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Research Article

Environmental DNA (eDNA) detection of marine aquatic invasive species (AIS) in Eastern Canada using a targeted species-specific qPCR approach

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Abstract

Aquatic invasive species (AIS) represent a significant threat to aquatic ecosystems, and early detection is a crucial step in mitigating risks of establishment and spread. In this study, we designed and/or optimized species-specific qPCR assays for 9 marine invertebrates considered invasive in Eastern Canada for use in conjunction with environmental DNA (eDNA) sampling. All qPCR assays showed high analytical sensitivity with theoretical limit of detection (LOD) ranging from 0.045 to 9.24 picogram (pg) of DNA. Comparison of two eDNA water sampling methods showed an increased detection sensitivity using the grab sample approach. eDNA AIS detections were also compared to results obtained using a traditional *in situ* biofouling monitoring method (i.e., biofouling monitoring lines), and a high concordance between the two was observed. We also determined temporal variability in the amount of DNA found in the environment for some of the targeted AIS, which will help target seasonal monitoring efforts. The results provided herein clearly demonstrate the usefulness of including eDNA for early AIS detection in ongoing monitoring, rapid response investigations and mitigation/eradication efforts.

Key words: early detection, monitoring, species-specific qPCR assays

Introduction

Aquatic invasive species (AIS) are a substantial threat to aquatic ecosystems and indigenous species and can have significant negative impacts on recreational and commercial activities in coastal communities (Colautti et al. 2006). In Eastern Canada, the establishment of several marine invasive species, including the solitary tunicates *Ciona intestinalis* (Linnaeus, 1767) and *Styela clava* (Herdman, 1881), as well as the colonial tunicates *Botrylloides violaceus* (Oka, 1927) and *Botryllus schlosseri* (Pallas, 1766), has occurred in various regions (Locke et al. 2007; Ramsay et al. 2008; Simard et al. 2013; McKenzie et al. 2016; Sephton et al. 2016). These biofouling organisms have had a detrimental effect on the shellfish aquaculture industry through increased production costs and reduced shellfish growth (Locke et al. 2009; Carman et al. 2010). The cost to the

Canadian economy of three marine AIS (*Styela clava*, *Carcinus maenas* (Linnaeus, 1758) and *Codium fragile* (Hariot, 1889)) found in Eastern Canada has been estimated to be about 100 million annually (Colautti et al. 2006). The introduction and spread of AIS can occur through various vectors, such as hull biofouling, ballast water and ship movements, as well as aquaculture activities (Carlton 1985; Ramsay et al. 2008; Darbyson et al. 2009; Simard et al. 2017). Consequently, there continues to be a considerable risk for the introduction and spread of additional species. A "watch list" of 17 tunicate species considered most likely to successfully invade Atlantic Canada based on shipping and climate match risks has been identified (Locke 2009).

In response to the threat posed by AIS in aquatic ecosystems, Fisheries and Oceans Canada (DFO) established a coastal AIS Monitoring Program in 2006 (Sephton et al. 2011). Recently, AIS Regulations were added to the *Canadian Fisheries Act* to provide regulatory tools aimed at preventing the introduction of AIS and mitigating their establishment and spread if introduced (Aquatic Invasive Species Regulations 2015). Given the importance of early detection, various monitoring and rapid response methods have been employed, including biofouling monitoring lines, dive surveys, underwater imaging and molecular analysis of water samples (Sargent et al. 2013; Ma et al. 2016; Marraffini et al. 2017). These methods are usually conducted at sites where a high risk of introduction exists, such as marinas, fishing and commercial ports and aquaculture sites (Ma et al. 2016; Sephton et al. 2016). The molecular analysis of water samples for the early detection of AIS is a particularly promising approach, which is gaining popularity in aquatic ecology. All organisms release genetic material into their environment through various biological sources, including feces, skin, mucus and gametes, which is collectively known as environmental DNA (eDNA). Detection of this eDNA can be used as a proxy for the presence of a species within an ecosystem (Goldberg et al. 2015). The molecular detection of non-indigenous tunicates in Eastern Canada from environmental water samples has predominantly been conducted using concentrated-sieved samples (using a 64 or 75 µm mesh sieve) designed to collect tunicate larvae and gametes (Gill et al. 2007; Willis et al. 2011; Stewart-Clark et al. 2013; Ma et al. 2016), although a grab sampling method targeting both extra- and intracellular DNA has also been investigated (Stewart-Clark et al. 2013). Both sampling methods have been used in conjunction with conventional PCR species testing. Recent advances have been made regarding eDNA capture methods, and molecular detection of eDNA is now commonly done using either real-time PCR (qPCR), which specifically targets single species, or DNA metabarcoding, which uses a more universal high-throughput approach to identify multiple species. Both have been used successfully for eDNA AIS detection (Gillum et al. 2014; Gingera et al. 2016, 2017; Balasingham et al. 2018),

with qPCR generally accepted as being more sensitive and enabling greater quantitative power.

The goal of this study was to develop species-specific qPCR assays and evaluate their utility on eDNA samples for the detection of marine AIS already established or at risk of establishing in Eastern Canada. Our assessment included performance characteristics of the assays and comparison of two water sampling methods. Results were also compared to a traditional field monitoring method (biofouling monitoring lines). Finally, temporal variations in eDNA abundance were examined to assess optimal seasonality for eDNA-based AIS monitoring.

Materials and methods

AIS specimen acquisition and DNA barcoding

Specimens of the targeted AIS (Supplementary material Table S1) collected from various locations in Eastern Canada between 2011 and 2018 were obtained to assess any haplotype diversity of local specimens and to ensure sensitive and specific qPCR assay design. An effort was also made to obtain closely related species that had a high nucleotide sequence similarity (≥ 90%) and a similar geographic range to the targeted AIS. The mitochondrial gene cytochrome *c* oxidase subunit 1 (CO1) was chosen for DNA barcoding purposes for all species. Total genomic DNA was extracted from tissue samples preserved in 95% non-denatured EtOH using the MN NucleoSpin Tissue Kit (Macherey-Nagel, PA, USA) following manufacturer's instructions, and DNA extracts were stored at −20 °C until needed. Universal primers designed for CO1 amplification of marine invertebrates (Geller et al. 2013) were used for DNA barcoding of most species. PCRs were performed using the AmpliTaq Gold 360 PCR Master Mix (Life Technologies, ON, Canada) and cycling conditions consisted of an initial incubation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 48 °C for 30 s and 72 °C for 30 s. For some non-amplifiable specimens, species-specific DNA barcoding primers were designed (Table S1), and PCRs were conducted as above except at a higher annealing temperature (55 or 60 °C). PCR products were visualized on a 1.5% agarose gel followed by PCR product cleanup using ExoSAP-IT (Affymetric, CA, USA) or the Monarch PCR & DNA Cleanup Kit (New England Biolabs, MA, USA). Samples were sent for Sanger sequencing using both the forward and reverse primers at the McGill University and Génome Québec Innovation Centre (Montreal, Canada). DNA sequences representing unique haplotypes found at each location were submitted to GenBank. (https://www.ncbi.nlm.nih.gov/genbank/).

