Targeted detection of European green crab (*Carcinus maenas*) and Chinese mitten crab (*Eriocheir sinensis*) using environmental DNA

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ABSTRACT

Chevrinais, M., Demers, A., Dumoulin, L.-A., Gagné, N., Larivière, J., LeBlanc, F., Simard, N., Therien, A., Weise, A. M., and Parent, G. J. 2023. Targeted detection of European green crab (*Carcinus maenas*) and Chinese mitten crab (*Eriocheir sinensis*) using environmental DNA. Can. Tech. Rep. Fish. Aquat. Sci. 3539: vii + 32 p.

Mitigating the impact of Aquatic Invasive Species (AIS) is critical because of their potential to disturb ecosystems. AIS introduction involves low abundances, which can limit detection with traditional monitoring. Environmental DNA detection (eDNA, or DNA from organisms in the environment) is a relatively sensitive tool for monitoring AIS at low abundances. We present targeted assays for eDNA detection based on quantitative PCR for two AIS, the European green crab (*Carcinus maenas)* and the Chinese mitten crab (*Eriocheir sinensis)*. *In silico* validation showed that the *C. maenas* assay was species-specific whereas the *E. sinensis* assay also detected two other *Eriocheir* species (not reported in Canada but potential invaders). Specificity of the 2 assays was assessed *in vitro* by testing against phylogenetically related species commonly encountered in the Gulf of St. Lawrence, none of which showed DNA amplification. Assay sensitivity was high for both species (limit of detection <2.5 DNA copies/qPCR reaction). Detection of eDNA *in situ* confirmed findings from traditional monitoring for *C. maenas* in the Gulf of St. Lawrence. *E. sinensis* eDNA was detected in the St. Lawrence River but its presence was not confirmed (no active sampling). This study provides targeted eDNA detection protocols with high sensitivity, adapted to Atlantic Canadian waters, and validated to level 4 according to the DFO minimum requirements for eDNA science. These protocols have been implemented for monitoring by the DFO Quebec region since 2019.

RÉSUMÉ

Chevrinais, M., Demers, A., Dumoulin, L.-A., Gagné, N., Larivière, J., LeBlanc, F., Simard, N., Therien, A., Weise, A. M., and Parent, G. J. 2023. Targeted detection of European green crab (*Carcinus maenas*) and Chinese mitten crab (*Eriocheir sinensis*) using environmental DNA. Can. Tech. Rep. Fish. Aquat. Sci. 3539: vii + 32 p.

L'atténuation de l'impact des Espèces Aquatiques Envahissantes (EAE) est critique à cause de leur potentiel de perturbation des écosystèmes. L'introduction des EAE implique de faibles abondances, ce qui peut limiter leur détection par du monitorage traditionnel. La détection de l'ADN environnemental (ADNe, ou ADN d'organismes dans l'environnement) est un outil relativement sensible pour monitorer les EAE en faible abondance. Nous présentons une approche ciblée pour la détection d'ADNe de 2 EAE, le crabe vert européen (*Carcinus maenas)* et le crabe chinois à mitaines (*Eriocheir sinensis*). La validation *in silico* a montré que l'essai *C. maenas* était spécifique alors que l'essai *E. sinensis* permettait aussi la détection de deux autres espèces du genre *Eriocheir* (jamais rapportées en eaux canadiennes, mais potentiellement envahissantes). La spécificité de chaque essai a été évaluée *in vitro* en testant des espèces phylogénétiquement proches retrouvées dans le Saint-Laurent, aucune de ces espèces ne présente d'amplification. La sensibilité de chaque essai était forte (limite de détection <2,5 copies d'ADN/réaction). La détection d'ADNe *in situ* confirme les résultats de monitorage traditionnel pour *C. maenas* dans le Golfe du Saint-Laurent. L'ADNe d'*E. sinensis* a été détecté dans l'estuaire du Saint-Laurent, mais leur présence n'a pas été confirmée (absence d'échantillonnage actif). Ces protocoles, avec de fortes sensibilités, adaptés à l'Atlantique canadien, ont été validés au niveau 4 selon les conditions minimales requises dans les études d'ADNe par le MPO. Ces protocoles ont été appliqués pour un monitorage par le MPO (région du Québec) depuis 2019.

1. INTRODUCTION

Aquatic invasive species (AIS) are a major threat to Canadian and worldwide ecosystems (Bax et al. 2003; Molnar et al. 2008; Havel et al. 2015). Efforts have focused on early detection of AIS for effective management responses, such as to mitigate the risk of establishment and spread (Anderson 2005; Lodge et al. 2006; Wimbush et al. 2009; Vander Zanden et al. 2010; Jerde et al. 2011). AIS are inherently difficult to detect, and detection probability increases with sampling effort. Increased sampling effort represents a challenge as resources are often limited by time, funding and human resources (Hayes et al. 2005).

Amongst the tools available for early AIS detection, environmental DNA (eDNA) sampling combined with quantitative real-time polymerase chain reaction (qPCR) detection has emerged as a useful technique (e.g., Jerde et al. 2011; Goldberg et al. 2013; Klymus et al. 2015). eDNA sampling allows researchers to collect DNA from an aquatic environment and then concentrate the DNA suspended in the water through various methods *e.g.*, membrane filtration (Ficetola et al. 2008). DNA extraction and subsequent qPCR analysis can then determine the presence and relative quantity of DNA from a target species (Goldberg et al. 2013). This approach is considered specific, relatively sensitive, non-invasive, and does not rely on taxonomic expertise.

Some issues prevent the wide adoption of eDNA methods to guide decision-making. The lack of validated methods and susceptibility to false-positive and false-negative detections for rare targets are some examples (Abbott et al. 2021). To address these issues, this work describes methods and the validation data to support their use for the eDNA-based detection of two non-indigenous crabs: the European green crab (*Carcinus maenas*) and the Chinese mitten crab (*Eriocheir sinensis*). For both species, eDNA detections can be useful for early detection and monitoring purposes and support management decisions at Fisheries and Oceans Canada (DFO).

The European green crab is native to Europe (North Sea) and North African coasts (Roman 2006). This invasive crab is a voracious predator of mollusks, known to compete for space and food with local species, and able to uproot eelgrass beds when feeding (Davis et al. 1998; Rossong et al. 2006; Williams et al. 2006; Tan and Beal 2015; Lutz-Collins et al. 2016; Matheson et al. 2016). Consequently, the green crab causes major ecological and economical challenges in invaded regions (Gillespie et al. 2007; Therriault et al. 2008). Introduced in North America in 1817 (Long Island, New York), the European green crab was first reported in Canada in 1951 in Passamaquoddy Bay, NB, in 1998 in Prince Edward Island, and in 2004 in the Magdalen Islands, QC (reviewed in Audet et al. 2003; Simard et al. 2013). At least two independent introductions of green crab have occurred since the 1800s along the North American East coast (Roman 2006; Audet et al. 2008; Blakeslee et al. 2010), resulting in the establishment of genetically distinct northern and southern ecotypes (Tepolt and Palumbi 2015; Jeffery et al. 2017, 2018). These ecotypes correspond to northern and southern populations in the native range (Roman 2006). Following its introduction in the Magdalen Islands in 2004, the green crab population rapidly spread in all lagoons to reach a maximal annual capture in 2011 (9,617 crabs). Between 2008 and 2012, there were efforts to control the green crab population by DFO, Merinov, and eel fishermen. No crabs were captured in 2015. Since then, only a few crabs (1-3 crabs per year) have been captured in the entire archipelago (DFO monitoring data, Simard N., pers. comm.). Detection of eDNA is a promising tool to monitor green crab in the Magdalen Islands and for detecting changes in population size.

The Chinese mitten crab is a catadromous crab native to freshwater and estuarine habitats of eastern Asia. It is listed as one of the 100 worst invasive alien species in the world (Lowe et al. 2000). It can disrupt local ecosystems, cause the erosion of riverbanks due to its burrowing behavior, and result in substantial ecological damage including negative impacts on fisheries (e.g., crayfish, see review by Dittel and Epifanio 2009). The Chinese mitten crab was first reported in Canada in 1965 in the Detroit River at Windsor, Ontario (Nepszy and Leach 1973; Veilleux and de Lafontaine 2007). The species is thought to have been introduced in Canada accidentally through ship ballast discharge or intentionally through illegal live food imports (Cohen and Carlton 1997; Veilleux and de Lafontaine 2007). In Quebec, this invasive crab was found for the first time in the St. Lawrence River in 2004 close to Quebec City (de Lafontaine 2005) and 8 additional crabs were caught in the fresh and brackish waters of the St. Lawrence River between 2004 and 2007 by commercial fishermen (de Lafontaine et al. 2008). Since then, only 2 individuals were found in the St. Lawrence River in 2020 (MELCCFP, Morissette O., pers. comm.). Additional monitoring efforts are needed to determine whether this species is established in the St. Lawrence River, and eDNA methods are particularly suitable for this application.

In this study, we present targeted approaches for eDNA detection of both species using a qPCR probe-based protocol, and validation data obtained *in silico*, *in vitro* and *in situ*. We provide evidence of validation to level 4 according to the DFO minimum requirements in eDNA studies (Abbott et al. 2021). The 2 protocols presented in this report are currently used as a monitoring tool for eDNA-based detection of these species by the DFO Quebec region on an annual basis since 2019. A qPCR assay targeting European green crab DNA has already been published to help in its early detection in Pacific Ocean waters (Roux et al. 2020). This assay was only validated in the Pacific Ocean waters and was not published at the beginning of the Quebec region monitoring activities in 2019. The publication of a second qPCR probe-based protocol for the European green crab and validated for Atlantic Canadian waters is consequently necessary to support the robustness of the Quebec monitoring program.

2. METHODS

2.1 DESIGN AND OPTIMIZATION OF QPCR ASSAYS

2.1.1 *In silico* specific qPCR assay design

The mitochondrial gene cytochrome c oxidase subunit 1 (COI) used as the Barcode of Life for animals was selected for eDNA detection of *C. maenas* and *E. sinensis.* We retrieved COI sequences from NCBI and BOLD for the target species, closely related species, and sympatric species (Table 1). COI sequences were then aligned in Geneious Version 9.1.8 and primers and probes were designed for *C. maenas* and *E. sinensis* to amplify regions of 174 and 137 base pairs (bp), respectively (Table 2). Mitochondrial DNA fragments under 200 bp have been targeted to maximise the likelihood of finding those DNA in the environment (Jo et al. 2022). To maximize specificity, assays were designed targeting DNA sequences containing high numbers of nucleotide differences between the targeted AIS and closely related or common sympatric species (Table 1). The assays were optimized for a qPCR annealing temperature of 60°C. Primer-BLAST (Ye et al. 2012) was also used to ensure that assay primers were target specific.

