




## Optimized and validated protocol to the detection of the invasive bivalve *Limnoperna fortunei* from eDNA plankton samples

Protocolo otimizado e validado para detecção do bivalve invasor *Limnoperna fortunei* de amostras de DNA ambiental de plâncton

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**Abstract:** We optimized a methodology for plankton environmental DNA detection of the invasive golden mussel and validated it in samples from a Southern Brazil reservoir. *Limnoperna fortunei* is a successful invasive alien species that causes significant impacts on freshwater ecosystems. We adjusted and validated the methodology to detect *L. fortunei* in plankton samples, with a SYBR Green assay. Based on the standard curve analysis, the observed theoretical minimal qPCR detection level was 0.0005625 ng.µL<sup>-1</sup> (R<sup>2</sup> = 0.99) at a PCR quantification cycle of 14.09–29.56. We also presented a practical guide to be used in monitoring and detection of *L. fortunei*. The optimized protocol was efficient in detecting *L. fortunei* and can be used to monitor already infested environments or invasions in new environments.

**Keywords:** biological invasion; biomonitoring; golden mussel; qPCR; reservoir.

**Resumo:** Nós otimizamos uma metodologia para detecção do mexilhão-dourado em amostras de DNA ambiental de plâncton e a validamos em amostras de um reservatório no sul do Brasil. *Limnoperna fortunei* é uma espécie exótica invasora bem-sucedida que promove impactos significativos nos ecossistemas de água doce. Nós ajustamos e validamos a metodologia para detectar *L. fortunei* em amostras de plâncton em reservatório, através de ensaios com SYBR Green. O nível teórico mínimo de detecção de qPCR observado, com base na análise de curva padrão, foi de 0,0005625 ng.µL<sup>-1</sup> (R<sup>2</sup> = 0,99) em um ciclo de quantificação de 14,09–29,56. Nós também apresentamos um guia prático



a ser utilizado no monitoramento da invasão de *L. fortunei* em bacias hidrográficas. A metodologia ajustada foi eficiente na detecção de *L. fortunei* e, portanto, pode ser usada para monitorar ambientes já infestados ou invasões em novos ambientes em larga escala.

**Palavras-chave:** invasão biológica; biomonitoramento; mexilhão-dourado; qPCR; reservatórios.

## 1. Introduction

Invasive alien species (IAS) are changing environments worldwide at an unprecedented rate, threatening to deplete and to homogenize the ecosystems and their respective environmental services (Mack et al., 2000; Rahel & Olden, 2008). The biological invasions are associated with biodiversity loss and the provision of ecosystem services, as supporting, provisioning, regulating and cultural services (Vilà & Hulme, 2017). Therefore, early detection and rapid response to early stages of invasive species infestation is critical for management and conservation of aquatic ecosystems (Goldberg et al., 2013; DePaula et al., 2020). The detection and quantification of IAS using visual or microscope identification methods can be difficult and time-consuming (Lucy, 2006; MacKenzie et al., 2006). Specially in the case of small or microorganisms, where the similarity found between species or in their initial life stages may result in uncertainties, adding an extra effort to the identification step (Baldwin et al., 1996).

The combination of real-time quantitative PCR (qPCR) with samples of environmental DNA (eDNA) has been used in the characterization, detection, and assessment of aquatic biodiversity (Deiner et al., 2017; Harrison et al., 2019). The use of eDNA has experienced considered growth and has been used in community and invasion ecology, because it allows you to obtain information from a bulk DNA sample (Taberlet et al., 2012). For this reason, the combination of qPCR and eDNA has been used as a technique to monitor aquatic environments (Jerde et al., 2011; Goldberg et al., 2013; Stoeckle et al., 2017), speeding up and bringing reliability to the use of non-lethal methods.

