Development and validation of a qPCR method for the Detection of the European eel (*Anguilla anguilla*) in Cyprus freshwater habitats through eDNA monitoring

Submitted to Swansea University in fulfilment of the requirements for the Degree of Master of Research"

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Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed...  .....

Date 03/01/2023

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for electronic sharing

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The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

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1.0 Introduction

1.1 Species biology, distribution, and its conservation problem

The European eel (*Anguilla anguilla*) is a catadromous teleost fish of the Anguillidae family. It exhibits a complex life cycle with seven distinct life stages across oceanic and freshwater habitats (Kettle, Asbjørn Vøllestad, & Wibig, 2011). Based on molecular markers, their population is panmictic, spawning in the Sargasso Sea and subsequently migrating to the Mediterranean, Baltic and other seas (Als et al. 2011). However, the panmixia hypothesis is still disputed by some researchers suggesting that non-random mating occurs thus differentiation through separate populations is still possible (Wirth and Bernatchez 2001). Their life cycle begins with their eggs being transformed into leptocephalus larvae that travel within oceanic currents to the African and European continents on a journey of 6,000 km triggered by several environmental factors. Subsequently, the larvae metamorphose into unpigmented glass eels reaching the continental entrance to estuaries. Glass eels are then transformed into yellow eel/elver and follow an upstream migration to different freshwater bodies. The last stage of their life cycle consists of metamorphosing again into silver eels (Silvering) undergoing visible anatomical and physiological changes such as enlargement of the eyes and fins (Baan, De Meyer, De Kegel, & Adriaens, 2020). Furthermore, upon reaching their continental freshwater habitats they will remain until their full maturation depending on their sex (males: 8-15y, females 10-18y) (Figure 1) (Bruijs and Durif 2009).

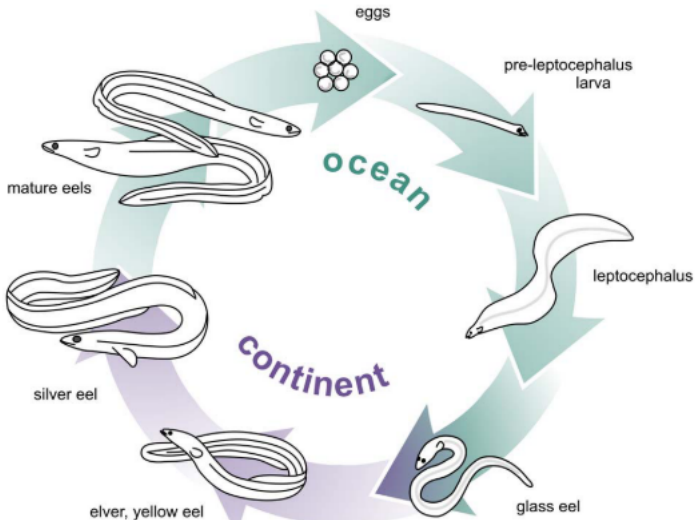


Figure 1 Seven distinct life stages of the European eel across its continental and oceanic migrations (Henkel et al., 2012)

Due to the complexity of the life cycle and difficulty in monitoring their behaviour, migration patterns are poorly understood thus reducing the efficiency of existing conservation efforts (Henkel et al. 2012). Furthermore, through the past decades, populations of European eel (*Anguilla anguilla*) have been severely reduced across the majority of their range, with some reports indicating reductions of up to 95% (Kettle et al. 2011). In response to that, the European eel has been listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Crook et al. 2018) with also having a “critically endangered” status by the International Union for Conservation of Nature (IUCN) red list (Jacoby and Gollock 2014). In contrast with all the above regulations the European eel still faces severe threats caused by anthropogenic activities. Their exploitation as food in illicit trade (Richards et al. 2020), habitat fragmentation (Griffiths et al. 2020), overfishing and parasitism are some of the main factors that influence this decline (Gollock, Kennedy, Quabius, & Brown, 2004)

1.2 Threats to eel populations

Habitat fragmentation

Habitat fragmentation has been one of the main factors in worldwide biodiversity decline (Haddad et al., 2015; Nilsson et al., 2005). Habitat engineering of freshwater bodies through dams and other small barriers used for irrigation and electricity production directly disturb local ecosystem dynamics (Lundqvist et al. 2008). An increase in barrier density results in the reduction of free-flowing rivers, with Europe having the most fragmented freshwater ecosystems in the world, with more than 1 million barriers present (Belletti et al., 2020). Moreover, barriers according to their type and morphology are likely to pose evolutionary pressures on current species assemblages. For example, the barrier passage rate of fish can be determined by specific traits such as body size and shape (Jones et al., 2020). As such, fish especially from the anguillid family, have an elongated body that significantly decreases the probability of passing through hydro turbines in compared to other fish species (Griffiths et al. 2020). To conclude, their two transatlantic migrations and later upstream migration through several fragmented lentic and lotic habitats in nearly all their range will directly decrease their fitness.

1.3 Pollution

Pollution is also an important cause of eel decline as it can significantly decrease overall fitness through a plethora of mechanisms. Pesticide runoff and heavy metal pollution can bioaccumulate in the tissues and organs and in some cases cause the mortality of eels (Geeraerts and Belpaire 2010). In response to polluted conditions, European eels have shown reduced expression of genes involved in their metabolism. Highly polluted areas can reduce the production rate of important enzymes involved in mitochondrial respiration and oxidative phosphorylation (Jose M. Pujolar et al., 2012). Moreover, increased energy expenditure for detoxification has been observed in sub-adult eel found in heavy metal-polluted environments (Maes et al. 2013). Increased energetic expenditure will directly affect the necessary fat reserves that eels must possess in order to successfully complete their 6000km migration back to the Sargasso Sea as mature eels. Some estimations have revealed that an individual with less than 13% of body weight in fat reserves in relation to body weight will not complete or delay migration until adequate lipid energy is obtained. This lipid insufficiency can also affect the endocrine system having detrimental effects on gonads and embryonic development (Geeraerts & Belpaire, 2010; J. M. Pujolar et al., 2013).

1.4 Synergistic effects of impacts on eel populations

Invasive species and parasitism

Altered abiotic conditions in lentic and lotic habitats can favour the introductions of aquatic invasive species (AIS) (Dudgeon et al., 2006; Nilsson et al., 2005) and therefore, impact European eel populations through competition for resources (Bevacqua et al. 2011). For example, thermophilic non-native species can exploit new niches in higher longitudes due to a poleward shift in worldwide average temperature. This phenomenon allows their native abiotic conditions to be shifted or expanded in different regions thus increasing the possibility of invasion. In the case of the European eel, the synergistic effects of parasitism and AIS have been observed with the introduction during the 1980s of *Anguillicoloides crassus*, a parasitic nematode of the Japanese eel *Anguilla japonica* (Costa-Dias, Dias, Lobón-Cerviá, Antunes, & Coimbra, 2010). The parasite has a variety of intermediate hosts, including prey of the European eel such as molluscs and other fish. Following infection, the larvae and adult worms are established in the swim bladder thus disrupting the main buoyancy

regulation organ of the fish. Moreover, the feeding of *A. crassus* on blood is likely to pose a negative physiological response resulting in haemorrhages and reduced overall fitness (Lefebvre, Fazio, Mounaix, & Crivelli, 2013).

