedius			1[2]	0.25		
Copepoda						
Acartia tonsa			1[1]	0.25		
Acartia omorii	1[3]	17.75				
Acartia pacifica	2[2,3]	6-116.75 (61.37)				
Calanus euxinus					4[1]	54-194 (157.88)
Ctenocalanus vanus	1[3]	26.75				
Diacyclops thomasi			1[1]	31.5		
Eurythemora affinis					8[1,2]	1.75-652.5 (87.12)
Leptodiaptomus siciloides			1[2]	5		

Calanoida, unidentified	25[1,2,3]	0.25-45 (4.25)	11[1,2]	0.75-41.5 (7.5)	38[1,2,3]	0.25-872.25 (20.87)
Harpacticoida, unidentified					9[2,3]	5.5-384 (14)
Amphipoda						
Americorophium sp.					1[1]	11
Eurythenes sp.	1[2]	5				
Parhyale sp.	1[3]	1				
Decapoda						
Carcinus maenas					2[3]	2*
Rhithropanopeus harrisii					1[2]	1*
Annelida						
Neanthes virens					1[3]	1*
Platynereis sp.					2[2]	2-3 (2.5)*
Gastropoda						
Littorina Littorea					1[3]	2*

Bivalvia						
Hyotiss numisma					2[2,3]	1-2 (1.5)*
Mya arenaria					4[1,2,3]	1*
Mytilus galloprovincialis					1[3]	1*
Scapharca sp.	1[3]	1.25				
Ascidia						
Botryllus schlosseri			1[1]	0.25		

[1] Transoceanic ship (TOE)

[2] Coastal ship performed mid-ocean exchange (CE)

[3] Coastal ship exempt from mid-ocean exchange (CNE)

Appendix 5.3 List of nonindigenous species (NIS) identified in this study, arranged taxonomically. Occurrence lists the number of ships of the 135 that were sampled that each species was collected from. Abundance lists the range (median) number of dormant eggs identified from 40 g sediment or range (median) number of active macroinvertebrates per tank (*). Sampled NIS capable of tolerating ambient salinity in the recipient port are identified in bold.

Taxon	Pacific region		Great Lakes		Atlantic region	
	Occurrence	Abundance	Occurrence	Abundance	Occurrence	Abundance
Bryozoa						
Hyalinella punctata					1[2]	4
Plumatella emarginata					28[1,2,3]	0.25-12.25 (1.5)
Anomopoda						
Bosmina freyi	3[2]	0.25-8.5 (6.25)				
Ceriodaphnia dubia	3[2]	3-46.8 (4.255)				
Daphnia magna			**2[1,2]**	**0.25-51.5 (25.87)**	11[1,2,3]	0.25-21 (2.25)
Daphnia retrocurva	5[1]	0.25-2.25 (0.25)				

Daphnia galeata	**1**[1]	**2**	2[2]	1.5-4 (2.8)
Daphnia cucullata			1[1]	1.8
Onychopoda				
Cercopagis pengoi	**2**[1]	**0.25-0.5 (0.37)**		
Pleopis polyphemoides	1[1]	0.25		
Podon intermedius	1[2]	0.25		
Copepoda				
Acartia tonsa	**1**[1]	**0.25**		
Calanus euxinus			**4**[1]	**54-194 (157.88)**
Decapoda				
Carcinus maenas			**2**[3]	**1***
Gastropoda				
Littorina Littorea			**1**[3]	**2***
Bivalvia				

Hyotiss numisma		**2**[2,3]	**1-2 (1.5)***
Ascidia			
Botryllus schlosseri	1[1]	0.25	

[1] Transoceanic ship (TOE)

[2] Coastal ship performed mid-ocean exchange (CE)

[3] Coastal ship exempt from mid-ocean exchange (CNE)

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