The golden mussel *Limnoperna fortunei* (Dunker, 1857) is an invasive bivalve originated from Southeast Asia (Ricciardi, 1998). It was detected for the first time in September 1991, in the Río de La Plata estuary (located between Argentina and Uruguay) (Pastorino et al., 1993). This species is spread throughout Brazil, Argentina, Paraguay, Uruguay, and Bolivia (Darrigran, 2002). In Brazil, *L. fortunei* is distributed along the Pantanal region, Paraná River watershed to the estuary of the La Plata River (Oliveira et al., 2015), and also can be found in the São Francisco River Basin

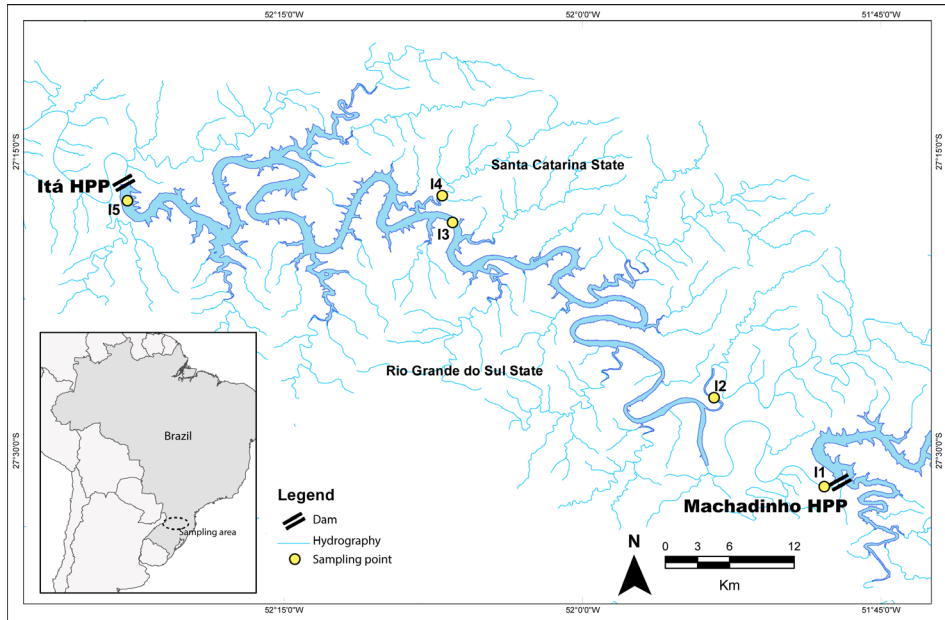
(Barbosa et al., 2016). However, according to the last update (Hermes-Silva et al., forthcoming), seven hydrographic basins in South America (Uruguay, South Atlantic, Paraná, Paraguay, San Francisco, Southeast Atlantic Basin and the Eastern Northeast Atlantic Basin) already had records of the invasion of *L. fortunei*.

The occurrence of *L. fortunei* can cause ecological and economical impacts in aquatic environments (for more information, see DePaula et al., 2020). Dispersion and colonization/establishment success of this species is associated with its biological attributes, such as high fecundity (Callil et al., 2012), free-living planktonic larvae (Cataldo, 2015), tolerance to prolonged periods of starvation (Cordeiro et al., 2016), and to different water temperatures (Andrade et al., 2018). As former preventive and controlling measures were inefficient, there is a demand to monitor golden mussel dispersion and establishment. eDNA analysis offers a cost-efficient approach, with high resolution and sensitivity to detect invasive species and monitoring (Ruppert et al., 2019; Beng et al., 2020). eDNA analysis relies on optimized protocols with species-specific primers that could avoid/address potential pitfalls (e.g., false negatives and false positives) (Roussel et al., 2015). Given this scenario, the present study aimed to optimize and validate a real-time PCR protocol for the detection of *L. fortunei* using eDNA plankton samples. Additionally, we tested two current protocols and discussed/addressed the limitations of each one, providing a new protocol for the detection of *L. fortunei* using environmental plankton samples.

## 2. Material and Methods

### 2.1. Sampling and DNA extraction

We used plankton samples and golden mussel adults collected at the Itá Reservoir, located in the upper section of the Uruguay River Basin (Figure 1). Five sampling sites were selected based on different water fluviometric characteristics under the reservoir area influence: I1 – Lotic environment with active water flow, located immediately downstream of Machadinho Hydroelectric Power Plant (HPP); I2 - Transitional lotic/lentic environment with moderate water movement, located on the Peixe River, a tributary of the Itá reservoir; I3 - Lentic



**Figure 1.** Sampling sites of eDNA plankton samples collected in the Itá reservoir, Upper Uruguay River, in Southern Brazil.

environment with slow-moving waters located in the reservoir body; I4 - Lentic environment with slow-moving waters located in one of the marginal area formed by the reservoir; and I5 - Lentic environment with still or slow-moving waters located in the reservoir body immediately upstream of the Itá dam.