qPCR assay design and validation

DNA sequences from local barcoded specimens as well as sequences found in NCBI (https://www.ncbi.nlm.nih.gov) and BOLD (http://www.boldsystems.org)

were aligned in Geneious (version 9.1.4) using the default Geneious alignment tool. Primer and probes were designed *in silico* to amplify a region ranging from 127 to 234 base pairs (bp) and optimised for an annealing temp of 60 °C (Table S2). Assays were designed by targeting DNA sequences containing high numbers of nucleotide differences between the targeted AIS and closely related species, to ensure assay specificity. Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was also used to help ensure that primers were target-specific. For certain species, such as *B. schlosseri* and *D. listerianum* (Milne Edwards, 1841), for which cryptic species exist (Perez-Portela et al. 2013; Nydam et al. 2017), qPCR assays were designed to ensure the detection of haplotypes from Eastern Canada. For each newly designed qPCR assay, serial genomic DNA dilutions were done to determine the assay efficiency

$$(E = 10^{[-1/slope]})$$

and to calculate the theoretical limit of detection (LOD). Two serial dilutions from 10^{0} to 10^{-8} were made for each species and each serial dilution was tested in duplicate for a total of 4 qPCR threshold cycle (Ct) values. The theoretical LOD was determined as the lowest dilution for which 4 of 4 qPCR assays were positive. Non-target DNA normalized at 5 ng/µL was used as a background when preparing the serial dilutions. This was done to assess the efficiency of the assays under conditions similar to its prescribed usage. The specificity of the qPCR assays was also tested using DNA from close relatives and other targeted AIS when *in silico* assessments showed the potential for cross-amplification.

eDNA field sampling

In 2017, an initial set of samples was collected at 5 marinas in New Brunswick (NB) and Prince Edward Island (PEI) where biofouling monitoring was conducted as part of DFO Science's AIS Monitoring Program (Figure 1; Table S3). The sites were selected based on historical presence and abundance levels of established AIS for which we designed species-specific qPCR assays. All sites were sampled 4 times from June to September, and two water collection methods were assessed. The first eDNA sampling method, herein referred to as "grab sample", consisted of collecting 0.5 L of seawater at a depth of ~ 0.5 m, followed by filtration in the field using 47 mm diameter 1.5 µm Whatman 934-AH glass microfiber filters (GE Healthcare, IL, USA). Filters were preserved in microtubes containing 200 uL of 95% non-denatured EtOH and frozen at −20 °C until processing. The second sampling method (herein "concentrated-sieved sample") consisted of pumping 150 L of seawater through a 64 µm sieve and collecting the retained material in 95% non-denatured EtOH for subsequent DNA extraction. The first collection method targeted the capture of both extracellular and intracellular DNA, while the concentrated-sieved samples targeted larger constituents, including larvae and gametes.

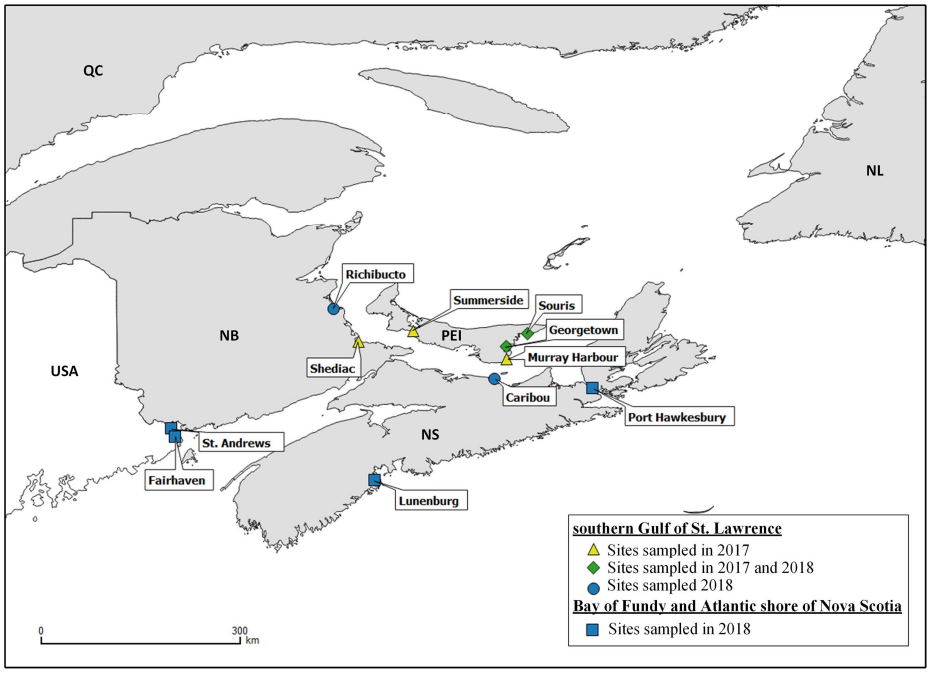


Figure 1. Map of the field sites from the Gulf of St Lawrence, Bay of Fundy and Atlantic shore of Nova Scotia in Canada sampled in 2017 and 2018.

In 2018, a second set of samples was collected using an optimized eDNA grab sample method at 8 marinas or commercial wharves in New Brunswick, Nova Scotia and Prince Edward Island in conjunction with traditional biofouling monitoring conducted at those sites (Figure 1; Table S3). As in 2017, these sites were selected based on historical presence and abundance levels of target AIS. Water samples (1 L) were collected at a depth of ~ 0.5 m at each site on two occasions between May and October to assess variability in eDNA quantities and detection rates. For each site and sampling date, 2 to 5 spatially distinct samples (separated by ≥ 10 m) were collected to further assess sample variability. The sampling frequency at the Georgetown, PEI site was increased to bi-weekly to help assess fine-scale *in situ* eDNA variability. All samples were filtered *in situ* or maintained at ~ 4 °C for transport and subsequent lab processing within 24 hours. All samples were filtered using 47 mm diameter 0.8 µm Whatman nylon membrane filters (GE Healthcare, IL, USA) and preserved in microtubes containing 200 µL of 95% non-denatured EtOH and frozen at −20 °C.

