Summary: This technical report presents the results of a Quantitative PCR method developed for the identification of the Aquatic Invasive Specie (AIS) *Dreissena polymorpha.* The method was developed using primers that have been already tested and validated in similar studies and adapted to a different amplification detection conditions and reagents. The report shows that the analysis of environmental samples using our eDNA amplification method provides a specific and sensitive technology for the detection of *D. polymorpha* DNA in water samples collected and preserved in 70%ethanol.

eDNA (AIS) Dreissena sp. detection

At the Enviro-Responsible eDNA Laboratory we analyze environmental samples using a genetic target approach to identify the invasive species *Dreissena polymorpha*. Results are classified at **LEVEL 3 (Essential)** according to the 5-level validation scale proposed by *Thalinger et al. 2021* to aid in evaluating eDNA assays and appropriate interpretation of results. The analysis method is based on a Quantitative Polymeric Chain Reaction (qPCR) using a set of amplification primers developed by *Williams et.al., 2017* for the identification of *Dreissena polymorpha* in environmental samples. This laboratory intends to continue improving the service by reaching an Operational Level 5 and extending its service to identify other invasive species.

The entire methodology follows the guidelines on using targeted eDNA samples to identify invasive species in the laboratory proposed by the Department of Fisheries and Oceans of Canada (DFO) as indicated in the table below.

Method internal validation (LEVEL 1 - LEVEL 2)

Validation Level	Variable blocks	Minimum criteria
Level 1 Assay designed	in silico analysis of PCR primer (and probe) sequences for the target species.	target species *Dreissena polymorpha* *Dreissena bugensis*
Level 2 Assay optimization	Comprehensive reporting of PCR conditions. Calibration curve for both species	DNA extract volume in PCR positive samples confirmed by sequencing analysis of the amplification product.
	in vitro testing on closely related non-target species	any in vitro non-target testing

William et. al, 2021 designed primers for qPCR detection as part of a Loop-Mediated Isothermal Amplification method (LAMP). For our method, we selected the same primers flanking the amplicon of the bivalve small subunit ribosomal RNA (18s), and the Cytochrome Oxidase I (COI) in the target species and adapted them to a quantitative PCR (qPCR) using SYBR fluorescence detection system (SYBR with a HotStart Taq DNA polymerase). Amplification for both genes was confirmed by DNA 1.5% Agarose gel electrophoresis.

Primer pair 1 *Dreissena sp.*/ 18S rRNA

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self complementarity	Self 3' complementarity
Forward primer F3	GTTAGCCCAGACCAACGC	Plus	18	152	169	58.43	61.11	4.00	2.00
Reverse primer B3	CTTCCTTGGATGTGGTAGCC	Minus	20	378	359	57.96	55.00	4.00	2.00
Product length	227								

Control target

>AF305702.1:152-378 *Dreissena polymorpha* 18S ribosomal RNA gene, partial sequence
GTTAGCCCAGACCAACGCGGTCGCCGCAAGGCGGCCGCAACTCGTGGTGACTCTGGACAACTTTGTGCCG
ATCGCACGGCCTCGCGCCGGCGACGTATCTTTCAAGTGTCTGCCCTATCAACTGTCGATGGTACGTGCTA
TGCCTACCATGGTGATAACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCATGAGACACGGC
TACCACATCCAAGGAAG

Primer pair 1 *Dreissena polymorpha*/ cytochrome c oxidase (COI)

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self complementarity	Self 3' complementarity
Forward primer	TAATGGGGGGATTCGGAA	Plus	18	182	199	54.67	50.00	5.00	5.00
Reverse primer	GCTCCCCCAATATGAAGAG	Minus	19	425	407	54.69	52.63	4.00	2.00
Product length	244								

Control target

>AF120663.1:182-425 *Dreissena polymorpha* cytochrome oxidase 1 (COI) gene, partial cds; mitochondrial gene for mitochondrial product
TAATGGGGGGATTCGGAAATTGATTGGTACCAATAATACTGAGTCTTCCTGATATAGGTTTCCCTCGTCT
TAATAATGTTAGTTTTTGGGTTTTACCTGTCTCTATAGGACTTCTATTTTGTTCAGCTTTTAGGGAAGGA
GGATTCGGGGGTGGTTGAACCTTATATCCTCCTTTATCTAGAGTTATAGGACATTCAGGCCCTGCGATAG
ATTTTTTGATTTTATCTCTTCATATTGGGGGAGC

Quantitative PCR (qPCR) amplification reactions were completed using a Strategene 3005P qPCR thermocycler and SYBR Master Mix following the manufacturer's recommendations for the preparation of the reaction mix. Each run consisted of an activation step at 95°C for 10 min and 40 amplification cycles (95°C-30sec, 58°C-60°C-30sec, 72°C -30sec) followed by a dissociation profile to detect the target's dissociation temperature (Tm).