Pure DNA from *L. fortunei* adult individuals was used as DNA positive control, whereas eDNA from plankton samples was used to validate the method. Adult individuals were preserved in alcohol 95% and stored in  $-20\text{ }^{\circ}\text{C}$  freezer. The extraction of the total DNA from adult individuals was performed using the saline method (Aljanabi & Martinez, 1997): 50 mg of the mantle were homogenized in 400  $\mu\text{L}$  of sterile salt homogenizing buffer (0.4 M NaCl 10 mM Tris-HCl pH 8.0 and 2 mM EDTA pH 8.0) for 10-15 s. Then 40  $\mu\text{L}$  of 20% SDS and 8  $\mu\text{L}$  of 20  $\text{mg}\cdot\text{ml}^{-1}$  proteinase K were added and mixed. The samples were incubated at  $60\text{ }^{\circ}\text{C}$  for 4 h, then 300  $\mu\text{L}$  of 6 M NaCl was added to each sample. Samples were vortexed for 30 s at maximum speed, and tubes spun down for 30 min at 14 000 g. Supernatant was transferred to new tubes, an equal volume of cold isopropanol was added to each sample, mixed and incubated at  $-20\text{ }^{\circ}\text{C}$  for 1 h. Samples were then centrifuged for 20 min,  $4\text{ }^{\circ}\text{C}$ , at 14 000 g, and the pellet was washed with 70% ethanol, dried and resuspended in 50  $\mu\text{L}$  of sterile  $\text{dH}_2\text{O}$ .

Plankton samples ( $n=20$ ) were collected seasonally in January, April, July, and October of 2019. Sampling was carried out with a plankton net (53  $\mu\text{m}$ ), using a motor pump to collect water just

below the surface line, following Tschá et al. (2012). To increase sample homogeneity, we pumped and filtered 200 L of water from each of two points located at a distance of 30 m, which were stored in the same bottle. Samples were fixed with ethanol (96%; 1:4 proportion) and were kept on ice to be transported to the laboratory, then stored in a  $-20\text{ }^{\circ}\text{C}$  freezer until further processing.

At the laboratory, water samples were initially filtered through a 100-micron nylon mesh and subsequently through a 0.22-micron membrane using a vacuum pump. Materials used in each filtration step were immersed in a bleach solution, rinsed with deionized water, and exposed to UV light for 20 min to avoid DNA contamination.

Total DNA was extracted using PureLink™ Microbiome DNA Purification Kit (Invitrogen™) according to the manufacturer's instruction. The filter paper with retained material was placed in a bead tube to perform the DNA extraction. DNA concentration and purity were analyzed in a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific) and stored at  $-20\text{ }^{\circ}\text{C}$ . All samples and specimens were collected under Brazilian environmental agency ICMBIO/SISBIO guidelines (License numbers 62772-1 and 62772-2).

## 2.2. Real-time PCR for *Limnoperna fortunei* detection

Real-time PCR protocols were evaluated for *L. fortunei* detection following the settings described by Endo et al. (2009) (named here as Protocol A)

and Pie et al. (2017) (Protocol B), briefly described below: Protocol A: Primers 5'-TCTCTTCATT TAGCTGGTGCGTC-3' (Limf-F) and 5'-CAAAACAGATGTA ACTGCAAGAGAC-3' (Limf-R); SYBR® Green QuantiFast PCR Kit (Qiagen). The reaction was performed with 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Protocol B: GGGACTGGTTGGACA GTTTAT (Forward); Probe 6FAMCCCAG CAGTTGACATAGCTGCTTTMGB-NFQ (Sense); ACGCACCAGCTAAATGAAGA (Reverse); 12.5- $\mu$ L total volume including 6.25  $\mu$ L of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 3  $\mu$ L of DNA as template, 10 nM of each primer, 25 nM of probe, 0.3  $\mu$ L of TaqMan® Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.15  $\mu$ L of TaqMan® Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems) and 2.8  $\mu$ L water. The reaction was performed with 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 1 min and annealing/extension at 65 °C for 1 min).

