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Detection of *Limnoperna fortunei* DNA From Plankton Samples: A New Protocol

Leandro Capurro, Ernesto Brugnoli, Edgardo Díaz-Ferguson, and Claudio Martínez

ABSTRACT

***Limnoperna fortunei* (golden mussel) is an invasive mollusk, native to Southeast Asia. The detection of *L. fortunei*, during early stages of the invasion, is an interesting strategy for its control and eradication. For these aims, traditional methods are used based on identifying the species in samples of zooplankton or benthos community. In addition, molecular methods based on the detection of DNA in different environmental samples (eDNA) have been proposed. We describe and discuss a protocol developed for the detection of *L. fortunei* based on mitochondrial eDNA in plankton samples from freshwater systems. Water samples were obtained during 2018-2019, from cooling systems of the hydroelectric power plant in Río Negro, Uruguay. eDNA was detected by end-time PCR and PCR products were resolved on polyacrylamide gels and silver staining. Primers specificity was determined in related limnic and brackish native species. Sensitivity was analyzed as genomic DNA concentration and number of larvae. Different methodologies were tested to remove inhibitors from samples. Finally, the eDNA PCR products were sequenced to confirm the specificity of the reaction. The results obtained by molecular techniques were more sensitive than traditional methods of species detection.**

Keywords: Biological invasion, end point PCR, environmental DNA, reservoir.

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I. Introduction

Limnoperna fortunei (golden mussel) is a freshwater bivalve native to Southeast Asia. This species was accidentally introduced into South America through ballast water and detected in the 1990s in the Río de la Plata estuary (Pastorino *et al.,* 1993, Scarabino & Verde, 1994). Currently, it is an invasive alien species (IAS) in South America, changing the whole structure of the aquatic ecosystems in the invaded regions (Silva *et al.,* 2020, Boltovskoy*et al.,* 2022). Furthermore, it causes economic damage to different industrial activities (e.g. agricultural and hydroelectric generation) due to the macrofouling process (Boltovskoy *et al.,* 2006, Brugnoli *et al.,* 2006, Olivera *et al.,* 2015). During its life cycle, it presents different planktonic non-valved larval stages (trochophore and pre-veliger: 80 to 115 µm long) and valved types (veliger D to plantigrade or post-larva, 100 to 320 µm long; dos Santos *et al.,* 2005, Cataldo *et al.,* 2005, Ezcurra de Drago *et al.,* 2006). The plantigrade larvae (post-larvae) lose their ciliated velum and develop their foot, settling on hard bottoms and developing as benthic individuals (Fig. 1, dos Santos *et al.,* 2005, Cataldo *et al.,* 2005).

Early detection of IAS at the initial stages of invasion is essential for control or eradication procedures (Reaser *et al.,*2020). Traditional methods of detection and monitoring of aquatic IAS are based on observation or capture of the organism during planktonic or benthic life stages (dos Santos *et al.,* 2012, Fabián *et al.,*2021). However, this methodology can be costly, time-consuming, hazardous to the investigator and the environment, and sometimes less sensitive than molecular techniques (Harper *et al.,*2019, Ravindran 2019, Sepulveda *et al.,*2020, Blattner *et al.,* 2021, Ito & Shibaike, 2021).

Several molecular studies have been carried out on the eDNA of the golden mussel, mainly in South America, based on the detection and quantification of larvae DNA or particulate and dissolved DNA (Pie *et al*., 2006; Endo *et al*., 2009; Díaz-Ferguson & Moyes, 2014; De Paula *et al*. 2020, Ribolli *et al*., 2021).

Recently, De Paula *et al.,* (2020) conducted a review of

molecular studies performed on *L. fortunei* including genomics, transcriptomics, population genetic studies, and the use of environmental DNA to detect the species in invaded environments. In spite of these advances, in Uruguay this kind of molecular study with eDNA in Eucariota organism, are absent.

The present work aims to describe a new protocol developed and standardized for the detection of *L. fortunei* larval DNA in zooplankton samples obtained from a cooling system of a hydropower station by end-point PCR. Moreover, it is the first eDNA study with a case applied to IAS in Uruguay.