To assess sample contamination issues in the field, at least 1 field blank (tap water) per site was included for each eDNA sample collection event. The field blanks were brought in the field and filtered alongside the seawater samples or kept in the same cooler as the seawater samples for subsequent lab processing. When samples were processed in the lab, lab filtration blanks (tap water) were also included to monitor for signs of contamination at the lab processing step. DNA extraction and qPCR negative controls were also included during the processing and testing of samples. To minimise cross-contamination and potential false positives, all reusable equipment (e.g., mason jars, forceps, vacuum flasks) was soaked

in a 1% commercial bleach solution for a minimum of 1 hour. The detailed eDNA field sampling protocol used for the 2018 field sampling can be found in Supplementary material Appendix 1.

DNA extraction and species-specific qPCR testing

DNA extraction from filters collected in 2017 (0.5 L of water filtered on 47 mm 1.5 µm Whatman 934-AH glass microfiber filters) and 2018 (1 L of water filtered on 47 mm 0.8 µm Whatman nylon membrane filters) was conducted using half of each filter with the MN NucleoSpin Tissue Kit (Macherey-Nagel, PA, USA) following a modified protocol (Appendix 2), and extracts were stored at −20 °C. The second half of each filter was retained as a back-up. DNA extraction from the preserved concentrated-sieved samples was conducted using the DNeasy PowerSoil Kit (Qiagen, Netherlands) following a modified protocol (Appendix 2), and extracts were stored at −20 °C.

qPCR testing of the eDNA samples was conducted with the species-specific qPCR assays. Briefly, 3 µL of template DNA, 480 nM of each primer, 200 nM of the probe, 1 µL of BSA 1%, and the 2x TaqMan Gene Expression kit (Thermo Fisher Scientific, MA, USA) were used in 25 µL reactions. All qPCR tests were conducted in duplicate on a StepOnePlus™ qPCR platform (Thermo Fisher Scientific, MA, USA) using the following cycling parameters: initial hold at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 seconds (s), 60 °C for 30 s and 72 C for 30 s, with fluorescence reading at the end of each elongation cycle.

To evaluate if PCR inhibitors were present in environmental samples, which could lead to potential false negative results, all samples (including blank controls) were spiked with a synthesised exogenous internal positive control (IPC) and tested using a qPCR assay specific to that IPC. The IPC consisted of a linearized DNA plasmid containing a DNA sequence not found in the targeted environments. Inhibition was considered present if a difference of more than 2 between the qPCR Ct of environmental samples and field blanks was observed. The IPC qPCR assay was done using the same parameters and reagents used for the species-specific qPCR assays.

Confirmatory DNA sequencing of positive detections

To confirm the specificity of the assays on field eDNA samples, a subset of the positive results was sent for Sanger sequencing at the McGill and Génome Québec Innovation Centre. For each species, at least one positive sample per site was sequenced by using the same primers and parameters used for the species-specific qPCR assays. Briefly, samples were amplified using the AmpliTaq Gold 360 PCR Master Mix (Thermo Fisher Scientific, MA, USA) and PCR products were visualized on a 1.5% agarose gel, followed by PCR product cleanup using ExoSAP-IT (Affymetric, CA, USA).

Biofouling monitoring lines

The presence of AIS at eDNA sampling sites was evaluated in conjunction with DFO Science's AIS Biofouling Monitoring Program in 2017 and 2018. AIS occurrence was assessed by deploying 6 to 10 biofouling monitoring lines at each study site in late May to early June of each year. Biofouling monitoring line configurations included either saucer-type collectors (Martin et al. 2011) and/or plate-type collectors with 1 to 3 plates per line (Sephton et al. 2011). Monitoring lines were attached to the underside of floating docks and suspended at a depth of 1 m below the water surface. In 2017, the average percent coverage of AIS on monitoring lines was assessed in June, July and August using only the line adjacent to the collected eDNA samples, while in September, all monitoring lines deployed at a site were used to determine AIS coverage. eDNA water samples were collected prior to verifying AIS presence on the monitoring lines to avoid the potential dispersion of DNA. In 2018, the average percent coverage of AIS on monitoring lines was assessed using all lines at the time of retrieval in late September to early October.

Results

DNA barcoding and assay design

One to six specimens per species were barcoded. The DNA sequences of the barcoded specimens were aligned with DNA sequences found in public databases and intra/inter-species variability was assessed. DNA sequences from local barcoded specimens matched the DNA barcodes found in public databases for the targeted AIS and minimal (≤ 2.1 %) to no variability was observed intra-species for the partial CO1 regions targeted by assays. In total, qPCR assays were designed and/or optimized for 9 AIS and the parameters were standardized to allow future multiplexing if desired. The risk of cross-species amplification was minimal *in silico*.

In vitro qPCR assay validation

In vitro validation performed for all qPCR assays demonstrated high sensitivity with theoretical LOD ranging from 0.045 to 9.24 pg of DNA, while efficiencies ranged from 88.2 to 99.8% (Table S2). Specificity testing conducted against non-targeted AIS revealed no non-specificity issues. Based on *in vitro* validation results, field detections were classified as follows: 1) not detected, 2) inconclusive (detection was only obtained in 1 of the 2 technical replicates of the qPCR assay performed, 3) suspected (detection was obtained in 2 of the 2 technical replicates of the qPCR assay performed, but the value was below the LOD) and 4) detected (detection was obtained in 2 of the 2 technical replicates of the qPCR assay performed and the value was above the LOD).

In situ qPCR assay validation

In situ validation of qPCR assays and a comparison of the 2 water collection methods (i.e., grab sample and concentrated-sieved sample) were achieved in 2017 by targeting sites with varying abundances of four aquatic invasive ascidian species established in Eastern Canada (*C. intestinalis*, *S. clava*, *B. violaceus* and *B. schlosseri*). Results for all four targeted species showed an overall higher detection sensitivity when using the grab sample method compared to the concentrated-sieved method with DNA detectable earlier in the field season with the grab sample method (e.g., *C. intestinalis*, *S. clava* and *B. violaceus* were all "suspected" in the eDNA grab sample in Souris, PEI in June, but "not detected" in the concentrated-sieved sample) (Table S4). The concentrated-sieved sample method did however detect higher amounts of DNA later in the field season (i.e., August/September) when compared to the grab sample method.

