DETECTION AND QUANTIFICATION OF AN ECOLOGICALLY IMPORTANT MARINE PATHOGEN, *PARAMOEBA INVADENS*

by

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ABSTRACT

A pathogenic amoeba, *Paramoeba invadens,* causes re-current mass mortalities of sea urchins (*Strongylocentrotus droebachiensis*) in coastal Nova Scotia, with major ecological consequences for the structure of the rocky subtidal ecosystem. Outbreaks of this disease (termed paramoebiasis) are sporadic, and due to a lack of reliable monitoring tools, the source population and epizootiology of this species is poorly understood. I developed and validated a PCR-based assay to detect and/or quantify *P. invadens* in sea urchin tissue, sediment, and seawater. Primers specific to *P. invadens* were designed to the nuclear SSU rRNA gene and were used in PCR and qPCR analyses to detect and quantify abundances of *P. invadens* during, following, and in absence of a natural disease outbreak. Using these methods, *P. invadens* was reliably detected in sea urchin tissue and a comparison of pathogen load (cells mg^{-1} tissue) in "asymptomatic" and "symptomatic" sea urchins indicated a minimum load of ~ 1 cell mg^{-1} tissue for observing overt signs of paramoebiasis in sea urchins. *Paramoeba invadens* was detected for the first time in sediment during and following an outbreak of disease. It also was detected in seawater collected in fall 2015 in the absence of sea urchin mortality or a strong storm event, when consequently *P. invadens* was not expected to be present. Abundance (cells l^{-1}) of *P. invadens* in seawater appeared to be associated with peaks in seawater temperature, but was low overall (range 0 – 9.74 cells l^{-1}), and possibly below the level required to encounter and infect small, localized populations of sea urchins. Evidence of a possible annual presence of *P. invadens* in Nova Scotia may shed new light on mechanisms of introduction, spread, and persistence of *P. invadens* along this coast and the role of large-scale meteorological events in these processes.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviation	Description
AGD	Amoebic Gill Disease
bp	Base pair(s)
BSA	Bovine Serum Albumin
cm	centimeter(s)
Ct	Critical Threshold
d	day(s)
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dTTP	Deoxythymidine Triphosphate
e.g.	for example
EtOH	Ethanol
Expt	Experiment
g	gram(s)
h	hour(s)
i.e.	that is
ITS	Internal Transcribed Spacer
km	kilometer(s)
l	liter(s)
LB	Lysogeny Broth
LI	Luke Island
LSU	Large Subunit
m	meter(s)
mg	milligram(s)
$MgCl_2$	Magnesium Chloride
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
mmHg	millimeter of mercury
MWW	Mann-Whitney-Wilcoxon
ng	nanogram(s)
nM	nanomolar
NN	Non-nutrient

Abbreviation	Description	
NTC	No-template Control	
R^2	Regression coefficient	
rDNA	Ribosomal Deoxyribonucleic Acid	
rpm	Rotations per minute	
RNA	Ribonucleic Acid	
rRNA	Ribosomal Ribonucleic Acid	
PCR	Polymerase Chain Reaction	
qPCR	Real-time Quantitative Polymerase Chain Reaction	
s	second(s)	
SP	Splitnose Point	
SSU	Small Subunit	
TAE	Tris-acetate-EDTA	
TL	The Lodge	
U	unit	
μg	microgram(s)	
μl	microliter(s)	
μm	micrometer(s)	
μM	micromolar	
wk	week(s)	
+ ve	Positive	
- ve	Negative	
%	Percent	

Symbol	Description	Units
g_0	Gravity of Earth (9.81)	$m\ s^{-2}$

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CHAPTER 1

INTRODUCTION

Coastal ecosystems are highly important components of the marine realm and are heavily relied upon by nearby economies and societies, as they provide valuable ecosystem services such as protection from coastal erosion, increased tourism revenue, and an important habitat for commercially important finfish and invertebrate species (Staudinger et al. 2012, Burge et al. 2014). Outbreaks of pathogens that cause epizootics play a critical role in shaping coastal marine ecosystems and have been documented to heavily influence community dynamics in various ecosystems including eelgrass beds (reviewed by Burge et al. 2013), coral reefs (reviewed by Done 1992), and sea urchin barrens (Scheibling 1986). Despite the critical role that marine pathogens can play in shaping natural ecosystems, examining the etiology and epizootiology of pathogens in the marine environment is often a difficult task and thus they are not well understood in most systems where infectious diseases occur (Harvell et al. 2002, Burge et al. 2014). Due to a predicted increase in the frequency of disease outbreaks as a direct consequence of continual ocean warming and other aspects of climate change (Harvell et al. 2002), understanding the dynamics governing infectious diseases in important marine macrobiota is becoming an area of increasing interest to scientists and managers of ecosystems (Burge et al. 2014).

Causative agents of infectious diseases are often associated with bacteria and viruses, however in the marine environment, there are many instances in which microbial eukaryotes cause disease and mass mortality. For example, *Labyrinthula zosterae,* a slime-net labyrinthulomycete, is the etiological agent of wasting eelgrass disease that causes massive mortality in eelgrass meadows along the Atlantic coast of North America and in Europe (Muehlstein 1989, Muehlstein et al. 1991), and *Spironucleus salmonicida,* formerly known as *Spironucleus barkhanus* (Jorgensen & Sterud 2006), is an anaerobic flagellate that causes infection and mortality in populations of farmed fish, including Atlantic salmon (Mo et al. 1990), Arctic char (Sterud et al. 2003), and Chinook salmon (Kent et al. 1992). One group in particular that includes several (suspected) marine

pathogens is the *Paramoeba-Neoparamoeba* assemblage of Amoebozoan lobose amoebae, which has been recently proposed on phylogenetic grounds to represent a single genus, *Paramoeba* (Feehan et al. 2013). For example, *Paramoeba/Neoparamoeba perurans*, is the causative agent of amoebic gill disease (AGD), causing infection and mortality in Atlantic salmon (Young et al. 2007), and *Paramoeba/Neoparamoeba pemaquidensis* has been suggested as the causative agent of paramoebiasis in lobsters, resulting in mass mortalities of lobsters in Western Long Island Sound, USA (Mullen et al. 2005). Despite it being difficult to determine the etiology and epizootiology of marine pathogens, recent studies focusing on monitoring populations of *P. perurans* in the water column at Atlantic salmon farms using molecular tools (Douglas-Helders et al. 2003, Wright et al. 2015) have highlighted the potential for the detection and monitoring of pathogenic marine paramoebae in the environment outside of a diseased host.

Along the Atlantic coast of Nova Scotia, the rocky subtidal ecosystem is heavily impacted by recurrent outbreaks of a disease of the sea urchin *Strongylocentrotus droebachiensis*, which is caused by the amoeba *Paramoeba invadens* (reviewed by Scheibling et al. 2013). Outbreaks of this disease, termed 'paramoebiasis', cause mass mortalities of sea urchins and facilitate shifts in marine community structure from stable sea urchin barrens to highly productive kelp beds (Scheibling 1986). Kelp beds are the desired ecosystem state along Nova Scotia due to their high productivity, biodiversity, and the important ecosystem services they provide, however recurrent outbreaks of *P. invadens* have also had dramatic negative consequences for the sea urchin fishery along this coast (reviewed by Scheibling et al. 2013). Outbreaks of *P. invadens* appear to be associated with seawater temperature and the passing of large scale meteorological events, suggesting that *P. invadens* is an exotic species to this coast (Scheibling & Stephenson 1984, Scheibling & Lauzon-Guay 2010), however little is known about the source population(s) and transmission dynamics of this pathogen. Given that *P. invadens* is the only known agent to facilitate shifts from sea urchin barrens to kelp beds in Nova Scotia, it is important to understand the epizootiology of this disease in order to predict and manage the long-term persistence of kelp beds along this coast (Feehan et al. 2012). Furthermore, outbreaks of this pathogen appear to be localized to the coast of Nova Scotia and have not yet been observed to affect nearby sea urchin populations in the Gulf

of Maine (Mullen et al. 2005), where the introduction of this pathogen may be limited by physical ocean conditions and processes (Feehan et al. 2012) and the Gulf of St. Lawrence (Gagnon et al. 2004, Steneck et al. 2004) where colder seawater temperatures may limit the persistence and spread of this pathogen (Feehan et al. 2012). This makes it critically important to understand the dynamics governing this disease in nature in order to predict possible range expansions of this pathogen with changes in oceanographic processes (i.e. frequency and intensity of large storms, changes in ocean circulation and currents) and warming seawater temperature, with continual climate change (Hoegh-Guldberg & Bruno 2010, Feehan et al. 2012).

The overarching goal of my thesis was to develop and validate the use of a molecular assay to detect and monitor the presence and abundance of *Paramoeba invadens* in sea urchin tissues and the environment, with the long-term goal of providing the tools necessary to increase our understanding of the source population(s) and transmission dynamics of this species. In Chapter 2, I developed and optimized polymerase chain reaction (PCR) and quantitative real-time polymerase chain reaction (qPCR) assays to reliably detect *P. invadens* in sea urchin radial nerve tissue, sediment, and seawater. In Chapter 3, I validated the use of this assay by monitoring for *P. invadens* along the Atlantic coast of Nova Scotia in two consecutive years, both during and in the absence of an outbreak of disease.

CHAPTER 2

DEVELOPMENT OF A SPECIES-SPECIFIC PCR/QPCR ASSAY FOR

DETECTION AND QUANTIFICATION OF *PARAMOEBA INVADENS*

2.1. INTRODUCTION

Paramoeba invadens is the etiological agent of paramoebiasis in the green sea urchin *Strongylocentrotus droebachiensis* along the Atlantic coast of Nova Scotia (Jones 1985, Jones & Scheibling 1985). External symptoms of paramoebiasis include the loss of tube feet function, dishevelment and loss of spines, and a discoloured and gaping peristome (Scheibling & Stephenson 1984). The exact mechanism (physiological or chemical) by which *P. invadens* causes mortality in sea urchins is unknown, however histopathological studies have shown the degradation of muscle and connective tissue of the tube feet and spine bases in infected sea urchins (Jones et al. 1985). Paramoebiasis in sea urchins is likely dependent on pathogen load (i.e. the abundance of *P. invadens* in sea urchins must pass some critical threshold before paramoebiasis is observed), and water-borne transmission experiments have shown that sea urchins may quickly succumb to disease once exposed to diseased conspecifics (time to greater than 95 % mortality at 16 °C is 9 – 12 d) (Scheibling & Stephenson 1984, Feehan et al. 2012).

First observed in the early 1980s, paramoebiasis has caused recurrent widespread mass mortalities of sea urchins in Nova Scotia, reducing population biomass by up to 99 % (Miller & Colodey 1983). Mortality events play a major role in driving the transition between alternative stable states of urchin-dominated barrens and highly productive kelp beds (Scheibling 1986), and have a devastating effect on commercial sea urchin fishing operations in Nova Scotia (reviewed by Scheibling et al. 2013). Disease events in Nova Scotia are sporadic in nature and remain largely unpredictable in their magnitude and timing. With such uncertainty, it is important to understand the dynamics of this disease in the marine environment, including how the pathogen is introduced, where the source population(s) resides, and whether *P. invadens* has the ability to become a resident pathogen along this coast as the climate changes.

Knowing the presence and abundance of *P. invadens* in the environment and in sea urchin tissue is critical in understanding the transmission dynamics of this pathogen in coastal waters. The current method used to isolate and identify infection by *P. invadens* involves dissecting the radial nerves of infected sea urchins, establishing cultures of *P. invadens* on bacterial prey, and, for certainty, sequencing a section of the SSU rRNA gene of this species (Jones & Scheibling 1985, Feehan et al. 2013). Although this culture-based method has proven successful, it is time-expensive and restricted in its application: current methods are limited to detection of *P. invadens* in sea urchins exhibiting signs of paramoebiasis (Jellett & Scheibling 1988, Jellett et al. 1989). *P. invadens* is widespread and in low abundance within radial nerve and water vascular tissues of "symptomatic" sea urchins (Jones et al. 1985), likely making it difficult to detect this pathogen in "asymptomatic" urchins where *P. invadens* is expected to be present in even lower abundances. (The terms symptomatic and asymptomatic used here refer to sea urchins exhibiting clinical signs of disease and sea urchins with subclinical infections, respectively. For simplicity and to remain consistent with the terminology used in previous studies, the terms symptomatic and asymptomatic are used throughout this thesis). Meanwhile, attempts to isolate *P. invadens* from seawater and sediment samples using filtration and culturing methods have been limited to one study from the 1980s, and proved unreliable (Jellett et al. 1989, Jellett et al. 1990). Jellett et al. (1989) isolated organisms identified as *P. invadens* from surface and bottom-water seawater samples collected 0, 30, and 60 m away from an outfall pipe releasing treated effluent from Dalhousie University's Aquatron facility. The identification was made through microscopic observation of a kinetoplastid endosymbiont referred to as a parasome (Dykova et al. 2003); but this is a diagnostic feature of the genera *Paramoeba* and *Neoparamoeba* (Page 1987), not of *P. invadens* in particular. Results showed that isolation of *P. invadens* using this method was not consistent among all sampling sites and when an isolate originating from a single amoeba cell was injected into healthy sea urchins, paramoebiasis was not observed. Although it has been suggested that virulence varies between individual *P. invadens* cells released via the Aquatron effluent, it is also possible that the amoeba culture was another species of *Paramoeba*/*Neoparamoeba*. For example, *Paramoeba*/*Neoparamoeba pemaquidensis* and *Paramoeba*/*Neoparamoeba*

aestuarina, have been found around the world in a range of coastal marine habitats (Page 1973, Cann & Page 1982) and *Paramoeba atlantica* has been isolated from sandy bottom sediments at 270 m depth in the eastern Atlantic Ocean (Kudryavtsev et al. 2011). Jellett et al. (1990) also isolated *P. invadens* from sediment collected from an aquarium with *P. invadens*-infected sea urchins, however attempts to isolate *P. invadens* from sediment collected from the outfall sites were unsuccessful (Jellett et al. 1990). The inability to isolate *P. invadens* from sediment and the questionable accuracy of methods used to isolate and identify *P. invadens* from seawater, highlights the need for more reliable methods for detecting this pathogen. This has left the presence of *P. invadens* in the environment heavily under-explored.

Traditionally, identification of marine pathogens and diseases in hosts was accomplished through culturing, morphological identification, and histological examination. More recently however, the development of molecular techniques has presented the opportunity for more rapid detection of pathogens within hosts and in the environment (Cunningham 2002). The polymerase chain reaction (PCR) and derived approaches such as the real-time quantitative polymerase chain reaction (qPCR) are tools that have been widely used to diagnose diseases and detect pathogens in marine systems. For example, PCR-based techniques have been used to detect and/or quantify other ecologically important paramoebids such as *Paramoeba/Neoparamoeba perurans*, the causative agent of amoebic gill disease (AGD) in finfish (Young et al. 2008, Bridle et al. 2010, Fringuelli et al. 2012) and *Paramoeba/Neoparamoeba pemaquidensis*, the suggested agent of paramoebiasis in lobsters (Mullen et al. 2005). Molecular techniques such as PCR and real-time quantitative PCR are advantageous in that they can be designed to reliably detect a single species, they are highly sensitive, they can be used on crude and degraded DNA samples, and they are time-efficient (Rapley 1998). Consequently, they can be used on mixed DNA samples, usually eliminating the need for cultivation or complicated enrichment techniques.

The nuclear ribosomal RNA cistron, and especially the SSU rRNA gene, is the most widely used sequence region for the identification of micro-organisms (Pawlowski et al. 2012). For example, using predominantly SSU rRNA and some LSU rRNA gene sequences, Young et al. (2007) identified a previously undescribed species, *P. perurans*,

as the predominant etiological agent of AGD in finfish, separate from the previously suggested agents *P. pemaquidensis* and *Paramoeba/Neoparamoeba branchiphila*. Additionally, the putative agent of paramoebiasis in Western Long Island Sound lobsters was identified as *P. pemaquidensis* using SSU rRNA gene sequences (Mullen et al. 2005) and more recently, Feehan et al. 2013 sequenced the SSU rRNA gene of two *P. invadens* strains to establish that *P. invadens* was indeed a distinct species from *P. pemaquidensis* (Feehan et al. 2013). Advantages of this region that make it suitable for species identification and detection include its wide-spread presence in eukaryotes (making it widely applicable for species comparisons), a high genomic copy number (typically allowing for the identification of single cells), and the presence of interspersed highly conserved and polymorphic regions (allowing for the design of both universal primers, and group- or species-specific primers; Pawlowski et al. 2012). Additional regions that might be used to distinguish species with identical or near-identical SSU rRNA gene sequences are the internal transcribed spacer regions of the ribosomal RNA cistron ITS1 and ITS2 (Coleman & Vacquier 2002). The rapid evolution of these regions results in a high degree of variability among closely related species (White et al. 1990), making them possible candidate regions for species-specific primer design. In addition to these regions, the presence of a parasome in species from the genera *Paramoeba* and *Neoparamoeba* provides the advantage of having two nuclear SSU and ITS regions present for species-specific primer design (Dykova et al. 2003).

The aim of this study was to design and optimize PCR- and qPCR-based methods to detect and quantify an ecologically important pathogen, *P. invadens*, in sea urchin tissue as well as in sediment and seawater. This study identifies PCR primers that are specific for *P invadens*, and in combination with DNA extraction protocols, identifies suitable procedures for the detection and quantification of this species.

2.2. Materials and Methods

2.2.1. Sequencing *P. invadens* and Parasome ITS Regions

To test for genetic variation between strains and to characterize a possible region for primer design, the internal transcribed spacer (ITS) regions of both the *P. invadens* nuclear genome and its parasome nuclear genome were sequenced. Genomic DNA was extracted from 4 isolates of *P. invadens* collected in 2011 (Feehan et al. 2013) and 3 collected in 2012 (C.J. Feehan unpublished data) (Table 2.1.). DNA was extracted using the DNeasy Blood & Tissue Kit, following the manufacturer's protocol (Qiagen, Hilden, Germany). The ITS region of the *P. invadens* nuclear genome was amplified by polymerase chain reaction (PCR) using a SSU rDNA primer specific for *P. invadens,* PARNUC-SSU-1512F (5'-TGCTAATTAGTTGTGCAAATG-3'; J. Johnson-MacKinnon and A.G.B. Simpson, unpublished data) in combination with a LSU rDNA primer, GRev (5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3'; Coleman & Vacquier 2002). The ITS region of the parasome nuclear genome was amplified by PCR using a *P. invadens*-parasome-specific primer, PARP-SSU-1456F (5'GTGTGCTCCTTGTCCT-3'; J. Johnson-MacKinnon and A.G.B. Simpson, unpublished data) and LSU rDNA primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990). The thermocycling protocol for amplification of both gene regions was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 20 s, 58 °C for 2 min, 72 °C for 3 min, and one final step of 72 °C for 10 min. The PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen), ethanol-precipitated, and re-suspended in 8 μl distilled water. The resulting amplicons were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and 10 positive colonies were confirmed by a PCR screen using vector primers. Five positive colonies (randomly selected) were inoculated into 3 ml Lysogeny broth (LB) medium with 100 mg ml^{-1} ampicillin, and grown overnight on a shaker (150 rpm) at 37 °C. The plasmid DNA was purified using the Qiagen MiniPrep Kit (Qiagen) and Sanger-sequenced in both directions (at the McGill University and Genome Quebec Innovation Center). The nuclear ITS and parasome ITS sequences from the 4 or 7 strains of *P. invadens*, respectively, were analyzed using Geneious 6.1.6

(http://www.geneious.com, Kearse et al. 2012) and aligned using the software Seaview 4.0 (Gouy et al. 2010). Sequence similarity between clones of all strains was calculated separately for the nuclear and parasome ITS regions, using the online software "Sequence Manipulation Suite" (Stothard 2000).

Table 2.1. *Paramoeba invadens* isolates used to sequence ITS regions of the nuclear genome and parasome nuclear genome, and to test the specificity of designed primers. Isolates were obtained from sea urchins during disease outbreaks at various locations in Nova Scotia (see Fig. 2.1) in 2011 and 2012.

Year Isolated	Isolate Name	Location	Depth (m)	Source
2011	SMB-8	St. Margaret's Bay	8	Feehan et al. 2013
2011	SMB-60	St. Margaret's Bay	60	Feehan et al. 2013
2011	SPS	Sandy Point, Shelburne	8	Feehan et al. 2013
2011	SP	Splitnose Point	8	Feehan et al. 2013
2012	PP-2012	Point Pleasant	3 – 6	C. Feehan unpublished data
2012	SP-2012	Splitnose Point	8	C. Feehan unpublished data
2012	GI-2012	Gravel Island	8	C. Feehan unpublished data

2.2.2. *P. invadens*-Specific PCR Design

2.2.2.1. Primer Design

The SSU rRNA genes (previously sequenced and available in Genbank; Feehan et al. 2013) and ITS sequences of both the nuclear genome and parasome nuclear genome of *P. invadens* were examined for possible species-specific primer sites. Candidate primer sets that amplified fragments of approximately 100 – 200 bp were designed using the online software Primer3 (Koressaar & Remm 2007, Untergasser et al. 2012).

2.2.2.2. Primer Specificity Testing

The analytical specificity of primers to distinguish DNA from *P. invadens* from

non-target organisms was tested *in silico* using nucleotide alignments of the SSU rRNA gene of the nuclear genome and parasome nuclear genome of *P. invadens,* together with the homologous regions of closely related species. Basic local alignment search tool (BLASTn) queries (Altschul et al. 1990) of the primer sequences were also performed to search for highly similar nucleotide sequences in other species. Primer specificity was confirmed by PCR, using DNA from *P. invadens* isolates SMB-8, SMB-60, and SP (Feehan et al. 2013; Table 2.1); DNA from other *Paramoeba* species (*P. branchiphila*, *P. aestuarina*, and two strains of *P. pemaquidensis*); and DNA from a diverse sample of distantly related marine eukaryotes, including *Ancyromonas sigmoides, Cyranomonas australis, Developayella* sp., *Notosolenus urceolatus*, *Neobodo designis,* and *Percolomonas cosmopolitus* 'group 1' (provided by W.J. Lee and K. Miller). Reactions were carried out in 20 μl volumes containing 2 μl of 10X Reaction Buffer (Invitrogen); 0.2 mM each dATP, dTTP, dCTP, dGTP; 1.5 mM $MgCl_2$; 1 μM of each primer, 1 μl template, and 1 U Taq DNA polymerase (Invitrogen), using the following thermocycling protocol: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 58 °C for 30 s, 72 °C for 15 s, and then a final step at 72 °C for 1 min. PCR products were visualized using gel electrophoresis for the above and all subsequent PCR tests (1.5 % TAE agarose gel; 6 μl PCR product per well).