We also performed TaqMan assays using the primers and probe described in Protocol B: we tested higher probe and primers concentrations (0.15  $\mu$ M for probe and 1.0  $\mu$ M for each primer) and combined, in the same assay, higher concentration of probe (0.25  $\mu$ M) and lower concentration of primers (0.05  $\mu$ M), respecting the limits of the TaqMan assay. Besides that, we tested other probe quenchers (double quencher and NFQ quencher with MGB). In all these assays, we used a serial dilution of known amounts of target DNA from an adult *L. fortunei* individual, and eDNA plankton samples that tested positive for *L. fortunei* presence by conventional PCR (according to Boeger et al., 2007; data not shown).

Additionally, we tested an optimized version of the protocol, using QuantiFast SYBR Green PCR Mix (Qiagen) with primers described by Protocol B, but without the probe (6FAM-CCCAGCAGTTGACATAGCTGCTTT-MGB-NFQ-Sense) (Protocol C). The optimized qPCR assay was performed in a 20  $\mu$ L reaction volume containing: 10  $\mu$ L of QuantiFast SYBR Green PCR Mix (Qiagen), 1.0  $\mu$ L of each primer (Forward: GGGACTGGTT GGACAGTTTAT and Reverse: ACGCACCAGC TAAATGAAGA) (20  $\mu$ M), eight concentrations of DNA positive control /reaction (45, 4.5, 0.45, 0.045, 0.0045, 0.00225, 0.001125, 0.0005625 ng), and 3.0  $\mu$ L of RNase free water (Qiagen). A total of three replicates were carried out for each DNA concentration, and three replicates for negative control without the target DNA. The amplification was performed in a Rotor-Gene TM 6000 (Qiagen)

thermocycler, using the program as follows: 5 min at 95 °C, 35 cycles of 10 seconds at 95 °C, and 15 seconds at 60 °C. Then, we proceed to the validation of this protocol, detecting *L. fortunei* DNA in environmental plankton samples.

### 2.3. Validation of *Limnoperna fortunei* detection and quantitative estimation in eDNA plankton samples

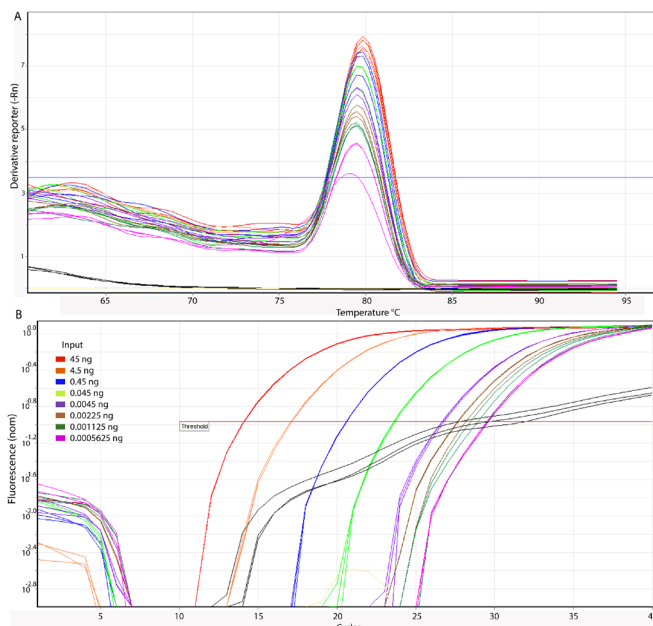
The qPCR assays were conducted using three replicates with 8.0 ng of eDNA/reaction from plankton samples and six replicates of no template control (NTC) reactions without a DNA template. We used the optimized reaction in a total 20  $\mu$ L containing 10  $\mu$ L of QuantiFast SYBR Green PCR Mix (Qiagen), 1.0  $\mu$ L of each primer (20  $\mu$ M), 8.0 ng of eDNA/reaction, and 3.0  $\mu$ L of RNase free water (Qiagen). The cycling conditions were: 5 min at 95 °C for polymerase hot-start activation followed by 35 cycles of 10 s at 95 °C denaturation, and 15 s at 60 °C annealing/extension. Rotor-Gene Q Series Software (version 2.3.4) was used to compare each sample with a standard curve produced with pure *L. fortunei* DNA. Melting curves (ramp from 60 to 95 °C, rising by 1 °C each step) were analyzed to nonspecific products and primer dimer formation.