When eDNA AIS detections were compared to the biofouling monitoring lines, the grab sample method gave a "suspected" or "detected" result every time a species was observed on the monitoring lines. Results were different for the concentrated-sieved samples, as they yielded "not detected" results on a few occasions (i.e., 3 out of 34 occasions) when species were observed on the monitoring lines (e.g., *B. violaceus* in Shediac, NB in September). At one site (Shediac, NB), *B. schlosseri* was only detected using eDNA (both grab sample and concentrated-sieved sample) (Table S4).

eDNA sampling was conducted again in 2018 to further validate the qPCR assays and to investigate detection rates and temporal differences using an optimized eDNA grab sample method. As in 2017, sites were chosen based on known presence of selected AIS for which we designed species-specific qPCR assays. At sites in the southern Gulf of St. Lawrence (sGSL), eDNA and biofouling monitoring line results were in general agreement for *C. intestinalis*, *S. clava*, *B. violaceus* and *B. schlosseri* when the species was known to be at high abundance at that site. However, when an AIS was not detected in the past or believed to occur at low abundance levels and had sporadically been detected in past years, eDNA was the only method that revealed a potential occurrence, through "suspected" or "detected" results (e.g., *B. violaceus* and *M. membranacea* (Linnaeus, 1767) in Richibucto, NB and *C. mutica* (Schurin, 1935) in Souris, PEI and Caribou, NS) (Table S5).

At sites in the Bay of Fundy and the Atlantic shore of Nova Scotia (NS), which targeted recently established AIS typically found at low abundance (< 25 % plate coverage) and not found in the sGSL sites (i.e., *D. listerianum*, *A. aspersa* (Müller, 1776), *D. vexillum* (Kott, 2002)), eDNA and biofouling monitoring line results were in concordance. *S. clava* and *M. membranacea* "detected" results were only obtained using eDNA (exception of Port Hawkesbury, NS where *M. membranacea* was also detected on the monitoring

lines), despite the fact that both species have been historically present at all sites. *A.aspersa* was "detected" with both methods at the only site out of the four where its presence has been confirmed (Lunenburg, NS) (Table S6).

Detection rates (i.e., percentage of positive water samples/total amount sampled at a specific site) were also assessed in 2018 by sampling 2 to 5 spatially distinct water samples per site. When the amount of DNA in the environment was above the LOD of each test, the detection rates for all species were 100%, whereas when it was below the LOD, detection rates varied between 25 and 100%.

In 2018, one site (Georgetown, PEI) was sampled bi-weekly to measure temporal variability in eDNA abundance for the various species. For some species, including *C. intestinalis*, *S. clava*, *B. violaceus,* a first DNA peak was observed at the end of June, while the highest eDNA quantities were observed in September for all species (Figure 2). The amount of DNA detected in the environment for *C. mutica* remained steady over time with detections being below or close to the LOD.

Confirmation of positive results and QC

Sanger sequencing of a subset of positive results at various sites confirmed the specificity of the qPCR assays. The presence of inhibitors that could result in false negatives was also assessed by including an exogenous internal positive control (IPC) in all samples. Results showed no signs of qPCR inhibition at any of the sites with all Ct values of environmental samples being within 2 Cts of what was obtained with the field blanks.

DNA contaminations were also monitored throughout this work by including field blanks, lab filtration blanks, as well as DNA extraction and qPCR negative controls. Field and lab filtration blank contaminations were only observed when a targeted AIS was either "suspected" or "detected" in seawater samples collected at a sampling event/site and not when a targeted AIS was either "inconclusive" or "not detected" which indicates that contaminations were likely not because of inadequate equipment decontamination. Contaminations were also not observed in qPCR negative controls, or DNA extraction blanks. Upon close review, it is likely that contaminations in a proportion of the numerous field and lab filtration blanks happened during the manipulation of the DNA plate extracts (i.e., foil removal from stored DNA extract plates prior to qPCR testing). Sequential qPCR testing was a risk factor explaining the increasing percentage of contaminated blanks (i.e., blanks were negative in the first 2 or 3 use of a DNA extract plate for qPCR, and contaminations increased gradually as the same DNA extract plates were re-used for qPCR testing of other AIS). In total, the percentage of grab sample field blanks contaminated based on the number of targeted AIS screened was 7.9% (21/266), concentrated-sieved field blanks contaminated was 6.6% (5/76) and lab