Table 1: Specificity assessment for primers/probe. European green crab (*Carcinus maenas*) and Chinese mitten crab (*Eriocheir sinensis*) qPCR assays using close relative and sympatric species. Nucleotide mismatches from the target species are underlined and in bold. M = total number of bp mismatches from the target species.

Species	GenBank #	Forward primer 5'-3'	Reverse Primer 5'-3'	Probe 5'-3'	M
Carcinus maenas	FJ581592	TCTGATTACTTCCTCCGTCTTTAACCTTA	CTAAAATAGAAGAAACCCCGGCTAAA	AGGAGTTGGAACAGGATGA	
Cancer irroratus	FJ581567	TCTGA**C**T**TT**T**A**CCTCC**C**TC**C**TTAAC**AC**T**C**	CTAA**G**ATAGA**G**GA**G**AC**T**CC**T**GCTAAA	**G**GGAGTTGGAACAGG**T**TG**G**	17
Chionoecetes opilio	FJ581598	T**T**TG**G**TTA**T**T**G**CCTCC**T**TCTTTAAC**AC**TA	CTAAAATAGA**G**GAAAC**T**CC**A**GCTAAA	AGGAGT**A**GGAAC**T**GGATG**G**	13
Ovalipes ocellatus	KU905781	T**T**TGA**C**T**T**CTTCC**C**CC**C**TC**AC**TAACCTTA	CTAA**G**ATAGAAGA**G**AC**T**CCGGCTAAA	AGGAGTTGG**T**ACAGGATGA	11
Lithodes maja	FJ581740	T**T**TGA**C**T**TT**T**A**CC**C**CC**C**TC**AC**TAAC**TC**T**T**	CTAAAATAGAAGA**T**AC**T**CC**A**GC**C**AAA	AGG**T**GT**A**GG**T**ACAGGATGA	18
Hyas araneus	MG319203	T**T**TG**G**TTACT**A**CCTCC**A**TCTTTAAC**A**TTA	CTAAAATAGA**G**GAAAC**A**CC**T**GC**C**AAA	AGG**T**GT**A**GG**T**AC**C**GG**T**TG**G**	15
Carcinus aestuarii	HF952830	TCTGA**C**TACTTCCTCC**A**TCTTTAACTTTA	CTAAAATAGAAGAAAC**T**CC**A**GC**G**A**G**A	**G**GGAGTTGG**G**AC**C**GG**G**TGA	10
Eriocheir sinensis	MH087510	TCTGA**C**T**TT**T**A**CC**C**CC**T**TCT**C**T**CT**C**TC**TT	CTAAAAT**T**GA**G**GAAAC**T**CCGGC**A**A**G**A	AGGAGTTGG**T**AC**T**GGATG**G**	19
Cancer borealis	AF060767	TCTG**G**TTACT**A**CC**C**CC**T**TC**A**TTAACC**C**TA	CTA**GG**AT**T**GAAGAAACCCC**A**GCTAAA	AGG**G**GTTGGAACAGG**T**TG**G**	13
Eriocheir sinensis	MH087510	CATCAGTTGATCTTGGTATCTTYTCTCTACA	TCAAACAAAAAGAGGTATTTGATCCATT	TCCTCAATTTTAGGAGCTGTT	
Eriocheir japonica	FJ750327	CATCAGTTGATCTTGGTATCTTTTCTCTACA	TCAAACAAAAAGAGGTATTTGATCCATT	TCCTCAATTTTAGGAGCTGTT	0
Eriocheir hepuensis	AF516699	CATCAGTTGATCTTGGTATCTTTTCTCTACA	TCAAACAAAAAGAGGTATTTGATCCATT	TCCTCAATTTTAGGAGCTGTT	0
Eriocheir ogasawaraensis	FJ750331	CATCAGTTGATCTTGGTATCTTTTCTCT**C**CA	TCAAACAAAAAGAGGTATTTG**G**TCCATT	TCCTCAATTTTAGGAGCTGTT	2
Eriocheir rectus	AF317332	C**C**TC**G**GTTGA**C**CT**G**GG**A**AT**T**TTTTCTCT**T**CA	TCAAACAAAAAGAGGTATTTG**G**TC**T**ATT	TC**T**TCAATT**C**TAGG**G**GC**C**GT**G**	14
Cancer irroratus	FJ581567	C**C**TCAGTTGAT**A**T**A**GGTAT**T**TTCTCT**T**TACA	TCA**G**ACAAAAAG**T**GGTATTTG**G**TC**T**A**AA**	TCCTC**T**AT**C**TTAGG**G**GC**C**GTT	15
Chionoecetes opilio	FJ581598	C**C**TC**C**GTTGAT**A**T**G**GG**G**AT**T**TTTTCTCTACA	TCAAACAAAAAG**T**GGTATTTGATC**T**A**GG**	TCCTC**T**ATTTTAGGAGCTGT**A**	12
Ovalipes ocellatus	KU905781	C**T**TCAGT**A**GA**CT**T**A**GG**A**ATCTTTTC**C**CT**T**CA	TCA**T**ACAAA**T**AGAGGTATTTG**G**TC**T**AT**A**	TC**T**TC**T**AT**C**TTAGG**T**GCTGTT	17
Lithodes maja	FJ581740	C**A**TCAGT**G**GAT**T**T**A**GG**A**AT**T**TTCTCT**T**T**G**CA	TCA**C**ACAAA**T**A**A**AGG**C**AT**AC**GATC**T**A**GG**	TC**T**TC**T**ATTTTAGGAGCTGT**A**	20
Hyas araneus	MG319203	C**T**TC**T**GTTGAT**A**T**G**GG**A**AT**T**TTCTC**CT**TACA	TCAAACAAAAAG**T**GGTATTTGATCAA**GA**	TCCTC**T**ATTTTAGG**G**GC**C**GT**A**	15
Carcinus maenas	FJ581592	C**T**TCAGTTGAT**T**T**A**GG**G**AT**T**TTCTCT**T**TACA	TCA**C**ACAAA**T**A**A**AGGTAT**C**TG**G**TC**T**ATT	TC**T**TC**T**ATTTTAGGAGCTGT**A**	15
Carcinus astuarii	HF952830	C**T**TCAGTTGA**CT**T**A**GG**G**AT**T**TTTTCT**T**TACA	TCA**C**AC**G**AA**T**A**A**AGGTATTTGATC**T**ATT	TC**T**TC**T**ATTTTAGGAGCTGT**A**	15
Cancer borealis	AF060767	C**T**TCAGT**G**GA**CA**T**G**GG**G**AT**T**TTTTCTCT**T**CA	**C**CAAACAAAAAG**T**GGTATTTG**G**TC**T**A**AG**	TC**T**TCAAT**CC**TAGGAGCTGT**G**	18

Table 2: Information on qPCR assays. A) Primers (F and R) and TaqMan® MGB probes (P). B) qPCR assays performance based on the protocol used for 2021. IPC: internal positive control.

A

Target	Primers and probes	Sequence 5'-3'
Carcinus maenas	COI_1772F_Cm	TCTGATTACTTCCTCCGTCTTTAACCTTA
	COI_1945R_Cm	CTAAAATAGAAGAAACCCCGGCTAAA
	COI_1827P_Cm	6FAM-AGGAGTTGGAACAGGATGA-MGBNFQ
E. sinensis	COI_375F_Es	CATCAGTTGATCTTGGTATCTTYTCTCTACA
	COI_512R_Es	TCAAACAAAAAGAGGTATTTGATCCATT
	COI_419P_Es	6FAM-TCCTCAATTTTAGGAGCTGTT-MGBNFQ
IPC	FF_12S_F	GTAAAACTCGTGAACAGAGC
	FF_12S_R	GGATTATTTAAAGCAACTGGC
	FF_12S_P	6FAM-TAGTCCTCGGCGTAAAGAGTGGTTAAGAAA-MGBNFQ

B

Target	Amplicon (bp)	Efficiency (%)	Regression equation	LOD95% (copies/reaction)
Carcinus maenas	174	113	-3.03(log(x))+35.11	1.08
E. sinensis	137	105	-3.21(log(x))+36.38	2.59
IPC	165	97	-3.39(log(x))+40.42	1.26

2.1.2 *In vitro* specificity

The *in vitro* specificity of the qPCR assays was tested using DNA from phylogenetically close species commonly encountered in the Gulf of St. Lawrence (Table 3). The sampling coordinates and year for *Cancer irroratus* and *Chionoecetes opilio* are unknown, however those specimens were selected either based on previous genetic analyses confirming the species identity (unpublished) or identification by taxonomic expert. Genomic DNA was extracted from each specimen using the DNeasy® Blood and Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. DNA extracts were stored at -20°C until qPCR testing.

The volumes and concentrations of each qPCR assay solutions were 10 µL of TaqPath™ ProAmp™ 2x master mix (Thermo Fisher Scientific™, Waltham, USA), 0.96 µL of forward and reverse primer (10 µM, Sigma-Aldrich, Saint-Louis, MO, USA), 0.4 µL of the TaqMan™ MGB probe (10 µM, Applied Biosystems®, Foster City, USA), 3 µL of DNA template, 0.8 µL of 1% Bovine Serum Albumin (Sigma-Aldrich, Saint-Louis, MO, USA) and 3.88 µl of nuclease-free water (Sigma-Aldrich, Saint-Louis, MO, USA) to yield a 20 µL final reaction. Cycling parameters were 10 min. at 95°C followed by 45 cycles of 15 sec. denaturation step at 95°C, 1 min. annealing step at 60°C, and 1 min. elongation step at 60°C. All qPCR reactions were conducted on the AriaMX qPCR system (Agilent Technologies, Santa Clara, CA, USA). Fluorescence was captured at the end of each elongation step.

Table 3: Information of specimens used to test *in vitro* specificity of qPCR assays. Geographic reference system WGS 84. MLI: Maurice Lamontagne Institute.