II. Material And Methods

A. Sample Collection

Limnoperna fortunei larvae were obtained by filtering 100 L of water from the cooling systems of the Constitución Hydroelectric Power Plant (HPP) (Río Negro, Uruguay) through a 100 µm mesh sieve. The sampling was made during 2018-2019 (Fabián *et al.,* 2021). Chosen samples showed a wide range of abundance valved larval stages (Fabian *et al.,* 2021): 0 (August 2018), 4340 (December 2018) 2900 and 33.000 larvae (January 2019a and 2019b). Samples were stored in 95% ethanol, refrigerated (4 °C), and in the dark until processing. Plankton samples were concentrated by filtering on a 4.5 mm diameter glass fiber filter (0.7 µm pore), which was washed with MilliQ quality water to remove excess alcohol and stored in the digestion buffer (ThermoFisher^{Tm}) at -20 °C.

B. Decontamination of Materials

Due to the high sensitivity of PCR-based molecular assays (Nathan *et al*., 2014, Xia *et al.,* 2018), 10% hypochlorite and 10% hydrochloric acid solutions, heat and UV radiation methods were used for decontamination of exogenous DNA as well as inactivation of enzymes (DNAases and proteases).

The surfaces and instruments to be used (e.g. pipettes, pipette tip boxes and, racks) were thoroughly cleaned before and after the work. Hypochlorite 10% was applied and left to act for 10 minutes, rinsed with alcohol 70% and the alcohol residues were air dried. The same method was applied to glass and metal supplies used in the extraction of tissues and plankton. Plastic materials such as containers for storing adult organisms and plankton samples, mesh sieves, zooplankton counting chambers and filtering equipment were deposited in containers with 10% hydrochloric acid (30 min) and rinsed with 70% alcohol and MilliRo quality water.

Dry heat or moist heat modalities were also applied. The dry heat was used to decontaminate glass fiber filters (500°C for 20 min). Moist heat (autoclaving) was applied to disposable material (i.e. pipette tips, 40 min). The products used for PCR reaction (e.g. Eppendorf tubes, water aliquots, pipette tips and the outside of automatic pipettes) were additionally decontaminated by ultraviolet radiation in a BIOSAN UVC/T-AR chamber and STERILIZER GM209 oven (at least 15 min).

C. DNA Extraction and Visualization

DNA extraction was performed with the Gene JET Genomic DNA Purification Kit (ThermoFisher^{TM}) according to the protocol provided by the manufacturer with some modifications. Sample digestion was performed for 24±2 h, then, 350 µL of Chloroform - isoamyl alcohol 24:1 (Chf-Ia) was added to the lysate. The sample was mixed for 15 s and then centrifuged for 2 min at maximum speed (>14000 g). The supernatant was recovered by pipette. As it was likely that the sample contains a high load of inhibitors (humic substances, lipids, proteins, polysaccharides) the organic extraction was repeated. The elution was performed in 2 steps, with a final volume of 100 µL (70 + 30 µL or 50 + 50 µL) at 70 °C.

DNA was extracted from different local not target species, considering benthic organisms (*L. fortunei*-four adult, *Corbicula fluminea*, *Pomacea canaliculata*) and pooled planktonic organisms. The adult benthic individuals were collected from Río Negro river and Santa Lucía watershed) to cover possible haplotipical differences (Ghamboo *et al*., 2013); planktonic samples were collected in Montevideo Bay (Río de la Plata estuary), with a 180 µm pore mesh net to collect zooplankton organism.

DNA total concentrations were determined by Nanodrop UV spectrophotometry. DNAs integrity were analyzed by 1% agarose gel electrophoresis (with the addition of Goodview^{TM}–SBS staining, Genetech- final concentration: 10 µL/100mL). Hyperladder^{TM} II (Meridian Bioscience®) was used as a molecular weight marker. Approximately 1 µg of genomic DNA and 220 ng of plankton extracted DNA were loaded into each well of the gel, due to the low yield of DNA extraction and the maximum loading volume allowed per well.

D. End-Point PCR

End-Point PCR was performed using *Limf* primers designed by Endo *et al.,* (2009). Two different conditions were assayed due to the possibility of PCR inhibitors: with and without Bovine Serum Albumin (BSA, final concentration of 0.4 µg/µL). The PCR conditions for a 25 µL reaction were: 1X PCR buffer (BIOLINE), 0.2 mM of each primer, 0.05 mM of each dNTP, 2.0 mM Mg2+, 0.4 µg/µL of BSA, 1 unit of Taq polymerase (HybriPol™ DNA Polymerase, BIOLINE) and 3 µL of DNA eluate. The cycling program was as follows: a first denaturation step, 4 min at 95°C; 30 cycles with denaturation at 95°C for 30 sec; hybridization at 60°C for 35 s; and elongation steps at 72°C for 30 s; with a final extension one at 72°C for 7 min.

