Spatial and seasonal distribution patterns of native and introduced aquatic species based on environmental DNA

Submitted by

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SUMMARY

The analysis of environmental DNA (eDNA; DNA recovered from environmental samples) is a recently developed non- invasive method used for detecting aquatic invasive and native species. Combined with barcoding or metabarcoding can be used to identify the presence of a target organism or to analyse an entire community, by estimating species presence/ absence and relative abundance, providing information for aquatic species management. The main aim was the optimisation of eDNA methods for assessing spatial and seasonal distribution of aquatic native and invasive species, focusing on hotspots of marine invasive species and on the distribution of fish species in rivers. An initial study on optimisation of eDNA capturing protocols, determined that sampling a large but feasible volume of water by combining syringe filtration with ethanol- sodium acetate precipitation was the most optimal strategy. Using this method of eDNA extraction and metabarcoding, a second study analysed river restoration success, after a recently removed weir, to identify changes in the abundance of freshwater fish, with no evidence of weir impacting fish discontinuity patterns, before or after removal. A similar approach analysing the role of obstacles on fish distribution in rivers with contrasting levels of fragmentation, indicated that both natural and artificial barriers resulted in limited fish community composition upstream compared to downstream, particularly for non-migratory species, with the migratory salmonid species being able of upstream passage in both of the rivers. Finally, the application of eDNA and barcoding for early detection and monitoring of invasive seaweed, indicated that native and invasive *Codium spp.* displayed significant seasonal and spatial differentiation, which could explain the establishment success of the non-native species. The results of this thesis establish the usefulness of environmental DNA spatial and seasonal dispersal assessment of aquatic species and suggests new avenues for eDNA future applications, such as providing data for spatial modelling.

DECLARATIONS AND STATEMENTS

I, **Teja Petra Muha**, certify that this work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ..... [redacted] ..... (candidate)

Date 28.3.2019

This thesis is the result of my own investigations except where otherwise stated.

Other sources are acknowledged by footnotes giving explicit references, with additional bibliography appended.

Signed ..... [redacted] ..... (candidate)

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Paper 5: Using Environmental DNA to Improve Species Distribution Models for Freshwater Invaders.

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TABLE OF CONTENTS

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LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Supplementary material

LIST OF ILLUSTRATIONS

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Supplementary material

DEFINITIONS OF ABBREVIATIONS

AIC	Akaike information criterion
AIS	Aquatic Invasive Species
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool for nucleotides
bp	Base pair
cm	Centimetres
Cq	Quantitation cycle
Ct	Quantitation cycle
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	qPCR efficiency
eDNA	Environmental DNA
H’	Shannon-Wiener diversity index
ha	Hectares
IAS	Invasive Aquatic Species
IUCN	International Union for Conservation of Nature
km	Kilometre
kpa	Kilopascal
L	Litre
LM	Linear model

m	Meter
M	Molar
m^3	Cubic meters
min	Minutes
mL	Millilitres
mm	Millimetre
MOTUs	Molecular operational taxonomic units
mtDNA	Mitochondrial Deoxyribonucleic acid
N	North
NCBI	National Center for Biotechnology Information
ng	Nanograms
nM	Nanomolar
PCR	Polymerase Chain Reaction
pmol	Picomole
qPCR	Quantitative Polymerase Chain Reaction
RefSeq	Reference Sequence
RFU	Relative fluorescence units
rRNA	Ribosomal ribonucleic acid
s	Second
SD	Standard deviation
SDM	Species Distribution Models
spp.	Species
U	Unified atomic mass unit
UK	United Kingdom

USA	United States of America
UV	Ultraviolet
W	West
µl	Microlitres
µM	Micromolar
°C	Celsius
µg	Microgram
µL	Microliters
µm	Micrometre

CHAPTER 1- Introduction

1.1 Environmental DNA

The use of non-invasive extraorganismal DNA- based species detection tools which enable the early detection and monitoring of aquatic species is increasing. Environmental DNA (eDNA), refers to intracellular and extracellular DNA, which can be extracted from environmental samples without physical detection of target organism (Taberlet et al., 2012). Diagnostic species identification tools have gradually lead towards more reliable barcoding methods for species recognition using endpoint PCR (Darling and Mahon, 2011), a method which assess amplification success after the plateau phase is reached, overcoming the difficulties of morphological identification. The eDNA detection technique can efficiently be applied as a tool for species' presence/ absence (Goldberg et al., 2016) and species relative abundance estimates (Evans et al., 2016). By detecting eDNA it is possible to define a target organism or even the entire community by using a barcoding or metabarcoding approach. Species specific or multiple species and community based eDNA detection techniques, using endpoint PCR, quantitative PCR (qPCR), droplet digital PCR (ddPCR) and next generation sequencing, have all been used as an efficient tool for aquatic invasive and native species detection (Doi et al., 2015, Takahara et al., 2013, Piaggio et al., 2014, Rius et al., 2015, Wilcox et al., 2013, Fukumoto et al., 2015). Applying presence/ absence and relative abundance estimates, a number of important issues can be addressed such as, defining invasive species presence (Nathan et al., 2014), population genetics (Sigsgaard et al., 2016), monitoring of infectious disease outbreaks (McManus et al., 2018), populations' use of space (Stewart et al., 2017), sequencing of whole metagenomes (Deiner et al., 2017c) and potential usage of mito-

metagenomics (Bista et al., 2018). Commonly, the species are identified by targeting short sequences (Pilliod et al., 2013, Evans et al., 2016) used for quantitative PCR or next generation sequencing. Yet, eDNA is not necessarily degraded and can be utilised for whole mitochondrial genome identification (Deiner et al., 2017c), providing information on population dynamics structure and identification of specimens movements (Sigsgaard et al., 2016).

Development of efficient eDNA based molecular protocols for detection of a number of species and whole communities is a high priority. Species-specific assays can be done with quantitative real-time PCR (qPCR) using primers that only amplify individual target species. qPCR is widely used for the quantification of DNA (Demeke and Jenkins, 2010, Rees et al., 2014), as the amplification of the target sequence allows us to quantify relative species density. eDNA metabarcoding takes advantages of the ability of next generation sequencing (NGS) techniques to detect short fragmented DNA (Shokralla et al., 2012). The next generation sequencing (NGS) platform is able to provide accurate results based on detection of targeted short DNA fragments with efficiency of up to one hundred thousands of replicates of reads per sample (Shokralla et al., 2012). It produces sequence reads that can be assigned initially to operational taxonomic units (MOTU) (Blaxter et al., 2005) and more precisely to species level using a referenced database. A community based metabarcoding approach is allowing us to understand the aquatic species network pathways on a broad spatial scale in time and could majorly transform our vision and understanding of ecosystem, maximising efficiency towards its conservation efforts.

The actual detection rate can vary between species, species specific production rates, target organism exposure time, species actual density, and also between sampling sites, seasons, environment, ecosystems, and eDNA capture strategies (Thomsen and Willerslev, 2015, Pilliod et al., 2013, Deiner and Altermatt, 2014), a difficult task for correlating eDNA abundance with actual species densities. Assessing population dynamics of aquatic species, requires quantifiable information, a good approximation

to absolute abundance. eDNA can arguably offer efficient estimations of relative abundance compared to results obtained by conventional sampling techniques (Lodge et al., 2012, Thomsen et al., 2012). Increased number of studies have found a linear relationship between actual species density and eDNA detection rate (Klymus et al., 2015, Takahara et al., 2012, Thomsen et al., 2016), offering a solution towards aquatic species distribution patterns.