Trade and export

Another threat to the populations of European eel is the efficiency of the European ban on the trade and export. Several reviews indicate that illicit trade across Europe and Asia is present and at a large scale (Stein et al. 2016). Through DNA barcoding it has been confirmed that the demand of the Japanese eel (*Anguilla japonica*) has been fulfilled by two different pathways (Cardeñosa et al. 2019). One being the illegal capture and trade within the European Union and the other the increase of export by non-European countries where the CITEs have no jurisdiction. Regions such as Morocco, Algeria and Indonesia have exported 10-fold the number of eels in regards to exports before the ban (Nijman 2015, 2017). Moreover, the demand is also covered by other Anguilla species shifting the pressure to species highly likely to follow the same population reduction as the European eel.

1.5 The case study: why in Cyprus?

Freshwater fish assemblages are under threat in Cyprus, due to their biogeographical isolation and human activities on freshwater bodies such as dams (Zogaris et al., 2012). Large dams and small obstacles can significantly disrupt stream movement (Ovidio & Philippart, 2002). Due to these highly modified freshwater habitats, the knowledge of the current distribution and ecosystem dynamics of the European eel will be critical for the development and application of any future conservational efforts. For example, evaluating the current known population across different sites can be the steppingstone towards future restoration efforts such as restocking.

1.6 Aim

There are notable differences in the physiology and anatomy of the *A.anguilla* that require extensive research to monitor the impacts of several stressors such as dams (Dainys et al., 2018). Consequently, the development of efficient and financially feasible techniques will aid any future conservational efforts. Through eDNA and barcoding, the presence or absence of *A. anguilla* can be monitored with greater efficiency than traditional methods such as electrofishing and netting (Zogaris et al., 2012). In this study, I will apply a species-specific eDNA-qPCR assay identifying the distribution of the catadromous European eel. The magnitude of the worldwide decline in *A. anguilla* populations has yet to be extensively studied in Cyprus. As of now only one survey regarding eDNA collection from freshwater habitats has been conducted in 2020 by utilizing a metabarcoding approach. This will be further expanded below as the samples of this methodology have been obtained after author approval so they can be compared and contrasted with the species-specific qPCR assay that has been applied. By that, the pathway for future eDNA monitoring methods will be further expanded providing empirical data to future researchers regarding the efficiency and effectiveness of each of these approaches.

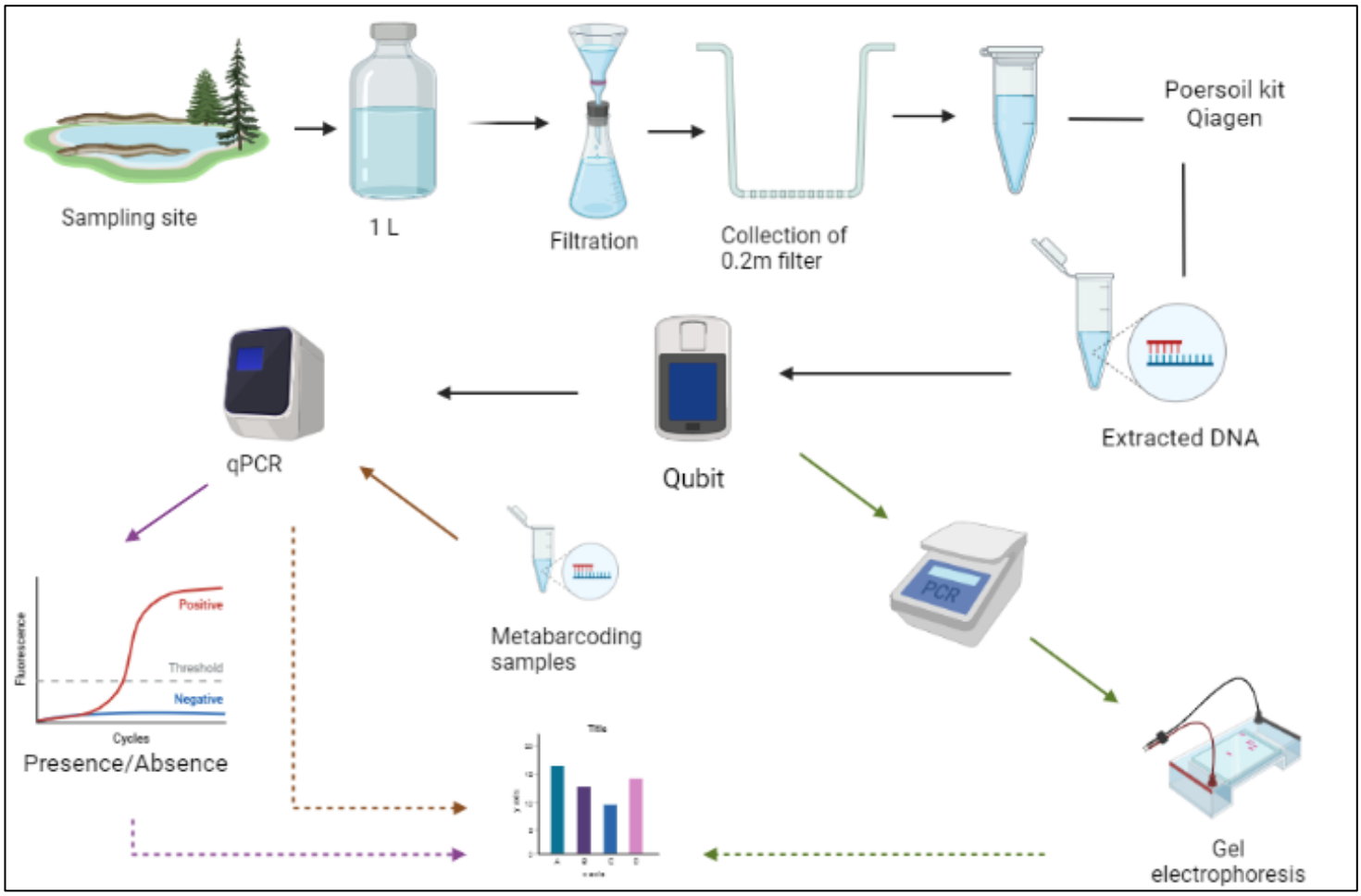


Figure 2: Flowchart of the three different approaches used in comparing eDNA samples. After sampling a qPCR (Purple) PCR (Green) were applied for the same samples. Furthermore, metabarcoding samples obtained from previous studies where then amplified through qPCR for later comparison. (Figure Made in BioRender.com)

MATERIALS AND METHODS

2.1 Site selection

A total of four lentic waterbodies were selected from public data on *A. anguilla* distribution. Two large capacity dams act as catchments of at least 2 rivers and two smaller waterbodies acting as entry points for migrating *A. anguilla*. All water bodies were classified according to the known status of *A. anguilla* presence (Present/Unknown) from existing records in scientific literature ([Zogaris et al. 2012a; Griffiths et al. 2022).