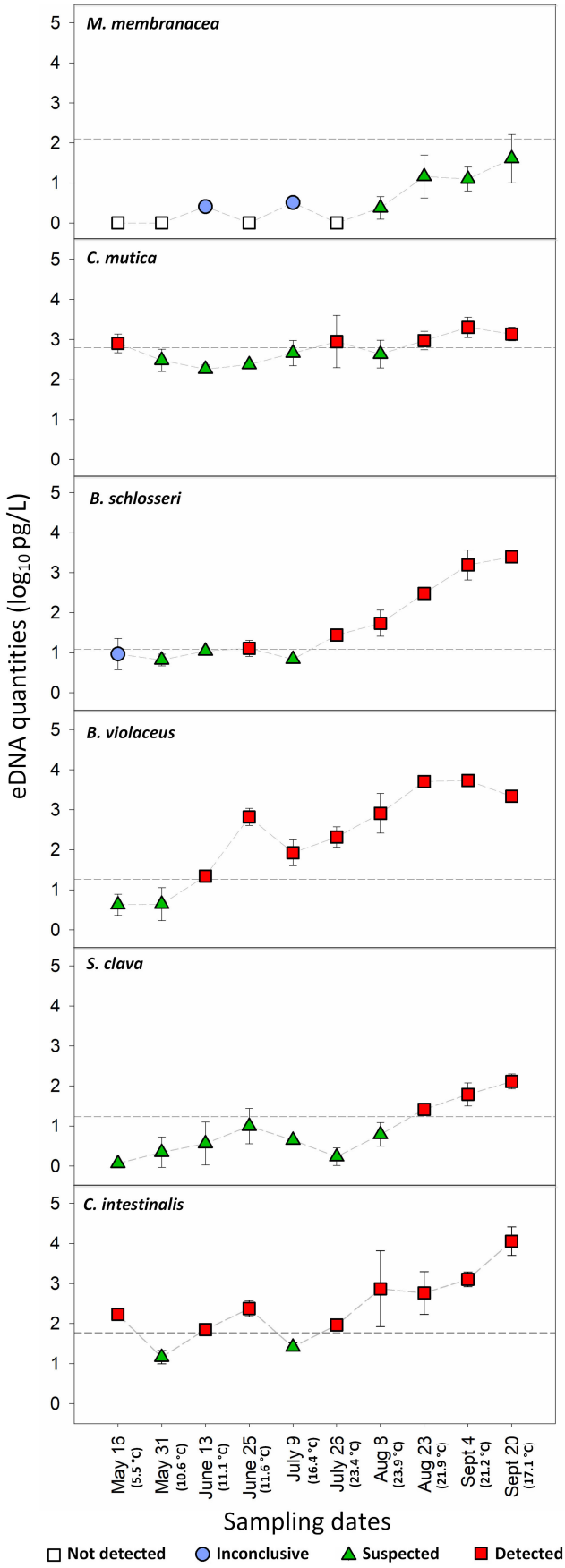


Figure 2. Temporal amount of eDNA (mean ± sd) (n = 3–5) detected in Georgetown, PEI. Water temperature at each sampling time-point are in parenthesis. Dashed line represents theoretical limit of detection (LOD).

filtration blanks contaminated was 4.4% (4/90). All contaminations were below the theoretical LOD of the qPCR assays with 47% being classified as "suspected" and 53% classified as "inconclusive" (i.e., only positive in one of the two technical qPCR replicates).

Discussion

Detection of aquatic invasive species using eDNA in conjunction with molecular tools represents a non-disruptive, sensitive and relatively cost-efficient way of gaining insight into the presence of an organism within an ecosystem. As such, monitoring for early detection of AIS, which is a crucial step when mitigating the risk of establishment and spread, could greatly benefit from the use of this technique. In this study, we designed and validated species-specific qPCR assays against 9 marine AIS established or considered at-risk of introduction in Eastern Canada and assessed optimal eDNA capture methods. Performance characteristics of the assays were assessed *in vitro* and in the field, with qPCR detection results compared to a visual method (i.e., biofouling monitoring lines). DNA barcoding of the CO1 of local AIS specimens in combination with publicly available sequences enabled us to assess intra/inter-species variability and design sensitive and specific assays. For some species, including *B. schlosseri* and *D. listerianum,* cryptic species are believed to exist with genetically divergent clades found in different geographic ranges (Perez-Portela et al. 2013; Nydam et al. 2017). Consequently, an effort was made to design assays that would detect as many haplotypes as possible, with a focus on those found in our region.

The targeted qPCR assays designed and optimised in this study have shown good performance characteristics *in vitro*. LODs ranging from 0.045 to 9.24 pg of DNA were similar to results from other studies (Diaz-Ferguson et al. 2014; Gillum et al. 2014; Farrington et al. 2015; Roy et al. 2017).

In 2017, *in situ* comparisons of two water sampling methods revealed increased sensitivity with the grab sample method, while higher amounts of DNA were detected in August and September with the concentrated-sieved approach. These discrepancies are likely due to differences in particle retention between each method. While the grab sample collects all eDNA, which includes extra- and intracellular DNA from all life stages, the concentrated-sieved sample with the 64 µm mesh would predominantly retain larvae and gametes. The increase in eDNA quantities observed later in the field season for *C. intestinalis*, (concentrated-sieved sample) coincides with a peak in recruitment observed in late August in PEI (Ramsay et al. 2009). Similarly, *S. clava* larvae concentrations at one study site in PEI have been shown to be at their highest in mid-August (Bourque et al. 2007). Results from a previous study that also compared grab and concentrated-sieved sampling using conventional PCRs targeting invasive tunicates (*C. intestinalis*, *S. clava*, *B. violaceus* and *B. schlosseri)* showed similar

results between the two sampling methods (Stewart-Clark et al. 2013). It is important to note, however, that only presence/absence was investigated in Stewart-Clark et al. (2013).

The higher sensitivity of eDNA coupled with qPCR compared to some traditional survey methods for detecting aquatic invaders has been previously demonstrated (Hunter et al. 2015; Smart et al. 2015). In this study, when compared to traditional AIS monitoring using biofouling monitoring lines, eDNA showed an increased detection sensitivity, with some species "suspected" or "detected" only by qPCR (e.g., *B. schlosseri* in Shediac, NB in 2017; *M. membranacea* in Souris, PEI and three sites in the Bay of Fundy or Atlantic shore of NS in 2018) and other species "suspected" or "detected" earlier in the field season (e.g., *C. intestinalis* in Georgetown, PEI in June 2017). While we have not visually confirmed the presence of AIS at sites where they were only "suspected" or "detected" by qPCR, they have been known to be present at these sites based on past monitoring done within DFO Science's AIS Biofouling Monitoring Program (Sephton et al. 2017; Fisheries and Oceans Canada 2019). In addition, no field blanks or lab filtration blanks were contaminated at those sites for the AIS in question indicating that the results are likely not false positives. Earlier detections of AIS provided by eDNA could lead to mitigation measures earlier in the season, resulting in a greater potential to reduce AIS establishment and spread.

