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Detecting eDNA of Invasive Dreissenid Mussels: Report on Capital Investment Project

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PURPOSE: This technical note focuses on the development of eDNA-based sampling capabilities for invasive Dreissenid mussels from environmental waters. Topics include the testing of two water sampling approaches, the potential use of propidium monoazide (PMA) in discerning between Dreissenid eDNA from live veliger versus dead cell sources, and the utilization of differing DNA markers for Dreissenid detection.

INTRODUCTION: The zebra mussel (*Dreissena polymorpha*) and the closely related quagga mussel (*Dreissena bugensis*) are two invasive species of bivalve that have become problematic in North American waterways, having many ecological and socioeconomic impacts. Originally native to the Ponto-Caspian region of Eastern Europe (Gelembiuk et al. 2006, May et al. 2006, Therriault et al. 2004), *D. polymorpha* is thought to have become more widespread due to several factors, including increased shipping traffic and greater connectivity among waterways due the construction of canals (Karatayev et al. 1998, May et al. 2006, Ricciardi 2003). Larval Dreissenids are free-floating veligers and may be easily dispersed by water current – a life history attribute that likely enhances the species' invasiveness (Borcherding 1991, Mackie 1991, Ricciardi 2003, Stepien et al. 2002).

D. polymorpha and *D. bugensis* were likely introduced into North America's Great Lakes in the early to mid-1980s by shipping ballast discharge of transoceanic liners (Mackie 1991, May et al. 2006, Ricciardi 2003, Stepien et al. 2002, Xu and Faisal 2008), and have become problematic for native ecology. Dreissenids differ from native bivalve species in their ability to attach to solid substrates via byssal threads. Attachment to native bivalves and other invertebrates can result in death or serious impairment of those species (Borcherding 1991, Mackie 1991, Wainman et al. 1996, Xu and Faisal 2008). Additionally, Dreissenids are prolific breeders and may out-compete native species for resources, affect water clarity, and change food web dynamics within the ecosystem. This ability to attach to solid substrates also allows Dreissenids to attach to one another and to form large "plaques." These plaques can block water intake pipes and drains, foul navigational buoys, cause erosion in submerged structures, and are a significant, costly problem for industrial and domestic facilities (Mackie 1991, May et al. 2006, McNickle et al. 2006, Ricciardi 2003, Stepien et al. 2002). Based on the most recent estimates, annual costs associated with Dreissenid mussels – from damages to infrastructure, recreation, and ecosystems, to control and eradication efforts -- likely range well above $100 million (Strayer 2009).

Standard techniques for detection of invasive Dreissenids from environmental sources have involved visual surveys, physical sampling, plankton trawling combined with microscopy, flow cytometry, or genetic identification of veligers. Assays for environmental DNA (eDNA) shed into the aquatic environment by target species are based on polymerase chain reaction (PCR), which amplifies DNA extracted from filtered water samples. These assays offer highly specific and efficient methods for

detection of invasive species (Darling and Mahon 2011, Dejean et al. 2011, Jerde et al. 2011). Recent applications of eDNA assays include detection and monitoring of invasive Asian carp (*Hypophthalmichthys molitrix* and *H. nobilis;* Jerde et al. 2011), invasive bullfrogs (*Rana catesbeiana*; Ficetola et al. 2008), and imperiled amphibians (*Ascaphus montanus* and *Dicamptodon aterrimus*; Goldberg et al. 2011).

One of the current weaknesses of eDNA as a monitoring or survey tool is that there are no means for determining whether the detected genetic material came from live or dead cells. However, such information could be particularly useful in some cases. For example, in surveys for invasive Dreissenids, this distinction could indicate the presence/absence of live veligers (relative to sloughed material from adult mussels). The exposure of water filtrates to propidium monoazide (PMA) prior to eDNA extraction and PCR might provide just such a capability. PMA is a membrane impermeable photoreactive dye that binds to DNA with high affinity and impedes PCR (Bae and Wuertz 2009, Nocker et al. 2007). Because PMA cannot infiltrate intact cells, DNA in living cells is protected from PMA activity and can serve as a functional template for PCR, whereas free-floating DNA and DNA associated with dead and degrading cells would be exposed to PMA, therefore being effectively non-functional for PCR. Because DNA in living veligers would be protected from PMA, while most other aquatic eDNA would be exposed, paired eDNA assays, with and without filtrate exposure to PMA, could be used to identify sampling sites with high likelihood of veliger presence. PMA has been proposed as having potential use in detecting the presence of live versus dead microbial cells in PCR-based assays of environmental samples (Bae and Wuertz 2009, Nocker et al. 2007). The use of PMA in assays for multicellular organisms, like veligers, and for assaying eDNA-type samples has not been previously characterized.