PCR products were visualized on a 12% polyacrylamide gel. 5 µL of each PCR product with the addition of 1 µL of loading buffer were loaded. The gels were run at 100-110 mV, 40-60 min. DNA amplicons were visualized by silver staining (Sanguinetti *et al.,*1994)

E. Specificity and Sensitivity

The lower limit of detection of the reaction, -defined as the minimum concentration of DNA that generates 50% positive reactions-, was determined by serially diluting DNA from adult organisms and testing by end-time PCR (Xia *et al.,* 2018). We started from a 60 ng/µlthat was diluted sequentially by an order of magnitude until 6 pg/µl. The experiment was performed in triplicates. The same trial was carried out with larvae of *L. fortunei*, where 35, 10 and 1 organisms were tested (stage Veliger D and Straight-hinged Veliger Fig. 1A), considering one experimental replicate for

each biological replicate. Subsequently, three biological replicates were performed with three experimental replicates for 1 larva and ½ larva as well as two experimental replicates for the three biological replicates of ¼ and 1/10 larvae. The standards that are less than 1 larva were obtained by serial dilution from extraction from 1 larva.

F. Test Samples

9 ng (August 2018), 16 ng (January 2019b), 24 ng (January 2019a) and 30 ng (December 2018) of complex sample eDNA were analyzed by PCR, as described in the D section.

PCR amplicons form genomic DNA (1 PCR product) and environmental samples from August 2018 (5) and January 2019a (5) and January 2019b (3) were purified with GeneJET PCR Purification Kit (ThermoFisher™) and their nucleotide sequences were determined by DNA sequencing at Macrogen Sequencing Service, Korea. The August 2018 sample was sequenced with the *Limf* R reverse primer, the genomic and January 2019b samples with *Limf* FR, and the January 2019a sample with both primers. Retrieved DNA sequences were analyzed using Mega 7 bioinformatics software (Kumar *et al.,* 2016) and also analyzed for sequence homologies with the BLASTn algorithm from the free NCBI database.

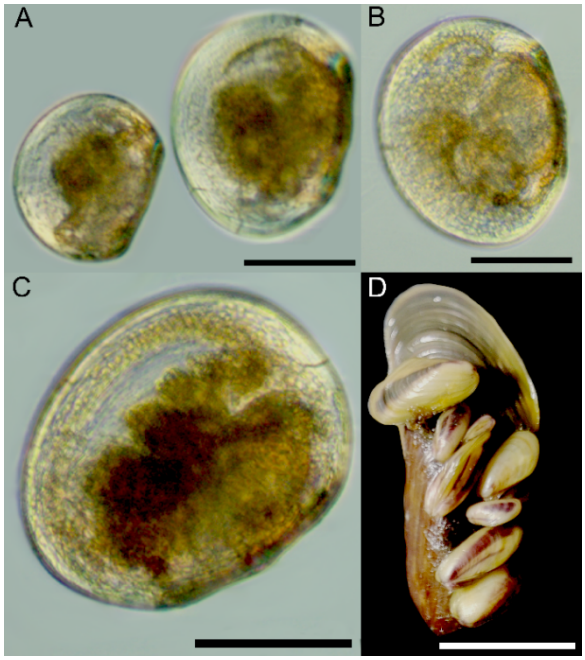

Fig. 1. A) veliger D (left) and Straight-hinged veliger (right). B) Umboned veliger. C) Pediveliger larvae. D- Adult *L. fortunei* with juveniles fouled over it. Bar scale: A-C: 100 µm, D: 0.25 mm.

III. Results

A. DNA Extraction

An increased amount of DNA obtained from tissues was observed when the Chf-Ia step was applied, as in DNA Extractions 3 and 4 (Fig. 2). All plankton samples from Constitución HPP were treated with Chf-Ia except January 2019b, which was treated in duplicate due to its high suspended organic load.