Figure 3: Kannaviou dam and locations that 1L samples were collected

One of the largest in capacity dams found in Cyprus constructed in 2006 at Paphos district mainly for irrigation and water supply purposes. Ezousa river basin is drained in to the 18.000.000m^3 capacity dam. No listed records of *A. anguilla* presence were found however due to its large capacity and relatively recent construction it was selected as one of the sampling sites (Zogaris et al. 2012b).

Table 1: Known species of fish found in Kannaviou dam

Species	Source
Rainbow trout (*Oncorhynchus mykiss*)	Online fishing sources
European carp (*Cyprinus carpio*)	Online fishing sources
Bleak (*Alburnus alburnus*)	Online fishing sources
Roach (*Rutilus rutilus*)	Online fishing sources
Mosquitofish (*Gambusia affinis*)	Online fishing sources
Largemouth bass (*Micropterus salmoides*)	Online fishing sources
Pumpkinseed (*Lepomis gibbosus*)	Local anglers
Three Catfish (*Siluriformes*)	Online fishing sources

Figure 4: Lefkara and selected sampling sites were 1L of water was collected.

Lefkara dam is located in the mountainous regions of Larnaka district and has seasonal supply of water from the Setrachos river. It has a 368.000m3 capacity and it's a common spot for local anglers to fish (Table 2). There are no known records of *A.anguilla* presence but several other know species that can be found in similar abiotic conditions where *A.anguilla* is found are present.

Table 2: Known species of fish found in Lefkara dam

Species	Source
Rainbow trout (*Oncorhynchus mykiss*)	Online fishing sources
European carp (*Cyprinus carpio*)	Online fishing sources
Bleak(*Alburnus alburnus*)	Online fishing sources
Roach (*Rutilus rutilus*)	Online fishing sources
Mosquitofish (*Gambusia affinis*)	Online fishing sources
Zander (*Sander lucioperca*)	Online fishing sources
Largemouth bass (*Micropterus salmoides*)	Online fishing sources
Pumpkinseed (*Lepomis gibbosus*)	Local anglers

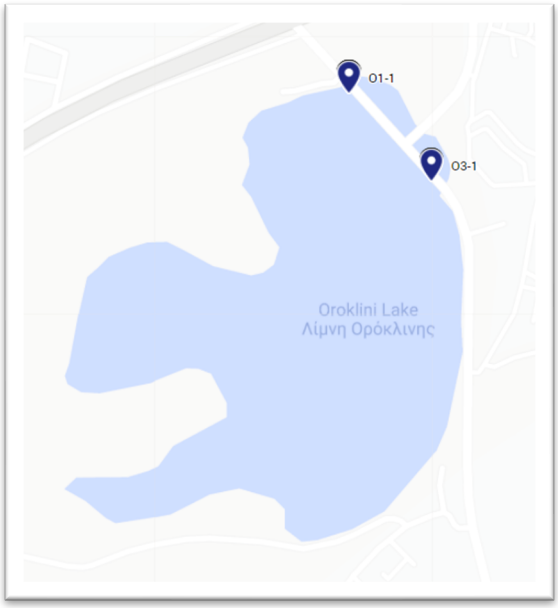


Figure 5: Oroklini lake and selected sampling sites were 1L of water was collected.

A natural wetland and Special Protection Area (SPA) (CY6000010) as it provides shelter and food source for an abundance of Annex 1 avians. Oroklini lake has great importance in *A.anguilla* conservation as it is one of the few protected refugia with access to the sea. Estimates of *A.anguilla* population come to hundreds of individuals entering the wetland from the two canals present. A recent drought event in 2013 along with possible pollution, had desiccated the local eel population with estimated losses of 1200 individuals (LIFE project Oroklini). One of the main entry points for eels in the wetland system has been chosen as well as a sampling point in the main water basin.

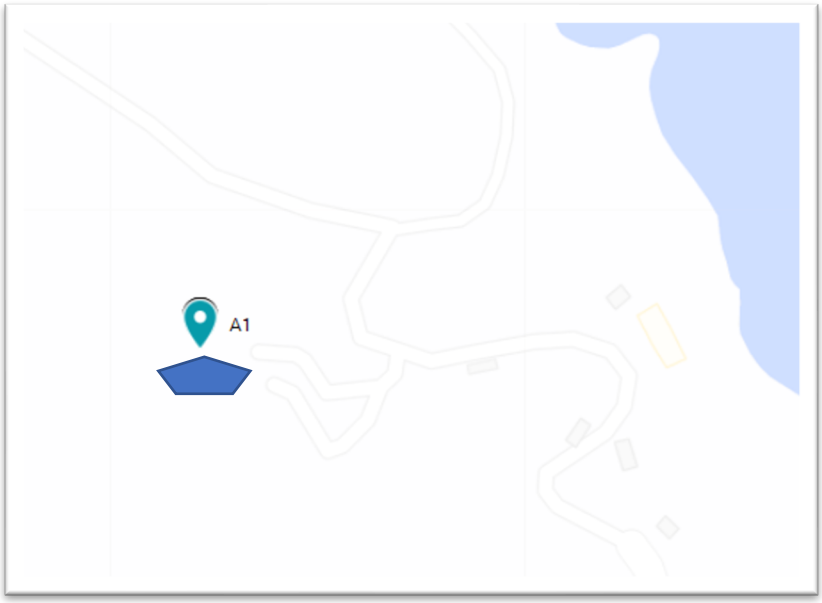


Figure 6: Baths of Aphrodite Pond (Loutra) and selected sampling sites were 1L of water was collected.

Baths of Aphrodite Pond is located 1km from the sea and is a small manmade pond of approximately 2000m3. The water is supplied during the dry months of June-September through an irrigation system. The presence of mature *A. anguilla* eel has been confirmed on-site by visual detection of at least one individual. It is also a tourist attraction, and the presence of humans is constant especially during the summer months however the eels can be found in a proximity of 1m from the pond barrier.

Experimental design & Sampling methodology

Sampling Methodology

All field samplings and filtrations were conducted on the same day in September of 2021 with a total of 12 1 L of samples being collected from all four sites. A 1 L sample of water was collected from the shore using single-use nitrile gloves and shoe covers replacing them between each sampling to avoid cross-contamination. The water bottle was fully submerged close to the water surface to prevent any sediment input by immediately closing the cap and placing over ice packs in a sterilised cool box. A label with a unique identification code was used consisting of the sample site ID (Name of location), 1 L sample ID (Initial letter of location and numbered sample sequence). The GPS coordinates in Latitude and Longitude format were then recorded for each sample. Each sampling point from every waterbody was deliberately chosen with a relatively distant separation of 5-10 meters to ensure a homogeneous coverage of the waterbody's surface. For each sampling point, 1 L water replicate samples were collected 1-10 meters from areas where access by foot was possible **Figures 3-6**.