2.2.2.3. Primer Sensitivity Testing

The analytical sensitivity of PCR with these primers (i.e. limit of detection) was tested using a dilution series of DNA extracted from a known number of cells of *P. invadens*. Briefly, 2 ml of sterile seawater was pipetted onto a 7-d old monoxenic culture of *P. invadens* strain SMB-60 and gently scraped using a sterile scraper. The resultant suspension of amoebae in seawater was transferred to a sterile 15 ml falcon tube. The concentration of amoeba cells was calculated using a hemocytometer and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). A dilution series was performed on the extracted DNA to yield material from 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 amoeba cells μl^{-1} and tested using the same PCR conditions described in Section 2.2.2.2 (such that cell equivalents μl^{-1} is the same as cell equivalents per PCR reaction).

2.2.2.4. PCR Optimization of Selected Primer Sets

The PCR assay was optimized for primer annealing temperature and magnesium chloride ($MgCl_2$) concentration using the primer set identified as the most specific and sensitive in detecting *P. invadens* DNA (Nuc1: primers ParNssu905F and ParNssu1094R; see Section 2.3.2.1). Annealing temperatures tested included 50.0 °C, 52.4 °C, 53.8 °C, 56.7 °C, 58.1 °C, 59.6 °C, and 62.0 °C, and PCR conditions were as described in Section 2.2.2.2. Details of optimization of $MgCl_2$ concentration are given in Appendix A.1.

2.2.3. Host Tissue and Environmental Sample Processing

2.2.3.1. Sample Collection

Sea urchins, sediment and seawater used to test initial DNA extraction protocols were obtained from aquaria maintained in the Aquatron at Dalhousie University, which contained sea urchins, sediment and seawater putatively infected with *P. invadens*. Once reliable DNA extraction techniques were established (see Section 2.2.3.3. – 2.2.3.5.), sea urchins, sediment, and seawater sourced from the environment were used to confirm amplicon identity and optimize PCR conditions. Sea urchins and sediments were sourced during a disease outbreak in 2014 from four sites in and around St. Margarets Bay, Nova Scotia (Splitnose Point, Luke Island, The Lodge-8m and The Lodge-18m; Fig. 2.1.; Chapter 3, Section 3.2.1.). Seawater was collected from two sites (Splitnose Point and Luke Island) during late summer/early fall of 2015, in the absence of diseased sea urchins (Chapter 3, Section 3.2.1.). Sediment was collected by divers into 50 ml falcon tubes from both inside and outside (within a 5 m radius) of experimental cages containing sea urchins. Seawater (~ 4 l) was collected in previously unopened 'Ziploc' plastic bags from above sea urchin cages, prior to any sediment disturbance.

Fig. 2.1. Map of study sites along the Atlantic coast of Nova Scotia showing The Lodge-8m and The Lodge-18m (TL) and Luke Island (LI) in St. Margarets Bay, and Splitnose Point (SP) along the headland southwest of Halifax Harbour. The basemap and scale bar for this figure were created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved. For more information about Esri® software, please visit www.esri.com.

2.2.3.2. Culturing P. invadens

Paramoeba invadens was obtained from infected sea urchins following the methods of Jones & Scheibling (1985) and Feehan et al. (2013). Briefly, 5 – 10 mm sections of radial nerve and associated water-vascular canal were excised and placed onto 0.6 % non-nutrient (NN) seawater agar. Plates were incubated at 18 °C for 1 – 3 wk to allow amoebae to migrate away from the tissue. 1.0 cm^2 pieces of 0.6 % agar were then sub-cultured onto 1.2 % NN agar with 400 µl of a liquid overlay containing *Escherichia coli* as food. The liquid overlay was prepared by inoculating *E. coli* in LB and incubating at 37 °C for 24 h on a moving platform (150 rpm). The resultant *E. coli* and LB mixture

was centrifuged at 3000 x g_0 for 5 min to pellet cells of *E. coli* and the supernatant was replaced with sterile seawater in a 1:1 ratio. Cultures were maintained at 16 – 18 °C and sub-cultured monthly onto new 1.2 % NN agar with liquid overlay.

2.2.3.3. Culturing versus Molecular Identification of Infection with P. invadens, *and Radial Nerve DNA Extraction*

Detection of *P. invadens* by PCR with *P. invadens*-specific primers was compared to detection by tissue culturing for sea urchins collected from three sites (Splitnose Point, The Lodge-8m, and The Lodge-18m; Fig. 2.1.) during a disease outbreak in 2014 (sea urchins at Luke Island were moribund prior to this part of the study). One radial nerve from each sea urchin was plated following the methods described above (Section 2.2.3.2.). Two to four of the remaining radial nerves (10 – 20 mg total weight) were excised and stored in 1.6 ml centrifuge tubes at −80 °C until DNA was extracted. DNA extractions were performed using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions with the following modifications: upon DNA extraction, radial nerve tissue was allowed to thaw at room temperature for 5 min and was subsequently suspended in 180 μl buffer ATL (Qiagen) and 20 μl proteinase K (Qiagen); it was then incubated at 56 °C for 1 h on a rocking platform, and vortexed every 15 min. Subsequent steps were as per the manufacturer's protocol. Radial nerve DNA was eluted in 100 μl AE buffer (Qiagen) and stored at −20 °C. The 260/280 ratio was measured using NanoDrop (Thermo Fisher Scientific) and ranged between 1.9 and 2.06.

2.2.3.4. Sediment DNA Extraction

Approximately 3 – 7 g of sediment was added to 2.5 ml bead solution (MoBIO), 0.2 ml buffer SR1 (MoBIO), 0.8 ml buffer SR2 (MoBIO), and 3.5 ml phenol:chloroform:isoamyl alcohol (25:24:1; Thermo Fisher Scientific). This was performed soon as possible upon collection (within 1 h) to minimize changes in the microbial community assemblage. Reactions were transferred on ice, stored at 4 °C overnight, and DNA was extracted the following day. Sediment RNA and DNA was

extracted using the MoBIO PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBIO) following the manufacturer's protocol. DNA was eluted in 50 μl buffer SR7 and stored at −20 °C. The 260/280 ratio ranged between 1.85 – 1.93.

2.2.3.5. Seawater DNA Extraction

4 l of seawater was pre-filtered through 100-μm mesh, and subsequently filtered onto 2.0 μm Isopore membranes (Merck Millipore). A maximum filtration time of 30 min and/or 1.5 l seawater per membrane was used. Seawater was filtered at a maximum pressure of 100 mmHg. All filtering equipment was cleaned using a 10 % bleach solution and rinsed with autoclaved distilled water prior to filtration. Immediately following filtration, membranes were placed into cryogenic tubes, flash-frozen in liquid nitrogen, and stored at −80 °C until DNA extraction. Based on a comparison of three seawater DNA extraction techniques (see Appendix A.2 for details), seawater DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol for "Purification of Total DNA from Animal Tissues" with modifications. Seawater filters were removed from storage at − 80 °C, allowed to thaw for 5 min and then were suspended in 400 μl sterile distilled water (dH$_2$O), 360 μl buffer ATL (Qiagen), and 40 μl proteinase K (Qiagen). Reactions were mixed by vortexing and allowed to incubate at 56 °C for 1 h. Following incubation, reactions were mixed again, and 400 μl buffer AL (Qiagen) and 400 μl, 96 – 100% ethanol (EtOH) were added. Subsequent steps were as per the manufacturer's protocol. Seawater DNA was eluted in 200 μl AE buffer and stored at −20 °C. The 260/280 ratio ranged from 1.94 to 2.07.

2.2.3.6. PCR Reaction Conditions for the Detection of P. invadens *in Radial Nerve, Sediment and Seawater DNA Samples*

MgCl$_2$, primer, and template DNA concentrations were optimized for PCR on each of the three sample types (see Appendix A.3. for details). Based on these results, PCR reactions using radial nerve, sediment, and seawater DNA were conducted in 20 μl volumes containing 2 μl of 10X Reaction Buffer (Invitrogen); 0.2 mM each dATP, dTTP,

dCTP, dGTP; 1.5 mM (radial nerve) or 2.0 mM (sediment and seawater) MgCl$_2$; 0.5 μM of each primer, 1 μl template at 1/2 (radial nerve and sediment) or 1/1 (seawater) dilution of the original DNA concentration, and 1 U Taq DNA polymerase (Invitrogen). Additionally, 1.2 μg of Bovine Serum Albumin (BSA) was added to PCR reactions with seawater DNA. The thermocycling protocol was as follows: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 58 °C for 30 s, 72 °C for 15 s, and then a final step at 72 °C for 1 min. PCR products were visualized using gel electrophoresis (1.5 % TAE agarose gel; 6 μl PCR product per well).

2.2.3.7. Confirmation of Amplicon Identity from Environmental DNA Samples

Amplicons obtained from radial nerve, sediment, and seawater DNA using *P. invadens*-specific primers ParNssu905F and ParNssu1094R were sequenced to confirm the specificity of the designed primers within the 'background' of each sample type. PCR products from two positive radial nerve samples, four positive sediment samples, and three positive seawater samples were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), or the StrataClone PCR Cloning Kit (Agilent Technologies, La Jolla, CA, USA) as per the manufacturer's protocols. 5 – 17 positive colonies per sample were identified by a PCR screen using vector primers, and 1 – 10 clones per sample were selected for sequencing. In total, 2 radial nerve clones, 29 sediment clones, and 8 seawater clones were sequenced from 2, 4, and 3 DNA samples, respectively. PCR products from the selected colonies were purified using the Qiagen MiniPrep Kit (Qiagen) and Sanger-sequenced in one direction using a vector primer (at the McGill University and Genome Quebec Innovation Center). Sequence identity was confirmed using nucleotide alignments against the nuclear SSU rRNA gene from *P. invadens* strains SP and SMB-8 and by BLASTn.

2.2.4. Inferring PCR Assay Sensitivity for Environmental Samples

The sensitivity of detecting *P. invadens* in environmental samples was inferred by adding known quantities of *P. invadens* cells to radial nerve, sediment, and seawater

samples, either following, or prior to, DNA extraction. In Expt 1, DNA extracted from radial nerve, sediment, and seawater (seawater was sourced from Sandy Cove, Terence Bay, NS [44° 27' 6" N, 63° 42' 4" W] for this experiment), that tested negative for *P. invadens* using PCR, was 'spiked' with *P. invadens* DNA extracted from a known number of cultured amoeba cells. In Expt 2, radial nerve, sediment, and seawater expected to be negative were 'spiked' with a known number of cultured *P. invadens* cells immediately prior to DNA extraction or immediately prior to filtration for seawater samples. Triplicate radial nerve, sediment, and seawater samples were used for both experiments.

For Expt 1, *P. invadens* cells from 1 – 4 wk old cultures (strain SMB-60) were suspended in sterile seawater and the concentration estimated using a hemocytometer. DNA was extracted from the total population of cells in the amoebae and seawater stock solution using the DNeasy Blood and Tissue DNA extraction kit (Qiagen) as per the manufacturer's protocol, and the final concentration of cells per μl of DNA was calculated. Appropriate dilutions were then prepared from this stock and *P. invadens* DNA was added to radial nerve, sediment, and seawater DNA in a 1:1 ratio. PCR was performed on radial nerve, sediment, and seawater DNA spiked with *P. invadens* DNA corresponding to 16, 8, 4, 2, and 1 *P. invadens* cells per PCR reaction.

For Expt 2, an amoeba stock solution with known cell concentration was obtained using the same method as described for Expt 1. A known number of cells of *P. invadens* was added to radial nerve, sediment, and seawater samples prior to DNA extraction to yield a final concentration of 32 or 16 cells μl^{-1} of DNA solution. The DNA was diluted to 1/2 the original DNA concentration, and PCR was performed. PCR results from Expt 1 and Expt 2 were compared visually to identify possible biases in sensitivity estimates due to spiking with *P. invadens* DNA after DNA extraction.

2.2.5. Quantitative Real-Time PCR with SYBR Green

2.2.5.1. Quantitative PCR Reaction Conditions and Optimization

Paramoeba invadens was quantified using *P. invadens*-specific primers, ParNssu905F and ParNssu1094R, in duplicate reactions. Quantitative real-time PCR (qPCR) was performed using SYBR Green reagents and analyzed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Reactions were carried out in 20 μl volumes containing 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer (see Appendix A.4 for details of optimization of primer concentrations), 1.2 or 2.5 μg BSA for radial nerve and seawater DNA respectively, and 1 μl template DNA with the following thermocycling conditions: 95.0 °C for 10 min, followed by 45 cycles at 95.0 °C for 15 s and 60.0 °C for 1 min. Additionally, a melt curve analysis was performed immediately after PCR to confirm that only the target region was amplified, and reactions containing multiple peaks were excluded from all analyses.

An optimization was performed varying the concentration of template DNA and BSA to yield optimal PCR conditions for quantifying *P. invadens* in each sample type. BSA was tested at final concentrations of 1.2 μg, 2.5 μg, and 5.0 μg BSA per reaction. Template DNA was added in 1 μl volumes and tested at final concentrations of 1/4, 1/2, and 1/1 the original DNA concentration. Additionally, sediment DNA was tested at final concentrations of 1/16 and 1/8. Radial nerve, sediment, and seawater DNA that had tested negative for *P. invadens* was spiked with DNA from 10 cells of *P. invadens*, and used as template DNA for these experiments.

2.2.5.2. Calibration Curves for Quantification of P. invadens

Calibration curves were established by spiking radial nerve and seawater DNA with DNA extracted from a known quantity of *P. invadens* cells, as per methods described in Section 2.2.4. A 1:10 dilution series was used, with final DNA concentrations of 500, 50, 5, 0.5, 0.05, and 0.005 *P. invadens* cell equivalents per

reaction. Dilutions were created by adding 4 µl DNA from 125, 12.5, 1.25, 0.125, 0.0125, and 0.00125 cells µl^{-1} to 1 µl radial nerve or seawater DNA (that was negative for *P. invadens* using PCR) at 1/2 the original DNA concentration. Dilution series were run in triplicate reactions. The slope, R^2, and efficiency values were -3.41, 0.998, 96.4 % and -3.41, 0.985, 96.5 % for radial nerve and seawater calibration curves, respectively.

2.2.5.3. Genomic Copy Number of 18S rRNA Gene in P. invadens

To determine the 18S rRNA gene copy number in *P. invadens,* a 190 bp PCR-generated fragment of the gene using the *P. invadens* specific primers ParNssu905F and ParNssu1094R, was cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Recombinant plasmid DNA was purified using the Qiagen MiniPrep Kit (Qiagen). After purification, a PCR using primers ParNssu905F and ParNssu1094R was conducted and the DNA concentration in the resultant product was determined using Nano-drop (Thermo Fisher Scientific). The number of construct copies in the PCR product was calculated using the standard equation: copy number = (DNA concentration in ng µl^{-1}) x (6.022 x 10^{23}) / molecular weight of double-stranded fragment x (1 x 10^9)), based on a fragment size of 190 bp. A calibration curve was generated using a 10-fold dilution series ranging from 10^5 to 10^1 copies per PCR and run in triplicate reactions. DNA from 20 and 50 cells of *P. invadens* was run against copy number calibration curves to generate an estimate of copy number per cell. The slope, R^2, and efficiency values were -3.392, 1.0, and 97.168 %, respectively.

2.2.5.4. Reliability of Cell Number Estimates Obtained using qPCR

To identify the reliability of cell number estimates obtained by qPCR, radial nerve and seawater samples spiked with 32 or 16 cells of *P. invadens* prior to DNA extraction (Section 2.2.4.) were quantified. Calibration curves were generated by spiking radial nerve and seawater DNA with DNA from known numbers of *P. invadens* cells following DNA extraction. Cell number may be under- or over-estimated with this method if there is a difference in results obtained when spiking DNA before vs. after extraction (e.g. if

the recovery of cells differs when spiking with *P. invadens* before DNA extraction). Reaction conditions used for this study were as described in Section 2.2.5.1 and calibration curves were generated using the methods described in Section 2.2.5.2. The slope, R^2, and efficiency values were -3.524, 0.995, 92.2 % and -3.57, 0.982, 90.6 % for radial nerve and seawater calibration curves, respectively.

2.3. RESULTS

2.3.1. Sequencing *P. invadens* and Parasome ITS Regions

A 1318 – 1330 bp fragment was amplified to sequence the ITS regions of the *P. invadens* nuclear genome. ITS1 was 198 – 204 bp in length and ITS2 was 347 – 356 bp in length. A 750 – 753 bp fragment was amplified to sequence the ITS regions of the parasome nuclear genome. ITS1 was 90 – 91 bp and ITS2 was 76 bp in length. No clear evidence was found for genetic variation between strains of *P. invadens* isolated from along the coast of Nova Scotia. Comparison of the parasome ITS regions of the 2011 and 2012 strains of *P. invadens* (7 strains in total) indicated 96.4 – 100 % sequence identity, with zero conserved differences (i.e. substitutions or indels found in all clones from one or more strains). Multiple sequence types of the parasome ITS region were observed, however the two main sequence types were found together in all or almost all strains, suggesting these types represent differences among ITS regions within the parasome genomes of all strains, rather than differences between strains. Similarly, no conserved differences were observed within the nuclear ITS regions of the four 2011 strains. However, variation between clones was greater for the nuclear ITS regions, with 93.9 – 100 % sequence identity.

2.3.2. *P. invadens*-Specific PCR: Design and Optimization

2.3.2.1. Primer Design

A total of three primer sets were designed and tested for specificity and sensitivity of detecting *P. invadens* (Table 2.2). Two primer sets, designated as Nuc1 (primers ParNssu905F and ParNssu1094R) and Nuc2 (primers ParNssu1571F and ParNssu1677R) were designed to amplify portions of the SSU rRNA gene of the *P. invadens* nuclear genome (see Table 2.2 for primer details). One primer set, designated as IRE1 (primers ParPssu1017F and ParPssu1188R), was designed to amplify a portion of the SSU rRNA gene of the parasome nuclear genome (see Table 2.2 for details).

Table 2.2. Details of primer pairs designed against the *P. invadens* nuclear SSU rRNA gene and parasome SSU rRNA gene. Primer set indicates name given to that primer pair. Numbers embedded in primer names indicate the binding position of the primer, from its 5' end, in the published SSU rRNA sequence data for *P. invadens* isolate SP (accession number KC790385.1 for the *P. invadens* nuclear genome and accession number KC790388.1 for the parasome nuclear genome).

Primer Set	Genome type	Amplicon size (bp)	Primer Name	Primer Direction	Primer sequence
Nuc1	*P. invadens* nuclear genome	190	ParNssu905F	Forward	5'-TCAAGGCAAGCATAATTTTAATG-3'
			ParNssu1094R	Reverse	5'-CAAGAATTTCACCTCTGACATC-3'
Nuc2	*P. invadens* nuclear genome	107	ParNssu1571F	Forward	5'-GGAAGGGGGTTTCTTTGTT-3'
			ParNssu1677R	Reverse	5'-ATTATCGGAATCRTCAARCGT-3'
IRE1	Parasome nuclear genome	172	ParPssu1017F	Forward	5'-AGAGGTGAAATTCTGTGATCTTC-3'
			ParPssu1188R	Reverse	5'-CCCATCGGAATAAGATGCTA-3'

2.3.2.2. Testing Primer Specificity

All three primer sets (Nuc1, Nuc2, and IRE1) tested positive against DNA from *P. invadens* strains SMB-8, SMB-60, and SP, and yielded a product of the appropriate size (190 bp, 107 bp, and 172 bp respectively; Fig. 2.2). When tested against DNA from various *Paramoeba/Neoparamoeba* species and other common marine eukaryotes, PCR results were all negative at the same annealing temperature (58 °C: Fig. 2.2).
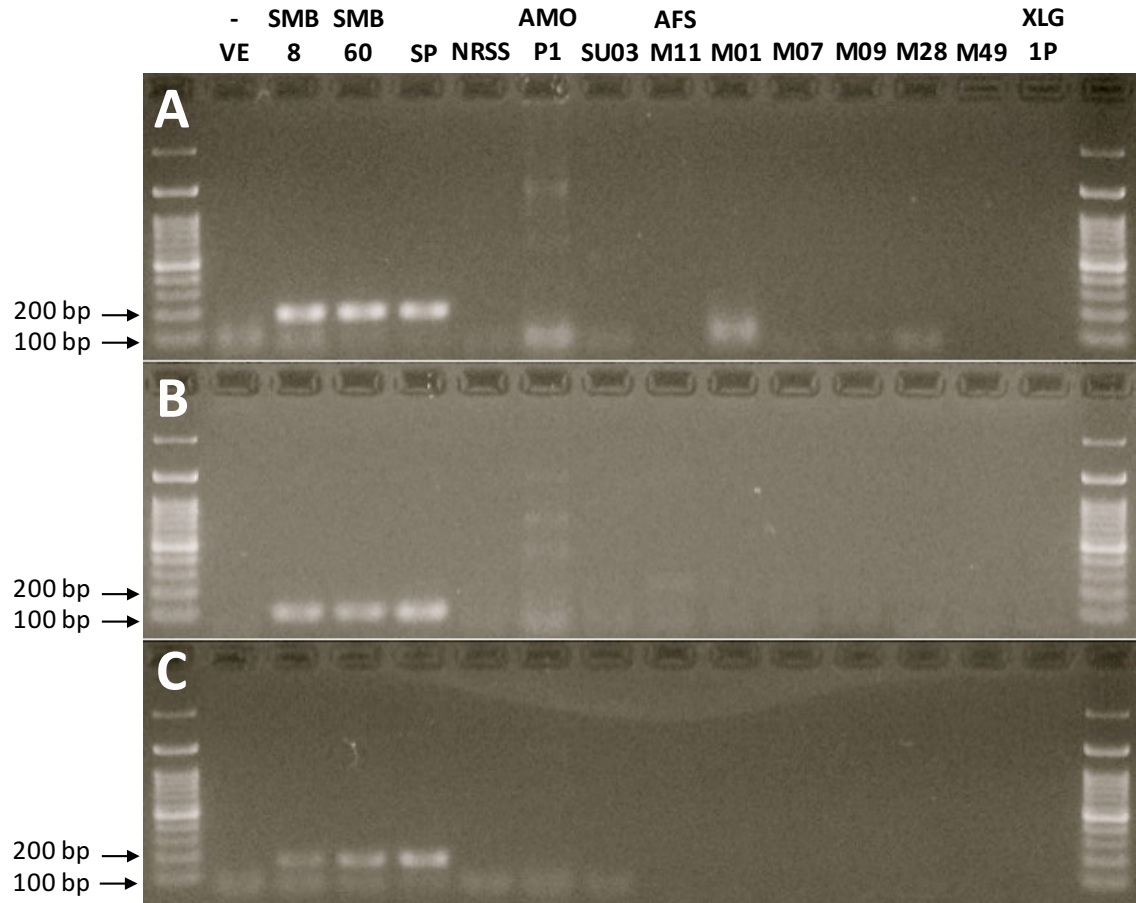
Fig. 2.2. Testing the specificity of *P. invadens*-specific primer sets: A) Nuc1 (ParNssu905F & ParNssu1094R; 190 bp fragment) designed to a region of the *Paramoeba* nuclear SSU rRNA gene, B) Nuc2 (ParNssu1571F & ParNssu1677R; 107 bp fragment) designed to a region of the *Paramoeba* nuclear SSU rRNA gene, and C) IRE1 (ParPssu1017F & ParPssu1188R; 172 bp fragment) designed to a region of the parasome nuclear SSU rRNA gene. Primer sets were tested against 3 strains of *P. invadens* [SMB8; SMB60; SP], 4 strains representing 3 other *Paramoeba* species [NRSS: *P. branchiphila*; AMOP1: *P. aestuarina*; SU03 & AFSM11: two strains of *P. pemaquidensis*], and various other marine eukaryote species [M01: *Ancyromonas sigmoides*; M07: *Neobodo designis*; M09: *Cyranomonas australis*; M28: *Developayella* sp.; M49: *Notosolenus urceolatus*; XLG1P: *Percolomonas cosmopolitus* 'group 1'].