1.2 eDNA challenges and limitations

The number of studies focusing on the benefits and limitations of eDNA techniques is currently on a rise (Goldberg et al., 2016, Bohmann et al., 2014, Deiner et al., 2015, Turner et al., 2014b, Bista et al., 2017b). There is a multitude of possibilities that can be achieved by eDNA detection application, but clear and consistent measure procedures need to be addressed for a reproducible, comparable and efficient long-term aquatic species management (Goldberg et al., 2016). Due to eDNA variable nature and its viability in different environments (Barnes et al., 2014), there are a number of challenges that need to be addressed to comprehend eDNA threshold capabilities, starting with the eDNA sampling and capture technique and its usage within the different aquatic environments. Species specific biomass correlation with eDNA density is another challenging step for efficient relative abundance estimations, where adhering eDNA detection to results obtained through conventional sampling is another important and wise aspect of cross- referencing of actual species confirmation (Civade et al., 2016). Accurate presence/ absence and relative abundance of native and non- native species eDNA based assessments, are a novel contribution towards species distribution models (Muha et al., 2017), implied in aquatic invasive species (AIS) management and policies requirements. In order to combine eDNA studies with the conventional surveys (Deiner et al., 2017a), it is crucial to standardise the methodologies to make it useful for long term monitoring. Defining detection of species specific eDNA as a result of actual species presence at certain location, is the most important limitation needed for the identification of species spatial distribution and seasonal dispersion patterns. In the aquatic environment DNA is prone to degradation (Rees et al., 2014), susceptible to UV light, microbial activity, temperature and pH variation (Rees et al., 2014, Strickler et al., 2015), providing species specific detection constraint in time and space. For instance, in rivers the challenge of eDNA detection reflecting actual species density estimation remains challenging, as higher

eDNA density at specific location does not necessarily mean actual higher species density, as eDNA could be transported from upstream localities (Deiner et al., 2017a). Long downstream persistence of eDNA detection was evaluated in rivers (Deiner and Altermatt, 2014, Shogren et al., 2017a), defining difficult precise spatial estimates of actual species presences corresponding to detected organismal eDNA at particular location. Assessing longitudinal eDNA dispersion in fragmented rivers can contribute towards spatial eDNA precise estimates of actual species presence, with limited dispersal possibilities. A number of parameters can influence detection rates of eDNA such as stream velocity (Jane et al., 2015) or inconsistent transport in flowing waters by different eDNA retention to benthic substrate (Shogren et al., 2017b). Comparing species relative abundance on seasonal and temporal scale presents another limitation, as eDNA may vary between different water temperatures, light and ultraviolet radiation (UV) (Klymus et al., 2015, Pilliod et al., 2014). Despite a number of eDNA detection variations, a strong correlation between the eDNA detection rate and organismal behaviour, seasonal patterns or certain activity, such as spawning, has been reported (de Souza et al., 2016, Bylemans et al., 2017). Inappropriate usage of eDNA detection tools can result in unreliable information, a difficult limitation to overcome, normally requiring a novel approach from the start of the experiment. Assessing both, impacts of barrier on fish dispersal, including barriers' removal would be highly beneficial, contributing to eDNA detection thresholds identification, dispersal disruption assessment of aquatic species, restoration action success measures and AIS mitigation of spread.

1.2 Use of eDNA to identify freshwater fish dispersal in fragmented rivers

Aquatic species population dynamic is defined by their dispersal and dispersal limitations (Shurin et al., 2009, Lowe and Allendorf, 2010). Natural barriers in lotic systems are a provision of natural intraspecific selection processes in stream communities (Bunn and Hughes, 1997), whereas artificial barriers contribute to sudden discontinuity patterns to the previously established natural flow of organisms. Both types of barriers can impact aquatic species dispersal, consequently harming local populations' survival rate and resistance towards adverse conditions in rivers, such as reduced river flow or introduction of more resistant invasive species (Kanehl et al.,

1997, Schaller et al., 1999). As the knowledge on dams' negative effects over the native populations is increasing (Morita and Yamamoto, 2002), a number of restoration processes are taking place, lacking an appropriate tool for restoration success measures, leading to poorly understood species dynamics after renovations took place.

Evaluation of aquatic species presence or absence, with additional accountable measure of their dispersal in rivers is a challenging task. The traditional surveillance of aquatic species detection and dispersal assessment can successfully identify species by implying different tools such as catch and release (Steinhart et al., 2004), electrofishing (Kamerath et al., 2008) and visual inspections (Johnson et al., 2001), all requiring actual catch or visual encounter of the specimen for its morphological identification. Environmental DNA metabarcoding approach could be used for freshwater species dispersal assessment in lotic bodies.

1.3 Spatial and seasonal distribution of aquatic invasive species

Aquatic biological invasion is defined as the introduction of non- indigenous species into a new habitat outside their natural range, potentially causing numerous problems in the environment and society (Pimentel et al., 2005, Leung et al., 2002, Lovell et al., 2006). AIS are one of the major causes of decline in worldwide aquatic biodiversity, putting at risk human water resources and economic interests (Havel et al., 2015, Lovell et al., 2006). Globalisation has expanded AIS global distribution (Hulme, 2009), by increased transport networks, providing numerous pathways of introduction. Freshwater and marine AIS dispersal assessment can be initially approached from the point of understanding invasive species dispersion constrains; abiotic parameters in marine coastal zone (Gray et al., 2007), habitat preferences in lentic bodies (Ricciardi, 2003), and in majority single direction of dispersal for in lotic waterbodies (Dana et al., 2011). It is crucial to identify AIS dispersion disruptions, whether these are natural obstacles, E.g. salinity in marine, man- made obstacles or waterfalls in rivers, to

mitigate their further spread. Artificial as well as natural barriers might pose a limit to further spread of AIS and can be accounted for a control measure. In lotic environments, the containment of AIS can be limited by actual building of exclusion barrier (Bylemans et al., 2016), a result of successful collaboration between researches and managers. Understanding key limitations of local species dispersion, depending on their biology and ecology, can also affectively contribute towards AIS management, which can be prone to same limitations of movements by same obstacles. In lentic bodies, the idea of AIS species containments is an early detection and early elimination rather than prevention (Vander Zanden et al., 2010), a sufficient approach also for marine coastal environment.

Marine invasive species are a dominant threat to marine biodiversity (Molnar et al., 2008); even small portion of introduced species are able to thrive in recipient habitats being accountable for substantial damage (Mack et al., 2000). Estimating the spread of marine invasive species is an ambitious challenge. Ports are known as global hotspots for invasions (Drake and Lodge, 2004), thus the most convenient starting point for primary and secondary AIS dispersal evaluation. After the initial primary introduction of species within a new geographic location, AIS can be dispersed further by natural and anthropogenic processes, colonising new habitats, resulting in a secondary introduction (Vander Zanden and Olden, 2008). AIS management dealing with primary and secondary introductions relies on efficient early detection strategies and assessment of their spatial dispersal, are currently based on challenging and time consuming approaches (Delaney et al., 2008).