Sample filtration

All filtrations took place in a dedicated eDNA molecular biology laboratory at the Cyprus University of Technology (CUT), with an average time after filtration of 5-7 hours. A 300 ml filtering cup and 1000 ml conical flask were used along with a 0.2 μm filter. A plastic tube was attached to a laboratory vacuum hose, with each connection sealed using clear tape to further improve filtering efficiency. The filters were then removed with bleached forceps, folded x 3 and placed in 1.5 μl Eppendorf tubes along with 500 μl of 95% molecular grade ethanol and stored at a 4 °C freezer in separate storage boxes. For samples with high sediment concentration the filter was replaced when clotted until the sampled 1 L volume was filtered. A negative control filtration was applied after each filtration with all apparatus submerged in 10% bleach and 70% ethanol for 10 min before and after each filtration.

eDNA extraction

All eDNA extractions were carried out in a laminar flow cabinet with HEPA filtration exclusively used for the purposes of this study between the first and last extraction. All working apparatus and working bench were sterilized with 10% bleach, 70% ethanol solutions and placed under UV radiation for 15 minutes prior each use. Moreover, all working equipment was exclusively used for eDNA extraction and placed separately in sterilized compartments between each extraction. A Qiagen DNeasy PowerSoil Pro Kit was used for all eDNA extractions following manufacturer's instructions, with a total of 17 filters between 12 1L water samples from field sites and 3 negative controls were being processed. All samples were stored at -20 °C until PCR and qPCR amplification.

Control tissue DNA extraction protocol

For primer validation and optimisation regarding its specificity towards *A. anguilla* a series of DNA extractions from fin clips was carried out prior to other analysis described below. The fin clips were provided by Water Development Department in 1.5 Eppendorf tubes with 95% molecular grade ethanol and they were collected in 2020 Table 1. THE Mu-DNA: tissue kit was used following all manufacturer's instructions after rehydration and adjusting the incubation duration for tissue lysis to overnight. Additionally, to avoid any cross-tissue contamination all clips were processed in batches of maximum 12 samples of the same species. All working apparatus, such as forceps and grinding pestles, were bleached and autoclaved prior to the processing of each batch. All DNA samples were then labelled and stored at -20 °C fridge. A nanodrop was used to confirm the presence and quantity of DNA.

Table 3: DNA extraction from 106 fin clips 12 fish species found in Cyprus rivers in 2020.

Common name	Species	Number of Individuals
Rainbow trout	*Oncorhynchus mykiss*	32
Common roach	*Rutilus rutilus*	15
Brown trout	*Salmo truta*	9
European perch	*Perca fluvitialis*	3
Eastern mosquitofish	*Gambusia holbrooki*	3
Goldfish	*Carassius auratus*	3
Crusian carp	*Carassius carassius*	4
European eel	*Anguilla anguilla*	22
Largemouth bass	*Micropterus salmoides*	9
Eurasian carp	*Cyprinus carpio*	3
Channel catfish	*Ictalurus-puctuatus*	1
Thicklip grey mullet	*Chelon labrosus*	2

Metabarcoding samples template DNA amplification

For the selection of metabarcoding samples, a random stratified approach was followed. All samples were sorted into 5 catergories accordig to their eDNA reads and 2 samples were randomly selected from each pool of Table 2. A total of 7 samples of extracted DNA using Mu-DNA water protocol were used. They were later analysed using the qPCR assay described below with CYT B primers.Table 4: Metabarcoding samples template DNA amplification

Table 4: Total number of samples selected from metabarcoding survey with the respected A.a DNA reads.

Sample Number	*A.anguilla* reads
1	153850
2	86873
3	9320
4	6381
5	3331
6	507
7	342

PCR validation of primers

A total of 7 species-specific primers were designed targeting three regions of the mitochondrion (CO1, CYTB, 16s) derived from reference sequences from GenBank. All sequences were then uploaded in Primer-BLAST and tested for any cross-species amplification. The specificity of all 7 primers (Table:4) was initially tested through PCR triplicates and gel electrophoresis with template DNA extracted of fin clips from *A. anguilla, O. mykiss and M. salmoides*. An initial annealing temperature of 56 °C was applied and then further increased to 60 °C if the amplification of the two non-target species was observed (Table 3). The results were then visualised through 1.6 % agarose gel with Gel red dye.

Table 5: PCR protocol followed for primer validation,

Step	PCR temp (C)	Time	Cycles
Initial denaturation	95	2:00	1x
Denaturation	95	0:30	35x
Annealing	**56-60**	0:30	
Extension	72	0:30	
Final extension	72	10:00	1x

Table 6: Forward and Reverse primer sequences

Primer Number	Forward	Reverse
1	ACACACCCCGCTAATTCCAG	CGTACCGAAATCGGGGGTAG
2	GCACTGCACTGAGCCTTCTA	GTCTGGGGCGCCGATTATTA
3	ATTTCCACGACCATGCGCTA	GGGGAGCGCGATGAGAATTA
4	ATAGTTACTCCGCCGCACAT	AAATTCAGAATAGTAGTTGG
5	CTTCCCAGTACGAAAGGACCG	TTTTCAGCAGGTAGAGGTGGG
6	TTCCCAGTACGAAAGGACCG	TTTCAGCAGGTAGAGGTGGG
7	TCCATAGGGCAGTATTTGC	AGGCATCTGGGTAATCTGAG

qPCR validation of primers

A species-specific quantitative PCR (qPCR) along with High-resolution melt (HRM) analysis was applied with SYBR Green technology (Bio-Rad). A final quantity of 25 ul was used for qPCR with adjusted amounts of SYBR green, FW and RV primers, molecular grade water and 2ul of template DNA. An initial test was conducted by obtaining a 1 L sample from an ex-situ 1000 L container containing an adult *A. anguilla* individual and 15-20 European carp (*Cyprinus carpio*). All environmental 1 L samples were then replicated x 6 and the presence of *A. anguilla* was recorded and verified according to the HRM temperature of previous tissue and ex-situ extractions. A serial dilution from 10^{-2} to 10^{-8} with template tissue DNA was applied for qPCR run to determine the efficiency and detection threshold.