Species	Longitude	Latitude	Year	Source	Specimen ID
Eriocheir sinensis	W 70° 2' 36.362''	N 47° 25' 38.996''	2007	DFO sampling program	S_21_04842 S_21_04841
Cancer irroratus	Gulf of St. Lawrence		NA	MLI collection	S_21_04845
Chionoecetes opilio	Gulf of St. Lawrence		NA	MLI collection	S_21_04840
Lithodes maja	W 62° 5' 52.44''	N 48° 13' 39.36''	2021	DFO sampling program	S_21_04836
Hyas alutaceus	W 57° 9' 8.742''	N 51° 9' 19.534''	2021	DFO sampling program	S_21_04838
Hyas araneus	W 57° 9' 8.742''	N 51° 9' 19.534''	2021	DFO sampling program	S_21_04837
Carcinus maenas	W 61° 54' 9.359''	N 47° 13' 45.876''	2012	MLI collection	S_22_08135

2.1.3 *In vitro* sensitivity

Serial dilutions of synthetic DNA were used to determine the *in vitro* sensitivity of each qPCR assay. In 2019 and 2020, 2 double-stranded gBlocks® (Integrated DNA Technologies, Coralville, IA, USA) containing the target DNA sequences from *C. maenas* (174 bp) and *E. sinensis* (137 bp) were used. In 2021, a plasmid, pDNA (Genewiz, South Plainfield, NJ, USA) containing the 2 target DNA sequences from each species was used.

Serial dilutions from 2,000,000 to 2 DNA copies per reaction were tested in triplicate by qPCR to determine the assay efficiency using the equation E = -1 + 10[-1/slope] and to calculate the theoretical limit of detection (LOD). The LOD95% is defined as the lowest standard concentration at which 95% of the replicates produce positive detection of the target DNA. The LOD95% was determined following the method developed by Klymus et al. (2019).

We tested the validity of the LOD95% by estimating false positive and false negative rates for each qPCR assay. The false positive rate was determined by running a plate containing only non-template controls (no DNA added) whereas the false negative rate was determined by running a plate containing target pDNA at a concentration of 3 copies/reaction above the LOD95%. The concentration of 3 copies/reaction above the LOD95% is used because of the variability occurring at low concentrations due to pipetting variations notably. An assay with a false positive rate above 5% would indicate contamination during the laboratory analyses. In contrast, an assay with a false negative rate above or below 5% would indicate that the LOD95% may be over or underestimated, respectively.

2.2 EDNA DETECTIONS *IN SITU*

Samples were processed following the qPCR detection protocols with specific steps (Figure 1B) and procedures to prevent contamination (Appendix 1).

2.2.1 Sampling and filtration

eDNA samplings were planned at multiple levels (Figure 1A) including survey (occurring at a specific time period like a season or a month, V), site (geographic region or area, W), station (one geographic location identified by unique GPS coordinates, X) and sample (water volume replicate from one station, Y).

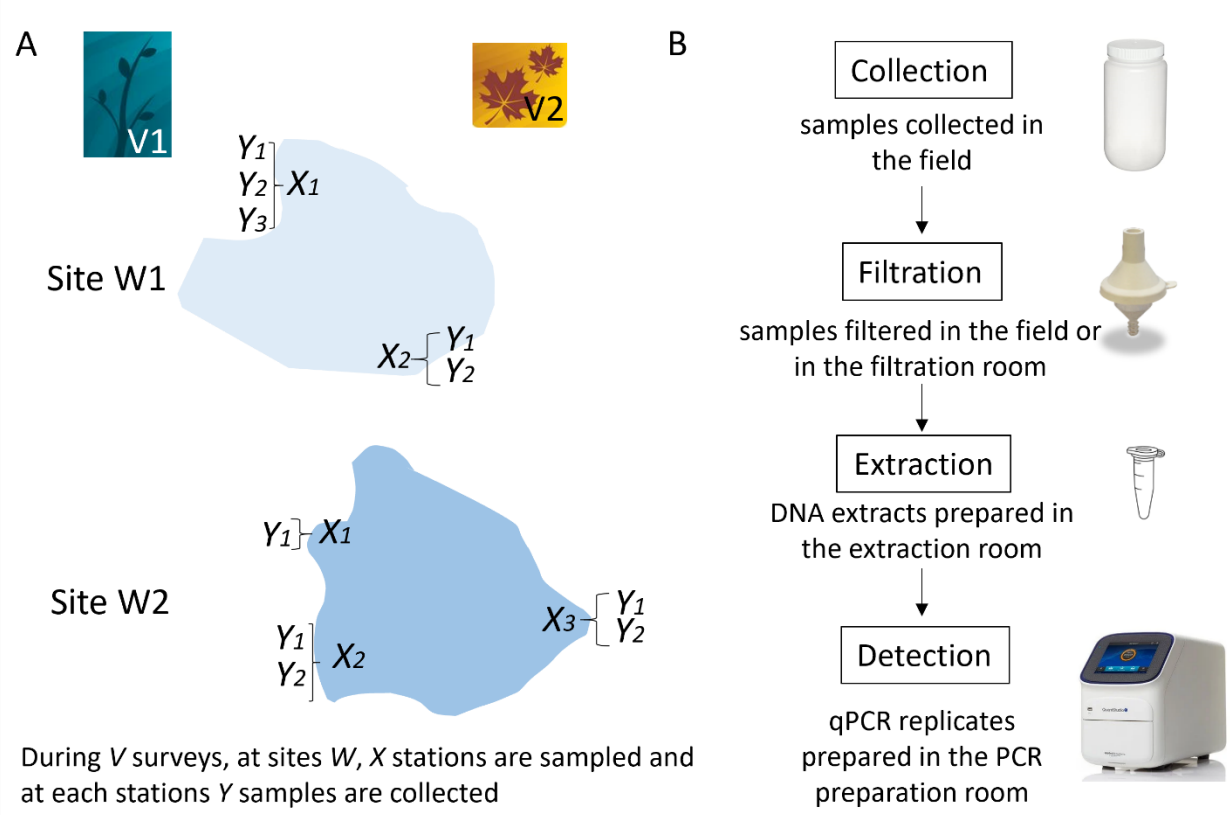

Figure 1: Field sampling design (A) and main steps of a qPCR-based eDNA detection protocol (B).

For *C. maenas,* sampling stations (Figure 2) were determined based on 1) an existing monitoring program in the Magdalen Islands, 2) the knowledge of *C. maenas* presence in Prince Edward Island and New Brunswick (positive controls), and 3) prospecting along the Gaspé Peninsula and the North Shore. For *E. sinensis*, sampling stations (Figure 2) were determined based on 1) previous sites where specimens were captured in the St. Lawrence River (Veilleux and de Lafontaine 2007; Simard et al. 2013), and 2) prospecting in typical habitats of this species. Based on DFO green crab monitoring data (2005-2020), capture success was higher at the end of the summer (August/September) when the water temperature is higher in the

Magdalen Islands and when crabs are probably more active. The choice of eDNA sampling period for green crab was based on this rationale. Three to 6 water samples (2L) were collected at each station from the coastline or from a floating pontoon with a 2L Nalgene® (Nalge Nunc International, Rochester, USA) sterile bottle.

Water samples were kept on ice and filtered within 6 hours after collection (Table 4). Samples were either filtered with 1) a vacuum pump (Appendix 4) in the field, 2) a vacuum pump in the ultraclean laboratory, or 3) a eDNA filter pack connected to an eDNA sampler (Smith Root, Vancouver, USA) (Table 4). Two types of filters were used, i.e., polyethersulfone filters (PES, 47 mm, 1.2 µm, Hoskin Scientific LTD, Burnaby, Canada) or glass fiber filters (GF, 47 mm, 1.5 µm, Hoskin Scientific LTD, Burnaby, Canada). Filter pore sizes were chosen based on the literature (Turner et al. 2014; Jo and Minamoto 2021) and to maximize the volume of water filtered knowing that estuaries are environments with high sedimentary particles.

For each sample, the full volume of water was filtered except when filters clogged (Table 4). For *E. sinensis*, filtering 2L of water was only possible in 45% of the samples during the 2019 survey and 0% of samples during the 2020 survey. Filter clogging is likely due to the abundance of sedimentary particles in suspension in the water column in sampling areas. Therefore, in 2021, a decision was made to reduce the filtered water volume from 2L to 1L but to increase the number of samples collected at a station (from 3 to 6). Also, filters were changed from PES (2019-2020) to GF (2021).

Filters processed in the ultraclean laboratory were preserved in microtubes at -20°C whereas filters processed in the field were preserved in a paper envelope in silica beads until DNA extraction (Table 4).

2.2.2 DNA extraction

Filters were cut in half and DNA was extracted from 1 half using the DNeasy® PowerWater kit (PW, QIAGEN, Hilden, Germany) following manufacturer's instructions or the DNeasy® Blood and Tissue kit (BTT) with minor modifications to the manufacturer's instructions, i.e. filters were digested for 2 hours at 56 °C in a mixture of 756 µL AL buffer and 84 µL of proteinase K, and five minutes incubation of the silica column with the elution buffer. The elution step of the DNA BTT was modified; the solution was incubated for five minutes using a TRIS buffer (10 mM) instead of the elution with the AE buffer containing EDTA, that may inhibit the qPCR reaction (QIAGEN 2017). The elution volume was 80 and 100 µL for BT and PW, respectively. DNA extracts were preserved at -20°C until qPCR and at -80°C for long-term preservation.