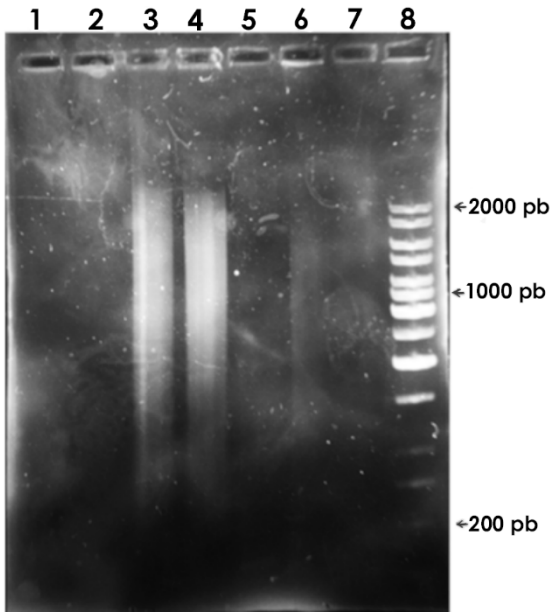

Fig. 2. 1% agarose gel with genomic or total DNA extraction. 1: *L. fortunei* 4, 2: *L. fortunei 3*, 3: *L. fortunei 1*, 4: *L. fortunei 2*, 5: C. *fluminea 6*: Montevideo Bay Zooplankton 2, 7: *Pomacea canaliculata*, 8: MW marker.

An absence of signal was observed in lanes 1, 2 and 5; and in lanes 6 and 7 only faint bands are visualized.

B. Specificity and Sensitivity

DNA obtained from non-target species was not amplified under our working conditions.

The minimum detection limit of the technique was determined as 60 pg of genomic *L. fortune*i DNA. Positive reactions were observed till 1/2 larvae of *L. fortunei.* However, no positive reaction was observed for ¼ and 1/10 *L. fortunei* larva. Clean sequences (100% Identity Percentage with *L. fortunei*) were obtained from the Edna samples (Table I) and BLASTn analyses matched exclusively with the Cytochrome Oxidase I gene from *L. fortunei*. Fragments length were found to be between 70 pb for January 2019b and 94 pb for August 2018, and the DNA sequence from the tissue sample was 86 bp long. The percentages of identity were greater than 97.33%, with E-values ranging from 2.00E-46 to 3.00 E-32. Results of this BLASTn analysis are shown in Table I. DNA sequence chromatograms showed a single sequence, although some also showed a double signal at some sites (data not shown).

C. Optimizing PCR reactions

BSA treatment allows amplification in samples that did not amplify without its addition (Fig. 3, January 2018a) and some bands presented higher intensity (August 2018, January 2019b, Fig. 3).

TABLE I: Results of BLASTn Analysis for the Different Environmental Sequences and Parameters Obtained.

Samples	Sequencing Primer	Sequence length	Maximum homology	E-value	Per. Ident
August 2018	*Limf* R	94	AB828681.1	2,00E-46	100%
January 2019a	*Limf* R	85	AB828681.1	2,00E-41	100%
January 2019a	*Limf* F	85	AB828679.1	3,00E-38	97.65%
January 2019b	*Limf* F	74	MT408047.1	3,00E-32	97.33%
Genomic	*Limf* F	86	AB828679.1	4,00E-42	100.00%

Per. Ident= Percentage of Identity

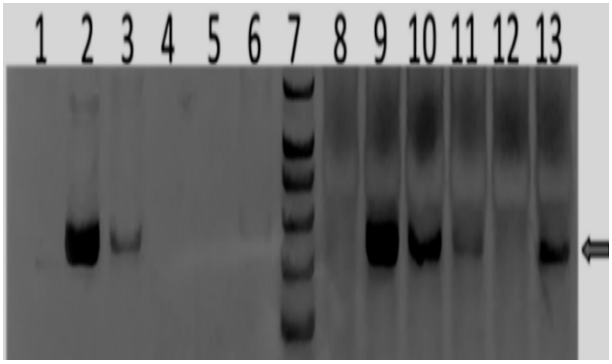

Fig. 3. Post-PCR gel with and without BSA from environmental samples 1: C-, 2: C+, 3: January 2019b, 4: January 2019a, 5: December 2018, 6: August 2018, 7: ladder, 8: C- BSA, 9: C+ BSA, 10: January 2019b BSA, 11: January 2019a BSA, 12: December 2018 BS.