Samples that test positive for D. polymorpha COI show Cq values between 28 and 36 amplification cycles and a single peak in the dissociation profile with a Tm between 68 and 70°C. Samples testing positive also show a positive result for the 18s with Cq values between 26 and 31 amplification cycles and dissociation profile with a double peak between 60°C and 80°C.

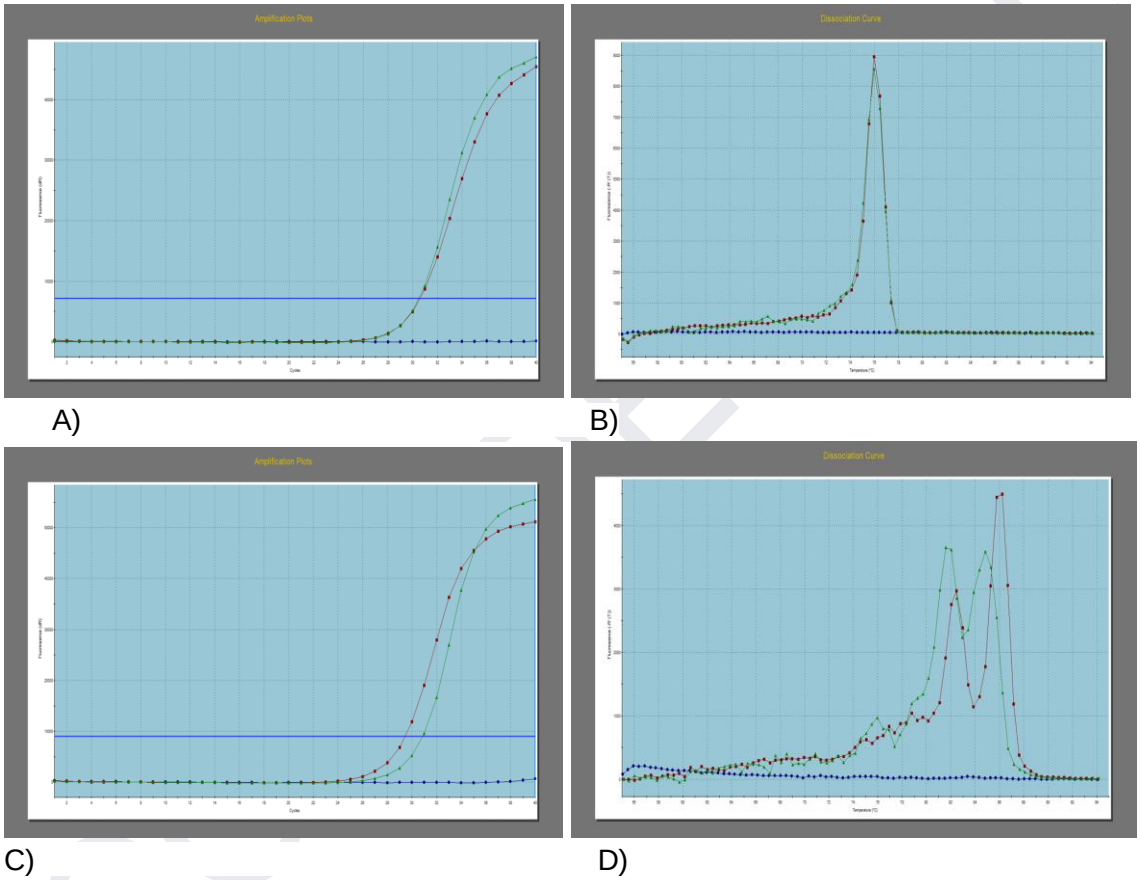
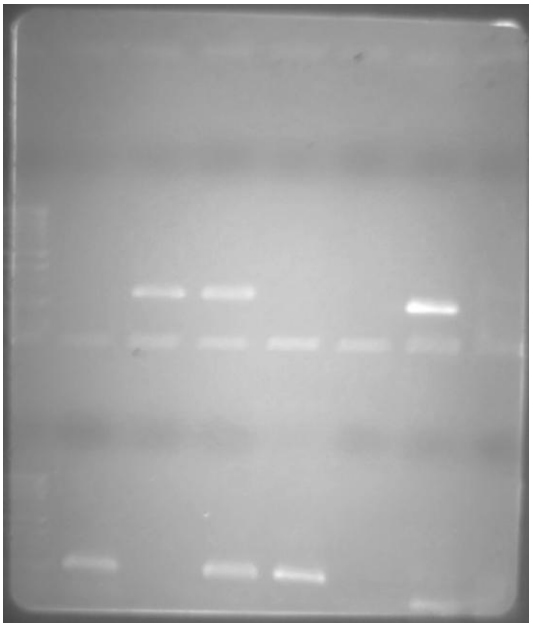


Fig 1 Amplification plots and dissociation profiles for the COI and 18s DNA segments using the primer pairs developed by William et. al, 2021. A) Amplification of D. polymorpha COI in eDNA samples V46 and V47 and NTC, B) Dissociation profile for the COI target in samples V46 and V47 and NTC; C) Amplification of the bivalve small subunit ribosomal RNA (18s) in eDNA samples V46 and V47 and NTC; D) Dissociation profile for the 18s target in samples V46 and V47 and NTC

Two qPCR-positive samples (V46 and V47) were run in 1.5% agarose gel in TAE buffer at 100mV to verify the purity of the amplification product. The size of the amplicon was estimated by comparing the results to a 100bp DNA ladder.