2.3.2.3. Testing Primer Sensitivity

PCR reactions for primer set Nuc1 (ParNssu905F and ParNssu1094R) showed strong amplification of DNA from $\geq$ 4 cells of *P. invadens* (Fig. 2.3). Weak amplification was observed when tested against DNA from 2 and 1 cells. Little or no amplification was observed of DNA from 0.5 and 0.25 cells. PCR reactions for primer set Nuc2 (ParNssu1571F and ParNssu1677R) showed strong amplification of DNA from $\geq$ 8 cells of *P. invadens* (Fig. 2.3). Amplification could be observed with difficulty of DNA from 4 cells. No amplification was observed with DNA from $\leq$ 2 cells. PCR reactions for primer set IRE1 (ParPssu1017F and ParPssu1188R) amplified DNA from $\geq$ 64 cells of *P. invadens*. Amplification was observed with difficulty for DNA from 32 cells. No amplification was noted from $\leq$ 16 cells. Based on these results, primer set Nuc1 was selected as the optimal primer set for detecting *P. invadens* DNA.
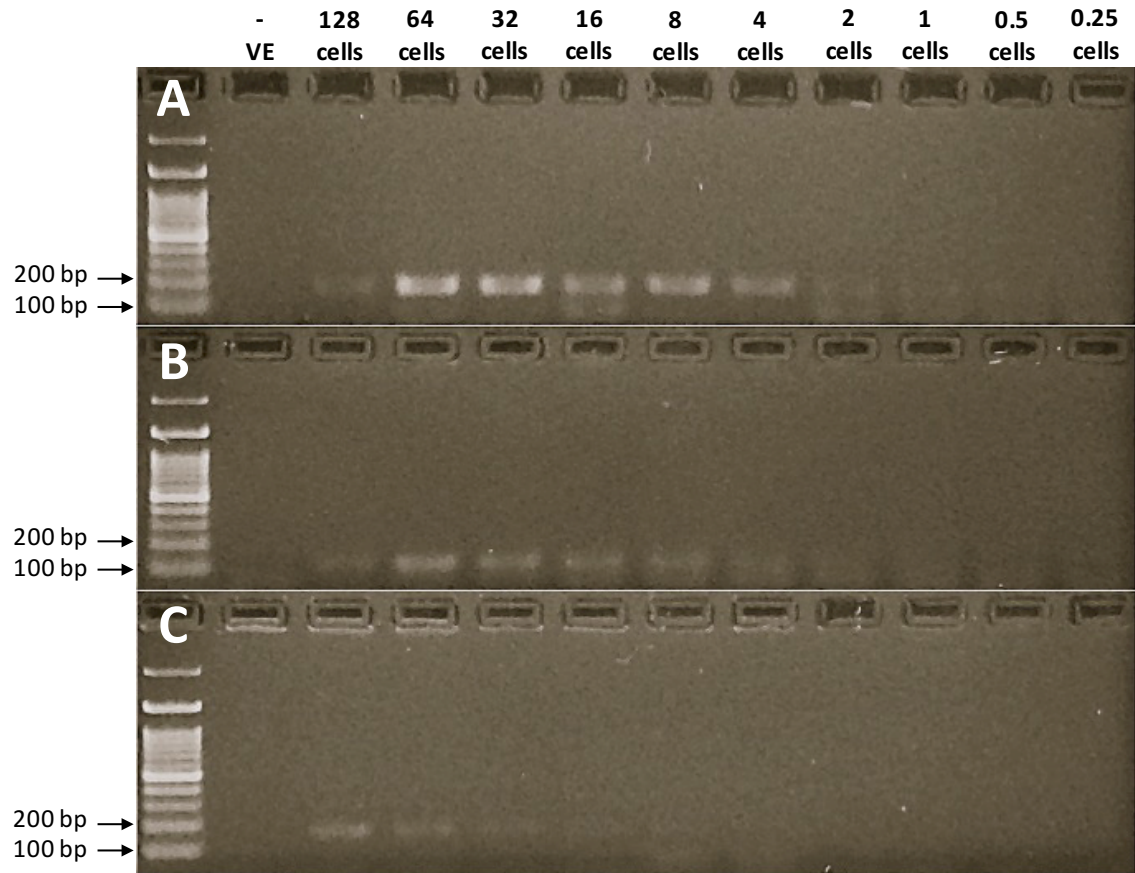
Fig. 2.3. Testing the sensitivity of *P. invadens*-specific primer sets: A) Nuc1 (ParNssu905F & ParNssu1094R; 190 bp fragment) designed to a region of the *Paramoeba* nuclear SSU rRNA gene, B) Nuc2 (ParNssu1571F & ParNssu1677R; 107 bp fragment) designed to a region of the *Paramoeba* nuclear SSU rRNA gene, and C) IRE1 (ParPssu1017F & ParPssu1188R; 172 bp fragment) designed to a region of the parasome nuclear SSU rRNA gene. PCR reactions were tested using DNA from 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 cells of *P. invadens* per PCR reaction.

2.3.2.4. PCR Optimization of Selected Primer Sets

Amplification of primer set Nuc1 (ParNssu905F and ParNssu1094R) was observed at all annealing temperatures tested (range: 50.0 °C – 62.0 °C; Fig. 2.4.). Based on amplification intensity, an annealing temperature of 58.1 °C was the highest annealing

temperature at which amplification was reliably observed across duplicate DNA samples (Fig. 2.4.), and was chosen as the optimal annealing temperature for subsequent PCR.
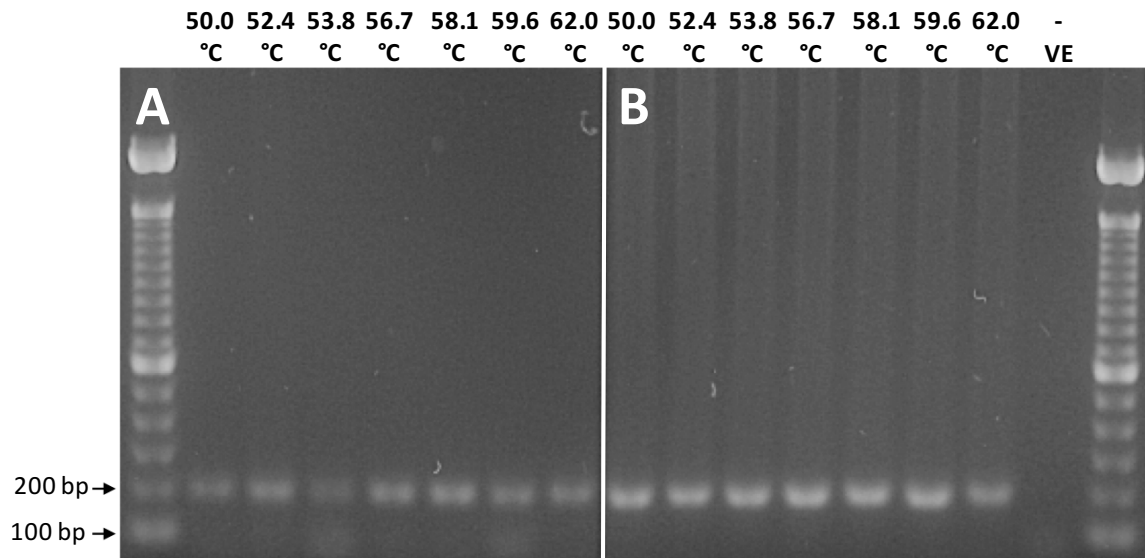
Fig. 2.4. Annealing temperature optimization of primer set Nuc1 (190 bp product) using: A) DNA extracted from cultures of *P. invadens* isolate SMB-60 and B) sediment DNA spiked with *P. invadens* DNA from 16 cells of isolate SMB-60. Annealing temperatures tested included 50.0, 52.4, 53.8, 56.7, 58.1, 59.6, and 62.0 °C.

2.3.3. Host Tissue and Environmental Sample Processing

2.3.3.1. Culturing versus Molecular Identification of P. invadens Infection

Sea urchins collected during and following a disease outbreak in 2014 were used to compare the existing method of culturing *P. invadens* to detection of *P. invadens* using PCR with primer set Nuc1. For all sea urchins sampled, presence/absence results obtained via culturing methods were in agreement with results obtained using PCR approaches, with one exception. Sea urchin number 4, collected from Splitnose Point on 6 November

2014, was negative for *P. invadens* using culturing methods, however amplification of *P. invadens* DNA was observed when using PCR (Table 2.3). In addition to sea urchins sampled in 2014, results obtained using both methods were in agreement for all sea urchins sampled between July 2015 and May 2016 (Chapter 3, Section 3.3.1).

Table 2.3. Detection of *P. invadens* in radial nerve tissue collected from Splitnose Point, The Lodge-8m and The Lodge-18m during a disease outbreak in 2014 using i) culturing methods by plating radial nerves onto 0.6 % non-nutrient (NN) agar, and ii) PCR amplification using primer set Nuc1. '+' indicates the presence of *P. invadens* cells or positive PCR amplification. '−' indicates the absence of *P. invadens* cells or no PCR amplification.

Collection Date	Collection Site	Urchin	*P. invadens* presence on 0.6 % NN agar	*P. invadens* amplification using primer set Nuc1
06-Nov-14	Splitnose Point	1	+	+
		2	+	+
		3	+	+
		4	-	+
10-Nov-14	The Lodge - 8 m	1	-	-
		2	-	-
		3	-	-
		4	-	-
10-Nov-14	The Lodge - 18 m	1	-	-
		2	-	-
		3	-	-
		4	-	-
26-Nov-14	Splitnose Point	1	-	-
		2	-	-
		3	-	-
		4	-	-
26-Nov-14	The Lodge - 8 m	1	-	-
		2	-	-
		3	-	-

2.3.3.2. Confirmation of Amplicon Identity from Environmental DNA Samples

All positive clones examined for the two radial nerve DNA samples, and the three seawater DNA samples had inserts of the expected size. The same amplicon was observed amongst positive clones for all four sediment DNA samples, however an additional amplicon, ~ 50 bp smaller, also was observed for one sediment sample collected on 6 November 2014 from inside sea urchin cages at Luke Island. As it was unexpected, five clones of this smaller band size were included for sequencing. All amplicons of the expected size for radial nerve, seawater, and sediment clones, showed 98.3 – 100 % sequence identity to one of the known sequence variants of the nuclear SSU rRNA gene of *P. invadens*. The smaller amplicons from sediment were identical to each other; a BLASTn query indicated no significant similarity to any sequences in the NCBI database.

2.3.4. Inferring PCR Assay Sensitivity for Environmental Samples

2.3.4.1. Radial Nerve DNA

Amplification was observed for all three radial nerve DNA samples spiked with DNA from ≥ 1 cell of *P. invadens* (Fig. 2.5). As *P. invadens* concentration decreased, only a marginal decrease in amplification was observed, suggesting a greater sensitivity of detecting *P. invadens* in radial nerve DNA than was tested in this study (i.e. *P. invadens* is likely detectable from less than 1 cell equivalent). All three radial nerve DNA samples were negative for *P. invadens* when not spiked with *P. invadens* DNA.

To determine if there was a bias in estimating sensitivity as a result of spiking radial nerve DNA after DNA extraction, three radial nerve samples were spiked with *P. invadens* cells prior to DNA extraction to yield a final concentration of 16 cells μl^{-1} at a DNA background concentration of 1/2 the original DNA concentration. Greater amplification was observed for all three radial nerve samples spiked prior to DNA extraction when compared to those spiked with DNA from the same number of cells after DNA extraction (Fig. 2.6). These results suggest that sensitivity estimates inferred from

samples spiked after DNA extraction are likely underestimates, further suggesting that *P. invadens* is likely detectable from less than one cell equivalent.
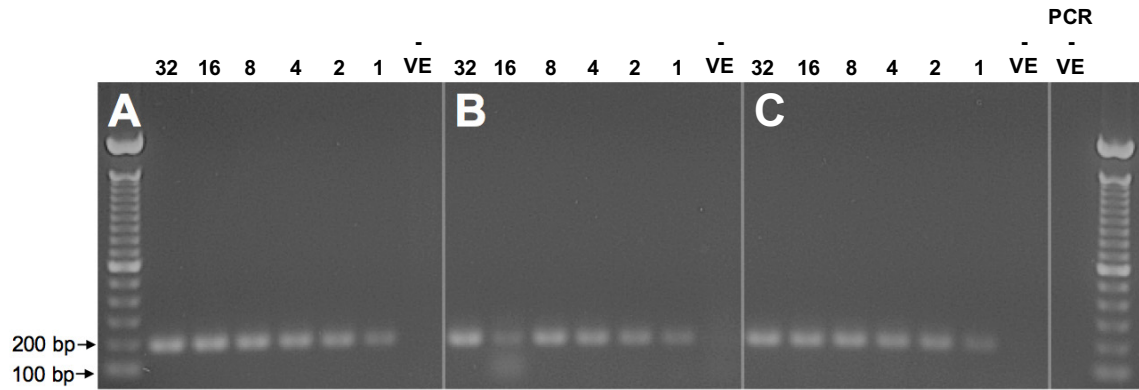
Fig. 2.5. PCR amplification (Nuc1 primer set) from three radial nerve DNA samples spiked after extraction with *P. invadens* DNA: A – collected from The Lodge-8m on November 10, 2014; B – collected from The Lodge-8m on November 26, 2014; and C – collected from Splitnose Point on November 26, 2014. Radial nerve samples were spiked with DNA from 32, 16, 8, 4, 2, and 1 *P. invadens* cells. Negatives (-VE) are radial nerve DNA samples without any *P. invadens* DNA and PCR negatives (PCR –VE) contain no added DNA.
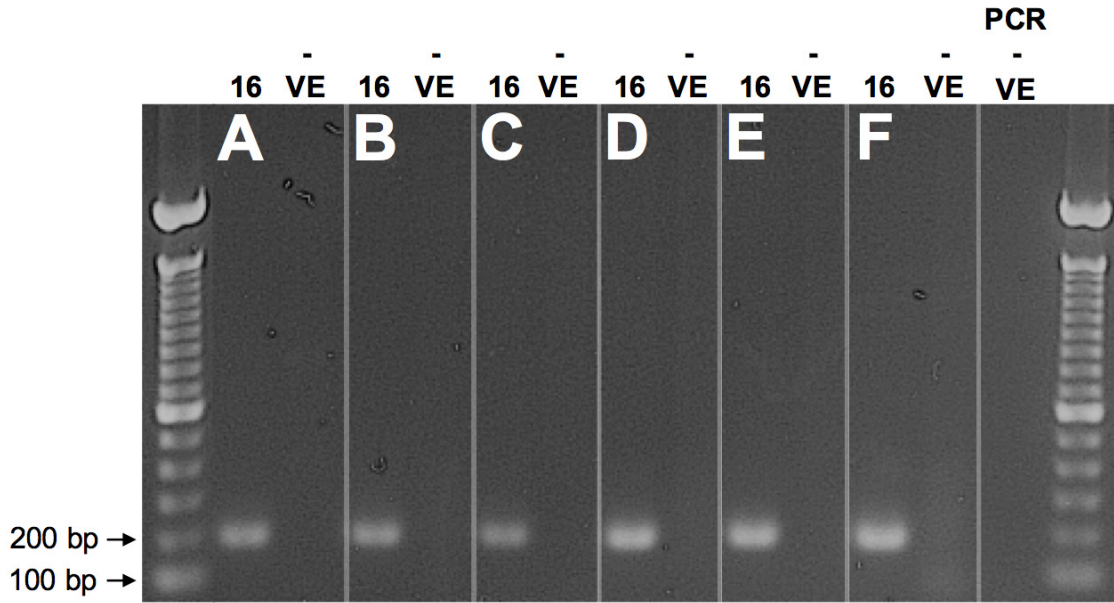
Fig. 2.6. Panel comparing radial nerve DNA samples spiked with DNA from 16 *P. invadens* cells before and after DNA extraction took place. Panels A, B, and C represent three radial nerve DNA samples spiked with *P. invadens* DNA after DNA extraction: A – collected from The Lodge-8m on November 10, 2014; B – collected from The Lodge-8m on November 26, 2014; and C – collected from Splitnose Point on November 26, 2014. Panels D, E, and F represent three radial nerve DNA samples spiked with *P. invadens* cells before DNA extraction. Samples D, E, and F were all collected from Splitnose point on May 21, 2015. Negatives (-VE) are radial nerve DNA samples without added *P. invadens* DNA and PCR negatives (PCR –VE) contain no added DNA.

2.3.4.2. Sediment DNA

Positive amplification was observed for all three sediment DNA samples spiked with DNA from ≥ 2 cells of *P. invadens* (Fig. 2.7). As *P. invadens* concentration decreased, a consistent decrease in amplification was observed. When spiked with DNA from 1 cell, amplification was only observed for one of three sediment samples (Fig. 2.7., panel C), suggesting a detection limit of > 1 cell/reaction for reliable detection of *P. invadens* in sediment. All three sediment samples tested negative for *P. invadens* when not spiked with *P. invadens* DNA.

As per radial nerve results, stronger amplification was observed for all three sediment samples spiked prior to DNA extraction with cells of *P. invadens* versus after DNA extraction with DNA from cells of *P. invadens* (Fig. 2.8). Again, these results suggest an underestimation of the detection limit when inferred by spiking sediment DNA with *P. invadens* DNA after DNA extraction.
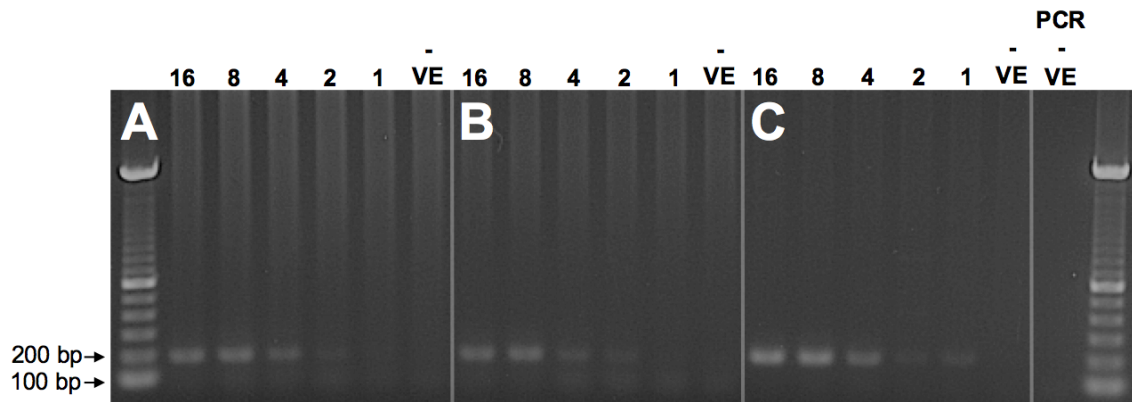
Fig. 2.7. PCR amplification (Nuc1 primer set) from three sediment DNA samples spiked after extraction with *P. invadens* DNA: A – collected from The Lodge-8m outside sea urchin cages on October 21, 2014; B – collected from The Lodge-18m inside sea urchin cages on November 4, 2014; and C – collected from The Lodge-18m inside sea urchin cages on November 26, 2014. Sediment samples were spiked with DNA from 16, 8, 4, 2, and 1 *P. invadens* cells. Negatives (-VE) are sediment DNA samples without any *P. invadens* DNA added and PCR negatives (PCR –VE) contain no added DNA.
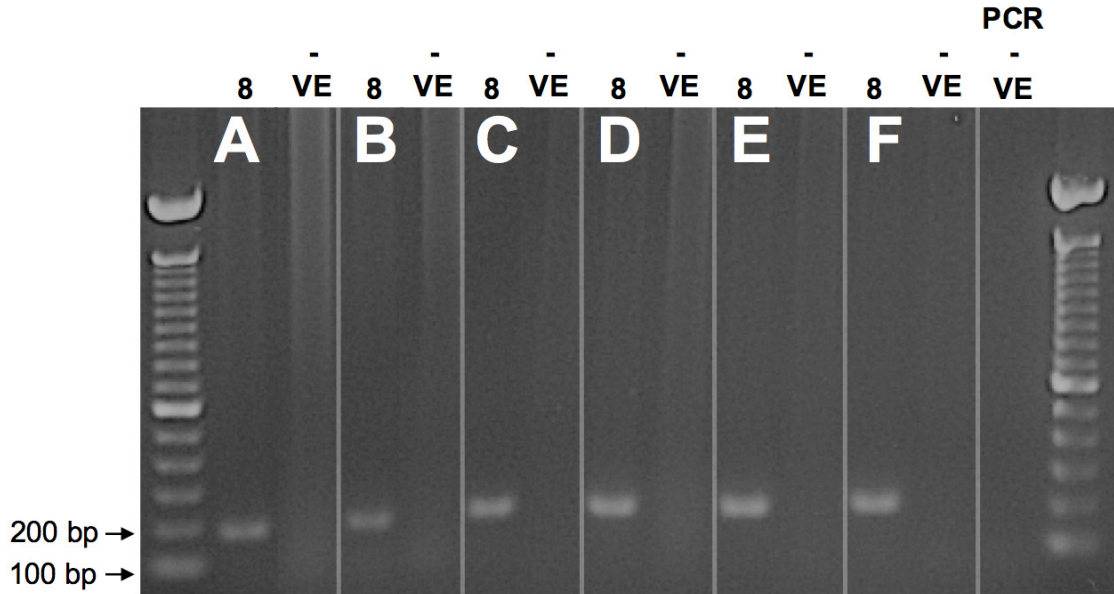
Fig. 2.8. Panel comparing sediment DNA samples spiked with DNA from 8 *P. invadens* cells before and after DNA extraction took place. Panels A, B, and C represent three sediment DNA samples spiked with *P. invadens* DNA after DNA extraction: A – collected from The Lodge-8m outside sea urchin cages on October 21, 2014; B – collected from The Lodge-18m outside sea urchin cages on November 4, 2014; and C – collected from The Lodge-18m outside sea urchin cages on November 26, 2014. Panels D, E, and F represent three sediment DNA samples spiked with cells of *P. invadens* before DNA extraction: D – collected from The Lodge-8m outside sea urchin cages on April 18, 2015; E – collected from The Lodge-18m from inside sea urchin cages on April 18, 2015; and F – collected from The Lodge-18m from outside sea urchin cages on April 18, 2015. Negatives (-VE) are sediment DNA samples without added *P. invadens* DNA and PCR negatives (PCR –VE) contain no added DNA.

