



UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM
FACULTAD CIENCIAS DE LA VIDA
CARRERA DE ECOSISTEMAS

**FISH BIOMASS, DNA DEGRADATION AND CONCENTRATION
AS FACTORS INFLUENCING, EXPERIMENTALLY, THE
RELIABILITY OF THE eDNA TECHNIQUE FOR THE DETECTION
OF *Oreochromis niloticus***

Proyecto de investigación previo a la obtención del Título de
INGENIERA EN ECOSISTEMAS

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DEDICATORIA

A mis padres, Julio Oligario y María Isabel, mis hermanos, Pedro Israel y Camila Sofía, pilares fundamentales en mi vida, fuente de inspiración, constancia y paciencia. Gracias por nunca dudar de mis decisiones y confiar siempre en mis capacidades, este y todos mis logros irán siempre en su nombre.

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TABLA DE CONTENIDO

DECLARACIÓN DE DERECHO, AUTENTICIDAD Y RESPONSABILIDAD	ii
AUTORIZACIÓN DE PUBLICACIÓN EN EL REPOSITORIO INSTITUCIONAL	iii
CERTIFICADO DE DIRECCIÓN DE TRABAJO DE TITULACIÓN	iv
DEDICATORIA.....	v
AGRADECIMIENTOS	vi
RESUMEN	ix
ABSTRACT	x
INTRODUCTION.....	1
MATERIALS AND METHODS.....	2
Experimental materials.....	3
Adequacy of fish tanks and acclimatation of individuals of <i>Oreochromis niloticus</i>	3
Experimentation	3
<i>First section: Relationship between DNA concentration and biomass</i>	3
<i>Second section: DNA degradation over time</i>	4
<i>Third section: Determine the minimum DNA concentration required to be detectable by eDNA</i>	4
Filtration	4
Extraction and quantification of DNA, PCR and agarose gel visualization	4
Data analysis.....	5
RESULTS	6
Relationship between DNA concentration and biomass	6
<i>DNA degradation over time.</i>	6
<i>Lowest concentration detectable by eDNA technique</i>	7
DISCUSSION.....	8
CONCLUSIONS.....	10

ÍNDICE DE FIGURAS

Figure 1. Relationship between the natural logarithm of biomass (g) and logarithm of DNA concentration (ng/μl).....	6
Figure 2. DNA degradation over time during the experiment. Relationship between the natural logarithm of DNA concentration (ng/μl) and time (1, 5 and 21 days).....	7
Figure 3. Minimum DNA concentration required to be detectable by eDNA.....	8

RESUMEN

En la última década, la técnica de ADN ambiental ha sido ampliamente empleada ya que permite detectar y monitorear especies de manera no invasiva. Hasta el momento, varios estudios describen la eficacia de la técnica al alterar diversos factores como la temperatura, pH, degradación de ADN, entre otros, empleando diversas especies de peces. Este estudio se realizó en un ambiente controlado donde se analizó la fiabilidad de la técnica de ADN ambiental para detectar la tilapia de Nilo (*Oreochromis niloticus*), una especie altamente invasiva, que representa un recurso alimenticio importante en la amazonía ecuatoriana. Para ello, los objetivos del presente estudio fueron: i) Conocer cómo la densidad de individuos (biomasa) altera la cantidad de ADN recuperado usando la técnica de ADN ambiental; ii) Determinar cómo influye el tiempo en la degradación de ADN para ser detectado mediante la técnica de ADN ambiental y iii) Determinar la concentración mínima requerida de ADN para ser detectado empleando esta técnica. Los resultados de este estudio mostraron una relación positiva y estadísticamente significativa entre la densidad de individuos, medida como biomasa y la concentración de ADN ($r^2 = 0.62$; $p = 0.001$). Por otro lado, se determinó que, a los 21 días de retirar los individuos, la detección mediante ADN ambiental es casi nula. Finalmente, se encontró que la concentración mínima necesaria para detectar ADN de tilapia es de 0.1 ng/ul. Estos hallazgos permiten comprender cuán importante son estos factores para tener en consideración al detectar especies invasoras para tomar decisiones que permitan su manejo, evitando así la alteración de los ecosistemas.

Palabras clave: ADN ambiental, *Oreochromis niloticus*, primer MiFish U/E, degradación de ADN, concentración de ADN.