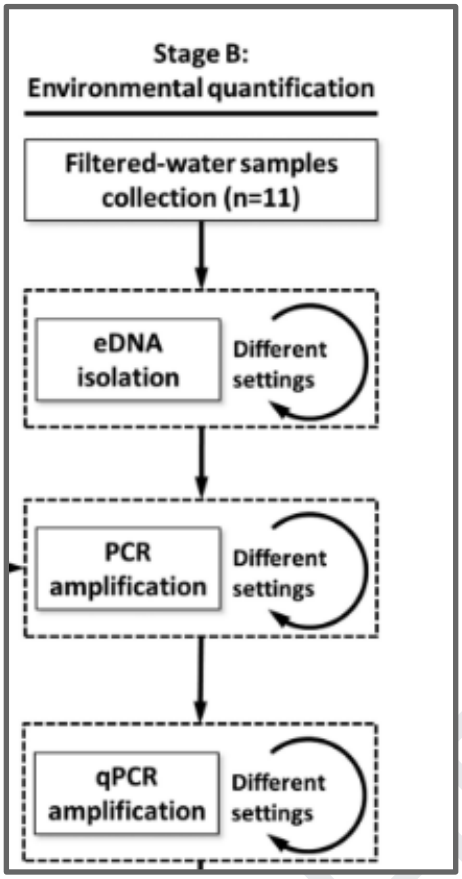


Top comb
Lane 1 - 100bp ladder
Lane 2- NTC for DP-COI primers
Lane 3 - Sample V46 (*D.polymorpha*)
Lane 4 - Sample V47 (*D.polymorpha*)
Lane 5 - Sample V80 (Unknown)
Lane 6 - NTC for 18s primers
Lane 7 - Sample V46
Lane 8 - Simple V80
Bottom comb
Lane 1 - Sample V47
Lane 2- NTC for *Bythotrophes*-COI primers
Lane 3 - Sample V4 (*Bytothrophes*)
Lane 4 - Sample V5 (*Bytothrophes*)
Lane 5 - NTC for 18s primers
Lane 6 - V4
Lane 7 - V5
Lane 8 - empty

Fig1. Electrophoresis of qPCR amplification products in 1.5% agarose.

Single bands were observed for both *D polymorpha* COI and 18s genes with sizes around 200 bp with no amplifications in the non-template control samples (NTC). These results were expected since both primers amplify segments of 244bp for the COI and 227 bp for the 18s.

The *D. polymorpha* **COI** primers were tested on 29 environmental samples collected from different sites in 2024 for environmental monitoring of invasive species.
All samples were preserved in 70% Ethanol and processed for eDNA extraction using the workflow shown below.



Samples filtration - Environmental samples were passed through a 26 mm pore-size paper filter using a vacuum filtration system with a manifold to collect any free DNA in the sample. Filters were stored at -20 Celsius degrees until processing.

eDNA extraction - Extraction of eDNA from the filters was done using the DNeasy PowerWater Kit. This kit isolates genomic DNA from filtered samples, free from salts, metals, humic substances and other organic materials.

eDNA concentration- The concentration of the eDNA in the solution after purification was measured as dsDNA OD260nm and purity as the ratio OD260/OD280 using a NanoDrop instrument.

qPCR amplification - The presence of the target invasive species was detected by running eDNA samples in duplicate in the qPCR reaction mix with *D. polymorha* COI primers and the same amplification conditions explained before. Data from the qPCR machine was processed using the Instrument software to establish the cut-off value (Cq) and Tm. A positive sample was considered as one that shows a Cq value, and the Tm is between 65-70°C in at least one of the replicates.

The analysis showed the presence of *D.polymorpha* DNA in 22 of the 29 locations (second call out) confirming 3 out of 4 sites reported for AIS for a 75% specificity.

Number of sites	Positive	Negative
AIS reported	4	25
qPCR	3	7

The average Cq value was 33 +/- 3 cycles with Tm 69.4°C +/-2.8°C.

References

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5643059/
Williams, M. R., Stedtfeld, R. D., Engle, C., Salach, P., Fakher, U., Stedtfeld, T., Dreelin, E., Stevenson, R. J., Latimore, J., & Hashsham, S. A. (2017). Isothermal amplification of environmental DNA (eDNA) for direct field-based monitoring and laboratory confirmation of *Dreissena sp*. PloS one, 12(10), e0186462. https://doi.org/10.1371/journal.pone.0186462

Abbott, C., Coulson, M., Gagné, N., Lacoursière-Roussel, A., Parent, G.J., Bajno, R., Dietrich, C., May-McNally, S. 2021. Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk. DFO Can. Sci. Advis. Sec. Res. Doc. 2021/019. iv + 42 p.