## 3. Results

### 3.1. DNA extraction and qPCR reaction optimization

The eDNA samples concentration varied between 0.6 and 82.0 ng. $\mu$ L<sup>-1</sup>, and Optical Density (OD) 260/280 varied between 1.05 and 1.99. Following the original method described in Protocol A, reactions did not show specificity, suggesting the amplification of other species. The samples melting curve peaks were different from the expected for pure *L. fortunei* DNA or *L. fortunei* positive plankton samples. Following Protocol B, no amplification was achieved, even with modifications in reagents concentrations and probe chemistry. Our optimized protocol was able to amplify samples from adult *L. fortunei* individuals and plankton samples, with a minimum limit of 0.0005625 ng of *L. fortunei* DNA/reaction (Figure 2a). The melting curves analysis demonstrated a single peak at 79.6 °C, confirming *L. fortunei* DNA amplification and no peaks in negative controls (Figure 2b). A visual scheme of the sampling and laboratory protocols was provided (Figure 3).

The sensitivity of the qPCR-optimized assay (Figure 4), which represents the relationship between the PCR Quantification cycle (C<sub>q</sub>) and concentration of DNA for the standard curve,



**Figure 2.** (A) Quantitation curves of known amounts and (B) melting curves of known amounts to 45 (red), 4.5 (orange), 0.45 (blue), 0.045 (light green), 0.0045 (purple), 0.00225 (brown), 0.001125 (dark green), 0.0005625 (pink) ng of DNA/reaction of *L. fortunei* DNA. Negative controls are shown in black lines.

depicted by a straight line with a very high coefficient of determination ( $R^2$ ), was 0.9983. The  $C_q$  of 29.56 (14.09–29.56) was required to detect the minimum amount of DNA (Figure 4).

### 3.2. Detection of *L. fortunei* in plankton samples

All plankton samples were standardized for a quantity of 8.0 ng of DNA/reaction. Melting curves analysis demonstrated a single peak at 79.6 °C in 17 of 20 plankton samples, confirming *L. fortunei* DNA in the vast majority of samples, as shown in Figure 5. In the positive samples, the *L. fortunei* DNA quantification ranged between 0.00084 and 1.304179 ng of DNA/reaction.

The highest quantity of *L. fortunei* DNA per reaction (1.304 ng of DNA) was detected in April (site I4), followed by the second highest DNA load per reaction (0.949 ng of DNA) collected in October (site I2). The other samples showed loads below 0.2 ng of *L. fortunei* DNA, and only in three sampling sites species presence was not detected (I2/April, I3/July, and I4/July). *Limnoperna fortunei* was detected in all samples collected in spring and summer.

## 4. Discussion

The present study optimized and validated an SYBR Green qPCR protocol to detect the invasive golden mussel *Limnoperna fortunei* in eDNA plankton samples. Twenty samples were collected in the Upper Uruguay River throughout the year to assess the presence of *L. fortunei* in environmental samples

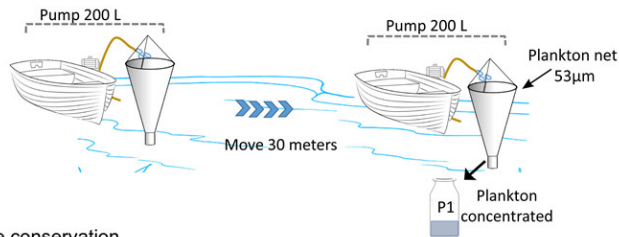
A former protocol proposed by Endo et al. (2009) showed no specific amplification in our samples from the Upper Uruguay River. Comparing the melting curves of pure *L. fortunei* (DNA positive control) and eDNA, the occurrence of no specific peaks in eDNA indicated amplification of other species. Therefore, this assay was inappropriate to evaluate environmental samples from the Upper Uruguay River. The second qPCR assay tested used hydrolysis probe as qPCR detection chemistry and primers developed by Pie et al. (2017) and also failed to amplify pure *L. fortunei* DNA even with adaptations in reagent concentrations. However, it was possible to detect *L. fortunei* DNA using primers described by Pie et al. (2017) with SYBR green as qPCR detection chemistry.