ABSTRACT

In the last decade, the environmental DNA technique has been widely used since it allows the detection and monitoring of species in a non-invasive way. So far, several studies have described the efficacy of the technique by altering different factors such as temperature, pH, DNA degradation, among others, using different fish species. This study was conducted in a controlled environment to analyze the reliability of the environmental DNA technique for detecting Nile tilapia (*Oreochromis niloticus*), a highly invasive species that represents an important food resource in the Ecuadorian Amazon. The aim of this study was to: i) determine how the density of individuals (biomass) alters the detectability of the environmental DNA technique; ii) determine how time influences the degradation of DNA to be detected by the environmental DNA technique; and iii) determine the minimum concentration of DNA required to be detected using this technique. The main results of this study showed a positive and statistically significant relationship between the density of individuals, measured as biomass and DNA concentration ($r^2 = 0.62$; $p = 0.001$). Furthermore, it was determined that 21 days after the removal of the individuals from the experimental tanks, the detection by environmental DNA is almost null. Finally, it was found that the minimum concentration required to detect tilapia DNA is 0.1ng/μl. These findings allow us to understand how important these factors are to take into consideration when detecting invasive species in order to make decisions that allow their management, thus avoiding the alteration of ecosystems.

Key words: environmental DNA, *Oreochromis niloticus*, MiFish U/E primer, DNA degradation, DNA concentration.

INTRODUCTION

Globally, invasive species are one of the main causes of biodiversity loss. It is estimated that their presence alters ecosystems, causing the displacement of native species and reducing species diversity, which implies alterations in the food chain [1]. The Amazon basin is considered a biodiversity hotspot [2] that is very sensitive to anthropogenic impacts such as, among others, overfishing and species invasions, leaving its richness and diversity vulnerable [3]. A recent study showed that the Napo and Nanay river basins, tributaries of the Amazon basin, have the highest richness of introduced fish species per unit area [4]. Some of the introductions in these basins are caused by fish farming including the case of Nile tilapia (*Oreochromis niloticus*) [5].

O. niloticus, a member of the Cichlidae Family, originally from Africa, has been introduced in at least 85 countries [6] and is considered a highly invasive species due to its rapid reproduction and growth rate. On the other hand, it also has a high protein value, which is key for aquaculture [7] and is the third species with the highest aquaculture production worldwide [8]. In Ecuador, *O. niloticus* represents the basis of aquaculture production and its cultivation stands out in the Amazon region for local consumption [9]. However, local production in the region usually employs traditional rearing methods in fish ponds that do not have an appropriate water outlet system, running the risk of individuals moving from the ponds into water bodies. By possessing a high invasive potential, the establishment of *O. niloticus* in natural water bodies is feasible and poses itself as a threat to the conservation of aquatic biodiversity [10].

Quantifying, identifying and monitoring invasive species is key to conserving ecosystems. Unlike the terrestrial environment, carrying out these actions in the aquatic environment is a challenge for researchers, since traditional methods such as nets or fishing rods are used for detection, which involve greater effort, sampling time and labor. The use of the environmental DNA technique (eDNA) is considered as an alternative to traditional monitoring methods (i.e. collection of specimens, visual surveys) that allows recording organisms through DNA fragments present in abiotic components such as water, soil or sediments, implying less labor and collection time [11]. It has been determined that biotic factors as metabolic rate, stress, diet or migration and abiotic factors as UV radiation, pH or temperature influence the presence of DNA on the ecosystems [12]. However, it is important to determine the efficiency and sensitivity of environmental DNA depending on factors such as the density of individuals in the

environment (biomass) and DNA degradation over time [13] [14]. Furthermore, a specific amount of DNA to be detected by environmental DNA has not been assessed for *O. niloticus*.

For a reliable recovery of eDNA, it is required that the DNA is of high quality, i.e. that despite being fragments, these are not so degraded and this will depend on the level of DNA excretion of each species, where size, age, reproductive stage and environmental conditions of the environment will also influence [15]. Additionally, in relation to DNA degradation time, it has been determined that in artificial ponds for Siberian sturgeon (*Acipenser baeri*) the detectability of DNA decreased over time, maintaining a probability of detection of less than 5% at 17 days after removing the individual, i.e., after 17 days it is very unlikely that the presence of the fish will be detected by the environmental DNA technique [16]. Following the same pattern, but using mesocosms with common carp (*Cyprinus carpio*), there was a reduction in the concentration of DNA recovered over time [17]

In order to contribute to the standardization of the environmental DNA technique for the detection of *Oreochromis niloticus*, as an invasive species in the Amazonian region, we developed laboratory experiments to evaluate the effectiveness of environmental DNA as a function of the density of individuals (biomass) in the experimental environment, DNA degradation over time and the minimum DNA concentration needed to be detected by this technique.

MATERIALS AND METHODS

The experimental development will be divided into three sections, each one corresponding to the specific objectives: i) determine how the density of individuals (biomass) alters the detectability of the environmental DNA technique; ii) determine how time influences the degradation of DNA to be detected by the environmental DNA technique; and iii) determine the minimum concentration of DNA required to be detected using this technique.