2.3.4.3. Seawater DNA

Amplification was observed for all three seawater samples spiked with DNA from ≥ 1 *P. invadens* cells (Fig. 2.9). As concentration of *P. invadens* decreased, amplification brightness decreased only marginally, if at all, suggesting a greater sensitivity of detecting *P. invadens* in seawater DNA than was tested in this study. All three radial nerve samples tested negative for *P. invadens* when not spiked with *P. invadens* DNA.

In contrast to what was observed for radial nerve and sediment DNA, less amplification was observed for all three seawater samples spiked prior to DNA extraction with cells of *P. invadens* versus after DNA extraction with DNA from cells of *P. invadens* (Fig. 2.10). These results suggest that sensitivity estimates identified by spiking after DNA extraction, are likely to be overestimates.
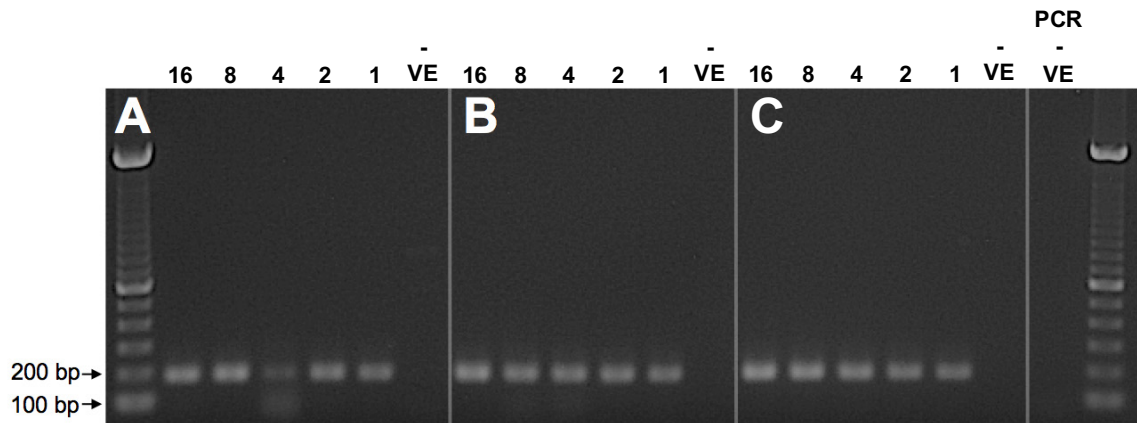
Fig. 2.9. PCR amplification (Nuc1 primer set) from three seawater DNA samples spiked after DNA extraction with *P. invadens* DNA: A – collected from Splitnose Point on July 13, 2015, B – collected from Splitnose Point on July 27, 2015, and C – collected from Luke Island on July 27, 2015. Seawater samples were spiked with DNA from 16, 8, 4, 2, and 1 cell(s) of *P. invadens*. Negatives (-VE) are seawater DNA samples without any *P. invadens* DNA and PCR negatives (PCR –VE) contain no added DNA.
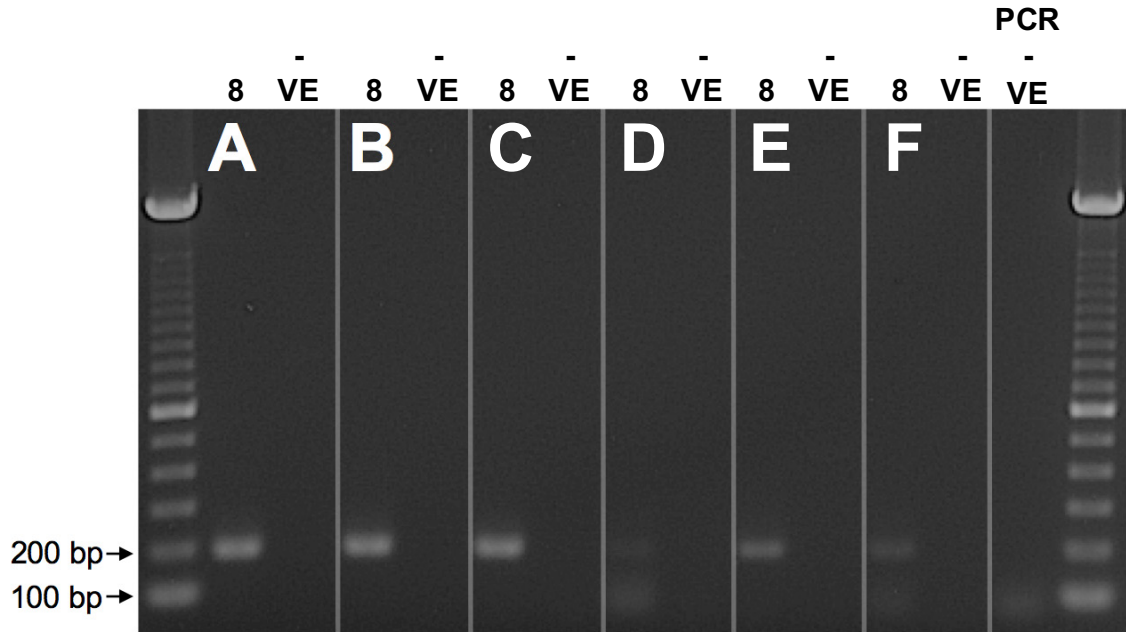
Fig. 2.10. Comparison of seawater DNA samples spiked with DNA from 8 cells of *P. invadens* either before or after DNA extraction. Panels A, B, and C show three seawater DNA samples spiked with *P. invadens* DNA after DNA extraction: A − collected from Splitnose Point on July 13, 2015; B − collected from Splitnose Point on July 27, 2015; and C − collected from Luke Island on July 27, 2015. Panels D, E, and F show three seawater DNA samples spiked with cells of *P. invadens* before DNA extraction. Samples D, E, and F were all collected from Sandy Cove on June 3, 2016. Negatives (-VE) are seawater DNA samples without any added *P. invadens* DNA and PCR negatives (PCR − VE) contain no added DNA.

2.3.5. Quantitative Real-Time PCR

2.3.5.1. qPCR Optimization

Based on a target efficiency of 90 % or greater, radial nerve DNA was suitable for qPCR at all DNA and BSA (μg per 20 μl reaction) concentrations tested (Table 2.4). The Ct values for radial nerve samples with BSA concentrations of 1.2, 2.5, and 5.0 μg ranged from 19.55 − 21.93, with a positive correlation between decreasing DNA concentration

and decreasing Ct value (Pearson's r > 0.95). A template DNA concentration of 1/2 and BSA concentration of 2.5 µg per reaction were selected as the most suitable conditions for quantitation of *P. invadens* in radial nerve DNA.

Sediment DNA was unsuitable for qPCR at all DNA and BSA (µg per 20 µl reaction) concentrations, with one exception (Table 2.4). At a background DNA concentration of 1/16 and BSA of 2.5 µg the average efficiency was 90.5 % and the average Ct value was the lowest of all combinations tested (Ct = 23.91). At natural abundances, *P. invadens* was undetectable in sediment at DNA concentrations of 1/8 (see Appendix A.3; Fig. A.3.8), thus qPCR using the specifications described, was deemed unsuitable to detect *P. invadens* in sediment DNA. With this information, qPCR examination of sediment DNA samples was not pursued further.

Seawater DNA was unsuitable for qPCR in all reactions containing 5.0 µg of BSA and all reactions with a DNA concentration of 1/1 (Table 2.4). All other combinations were suitable for qPCR and had an average efficiency between 90.2 – 91.9 % and critical threshold (Ct) value between 22.92 – 23.38. Natural abundances of *P. invadens* are undetectable at seawater DNA concentrations below 1/2, thus a DNA concentration of 1/2 and BSA of 1.2 µg was selected as the most appropriate for qPCR.

Table 2.4. DNA concentration and BSA optimization results for radial nerve, sediment, and seawater DNA samples spiked with DNA from 10 *P. invadens* cells. DNA concentration is based on the addition of 1 μl DNA and BSA concentration is the final concentration of BSA per 20 μl qPCR reaction. Efficiency (%) is a measure of how well the reaction ran, with a target efficiency of 100 %, and is calculated based on the slope of the amplification curve and an amplification factor of 2 (i.e. a doubling in the fluorescence with each subsequent cycle). The Ct value is the cycle number at which amplification is first observed (i.e. the fluorescent signal is significantly greater than the background fluorescence). Averages for radial nerve samples are calculated from triplicate reactions and averages for sediment and seawater samples are calculated from duplicate reactions.

DNA Type	DNA Concentration	BSA Concentration (µg)	Average Efficiency (%)	Average Ct Value
Radial Nerve	1/4	1.2	96.3	19.58
		2.5	101.4	19.55
		5	100.9	19.95
	1/2	1.2	96.5	20.26
		2.5	100.3	20.12
		5	108.2	20.47
	1/1	1.2	93.9	21.67
		2.5	99.4	21.93
		5	102.2	21.78
Sediment	1/16	1.2	85.4	24.18
		2.5	90.6	23.91
		5	86.9	24
	1/8	1.2	73.0	25.66
		2.5	83.1	25.14
		5	77.1	25.31
	1/4	1.2	65.0	30.97
		2.5	66.7	28.44
		5	66.3	27.844
	1/2	1.2	22.9	42.84
		2.5	31.4	33.01
		5	32.5	32.33
	1/1	1.2	-	-
		2.5	-	-
		5	-	-
Seawater	1/4	1.2	91.9	23.14
		2.5	91.2	22.92
		5	89.3	23.14
	1/2	1.2	90.5	23.38
		2.5	90.2	23.2
		5	88.9	23.44
	1/1	1.2	86.7	22.86
		2.5	87.7	21.81
		5.0	85.3	22.79

2.3.5.2. Estimation of Copy Number in P. invadens *Cells*

As estimated by copy number calibration curves, it was inferred that there are ~ 475 copies of the nuclear SSU rRNA gene per *P. invadens* cell (estimates derived from 20 cell equivalents and 50 cell equivalents were 474 and 471 copies, respectively).

2.3.5.3. Reliability of Cell Number Estimates Obtained using qPCR

To ensure that calibration curves were as similar as possible to the experimental samples, calibration curves were generated by adding sample background DNA to DNA extracted from known numbers of cells of *P. invadens*. Due to generating calibration curves by adding the two components together after DNA extraction had already occurred, I checked the direction and approximate extent of inaccuracy in cell number estimates generated by qPCR by quantifying *P. invadens* in samples that had been spiked with a known number of cells of *P. invadens* prior to DNA extraction. Estimates of cell number obtained using qPCR on three radial nerve samples spiked with 16 cells of *P. invadens* prior to DNA extraction ranged from 24.4 – 29.6 cells, representing a moderate (~ factor of 2) overestimation of true cell number. Estimation of the number of cells of *P. invadens* in three seawater samples spiked prior to DNA extraction with a final abundance of 8 cells per reaction ranged from 0.62 – 0.92 cells. This indicates a significant underestimation, of more than an order of magnitude, when estimating numbers of *P. invadens* cells in seawater samples, when using the filtration and DNA extraction protocols followed here.

2.4. DISCUSSION

2.4.1. Contributions of the Present Study

The presence of *Paramoeba invadens* in its sea urchin host has been recorded along the Atlantic coast of Nova Scotia for more than 3 decades (Jones & Scheibling 1985, Feehan et al. 2012, Feehan et al. 2016), however prior to this study, *P. invadens* had not been reliably detected or monitored in any form of environmental sample. The PCR-based protocols described here provide a reliable method to detect *P. invadens* in sea urchin radial nerve tissue, as well as in sediment and seawater, at abundances found naturally in the samples collected. When compared to the traditional method of culturing, this PCR-based approach appears to be as sensitive, if not more sensitive, at detecting *P. invadens,* and provides the advantages of being a more time-efficient approach and

having the potential for broader application. This is the first study to identify protocols to quantify *P. invadens* in radial nerve and seawater samples and to estimate copy number of the SSU rRNA gene in *P. invadens*. Interestingly, copy number was estimated to be an order of magnitude smaller than for the related species, *Paramoeba perurans* (2880 copies per cell; Bridle et al. 2010).

2.4.2. Selection of DNA Extraction Techniques

The quality and purity of radial nerve and seawater DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen), and of sediment DNA extracted using the MoBio PowerSoil Kits (specifically the Total RNA Isolation Kit combined with the DNA Elution Accessory Kit), were confirmed by PCR controls. The commercial DNA extraction kits used in this study were selected primarily because of popularity or previous use for similar material (e.g. sediment and seawater; Bonilla et al. 2015, Mueller et al. 2014). Initial attempts to isolate radial nerve and seawater DNA using the DNeasy Blood and Tissue kit were unsuccessful, and various modifications of the tissue/cell lysis step were required before the extraction of high quality DNA was achieved. Consequently, it is important to follow the radial nerve and seawater DNA extraction protocols as described here for reproducibility. Sediment DNA suitable for conventional PCR was successfully extracted in initial attempts using the selected kit under standard protocols, and modification of the manufacturer's protocol to store sediment in buffers overnight was based on logistical constraints of sampling, rather than to improve yield or quality.

2.4.3. PCR Sensitivity in Radial Nerve, Sediment, and Seawater DNA

Using the methods described here, *P. invadens* was detected in radial nerve and seawater DNA with as little as 1 cell (equivalent) present and reliably detected in sediment DNA with as little as 2 cells. With an elution volume of 100, 50, and 200 μl for radial nerve, sediment, and seawater samples respectively, this would translate to an approximate detection limit of 200, 200, and 400 *P. invadens* cells total per sample (total cells = cells μl^{-1} x 2 x total elution volume of DNA sample). The sample sizes used in my

study (~ 20 mg radial nerve tissue, ~ 5 g sediment, 4 l seawater) translates to a detection limit of 10 cells mg^{-1} tissue, 40 cells g^{-1} sediment, and 100 cells l^{-1} seawater. Amplification strength at these low cell abundances, and results from experiments where radial nerve and sediment were spiked prior to DNA extraction, indicate that the sensitivity of detecting *P. invadens* using these methods is likely to be greater than that demonstrated in this study. Since the nuclear SSU rRNA gene is estimated to be present in hundreds of copies per *P. invadens* cell, it is reasonable to assume the reliable detection of *P. invadens* at fractions of cells per PCR reaction, given that all other PCR conditions are optimized. Sensitivity of the assay may be improved by decreasing the DNA elution volume (i.e. increasing concentration), although this would counter the recommended limits of the DNA extraction protocols (DNeasy Blood and Tissue Handbook, Qiagen), or re-eluting in a smaller volume (e.g. following an ethanol precipitation). Increasing sensitivity was deemed unnecessary for this present study because *P. invadens* was detected in radial nerve, sediment, and seawater samples, at abundances found naturally in the environment (see Chapter 3, Section 3.3.1. & 3.3.2.).

Spiking experiments involving seawater DNA (Section 2.3.4.3.) suggested a substantial loss of *P. invadens* DNA when samples were spiked prior to DNA extraction (as cells) versus after (as DNA). Filters used in this study had a pore size of 2.0 μm, which is less than the smallest cell length (8 – 57 μm) or width (3 – 30 μm) of *P. invadens* recorded in previous studies (Jones 1985, Feehan et al. 2013). Therefore it is unlikely that intact cells of *P. invadens* were lost through filters during filtration, although it is possible that some cells were damaged by the process, resulting in loss of cell fragments and DNA. Some loss of DNA may have also occurred upon transfer of the lysis buffers and lysed DNA to spin columns during the DNA extraction protocol (combining three filters into the 2-ml Eppendorf tubes made it difficult to ensure that buffers were homogenized within the tube and that all solution was pipetted off upon transfer). In an attempt to identify variables influencing DNA extraction from microbial plankton filtered onto Nanoporpous Aluminum Oxide Filters, Mueller et al. (2014) observed an increase in DNA yield when using the MasterPure total DNA and RNA extraction kit (Epicentre) compared to the DNeasy Blood and Tissue DNA extraction kit. Also, Mueller et al. (2014) used a back-flushing technique (i.e. flushing filters with cell lysis solution and

proteinase K through the outlet side of the filter) before DNA extraction to reduce the potential loss of DNA and cells during DNA extraction. This procedure and use of the MasterPure DNA extraction kit may further improve extraction efficiency of *P. invadens* DNA.

2.4.4. Reliability of Cell Estimates Obtained using qPCR

Calibration curves for qPCR were generated by spiking radial nerve and seawater DNA with DNA extracted from a known number of cultured cells of *P. invadens*. When testing the reliability of estimates of *P. invadens* abundance obtained using this approach, biases were identified. An overestimation by a factor of about 2 was observed when quantifying *P. invadens* in radial nerve DNA samples; there is no clear explanation for this modest discrepancy. An underestimation by more than an order of magnitude was observed when quantifying *P. invadens* in seawater DNA samples, which as discussed in the previous section, may be linked to DNA loss during filtration or DNA extraction. Ideally, calibration curve dilutions would be generated by spiking radial nerve and seawater samples with *P. invadens* before DNA extraction, as performed in a recent similar study that generated a qPCR assay to detect *Paramoeba perurans* in tissue from mussels (genus *Mytilus*; Rolin et al. 2016). However, this would have required the construction of two calibration curves, one for each sample type, which was not done because of logistical constraints. Instead over- and under-estimations identified in this study should be taken into consideration when calculating abundances of *P. invadens* in radial nerve and seawater samples in future studies using this qPCR assay.

2.4.5. qPCR Inhibition and Optimization

PCR inhibition is a well-known limitation of using PCR-based approaches on environmental DNA (Schrader et al. 2012). Substances referred to as inhibitors are extracted along with target DNA and may decrease sensitivity and/or result in false-negatives by interfering with DNA polymerase activity, reducing reaction efficiency, or competitively binding to and reducing the availability of template DNA (Opel et al. 2010,

reviewed by Schrader et al. 2012). Quantitative PCR is sensitive to even low concentrations of inhibitors within DNA samples and often requires extensive optimization to identify appropriate reaction conditions for individual sample types and target sequences. PCR inhibition can be overcome by diluting DNA to reduce inhibitor concentration (Lloyd et al. 2010), however this naturally sacrifices sensitivity, and may be unsuitable when trying to detect a species that occurs in low abundance in the environment. The qPCR methods described here were optimized to provide estimates of cell number of *P. invadens* at natural abundances in radial nerve tissue and seawater. However, they could not be used to quantify natural abundances of *P. invadens* in sediment DNA. An increase in qPCR efficiency with decreasing sediment DNA concentration indicates this is likely a result of high concentrations of PCR inhibitors in combination with a low abundance of DNA from the target organism. Some commercial DNA extraction kits are specifically designed to remove PCR inhibitors (e.g. MoBio PowerSoil Kits), and may be adequate for use in traditional PCR (Lloyd et al. 2010). However, alternative DNA extraction protocols and/or additional DNA purification steps need to be explored for quantitation of *P. invadens* within sediment DNA.