Limiting the dispersal of AIS requires an appropriate management strategy, focused on i) effective early detection, to eradicate or limit the spread of AIS (Jerde et al., 2011), ii) effective evaluation of their dispersal potential and iii.) evaluation of the

recolonization process after eradication (Hughes, 2007). In areas with known or potential presence of harmful marine invasive species, a species specific target approach would substantially benefit AIS management. A community-based approach allowing to study the network pathways between invaders and natives could help understanding ecosystem changes caused by invasions, maximising the efficiency of conservation efforts. eDNA is increasingly being used in freshwater and marine environments to detect the presence of AIS target species (Dejean et al., 2012, Piaggio et al., 2014, Takahara et al., 2013, Tréguier et al., 2014, Xia et al., 2018).

Assessing aquatic freshwater and marine native and non- native species spatial and seasonal distribution patterns through the detection of eDNA was fundamental part of the current PhD thesis, focusing on the following study cases a.) assessing eDNA capture variations between three different freshwater bodies, b.) eDNA freshwater fish disruptive pathways assessment in rivers and c.) eDNA spatial and seasonal invasive and native seaweed distribution assessment.

1.4 Aims and objectives

Main aim

The main aim of the following thesis is to assess the spatial distribution of aquatic native and invasive fish and seaweed species, focusing on dispersion limitations occurring in fragmented rivers and coastal zones, including seasonal dispersion, by optimising eDNA barcoding and metabarcoding techniques, both used as eDNA species specific and community based detection tools, respectively. For that, firstly, I optimised the eDNA detection technique, assessing eDNA capture and amplification rate efficiencies between lentic and lotic water bodies, focusing on water volume, filtration method and DNA extraction kit, by establishing clear eDNA capture pathways for further assessment of the presence/ absence and relative abundance for both, species specific and community based targeted approach. Secondly, I estimated correlations between the eDNA detection rates with actual species densities of the target species. Thirdly, I estimated species seasonal and spatial dispersion, focusing on hotspots of marine invasive species introduced pathways and also identify dispersal limitations for both, native and invasive fish species in rivers. A perspective on the use of eDNA for spatial distribution modelling is also included in the last chapter.

Objectives per each chapter:

Chapter 2:

The main aim of Chapter 2 was to optimise a highly efficient and practical eDNA sampling technique with similar efficiency in both, lentic and lotic water bodies, focusing on the three most important steps with high impact on eDNA capture rate, water volume, filtration method and DNA extraction kit. Chapter 2 is a methodological study which contributed towards the improvement of eDNA capture techniques. QPCR approach using vertebrate primers has been used to avoid species-specific bias and to avoid limitations between three different water bodies distinctive community compositions.

Muha T. P., Robinson C. V., Garcia de Leaniz C., Consuegra S. An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. (PLOS ONE- Major changes).

Chapter 3:

The main aim of Chapter 3 was to evaluate, using eDNA metabarcoding, to what extent different types of barriers, natural and artificial, pose an impact on migratory salmonids and other non- migratory fish species. eDNA metabarcoding approach was used to assess fish community structure being affected by river fragmentation in order to assess dispersal limitations of migratory and non-migratory species.

Muha T. P., Rodriguez Barreto D., Garcia de Leaniz C., Consuegra S. Impacts of river fragmentation on fish dispersal assessed using environmental DNA. (Intending to submit to Freshwater Biology journal).

Chapter 4:

The main aim of Chapter 4 was to assess the suitability of the eDNA metabarcoding approach for evaluating the spatial distribution of migratory and non- migratory fish species affected by barrier removal.

Muha T. P., Rodriguez Barreto D., Garcia de Leaniz C., Consuegra S. Assessment of fish connectivity after weir removal by environmental DNA metabarcoding.
(Intending to submit to Ecological Indicators journal).

Chapter 5:

The main aim of Chapter 5 was to define seasonal and spatial patterns of native and invasive *Codium sp*. in coastal and port environments, using eDNA barcoding. QPCR approach was used as it was important to assess invasive species dispersion, with only four species of interest being present in the area.

Muha T. P., Skukan R., Borrell Y. J., Rico J. M., Garcia de Leaniz C., Garcia-Vazquez E., Consuegra S. eDNA barcoding reveals contrasting seasonal and spatial distribution of native and invasive *Codium* seaweed. (Ecology and Evolution- Major changes).

Chapter 6:

The main aim of Chapter 6 was to define usefulness of eDNA presence/ absence assessments within spatial distribution models for aquatic invasive species.

Muha T.P., Rodríguez-Rey M., Rolla M., Tricarico E. 2017. Using Environmental DNA to Improve Species Distribution Models for Freshwater Invaders. *Front. Ecol. Evol*. 5:158. doi: 10.3389/fevo.2017.00158.

CHAPTER 2- An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume

Abstract

Environmental DNA is increasingly being used for assessing the presence and relative abundance of fish in freshwater, but existing protocols typically rely on filtering large volumes of water which is not always practical. We compared the effects of water volume, filtration type and eDNA extraction procedures in the detection of fish in three freshwater bodies (pond, lake and river) using a short fragment of the 12s rRNA mtDNA gene. Quantification of eDNA capture efficiency after DNA extraction, as well as amplification efficiency, were evaluated by conventional PCR and quantitative real-time PCR. No significant differences on eDNA capture yield or amplification rates were found among freshwater bodies, but increasing water volume had a positive effect on eDNA capture and amplification efficiency. Although highest eDNA capture rates were obtained using 2 L of filtered water, 100 mL syringe filtration in combination with ethanol precipitation proved to be more practical and increased quantitative PCR amplification efficiency by 6.4 %. Our results indicate that such method may be enough to detect fish species effectively across both lotic and lentic freshwater environments.

2.1 Introduction

Environmental DNA (eDNA) is increasingly being used in freshwater environments to detect the presence of target invertebrate and vertebrate species, based on the detection of short extracellular DNA fragments released into the environment (Larson et al., 2017, Valentini et al., 2016). eDNA detection can be used for management purposes, such as monitoring of species' presence/absence (Goldberg et al., 2016), invasive species detection (Nathan et al., 2014), relative abundance estimates (Evans et al., 2016), population genetics (Sigsgaard et al., 2016) and use of space (Stewart et al., 2017). In some cases it can offer more efficient estimations of relative abundance than conventional sampling techniques (Lodge et al., 2012) as it can provide higher detection sensitivity (Biggs et al., 2015). Examples of accurate eDNA presence/ absence detection rates include the American bullfrog *Lithobates catesbeianus* (Dejean et al., 2012), the smooth newt *Lissotriton vulgaris* (Smart et al., 2015) and great crested newt *Triturus cristatus (*Harper et al., 2018).