Experimental materials

13 fish tanks were placed on the laboratory, one of 60 L capacity and twelve of 40 L capacity. The one glass fish tank of 60 L capacity was used to store tilapia fry. The first two sections (eDNA vs Biomass and time) were developed on twelve fish tanks of 40 L capacity, which were filled with a volume of 20 L with tap water. The third section, dedicated to detect the minimum DNA concentration needed by the technique, was developed in 500 ml polypropylene bottles. The 12 smaller fish tanks (20 L capacity) and the 500 ml bottles were disinfected using water, soap, 1% chlorine, type II autoclaved water, and 70% alcohol to eliminate any DNA residue. Additionally, bottles were autoclaved at 121°C for 60 min.

Adequacy of fish tanks and acclimatation of individuals of *Oreochromis niloticus*.

The adaptation of 13 glass fish tanks was carried out in the biology laboratory of the Universidad Regional Amazónica Ikiam. Three water filters were placed in the largest fish tank, which had a capacity of 60 L: physical (sponge/cotton), chemical (activated carbon), and biological (ceramic, used for the establishment of nitrifying bacteria). A water circulation pump JAD® was also installed in this tank, and the water was cycled. To achieve acclimatization, an adult individual of *O. niloticus* (approximate weight: 0.60 kg) was placed in the tank for 15 days. Physicochemical parameters such as temperature, pH, electrical conductivity, dissolved oxygen, nitrates, and ammonium were measured twice a week to monitor water quality. After 15 days of acclimatization, the adult individual was removed, and the 100 tilapia fry were placed.

Experimentation

For the first and second sections the fry were extracted randomly from the experimental fish tank.

First section: Relationship between DNA concentration and biomass

Three treatments were established with different numbers of individuals (1, 5, and 10) on the twelve fish tanks of 40 L capacity filled with 20 L of tap water. Additionally, a control treatment without the addition of fish was also established. All treatments had three replicates, totaling 12 tanks and 48 fry. The individuals were kept for a period of 24

hours and were then removed to take a 500 mL water sample, resulting in a total of 12 water samples collected.

Second section: DNA degradation over time

Three treatments were established with different collection times (1, 5, and 21 days) using five individuals on the twelve fish tanks of 40 L capacity filled with 20 L of tap water, after removed the fishes a plastic cover was collocated over each fish tank. Additionally, a control treatment was also established without the addition of fish. All treatments had three replicates, totaling 12 tanks and 45 fry. The individuals were kept for a period of 24 hours and then were removed to take a 500 mL water sample, depending on each time required (1, 5, or 21 days), resulting in a total of 12 water samples collected.

Third section: Determine the minimum DNA concentration required to be detectable by eDNA

Five treatments were established with the same volumes of type II autoclaved water (500 ml) using five microliters of a known concentration of pure liver DNA of *O. niloticus* (0.2, 0.4, 0.6, 0.8, and 1 ng/μl), previously extracted according to the protocol [18]. Additionally, three control treatments without the addition of fish DNA were also established. Each of the treatments had three replicates, totaling 15 bottles. After 24 hours, all the 500 ml water samples were filtered.

Filtration

We used filters with 47 mm diameter and pore size of 0.45 μm Nitrocellulose Mixed Ester (MCE) in a glass vacuum filtration system to eDNA water filtration. Before use, the filtration system was sterilized by washing it with soap and water, followed by soaking it in 1% chlorine for three minutes. After that, it was rinsed with autoclaved distilled water, soaked in 70% alcohol for three minutes, and finally autoclaved.

Extraction and quantification of DNA, PCR and agarose gel visualization

For the extraction of DNA from the filters, the protocol of Carrera-González et al., 2021, was followed [19]. Once the eDNA sample was extracted and suspended with 40 μL of nuclease-free water, it was stored at -20°C. For quantification, 2 μL of each sample was

vortexed and placed in the UV-Vis Thermo Scientific™ NanoDrop™. For DNA amplification, the protocol of Carrera-González et al., 2021 [20] was followed with some modifications, utilizing the MiFish-U/E primers targeting a hypervariable region of the 12S rRNA gene (163-185 bp), the sequence of the first forward MiFish-U-F is TTTCTGTTGGTGCTGATATTATTGC GTC GGT AAA ACT CGT GCC AGC and the sequence of the first reverse MiFish-U-R is ACTTGCCTGTCGCTCTCTCTATCTCTTC CAT AGT GGG GTA TCT AAT CCC AGT TTG. The reagents that constituted the Master Mix solution were: water nuclease-free 6 µL, GoTaq Green Master Mix 12.5 µL, primer forward 1 µL, primer reverse 1 µL, BSA 2.5 µL and DNA 2 µL.