2.4.6. Limitations of qPCR using SYBR Green and Recommendations

Basic quantitative PCR methods using SYBR Green reagents in this study were an initial attempt to quantify *P. invadens* in radial nerve, sediment, and seawater DNA, however an investment into more sensitive and specific qPCR approaches, such as a TaqMan Probe assay, is recommended. SYBR green reagents are non-specific, i.e. they will bind to any double stranded DNA available (Arya et al. 2005). Therefore, assays using SYBR Green reagents often are limited to the initial design of species-specific PCR primers and need extensive optimization to ensure that amplification of off-target products does not occur (Smith & Osborn 2009). Using *P. invadens*-specific primers ParNssu905F and ParNssu1094R, non-specific binding was observed in no-template controls (NTC) and at low abundances of *P. invadens* (0.5-0.005 cells per sample) in some, but not all replicates, for both radial nerve and seawater calibration curves. The melting temperature of non-specific products ranged from 70.21 – 71.1 °C, approximately

5 – 6 °C lower than the melting temperature of the target sequence (~76 °C), which could suggest the formation of primer dimers. The presence of non-specific binding at these low concentrations limits the sensitivity of this assay. With the expectation that natural concentrations of *P. invadens* in radial nerve and seawater DNA are likely low (see Chapter 3, Section 3.3.2.), this could lead to the exclusion of replicates or entire samples due to non-specific amplification. To combat this, increased replication or repeats of entire qPCR runs may be required, which is neither time-efficient nor cost-effective. In comparison to SYBR green chemistry, TaqMan Probes are designed to complement an additional region between flanking primers, and contain a fluorescent signal that is only expressed upon removal of the probe from the template DNA (Arya et al. 2005). This allows for increased specificity as increased fluorescence will only be observed when a primer and the probe anneal to the target DNA strand and are amplified (Arya et al. 2005). Using *P. invadens* specific primers ParNssu905F and ParNssu1094R, a candidate internal probe sequence was designed using the software Primer3 (5'-ACAGTTGGGGGCATTCGTAT-3'). TaqMan probe assays typically amplify a region between 100 – 150 bp in length to allow for almost complete coverage of the targeted amplicon, therefore it may be necessary to design new primers for use with a TaqMan probe as the primers used in this study amplify a fragment that is 190 bp long. Regions within the SSU rDNA gene and ITS regions suitable for primer design were limited due to the violation of primer design guidelines or high sequence similarity to closely related species, therefore it may be necessary to explore additional gene regions for TaqMan probe design. One possibility is the cytochrome c oxidase I (CO1) gene, which has been proposed as a universal region for barcoding of all animals (Hebert et al. 2003) and has been successfully used to differentiate between species and isolates of amoebae in the genera *Cochliopodium* and *Vannella*, which belong to the same phylogenetic supergroup as *Paramoeba* (Nassonova et al. 2010, Tekle 2014).

2.4.7. Suggestions for Future Research

With these methods in hand, *P. invadens* presence and abundance may be monitored in the environment and used to increase our understanding of the transmission,

progression, and survival of this pathogen along the Atlantic coast of Nova Scotia. Future studies should focus on monitoring populations of *P. invadens* in the environment to gain a better understanding of how the abundance of *P. invadens* varies along the Atlantic coast of Nova Scotia, before, during, and after a disease outbreak, and as seawater temperature declines towards the proposed lower thermal threshold for survival of *P. invadens* (2.5 °C; Buchwald et al. 2015). Additionally, the methods described here may be used to compare pathogen load in symptomatic and asymptomatic sea urchins and to quantitatively test the hypothesis that below 10 – 12 °C, sea urchins are able to recover from infection by *P. invadens* (Scheibling & Hennigar 1997, Scheibling & Stephenson 1984), providing insight into the host-pathogen dynamics of this disease.

CHAPTER 3

DETECTION AND QUANTIFICATION OF *PARAMOEBA INVADENS* ALONG THE ATLANTIC COAST OF NOVA SCOTIA

3.1. INTRODUCTION

Epizootics, caused by marine pathogens, play a major role in structuring marine ecosystems (Lafferty et al. 2004, Ward & Lafferty 2004, Feehan & Scheibling 2014), yet despite an increase in the frequency of these events in recent decades (Ward & Lafferty 2004, Wilcox & Gubler 2005), the host-pathogen interactions, reservoirs and origins, and transmission dynamics of marine pathogens remain difficult to determine and are poorly understood (Harvell et al. 2004, Burge et al. 2014). The advancement of molecular techniques has begun to bridge this gap by providing tools to rapidly and effectively track the distribution and abundance of pathogens in the environment. For example, quantitative real-time PCR techniques (qPCR) have been effectively used to detect and monitor *Paramoeba/Neoparamoeba* species (causative agents of amoebic gill disease) in gill tissue of farmed Atlantic salmon in Ireland (Downes et al. 2015) and in the water columns at salmon farms in Tasmania (Wright et al. 2015), where they have informed management strategies to prevent the spread of the pathogen. The frequency of marine epizootics is predicted to increase with climate change (Burge et al. 2014) making it increasingly important to understand the dynamics of these disease outbreaks to manage and conserve ecologically and economically important marine species and ecosystems (Burge et al. 2014).

The green sea urchin *Strongylocentrotus droebachiensis* plays a key ecological role in determining the structure of the shallow rocky subtidal ecosystem (< 25 m) along the Atlantic coast of Nova Scotia, where increases in sea urchin density can drive phase-shifts from a kelp bed to an urchin barren state (Mann 1977, Scheibling et al. 1999). Since the early 1980s, recurrent outbreaks of the marine amoeba *Paramoeba invadens* have significantly reduced sea urchin populations in Nova Scotia, resulting in re-establishment of the kelp-bed state and collapse of the sea urchin fishery along this coast (reviewed by

Scheibling et al. 2013). A direct relationship between seawater temperature and the growth and progression of *P. invadens* in coastal waters is well documented in field and laboratory studies. Outbreaks of *P. invadens* typically occur between August to October, when surface seawater temperatures are above 10 to 12 °C (the proposed lower thermal threshold for propagation of paramoebiasis) and attain an annual peak around 16 – 18 °C (reviewed by Scheibling et al. 2013, Feehan et al. 2016). Below 5 °C *P. invadens* ceases to grow in culture, and 2.5 °C has been proposed as the lower threshold for survival of the pathogen in shallow coastal waters (Jellett & Scheibling 1988, Buchwald et al. 2015).

These studies suggest that *P. invadens* is typically eliminated from coastal waters during winter, when minimum temperatures usually range from –1 to 2 °C (Jellett et al. 1989, Feehan et al. 2012, Buchwald et al. 2015, Feehan et al. 2016). The sporadic nature of disease events further supports this hypothesis, as seawater temperatures in Nova Scotia extend above the lower thermal threshold for propagation of paramoebiasis each year (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010, Scheibling et al. 2013). Recurrent outbreaks of the disease have been statistically linked to strong storms and hurricanes, suggesting that *P. invadens* is re-introduced to Nova Scotia from remote source population(s) (Scheibling & Lauzon-Guay 2010, Feehan et al. 2016). The probability of a mass mortality event occurring following the passage of a large storm or hurricane can be reliably predicted using a logistic regression model with parameters including the distance of passing storms to the coast, the maximum sustained wind speed when closest to the coast, and the post-storm seawater temperature (i.e. seawater temperatures above 10 °C) in the two weeks following the passing of a candidate storm (Scheibling & Lauzon-Guay 2010; Feehan et al. 2016). Although, the mechanism of introduction remains elusive, using the parameters above and additional parameters including atmospheric pressure, hourly winds, air temperature, and bottom orbital velocity (μ_b, m s^{-1}), there is evidence that *P. invadens* may be transported to Nova Scotia by horizontal advection of surface seawater from an offshore source population(s) where temperatures are more conducive to year-round survival (Feehan et al. 2016).

To date, *P. invadens* has only been documented in its sea urchin host and only during outbreaks of disease, either through histological observation (Jones et al. 1985) or tissue culture of infected individuals (Jellett & Scheibling 1988, Jellett et al. 1989, Feehan

et al. 2012, Feehan et al. 2016). Histological observations indicate that *P. invadens* is present in low abundances in tissues of infected sea urchins (Jones et al. 1985), and water-borne transmission experiments, using symptomatic individuals as an upstream source, indicate that infection rate of healthy downstream individuals depends on the number of infected sea urchins at the source (Scheibling & Stephenson 1984). *Paramoeba invadens* has been isolated only from sea urchins showing clinical signs of paramoebiasis (Jellett & Scheibling 1988, Jellett et al. 1989), suggesting that culturing methods may not be sufficiently sensitive to detect amoebae during initial (pre-symptomatic) stages of infection or during recovery of individuals at low temperatures, when growth rate of *P. invadens* in culture is slow (Jellett & Scheibling 1988). Also, since *P. invadens* is likely transported to Nova Scotia via seawater (Feehan et al. 2016), tracking the presence of *P. invadens* in the environment (sediment and seawater) as well as in sea urchins is critical to understanding the transmission and progression of this pathogen and disease.

This study uses a PCR/qPCR-based assay (Chapter 2), to monitor the presence and abundance of *P. invadens* in sea urchins, sediment, and seawater in Nova Scotia during two consecutive years (2014 and 2015). The main objectives were 1) to identify patterns of distribution and abundance of *P. invadens* to gain insight into the natural transmission of *P. invadens* and propagation of paramoebiasis on this coast, and 2) to quantify and compare pathogen load in asymptomatic and symptomatic sea urchins and determine the utility of PCR-based tools in detecting populations of *P. invadens* in environmental samples.

3.2. MATERIALS AND METHODS

3.2.1. Sampling Design

In 2014, as part of a broader field experiment to examine mechanisms of introduction of *P. invadens* to the Atlantic coast of Nova Scotia, we monitored the presence of *P. invadens* in sea urchins (*S. droebachiensis*) transplanted from a source population (Splitnose Point), where sea urchins had persisted in barrens for more than a

decade, to cages in kelp beds at two sites at 8 m depth (Luke Island, The Lodge) in St. Margarets Bay, where sea urchins had experienced recurrent mass mortalities due to paramoebiasis. We also monitored sea urchins in cages at 18-m depth at one of the bay sites (The Lodge) as well as those in a back-transplant control group at Splitnose Point. Transplantation took place in August 2014, just prior to an increase in seawater temperature above the lower thermal threshold for propagation of paramoebiasis (~ 10 °C; temperature was recorded at 8-m depth at The Lodge). At each location, 20 adult sea urchins (> 30 mm test diameter) were added to each of 2 or 4 cages placed 2 – 5 m apart on the rocky seabed, and fed kelp from the surrounding area (for details see Feehan et al. 2016). Divers monitored the cages biweekly to count surviving sea urchins, remove dead individuals, and collect those showing signs of paramoebiasis (drooping spines, gapping peristome, loss of attachment to substratum).

Sampling of caged sea urchins for *P. invadens* began 21 October 2014, amid an outbreak of paramoebiasis following the passing of a strong storm on 22 September 2014 (Feehan et al. 2016). At each sampling interval, 4 symptomatic sea urchins (1 per cage) were collected to test for *P. invadens* using the PCR-based assay described in Chapter 2. If 4 symptomatic sea urchins were not available at the time of sampling, asymptomatic sea urchins were haphazardly selected to make up the difference. In addition to sea urchins, 2 surface sediment samples were collected by divers into 50 ml centrifuge tubes and tested for *P. invadens* at each sampling interval, beginning 7 October 2014. One sediment sample was collected from within a randomly selected cage, and the other ~ 5 m from the cage site.

In November 2014, once the seawater temperature dropped below 10 °C, the main experiment was terminated. Surviving sea urchins were left to overwinter in cages at Splitnose Point. All sea urchins in cages at Luke Island had died or were sampled prior to this date. At The Lodge, there was little or no mortality or morbidity in cages at either 8 m or 18 m depth (Feehan et al. 2016). When sampling was resumed in April 2015, it was discovered that the cages at Splitnose Point had been swept away over winter, and only sediment was sampled in the experimental area.

In June 2015, 4 cages at Luke Island and Splitnose Point were replaced and restocked with wild sea urchins from Splitnose Point (20 per cage). Monitoring of caged

sea urchins and sampling of sea urchins and sediments to test for the presence of *P. invadens* was resumed at biweekly intervals (as above) until October 2015. At each interval, 4 haphazardly selected sea urchins were collected (1 per cage) along with 4 sediment samples (1 from within each of 2 randomly selected cages and 2 at ~ 5 m from the cage site in opposing directions). In addition, two 4-l seawater samples were collected from ~ 1 m above the cage site to test for *P. invadens* in the water column (see 3.2.2.1 for more details). After October 2015, once seawater temperature had again dropped below 10 °C, sea urchins, sediment and seawater were sampled every 2 to 3 months until May 2016. Sea urchin cages at Splitnose Point had once again been swept away upon sampling on 17 December 2015, concluding sampling at this site.

3.2.2. Sample Analysis

3.2.2.1. Radial Nerve Plating and Radial Nerve, Sediment, and Seawater DNA Extraction

Sea urchin samples were prepared and tested for *P. invadens* following the methods described in Chapter 2, Section 2.2.3.3. Briefly, one radial nerve from each urchin was plated onto 0.6 % NN agar, and subcultured onto 1.2 % NN agar if *P. invadens* was present. The remaining 2 – 4 radial nerves, weighing 10 – 20 mg total, were placed into 1.6 ml Eppendorf tubes and stored at −80 °C until DNA extraction. Radial nerve DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions, with a modified incubation time for 1 h at 56 °C. Radial nerve DNA was eluted in 100 µl buffer AE and stored at −20 °C.

Sediment samples were collected and tested for *P. invadens* following the methods described in Chapter 2, Section 2.2.3.4. Immediately upon collection, ~ 5 g of surface sediment was placed into phenol:chloroform:isoamyl alcohol (25:24:1; Fisher Scientific), and buffers SR1, SR2, and bead solution, following the manufacturer's protocol for the PowerSoil RNA Extraction Kit and DNA elution accessory kit (MoBio). Sediment samples were stored overnight in buffers at 4 °C and DNA was extracted the next day, following the manufacturer's protocol. DNA was eluted in 50 µl buffer SR7 and stored at −20 °C.

Seawater samples were collected and tested for *P. invadens* following the methods described in Chapter 2, Section 2.2.3.5. Duplicate, 4-l seawater samples were collected from above sea urchin cages into clean Ziploc bags, prior to any sediment disturbance. Seawater samples were transported on ice and filtered immediately upon arrival. Seawater was pre-filtered through 100 μm mesh and subsequently filtered onto 2.0 μm Isopore membranes. Immediately upon filtration, filters were flash-frozen in liquid nitrogen and stored at −80 °C until DNA extraction. DNA was extracted using the DNeasy Blood and Tissue Kit, following the manufacturer's protocol with minor modifications (see Section 2.2.3.5 for details).

3.2.2.2. Detection of P. invadens *by PCR*

Radial nerve, sediment, and seawater DNA samples were tested for *P. invadens* using 20 μl PCR reactions containing 2 μl of 10X PCR Buffer – Mg (Invitrogen), 1.5 mM MgCl$_2$ (radial nerve DNA) or 1.0 mM MgCl$_2$ (sediment and seawater DNA), 0.2 mM each of dATP, dTTP, dCTP, dGTP, 0.5 μM of each primer (ParNssu905F and ParNssu1094R), 1 μl template DNA at final concentrations of 1/2 (radial nerve and sediment) or 1/1 (seawater) the original DNA concentration after elution, and 1 U Taq DNA polymerase (Invitrogen). Additionally, 1.2 μg of Bovine Serum Albumin (BSA) was added to PCR reactions with seawater DNA. PCR reactions were conducted using the following thermocycling protocol: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 15 s, and a final step at 72 °C for 1 min. PCR products were visualized using gel electrophoresis (1.5 % TAE agarose gel; 6 μl PCR product per well).

3.2.2.3. Quantification of P. invadens *in Radial Nerve Tissue and Seawater*

Pathogen load (i.e. the total number of *P. invadens* cells mg^{-1} of radial nerve tissue) or abundance (cells l^{-1} of seawater) was quantified using real-time quantitative PCR (qPCR) following the methods described in Chapter 2, Section 2.2.5. Given the low precision of measurement for radial nerve weight (+/- 10 mg), we used a conservative estimate of 20 mg tissue (upper limit of possible weight) to calculate pathogen load for all

samples. qPCR was performed using SYBR Green chemistry and analyzed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Briefly, calibration curves for radial nerve and seawater DNA were generated by adding DNA from known quantities of *P. invadens* cells (500, 50, 5, 0.5, 0.05, and 0.005 cells per reaction) to radial nerve and seawater DNA (at 1/2 the original DNA concentration) that had tested negative for *P. invadens,* to act as a relevant 'background'. Calibration curves were conducted using triplicate reactions, and experimental samples were analysed in duplicates. Quantitative PCR reactions were carried out in 20 μl volumes containing 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer (ParNssu905F and ParNssu1094R), 1.2 μg BSA for radial nerve samples or 2.5 μg BSA for seawater samples, and 1 μl template DNA, with the following thermocycling conditions: 95.0 °C for 10 min, followed by 45 cycles of 95.0 °C for 15 s and 60.0 °C for 1 min. Template DNA was added at a concentration of 1/2 the original DNA concentration. A melt-curve analysis was included to confirm specific amplification; samples or replicates with double peaks were excluded from the analysis. The slope, R^2, and efficiency values were -3.41, 0.998, 96.4 % and -3.41, 0.985, 96.5 % for radial nerve and seawater calibration curves, respectively. A Mann-Whitney-Wilcoxon (MWW) test was used to compare pathogen load between symptomatic and asymptomatic sea urchins.

3.3. RESULTS

3.3.1. Detection of *P. invadens* in Sea Urchins, Sediments and Seawater using PCR

Following a strong storm (nor'easter) on 22 September 2014, *P. invadens* was detected in all sea urchin tissue (n = 4) and sediment (n = 2) samples collected from Luke Island and Splitnose Point during a disease outbreak that was observed 2 – 6 weeks post-storm (Fig. 3.1). As temperatures declined in November towards the lower thermal threshold for propagation of paramoebiasis (10 – 12 °C), a decrease in the proportion of sea urchins infected with *P. invadens* was observed at both sites, although *P. invadens* was still detected in sediment samples. *Paramoeba invadens* was not detected at The Lodge-8m until the final sampling date (26 November 2014), suggesting a lag in

introduction of the pathogen to the western shore of St Margarets Bay. At this time, *P. invadens* was detected in both sediment samples, but in only 1 out of 4 sea urchins sampled. *P. invadens* was not detected in any of the sediment and sea urchins sampled from The Lodge-18m in 2014.

Following an unusually cold winter, with temperatures at 8 m dropping to 0 °C in February 2015, *P. invadens* was not detected in sediment or sea urchins at any of the 4 sites sampled in April 2015. Additionally, *P. invadens* was not detected in sediment or sea urchins at either of the 2 remaining sites (Luke Island and Splitnose Point) during the summer/fall period of peak temperatures in 2015, or throughout the following winter at Luke Island (December 2015 – May 2016), when minimum temperatures were relatively warm (~ 2.5 °C).

Interestingly, however, *P. invadens* was detected in seawater samples collected from Luke Island and Splitnose Point during the temperature peak between July and October in 2015, when temperature at 8 m depth was > 10 °C. It was also detected in seawater samples collected in December 2015 at Splitnose Point and in one replicate at Luke Island, when temperature had dropped to 7.4 °C (Fig. 3.1). *P. invadens* was not detected in subsequent seawater samples collected at Luke Island in March and May, 2015 when seawater temperatures were 2.6 °C and 8.2 °C, respectively.

To exclude the possibility of contamination, duplicate 4-l distilled water samples, putatively free of *P. invadens*, were obtained from a *P. invadens*-free laboratory on 19 April 2016 and subjected to the same filtering and extraction protocols as the seawater samples from our field sites. Additionally, 3 seawater samples, putatively uninfected with *P. invadens*, were collected on 3 June 2016 from Sandy Cove, Terence Bay (44° 27' 6" N, 63° 42' 4" W), located ~ 20 km northeast of St. Margarets Bay, where sea urchins have not been observed for more than a decade. PCR results for all 5 water samples were negative for *P. invadens* using *P. invadens*-specific primers ParNssu905F and ParNssu1094R. This suggests that contamination did not occur and that *P. invadens* was indeed present in seawater collected from Luke Island and Splitnose Point in 2016.