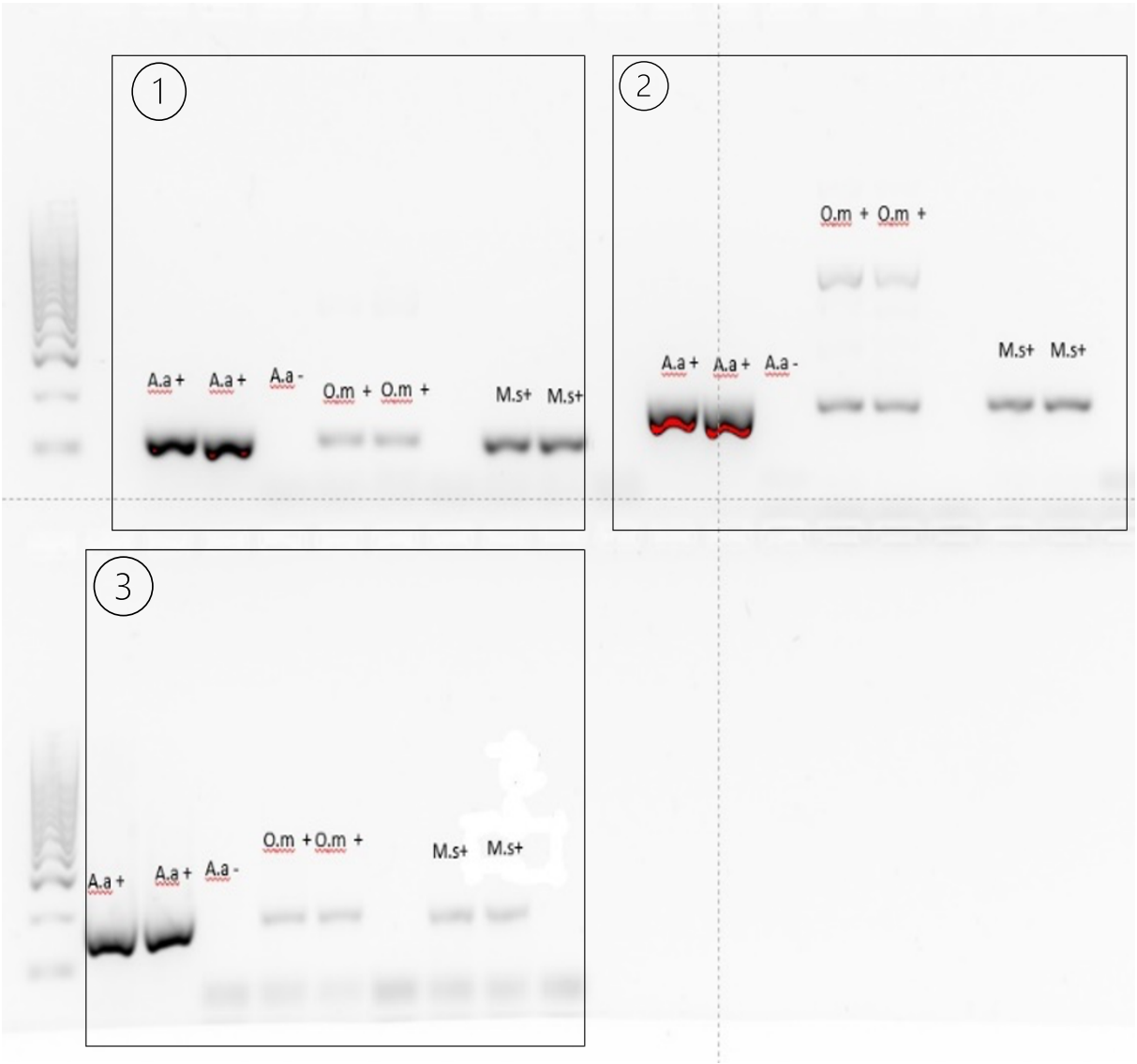
Table 7: qPCR – HRM protocol

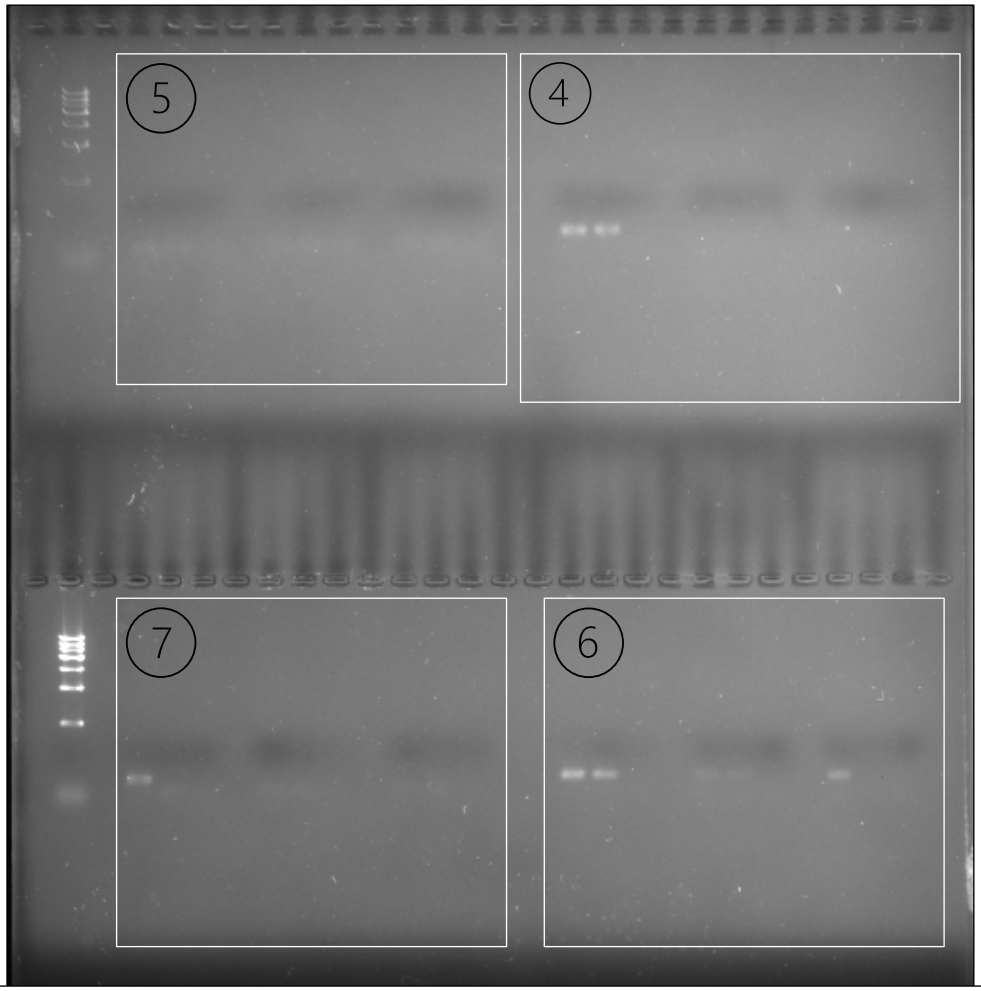
Step	qPCR temp (C)	Time	Cycles
Initial denaturation	95	5:00	1x
Denaturation	95	0:30	45x
Annealing	60	0:30	
HRM	60-95	0:05	61x

Results

Assay development

Through the initial PCR testing of all seven primer pairs, CYTB primers fit all parameters of only amplifying our target species sequence (Figures 7&8). This was later confirmed by following extraction and amplification protocols using ex-situ eDNA through qPCR assay that confirmed the 80 °C HRM temperature observed in *A. anguilla* tissue amplification with no amplification of non-target DNA.





Figures 7 (top)& 8 (Botomm): CYTB well pair 4 have shown amplification of A. anguilla with no non-target species amplification. All other primers have amplified A. anguilla along with non-target species eDNA of O. mykiss and M. salmoides For each primers a total of two positive and one negative cotrol well where used in this order A. anguilla, O. mykiss and M. salmoides.