MATERIALS AND METHODS:

Study locale. Sampling for the study took place on 1) Lake Pepin, a natural impoundment of the Mississippi River on the border of Wisconsin and Minnesota, approximately 95 km downstream from St. Paul, Minnesota, and 2) just below Mississippi River Lock and Dam 4 at Alma, Wisconsin, approximately 105 km downstream from St. Paul.

Sampling procedure. At each of nine locations on Lake Pepin (Figure 1), two or three "sieve" samples were collected, while three sieve samples were collected at each of five sites downstream of the Pool 4 Dam (Figure 2), for a total of 39 sieve samples. At each sampling location, an 8.2-cm-diam sieve cloth with a 40-µm pore size was placed on the end of a 5-cm-(2-in.-) diam PVC pipe cut to 30 cm in length and secured with a 5-cm (2-in.) PVC coupler. The PVC pipe was used to contain potential backflow of sample water in case of sieve cloth pore blockage by particulates during filtering of highly turbid samples. A 10-L volume of sample water from within the photozone was pumped through the sieve cloth using battery-operated peristaltic pumps. Following filtering, each sieve cloth sample was transferred to a sterile 50-mL collection tube and kept on ice for later processing. All PVC sampling units were sterilized between uses by disassembling them, soaking and scrubbing them in a 5-gal bucket containing dish soap water, rinsing in sterilized water, and a final 5-minute soak in a 5-gal bucket containing 10% bleach solution. Before sampling at each site, the tubing for the peristaltic pumps was flushed with lake water for 2 min a few meters away from each sampling location to avoid any cross-contamination from previous samples.