IV. Discussion

A protocol for the detection of *L. fortunei* DNA in environmental zooplankton samples was successfully developed and applied using an end-point PCR assay (for the first time in Uruguay), which has already been applied in other regions (Xia *et al.,* 2018; Andrade *et al.,* 2021). This detection method had been previously used also with qPCR (Endo *et al.,* 2009, Pie *et al.,* 2017, Ribolli *et al.,*2021). However, this well-established method requires special equipment that not all laboratories can fulfill. Indeed, the present protocol could serve as a more affordable alternative for eDNA detection in this species.

The specificity of *Limf* F and R primers has shown to be consistent with the results by Endo *et al*. (2009). The absence of cross-reaction with other Mytilidae species suggests that this method could also be used in limnic and brackish habitats, since, given their phylogenetic relationships, other untested species should also fail to generate such cross-reaction under or working conditions.

This would allow monitoring non-invaded areas, which are highly vulnerable to the spread of aquatic invasive species, thereby promoting early warning, control measures and eradication responses.

The eDNA sequences obtained were clean and with little background noise. This means that no other species or region of the *L. fortunei* genome was non-specifically co-amplified under the given working conditions. This opposes the findings of Ribolli *et al.* (2021), who were unable to use these primers for being non-specific considering their working conditions. making it easier to detect contamination. The main difference with our work is that Ribolli *et al.* (2021) used a qPCR assay, which, aside from being more sensitive also used longer cycling (Ct=35),

Another aspect worth considering is their primers final concentration 5 pmol/L while ours is 2 pmol/L. Primers performance for qPCR should be analyzed and validated in terms of their specificity, sensitivity, and self and cross-hybridized capacity to detect small amounts of DNA. The chromatograms showed little background noise and little or no presence of double peaks, which could imply the presence of a dominant haplotype or a bias in the primers towards said haplotype. Despite the foregoing, the Constitución dam is expected to generate a bottleneck effect on the haplotype diversity of the invasive species (Ghabooli *et al.,* 2013, Kennedy *et al.,* 2016), so it is not likely to find a high genetic diversity. In the chromatograms, the rare sites with two overlapping signals generally had at least one match with a known *L. fortune* haplotype. This would indicate that the double signals might not be sequencing errors but rather the presence of more than one haplotype, which is to be expected in environmental samples. In view that few studies deal with the genetic diversity of species in Uruguay (Ghabooli *et al., 2013*), it is very likely that some haplotypes remain to be described. Therefore, some sequences do not show 100% identity to the available ones in GenBank database.

The sensitivity of the *Limf* F and R primer pair proved to be sufficient to detect the presence of less than one D larva of *L. fortunei* in the samples analyzed. This is a key feature when it comes to diagnostic techniques, because the larger the volume filtered, the greater the number of impurities and contaminants that can be co-purified and potentially inhibit PCR testing (Turner *et al.,* 2014, Sanches & Schreier, 2020). Indeed, the "half larva" standard is not an actual biological parameter; still we have corroborated that this technique is sensitive enough to detect amounts of eDNA that is less than the DNA present in a full larva. This means that this technique is sensitive enough as to be used during winter when larvae presence is not reported in the invaded systems of the Rio Negro (Brugnoli *et al.,* 2021, Fabian *et al.,* 2021), but when true positives could potentially occur. Moreover, it could detect non-valved stages, which are harder to recognize with traditional methods (Cataldo *et al.,* 2005).

Turner *et al.* (2014) postulated that the highest amount of eDNA from the invasive species *Cyprinus carpio* (common carp) was retained on filters between 1-10 µm pore size. Consequently, smaller sizes would not result in better yields, especially in qualitative studies. It has been observed that eDNA would be better preserved within the mitochondria or fine organic material (Turner *et al.,* 2014, Jo & Yamanaca, 2022). Notwithstanding this, the present protocol performed well with zooplankton samples. Moreover, after fixation, they can break or empty their digestive tract, leading to DNA presence in the medium that can be retained by using small pore size filters.

Several protocols for eDNA extraction are currently available. Nonetheless, commercial kits provide cleaner extractions (lower level of inhibitors) and a higher amount of subsequent DNA (Deiner *et al.,* 2015, Sanches & Schreier, 2020). The present technique has shown to be highly sensitive, detecting up to 60 pg of *L. fortunei* genomic DNA. Then the DNA concentration to be added to the PCR reaction can be minimized, taking into account that the number of inhibitors increases as the amount of DNA does. The presence of contaminants or highly degraded DNA can translate into an overestimation of the amount of DNA measured by spectrophotometry (e.g., Nanodrop), so the visualization of DNA in 1% agarose gel (Fig. 2) is a useful tool for estimating actual DNA concentration. Xia *et al.* (2018) had developed other reactions with others primers and reached lower sensibilities. However, we demonstrated that our sensibility is enough to detect D veliger and even no-valved larvae in water column.