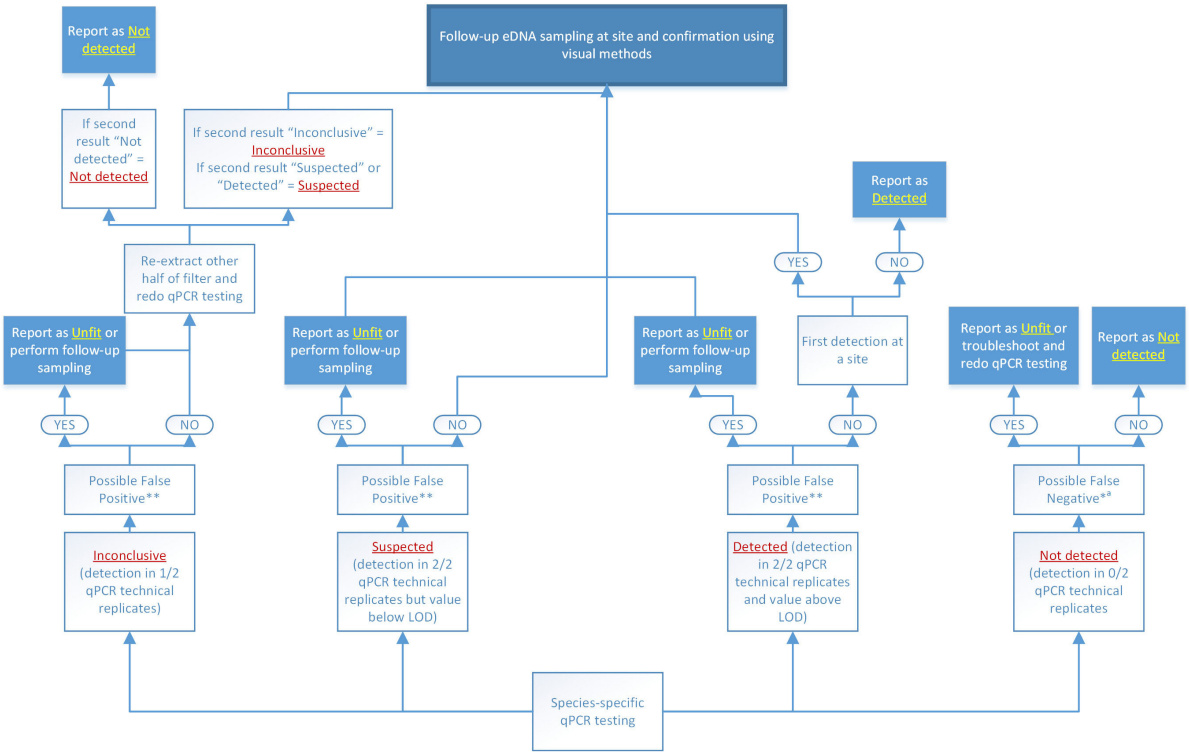
eDNA detection rates investigated in 2018 by collecting 2 to 5 spatially distinct eDNA samples per site agreed 100 percent of the time when the amount of DNA of a species in the water was above the assay's LOD. More work is needed, however, to better understand the minimal abundance or biomass of a species that is required to obtain an amount of DNA above the LOD, and to determine sampling strategies that could improve detection. Further studies on the effects of various environmental variables (e.g., temperature, salinity, water dynamics) and life history characteristics of a species on the amount of eDNA found in the water are also required to better understand detection probabilities. Temporal differences in eDNA quantity were assessed for 6 AIS established in Georgetown, PEI with results for most species, including *C. intestinalis*, *S. clava* and *B. violaceus,* showing a first peak in eDNA abundance at the end of June. For all species, eDNA quantities were at their highest in late August/September, which is likely the result of an increased number and size of individuals. AIS monitoring using eDNA during this time would provide increased likelihood of detection, although earlier sampling could be useful if mitigation measures to prevent AIS establishment/spread are required.

As observed in this study, AIS monitoring efforts can be hindered by limitations of sampling techniques. Limitations associated with biofouling monitoring lines that could help explain detection discrepancies with eDNA include: a late deployment (i.e., late spring/early summer, according

to ice conditions and access to floating docks at marinas), the requirement for larval settlement/visual observation to detect fouling species, the occurrence of space competition (overcrowding of monitoring substrate by certain AIS, thus limiting settlement of other species), limited detection of non-fouling/mobile species (e.g., *C. mutica*), and the possibility of being tampered with (whether intentional or not). Settlement panel composition used to monitor fouling organisms has also been shown to have an effect on species detected, and, as such, the choice of substrate could reduce the detection of some species (Chase et al. 2016).

While the eDNA technique can correct for these limitations, its monitoring capability can also be influenced by different factors, which can lead to the occurrence of false positive and false negative results. A false positive result can occur when the qPCR assay specificity is not sufficient and/or when DNA contaminations related to issues with sample collection and/or processing exist. To avoid such erroneous results, appropriate measures must be taken, such as the inclusion of process controls and standards. Some contaminations were observed in this study in both field and lab filtration blanks, but only at sites where the targeted AIS were either "suspected" or "detected" in seawater samples. The majority of observed contaminations were suspected to have occurred due to sequential qPCR testing for multiple AIS on the same DNA extract plates, and, as such, laboratories should be cautious when DNA plates are used, and prevention of cross-contamination should be monitored closely. All contaminations were also below the LOD of the assays, which reinforces the use of weighted categories ("inconclusive", "suspected" and "detected") when presenting eDNA qPCR results. It is also important to realize that even a true positive result only represents a proxy for the presence of a species, which means that in most cases, follow-up confirmatory work should be done. Another essential consideration relating to the use of eDNA qPCR detection of a species includes the importance of doing continued *in silico* and potentially *in vitro* assessments of assays to ensure that all haplotypes of a targeted AIS are detected and that no non-specificity issues exist. Sanger sequencing on a portion of the positive detections should also be done on a continuous basis to help ensure the specificity of the assays, as well as monitoring for signs of inhibition to minimize the occurrence of false negative results.

Based on the results obtained in this study, a preliminary sampling strategy recommendation for eDNA use in marine AIS monitoring for early detection would be to perform one or two sampling events per field season (spring to fall). More precisely, we suggest a first sampling early in the field season (May/June) and a second one in late August/September (i.e., when the highest eDNA quantities from most species was observed). Sampling at each site should consist of collecting multiple (i.e., three to five spatially distinct) one litre samples and should include at least one field blank. We also propose the use of a decision framework (Figure 3) to help



* Possible False Negative: answer "NO" when no signs of inhibition are observed, no method/processing errors are observed (through the use of appropriate positive controls and/or successful detection of another species in the sample) and qPCR assay sensitivity has been validated with local specimens.

[a] False Negative caused by amounts of DNA in the environment below the LOD of the qPCR assay are not considered in this framework unless other assessment methods have confirmed the presence of the species.

** Possible False Positive: answer "NO" when no DNA contamination is observed in any of the blanks/negative controls (field blanks, lab filtration blanks, DNA extraction blanks and qPCR negative controls) and qPCR assay specificity as been confirmed through stringent assay validation and/or Sanger sequencing of positive detection.

Figure 3. Decision framework for AIS eDNA results.

determine logical next steps based on AIS eDNA results obtained. In accordance with this framework, an eDNA result of "inconclusive", "suspected" or "detected" at a site where an AIS has never been detected (e.g., "inconclusive" result for *C. intestinalis* in Richibucto, NB in August 2018) should be followed-up with repeated eDNA sampling and visual confirmation of the species.