Fig. 3.1. Mean (± SE) proportion of samples in which *P. invadens* was detected in sea urchins (n = 4), sediment (n = 2), and seawater (n = 2) DNA using PCR at 4 sites A: Luke Island, B: Splitnose Point, C: The Lodge-8m, D: The Lodge-18m, between September 2014 and May 2016. Top panel shows daily average seawater temperature at The Lodge-8m. Vertical grey arrow indicates the passing of a strong storm (nor'easter) on 22 September 2014. Solid horizontal line indicates the lower thermal threshold for propagation of paramoebiasis (10 °C; Feehan et al. 2016); dashed horizontal line indicates the lower thermal threshold for survival of *P. invadens* (2.5 °C; Buchwald et al. 2015).
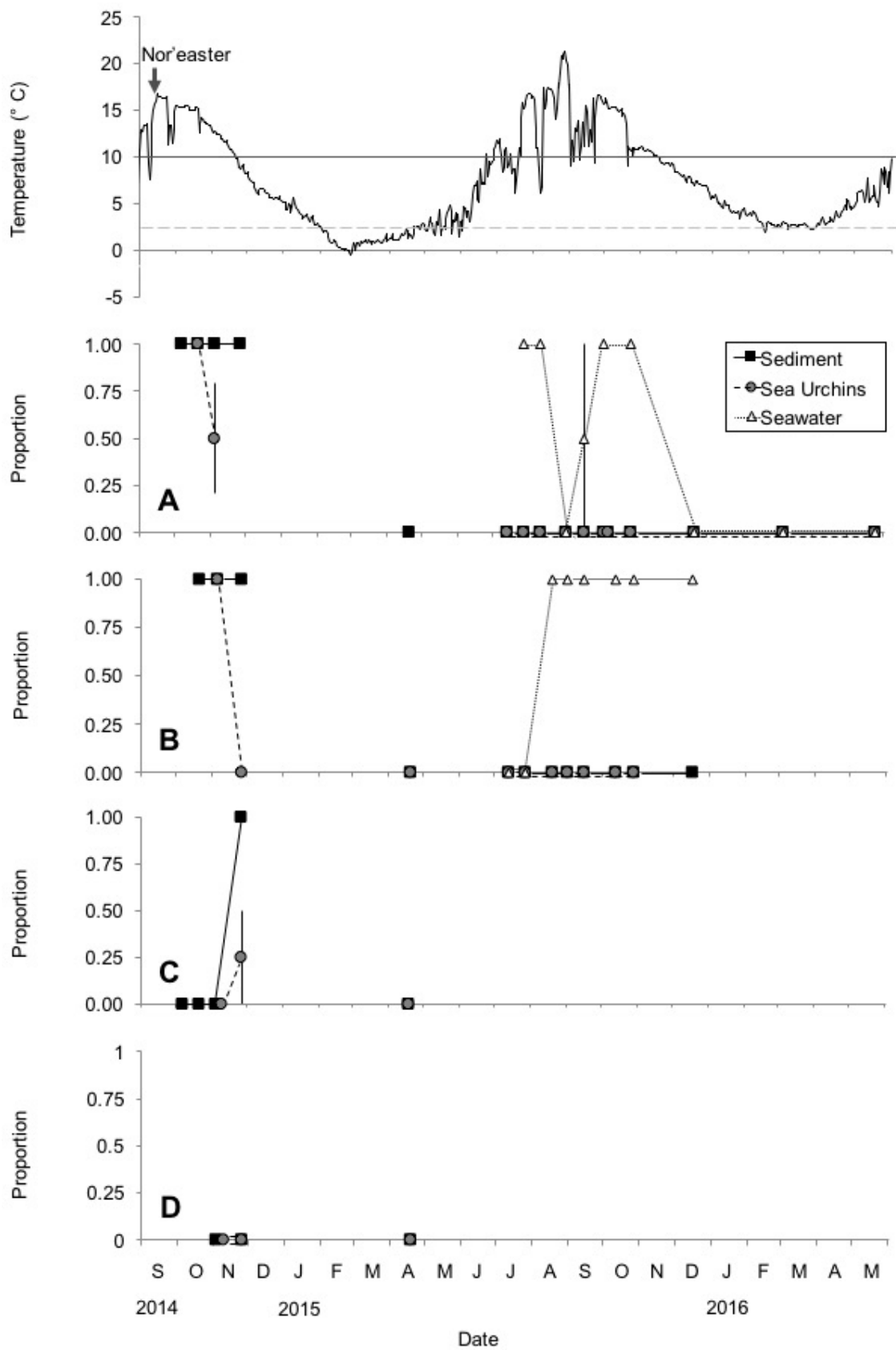
3.3.2. Quantification of *P. invadens* in Sea Urchins and Seawater Using qPCR

3.3.2.1. Sea Urchins

Pathogen load was low in radial nerve samples from 7 infected sea urchins (i.e. detected as positive for *P. invadens* DNA using PCR and qPCR), collected at Luke Island or Splitnose Point during an outbreak of paramoebiasis in 2014. The mean load was 2.25 cells mg^{-1} tissue (range: 0.20 – 6.72 cells mg^{-1}), excluding an outlier (94.55 cells mg^{-1} tissue) from Luke Island (Table 3.1). Once temperatures dropped below the lower thermal threshold for propagation of the disease (~ 10 °C), the pathogen load measured in radial nerve tissue from 2 infected sea urchins (from Splitnose Point) was 0.05 and 0.08 cells mg^{-1} tissue. This represents a decrease of 2 orders of magnitude (3 orders of magnitude if the outlier is included), although the data are too sparse to conduct a meaningful statistical analysis. There was a statistically significant difference in pathogen load between symptomatic and asymptomatic sea urchins (MWW test; U = 28, p = 0.011) during the disease outbreak, and the qPCR data in Table 3.2 are consistent with a threshold of ~ 1 cell mg^{-1} tissue, above which sea urchins display overt signs of paramoebiasis. With the exception of 1 sample, sea urchins with pathogen loads of < 1 cell mg^{-1} tissue were scored negative for *P. invadens* using PCR, also suggesting that ~ 1 cell mg^{-1} tissue is the detection limit for PCR. Real-time quantitative PCR is the more sensitive method of detection, yielding positive results from as little as 0.05 cells mg^{-1} tissue (or 1 cell in ~ 20 mg of radial nerve tissue).

Table 3.1. PCR (presence/absence) and qPCR (cells mg^{-1} tissue) analysis of *P. invadens* in radial nerve tissue of sea urchins from 3 sites: Luke Island (LI), Splitnose Point (SP), and The Lodge-8m (TL-8m). 'Outbreak status' indicates whether the sample was taken during or after an outbreak of paramoebiasis in 2014; there was no evidence of disease at TL-8m or later dates at LI and SP. Symp. (symptomatic) indicates whether sea urchins exhibited overt symptoms of paramoebiasis upon collection (+) or did not (-).

Date	Outbreak Status	Location	Sample	Symp.	PCR	qPCR (cells mg^{-1})
21-Oct-14	During	LI	1	-	+	1.66
			2	+	+	1.29
04-Nov-14	During	LI	1	-	-	0.25
			2	-	-	0
			3	-	+	0.2
			4	+	+	94.55
06-Nov-14	During	SP	1	+	+	3.4
			2	+	+	6.72
			3	+	+	1.08
26-Nov-14	After	SP	1	-	-	0.08
			2	-	-	0.05
26-Nov-14	None	TL-8m	1	-	-	0
			2	-	-	0
13-Jul-15	None	SP	1	-	-	0
			2	-	-	0
07-Mar-16	None	LI	1	-	-	0
			2	-	-	0

3.3.2.2. Seawater

Mean estimated abundance of *P. invadens* in seawater samples pooled from Luke Island and Splitnose Point increased from 0.7 cells l^{-1} in July to as high as 3.3 – 5.3 cells l^{-1} in mid August and early October 2015, coinciding with peaks in seawater temperature during this period, although there was considerable variability within and between sites and across dates (range: 0 – 9.74 cells l^{-1}; Table 3.2; Fig. 3.2). Mean cell abundance was below ~ 0.6 cells l^{-1} in December when seawater temperature was ~ 7.4 °C and decreasing. *Paramoeba invadens* was not detected in samples from Luke Island in March 2016 (2.7 °C) and May 2016 (8.2 °C). The estimated abundance of *P. invadens* in

seawater collected from an aquarium containing infected sea urchins and sediment (7860.5 cells l^{-1}) was 3 orders of magnitude greater than abundances observed in seawater samples collected during the annual peak in seawater temperature. Based on qPCR estimates of *P. invadens* in seawater samples spiked with a known number of cells of *P. invadens* (Chapter 2, Section 2.3.5.3), abundances presented here may be underestimated by a factor of about 10.

Table 3.2. PCR (presence/absence) and qPCR (cells l^{-1}) analysis of *P. invadens* in seawater from 2 sites: Luke Island (LI) and Splitnose Point (SP). Na indicates the data was not available due to multiple melt curve peaks; nd indicates no data.

Date	Location	Sample	PCR	qPCR (cells l^{-1})
13-Jul-15	LI	1	+	na
		2	-	0
	SP	1	-	0
		2	-	0
27-Jul-15	LI	1	+	1.25
		2	+	1.48
	SP	1	-	0
		2	-	0
11-Aug-15	LI	1	+	na
		2	+	1.94
19-Aug-15	SP	1	+	1.67
		2	+	3.12
02-Sep-15	LI	1	-	1.00
		2	-	nd
	SP	1	+	9.74
		2	+	na
16-Sep-15	SP	1	+	na
		2	+	1.77
17-Sep-15	LI	1	+	2.44
		2	-	0
04-Oct-15	LI	1	+	5.77
		2	+	3.07
08-Oct-15	LI	1	+	na
		2	+	na
13-Oct-15	SP	1	+	1.05
		2	+	na
28-Oct-15	LI	1	+	0
		2	+	3.94
	SP	1	+	4.09
		2	+	2.73
17-Dec-15	SP	1	+	0.76
		2	+	0.34
21-Dec-15	LI	1	-	1.17
		2	-	0
07-Mar-16	LI	1	-	0
		2	-	0
24-May-16	LI	1	-	0
		2	-	0
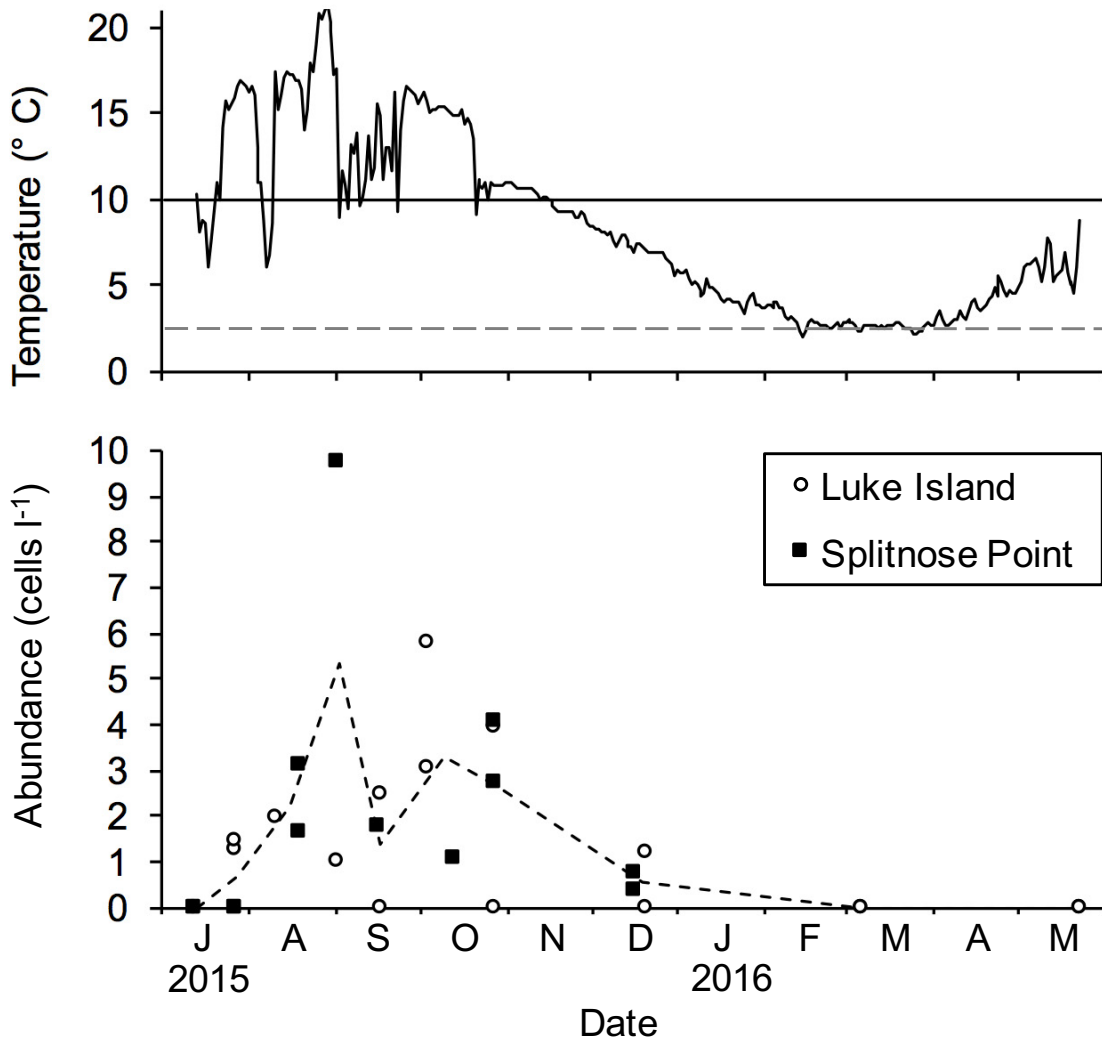
Fig. 3.2. Estimated abundance (cells l^{-1}) of *P. invadens* in seawater at Luke Island and Splitnose Point from July 2015 to May 2016. Data points are duplicate seawater samples at a given site and date, except single samples (see Table 3.2) due to exclusion of a replicate because of methodological problems (multiple melting temperature peaks). Dashed line represents the mean abundance of *P. invadens* (data pooled between sites). Top panel: daily average seawater temperature at 8-m depth at The Lodge. Solid horizontal line indicates lower thermal threshold for propagation of paramoebiasis (10 °C, Feehan et al. 2016); dashed horizontal line indicates lower thermal threshold for survival of *P. invadens* (2.5 °C, Buchwald et al. 2015).

3.4. DISCUSSION

3.4.1. Detection of *Paramoeba invadens* in Sea Urchins and Sediment

The PCR-based assay enabled rapid and reliable detection of *Paramoeba invadens* in radial nerve tissue of *Strongylocentrotus droebachiensis*, providing the most direct insight yet into temperature-mediated host-pathogen dynamics during and after a disease outbreak following the passage of a strong storm. My findings are consistent with predictions based on 3 decades of correlative field studies, laboratory experiments and statistical modeling, which link overt signs of morbidity and mortality in sea urchins to sea water temperature (reviewed by Scheibling et al. 2013, Feehan et al. 2016). Specifically, a decline in the proportion of infected sea urchins, and in the pathogen load of infected individuals, at Luke Island and Splitnose Point by late November, when seawater temperature was ~ 9 °C and had been below 10 °C for 12 days, is consistent with the putative lower thermal threshold of 10 °C for propagation of paramoebiasis (Scheibling & Stephenson 1984). The mechanism whereby the abundance of cells of *P. invadens* in sea urchin tissue declines at temperatures below 10 °C is unknown. A study conducted by Jellett et al. (1988) observed a decline in the abundance of coelomocytes in the coelomic fluid of urchins maintained at 16 °C, suggesting that the immune capacity of sea urchins may be compromised with extended exposure to the high temperatures that are observed during mass mortality events. Bearing this in mind, a reduction in the abundance of *P. invadens* in sea urchin tissues below 10 °C, may involve the temperature-dependent reduction in specific growth rate of the amoeba (Jellett & Scheibling 1988, Buchwald et al. 2015) and/or an increase in the ability of the sea urchin immune system to combat low levels of infection at these lower temperatures (Jellett et al. 1988).

Importantly, the assay also provides the first evidence of *P. invadens* in sediment samples from the natural environment during and after a disease outbreak. The presence of *P. invadens* in sediment samples at Luke Island and Splitnose Point, following a decline in the proportion of infected sea urchins and decrease in pathogen load at Splitnose Point as temperature dropped below 10 °C in late November 2014 (experimental sea urchins had been eliminated at Luke Island by this time), may suggest

that *P. invadens* can survive in the environment longer than within sea urchins immediately following a disease outbreak. Because we are targeting DNA in this study, the possibility of detecting residual DNA from dead cells should be considered, however detecting residual DNA from dead cells is unlikely as DNA is rapidly degraded upon cell death in the environment (Austin et al. 1997). Furthermore, the observation of the decline of *P. invadens* in sea urchins but not sediment by late November 2014 is consistent with the notion that the sea urchin immune system was actively reducing the abundance of *P. invadens* at these temperatures. A better understanding of host-pathogen interactions during recovery after a disease outbreak will require concurrent quantitative estimates of the abundance of *P. invadens* both in sea urchin tissues and in environmental samples, such as sediment and seawater, to resolve the relative effects of decreasing growth rate of the amoeba and a potential increase in immune capacity of the sea urchin host at lower temperatures.

The generally low abundance of *P. invadens* in tissues of infected sea urchins inferred by the qPCR assay is consistent with previous histological observations (Jones et al. 1985). A comparison of the abundance of *P. invadens* in radial nerve tissue between asymptomatic and symptomatic sea urchins indicates a threshold of ~ 1 cell mg^{-1}, where above this threshold sea urchins exhibit overt symptoms of paramoebiasis. The qPCR assay yielded positive results for *P. invadens* at abundances as low as 1 cell in 20 mg of radial nerve tissue, and was more sensitive in detecting infection than the PCR assay. These results indicate the utility of the PCR-based assay as a means of detecting low levels of infection in a sea urchin population prior to the onset of overt signs of disease, which could have important implications for population monitoring for scientific or management objectives. The maximum abundance of *P. invadens* recorded in a sea urchin, 95 cells mg^{-1} tissue (observed in a single individual from Luke Island in November 2014) may reflect a rapid increase in pathogen load immediately prior to the point at which *S. droebachiensis* succumbs to the disease. Interestingly, abundances of amoebae in 3 other specimens collected simultaneously ranged from 0 to 2.5 cells mg^{-1}, indicating high individual variability in the experimental population at this time.

An apparent lag in the introduction of *P. invadens* to The Lodge-8m on the western shore of St. Margarets Bay, is consistent with previous observations that

outbreaks of *P. invadens* can be patchy on a scale of 10 – 100s km of coastline (Feehan et al. 2016). Feehan et al. (2016) provided evidence that *P. invadens* may be introduced to this coast via horizontal advection of offshore surface waters associated with strong storms. Given that circulation in St. Margarets Bay is dominantly anti-clockwise (Heath 1973), we would expect a lag in the introduction of *P. invadens* to The Lodge on the western shore of St. Margarets Bay, compared to Luke Island on the eastern shore of the bay.

     Paramoeba invadens was not detected in sea urchins or sediment sampled from April 2015 until the end of my study in May 2016. The absence of the pathogen in sea urchins following an unusually cold winter in 2015/2016, when temperatures were < 2.5 °C for 8.5 wk, and the absence of a strong storm thereafter, conforms to observations in previous years and is consistent with a putative lower thermal threshold around 2.5 °C for over-winter survival of the amoeba along the coast of Nova Scotia (Buchwald et al. 2015). It has been proposed that outbreaks of disease after such cool winters require reintroduction of the pathogen by advection associated with strong storms (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010, Feehan et al. 2016). However if an outbreak of paramoebiasis is followed by a relatively warm winter, a recurrence of disease may ensue once summer temperatures exceed the 10 °C threshold for propagation of the disease (Buchwald et al. 2015, Feehan et al. 2016). My findings based on the PCR-based assay of *P. invadens* in sea urchins and sediments are consistent with this hypothesis. On the other hand, although it was as expected that *P. invadens* would not be detected in sediment collected in 2015 following an extremely cold winter, the detection of *P. invadens* in seawater in 2015 (see below) indicates that *P. invadens* is present in the environment and might also be present in the sediment, despite our failure to observe it. In comparison to seawater samples, the amount of sediment material used for DNA extraction was quite small (~ 5 g compared to 4 l; due to limitations of the commercial DNA extraction kit), therefore it is possible that the abundance of *P. invadens* in sediment in the absence of a recent disease event is below the detection limits of this assay.

3.4.2. Detection of *Paramoeba invadens* in Seawater

The PCR-based assay detected *P. invadens* in seawater collected from Luke Island and Splitnose Point between mid July and mid December 2015, during the period when seawater temperature was generally above 10 °C. *Paramoeba invadens* was patchy in seawater samples from Luke Island in late December 2015 and was not detected in seawater samples in early March to late May 2016, when temperatures were below 10 °C. This temporal pattern of presence is consistent with previous studies that have measured temperature-dependent growth of *P. invadens* in culture, suggesting that *P. invadens* DNA detected in seawater samples is from intact live cells experiencing growth. The alternative hypothesis, that the DNA detected in seawater in 2015 may have persisted from the disease outbreak in 2014 is unlikely. Without protection from enzymes DNA is heavily degraded upon death of an organism (Austin et al. 1997) and studies on DNA in seawater suggest residence times ranging from 10 days to 2 months (Nielsen et al. 2007).

It is important to note that abundances of *P. invadens* in seawater that are presented in this study may be large underestimates of actual abundance. Results from quantifying seawater samples spiked with a known number of cells of *P. invadens* using qPCR show >10-fold lower estimates of the cell number when compared to the actual number of cells added to the sample prior to filtration and DNA extraction (Chapter 2, Section 2.3.5.3). Although applying this transformation to obtained data seems straightforward, it would rely on the assumption that *P. invadens* is present in the natural environment in the same form as the cells added to seawater samples during the spiking experiment (i.e. free-living amoebae), however it is possible that the DNA detected in natural seawater samples was not from free-floating amoebae. Given that lobose amoebae are predominantly surface-associated (Rogerson & Gwaltney 2000, Rogerson et al. 2003), the DNA from *P. invadens* detected in this study may be from cells attached to suspended matter in the water column (e.g. suspended sediment and other particulate matter). Alternatively, *P. invadens* may be present within the body of another planktonic organism, and/or in an alternate life cycle stage. Any of these could plausibly reduce cell damage and loss of DNA during filtration, which in turn would reduce the magnitude of the underestimation.

3.4.3. Conclusions and Prospects for Future Research

My study presents the first evidence that *P. invadens* may be naturally present in the marine environment (at least in seawater) for extended periods during the annual temperature peak without infecting sea urchins. This does not refute the hypothesis that a hurricane or strong storm is needed to introduce sufficient quantities of the pathogen from a distant source population(s) to trigger a disease outbreak and mass mortality (Scheibling & Lauzon-Guay 2010, Feehan et al. 2013). Low abundances of *Paramoeba invadens* in environmental seawater samples in comparison to the abundance of *P. invadens* in an aquarium with infected sea urchins and sediment, indicate that *P. invadens* may be present at abundances that are too low to effectively encounter and infect localized populations of sea urchins, or that it inhabits an unknown planktonic host where it does not interact directly with sea urchin populations. The abundance of *P. invadens* in seawater from an aquarium with sediment and infected sea urchins was 3 orders of magnitude greater, indicating that *P. invadens* is likely to occur in much greater abundances in the water column during a disease outbreak. It is also possible that *P. invadens* is present year-round as an endemic pathogen in near-shore environments or alternative hosts that remain unstudied, and is minimally suspended into the water column with normal wave activity and currents (Buchwald et al. 2015). The ability to detect *P. invadens* in the absence of a storm sheds new light on the dynamics of this pathogen in nature and elucidating in what form *P. invadens* is present in seawater could be very important to our understanding of the dynamics of this disease in nature.

In this initial application of my PCR-based assay and attendant PCR/qPCR techniques, I was able to detect or quantify *P. invadens* in sea urchin tissue and environmental samples at multiple sites and over 1.5 years. This demonstrates the promise of these techniques as a tool to expand our understanding of the dynamics of *P. invadens* and transmission of paramoebiasis. Importantly, the ability to detect *P. invadens* in seawater and sediments provides an opportunity to expand our sampling range to explore offshore waters and sediments at greater depths in our search for potential source populations that might seed shallow coastal areas. It also allows us to examine the

potential of *P. invadens* to maintain local populations under environmental conditions (e.g. warm winters) predicted by climate change (Buchwald et al. 2015, Feehan et al. 2016). These sorts of expanded studies are needed to provide a better mechanistic understanding of the outbreak dynamics of this pathogen and the role of storms and sea temperature in mediating disease events of profound ecological and commercial significance.