The optimized SYBR Green assay and sampling method proposed here allowed us to detect and quantify low levels of *L. fortunei* DNA (0.0005625 ng of target DNA/reaction), similar to the 0.000225 ng of DNA detected in original method (Pie et al., 2017), allowing detection at all sampling sites in the study area. Most eDNA studies focusing on detecting aquatic species have used samples from controlled laboratory experiments or in small ecosystems, such as shallow lakes (Eichmiller et al., 2014; Stoeckle et al., 2017). In addition to the natural degradation of the samples, eDNA samples are also influenced by dilution effects and physical destruction on lotic systems, the detection in rivers can be more difficult (Balasingham et al., 2017). However, despite the

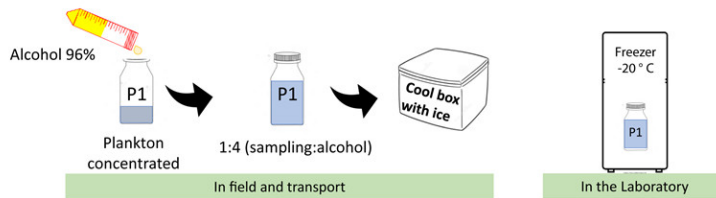
**Supplementary Material S1-** Optimized and validated protocol to detection and quantification of invasive *Limnoperna fortunei* environmental DNA from plankton samples.

**A practical guide: eDNA plankton sampling and qPCR analysis of golden mussel, *Limnoperna fortunei***

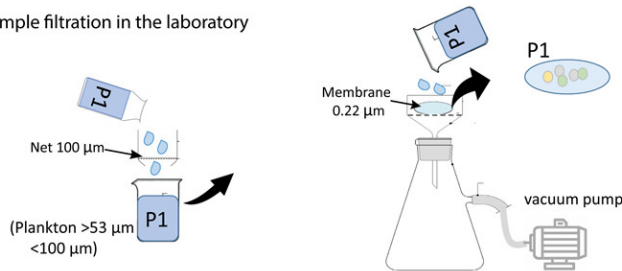
**1. Field sampling**



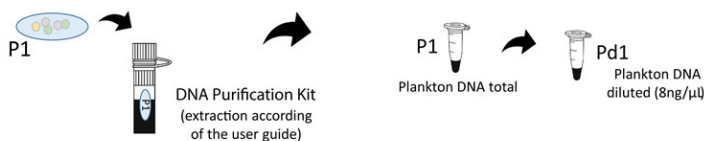
**2. Sample conservation**



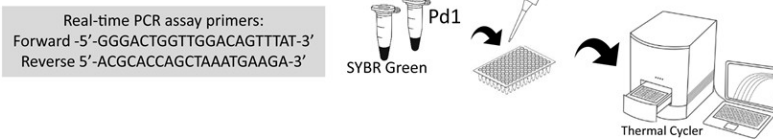
**3. Sample filtration in the laboratory**