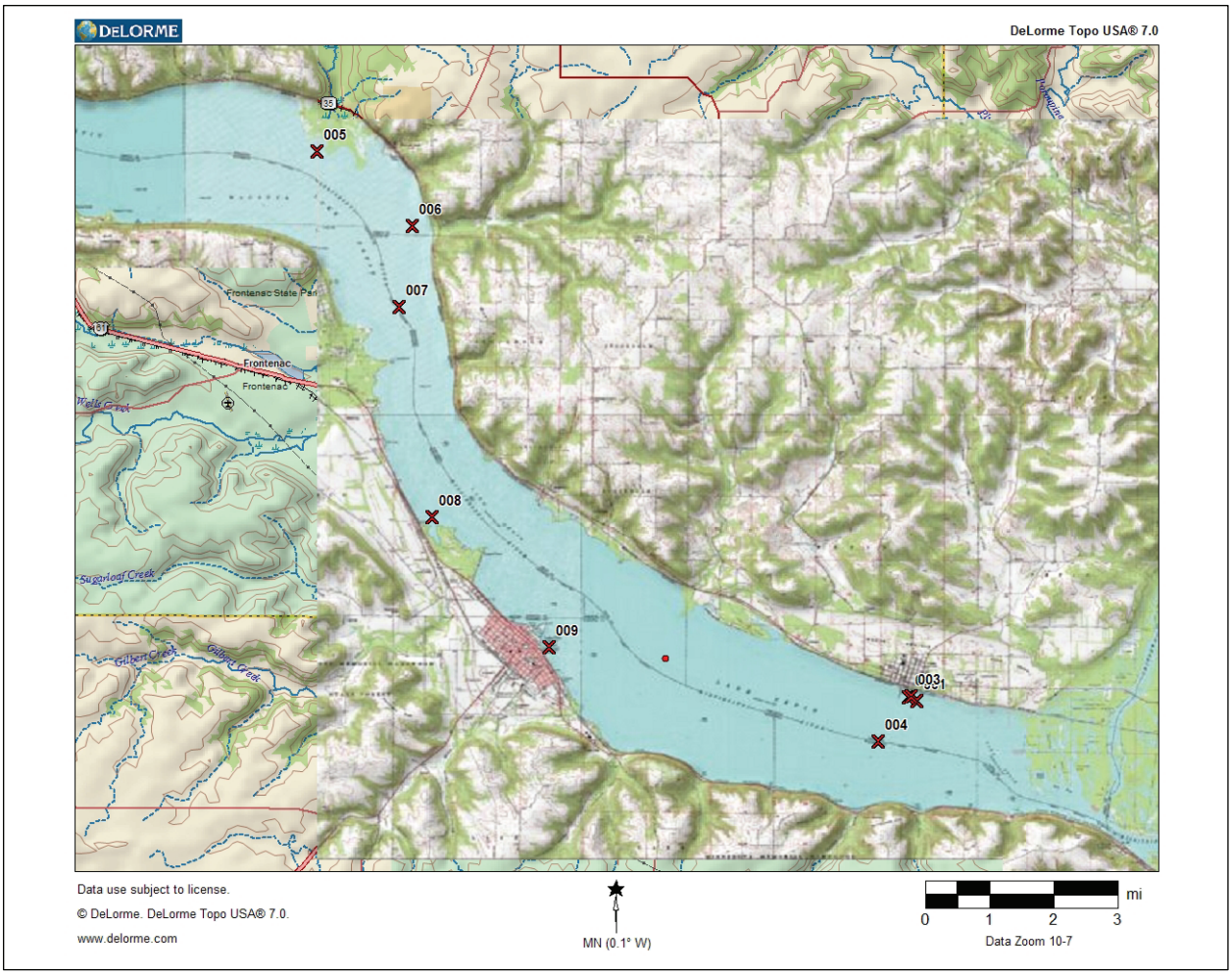

Figure 1. Lake Pepin study locales.

A total of 20 "grab" samples were collected by filling sterile disposable 2-L plastic roller bottles with surface water. At five of the Lake Pepin locations, two grab samples were collected (n = 10), while three grab samples were taken downstream of the Pool 4 dam (Figure 2) at sites 010, 011, and 012 (n = 9). One grab sample was also taken at site 013 (n = 1; N = 20). All grab samples were immediately labeled and placed in ice following collection.

Sample processing. All 2-L grab samples were filtered through 934-AH Whatman filters in 1-L aliquots using disposable 250-ml filter funnels. Before filtering, bottles were hand-shaken to homogenize contents. Processing time for grab samples varied according to the turbidity of the sample.

Grab sample filters and sieve cloths (and the associated filtrates) from each sample were randomly assigned a treatment: 1) PMA, 2) RNAlater® (Applied Biosystems/Ambion, Austin, Texas), which is a commercial tissue storage solution that stabilizes DNA and RNA against biochemical degradation, or 3) no treatment. For treatments 1 and 2, the assigned filter or sieve cloth was placed within a 50-mm petri dish, 300 µL of the select solution was pipetted onto the center of each cloth/filter, and the treatment solution was spread evenly over the cloth/filter using sterile disposable plate spreaders. The