Once the samples are prepared, the amplification process will commence. The thermal profile for the amplification, which will be set in the thermal cycler as: initial duration 95°C for 180 s, denaturation 95°C for 20 s and 35 cycles, annealing 60°C for 15 s and 35 cycles, extension 72°C for 15 s and 35 cycles, final extension 72°C for 300 s and the cooling 4°C until the samples were removed.

After the PCR was completed, the PCR products were stored at -20°C. A 2% agarose gel with Safe DNA gel stain was used to visualize the PCR products in the electrophoretic chamber Thermo Scientific™. A total volume of 5 µL of each sample and the ladder were carefully loaded into the gel. The configuration for the electrophoretic chamber Thermo Scientific™ was set to 100 Volts and 400 mA, and the process ran for a duration of 75 minutes.

The gel was transferred to the ENDURO™ GDS Gel Documentation System, once the electrophoresis run was completed. The resulting PCR image was captured using the system for further analysis and documentation.

Data analysis

Data were processed with RStudio, for the first section was developed a linear regression analysis to see how the variation of the biomass affects the eDNA concentration. For the second section an ANOVA analysis were developed to explore how the eDNA concentration change as a function of the time, showed on the Appendix A. For the last section it was applied a logistic regression to determine how the detectability of DNA measured as presence or absence, is related with the concentration of DNA (Appendix B).

RESULTS

Relationship between DNA concentration and biomass

The linear regression between the logarithms of biomass (g) and DNA concentration (ng/μl) showed a positive and significant relationship ($R^2 = 0.62$, $p = 0.001$; Figure 1). There is a general trend of increasing DNA concentration as biomass increases.

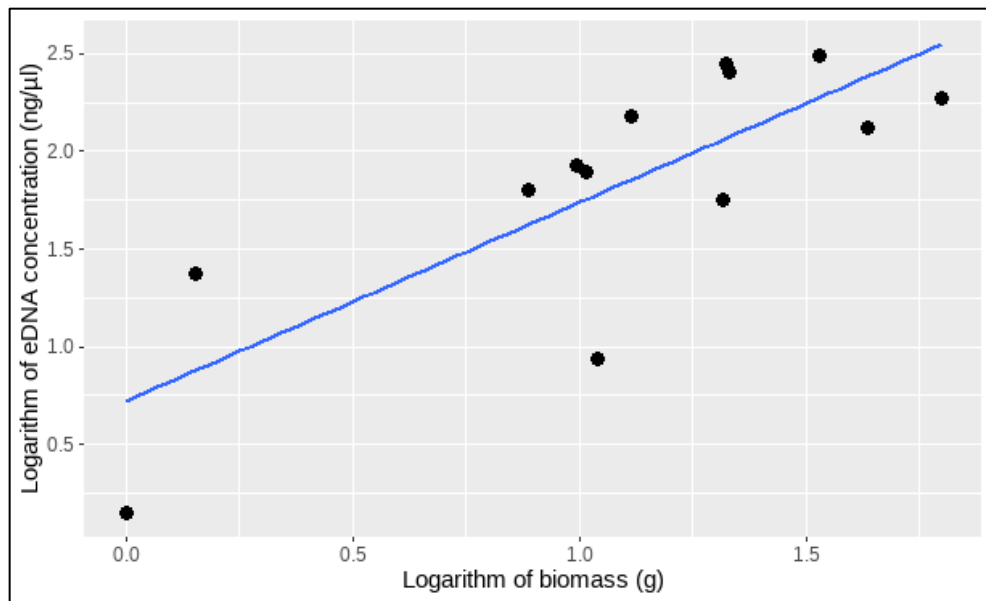


Figure 1. Relationship between the natural logarithm of biomass (g) and logarithm of DNA concentration (ng/μl).

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Additionally, results from the PCR, visualized in the horizontal electrophoresis gel, confirmed the presence of fish in all expected band marks for 1 (lanes 1- 6), 5 (lanes 7 - 12) and 10 individuals (lanes 13 - 18) in about 260 base pairs (bp). Also, the absence of tilapia eDNA was confirmed in the negative controls (lanes 19-26 and 28) (Appendix C).

DNA degradation over time.

The relationship between time and eDNA degradation measured as eDNA concentration is shown in figure 2, visually as time elapses the eDNA concentration decreases, so that by day 21 the DNA quantification is close to zero. However, the ANOVA showed that there are no significant differences between the independent variable (days) and the dependent variable (concentration of eDNA) (F value = 0.941, $p = 0.346$; Figure 2).