Several studies have focused on the benefits and limitations of eDNA techniques (Goldberg et al., 2016, Bohmann et al., 2014, Deiner et al., 2015, Turner et al., 2014b), and a number of comparative approaches have tested the efficiencies of eDNA capture by ethanol precipitation or filtration (Spens et al., 2017, Deiner et al., 2015), methods of preservation (Hinlo et al., 2017b, Williams et al., 2016), filter types and extraction kits (Djurhuus et al., 2017). It has been found, for example, that the protocol combination for capture and extraction of eDNA differ in capture efficiencies, between water bodies and target species sampling preferences, based on a study of four different invertebrate species using species specific primers (Deiner et al., 2015). It has also been discovered that environmental conditions in lotic bodies, particularly the acidity, accelerate eDNA decay, assessed between streams across an environmental gradient targeting species specific macroinvertebrates and vertebrates, with eDNA equal detection rate across taxonomic groups (Seymour et al., 2018). Yet, a consistent application of the same eDNA protocol across water bodies for species (Wilcox et al., 2013, Takahara et al., 2013) or relative abundance purposes (Takahara et al., 2012, Pilliod et al., 2013) is still lacking.

Two of the most widely employed techniques of eDNA capturing are the ethanol-sodium acetate precipitation (Ficetola et al., 2008) and the filtration method (Jerde et

al., 2011b), ethanol precipitation allowing for wider size range of eDNA detection, whereas filtering largely depends on the pore size (Minamoto et al., 2016). They have shown variable success rate in comparative studies (Minamoto et al., 2016, Spens et al., 2017), largely differing between volume of water, pore size, filter material and extraction methods used, including environmental and physical conditions (Spens et al., 2017, Deiner et al., 2015, Rius, 2018). Ethanol- sodium acetate precipitation becomes unfeasible on larger water volumes, and filtration largely depends on the type of filtered water before clogging appears, resulting in different eDNA capture success rate. eDNA extraction using ethanol- sodium acetate precipitation tends to be done on small (15 mL) water samples (Ficetola et al., 2008, Doi et al., 2015, Dejean et al., 2012) and appears suitable when target species are highly abundant (and hence there is a lot of eDNA) in small or closed freshwater systems (Minamoto et al., 2016), whereas filtration of larger volumes of water seems to be more efficient in larger systems (Jerde et al., 2011b, Goldberg et al., 2013b, Turner et al., 2014a). I have selected both methods for this study in order to compare their efficiencies in lotic and lentic systems. Additionally, a newly designed eDNA sampling method combining both techniques and its benefits, practicality on one hand and efficiency on the other, has been implemented, the syringe filtration with ethanol- sodium acetate precipitation. Using syringe filters in combination with ethanol- sodium acetate precipitation could reduce the risk of contamination and cross- contamination at the start of the eDNA processing pipeline (Ficetola et al., 2016), due to enclosed filtering environment compared to open filtration requiring several filter handling steps.

Additional experiment evaluating whether eDNA particle size based on filter pore selection plays an important role in final eDNA capture yield and amplification evaluation has been implemented. Comparing ethanol- sodium acetate precipitation including filtration with filtration only technique, using same filter material with same pore size, was key step for defining whether filtration only or ethanol- sodium acetate precipitation play an important part for eDNA capturing. Most commonly used filter materials in eDNA studies are glass fibre filters (Jerde et al., 2011b, Wilcox et al., 2013, Janosik and Johnston, 2015) and cellulose nitrate filters (Pilliod et al., 2013, Goldberg et al., 2011, Goldberg et al., 2013b) with different pore sizes, thus this two filter types were taken for the evaluation. The type of eDNA extraction kit also determines overall eDNA capture rate efficiency (Renshaw et al., 2015, Roh et al.,

2006, Deiner et al., 2015, Eichmiller et al., 2016b) but this can vary depending on the presence of inhibitors (Eichmiller et al., 2016b, Whitehouse and Hottel, 2007) and pollutants that can increase the number of extraction steps, unintentionally provide false positives by increasing exposure to potential contamination (Ficetola et al., 2016). Contamination is a known risk factor during eDNA sampling and/or laboratory handling (Ficetola et al., 2016) and can occur anytime, during water collection (Bohmann et al., 2014), filtration (Rees et al., 2014), DNA extraction (Rohland and Hofreiter, 2007), amplification (Ficetola et al., 2015a) and next- generation sequencing (Ficetola et al., 2016).

A fully optimised method should have low contamination risk and ideally allow the sampling of different water bodies. I carried out a comparison of different methods in both lentic and lotic freshwaters, to assess the importance of each of three key factors that determine eDNA capture efficiency, water volume, filtration method and DNA extraction kit.

2.2 Material and methods

2.2.1 Study sites

Water samples of various volumes were collected in April 2017 from three freshwater bodies (two lentic and one lotic) in Wales (UK): a small (15 m wide, 1 m deep) pond located at Swansea University, an artificial freshwater lake at Cardiff Bay and the River Tawe (Figure 2.1). Cardiff Bay is situated at the confluence of the Rivers Taff and Ely, it is approximately 200 ha and was impounded in 1999 (Burton et al., 2002). Water from the River Tawe was collected at the headwaters, close to the river source (latitude 51°46’0.276” N, longitude 3°46’35.514” W), and also at the river mouth (latitude 51°42'08.9"N, longitude 3°53'57.2"W). In the pond, water was collected at two different sampling points on opposite sides (longitude 51°36'26.5"N, latitude 3°58'52.5"W). The water samples in Cardiff Bay were collected from three different stations; the barrage (longitude 51°26'48.7"N, latitude 3°09'59.4"W); St David’s Hotel (longitude 51°27'39.1"N, latitude 3°10'01.1"W) and Cardiff International White Waters (longitude 51°26'52.6"N, latitude 3°10'57.1"W). Water samples were collected at different sites per water body in order to diminish sampling site variations.

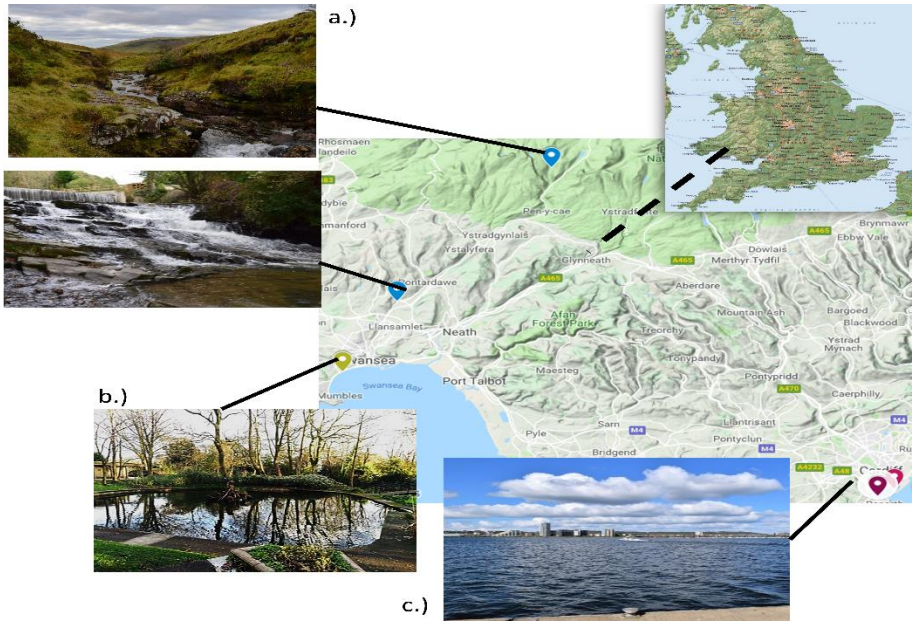


Figure 2. 1- Sampling locations at three water bodies, a.) River Tawe, b.) Swansea University pond and c.) Cardiff Bay, the lake.