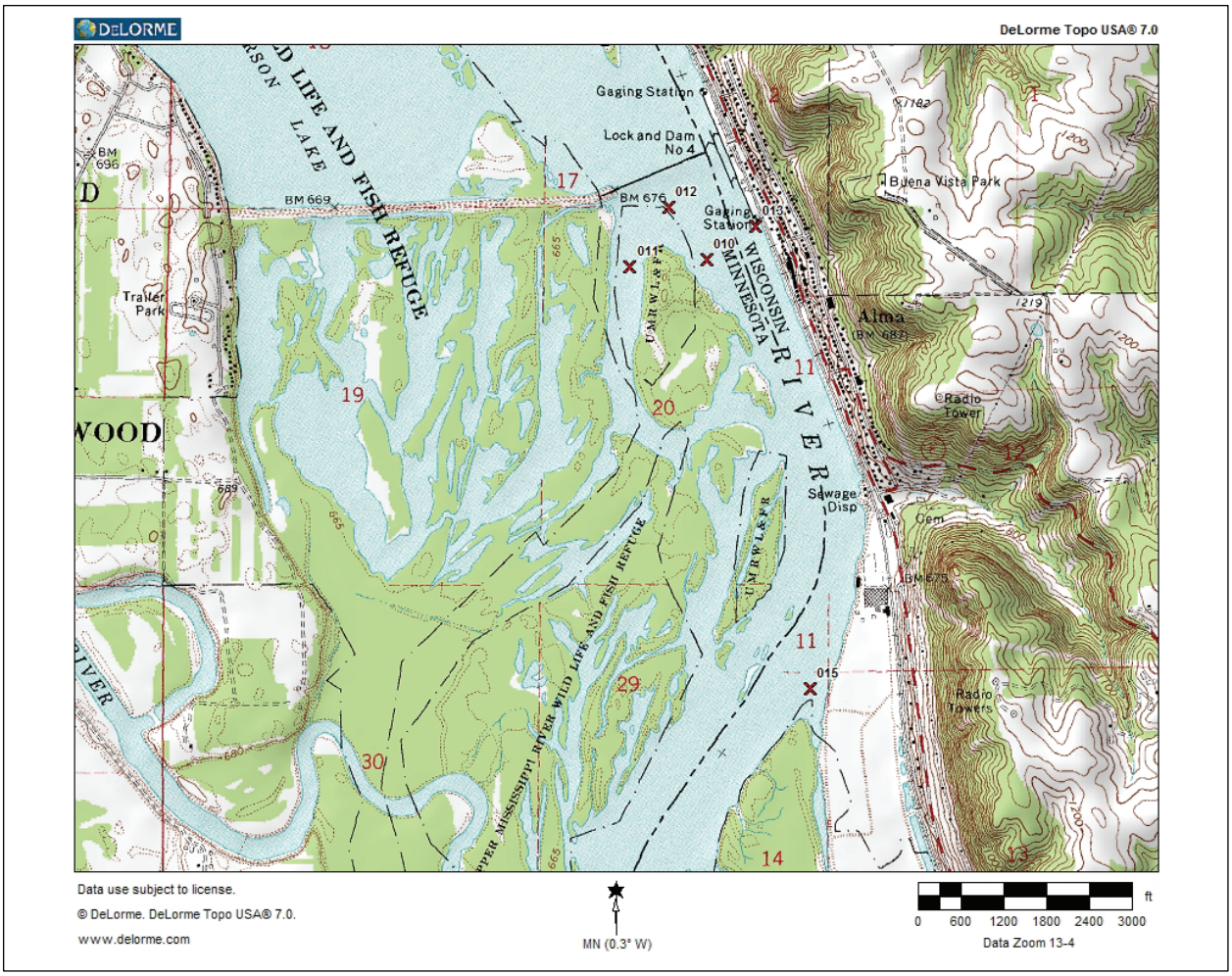

Figure 2. Pool 4 Dam study locales.

treated cloth/filter was then transferred to a dark drawer for 20 min (required for complete absorption of the PMA into the sample). Each sample was then removed and exposed to a blue LED lamp for 15 min (Current USA True Lumen 10-in. 4x 453-nm Blue Aquatic LED Strip) required for initiation of the PMA photochemical reaction permanent binding to any DNA in non-viable cells). All samples were transferred to labeled sterile 15-mL tubes containing 10 mL of RNAlater® solution, and were stored at -20 °C until DNA extraction.

RNAlater® was used here as a control treatment against which to compare the effects of PMA. RNAlater® and PMA treated samples were processed in identical fashion so that any differences in eDNA results would correspond to the presence/absence of PMA. Untreated samples served as a control to assess whether the additional handling and light exposure associated with PMA/RNAlater® treatments might impact the assay.

Samples were shipped overnight on ice to ERDC Vicksburg and transferred to a -20 °C freezer for storage. Later, eDNA was extracted from samples using the Power Water® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA).

Molecular analysis. Several published primer sets were assessed for use in PCR assays of eDNA samples. A universal primer set, Holl-F/R (Livi et al. 2006), which is expected to amplify a region of the 18S rDNA gene from nearly any animal species, was used to assess general sample viability. Another primer set, ZEB-715a/UnivF-15 (Frischer et al. 2002) was expected to be specific for 18S rDNA in zebra mussels, and was found to work well on positive controls (DNA from adult zebra mussel tissue). The 18S rRNA gene is fairly well-suited for eDNA surveys, as multiple copies are found within the nuclear DNA content of each cell. Mitochondrial DNA markers, if species-specific, are found in higher copy numbers per cell and are generally preferred over nuclear markers as eDNA targets. Another zebra mussel specific marker, DpolA6F/R (Naish and Boulding 2001), which is a single-copy microsatellite gene (nuclear DNA), worked well with positive controls. However, this marker failed to amplify from the environmental samples, likely due to much lower concentrations of target DNA in the environmental samples.