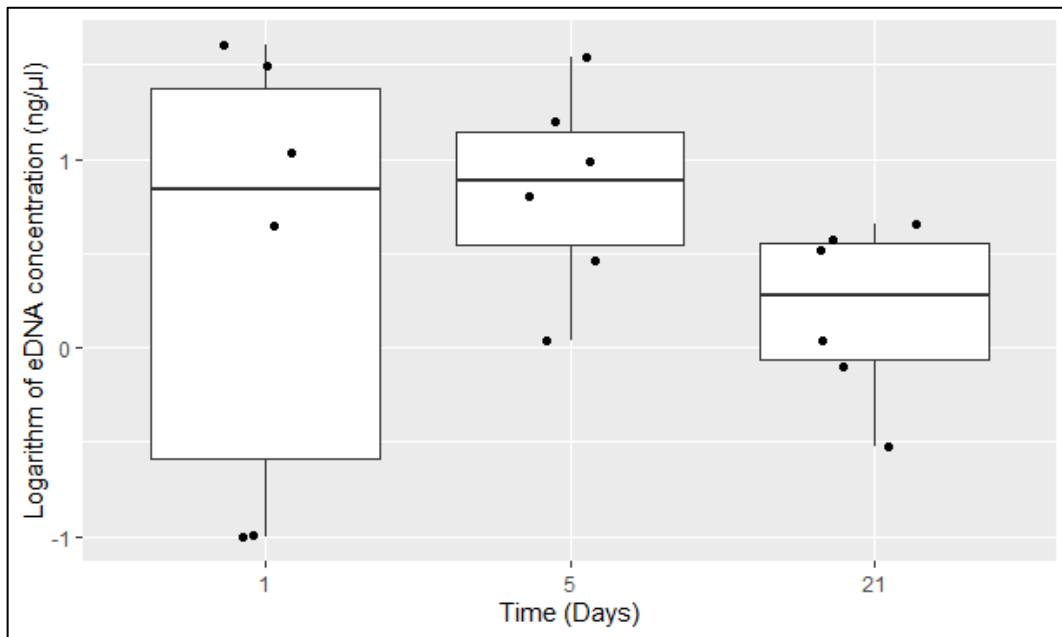


Figure 2. DNA degradation over time during the experiment. Relationship between the natural logarithm of DNA concentration (ng/μl) and time (1, 5 and 21 days).
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In the resulting electrophoresis gel, shown in Appendix D, we can observe that for days 1 (lanes 1-6) and 5 (lanes 7-12) the band is present, being a little fainter for day 5. However, by day 21 (lanes 13-18) the presence of the band is almost null. In the lower part of the gel no tilapia eDNA was detected in the negative controls.

Lowest concentration detectable by eDNA technique

The lowest concentration of DNA at which PCR no longer amplified in our experiment was 0.2 ng/μl (figure 3). It should be emphasized that 5 μl of that concentration was placed in the 500 ml, this implies that the final concentration at which DNA was no longer detected in our experiment was 1 μl.

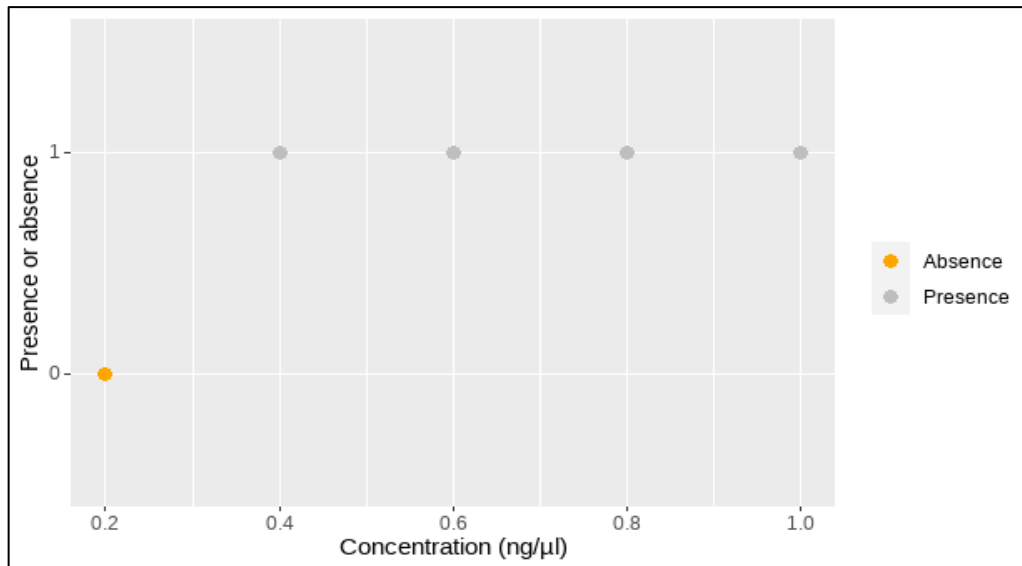


Figure 3. Minimum DNA concentration required to be detectable by eDNA.
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DISCUSSION

In the present study, we were able to determine that although the density of individuals varies, the detection by eDNA is maintained. However, the intensity of the detection is positively influenced by the fish biomass in the experimental tank. We also determined that after 21 days that the individuals were removed from the experimental tanks, the presence of DNA is almost undetected. Finally, to our knowledge, this is the first study determining the minimum concentration of tilapia DNA required to be detected via eDNA using the MiFishU/E primer. The one that, after calculations according to the concentration added in a certain volume of water (500 ml) was 1 ng/μL.