In conclusion, the work performed herein provides 9 species-specific qPCR assays for the molecular detection of marine AIS in Eastern Canada, in addition to a recommended eDNA sampling protocol. Performance characteristics of the assays showed good sensitivity and specificity when compared to biofouling monitoring lines, with detection probabilities of 100% (i.e., AIS observed on the lines were always detected with the qPCR assays). Furthermore, detection discrepancies between the two monitoring techniques (i.e., eDNA vs biofouling monitoring lines) highlighted the potential advantage of combining two or more methods when monitoring for AIS. Given these results, we conclude that the use of eDNA as a complementary tool to traditional AIS monitoring techniques (e.g., biofouling monitoring lines) would provide several advantages, including: 1) filling gaps in AIS distribution left by logistical challenges of traditional methods (e.g., remote/challenging locations, limited funding/time/manpower), 2) detecting

species at low densities and early life stages, thereby providing earlier detection, 3) enhancing the detection of non-fouling/mobile species, and, 4) correcting for the various factors influencing AIS presence on lines (e.g., intra/interspecific competition, removal by anthropogenic interference or weather-related damage).

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References

Aquatic Invasive Species Regulations (2015) Canada Gazette Part II. http://gazette.gc.ca/rp-pr/p2/2015/2015-06-17/html/sor-dors121-eng.html (accessed 24 June 2019)

Balasingham KD, Walter RP, Mandrak NE, Heath DD (2018) Environmental DNA detection of rare and invasive fish species in two Great Lakes tributaries. *Molecular Ecology* 27: 112–127, https://doi.org/10.1111/mec.14395

Bourque D, Davidson J, MacNair NG, Arsenault G, LeBlanc AR, Landry T, Miron G (2007) Reproduction and early life history of an invasive ascidian *Styela clava* Herdman in Prince Edward Island, Canada. *Journal of Experimental Marine Biology and Ecology* 342: 78–84, https://doi.org/10.1016/j.jembe.2006.10.017

Carlton JT (1985) Trans-Oceanic and Interoceanic Dispersal of Coastal Marine Organisms - the Biology of Ballast Water. *Oceanography and Marine Biology* 23: 313–371

Carman MR, Morris JA, Karney RC, Grunden DW (2010) An initial assessment of native and invasive tunicates in shellfish aquaculture of the North American east coast. *Journal of Applied Ichthyology* 26: 8–11, https://doi.org/10.1111/j.1439-0426.2010.01495.x

Chase AL, Dijkstra JA, Harris LG (2016) The influence of substrate material on ascidian larval settlement. *Marine Pollution Bulletin* 106: 35–42, https://doi.org/10.1016/j.marpolbul.2016.03.049

Colautti RI, Bailey SA, van Overdijk CDA, Amundsen K, MacIsaac HJ (2006) Characterised and projected costs of nonindigenous species in Canada. *Biological Invasions* 8: 45–59, https://doi.org/10.1007/s10530-005-0236-y

Darbyson E, Locke A, Hanson JM, Willison JHM (2009) Marine boating habits and the potential for spread of invasive species in the Gulf of St. Lawrence. *Aquatic Invasions* 4: 87–94, https://doi.org/10.3391/ai.2009.4.1.9

Diaz-Ferguson E, Herod J, Galvez J, Moyer G (2014) Development of molecular markers for eDNA detection of the invasive African jewelfish (*Hemichromis letourneuxi*): a new tool for monitoring aquatic invasive species in National Wildlife Refuges. *Management of Biological Invasions* 5: 121–131, https://doi.org/10.3391/mbi.2014.5.2.05

Farrington HL, Edwards CE, Guan X, Carr MR, Baerwaldt K, Lance RF (2015) Mitochondrial Genome Sequencing and Development of Genetic Markers for the Detection of DNA of Invasive Bighead and Silver Carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in Environmental Water Samples from the United States. *PLoS ONE* 10: e0117803, https://doi.org/10.1371/journal.pone.0117803

Fisheries and Oceans Canada (2019) Aquatic invasive species. https://www.dfo-mpo.gc.ca/species-especes/ais-eae/index-eng.html (accessed 11 November 2019)

Geller J, Meyer C, Parker M, Hawk H (2013) Redesign of PCR primers for mitochondrial cytochrome *c* oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources* 13: 851–861, https://doi.org/10.1111/1755-0998.12138

Gill K, MacNair M, Morrison A (2007) Investigation into the life cycle, impact on mussel culture and mitigation strategies for the vase tunicate (*Ciona intestinalis*), a new invasive species in the Montague/Brudenell River systems. PEI Department of Fisheries and Aquaculture Project # 062AR20

Gillum JE, Jimenez L, White DJ, Goldstien SJ, Gemmell NJ (2014) Development and application of a quantitative real-time PCR assay for the globally invasive tunicate *Styela clava*. *Management of Biological Invasions* 5: 133–142, https://doi.org/10.3391/mbi.2014.5.2.06

Gingera TD, Bajno R, Docker MF, Reist JD (2017) Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. *Management of Biological Invasions* 8: 287–300, https://doi.org/10.3391/mbi.2017.8.3.03

Gingera TD, Steeves TB, Boguski DA, Whyard S, Li WM, Docker MF (2016) Detection and identification of lampreys in Great Lakes streams using environmental DNA. *Journal of Great Lakes Research* 42: 649–659, https://doi.org/10.1016/j.jglr.2016.02.017

Goldberg CS, Strickler KM, Pilliod DS (2015) Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation* 183: 1–3, https://doi.org/10.1016/j.biocon.2014.11.040

Hunter ME, Oyler-McCance SJ, Dorazio RM, Fike JA, Smith BJ, Hunter CT, Reed RN, Hart KM (2015) Environmental DNA (eDNA) Sampling Improves Occurrence and Detection Estimates of Invasive Burmese Pythons. *PLoS ONE* 10: e0121655, https://doi.org/10.1371/journal.pone.0121655

Locke A (2009) A screening procedure for potential tunicate invaders of Atlantic Canada. *Aquatic Invasions* 4: 71–79, https://doi.org/10.3391/ai.2009.4.1.7

Locke A, Doe KG, Fairchild WL, Jackman PM, Reese EJ (2009) Preliminary evaluation of effects of invasive tunicate management with acetic acid and calcium hydroxyde on non-target marine organisms in Prince Edward Island, Canada. *Aquatic Invasions* 4: 221–236, https://doi.org/10.3391/ai.2009.4.1.23