Standard PCR amplification was performed on all eDNA samples. Each 25-µL reaction contained 2.5 µL of 10x Buffer solution with $MgCl_2$, 0.2 mM of a dNTP mix, 0.6 µM of each primer, 1 unit 5 PRIME Taq polymerase, 2 µL of sample DNA, and 16.75 µL of molecular grade nuclease-free water. PCR amplification was performed in a BIO-RAD tetrad thermocycler with an initial melting step at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 15 sec, 53°C for 15 sec, 72 °C for 30 sec). A final elongation step at 72 °C for 10 min followed. Gel electrophoresis was performed on Invitrogen E-Gel®48 2 % GP precast gels, and all gels were imaged using a BIO-RAD Molecular Imager® FX pro. All samples showing positive bands of the approximate target size were purified using Invitrogen E-Gel® SizeSelect™ 2% Agarose gels.

The purified products were then sequenced using Applied Biosystems BigDye® Terminator Cycle Sequencing Kit and ABI PRISM® 3100 Genetic Analyzer. All eDNA sequences were compared against known sequences in the National Center for Biotechnology Information's online database GenBank®.

RESULTS AND DISCUSSION:

Sieve samples. A total of 32 of 39 sieve samples produced PCR products with the universal primers (82% success), indicating that the method was a highly effective means for collecting eDNA. Copepods (e.g. *Acanthocyclops*, *Macrocyclops*) and rotifers (e.g. *Brachionus*, *Cephallodella*) dominated the sequence data resulting from these primers.

For the zebra mussel-specific primer set, 19 of 39 sieve samples showed bands at the expected target size (701 bp) and were considered putative positive hits. Of these 19 putative positives, 18 exhibited DNA sequence that matched *D. polymorpha*.

The difference between sampling method success rates for detection of *D. polymorpha* eDNA using the zebra mussel-specific primers was, as exhibited by logistic regression analysis, statistically significant ($p = 0.007$), and the chances for detecting *D. polymorpha* eDNA were 75% greater when using the sieve cloth protocol (Table 1).

Grab samples. Of the 30 grab samples, 10 samples produced PCR products using the universal primers. Only one of the 10 PCR positive samples produced good sequence data, which showed DNA associated with ciliated protists (e.g. *Tintinnidium*).

Table 1. Results describing the success rate of *Dreissena polymorpha* eDNA detection comparing sampling method and treatment.

Treatment	Zeb715 PCR	Sample Method	Site	Treatment	Zeb715 PCR	Sample Method	Site
RNA Later	Yes	Sieve Cloth	5	PMA	No	Sieve Cloth	10
PMA	No	Sieve Cloth		RNA Later	No	Sieve Cloth	
No Treatment	Yes	Sieve Cloth		No Treatment	No	Sieve Cloth	
RNA Later	No	Grab Sample		PMA	No	Grab Sample	
PMA	No	Grab Sample		RNA Later	No	Grab Sample	
No Treatment	No	Grab Sample		No Treatment	No	Grab Sample	
RNA Later	No	Sieve Cloth	6	PMA	No	Grab Sample	
PMA	No	Sieve Cloth		RNA Later	No	Grab Sample	
No Treatment	No	Sieve Cloth		RNA Later	No	Sieve Cloth	11
RNA Later	No	Grab Sample		PMA	No	Sieve Cloth	
PMA	Yes	Grab Sample		No Treatment	Yes	Sieve Cloth	
No Treatment	No	Grab Sample		No Treatment	No	Grab Sample	
RNA Later	Yes	Sieve Cloth	7	RNA Later	No	Grab Sample	
PMA	Yes	Sieve Cloth		PMA	Yes	Grab Sample	
No Treatment	Yes	Sieve Cloth		No Treatment	No	Grab Sample	
RNA Later	No	Grab Sample		RNA Later	No	Sieve Cloth	12
PMA	Yes	Grab Sample		PMA	No	Sieve Cloth	
No Treatment	No	Grab Sample		No Treatment	No	Sieve Cloth	
PMA	No	Sieve Cloth	8	RNA Later	No	Grab Sample	
RNA Later	Yes	Sieve Cloth		PMA	No	Grab Sample	
No Treatment	Yes	Sieve Cloth		No Treatment	No	Grab Sample	
PMA	No	Grab Sample		PMA	No	Grab Sample	
RNA Later	No	Grab Sample		RNA Later	No	Grab Sample	
No Treatment	No	Grab Sample		RNA Later	Yes	Sieve Cloth	13
PMA	No	Sieve Cloth	9	PMA	Yes	Sieve Cloth	
RNA Later	No	Sieve Cloth		No Treatment	Yes	Sieve Cloth	
No Treatment	Yes	Sieve Cloth		No Treatment	Yes	Grab Sample	
PMA	No	Grab Sample		RNA Later	Yes	Sieve Cloth	14
RNA Later	No	Grab Sample		PMA	Yes	Sieve Cloth	
No Treatment	No	Grab Sample		No Treatment	No	Sieve Cloth	