2.2.2 eDNA sampling procedure

Three replicates were obtained from each water body, collected approximately 30 cm under the surface. Water samples were kept refrigerated and transported to the laboratory for filtration within four hours of collection. To minimize the risk of cross-contamination, disposable nitrile gloves were used and Nalgene polyethylene bottles were treated with 10% bleach, left for 5 min and thoroughly rinsed with sterile distilled water before sampling at each station. All filtration was conducted on the day of the sampling. Water was thoroughly mixed between sampling stations before filtration in order to have one uniform representation for each specific water body. The eDNA captured on filter was later recovered from it by extracting DNA from the filter or by a combination of ethanol precipitation from the filter following DNA extraction.

2.2.3 eDNA capture and amplification efficiency experiment

The study evaluated the effects of different filtered volume, filter pore size and composition, and extraction kits (Figure 2. 2) individually. The efficiency of the experiments was assessed by eDNA capture yield (ng/ μL), conventional PCR amplification (ng/ μL), visual quantification by gel electrophoresis and amplification using qPCR (Cq values). DNA yield as well as the efficiency of PCR amplification was measured by Qubit 1.0 fluorometer (Thermo Fisher Scientific Inc., UK) applying

the high-sensitivity assay for DNA capture yield efficiencies and broad range assay for PCR products (Life Technologies, Carlsbad, CA, USA). Standard recommendations for work with eDNA in the laboratory were applied through all the study (Goldberg et al., 2016).

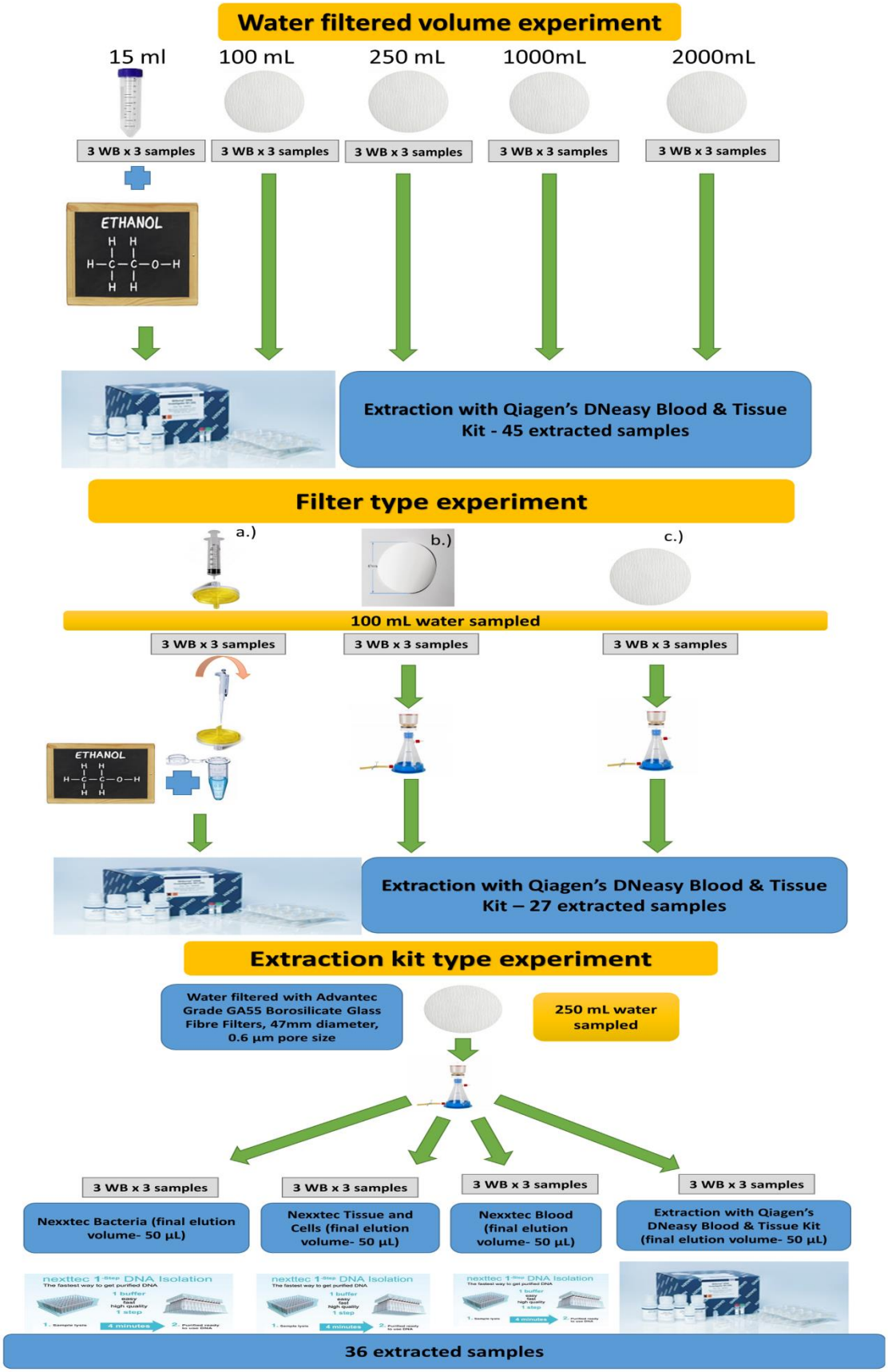


Figure 2. 2- Graphical representation of filtration volume, filter type and extraction kit experiments, where WB= Water body.

2.2.4 Water filtration volume comparison

For comparisons of water filtration volumes, three replicates of 15 mL, 100 mL, 250 mL, 1000 mL and 2000 mL water, were collected at each site (Figure 2. 2). For the 15 mL water samples I followed the protocol for ethanol precipitation described in (Ficetola et al., 2008) by adding 1.5 mL sodium acetate (3 M) and 33 mL of absolute ethanol. The mixture was centrifuged at 5000 g for 45 min at 6 ºC and the supernatant was discarded (Valiere and Taberlet, 2000). The precipitation itself was conducted on the day of water sampling, by centrifuging no more than four hours after collection. The falcon tubes with the DNA pellets were then stored at -20 °C without preservatives until the DNA extraction one week later. Negative control nuclease-free water was included.