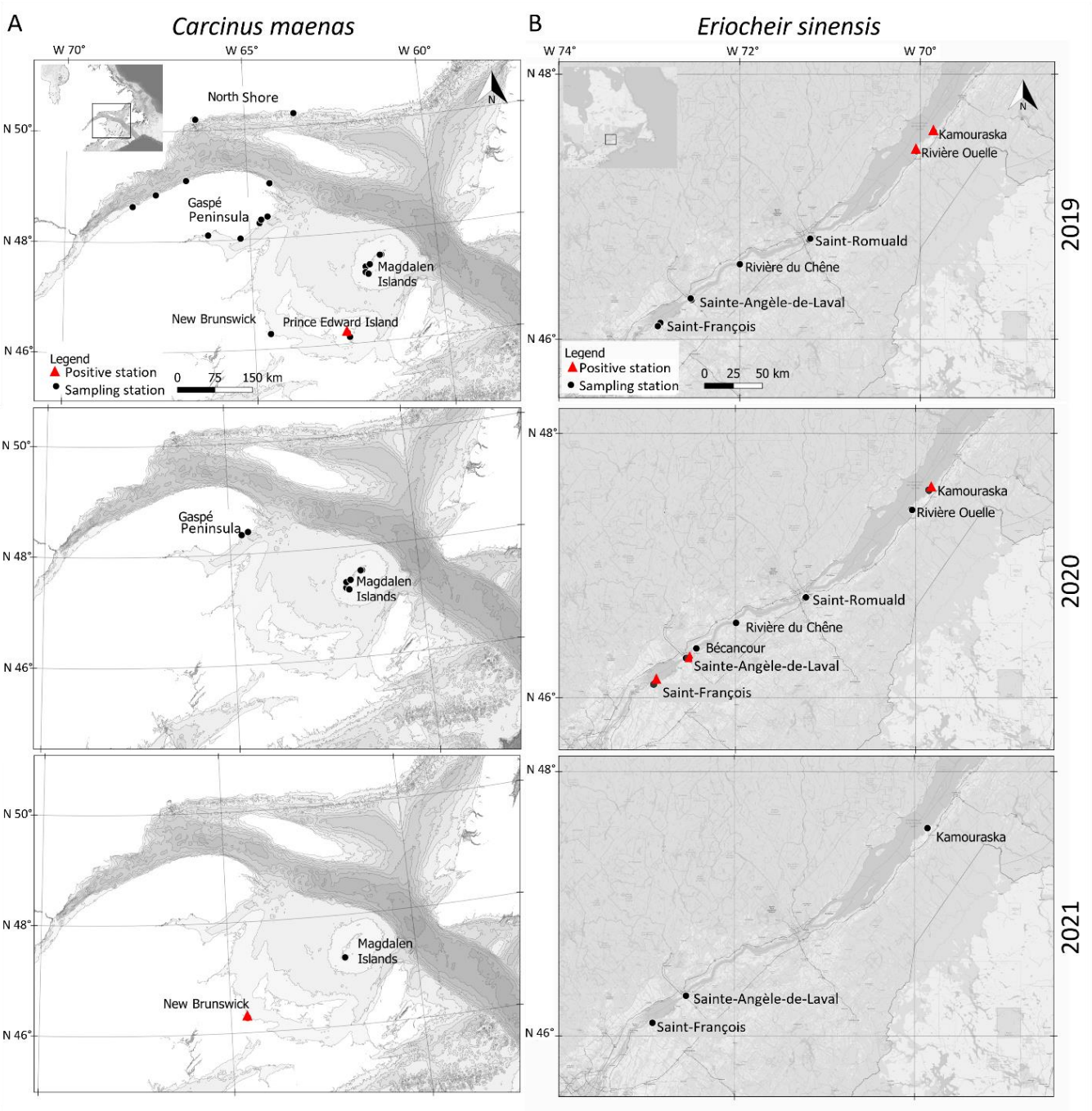

Figure 2: Sampling stations (dots) and positive stations (triangles) for A. *Carcinus maenas* [NAD83 (CSRS) Quebec Lambert, EPSG 6622] and B. *Eriocheir sinensis* (WGS 84/Pseudo-Mercator EPSG:3857) between 2019-2021. Stations abbreviations and GPS coordinates are provided in Appendices 2 and 3.

Table 4: eDNA sampling site data and laboratory treatment per sampling year (see Figure 2 for geographic locations of stations sampled each year and Appendices 2 and 3 for details). Vol.: volume, PES: polyethersulfone, GF: glass fiber, PW: Powerwater DNA extraction kit, BTT: modified DNeasy Blood and Tissue DNA extraction kit.

Species	Year	Surveys	Total # of sites	Stations per site	Samples per station	Vol. (L)	Filtration equipment	Filter and pore size (µm)	DNA extraction	qPCR per sample
Carcinus maenas	2019	August and September	5	1 to 10	3	2	Vacuum pump in the field	PES 1.2	PW	3
	2020	July	1	6	3	2	Vacuum pump in the field	PES 1.2	PW	3
		August	1	6						
		September	1	2						
	2021	August	2	1 to 2	3	2	Vacuum pump in the field	GF 1.5	PW	6
		September	1	2						
		October	1	1						
Eriocheir sinensis	2019	September	6	1 to 2	3	1-2	Vacuum pump in the field	PES 1.2	PW	3
	2020	July	1	2	3	0.4-0.9	Vacuum pump in the lab	PES 1.2	BTT	3
		October	7	1 to 2	3	0.2-1	Vacuum pump in the field	PES 1.2	BTT	3
	2021	August	3	1 to 2	6	1	eDNA sampler	GF 1.5	BTT	6
		September	3	1 to 2	6	0.5-1	eDNA sampler	GF 1.5	BTT	6

2.2.3 qPCR assay for inhibition verification

DNA extracts and all controls for contamination prevention were tested for the presence of PCR inhibitors. We used an internal positive control (IPC) to assess for qPCR inhibition in field samples. A double-stranded 165 bp gBlocks® gene fragment (FrankenFish, 5'-GTAAAACTCGTGAACAGAGCCGCGGTTATACGAGAGGCCCGAGTTGTTAGTCCTCGGCGTAAAGAGTGGTTAAGAAAAAAGAGAAAATATGGCCGAACAGCTTCAAAGCAGTTATACGCATCCGAAGTCACGAAGAACAATCACGCCAGTTGCTTTAAATAATCC-3') was designed as a chimeric sequence of several species using Geneious Version 9.1.8. Details for the primers and probe designed for this IPC are provided in Table 2. The IPC was added to the mastermix for a final estimate of 75,000 IPC copies per sample. The qPCR assay consisted of 10 µL of TaqPath™ ProAmp™ 2x master mix, 1.6 µL of forward and reverse primer (10 µM), 0.4 µL of the TaqMan™ MGB probe (10 µM), 3 µL of DNA template, 0.8 µL of 1% Bovine Serum Albumin and 2.6 µl of nuclease-free water to yield a 20 µL final reaction. Cycling parameters were 10 min. at 95°C followed by 45 cycles of 15 sec. denaturation step at 95°C, 1 min. annealing step at 60°C, and 1 min. elongation step at 60°C. Inhibition was considered present if a difference of more than 2 quantification cycles (Cq) was observed between eDNA samples and their corresponding SNC (LeBlanc et al. 2020).

2.2.4 Targeted qPCR assays

Testing of each DNA extract with either the *C. maenas* or the *E. sinensis* targeted qPCR assays was done using 3 to 6 qPCR replicates (Table 4). Each qPCR plate contained 89 to 91 DNA extracts and 3 to 5 qPCR negative controls (QNC, PCR grade water), and 1 qPCR positive controls (QPC, synthetic sequence of the species of interest with 6 extra nucleotides to identify putative laboratory contamination with the QPC) (Appendix 7). Details of the qPCR assays and the cycling parameters are provided in section 2.1.2.

qPCR replicates showing a sigmoidal amplification curve with a strong exponential phase and with a Cq > LOD95% were considered as positive detections (Figure 3). All positive detections were sequenced by Sanger DNA sequencing technology to confirm the specificity of the *C. maenas* and *E. sinensis* qPCR assays and the absence of contamination by the QPC. Products of the qPCR reactions were amplified using 1 µL of qPCR reaction product, 2 µL of each primer (10 µM), 10 µL of 10% trehalose and 0.5 µL of the Big Dye Terminator, 3.75 µL of the 5x buffer from the Applied Biosystems BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, MA, USA) and 3.88 µl of nuclease-free water to yield a 20 µL final reaction. Cycling parameters were 1 min. denaturation step at 96°C followed by 25 cycles consisting of a 10 sec. denaturation step at 96°C, a 5 sec. annealing step at 50°C and a 4 min. elongation step at 60°C. Following amplification, PCR products were cleaned using the Applied Biosystems BigDye® Xterminator™ purification kit (Thermo Fisher Scientific, MA, USA) and sequenced on the Applied Biosystems SeqStudio genetic analyzer system (Thermo Fisher Scientific, MA, USA).

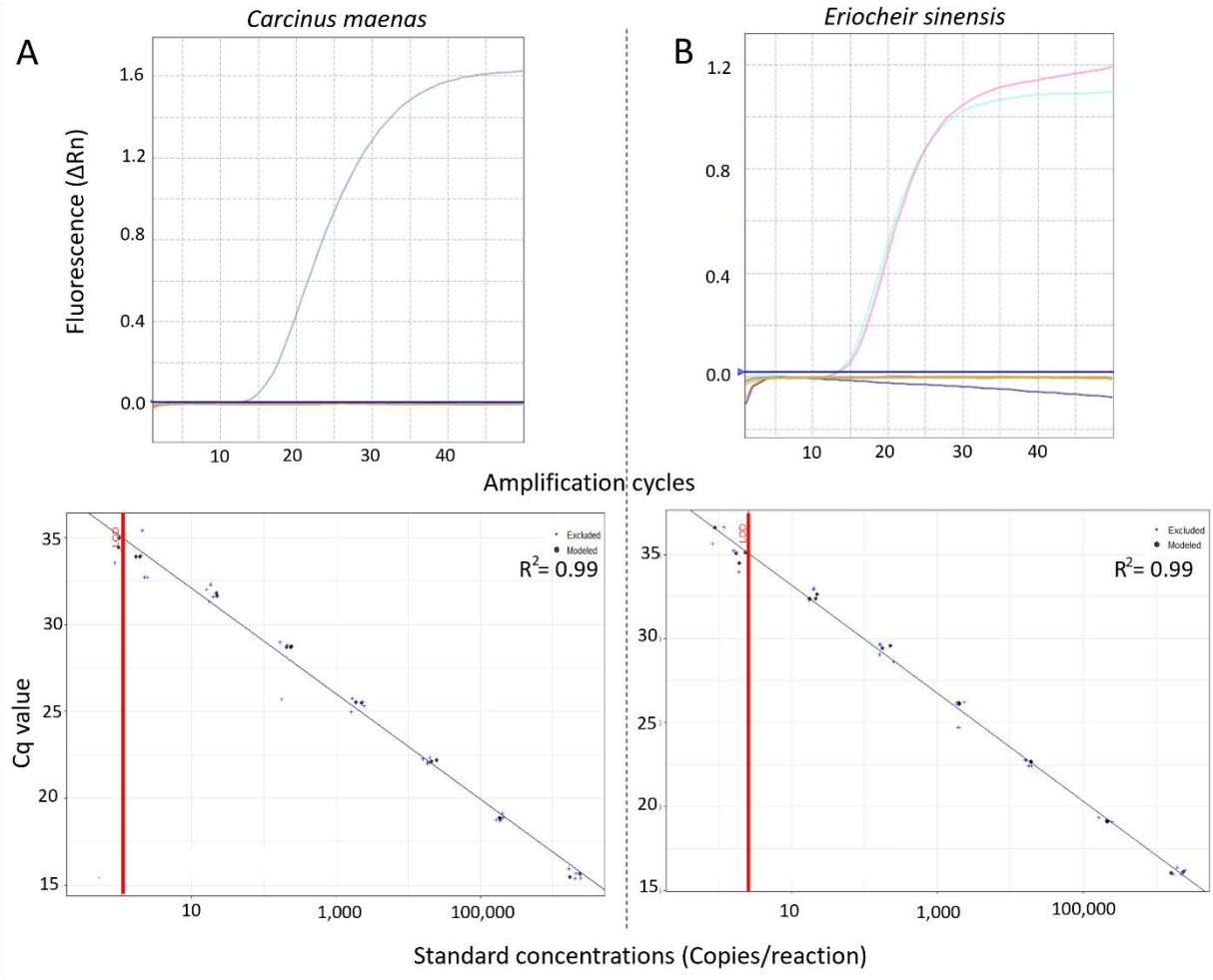

Figure 3: qPCR assays performance including specificity (upper panels) and sensitivity (lower panels) for *Carcinus maenas* (A) and *Eriocheir sinensis* (B). Specificity testing shows sigmoidal amplification curves with an exponential phase for both targeted species only (grey line for one reference specimen in A, blue and pink lines for two reference specimens in B), other species showing no DNA amplification (0 in fluorescence axis). Sensitivity testing shows linear standard curves of plasmid pDNA dilution series. LOD95% line (red line) was generated with Klymus et al. 2019 script.