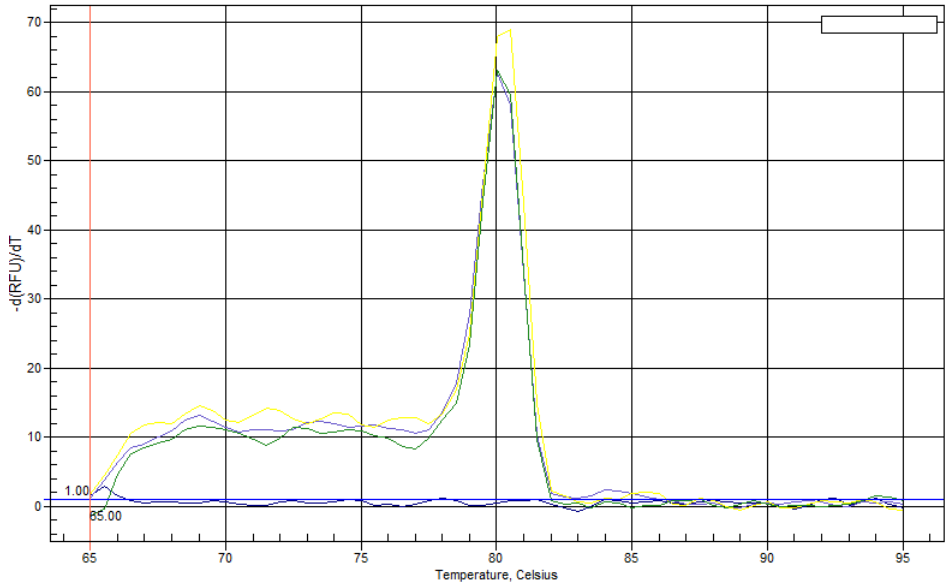


Figure 9: HRM melt curve illustrating the specificity of CYTB primers in ex-situ experiment. A total of 2 triplicates were processed. Two positives and one negative from the 1L water sample and two positives and one negative from A.a tissue template control.

Primer optimisation

Table 8: A total of 7 primers from four regions of the *A. anguilla* mitochondrion were designed from three different platforms. The DNA extracted from fin clips of *A. anguilla*, *O. mykiss* and *M. salmoides* was used for cross-validation of primer specificity.

No	Region	Product length (bp)	Species Amplification			Platform Designed	Validation Method
			A.a	O.m	M.s		
1	CO1	110	✓	✓	✓	NCBI Primer Blast	PCR - qPCR
2	CO1	185	✓	✓	×	NCBI Primer Blast	PCR
3	CO1	173	×	✓	✓	NCBI Primer Blast	PCR
4	**CYTB**	**169**	✓	×	×	**NCBI Primer Blast**	**PCR - qPCR**
5	16s	70	✓	×	✓	Snapgene - UGENE	PCR
6	16s	72	✓	✓	✓	NCBI Primer Blast	PCR
7	CO1	161	✓	✓	✓	Snapgene - UGENE	PCR

Positive samples and replicates form qPCR

For each 1L sample a total of 6 qPCR replicates were validated and in the case of a single positive replicate the 1L sample was considered positive. A total of four 1L were positive for A.a DNA out of 12 samples collected.

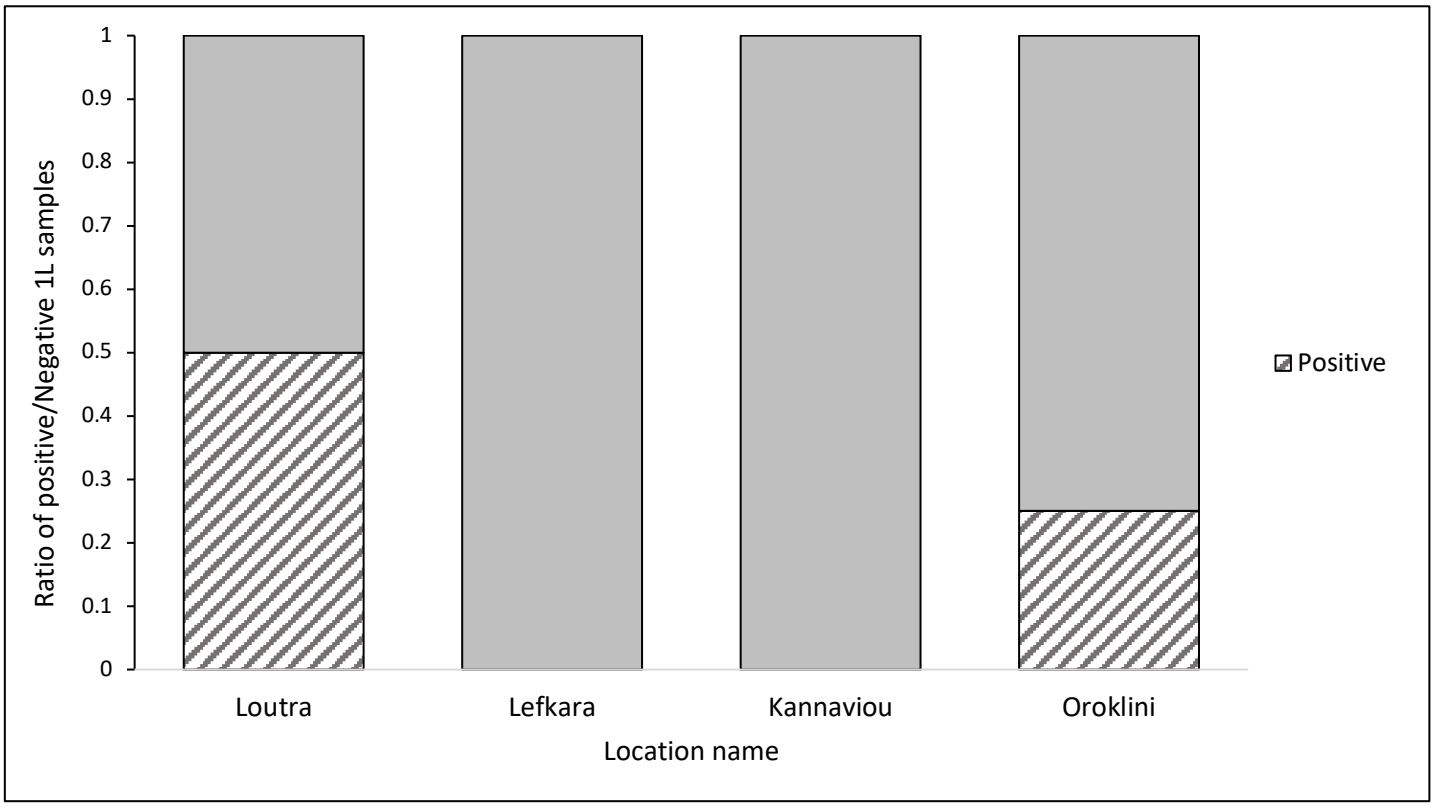


Figure 10: Ratio positive/negative 1L samples for each of the four locations.

