Title: *Retrospective Molecular Detection of Aquatic Invasive Species (AIS) from Ethanol-Preserved Plankton Samples*

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Abstract

Monitoring programs for aquatic invasive species (AIS), particularly for *Dreissena polymorpha* (zebra mussels) and *Bythotrephes spp.* (spiny waterflea), commonly rely on microscopy-based identification of preserved plankton samples. Although effective for confirming the presence of these organisms, microscopy may underestimate low-density established populations and is limited by sample processing constraints and taxonomic expertise. Molecular approaches such as polymerase chain reaction (PCR) can detect species-specific DNA signals and may provide complementary information for AIS surveillance. This study evaluated the feasibility of repurposing ethanol-preserved plankton samples for retrospective molecular detection of AIS. A total of 89 plankton samples collected from 21 Manitoba lakes between 2022 and 2024 were analyzed using microscopy and PCR assays targeting *Bythotrephes spp*. and *D. polymorpha*. Microscopy identified *Bythotrephes spp*. in 41 samples and *D. polymorpha* in 12 samples. PCR detected *Bythotrephes spp*. DNA in 49 samples, including 20 samples that were negative by microscopy. Agreement between microscopy and PCR for *Bythotrephes spp*. was fair (κ = 0.29), reflecting differences in the biological targets detected by the two methods. For *D. polymorpha*, PCR detected DNA in 55 samples. Late amplification (Ct >35) was also observed in filtration blank controls, whereas no-template controls remained consistently negative and filtration blanks contained no measurable DNA by fluorometric quantification. Because these findings could reflect either trace DNA below the fluorometric detection limit or late-cycle assay background amplification, a conservative Ct threshold (≤35) was applied, resulting in 34 samples being classified as positive. Agreement between microscopy and PCR was slight (κ = 0.19), and *D. polymorpha* detections should therefore be interpreted cautiously pending independent confirmation. These findings demonstrate that ethanol-preserved plankton samples can provide useful molecular information for retrospective AIS surveillance. The integration of microscopy and molecular approaches may enhance monitoring programs, although independent confirmation methods remain essential for reliable interpretation of molecular detections

Introduction

The objective of this study was to evaluate whether ethanol-preserved plankton samples could be repurposed for the molecular detection of AIS using DNA amplification reactions. We compared molecular detection outcomes with microscopy observations and assessed the extent of agreement between methods while recognizing that each approach measures different biological targets.

Methods

A total of 89 plankton samples collected from 21 lakes in Manitoba, Canada, between 2022 and 2024 as part of the annual routine AIS monitoring program were utilized in this study. Samples were originally preserved in 70% ethanol and stored at uncontrolled ambient temperature.

PCR assays

Species-specific target DNAs were amplified using polymerase chain reaction (PCR) assays employing primers targeting the cytochrome c oxidase subunit I (COI) gene of *Dreissena polymorpha*, and *Bythotrephes spp.* (Table 1). Primer sequences used in our study were taken from two original papers and optimized for two different amplification methods; SYBR Green, and hydrolysis probe-based PCR detections. Hydrolysis probes were designed using IDT PrimerQuest Tool in combination with the forward and reverse primers and template sequences for each species (accession numbers in Table 1).

Target Species	Primer ID	Primer sequence	Reference
Bythotrephes spp. **/cytochrome c oxidase COI** **AF435122**	Bytho-F	GCAGGAACTGGCTGAACA	Walsh et.al., 2019
	Bytho-R	AATAATAAAAGGAGGGCTGTAATACC	
	Internal probe	/5Cy3/AC TTT CTG CAG GTA TTG CTC ATG CAG GA/3IAbRQSp/	
Dreissena polymorpha **/cytochrome c oxidase COI** **Accession #: AF120663**	COI-DP -F3	TAATGGGGGGATTCGGAA	Williams et al. 2017
	COI-DP -B3	GCTCCCCCAATATGAAGAG	
	Internal probe	/5TET/TG GGT TTT A/ZEN/C CTG TCT CTA TAG GA/3IABkFQ/	

Table 1. Species-specific primers and hydrolysis probes used for the detection of *Dreissena polymorpha* (zebra mussel), and *Bythotrephes* spp. (spiny water flea). Primer and probe

Assay Verification

PCR amplification specificity and efficiency were evaluated through ten-fold serial dilutions of DNA extracted from confirmed specimens of *D. polymorpha* and *Bythotrephes spp*. obtained from field samples. Amplification reactions were completed using the Multiplex hydrolysis probe-based assays (PrimeTime™ Gene Expression Master Mix, Integrated DNA Technologies, Coralville, IA, USA). PCR products were verified by electrophoresis on 1.5% agarose gels prepared in 1× TAE buffer and run at 100 V. Amplicon sizes were checked using a 100-bp DNA ladder and compared with expected product sizes by in silico analysis of primers pairs.

Results

Preserved DNA

DNA was successfully recovered from all preserved plankton samples following filtration and extraction (Table. 2). Qubit fluorometric quantification indicated generally low DNA yields, with concentrations ranging from 0 to 62.57 ng μL^{-1} (mean ± SD = 1.85 ± 7.28 ng μL^{-1}; median = 0.125 ng μL^{-1}).

Species	Microscopy/PCR	Microscopy Positive	Microscopy Negative	Total
Bythotrephes sp	PCR Positive	29	20	49
	PCR Negative	12	28	40
Total		**41**	**48**	**89**
D. Polymorpha	PCR Positive	8	26	34
	PCR Negative	4	51	55
Total		**12**	**77**	**89**

Table 2. Comparison of microscopy and PCR detection results for *Bythotrephes* spp. and *Dreissena polymorpha* in preserved samples. Percentage agreement was calculated as the proportion of PCR results matching microscopy results within each category

PCR verification

The analysis of serial dilution of DNA extracted from confirmed specimens demonstrated strong and consistent amplification performance across a 10,000-fold dilution range for the selected pair of primers and fluorescent probes. The cycle threshold (Ct) values increased progressively with decreasing template concentration, showing the expected logarithmic amplification behavior characteristic of an efficient assay (Fig. 1).