CHAPTER 4

DISCUSSION

4.1. THE UTILITY OF MOLECULAR TOOLS FOR UNDERSTANDING THE EPIZOOTIOLOGY OF
PARAMOEBA INVADENS

The use of molecular tools to detect and monitor the presence, abundance, and distribution of pathogens in the marine environment is becoming increasingly popular and has the potential to answer critical and often difficult questions regarding the etiology and epizootiology of these pathogens. First observed in the 1980s, recurrent outbreaks of *Paramoeba invadens* have decimated populations of the green sea urchin *Strongylocentrotus droebachiensis* along the Atlantic coast of Nova Scotia (reviewed by Scheibling et al. 2013). However the lack of reliable tools to detect this pathogen outside of the urchin host has left the source population and transmission and propagation dynamics of this disease in nature poorly understood. The PCR-based assay developed (Chapter 2) and implemented (Chapter 3) in this thesis is a valuable tool that can be used to close this knowledge gap. The assay is highly sensitive, with the ability to detect *P. invadens* in sea urchins that are asymptomatic for paramoebiasis (from DNA from as little as one cell per tissue sample) and in seawater. The latter was especially noteworthy because the particular seawater samples examined were taken following a very cold winter and in the absence of a disease outbreak or strong storm, and thus the pathogen was not expected to be present at all (Scheibling & Stephenson 1984, Buchwald et al. 2015). This highlights the potential of this assay to detect *P. invadens* in the environment at very low abundances, which is necessary for monitoring changes in its distribution and abundance following a disease outbreak, when sea urchins recover as seawater temperature declines (Jellett & Scheibling 1988).

The results presented in my thesis are the first to identify *P. invadens* in sediment and seawater where artificial introduction of the pathogen is unlikely, and highlights the potential use of this assay to monitor the introduction, transmission, and changes in abundance of this pathogen in coastal waters along Nova Scotia, under differing

environmental conditions. For example, monitoring *P. invadens* in years following a strong storm and subsequent warm winter seawater temperatures can be used to test the over-wintering potential of this pathogen (Buchwald et al. 2015). Implementation of this assay also has the potential to identify offshore hot spots where *P. invadens* may reside year-round, either in asymptomatic sea urchins or in the broader benthic or pelagic environment.

4.2. PERSPECTIVES ON THE MECHANISMS OF INTRODUCTION OF *PARAMOEBA INVADENS* TO NOVA SCOTIA

The work presented in my thesis is the first to provide evidence that *P. invadens* may be present in seawater along the coast of Nova Scotia in the absence of a strong storm and sea urchin mortality. The absence of *P. invadens* during the winter months when seawater temperatures reached a thermal minimum of 0 °C is consistent with the existing hypothesis that *P. invadens* is eliminated from this coast during normal winters (Jellett et al. 1989, Feehan et al. 2012, Buchwald et al. 2015), and may suggest that *P. invadens* is annually re-introduced to this coast via some mechanism other than storm events. These results are not inconsistent with observations of a link between paramoebiasis events and the passing of strong storms, as the abundance of *P. invadens* found in the absence of a strong storm may be too low to encounter or infect sea urchins. Given this observation, large-scale meteorological events are still proposed to play a key role in facilitating mass mortalities of sea urchins along this coast, either by means of introducing greater abundances of *P. invadens* from offshore source populations as suggested by the "Killer Storm Hypothesis" (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010) or, in a modification of this hypothesis, by providing the necessary environmental conditions for proliferation of existing populations of *P. invadens* and propagation of paramoebiasis in coastal waters. Evidence of an annual presence of *P. invadens* in shallow coastal waters in Nova Scotia suggests that the pathogen may be more widespread and common in the environment than previously thought and may indicate that *P. invadens* is endemic to Nova Scotia rather than an exotic species. These results highlight the need for further investigation into alternative life-cycle stages in *P.*

invadens, possible alternative hosts for this amoeba (including pelagic animal species), and for long-term monitoring of populations of *P. invadens* in Nova Scotia and beyond.

A.1. Optimization of MgCl₂ concentration for PCR with primers ParNssu905F and ParNssu1094R

Materials and Methods: The PCR assay was optimized for $MgCl_2$ concentration using primers ParNssu905F and ParNssu1094R. $MgCl_2$ concentrations tested included 2.8 mM, 2.4 mM, 2.0 mM, 1.7 mM, and 1.5 mM and PCR conditions were as described in section 2.2.2.2.

Results: Amplification was similar at all $MgCl_2$ concentrations, with the exception of 2.4 mM and 2.0 mM for isolate SMB-60 (Fig. A.1.1). To minimize the risk of non-specific amplification, the lowest concentration tested (1.5 mM $MgCl_2$), was selected as the optimal $MgCl_2$ concentration to use with primer set Nuc1 (primers: ParNssu905F and ParNssu1094R). Higher $MgCl_2$ concentrations did not show dramatically increased yields.
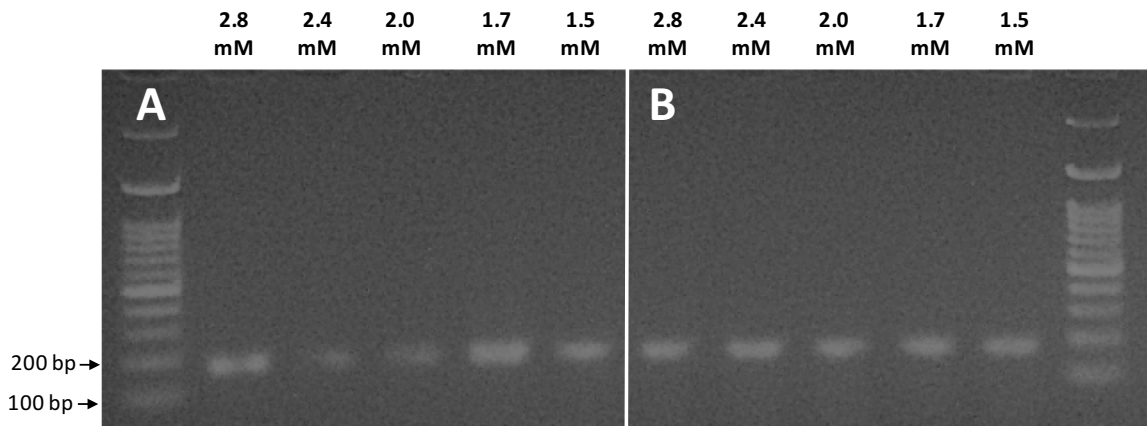

Fig. A.1.1. Magnesium chloride optimization of primer set Nuc1 (190 bp template) using: A) DNA extracted from cultures of *P. invadens* isolate SMB-8 and B) DNA extracted from cultures of *P. invadens* isolate PP-2012. Final concentrations of $MgCl_2$ in solution included 2.8 mM, 2.4 mM, 2.0 mM, 1.7 mM, and 1.5 mM per PCR reaction.

A.2. Comparison of seawater DNA extraction methods

Materials and Methods: Since establishing a working seawater DNA extraction method was difficult, three methods were compared to determine the most efficient DNA extraction technique for recovering *P. invadens* DNA. Triplicate seawater samples were obtained from the Aquatron aquaria and filtered in parallel. DNA was extracted from sample 1 using the DNeasy Blood and Tissue DNA extraction kit with minor modifications. Seawater filters were removed from storage at − 80 °C and allowed to thaw for 5 minutes. Upon thawing, filters were suspended in 400 μl sterile distilled water (dH$_2$O), 360 μl buffer ATL (Qiagen), and 40 μl proteinase K (Qiagen). Reactions were mixed by vortexing and allowed to incubate at 56 °C in a water bath for 10 minutes. Following incubation, reactions were mixed and 400 μl buffer AL (Qiagen) and 400 μl, 96 − 100 % ethanol (EtOH) were added. Subsequent steps were as per the manufacturer's protocol and seawater was eluted in 200 μl AE buffer. DNA from sample 2 was extracted using the DNeasy Blood and Tissue DNA extraction kit with the same modifications as described for sample 1, however with an incubation time of 1 hour at 56 °C. DNA was extracted from sample 3 using the DNeasy Plant Mini Kit (Qiagen) with minor modifications. Seawater filters were removed from − 80 °C and allowed to thaw at room temperature for 5 minutes. Upon thawing, 50 μl of a lysozyme and TE buffer mixture (20 mg/ml) was added to the reaction. Subsequently, 400 μl buffer AP1 (Qiagen) and 50 μl proteinase K (50 mg/ml) were added and the reaction was incubated at 52 °C for 1 hour. Following incubation, 4 μl RNase A (100 mg/ml; Qiagen) was added and the remainder of the protocol was as per manufacturer's protocol. Sample 3 was eluted in 200 μl AE buffer.

To compare the efficacy of the three DNA extraction techniques, the extracted DNA was tested by PCR using a dilution series of DNA at 1/2, 1/4, 1/8, and 1/16 of the original DNA concentration and compared. PCR was performed using i) universal primers 514F & 1055R, and ii) *P. invadens*-specific primers ParNssu905F and ParNssu1094R, following general protocols described in Section 2.2.2.2.

Results: Positive PCR amplification was observed for all three seawater DNA extraction methods using universal primers 514F and 1055R (Fig. A.2.1. A, B, C). Amplification using universal primers was comparable for both DNA extractions using the DNeasy Blood and Tissue kit (Qiagen) (Fig. A.2.1. A, B). DNA extraction using the DNeasy Plant Mini Kit appeared less efficient, with weaker amplification observed across the range of DNA concentrations tested (Fig. A.2.1. C; Note that the lack of detectable amplification of 1/8 original DNA concentration likely had another cause). Positive amplification of *P. invadens* DNA using primer set Nuc1 was observed for all DNA extraction methods (Fig. A.2.1. D, E, F). DNA extraction using the DNeasy Blood and Tissue kit with an incubation step of 1 hour and *P. invadens* primers Nuc1 yielded the strongest amplification at all dilutions tested (Fig. A.2.1. E). DNA extraction using the DNeasy Blood and Tissue Kit with an incubation step of 10 minutes yielded intermediate amplification (Fig. A.2.1. D). DNA extraction using the DNeasy Plant Mini Kit yielded the poorest amplification, particularly as DNA concentration decreased (Fig. A.2.1. F).
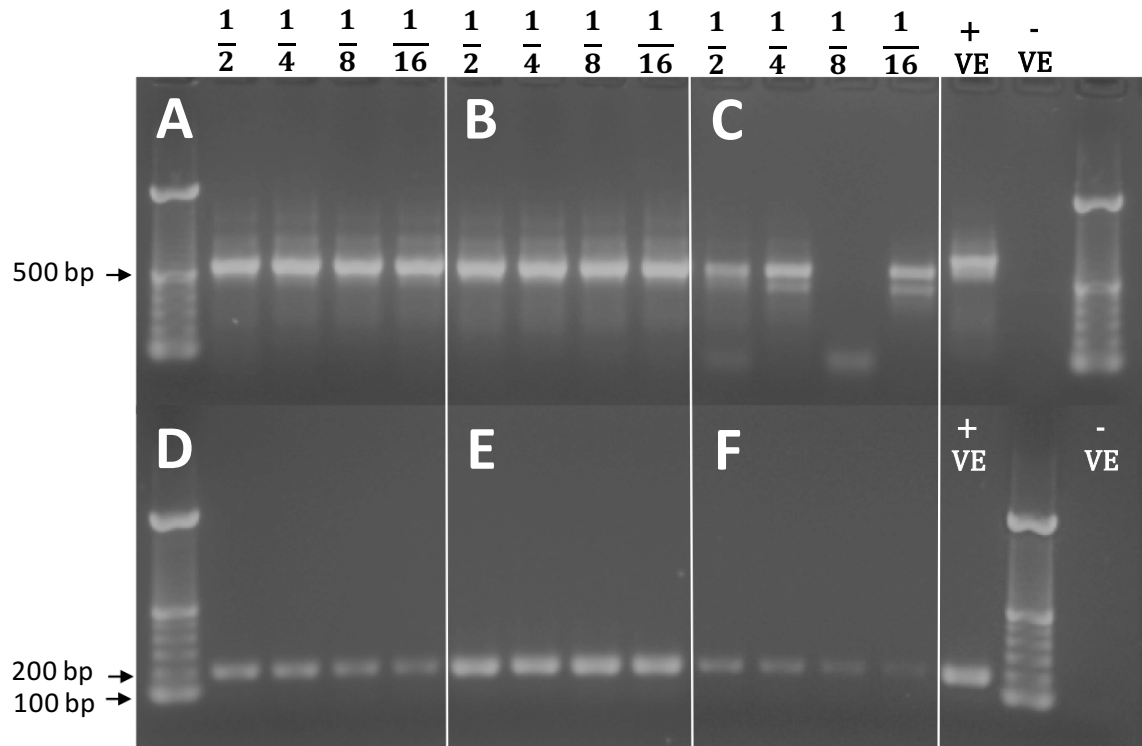
Fig. A.2.1. PCR dilution series comparing three seawater DNA extraction protocols: A & D) DNeasy Blood and Tissue Kit (Qiagen) with an incubation step of 10 minutes, B & E) DNeasy Blood and Tissue Kit (Qiagen) with an incubation step of 1 hour, and C & F) DNeasy Plant Mini Kit (Qiagen) using universal primers 514F and 1055R (A, B, C) and *P. invadens* primer set Nuc 1 (D, E, F). Dilutions were performed at 1/2, 1/4, 1/8, and 1/16 the original DNA concentration for each of the three DNA extraction methods. Positive controls (+VE) were performed using DNA from isolate SMB-60.

A.3. Molecular Assay Optimization for Radial Nerve, Sediment, and Seawater DNA

Materials and Methods: The following was performed on all 3 sample types (radial nerve, sediment and seawater DNA) using *P. invadens* specific primers. To optimize the detection of *P. invadens*, magnesium chloride ($MgCl_2$) and primer concentration dilution series were performed. $MgCl_2$ was tested at final concentrations of 2.5 mM, 2.0 mM, 1.5 mM, 1.0 mM, and 0.5 mM for sediment and seawater DNA. $MgCl_2$ was tested at final concentrations of 2.0 mM, 1.5 mM, 1.0 mM, and 0.5 mM for radial nerve DNA. Primer

concentrations tested included 1 μM, 0.75 μM, 0.50 μM, and 0.25 μM of each primer per reaction for all three DNA types. Following DNA extraction, DNA quality was assessed by PCR-amplification using universal primers 514F (5'-TCTGGTGCCAGCAGCCGCGG-3') and 1055R (5'-CGGCCATGCACCACC-3'). Additionally, DNA was tested for *P. invadens* using *P. invadens*-specific primer set 'Nuc1' (primers ParNssu905F and ParNssu1094R). A dilution series consisting of 1, 1/2, 1/4, and 1/8 of the original DNA concentration was performed on 2 - 4 DNA samples of each sample type using both universal primers 514F and 1055R and *P. invadens* specific primers to determine the optimal DNA concentration for PCR. PCR reactions were carried out in 20 μl reactions containing 2 μl of 10X PCR Buffer -Mg (Invitrogen), 1.5 mM $MgCl_2$ (radial nerve DNA) or 2.0 mM $MgCl_2$ (sediment and seawater DNA), 0.2 mM each of dATP, dTTP, dCTP, dGTP, 0.5 μM of each primer forward and reverse, 1μl template DNA, and 1 U Taq DNA polymerase per reaction. Additionally, 1.2 μg of Bovine Serum Albumin (BSA) was added to PCR reactions with seawater DNA. The thermocycling protocol was as follows: 94°C for 2 min., 35 cycles of 94°C for 15 sec., 56°C for 30 sec., 72°C for 15 sec., and a final step of 72°C for 1 minute.

Results: A short series of PCR experiments were conducted to optimize the PCR protocol for detecting *P. invadens* in radial nerve, sediment, and seawater DNA samples, using primer set Nuc 1 (four samples tested). *Radial Nerve:* Similar levels of amplification were observed using $MgCl_2$ concentrations of 2.0 mM and 1.5 mM. A decline in amplification was observed at $MgCl_2$ concentrations of 1.0 mM and no amplification was observed at $MgCl_2$ concentrations of 0.5 mM for all four radial nerve DNA samples (Fig. A.3.1.) Manipulating primer concentrations yielded similar PCR amplification at primer concentrations of 1.0 μM, 0.75 μM, and 0.5 μM. A decrease in amplification was observed for all four radial nerve DNA samples at a primer concentration of 0.25 μM (Fig. A.3.2). *Sediment:* Optimization of PCR conditions for sediment DNA showed similar amplification using $MgCl_2$ concentrations 2.5 mM, 2.0 mM and 1.5 mM, with marginally less amplification using $MgCl_2$ at 1.5 mM (Fig. A.3.3). There was poor amplification at a $MgCl_2$ concentration of 1.0 mM for both sediment DNA samples tested, and no amplification at a final $MgCl_2$ concentration of 0.5 mM. Optimization of

primer concentration yielded similar amplification for both sediment DNA samples when using primer concentrations of 1.0 μM, 0.75 μM, and 0.5 μM (Fig. A.3.4). A marginal decrease in PCR amplification was observed at a primer concentration of 0.25 μM. *Seawater:* Optimization of PCR conditions for seawater DNA showed similar amplification using $MgCl_2$ concentrations of 2.5 mM and 2.0 mM (Fig. A.3.5). There was a marginal decrease in amplification at a $MgCl_2$ concentration of 1.5 mM and poor amplification at a $MgCl_2$ concentration of 1.0 mM for both seawater DNA samples tested. No amplification was observed for either sample when using a final $MgCl_2$ concentration of 0.5 mM. Optimization of primer concentration yielded similar amplification for both seawater DNA samples using primer concentrations 1.0 μM, 0.75 μM, and 0.5 μM (Fig.A.3.6). A marginal decrease in PCR amplification was observed when using a primer concentration of 0.25 μM.

Dilution series were also performed on radial nerve, sediment, and seawater DNA samples to determine the optimal DNA concentration for detecting *P. invadens* using PCR. *Radial Nerve:* Amplification was observed at all concentrations tested using universal primers 514F and 1055R, indicating suitability of the DNA for PCR (Fig. A.3.7.). Amplification using *P. invadens* primer set Nuc1 was observed at concentrations of 1/1 and 1/2 for all four DNA samples, with slightly greater amplification at 1/2 in the case of sample E (Fig. A.3.7.). Amplification was not observed at lower dilutions of 1/4 and 1/8 for sample E (Fig. A.3.7.) and a decrease in amplification at concentrations of 1/4 and 1/8 was observed for samples G and H (Fig. A.3.7.). *Sediment:* Amplification was observed using universal primers 514F and 1055R at all concentrations tested, confirming the suitability of the DNA for PCR (Fig. A.3.8). Amplification using *P. invadens* primer set Nuc1 was comparable at concentrations 1/1 and 1/2 for all four DNA samples tested (Fig. A.3.8 panel E). A decrease in amplification, or absence of amplification, was observed at concentrations of 1/4 and 1/8 for all four samples. *Seawater:* Amplification was observed using universal primers 514F and 1055R at all concentrations tested, indicating suitability of the DNA for PCR (Fig. A.3.9). Amplification using *P. invadens* primer set Nuc1 was strongest for all four samples at the highest concentration of 1/1. A decline in amplification, or no amplification, was observed at lower dilutions of 1/2, 1/4, and 1/8.
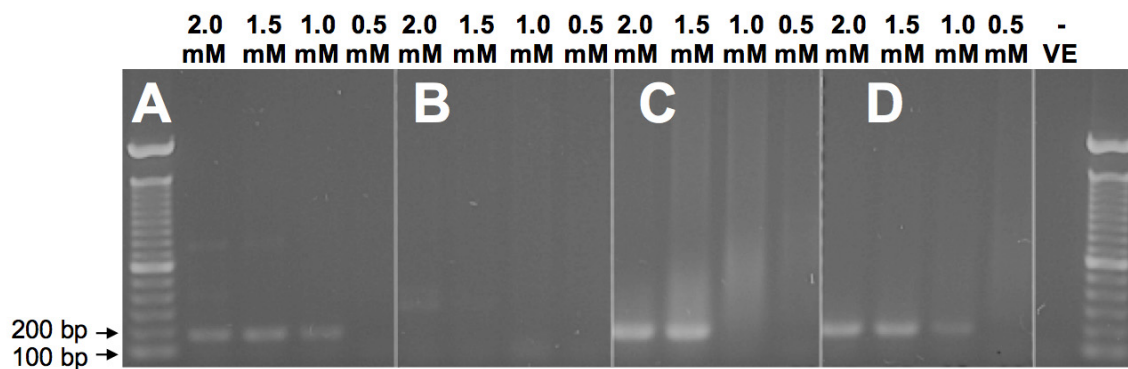
Fig. A.3.1. Effect of $MgCl_2$ concentration on PCR amplification using the Nuc1 primer set, on four radial nerve DNA samples collected from 3 sites in St. Margarets Bay: A) The Lodge-8m-U8, B) The Lodge-18m-U2, C) The Lodge-8m-U1, and D) Luke Island-U1 using *P. invadens* primer set Nuc1. $MgCl_2$ concentrations tested were 2.0 mM, 1.5 mM, 1.0 mM, and 0.5 mM.
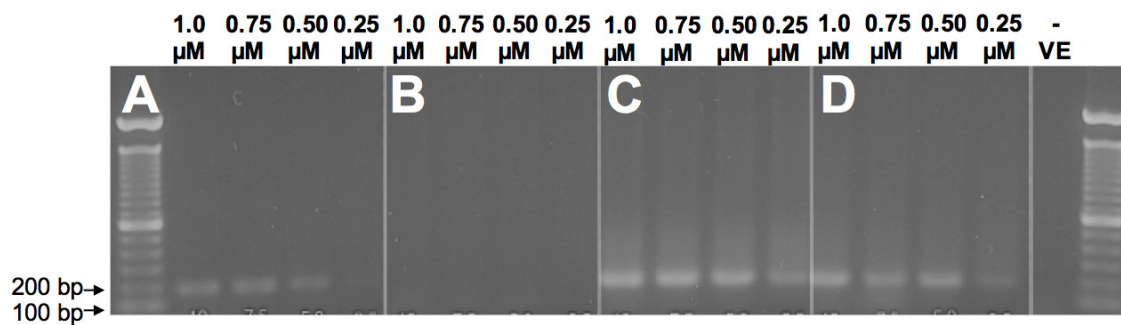

Fig. A.3.2. PCR optimization of primer concentration on four radial nerve DNA samples collected from 3 sites in St. Margarets Bay: A) The Lodge-8m-U8, B) The Lodge-18m-U2, C) The Lodge-8m-U1, and D) Luke Island-U1 using *P. invadens* primer set Nuc1. Primer concentrations tested included 1.0 μM, 0.75 μM, 0.5 μM, and 0.25 μM.