2.2.5 Reporting eDNA results

eDNA results were based on a decision flow chart to determine if the species DNA was detected (Figure 4, dashed arrows path) or not (Figure 4, plain arrows path) at the site level. qPCR replicates results were separated in two categories, namely qPCRs replicates with or without amplification curves (Figure 4, qPCR level). The species DNA was considered as not detected at a site if all qPCR replicates from all samples collected at that site showed no amplification curves (Figure 4, white rectangles). A sample was considered positive if at least 1 of its qPCR replicates was ≥ LOD95% (Figure 4, black rectangle, sample level) whereas a sample was considered inconclusive if at least 1 of its qPCR replicates had an amplification curve but the number of eDNA copies detected was below the LOD95% (Figure 4, grey rectangle). A station was considered positive if at least 1 sample was positive or 1 sample was

inconclusive during at least 2 different surveys (Figure 4, station level). A site was considered as positive (*e.g.*, green crab DNA detected) if at least 1 station was positive (Figure 4, site level).

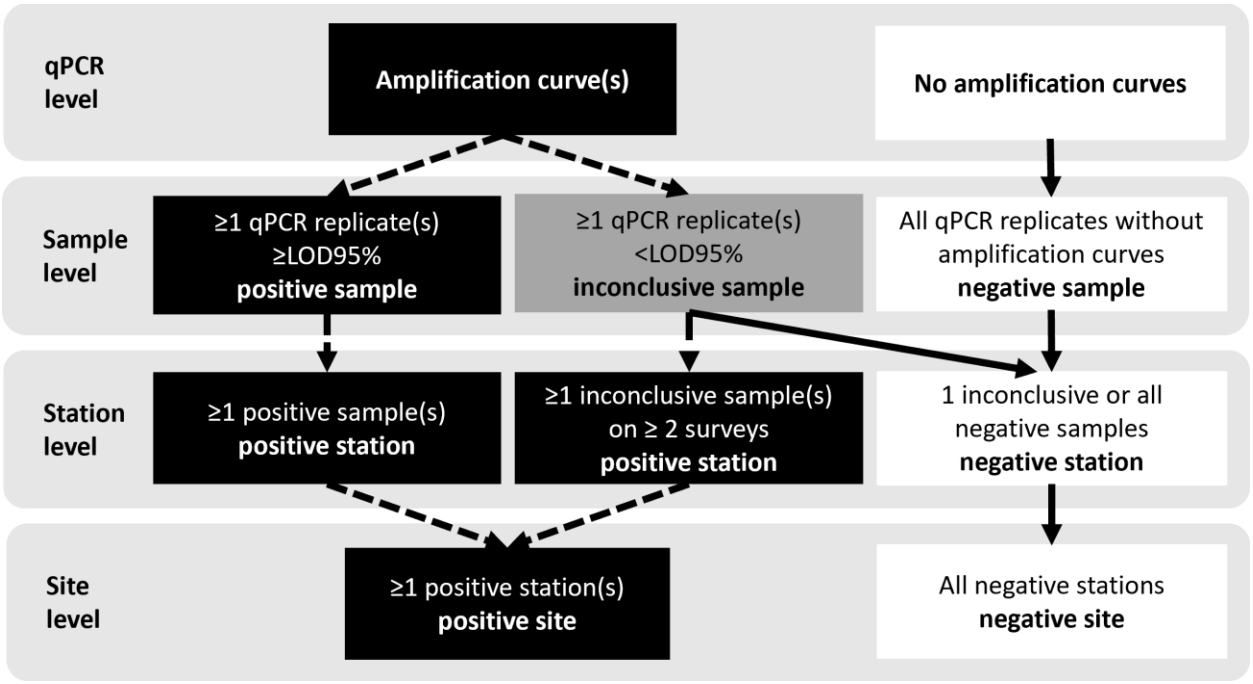

Figure 4: Decision flow chart for eDNA results reporting from qPCR results (amplification curve observed or not for qPCR replicate) to species detection at site-level.

3. RESULTS

3.1 QPCR ASSAYS PERFORMANCE

While it was possible to design species specific primers and probe for the detection of *C. maenas* based on the *in silico* validation step, it was not attempted to do so for *E. sinensis.* The design of *E. sinensis* qPCR assay allows for the detection of other non-indigenous *Eriocheir* species, (i.e., *E. japonica, E. hepuensis* and *E. ogasawaraensis*) due to the high COI nucleotide similarity among those species (Chu et al. 2003) (Table 1). Thus, we consider the assay targeting *E. sinensis* as unspecific although this could not be tested *in vitro* due to a lack of *E. japonica, E. hepuensis, and E. ogasawaraensis* samples*.* Note that all *Eriocheir* species are non-native to Canada and detection of one or the other is important from an AIS management standpoint. Amplicon sequencing could provide species confirmation, if *E. japonica, E. hepuensis* or *E. ogasawaraensis* are suspected to be present.

Both qPCR assays showed *in vitro* specificity to the targeted taxa (Figure 3). No DNA amplification of closely related species encountered in the Estuary and Gulf of St. Lawrence was observed with both assays.

Both qPCR assays were more sensitive to the targeted species when using the pDNA to infer copy numbers. For the *C. maenas* qPCR assay, the LOD95% was at Cq of 36.87, corresponding to 285 pDNA copies/qPCR reaction. Using gBlocks (2019-2020), the LOD95% was at Cq of 35.01, corresponding to 1 copy/qPCR reaction. For the *E. sinensis* assay, the LOD95% was at Cq of 37.48, corresponding to 6 gBlocks copies/qPCR reaction (2019-2020), and at Cq of 34.96, corresponding to 2.5 pDNA copies/qPCR reaction, in 2021.

False positive rates of 0% were observed for both *C. maenas* and *E. sinensis* assays using pDNA. The false negative rates were of 9.6% and 0% for the *C. maenas* and *E. sinensis* assays, respectively, using the pDNA.

3.2 EDNA DETECTIONS *IN SITU*

3.2.1 Controls

Controls for contamination prevention used at various steps of the protocol (Appendix 1) were examined for both assays. Negative controls failed if a qPCR amplification curve was observed and a positive control failed if QPC amplification was delayed by > 2 Cq. All the negative and positive controls passed.

3.2.2 Inhibition

No PCR inhibition was observed in any of the eDNA samples collected in this study. The IPC assay showed no delayed amplification i.e., > 2 Cq in the environmental samples compared to their SNC.

3.2.3 qPCR results

Carcinus maenas eDNA detections occurred in August (BRU in 2019 and SHE in 2021) and October (SHE in 2021) (Appendix 5, Figure 5). *Carcinus maenas* DNA was detected in 2 out of the 3 samples (6/9 qPCR replicates >LOD95%) collected at the station BRU in Prince Edward Island in 2019 and in 6 out of the 6 samples (7/36 qPCR replicates < LOD95% and 25/36 qPCR replicates > LOD95%) collected at the station SHE in New-Brunswick in 2021 (Appendix 5, Figure 5).

E. sinensis eDNA detections occurred in September (KAR) and October (SFO, KAM, ANR) (Appendix 6, Figure 6). *E. sinensis* DNA was detected in 2 samples (1/9 qPCR replicates >LOD95%) collected at the station KAR in 2019 and in 1 sample collected at KAM (1/9 qPCR replicates >LOD95%), ANR (1/9 qPCR replicates >LOD95%) and SFO (1/9 qPCR replicates >LOD95%) stations in 2020 (Appendix 6, Figure 6). Stations KAM and KAR belong to the site Kamouraska. *E. sinensis* DNA amplification curves <LOD95% were observed in one sample from CHE and POU (1/9 qPCR replicates) stations in 2019 (Appendix 6, Figure 6). The detection at CHE was considered negative based on the eDNA decision framework flow chart (Figure 4).