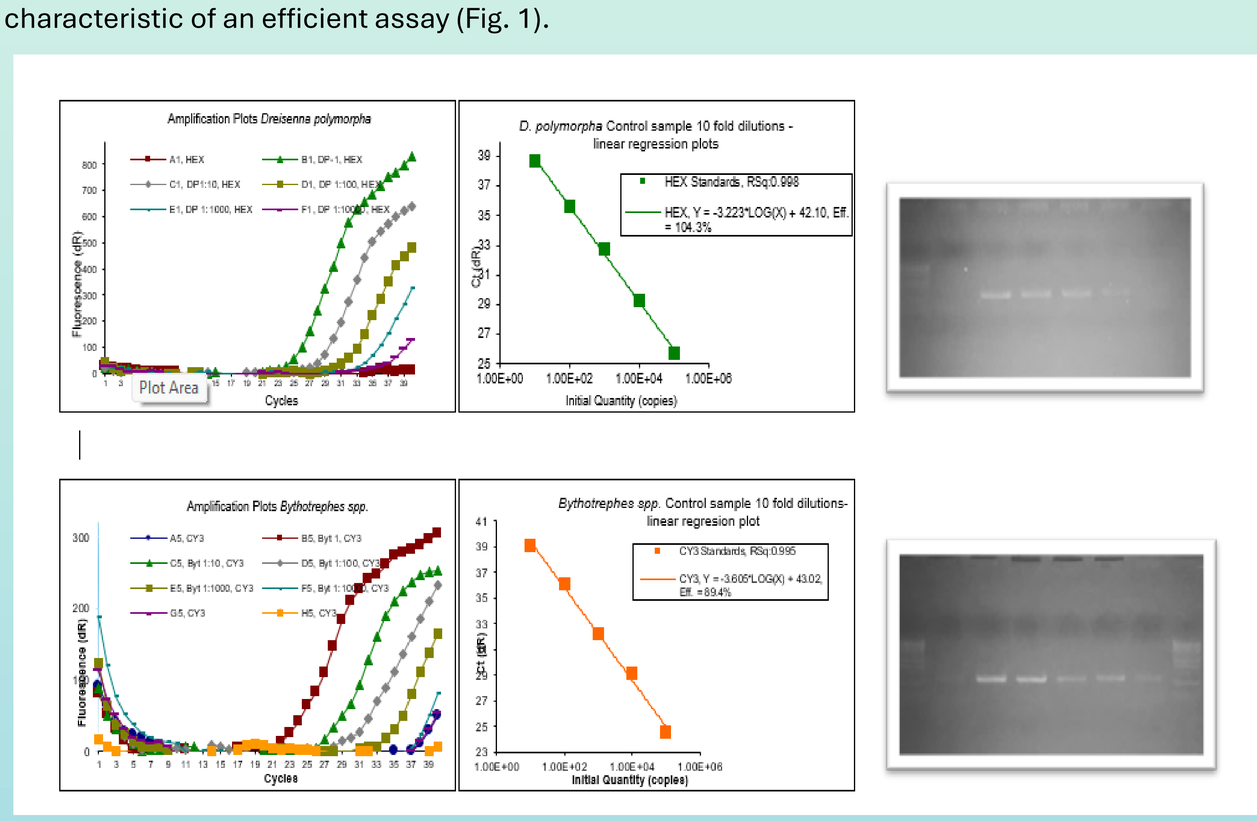

Figure 1. Real-time PCR assay validation for *Bythotrephes spp*. and *Dreissena polymorpha*, and Amplification plots (left), standard curves generated from ten-fold serial dilutions of positive control DNA (center), and agarose gel electrophoresis (right).

Overall, agreement between microscopy and PCR was fair for *Bythotrephes spp.* (κ = 0.29) and slight for *D. polymorpha (κ = 0.19)*, indicating differences in detection outcomes between the two methods. The relatively low kappa values observed reflect differences in the biological targets detected by each method, with microscopy identifying intact organisms and PCR detecting species-associated DNA signals (Table. 3).

Species	Observed Agreement (Po)	Expected Agreement (Pe)	Kappa (κ)
Bythotrephes spp.	0.640	0.496	0.29
D. polymorpha	0.663	0.586	0.19

Table 3. Cohen's kappa (κ) statistics describing the level of agreement between microscopy and PCR for the detection of *Bythotrephes spp*. and *Dreissena polymorpha* in ethanol-preserved plankton samples collected from Manitoba lakes (2022–2024).

Discussion

This study shows that ethanol-preserved plankton samples can provide useful molecular information for AIS surveillance one to two years after collection. Species-specific DNA signals were successfully recovered from preserved samples and detected using PCR assays. However, it is important to emphasise that because the DNA analyzed in this study originated from preserved rather than fresh plankton samples or direct environmental water samples, the results should be interpreted as retrospective molecular analysis of preserved biological material rather than conventional environmental DNA surveillance.

Conclusions

This study demonstrates that ethanol-preserved plankton samples can be successfully repurposed for retrospective molecular analysis of aquatic invasive species. Species-specific DNA signals were recovered and amplified from samples originally collected for microscopy-based monitoring, indicating that preserved collections represent a valuable source of historical biological information (Fig.2).

Fig. 2. One Plankton Sample, and two methodologies (microscopy and DNA) for better AIS monitoring.

References

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