The larger water volumes (100 mL, 250 mL, 1000 mL and 2000 mL) were filtered through the Advantec GA55 Borosilicate Glass Fibre Filters with 0.6 µm pore size (47 mm) (Figure 2. 2) with the intention to capture eDNA on filter itself. Each water sample was filtered through a filter funnel attached to a collection bottle and connected to the electronic vacuum pump with strength of 20 kpa for 15 s up to 75 s per sample. To avoid contamination, the filter funnel and handling tweezers were cleaned with a 10% bleach solution, rinsed with 99% molecular grade ethanol and then with sterile nuclease-free water between samples. For each different volume, a negative control consisting of nuclease-free water was used. In total, 120 samples were extracted with six and nine additional filtration and extraction control samples, respectively, controlling for false positives through the steps of sampling, filtration and extraction. The extraction and pre-PCR handling of eDNA water samples was carried out in a fume hood dedicated to eDNA analyses only. Before individual extractions, 10% bleach was used to clean up the fume hood as well as 45 min exposure to UV light. For eDNA extraction, the Qiagen DNeasy Blood & Tissue DNA extraction kit (Qiagen GmbH, Hilden, Germany) was used. For the 15 mL method the Qiagen protocol for blood (spin protocol) was used whereas for the filtration methods I used the protocol for dried blood spots. The manufacturer's protocol was followed in all cases, with the single modification of reducing the final elution volume to 50 µL in all three experimental designs.

2.2.5 Filter type comparison

For the comparison of filter types and pore size I used 100 mL of water and two different DNA capture methods: a pump filtration only method, and syringe filtration with additional ethanol precipitation. For the filtration only method, I used two different filter materials, Whatman Cellulose Nitrate Membrane Circle filters with 0.45 µm pore size (47 mm) and Advantec GA55 Borosilicate Glass Fibre Filters with 0.6 µm pore size (47 mm) (Figure 2. 2). The second method was based on a combination of filtration using closed syringe filters (Minisart® cellulose syringe filters with 0.45 µm pore size (Sartorius, Germany) with additional ethanol precipitation. For the syringe filtration, the water was pushed through by hand at an approximate flow rate of 50 mL per 30 s. After filtration, a mixture of 1350 µL absolute ethanol and 150 µL of sodium acetate was passed through the filters which were then centrifuged at 5000 g for 45 min at 6 ºC. For the other two types of filters, filtration was carried out as above. DNA was purified with the Qiagen DNeasy Blood & Tissue DNA extraction kit. For the 100 mL syringe filtration method, the Qiagen DNA purification protocol for blood (spin protocol) was used, whereas for the other two filtration techniques I applied the protocol for dried blood spots, designed for the DNA isolation out of filter paper.

2.2.6 Extraction kit comparison

Two hundred and fifty mL of water were collected and filtered through Advantec GA55 Borosilicate Glass Fibre Filters with 0.6 µm pore size (47 mm) for the extraction kit comparison (Figure 2. 2). The 250 mL water volume for the comparison between extraction kits was selected for the practicality itself as smaller volume results in higher differentiation between sampling triplicates. The Qiagen DNeasy Blood & Tissue DNA extraction kit (protocol for dried blood spots) was compared to three additional kits all from Nexttec (Nexttec™ Biotechnologie GmbH, Germany): the 1-step DNA Isolation Kit for Tissues & Cells, 1- step DNA Isolation Kit for Blood (200 µl) and 1-step DNA Isolation Kit for Bacteria. The reason for selecting Nexxtec kits was based on the potential advantages of reduced potential contamination, having a single step between the digestion of the sample and the final DNA elution. All extractions were carried out following the manufacturers' instructions, with the only modification of reducing the elution volume to 50 µL.

2.2.7 PCR amplification

In order to overcome the potential specificity bias, where potential mismatch of target species can occur using species-specific primers (Tedersoo et al., 2015) and to avoid differences based on single species representation in different lentic and lotic bodies, I used the vertebrate primer pair 12S-V5 developed by Riaz et al. (2011b), which amplifies a 144-bp long fragment of the 12s rRNA mtDNA gene and has been widely used previously (Kelly et al., 2014a, Miya et al., 2015, Port et al., 2016). The amplification reaction was performed in a total volume of 30 µl with, 12.5 µL Bioline BioMix Red PCR Mastermix (2X), 3 µL template, 1.5 µL of each primer (10 µM), adding sterile nuclease- free water to final total volume. PCR conditions were as follow, 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 52 °C 30 s and 72 °C for 30 s, with a final extension step at the 72 °C for 5 min. DNA yield as well as the efficiency of PCR amplification was measured by Qubit 1.0 fluorometer (Thermo Fisher Scientific Inc., UK) applying the high-sensitivity assay for DNA capture yield efficiencies and broad range assay for PCR products (Life Technologies, Carlsbad, CA, USA). PCR products were visualised on a 2% agarose gel. Positive controls were used for the evaluation of primer pair efficiency with DNA extracted from two different fish species commonly found in Tawe and Cardiff Bay, brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) testing for primer specificity. DNA was extracted from muscle or fin tissue from these target species using the Qiagen DNeasy Blood & Tissue DNA extraction kit. A negative control PCR with no DNA template was added at all PCR amplifications.

SYBR Green technology (Bio-Rad, US) was used in real-time PCR in a combination with 12S-V5 primer pair in a final reaction volume of 20 µl which included, 10 µL SsoAdvanced™ SYBR® Green Supermix (1x), 3 µL template, 0.4 µL of each 12S-V5 primer (10 µM) and 6.2 µL sterile nuclease- free water. The qPCR amplification was performed under the following conditions: 7 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 59 °C. Each of one of the three sampling replicates was amplified twice on a plate and final average Cq values of the duplicates was used for the statistical analysis. Each qPCR plate included three negative controls consisting of sterile nuclease- free water instead of the template. A standard curve with 8- point 10-fold dilutions with starting concentration of 1 ng/ µL of *Salmo trutta* DNA was used. *S. trutta* was chosen for the standard curve as it represents one of the most common fish species in Welsh freshwater bodies (Elliott, 1989).

2.2.8 Cloning

For species confirmation, four randomly selected samples from each water body and experimental design (twelve in total) were chosen and amplified with the 12S-V5 vertebrate primer pair using the same PCR protocol as above. The amplified PCR products (144 bp) were cloned into a pDRIVE Cloning Vector using Qiagen PCR cloning plus kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's recommendations. Three different concentrations of ligation- reaction mixture were plated on agar plates: 20 µL, 50 µL and 100 µL. Plasmid DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification kit (Promega, Madison, WI, USA). Sequencing was then carried out with T7 and Sp6 primers at the Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth. For sequencing 12, 7 and 12 clones were randomly selected from the river Tawe, the pond and Cardiff Bay respectively, with lower representation of pond samples due to low number of colonies, with maximum seven target colonies identified.

2.2.9 Statistical analysis

For analysis of eDNA capture yield (ng/ µL), PCR (ng/ µL) and qPCR (Ct values) amplification yields linear models were applied with water body, water volume, filtration type and extraction kit as predictors. Linear models were applied assessing three individual efficiency evaluation categories (eDNA capture, PCR, qPCR), accounting for each of the three individual experiments (water volume, filtration type, extraction kit), where water bodies were additionally included as a predictor within all the models. Models with and without interactions between the experimental category and water body were compared based on AIC criteria using the 'vegan' package, 'mass' function (Oksanen et al., 2007). The reported statistically significant differences are based on a model with the lowest AIC. For the post-hoc analysis the 'lsmeans' package was used (Lenth, 2016) based on Tukey contrasts. Technical qPCR duplicates were used for the analysis. Only samples with two technical working replicates were considered for further statistical analyses. Positive PCR and qPCR reactions without quantified DNA capture yield were only used for further comparison based on amplification efficiencies, excluding DNA yield. All statistical analyses were done with R, version 3.3.2 (Team, 2013).