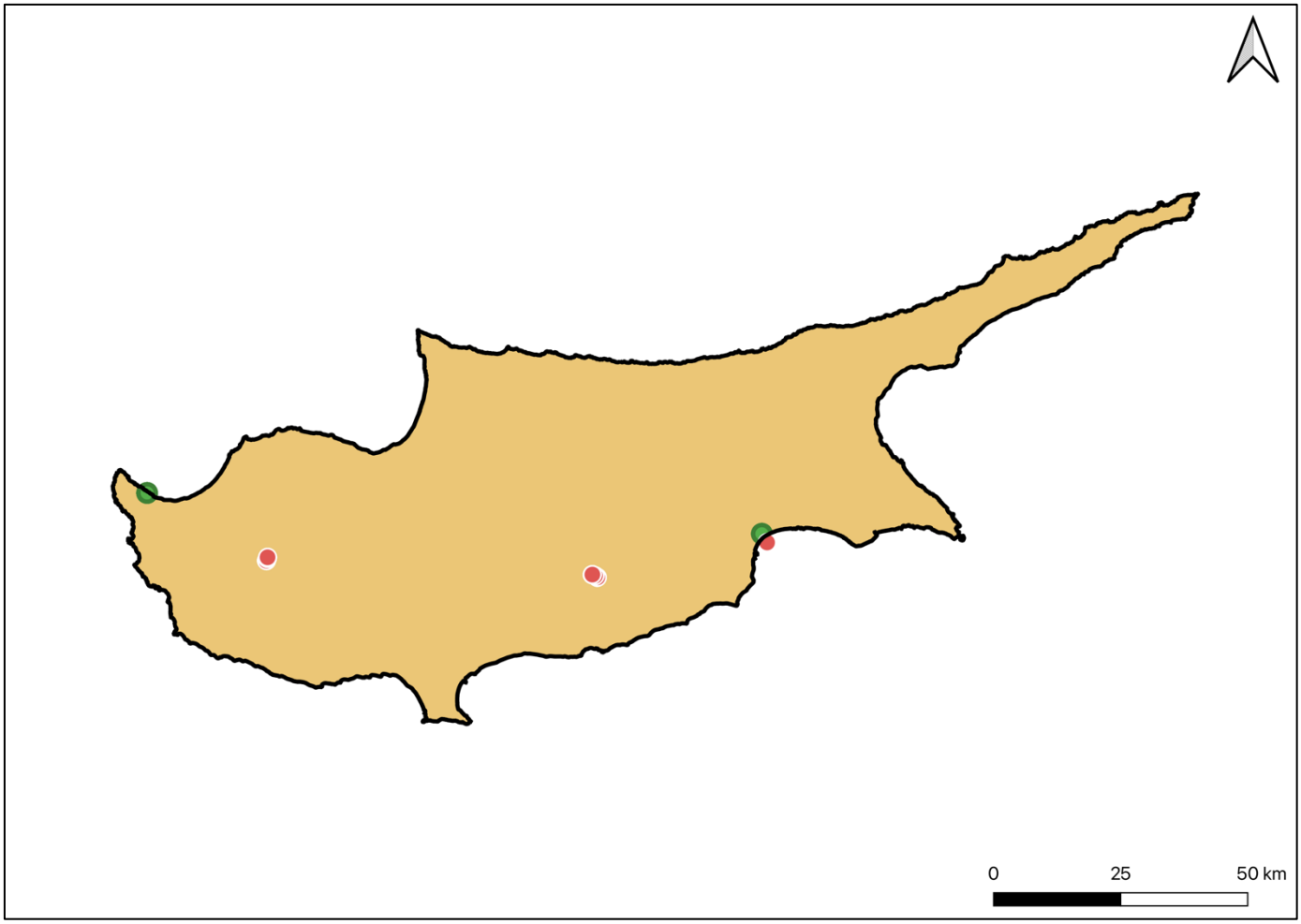


Figure 11: All four sampling locations along with 12 1L samples collected. Green= positive *A.anguilla*, Red= Negative.

A total of 84 replicates were analysed from 12 1 L samples across four different waterbodies. A total of 18 positive qPCR replicates from four 1 L samples were observed. Two replicates from the same location and sub-sample of 1 L water illustrated cross-amplification with different melting temperature from the HRM. All replicated from the above filter where not considered tin the overall analysis and reporting of results. No amplification was observed in all extraction and qPCR controls. Moreover, following the PCR assay and gel electrophoresis imaging a total of 7 PCR replicates out of the total 18 positives(qPCR) were detected (Figure 12).