The detection of eDNA was not affected by the variation in the density of individuals, since for one individual, five individuals and ten individuals the presence of the band in the electrophoresis gel is distinguishable (Appendix C). However, in the same figure we can observe that there is a variation in the intensity of the band that differs for the first experimental sample and the second experimental sample. This occurred due to the body size of the fish. Since the first experimental sample was taken when the fish were 3 months old and the second experimental sample when the fish were 7 months old, considering that the DNA emanated by fish comes from feces, body surface mucus, sperm, eggs and carcasses [21], therefore small fish extrude a lower amount of DNA compared to larger fish. Recent studies estimated that the rate of DNA emanation in

adult *Lepomis macrochirus* fish is 12 times higher than the rate of releasing in juveniles [22]. More fish individuals are reflected on higher intensity of the electrophoresis band [23].

As the amount of biomass increases, the concentration of eDNA also increases, and thus the intensity of the bands on Appendix C. Our results in experimental conditions were in accordance with those found in natural or mesocosms ecosystems, where low densities of individuals strongly influenced the detection by eDNA technique [13] [24].

As expected, the more time goes by, the eDNA concentration decreased in our experimental units. By day 21 the eDNA concentration is close to zero. Although there is DNA present on the quantification with NanoDrop™ (Figure 2), this is because the reliability and specificity of NanoDrop™ is not as high compared to other tools such as Qubit™ or qPCR to provide much more reliable information [25] and also the DNA quantified is most likely originated from DNA of other organisms such as humans, insects or bacterias present in the water [26]. In our case, we used tap water for experimental sections one and two, so the presence of DNA from other organisms most likely influenced the quantification. However, when obtaining the quantification of control samples that did not contain tilapia, we subtracted the control quantification minus the quantifications with tilapia to reduce the error generated. In other fish species such as *Acipenser baerii* detectability also decreases as the eDNA degrades, by day 17 there is a 5% chance of being detected, while by day 21 the chance is 1% [16].

DNA detectability varies among organisms, either due to the amount of DNA released or the conditions of the ecosystem in which they live. Thus, for example for *Dicamptodon aterrimus*, which is a giant salamander that it is a difficult to detect using traditional survey methods, it was determined that during sunny conditions, DNA degradation is faster than in shaded conditions [27]. Our experiment did not include modification of environmental variables such as temperature, UV rays or water flow, which influence DNA degradation, so we estimated degradation of tilapia DNA in a controlled environment. Estimating these values in experimental conditions allows us to understand the longest time frame that DNA remains detectable before being degraded after maintaining all other factors constant.

The use of environmental DNA allows us, in a non-invasive way to detect species inhabiting an ecosystem. In the developing this experiment we have confirmed that more biomass means more detectable DNA in the environment, we also acknowledge that

time is key to determine the presence of an organism and after 21 days, DNA degrades to a point that is undetectable under controlled conditions. However, the minimum amount of DNA that must be available in the environment to be detected by eDNA was still poorly understood. Our results show that the minimum concentration detectable was 1 ng/ μ L. However, this is a very sensible threshold, as the increase of only 1 ng/ μ L to that concentration, caused the band in the electrophoresis gel to be visible again, as observed in Appendix D. We also determined that 0.45 μ m nitrocellulose filters are effective for the retention of pure DNA molecules that are partially small compared to eDNA molecules that can be found in tissue, mucus, feces, etc. [28].

Knowing and understanding what and how external factors alter the reliability of the detectability of the eDNA technique is key for the application of this tool in the management and control of *O. niloticus* as an invasive species. This is crucial as tilapia is the most important species for aquaculture in many countries around the world. Monitoring the invasiveness of this species by applying non-invasive techniques such as eDNA is key to contributing to maintaining biodiversity of aquatic ecosystems.

CONCLUSIONS

We have determined that the density of individuals, degradation and DNA concentration are factors that influence the detection reliability of the eDNA technique, since altering one of these factors implies greater or lesser detection. Due to its sensitivity, eDNA technique should not be maintained as a single tool when monitoring species, but should be implemented as a complement tool with traditional techniques such as fishing. Our methodology and results allowed us to understand more about the behavior of *O. niloticus* when releasing DNA in a closed environment in a given time, which is important to estimate how it could occur in ecosystems. This work provides insights and data on the role of biomass, time and detection of this invasive species to calibrate eDNA as a tool for monitoring programs on biodiversity and health of aquatic ecosystems. For future investigations I strongly recommend to apply the same methodology on mesocosms, which would bring an approach of natural ecosystems, also with the implementation of different native species as *Piaractus brachipomus* to know if there are differences of the behavior in comparison with *O. niloticus* on the releasing of DNA and DNA degradation over time.