2.3 Results

In total 120 samples were extracted from all three freshwater bodies including negative and positive controls. There was no visible band and amplification curve, melt peak appeared in filtration and extraction negative controls during PCR and qPCR, respectively. All positive controls performed as expected, and species were confirmed by Sanger sequencing of 144 bp length products. Samples were only considered for analysis if at least two of the three replicates amplified. R^2 values for the qPCR standard curve ranged from 0.95 to 1.00, and the efficiency ranged from 97 to 104%, with a slope between -3.3 up to -3.2 (Figure S. 1, Figure S. 2, Figure S. 3). Average capture and amplification concentration success rate for all three experimental designs was analysed separately for the DNA capture yield, PCR and qPCR efficiency including confirmation through gel electrophoresis (Table S. 1- S. 3). The amplification success rate based on the number of visual confirmations by the gel electrophoresis was 70 – 100% out of 117 sampling replicates. The results based on linear models for each of the three individual experiments assessed by DNA capture yield (ng/ µL), PCR (ng/ µL) and qPCR (Cq) indicate statistically significant differences between the individual experiments (water volume, filter type, extraction kit) in all cases (Table 2. 1), with the exception of qPCR detection at extraction kit experiment. In general, there was no difference between the water bodies for all of the linear models, with the following exceptions: DNA capture yield at volume and extraction kit experiments due to higher efficiency in pond and Cardiff bay compared to Tawe river (Tukey's Post-hoc test, $p = 0.010$, $p = 0.005$) respectively, and PCR higher amplification in pond and Tawe at extraction kit experiment (Tukey's Post-hoc test, $p = 0.023$, $p = 0.019$) compared to Cardiff Bay, respectively. Only significant difference based on the interaction term of water body with the experimental categories resulted in eDNA capture efficiency during volume experiment due to 2000 mL higher capture efficiency in pond compared to all lower filtering volumes in Tawe (Tukey's Post-hoc test, $p < 0.001$), and qPCR amplification at filter type experiment with much higher efficiency of syringe filtration combined with ethanol precipitation compared to cellulose nitrate filtering in pond (Tukey's Post-hoc test, $p < 0.001$). All linear models are reported in (Table 2. 1).

Table 2. 1- Linear models analysing effects of filtration volume, filter type and extraction kit in correlation to water body type on successful eDNA extraction and amplification for each of the experimental category separately, including comparison between models with and without an interaction term between the tested categories and water bodies.

Model	**Dependent variable**	**Predictor**	**Model output statistics**	**AIC**
Capture Interaction = Volume * Water body	DNA capture yield (ng/ µL)	Volume x Water body	F (8,44) = 3.781, **p= 0.003**	
		Water body	F (2,44) = 4.441, **p= 0.020**	
		Volume	F (4,44) = 2.137**, p < 0.001**	
PCR Interaction= Volume * Water body	PCR efficiency (ng/ µL)	Volume x Water body	F (8,41) = 1.327, p= 0.275	237.8
		Water body	F (2,41) = 1.073, p= 0.356	
		Volume	F (4,41) = 6.447, **p < 0.001**	
PCR= Volume	PCR efficiency (ng/ µL)	Volume	F (4,41) = 6.049, **p < 0.001**	233.96
qPCR Interaction= Volume * Water body	qPCR (Cq values)	Volume x Water body	F (8,38) =1.167, p= 0.359	160
		Water body	F (2,38) =1.722, p= 0.200	
		Volume	F (4,38) = 3.602, **p= 0.019**	
qPCR= Volume	qPCR (Cq values)	Volume	F (4,38) = 3.330, **p= 0.020**	156.83

Model	Dependent variable	Predictor	Model output statistics	AIC
Capture Interaction= Filter type * Water body	DNA capture yield (ng/ µL)	Filter type X Water body	F (4,24) = 2.287, p= 0.105	-87.53
		Water body	F (2,24) = 1.402, p= 0.274	
		Filter type	F (2,24) = 4.294, **p= 0.032**	
Capture= Filter type	DNA capture yield (ng/ µL)	Filter type	F (2,24) = 3.379, **p= 0.050**	-85.87
PCR Interaction= Filter type * Water body	PCR efficiency (ng/ µL)	Filter type X Water body	F (4,25) = 0.737, p= 0.580	140.23
		Water body	F (2,25) = 0.544, p= 0.590	
		Filter type	F (2,25) = 3.990, **p= 0.037**	
PCR= Filter type	PCR efficiency (ng/ µL)	Filter type	F (2,25) = 4.362, **p= 0.024**	133.76
qPCR Interaction= Filter type * Water body	qPCR (Cq values)	Filter type X Water body	F (4,25) = 3.667, **p= 0.024**	101.37
		Water body	F (2,25) = 3.365, p= 0.058	
		Filter type	F (2,25) = 5.845, **p= 0.011**	

Model	**Dependent variable**	**Predictor**	**Model output statistics**	**AIC**
qPCR= Filter type	qPCR (Cq values)	Filter type	F (2,25) = 3.501, **p= 0.047**	110.56
Capture Interaction= Extraction kit * Water body	DNA capture yield (ng/ µL)	Extraction kit X Water body	F (6,31) = 2.363, p= 0.069	
		Water body	F (2,31) = 7.065, **p= 0.005**	
		Extraction kit	F (3,31) = 10.657, **p= 0.001**	
PCR Interaction= Extraction kit * Water body	PCR efficiency (ng/ µL)	Extraction kit X Water body	F (6,33) = 2.162, p= 0.086	
		Water body	F (2,33) = 6.412, **p= 0.006**	
		Extraction kit	F (3,33) = 4.159, **p= 0.018**	
qPCR Interaction= Extraction kit * Water body	qPCR (Cq values)	Extraction kit X Water body	F (6,31) = 2.042, p= 0.107	
		Water body	F (2,31) = 3.380, p= 0.054	
		Extraction kit	F (3,31) = 0.299, p= 0.825	

2.3.1 Water filtration volume comparison

In total 45 samples (15 per water body corresponding to three sampling replicates for each one of the five volume categories) were processed, of which all samples were used for the DNA capture yield, 42 samples for the PCR and 39 samples for the qPCR were used for statistical analysis. One 15 mL negative control, an additional one for all other filtered sampling volumes, and one negative extraction control were used only for controlling the contamination and were not part of the statistical analysis. eDNA capture yield increased with increase in filtered volume (Table 2.1, $F(4, 44) = 2.137$, $p < 0.001$), with the highest DNA yield obtained at 2000 mL of sampled water from pond (Tukey's Post-hoc test, $p < 0.001$). There were significant differences between the volume categories for both amplifications (Table 2.1, PCR, $F(4, 41) = 6.049$, $p < 0.001$; qPCR, $F(4, 38) = 3.330$, $p= 0.020$) with most efficient DNA capture method being 2000 ml (Tukey's Post-hoc test, $p = 0.002$) compared to 100 mL, and 2000 mL compared to 100 mL and 250 mL (Tukey's Post-hoc test, $p = 0.010$) respectively. The largest water volume filtered for the duration of experiment (2 L) showed the highest DNA capture yield (0.406 ± 0.497 ng/ µL), about tenfold higher compared to the other methods, followed by the 1 L (Figure 2.3). The capture yield for the 15 mL category was low compared to filtration with only 0.027 ± 0.009 ng/ µL yield. There was a gradual increase in the eDNA capture yield from smallest 100 mL category up to largest, 2 L. The PCR amplification rate was the highest for the largest filtered volume tested with an average of 15.111 ± 2.473 ng/ µL. The amplification rate for the 15 mL ethanol precipitation method was high compared to other filtered volumes (12.738 ± 4.203 ng/ µL). The qPCR amplification efficiency for the 2 L category resulted in an average of 31.242 ± 0.699 cycles, comparatively similar to the 15 mL category with an average of 32.978 ± 1.896 cycles.