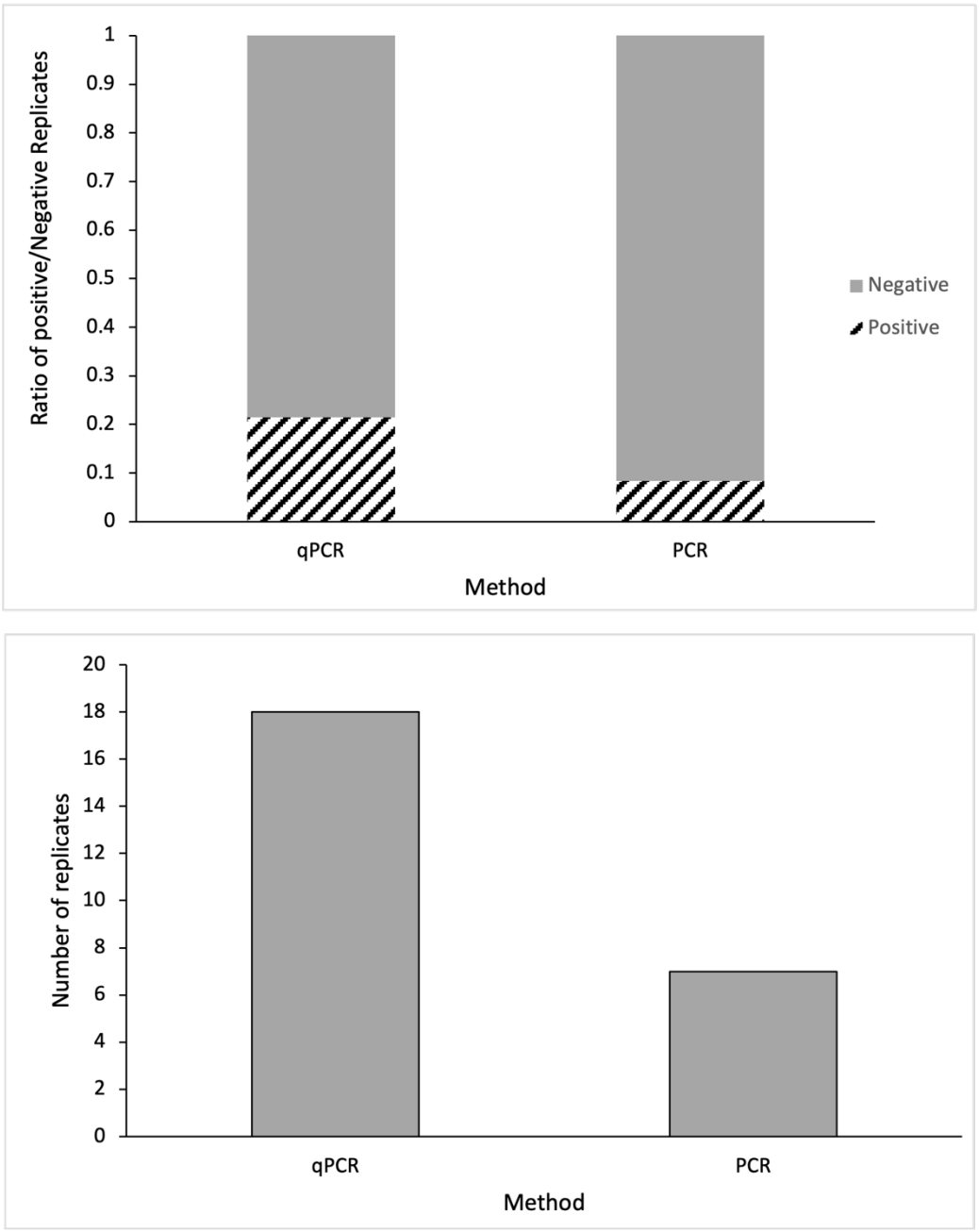


Figure 12: Ratio of positive/negative qPCR and PCR replicates(n=84), Top. Number of positive replicates between the two methods.

Metabarcoding & qPCR

Out of the seven *A. anguilla*-positive eDNA samples selected for qPCR, five were positive. Samples 3 and 4 (Figure 13) had no positive replicates.

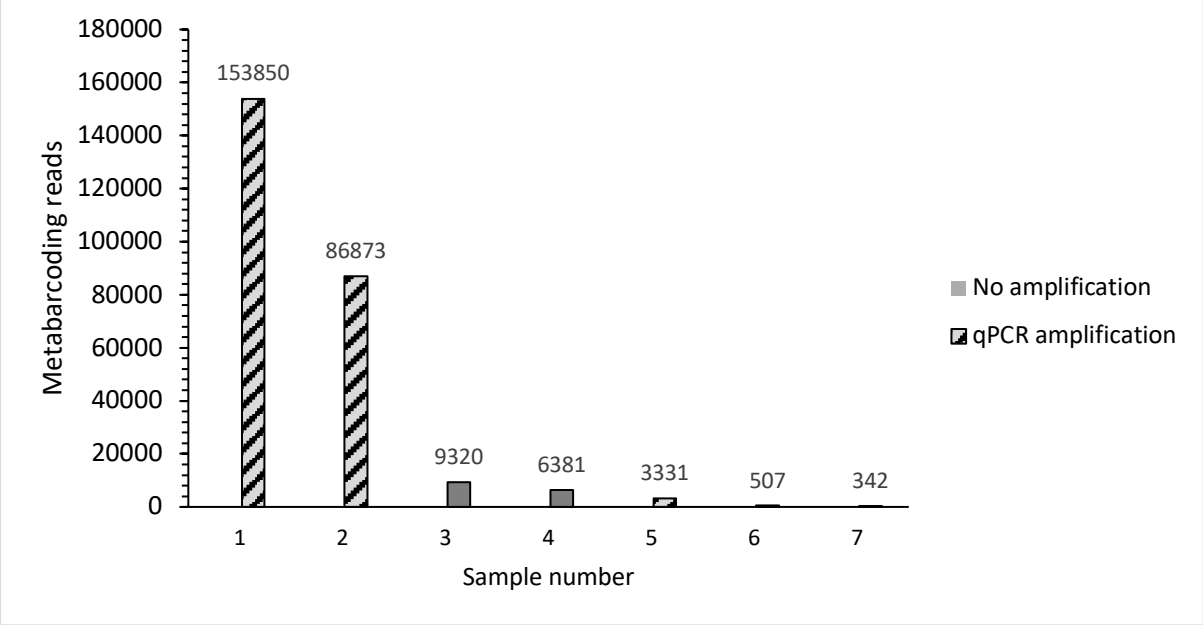


Figure 13: Metabarcoding sample reads and detection status of qPCR assay.

Discussion

A species-specific quantitative PCR (qPCR) along with High-resolution melt (HRM) analysis was applied thus allowing increased sensitivity and reduction in type 1 errors. DNA metabarcoding can provide a more comprehensive community structure (Ruppert, Kline, & Rahman, 2019), for the purposes of this study a total of four waterbodies were selected. Two low land entry points (Loutra & Oroklini) and two large dams in higher altitudes (Kannaviou & Lefkara). The reference data for the presence or absence of *A. anguilla* were obtained by the most recent study conducted by Griffiths et al. (2022). A metabarcoding eDNA assay was conducted and contrasted with electrofishing and conventional data thus, it stands as the most updated database regarding the spread of *A. anguilla* .

Following the results of the qPCR rapid surveillance and metabarcoding it is observed that the two assays can complement each other. The qPCR assay failed to detect eDNA in 2 out of the 7 samples tested however for lower concentrations both have been able to detect *A. anguilla* eDNA (Figure 9). However, the detection status between the three common sampling sites (Loutra, Oroklini, Kannaviou) monitored by metabarcoding was consistent along the two assays. Notably, the presence of *A. anguilla* in Loutra and Oroklini was confirmed and no detection of *A. anguilla* was observed in Kannaviou dam. Lefkara dam was not sampled by metabarcoding and no *A. anguilla* eDNA was detected by qPCR however reports of *A. anguilla* presence in the literature suggest that it can act as a refugium.

As there are only three common sites between the metabarcoding and qPCR methods with also having different sampling periods and years a comprehensive comparison between the two cannot be achieved. According to the current scientific literature there is a plethora of studies comparing species-specific qPCR and metabarcoding methods. There is also variability in the results within these studies as some suggest that qPCR and metabarcoding can complement each other regarding their limitations and strengths in detecting invasive and cryptic species (Wood et al. 2019; Pont et al. 2022). It is also argued however in the case that the level of detection is very similar to the holistic approach in describing fish communities in contrast with species-specific detection metabarcoding can be a more valuable tool (Harper et al. 2018). As of that,

the use of metabarcoding to replace species specific qPCR is still not clear as there are also studies that prove qPCR can have greater detection sensitivity as well as providing the quantitative aspect in regard to fish biomass and eDNA concentration (Gargan et al. 2022; McCarthy et al. 2022)

Figure 14: Map of four sampling locations along with positive (Green) and negative (Red) points of 1L samples. Watersheds and large water bodies (blue) were obtained by Cyprus Water Development Department database.

The field application of qPCR assay and the time from collection to result acquisition can also be a factor in choosing qPCR over other methods. Following the methodology described above and the ease of access to the sampling locations in Cyprus a total of three days is adequate. For example, the filtration and eDNA extraction of 4 1L samples can be conducted in a day and qPCR validation on the next day. Field sampling can be variable depending on conditions and different locations. To conclude qPCR assays can be a rapid and sensitive tool in providing insight to current distribution of A.a in freshwater habitats.

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