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SUPPORTING INFORMATION

Appendix A. ANOVA results calculated for the second section: DNA degradation over time.

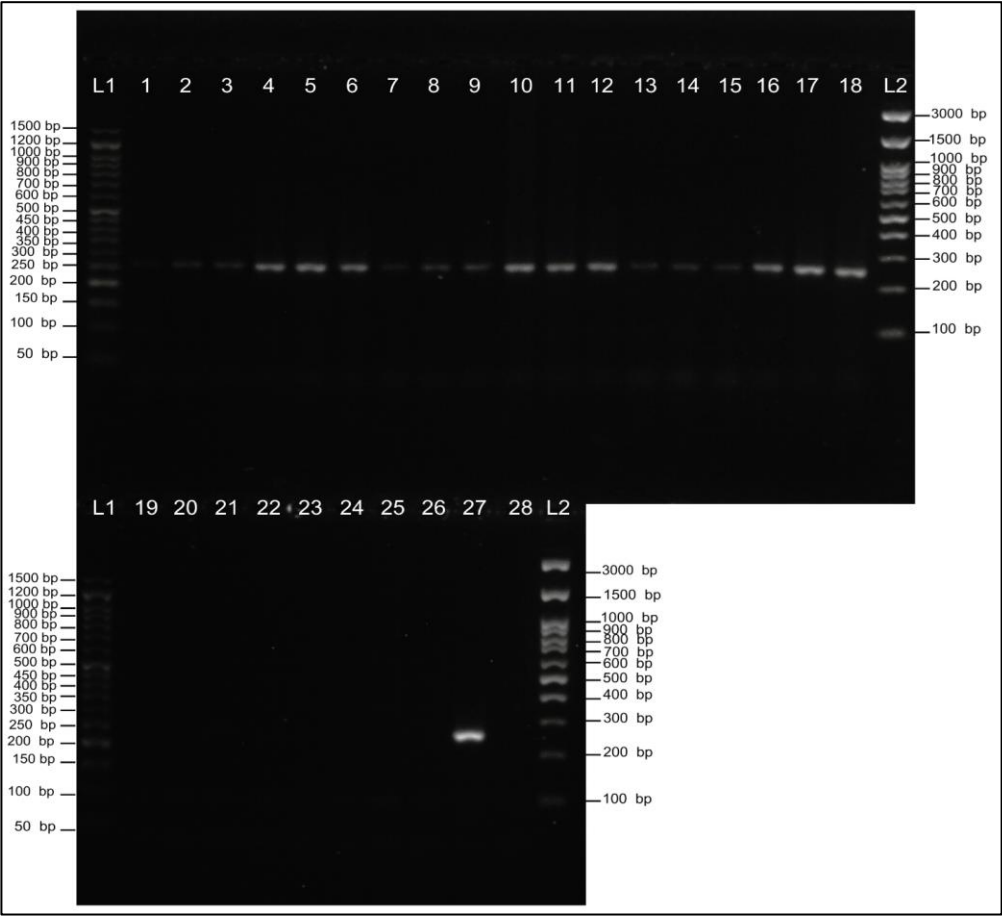
	Df	Sum Sq	Mean Sq	<i>F value</i>	<i>Pr (>F)</i>
Days	1	0.597	0.5969	0.941	0.346
Residuals	16	10.149	0.6343		

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Appendix B. Logistic regression to determine how the detectability of DNA is related with the concentration of DNA

	Estimate	Std.Error	z value	<i>Pr (> z)</i>
Intercept	-70.88	109479.28	-0.001	0.999
Concentration	16	10.149	0.001	0.999

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Appendix C-1. Lane L1 100 bp molecular weight marker (Biological Materials Inc.);
Lane L2 100 bp molecular weight marker (NIPPON Genetics)