Locke A, Hanson JM, Ellis KM, Thompson J, Rochette R (2007) Invasion of the southern Gulf of St. Lawrence by the clubbed tunicate (*Styela clava* Herdman): Potential mechanisms for invasions of Prince Edward Island estuaries. *Journal of Experimental Marine Biology and Ecology* 342: 69–77, https://doi.org/10.1016/j.jembe.2006.10.016

Ma KCK, Simard N, Stewart-Clark SE, Bernier RY, Nadeau M, Willis J (2016) Early detection of the non-indigenous colonial ascidian *Diplosoma listerianum* in eastern Canada and its implications for monitoring. *Management of Biological Invasions* 7: 365–374, https://doi.org/10.3391/mbi.2016.7.4.06

Marraffini ML, Ashton GV, Brown CW, Chang AL, Ruiz GM (2017) Settlement plates as monitoring devices for non-indigenous species in marine fouling communities. *Management of Biological Invasions* 8: 559–566, https://doi.org/10.3391/mbi.2017.8.4.11

Martin JL, LeGresley MM, Thorpe B, McCurdy B (2011) Non-indigenous tunicates in the Bay of Fundy, eastern Canada (2006–2009). *Aquatic Invasions* 6: 405–412, https://doi.org/10.3391/ai.2011.6.4.05

McKenzie CH, Matheson K, Caines S, Wells T (2016) Surveys for non-indigenous tunicate species in Newfoundland, Canada (2006-2014): a first step towards understanding impact and control. *Management of Biological Invasions* 7: 21–32, https://doi.org/10.3391/mbi.2016.7.1.04

Nydam ML, Giesbrecht KB, Stephenson EE (2017) Origin and Dispersal History of Two Colonial Ascidian Clades in the *Botryllus schlosseri* Species Complex. *PLoS ONE* 12: e0169944, https://doi.org/10.1371/journal.pone.0169944

Perez-Portela R, Arranz V, Rius M, Turon X (2013) Cryptic speciation or global spread? The case of a cosmopolitan marine invertebrate with limited dispersal capabilities. *Scientific Reports* 3: 3197, https://doi.org/10.1038/srep03197

Ramsay A, Davidson J, Landry T, Arsenault G (2008) Process of invasiveness among exotic tunicates in Prince Edward Island, Canada. *Biological Invasions* 10: 1311–1316, https://doi.org/10.1007/s10530-007-9205-y

Ramsay A, Davidson J, Bourque D, Stryhn H (2009) Recruitment patterns and population development of the invasive ascidian *Ciona intestinalis* in Prince Edward Island, Canada. *Aquatic Invasions* 4: 169–176, https://doi.org/10.3391/ai.2009.4.1.17

Roy M, Belliveau V, Mandrak NE, Gagné N (2018) Development of environmental DNA (eDNA) methods for detecting high-risk freshwater fishes in live trade in Canada. *Biological Invasions* 20: 299–314, https://doi.org/10.1007/s10530-017-1532-z

Sargent PS, Wells T, Matheson K, McKenzie CH, Deibel D (2013) First record of vase tunicate, *Ciona intestinalis* (Linnaeus, 1767), in coastal Newfoundland waters. *BioInvasions Records* 2: 89–98, https://doi.org/10.3391/bir.2013.2.2.01

Sephton D, Vercaemer B, Nicolas JM, Keays J (2011) Monitoring for invasive tunicates in Nova Scotia, Canada (2006-2009). *Aquatic Invasions* 6: 391–403, https://doi.org/10.3391/ai.2011.6.4.04

Sephton D, Martin JL, LeGresley M, Godin K (2016) Biofouling monitoring for aquatic invasive species (AIS) in DFO Maritimes Region, southwest New Brunswick: 2006-2011. Canadian Technical Report of Fisheries and Aquatic Sciences 3140, vi + 68 pp

Sephton D, Vercaemer B, Silva A, Stiles L, Harris M, Godin K (2017) Biofouling moitoring for aquatic invasive species (AIS) in DFO Maritimes Region (Atlantic shore of Nova Scotia and southwest New Brunswick): May-November, 2012-2015. Canadian Technical Report of Fisheries and Aquatic Sciences 3158, ix + 172 pp

Simard N, Pelletier-Rousseau M, Clarke Murray C, McKindsey CW, Therriault TW, Lacoursière-Roussel A, Bernier R, Sephton D, Drolet D, Locke A, Martin JL, Drake DAR, McKenzie

CH (2017) National Risk Assessment of Recreational Boating as a Vector for Marine Non-indigenous Species. DFO Canadian Science Advisory Secretariat Research Document 2017/006, vi + 95 pp

Simard N, Pereira S, Estrada R, Nadeau M (2013) State of marine invasive species in Quebec. Canadian Manuscript Report of Fisheries and Aquatic Sciences 3020, viii + 61 pp

Smart AS, Tingley R, Weeks AR, van Rooyen AR, McCarthy MA (2015) Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications* 25: 1944–1952, https://doi.org/10.1890/14-1751.1

Stewart-Clark S, Greenwood SJ, Davidson J (2013) Optimization and Validation of Molecular Assays for Invasive Tunicate Monitoring in Environmental Water Samples. *Aquatic Science and Technology* 1: 143–166, https://doi.org/10.5296/ast.v1i1.2979

Willis JE, Stewart-Clark S, Greenwood SJ, Davidson J, Quijon P (2011) A PCR-based assay to facilitate early detection of *Diplosoma listerianum* in Atlantic Canada. *Aquatic Invasions* 6: 7–16, https://doi.org/10.3391/ai.2011.6.1.02

Supplementary material

The following supplementary material is available for this article:

Appendix 1. eDNA field sampling protocol.
Appendix 2. DNA extraction protocol.
Table S1. List of targeted AIS for which species-specific qPCR assays were designed and DNA barcoding primers and sequences.
Table S2. Details of species-specific qPCR assays designed in this study including efficiency (%) and limit of detection (LOD).
Table S3. GPS coordinates of sites sampled.
Table S4. eDNA field detections of four AIS from grab samples and concentrated-sieved samples in comparison to biofouling monitoring lines in 2017 at five sites in the southern Gulf of St. Lawrence.
Table S5. eDNA field detections of six AIS from grab samples in comparison to biofouling monitoring lines in 2018 at four sites in the southern Gulf of St. Lawrence.
Table S6. eDNA field detections of six AIS from grab samples in comparison to biofouling monitoring lines in 2018 at sites in the Bay of Fundy and Atlantic shore of Nova Scotia.

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