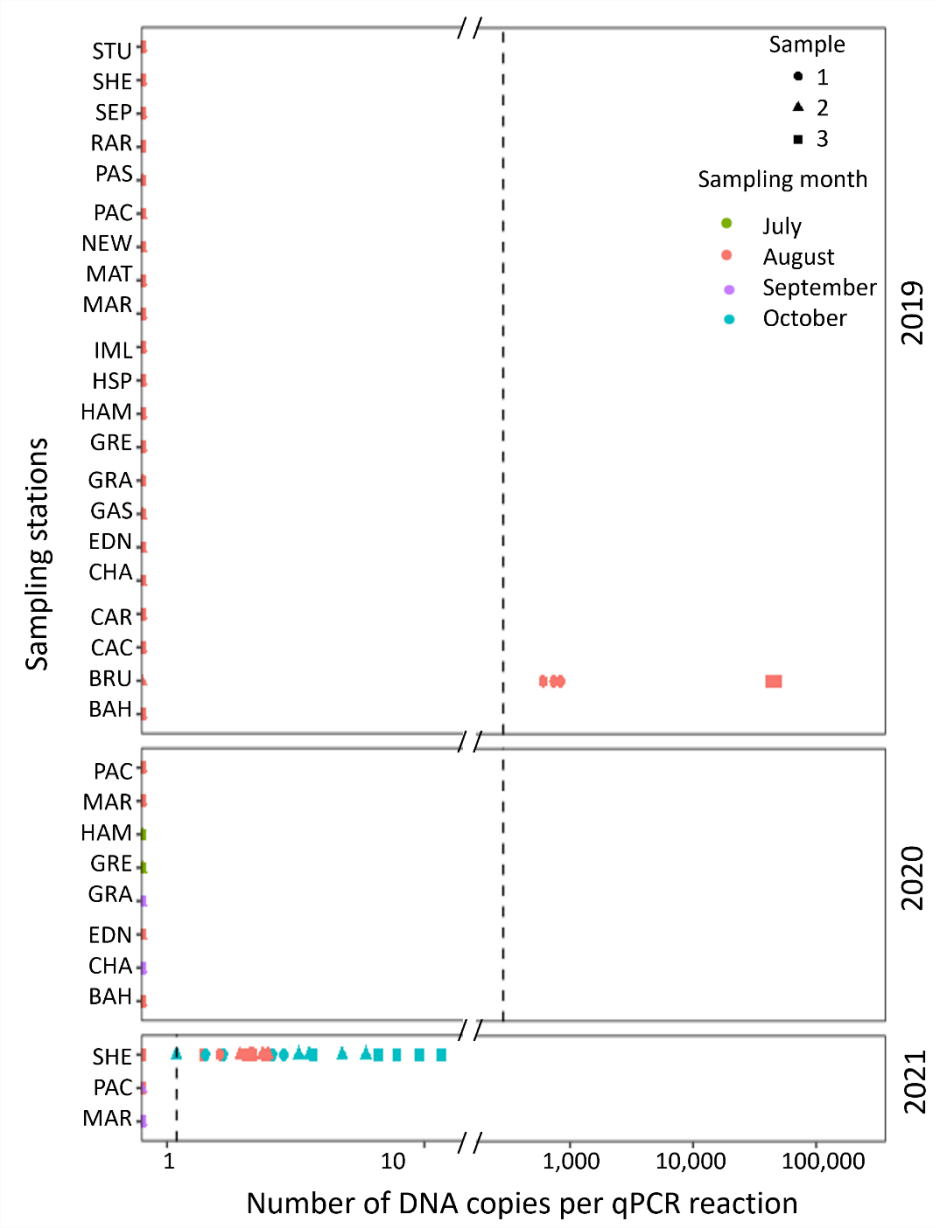

Figure 5: eDNA detection results for the *Carcinus maenas* qPCR assay. Each dot represents a qPCR replicate result. The dashed lines represent LOD95%. Superimposition occurs for negative qPCR detection.

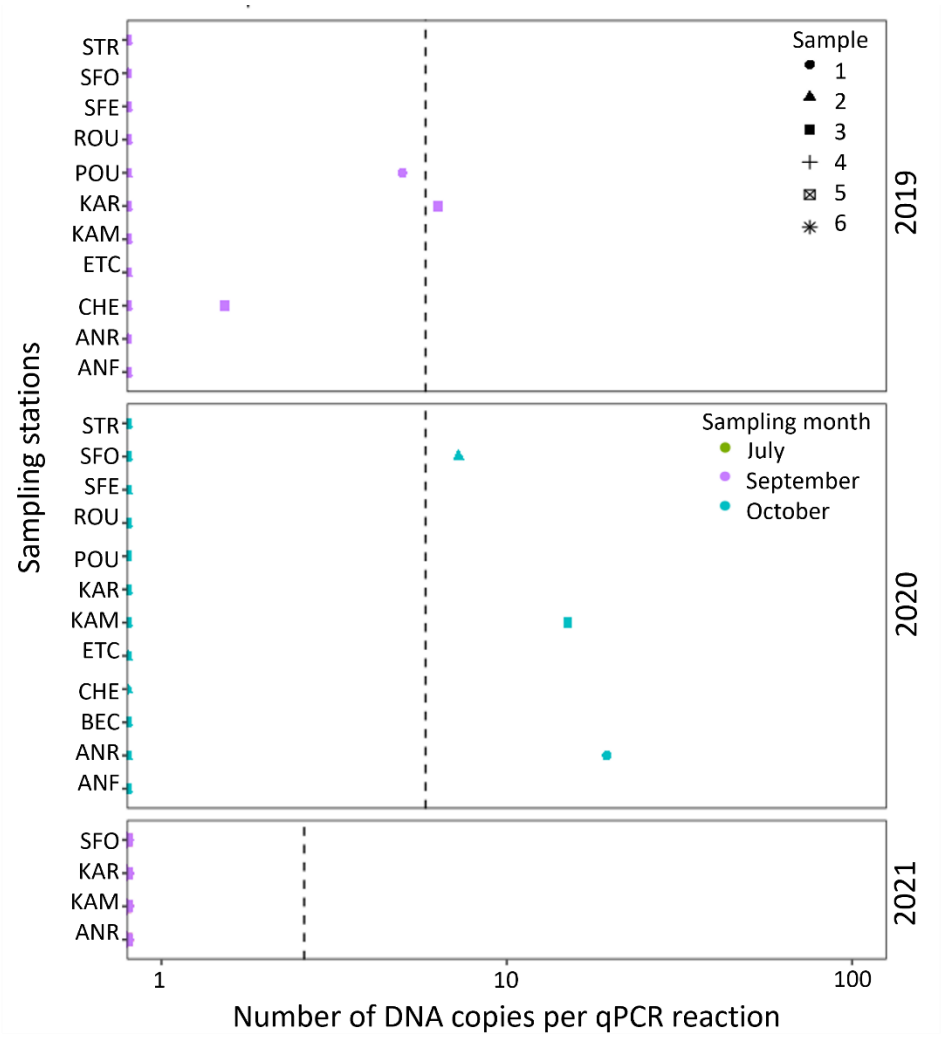

Figure 6: eDNA detection results for the *Eriocheir* qPCR assay. Each dot represents a qPCR replicate result. The dashed lines represent LOD95%. Superimposition occurs for negative qPCR detection.

4. DISCUSSION

This technical report provides an eDNA detection protocol to detect *C. maenas* and *Eriocheir* spp. on the eastern Canada (Atlantic coast and St. Lawrence River). This protocol has been tested in 2 DFO laboratories and showed similar performance for *in vitro* tests for sensitivity (LOD). The detection of both species in areas where the species were present or susceptible to be present provides evidence that those two targeted qPCR assays are reliable for species detections *in situ*. The *E. sinensis* assay has the potential of detecting *E. japonica, E. hepuensis,* and *E. ogasawaraensis*, although test performances for the detection of these species were not determined.

Detection results of eDNA for *C. maenas* and *E. sinensis* provide another piece of information for the management of these AIS. Environmental DNA detections are mostly perceived as important for AIS management (Bernos et al. 2022), being one of the most powerful additions to AIS managers' toolbox (Ricciardi et al. 2017). Some species are monitored with eDNA detection protocols with results guiding decision making, e.g., protected great crested newt in UK (Biggs et al. 2015); invasive bighead and silver carps in USA (Woldt et al. 2020); Asian carp in USA (Jerde et al. 2013); and zebra mussel in USA (Sepulveda et al. 2020).

We detected eDNA from *C. maenas* in sites where traditional surveys also captured specimens in recent years and eDNA from *E. sinensis* in sites where traditional surveys observed specimens more than a decade ago. For *C. maenas*, both eDNA and traditional methods provided similar results whereas for *E. sinensis* only eDNA allowed the detection of that species. Using both methods would improve the AIS surveys at DFO. A recent study showed advantages of using a combination of detection methods, including eDNA, to monitor *C. maenas* at marginal zones of invasion (Keller et al. 2022). On the Pacific coast of the USA, eDNA detection greatly improves the detection of European green crabs at low population densities (~0.05–0.50 crabs/trap), or if low traditional sampling effort is used, when using a joint model with both proxies (Keller et al. 2022). These conditions are commonly found in leading edge locations where AIS spread. The results of this study reinforces the use of eDNA for monitoring *C. maenas* and *E. sinensis*.

4.1 QPCR ASSAYS PERFORMANCE

C. maenas and *E. sinensis* qPCR assays show high specificity and sensitivity to the targeted taxa. We did not observe nonspecific amplification of DNA from crab species that are present on the Atlantic Coast. Our *C. maenas* assay has been validated for Atlantic waters and therefore is optimal for this region. The *C. maenas* and *E. sinensis* assays can detect as little as 1 and 2.5 copies per qPCR reaction, respectively, with a 95% reliability, which is highly sensitive for rare species (Klymus et al. 2019). We observed differences in the copy numbers at LOD95% for the *C. maenas* assay, when we used different starting material. This can be explained by differences in pipetting, and quantification of the starting material. Nonetheless, the Cq at which the LOD95% was obtained using pDNA or gBlock were close (*i.e.*, Cq of 36.87 and 35.01 respectively), which is another indication that the assay was performing the same. The *C. maenas* qPCR assay published for Pacific Ocean waters (Roux et al. 2020) shows a LOD50% of 0.6 copies per qPCR reaction, which is similar to our *C. maenas* assay showing a LOD50% of 0.86 copies per qPCR reaction.

We estimated false positive (type I error) and false negative rates (type II error) by preparing plates with no DNA for qPCR negative control and 3 copies per reaction above the LOD95% for qPCR positive control. We did not detect systematic contamination for both assays in this plate control. We did observe false negative rates higher than 5% for *C. maenas* (9%) and lower than 5% for *E. sinensis* (0%). Those results suggest that the LOD95% may be higher and lower than predicted for *C. maenas* and *E. sinensis* qPCR assays, respectively. Estimations of LOD95% are based on a curve-fitting method (Klymus et al. 2019). A newly developed approach based on a Binomial-Poisson statistical model relating the number of qPCR detected replicates to the copy number (Lesperance et al. 2021) is currently being assessed in our laboratory and appears to estimate the LOD with increased precision.

4.2 EDNA DETECTIONS *IN SITU*

We detected *in situ* eDNA from *C. maenas* and *E. sinensis*. For the European green crab, eDNA detections were consistent with traditional field observations. In Prince Edward Island, a few European green crab exuviae were present on the beach while sampling (BRU and STU stations) and eDNA was detected at the BRU station. Green crab eDNA was detected in 2021 in SHE (New Brunswick) where green crab was initially reported to be present in 2009 and has been known to be present since that time (Shediac Bay Watershed Association, pers. comm.). In the Magdalen Islands, only 1 crab was captured by a fisherman in 2019, 6 days after eDNA sampling at the PAC station. No eDNA was detected at this station.