Four of the 30 grab samples showed bands of the approximate target size using the zebra mussel-specific primer set. Of these four putative positives, two exhibited DNA sequences that matched *D. polymorpha*.

PMA. In the eight tests where RNAlater®-treated samples showed putative positive hits for *D. polymorpha* eDNA, four failed to exhibit corresponding PCR product when treated with PMA. All four of these putative positives were sequence verified. With the RNAlater®-treated samples, seven of eight putative positives were sequence verified.

For three samples, only the PMA-treated, and not RNAlater®-treated, samples showed a putative positive hit, which is a counterintuitive result. Of these three samples, only one could be sequence verified.

For sample sets that included untreated samples within which at least one of two samples exhibited a putative positive hit, there were four times when both RNAlater®-treated and untreated samples showed PCR product, whereas in only one set was the RNAlater®-treated sample the only hit and in only two sets were the untreated samples the only hits. Positive hits from all seven sets were sequence verified. Untreated samples showed, in total, six positive hits, including three sets where both untreated and PMA-treated, but not RNAlater®-treated, samples were positive (Table 1).

The 10-L sieve cloth samples produced a significantly higher number of sequence-confirmed positives than the 1-L grab/filter samples. This is likely a simple function of the higher total volume of water filtered per sample. While there is a significant (75%) increase in the likelihood of detecting *D. polymorpha* eDNA using the 10-L sieve method, this method requires a significantly greater time investment per sample. With the equipment on hand and three personnel, three 10-L samples were collected, simultaneously, in about 15 minutes. In contrast, each grab sample took approximately 1 min to collect, and could have been collected with one field person. While this is a significant difference in time required for each technique, the actual sampling time was only a fraction of the overall time required for field collection; travel time between sampling locales was more time-consuming and the time required to take samples may only be a minor concern in many cases. Additionally, the sieve method could be made more efficient with faster pumps or, potentially, by pouring water through sieves. Also note that the stowage requirement for sieve samples is much less than that of grab samples, which requires keeping large numbers of bottles on the boat and large ice chests for sample storage. Finally, pending further optimization of sieve sampling protocols, minimizing cross-contamination of grab samples may require relatively less effort (if disposable bottles are used) than required for sieve sampling. In this study, buckets of soapy water, sterilized water, and 10% bleach solution were kept on the boat so that the sieving equipment could be sterilized between samples. Assembling a large enough set of sampling tubes so that each tube is used only once per field trip could significantly reduce the effort and inconvenience required to prevent cross-contamination.

No firm statements can as yet be made on the use of PMA to discriminate samples containing live veligers from those containing only extracellular and degrading genetic material. It can be said that the current results are interesting and require further research. In some cases, PMA treatment resulted in the loss of PCR amplification, as would be expected if all eDNA in a sample were associated with free-floating DNA or degrading cells, and not with live veligers. However, the amplification of some samples in the presence of PMA, where no amplification was present in RNAlater®-treated or an untreated sample, is a confounding result. Future trials involving environmental samples known to contain live veligers would greatly further the development and application of PMA as a diagnostic tool for live versus dead cell eDNA sources.

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