Fig. A.3.3. Optimization of MgCl$_2$ concentration on two sediment DNA samples collected from an aquarium in the Aquatron at Dalhousie University identified as A) Disease Pool sediment 1 (DP Sed. DNA 1) and B) Disease Pool sediment 2 (DP Sed. DNA 2). MgCl$_2$ concentrations tested included 2.5 mM, 2.0 mM, 1.5 mM, 1.0 mM, and 0.5 mM.

Fig. A.3.4. PCR optimization of primer concentrations for PCR on two sediment DNA samples collected from an aquarium in the Aquatron at Dalhousie University using *P. invadens* primer set Nuc1. A) Disease Pool sediment 1 (DP Sed. DNA 1) and B) Disease Pool sediment 2 (DP Sed. DNA 2). Primer concentrations tested included 1.0 μM, 0.75 μM, 0.5 μM, and 0.25 μM.
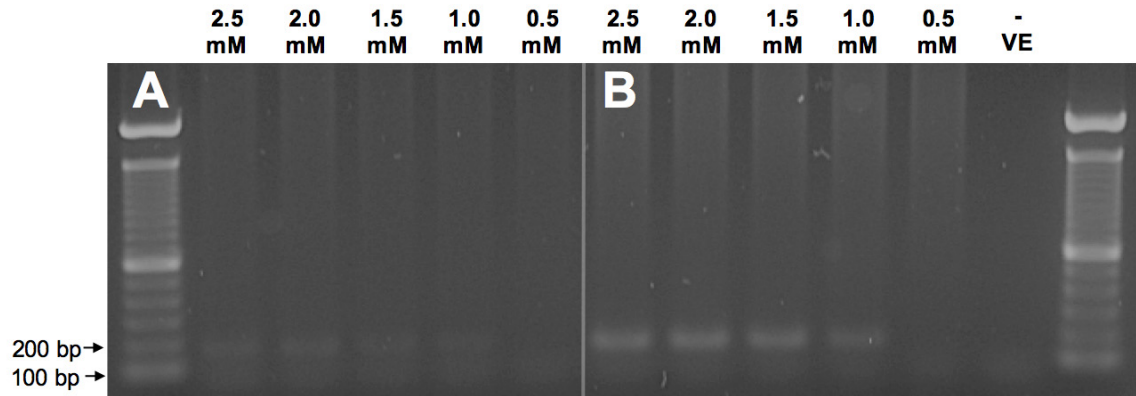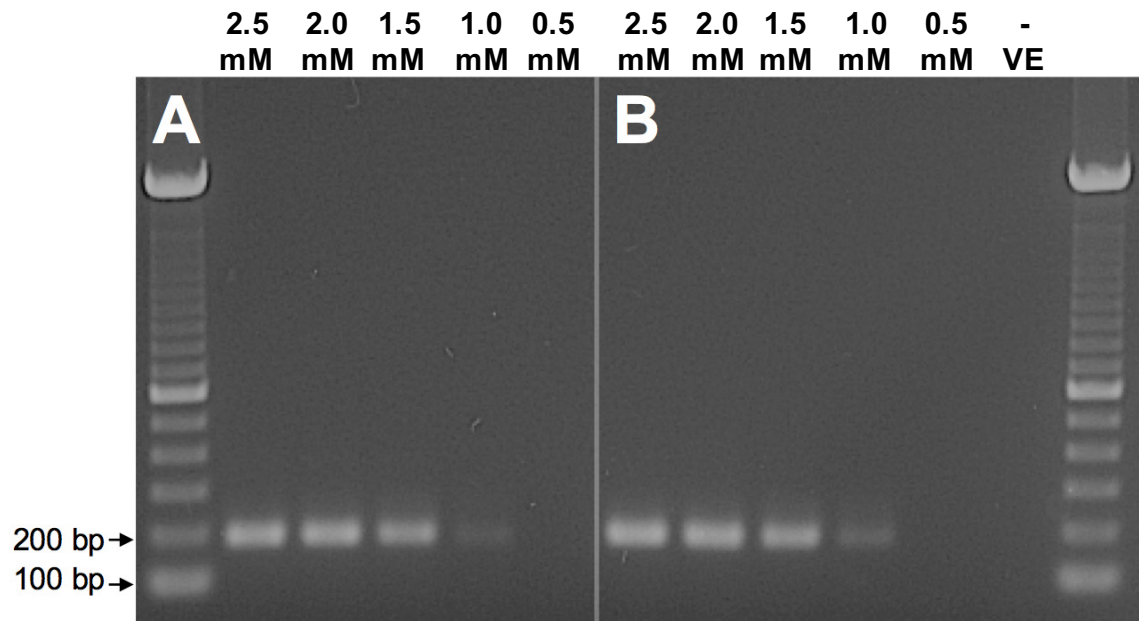
Fig. A.3.5. Optimization of MgCl$_2$ concentration on two seawater DNA samples collected from an aquarium in the Aquatron at Dalhousie University identified as A) Disease Pool seawater 1 (DP SW DNA 1) and B) Disease Pool seawater 2 (DP SW DNA 2). MgCl$_2$ concentrations tested included 2.5 mM, 2.0 mM, 1.5 mM, 1.0 mM, and 0.5 mM.
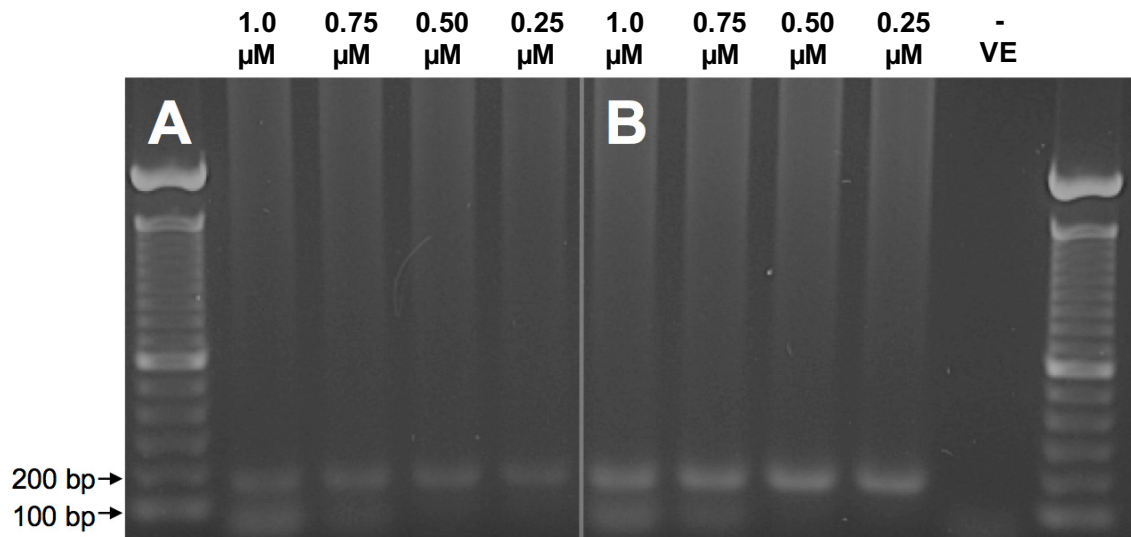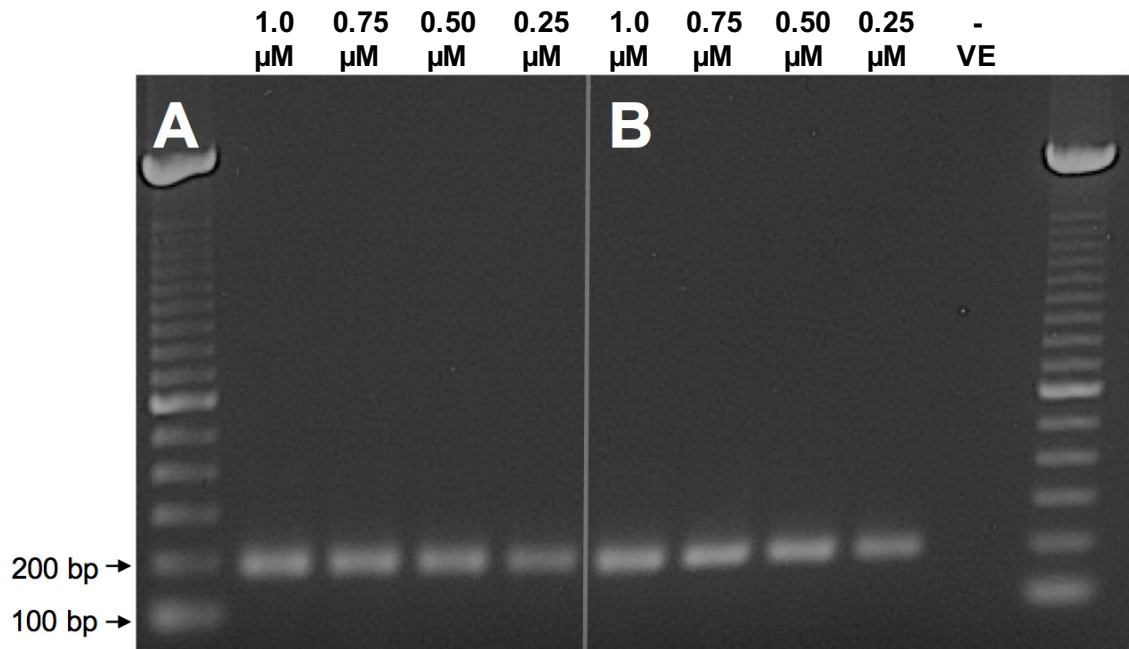
Fig. A.3.6. PCR optimization of primer concentrations for PCR on two seawater DNA samples collected from an aquarium in the Aquatron at Dalhousie University using *P. invadens* primer set Nuc1. A) Disease Pool seawater 1 (DP SW DNA 1) and B) Disease Pool seawater 2 (DP SW DNA 2). Primer concentrations tested included 1.0 µM, 0.75 µM, 0.5 µM, and 0.25 µM.
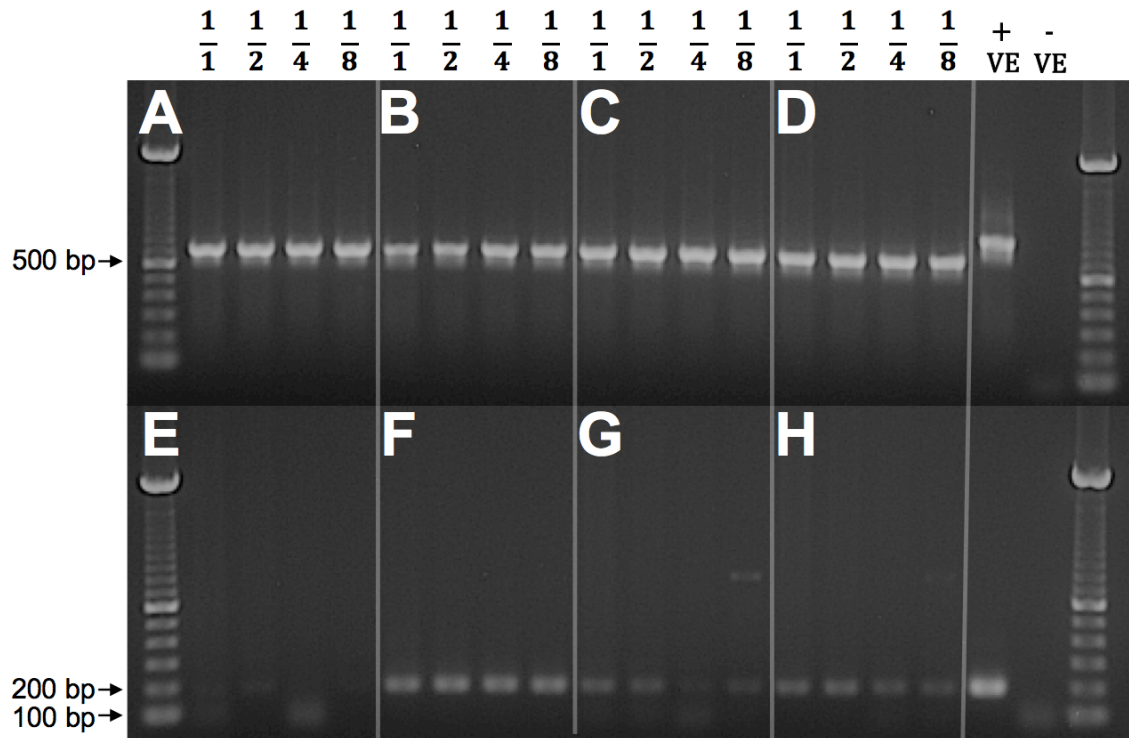
Fig. A.3.7. PCR dilution series on four radial nerve DNA samples collected on 4 or 6 November 2014 from sea urchin cages at Luke Island (panels A & E, B & F) and from sea urchin cages at Splitnose Point (panels C & G, D & H). Panels A, B, C & D display dilution series results using universal primers 514F & 1055R. Panels E, F, G & H display dilution series results using *P. invadens* primer set Nuc1. Dilutions tested included 1, 1/2, 1/4, and 1/8 the original DNA concentration.
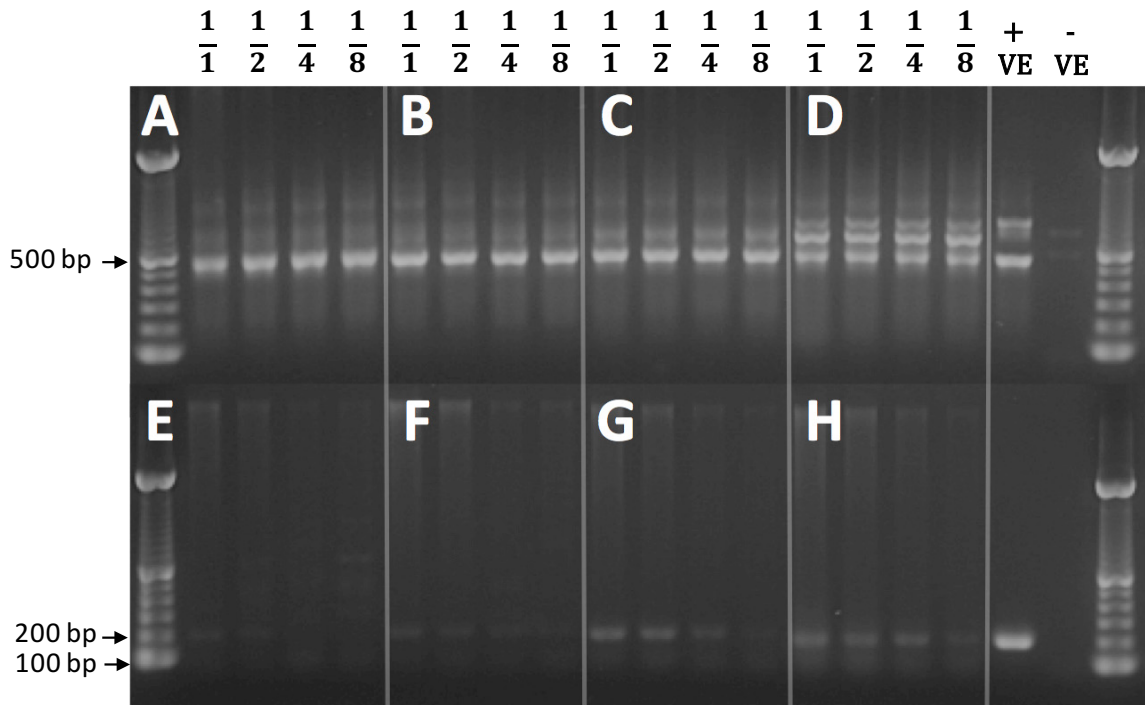
Fig. A.3.8. PCR dilution series on four sediment DNA samples collected on 4 or 6 November 2014 from inside sea urchin cages at Luke Island (panel A & E), outside sea urchin cages at Luke Island (panel B & F), inside sea urchin cages at Splitnose Point (panel C & G) and outside sea urchin cages at Splitnose Point (panel D & H). Panels A, B, C & D display dilution series results using universal primers 514F & 1055R. Panels E, F, G & H display dilution series results using *P. invadens* primer set Nuc1. Dilutions tested included 1, 1/2, 1/4, and 1/8 the original DNA concentration.
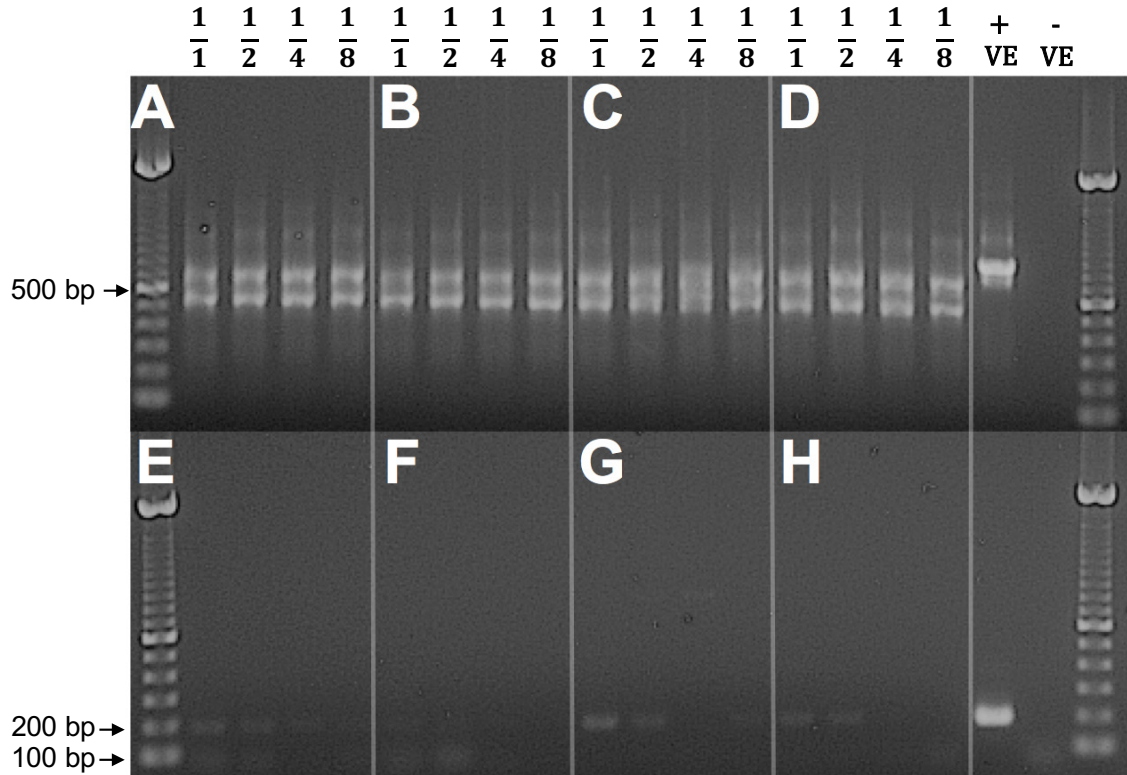
Fig. A.3.9. PCR dilution series on four seawater DNA samples collected above sea urchin cages on 4 October 2015 at Luke Island (panel A & E), on 8 October 2015 at Luke Island (panel B & F), on 13 October 2015 at Splitnose Point (C & G), and on 28 October 15 at Splitnose Point (panel D & H). Panels A, B, C & D display dilution series results using universal primers 514F & 1055R. Panels E, F, G & H display dilution series results using *P. invadens* primer set Nuc1. Dilutions tested included 1, 1/2, 1/4, and 1/8 the original DNA concentration.

A.4. Quantitative real-time PCR Primer Optimization

Materials and Methods: Primer concentrations were optimized by qPCR following the manufacturer's instructions testing a 3 x 3 matrix with primer concentrations of 50 nM, 300 nM, or 900 nM for each primer. Optimal primer concentrations were determined by selecting the primer combination with the lowest Ct value and best efficiency. Efficiency of individual reactions were determined using the software LineRegPCR 11.0 (Ramakers et al. 2003).

Results: Based on a target efficiency of 90 % or greater, primer combinations containing one or both primers at a concentration of 50 nM are unsuitable for qPCR (Table A.4.1). Ct values for primer combinations containing one or both primers at 50 nM were 3 – 8 cycles greater than Ct values for primer combinations containing primers at 300 nM and 900 nM, further indicating that concentrations of 50 nM are unsuitable for qPCR. Primer combinations containing one or both primers at 300 nM or 900 nM all yielded efficiency values greater than 90 %, with the highest efficiency obtained when both primers were at a concentration of 900 nM (96.12 %). Ct values for primer combinations with primers at 300 nM and 900 nM were all in the range of 20.17 – 20.55. Based on these results, the primer combination with both primers at 900 nM was selected as the most suitable for qPCR.

Table A.4.1. Primer optimization results testing 9 primer combinations on DNA from 50 *P. invadens* cells. ParNssu905F (forward) and ParNssu1094R (reverse) primers were tested at final concentrations of 50, 300, and 900 nM. Efficiency is a measure of how well the reaction ran with a target efficiency of 100 % and is calculated based on the slope of the amplification curve and an amplification factor of 2 (i.e. a doubling in the fluorescence with each subsequent cycle). The Ct value is the cycle number at which amplification is first observed (i.e. the fluorescent signal is significantly greater than the background fluorescence). Averages are calculated from triplicate reactions, with the exception of primer combination 300nM/300nM, which is calculated from duplicate reactions.

ParNssu905F Concentration	ParNssu1094R Concentration	Average Efficiency (%)	Average Ct value
50 nM	50 nM	82.95	28.72358
	300 nM	85.92	24.73741
	900 nM	84.97	25.45773
300 nM	50 nM	88.88	23.64197
	300 nM	94.1	20.24318
	900 nM	95.42	20.16522
900 nM	50 nM	87.42	24.10796
	300 nM	92.47	20.55362
	900 nM	96.12	20.54081

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