As zooplankton community was used in this work, many of the inhibiting factors that may be present in water (e.g., dissolved organic matter, salts) were not retained. Still inhibition would be observed. The identification of potential inhibitors and their effect on the reaction are key elements

when determining their most suitable removal method. Schrader *et al.* (2012) reviewed the different types of inhibitors, their mechanisms of action, and removal processes. BSA is used to reduce PCR inhibitors in environmental samples, e.g., humic acids (Kreader, 1996, Boeger *et al.,* 2007, Schrader *et al.,* 2012, Harper *et al.,* 2019). A BSA final concentration of 0.4 µg/µL is recommended (Kreader, 1996), because higher concentrations could interfere with silver staining.

Another treatment that has been shown to be successful in diminishing the effect and concentration of inhibitors from environmental samples is the use of more than one purification step with chloroform:isoamyl alcohol. Despite this, one sample in the present work still showed inhibition (December, 2018). In other samples (data not shown) we took the eluted DNA sample and then applied a chloroform:isoamyl alcohol step, and we treat them as it was a lysis sample and then proceeded with its passage through binding column from the kit, thus allowing to taking out the inhibitors. Other options are ethanol precipitation (Diaz-Ferguson *et al.,* 2014), commercial inhibitor removal kits in this scenario (Harper *et al.,* 2019) or purification with polyvinylpyrrolidone (Young *et al.,* 1993).

The silver staining process in polyacrylamide gels is more sensitive and provides higher resolving power than intercalating DNA dyes in agarose gels (Fierro, 2014).

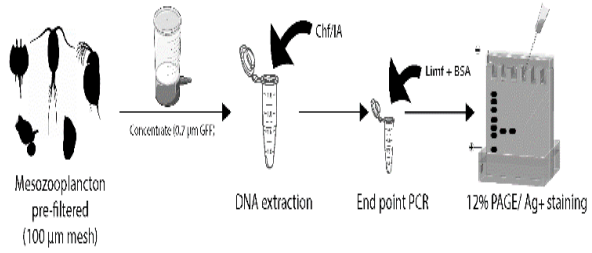

Fig. 4. Proposed protocol of eDNA detection of *L. fortunei* from plankton samples (Blommaert, 2022; Kodis, 2022; Losch De Oliveira, 2020).

In addition, it is safer for the technician due to the low toxicity of its reagents (Sanguinetti *et al.,* 1994, Fierro, 2014). One disadvantage showed by polyacrylamide gel electrophoresis and silver staining processes is their high sensitivity, which could result in the detection of DNA traces irrelevant to the analysis, such as primer dimers and other secondary structures, or unspecific reactions that occur at low yields (Fierro, 2014). Likewise, these DNA traces do not necessarily interfere with the methodology (specificity) or with subsequent steps to be performed (e.g., DNA sequencing). Consequently, a clean negative and a positive control reaction with a band intensity greater than the DNA traces is recommended along with a well-resolved ladder.

V. Conclusion

This is the first eDNA study in an aquatic Eukaryote IAS in Uruguay, in an scenario where eDNA based methodologies are acquiring relevance worldwide, due to its usefulness in the detection of low abundant species, such as IAS in the early stages of invasion or endangered species (Nathan *et al.,* 2014; Díaz-Ferguson & Moyes, 2014; Reaser *et al.,* 2020; Sepulveda *et al.,* 2020). The protocol presented here (Fig. 4), has been shown to be efficient, sensitive and specific for the detection of *L. fortunei* larvae in freshwater zooplankton samples, even in those samples where traditional methods did not detect their presence. This allows filtering less amount of plankton or working in systems with low abundances. Other primers had been developed for end-time PCR, however this one had been tested in South America and is short enough to be used in qPCR, but some other, more sensitive, are too long to be used in that platform. Indeed, we verify the protocol specificity, thus we would not need to sequence all the results to verify the identity of a positive reaction (Andrade *et al.,* 2021).

Finally, we suggest some fast and economical methodologies for the elimination of PCR inhibitors in complex environmental samples.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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