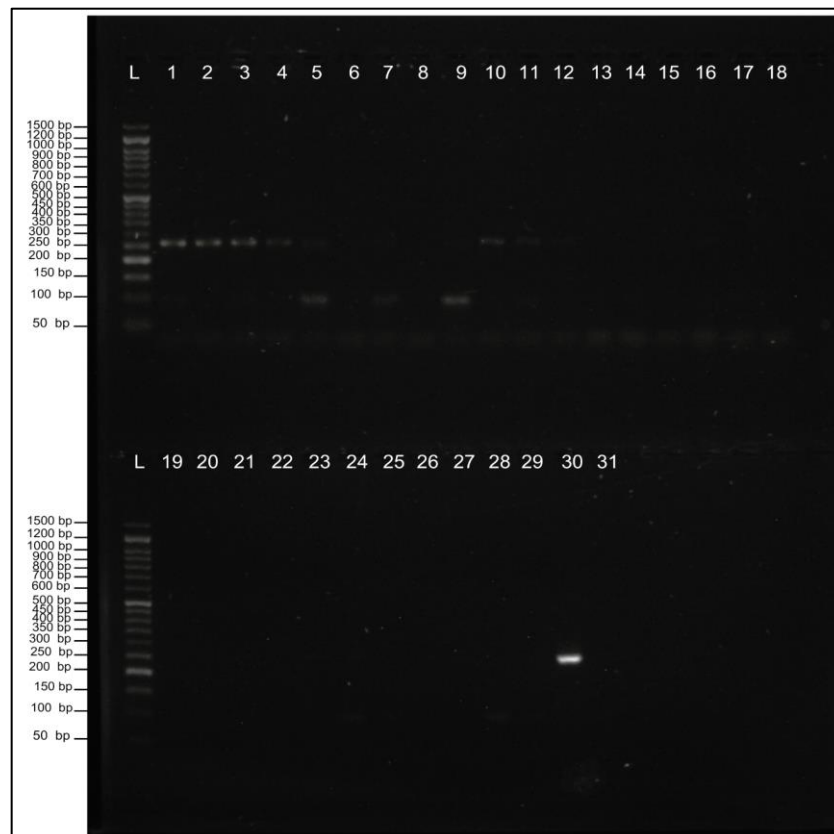
Made by: Quizhpi, Karina Belén, 2023

Appendix C-2. Description lanes.

Upper part of the gel	Lower part of the gel
Lane L1: Ladder; Lane 1: D1 I	Lane L1: Ladder; Lane 19: Cn D1
Lane 2: D1 II; Lane 3: D1 III	Lane 20: Cn D5; Lane 21: Cn D10
Lane 4: De1 I; Lane 5: De1 II	Lane 22: Ct D; Lane 23: Cn De1
Lane 6: De1 III; Lane 7: D5 I	Lane 24: Cn De5; Lane 25: Cn De10
Lane 8: D5 II; Lane 9: D5 III	Lane 26: Ct De
Lane 10: De5 I; Lane 11: De5 II	Lane 27: Positive control
Lane 12: De5 III; Lane 13: D10 I	Lane 28: Negative control
Lane 14: D10 II; Lane 15: D10 III	
Lane 16: De10 I; Lane 17: De10 II	
Lane 18: De10 III; Lane L2: Ladder	

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Lane L1 100 bp molecular weight marker (Applied Biological Materials Inc.); Lane L2 100 bp molecular weight marker (NIPPON Genetics); Lanes 1-28: eDNA PCR products with MiFish-U/E target 12S rRNA gene (163-185 bp); Lanes 19-26: Experimental and transport control; Lane 27: positive reaction control; Lane 28: negative reaction control.



Appendix D-1. Lane L1 100 bp molecular weight marker (Applied Biological Materials Inc.).

Made by: Quizhpi, Karina Belén, 2023

Appendix D. Description lanes

Upper part of the gel	Lower part of the gel
Lane L1: Ladder; Lane 1: Tc24 I	Lane L1: Ladder; Lane 19: Cn 24
Lane 2: Tc24 II; Lane 3: Tc24 III	Lane 20: Ct 24; Lane 21: Cn 5
Lane 4: Te24 I; Lane 5: Te24 II	Lane 22: Ct 5; Lane 23: Cn 21
Lane 6: Te24 III; Lane 7: Tc5 I	Lane 24: Ct 21; Lane 25: CnTe 24
Lane 8: Tc5 II; Lane 9: Tc5 III	Lane 26: CtTe 24; Lane 27: CnTe 5
Lane 10: Te5 I; Lane 11: Te5 II	Lane 28: CtTe 5; Lane 29: CnTe 21
Lane 12: Te5 III; Lane 13: Tc21 I	Lane 30: Positive control
Lane 14: Tc21 II; Lane 15: Tc21 III	Lane 31: Negative control
Lane 16: Te21 I; Lane 17: Te21 II	
Lane 18: Te21 III	

Made by: Quizhpi, Karina Belén, 2023

Lane L1 100 bp molecular weight marker (Applied Biological Materials Inc.). Lanes 1-31: eDNA PCR products with MiFish-U/E target 12S rRNA gene (163-185 bp); Lanes 19-29: Experimental and transport control; Lane 30: positive reaction control; Lane 31: negative reaction control.