For *E. sinensis*, no active sampling (using traps) has been done in any of the eDNA sampled sites since 2019. However, few visual observations of *E. sinensis* have previously been reported in rivers where we detected *E. sinensis* eDNA (i.e., 1 crab observed at the Sainte-Angèle-de-Laval site during the fall 2004, 1 crab observed in Lake Saint-Pierre near the Saint-François River in September 2005, 1 crab found in Kamouraska River in July 2007, 1 crab found in the Ouelle River in October 2007, Annex 1, de Lafontaine et al. 2008). The few number of observations could be due to low density or small number of individuals but also to the life stage (*i.e*., larval individuals are difficult to catch). More recently (May 26th, 2020), a female of *E. sinensis* was captured at the mouth of the Bécancour River (MELCCFP, pers. comm.). This site was then added for eDNA monitoring during October 2020 but there were no eDNA detections of *E. sinensis*. A larger sampling effort (*i.e.,* more surveys and more sampling stations) such as a better understanding of *E. sinensis* life cycle and the timing of migration between freshwater to saltwater in the St Lawrence System, would help to reduce the false negatives and inconclusive detections. Detections of *E. sinensis* eDNA provides a new proxy for the species' detection which is paramount since very little visual observations of specimens were made recently.

This technical report also shows that both qPCR assays are validated to level 4 according to the minimum requirements for DFO standards (Abbott et al. 2021). This validation level guarantees that our qPCR assays and eDNA detection protocols went through *in vitro* specificity and sensitivity testing, LOD95% determination and *in situ* testing. The annual *in situ* testing since 2019 allowed us to make several improvements in the eDNA detection protocol steps such as the filtration and extraction steps. Given that a sufficient sampling effort is made, the level 4 validation ensures the reliability of results: when eDNA from a target species is not detected, the target is likely absent, whereas detection of eDNA indicates that the target is very likely present (Abbott et al. 2021; Thalinger et al. 2021).

Both species were detected at low DNA copy number per reaction (*C. maenas*: 1.41 to 835.59; *E. sinensis*: 1.52 to 19.47), except for a sample from Prince Edward Island reaching more than 40,000 *C. maenas* DNA copies per reaction. Those low copy numbers and the false negatives present a challenge for eDNA detection of crabs. Adams et al. (2019) presented a "shedding hypothesis", where animals with hard integument (*e.g.,* chitinous, keratinized) do not shed as much DNA as mucus-covered organisms such as fish. This hypothesis is based on laboratory-controlled experiments on eDNA shedding and decay. In marine controlled environments, *C. maenas* eDNA concentration was about an order of magnitude lower than that of shanny fish eDNA (*Lipophrys pholis*) (Collins et al. 2018). The composition of their exoskeleton, their behaviour and breeding condition may also limit eDNA detection at particular times of the year (Collins et al. 2018).

The presence of DNA detections across temporal surveys increases the reliability of eDNA results by reducing the risk of detecting DNA from contamination, dead organisms, or transported from other areas (Abbott et al. 2021). Furthermore, collecting eDNA in multiple surveys at different times of the year increases the detection power of an eDNA assay because of temporal variability in eDNA shedding rates (e.g., during moulting or spawning), transport (e.g., with tides and freshwater input), and degradation within the same ecosystem for a given species (Buxton et al. 2017; Wacker et al. 2019; Jia et al. 2020).

Very little is known about the temporal variation in eDNA concentration for *E. sinensis,* but it has been studied for *C. maenas* in controlled experiments. Soft-shelled, ovigerous, and non-ovigerous females and males, as well as hard-shelled *C. maenas* were compared to understand the effect of life stages in eDNA detections (Crane et al. 2021). During most of their life cycle, *C. maenas* shed low levels of DNA except when ovigerous (Crane et al. 2021). Contrary to expectations, eDNA detection rates were not greater for soft- versus hard-shelled crabs leading the authors to suggest that the effects of crab activity and carapace hardness should be studied together. Based on this study, European green crabs may be easier to detect during fall and winter in the Estuary and Gulf of St. Lawrence, when crabs are ovigerous. For *E. sinensis*, the combination of having a catadromous species with limited shedding during summer (non ovigerous females) could limit detections. As *E. sinensis* move to saltwater to spawn, targeting fall (our results show eDNA detections in September and October) or spring for water sampling in the estuarine portions of the rivers could help to detect *E. sinensis* eDNA. Based on Eberhardt et al. (2016), the best time to observe *E. sinensis* is during the adult migration from freshwater into the estuary (fall) or the juvenile migration upstream to freshwater in late summer/early fall (Rudnick et al. 2005). Consequently, we assume that the probability to detect eDNA should be higher in estuaries during these periods.

4.3 FUTURE RESEARCH NEEDS AND GOALS

A limitation of eDNA detection is the lack of abundance quantification, i.e., results generally only indicate the presence/absence of species eDNA. A recent study with *C. maenas* showed no correlations between eDNA concentrations and biomass for this species (Danziger et al. 2022). However, correlations between eDNA quantification and vertebrate species biomass have been established, especially in experimental conditions (Thomsen et al. 2012; Takahara et al. 2012; Pilliod et al. 2013; Kelly et al. 2014; Klymus et al. 2015; Lacoursière-Roussel et al. 2016a; Doi et al. 2017). In environmental conditions, eDNA concentrations account for 50% of the observed variation in abundance suggesting that variability depend on collection protocols and environmental factors (Lacoursière-Roussel et al. 2016b; Yates et al. 2019).

The multiplexing of qPCR assays would allow the detection of the target and the IPC in the same qPCR reaction which conduct to a gain in reaction components and time.

Real-time detection of eDNA might be desirable and valuable in some specific circumstances. The use of portable qPCR devices (e.g., Franklin™ Real-Time PCR Thermocycler, Biomeme Inc., Philadephia, PA, USA) should be explored. Although the technology is similar, validation and comparability studies will be required to implement this real-time equipment. Tests for onsite detections are undertaken at the moment by multiple teams across DFO regions.

5. CONCLUSION

There is an increased interest to include eDNA for European green crab and Chinese mitten crab as part of the AIS monitoring program in DFO. This tool has a great potential for both scientists and managers, to enable expansion of monitoring efforts and prevention measures, or to develop response planning. For both species, eDNA monitoring on a larger scale along the East coast of Canada could provide an effective way to detect these AIS when they are in low abundance, such as at the onset of an invasion. Early detection may increase the ability to maintain ecosystem integrity (because they cause damage to habitat and compete with native species) which may ultimately preserve the integrity of the species communities in Marine Conservation Areas. It would however require that permanent resources be allocated to pursue eDNA monitoring of invasive species.

6. ACKNOWLEDGEMENTS

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8. APPENDICES

Appendix 1. Procedures for contamination prevention

Water collection and filtration in the field or at the Maurice Lamontagne Institute (MLI, Fisheries and Oceans Canada)

Water collection for eDNA was done before any other task when collection occurred in the field. Persons responsible of the water collection are trained by eDNA professional users before fieldwork. Any equipment contacting water before and after sampling (e.g., boots) is decontaminated using a 0.3% sodium hypochlorite solution. Water was collected by submerging bottles, oriented against the current, while wearing disposable gloves. A sampling negative control (SNC) is collected by transferring Milli-Q® water (MilliporeSigma, Darmstadt, Germany) in the field (when filtration occurred at MLI) or by filtering Milli-Q® water in the field. When sampling and filtration were done with an eDNA filter pack (Smith-Root, Vancouver, USA) connected to an eDNA sampler, only a SNC was processed (no filtration negative control, FNC).

MLI ultraclean eDNA laboratory

Samples were processed in an ultraclean laboratory dedicated to eDNA, located at the MLI. The laboratory consists of a special airlock system (SAS) room and three main rooms, namely a “filtration”, an “extraction” and a “PCR preparation” room. Filtered air (HEPA, high-efficiency particulate air) from the outside feeds independently in the three main rooms which then feeds into the SAS room due to positive air pressure. All laboratory users are trained to work in clean conditions according to a standard operating procedure for eDNA general procedures. These procedures include specific instructions about when to wear and change sterile gloves, coats, mobcaps, surgical masks, and overshoe protection. Rooms are also decontaminated between projects or every week with a 0.3% sodium hypochlorite solution. All the material, consumables and samples entering the three rooms are cleaned in the SAS room with 0.3% sodium hypochlorite solution.

Filtration and DNA extraction

Work surfaces, equipment, supplies and samples containers were cleaned with a 0.3% sodium hypochlorite solution prior to sample processing. Water samples were first treated in the filtration room. To detect potential contamination occurring during water filtration. A filtration negative control (FNC) was processed by filtering 2L of Milli-Q® water using the same protocols used for eDNA water samples. In the extraction room, sample cross-contamination was controlled by using a microcentrifuge tube opener, and by having a single sample tube open at a time. To detect potential contamination occurring during DNA extraction, an extraction negative control (ENC) (filter with Milli-Q® water) was processed each extraction day using the same protocols used for eDNA filter samples.

qPCR detection

qPCR plates were prepared in the “PCR preparation room” in a laminar flow hood which is decontaminated with a 0.3% sodium hypochlorite solution between uses. All reagents, except eDNA extracts, were first mixed in one sterile reservoir and then dispensed into the plate wells using a multichannel pipette (Eppendorf™, Hamburg, Germany) to minimize variation between qPCR replicates (Ellison et al. 2006). All liquid handling for qPCR used DNA low binding tubes

and aerosol barrier pipette tips. The plate containing the master mix was then moved to the bench to limit contamination of the hood. The eDNA template, qPCR negative control (QNC, PCR grade water), and positive control (QPC, synthetic sequence of the species of interest with 6 extra nucleotides to identify putative laboratory contamination with the QPC) were then added, and the sealed qPCR plates were carried from the preparation room to the general genomics laboratory for amplification.