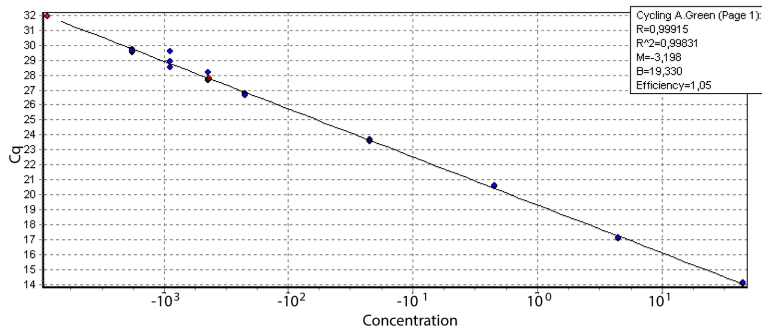
**4. DNA extraction and dilution**



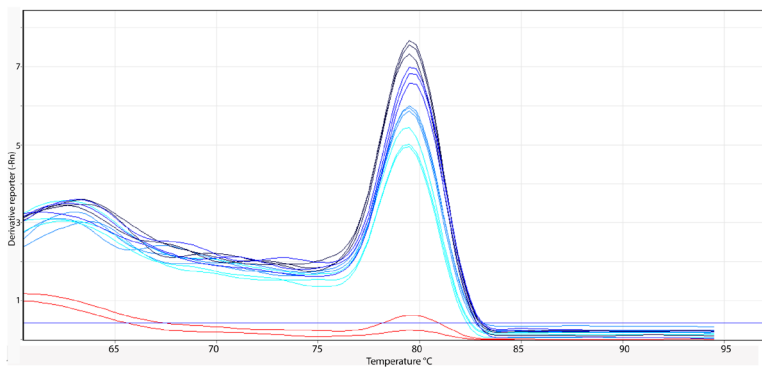
**5. Real-Time PCR**



**Figure 3.** A schematic figure with a practical guide to eDNA plankton sampling and qPCR analysis of *Limnoperna fortunei*: “A practical guide: eDNA plankton sampling and qPCR analysis of golden mussel, *Limnoperna fortunei*”. (1) Field sampling: Environmental DNA sampling using a plankton net (mesh opening of 53 µm), with a motor pump and a hose. Collect twice at the same sample site (filtering 200 L each) at two points (30 meters between each one) totaling 400 L of water. Concentrate the sample in the same bottle; (2) Sample conservation in the field: Fix the collected sample with 96% ethanol in the proportion of 1:4 (sampling:ethanol). Keep the flasks with the samples in ice until getting to the laboratory. Store the sample in a freezer at -20 °C; (3) Sample filtration at the laboratory: Pre-filter the samples with a 100 µm net. Use the filtrate to filter in 0.22 µm smooth membrane filtrates, with the aid of a vacuum pump. Sterilize the materials between one filter and another (UV light); (4) DNA dilution: Extract the DNA from the membrane using a purification kit, following the manufacturer’s recommendation. Quantify the total DNA extracted and standardize at 8.0 ng of DNA.µL<sup>-1</sup>; (5) Real-time PCR: Perform the PCR reaction in a 20 µL in a final volume containing 10 µL of QuantiFast SYBR Green PCR Mix (Qiagen), 1.0 µL of Forward primer (20µM), 1.0 µL of Reverse primer (20 µM), 8.0 ng of eDNA/reaction, and 3.0 µL of RNase free water (Qiagen) and the program as follows: 5 min at 95 °C, 35 cycles of 10 seconds at 95 °C, and 15 seconds at 60 °C.



**Figure 4.** PCR Quantification cycle (Cq) vs. DNA concentration of known amounts (45, 4.5, 0.45, 0.045, 0.0045, 0.00225, 0.001125, 0.0005625 ng of DNA/reaction) of *L. fortunei*.



**Figure 5.** Melting curves of eDNA plankton samples collected in the Itá reservoir, Upper Uruguay River, Southern Brazil. Blue lines represent positive samples, and red lines represent a negative sample for *L. fortunei*

presence of both lentic, lotic and transitional lotic/lotic environments in this study, the method's new settings allow, besides the detection, the quantification of the target DNA concentration in the eDNA sample, obtained through the value of Cq. The Cq curve can be used to measure the relative amount of target sequence in the qPCR reaction (Wilcox et al., 2013), indicating the density or abundance of the species in the environment (Takahara et al., 2012). The samples with higher Cq value indicate the smaller amount of the target DNA present in the sample because reactions with fewer initial copies of the target DNA require more amplification to degrade enough probe and emit fluorescence (Heid et al., 1996).

The methodology adjustment, such as the inclusion of a pre-filter step with a 100-micron mesh at the laboratory (Figure 3) allowed to reduce the concentration of non-target organisms in the study, such as some zooplankton species, and reduced organic matter, such as wood fragments and leaves. Cataldo (2015) showed that eggs size and first trochophore of *L. fortunei* phases falls between 80-100  $\mu\text{m}$  and 95-115  $\mu\text{m}$ , respectively, probably improving the concentration of the eDNA of the target species.

Sampling from varied environments of the reservoir at different seasons allowed us to validate the qPCR methodology, using eDNA plankton samples, because distinct environments may have different concentrations of the target DNA in the plankton and different composition and concentrations of other plankton organisms that could alter the success of amplification. eDNA methodology permits an early detection, is highly reproducible, fast, and technically an easy method to detect aquatic invasive species (Thomas et al., 2020). Early detection methods allow the adoption of adequate response measures in case of invasive species monitoring plans. The method described here can be used as a monitoring protocol in newly invaded environments and/or with low *L. fortunei* concentrations, helping to define adequate management and control plans focusing on preventing the invasion and establishment of the golden mussel in new environments.

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Barra Grande, Campos Novos Energia, Foz do Chapecó Energia.

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