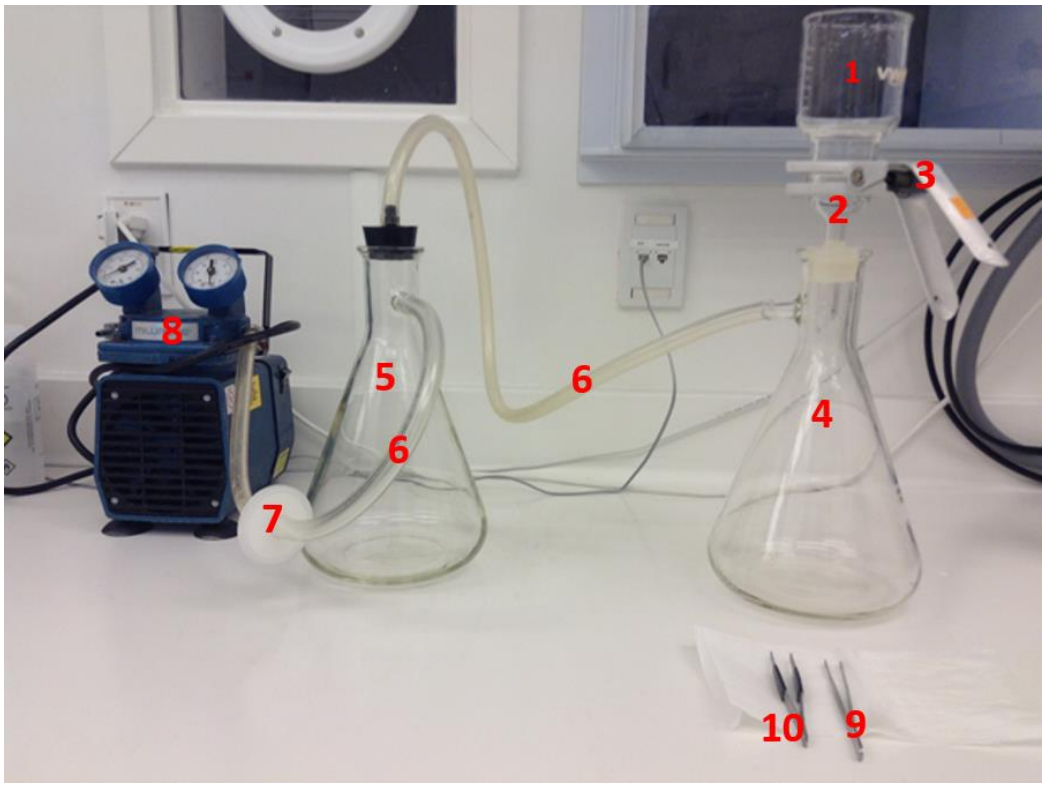
Appendix 2. Sampling sites and GPS coordinates (WGS 84) for *Carcinus maenas*.

Site	Station	Longitude	Latitude
Magdalen Islands	Havre-de-la-Grande- Entrée (GRE)	W 61° 33’ 21.96’’	N 47° 33’ 12.672’’
	Bassin aux Huîtres (BAH)	W 61° 30’ 21.347’’	N 47° 33’ 32.688’’
	Lagoon La Cuesta (HAM)	W 61° 50’ 45.888’’	N 47° 23’ 55.86’’
	Étang-du-Nord (EDN)	W 61° 57’ 35.028’’	N 47° 21’ 54.18’’
	Baie de Bassin - Marichite (MAR)	W 61° 54’ 7.776’’	N 47° 13’ 43.428’’
	Pointe-aux-Canots (PAC)	W 61° 54’ 9.359’’	N 47° 13’ 45.876’’
Prince Edward Island	Brudenell (BRU)	W 62° 35’ 13.235’’	N 46° 12’ 0.072’’
	Sturgeon wharf (STU)	W 62° 31’ 52.571’’	N 46° 7’ 22.656’’
New Brunswick	Shediac (SHE)	W 64° 34’ 30.18’’	N 46° 16’ 23.16’’
Gaspé Peninsula	Carleton (CAR)	W 66° 7’ 40.188’’	N 48° 6’ 0.0’’
	Paspébiac (PAS)	W 65° 15’ 23.58’’	N 48° 1’ 16.248’’
	Newport (NEW)	W 64° 43’ 17.759’’	N 48° 17’ 11.4’’
	Chandler (CHA)	W 64° 40’ 16.967’’	N 48° 20’ 45.384’’
	Grande-Rivière (GRA)	W 64° 29’ 41.603’’	N 48° 23’ 40.728’’
	Gaspé (GAS)	W 64° 28’ 33.96’’	N 48° 49’ 39.144’’
	Rivière-au-Renard (RAR)	W 64° 23’ 31.596’’	N 48° 59’ 51.576’’
	Cap-Chat (CAC)	W 66° 41’ 19.068’’	N 49° 5’ 55.716’’
	Matane (MAT)	W 67° 31’ 48.972’’	N 48° 51’ 14.832’’
	Institut Maurice Lamontagne (IML)	W 68° 9’ 49.32’’	N 48° 38’ 28.104’’
North Shore	Sept-îles (SEP)	W 66° 23’ 12.803’’	N 50° 12’ 11.7’’
	Havre-Saint-Pierre (HSP)	W 63° 36’ 18.827’’	N 50° 14’ 12.912’’

Appendix 3. Sampling sites and GPS coordinates (WGS 84) for *Eriocheir sinensis*.

Site	Station	Longitude	Latitude
Kamouraska	KAM	W 69° 51’ 23.266’’	N 47° 34’ 42.553’’
	KAR	W 69° 51’ 14.331’’	N 47° 34’ 19.067’’
Saint-Romuald	ETC	W 71° 13’ 52.96’’	N 46° 45’ 36.119’’
	STR	W 71° 13’ 10.772’’	N 46° 46’ 3.886’’
Rivière du Chêne	CHE	W 71° 59’ 54.488’’	N 46° 34’ 25.943’’
Sainte-Angèle-de-Laval	ANF	W 72° 32’ 34.742’’	N 46° 18’ 45.994’’
	ANR	W 72° 31’ 54.454’’	N 46° 18’ 6.365’’
Saint-François	SFE	W 72° 52’ 41.52’’	N 46° 7’ 34.403’’
	SFO	W 72° 54’ 24.93’’	N 46° 6’ 2.344’’
Bécancour	BEC	W 72° 26’ 9.276’’	N 46° 22’ 39.036’’
Rivière Ouelle	POU	W 70° 3’ 5.479’’	N 47° 26’ 1.028’’
	ROU	W 70° 2’ 36.362’’	N 47° 25’ 38.996’’

Appendix 4. Vacuum pump filtering system. 1: Filtration funnel, 2: Glass filter holder, 3: Pliers for tightening, 4: filtration flask, 5: back-up flask, 6: tubing, 7: back-up filter, 8: Vacuum pump, 9-10: Pliers for filter.



Appendix 5. eDNA detections of *Carcinus maenas. C. maenas DNA* confirmed by DNA sequencing (in bold). a = number of qPCR replicates >LOD95%, b = number of qPCR replicates <LOD95%, c = number of positive samples, d = total number of samples, e = total number of qPCR replicates.

Station	Year	Survey	a/e	b/e	c/d	Specimen observation	Researcher's finding at the station level
GRE	2019	Summer	0/9	0/9	0/3		Negative
	2020	July	0/9	0/9	0/3		
BAH	2019	Summer	0/9	0/9	0/3		Negative
	2020	July	0/9	0/9	0/3		
HAM	2019	Summer	0/9	0/9	0/3		Negative
	2020	July	0/9	0/9	0/3		
EDN	2019	Summer	0/9	0/9	0/3		Negative
	2020	July	0/9	0/9	0/3		
MAR	2019	Summer	0/9	0/9	0/3		Negative
	2020	August	0/9	0/9	0/3		
	2021	August	0/18	0/18	0/3		
		September	0/18	0/18	0/3		
PAC	2019	Summer	0/9	0/9	0/3	1 captured 6 days after sampling	Negative
	2020	August	0/9	0/9	0/3		
	2021	August	0/18	0/18	0/3		
		September	0/18	0/18	0/3		
BRU	2019	Summer	**6/9**	0/9	**2/3**	Summer (2 exuviae on the beach)	Positive
STU	2019	Summer	0/9	0/9	0/3	Summer (some exuviae on the beach)	Negative
SHE	2019	Summer	0/9	0/9	0/3		
	2021	August	**10/18**	3/18	**3/3**	September	Positive
		October	**15/18**	4/18	**3/3**	September	
CAR	2019	Summer	0/9	0/9	0/3		Negative
PAS	2019	Summer	0/9	0/9	0/3		Negative
NEW	2019	Summer	0/9	0/9	0/3		Negative
CHA	2019	Summer	0/9	0/9	0/3		Negative
	2020	September	0/9	0/9	0/3		
GRA	2019	Summer	0/9	0/9	0/3		Negative
	2020	September	0/9	0/9	0/3		
GAS	2019	Summer	0/9	0/9	0/3		Negative
RAR	2019	Summer	0/9	0/9	0/3		Negative
CAC	2019	Summer	0/9	0/9	0/3		Negative
MAT	2019	Summer	0/9	0/9	0/3		Negative
IML	2019	Summer	0/9	0/9	0/3		Negative
SEP	2019	Summer	0/9	0/9	0/3		Negative
HSP	2019	Summer	0/9	0/9	0/3		Negative

Appendix 6. eDNA detections of *Eriocheir sinensis. E. sinensis DNA* confirmed by DNA sequencing (in bold). a = number of qPCR replicates >LOD95%, b = number of qPCR replicates <LOD95%, c = number of positive samples, d = total number of samples, e = total number of qPCR replicates.

Station	Year	Survey	a/e	b/e	c/d	Specimen observation	Researcher's finding at the station level
KAM	2019	September	0/9	0/9	0/3		Positive
	2020	July	0/9	0/9	0/3		
		October	**1/9**	0/9	**1/3**		
	2021	August	0/36	0/36	0/6		
		September	0/36	0/36	0/6		
KAR	2019	September	**1/9**	0/9	**1/3**		Positive
	2020	July	0/9	0/9	0/3		
		October	0/9	0/9	0/3		
	2021	August	0/36	0/36	0/6		
		September	0/36	0/36	0/6		
ETC	2019	September	0/9	0/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
STR	2019	September	0/9	0/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
CHE	2019	September	0/9	1/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
ANF	2019	September	0/9	0/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
ANR	2019	September	0/9	0/9	0/3		Positive
	2020	October	**1/9**	0/9	**1/3**		
	2021	August	0/36	0/36	0/3		
		September	0/36	0/36	0/3		
SFE	2019	September	0/9	0/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
SFO	2019	September	0/9	0/9	0/3		Positive
	2020	October	**1/9**	0/9	**1/3**		
	2021	August	0/36	0/36	0/3		
		September	0/36	0/36	0/3		
BEC	2020	October	0/9	0/9	0/3	1 specimen found in May, 26th	Negative
POU	2019	September	0/9	1/9	0/3		Negative
	2020	October	0/9	0/9	0/3		
ROU	2019	September	0/9	0/9	0/3		Negative
	2020	October	0/9	0/9	0/3		

Appendix 7. Sample arrangement on a qPCR plate