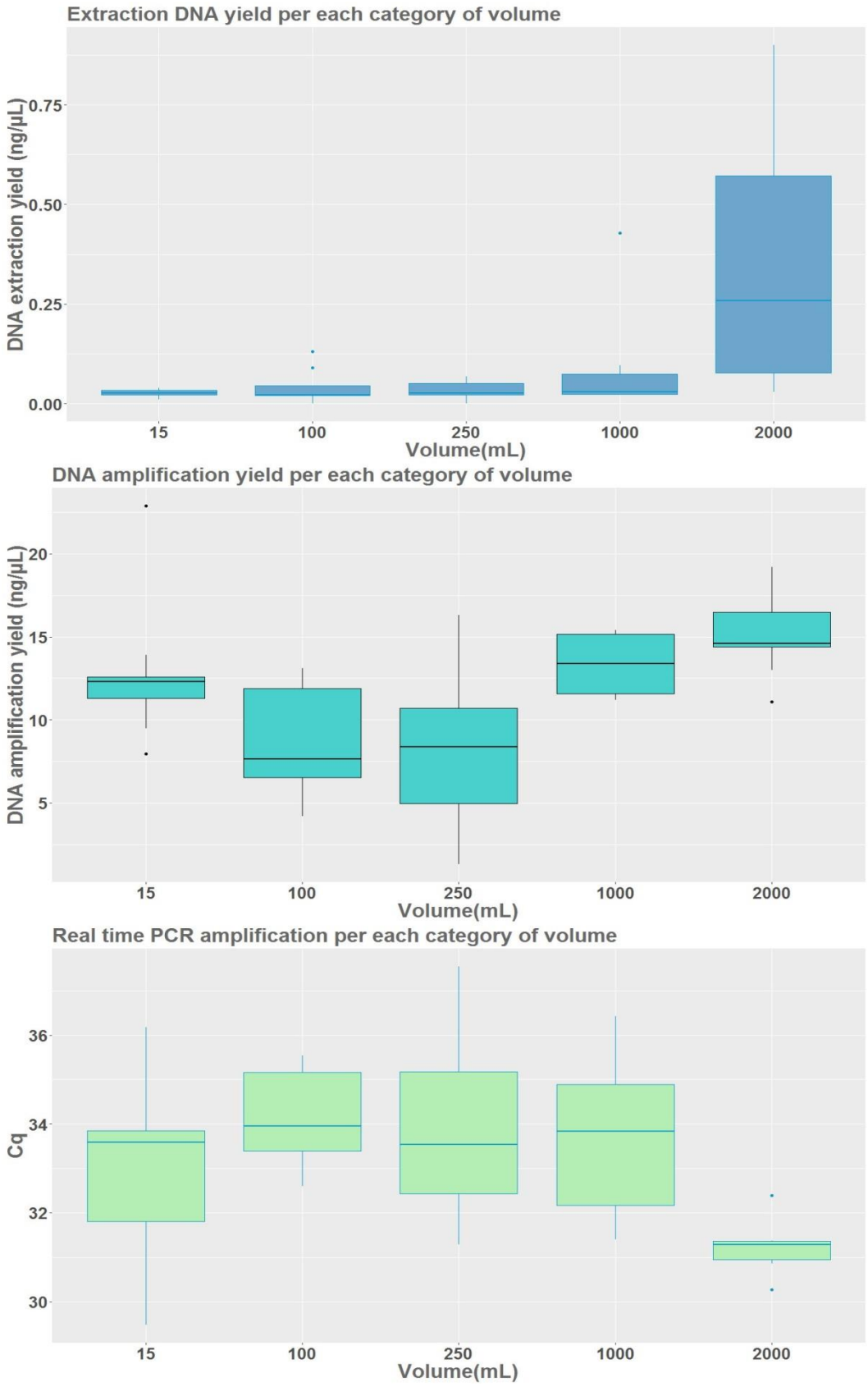


Figure 2. 3- eDNA capture yield (ng/ µL) and amplification efficiencies by filtration volume experiment. Differences in eDNA capture yield (ng/ µL), and amplification efficiencies by PCR (ng/ µL) and qPCR (Cq) divided by five different categorical groups (15 mL, 100 mL,

250 mL, 1000 mL and 2000 mL) for filtration volume experiment, where each category is represented by three sampling replicates per three water bodies (9). For the amplification efficiencies the technical triplicates of each sampling replicate was averaged before plotting. The 15 mL volume is based on ethanol- sodium acetate precipitation whereas the rest are based on water filtration. The lowest Cq value corresponds to the highest efficiency. For all categories the same two fixed factors were used, glass fibre filter with Qiagen extraction kit. The whisker plots represent the standard deviation.

2.3.2 Filter type comparison

Twenty-seven samples were extracted for the comparison between the filtration types excluding three negative filtrations and one extraction controls used for each individual water body. For statistical analysis 25 samples were evaluated from DNA capture yield, 26 for the PCR and 26 for the qPCR samples. Nine samples represented each individual water body, as sample triplicates for each of the three individual filter types were examined. I found statistically significant differences between filter type categories for DNA capture yield (Table 2.1, $F(2, 24) = 4.294$, $p = 0.032$), PCR ($F(2, 25) = 4.362$, $p = 0.024$) and qPCR ($F(2, 25) = 5.845$, $p = 0.011$) without differences between water bodies. DNA extraction yield was the highest for the ethanol precipitation in combination with filtration (0.070 ± 0.058 ng/ µL) in comparison to other two solely filtration procedures. Cellulose nitrate and glass fibre filter both performed poorly during DNA filtration (Figure 2. 4). Cellulose nitrate filters were the only filtration method where some of the filters failed to yield any eDNA and those samples were excluded from further statistical analysis. PCR amplification efficiency using the combined method of syringe filtration and precipitation yielded the highest DNA concentrations (average value of 12.593 ± 3.45 ng/ µL). A slightly better amplification performance was produced by glass fibre filter (average value of 9.280 ± 3.293 ng/ µL) in comparison to cellulose nitrate filter with an average of 0.635 ng/ µL lower amplification rate. The syringe filtration in a combination with ethanol precipitation resulted in low Cq values with an average of 33.235 ± 1.925 cycles evaluated by qPCR. QPCR provided similar results to PCR regarding performance of the glass fibre filter versus the cellulose nitrate filter with an average of 1.511 cycles higher for the glass fibre filter.

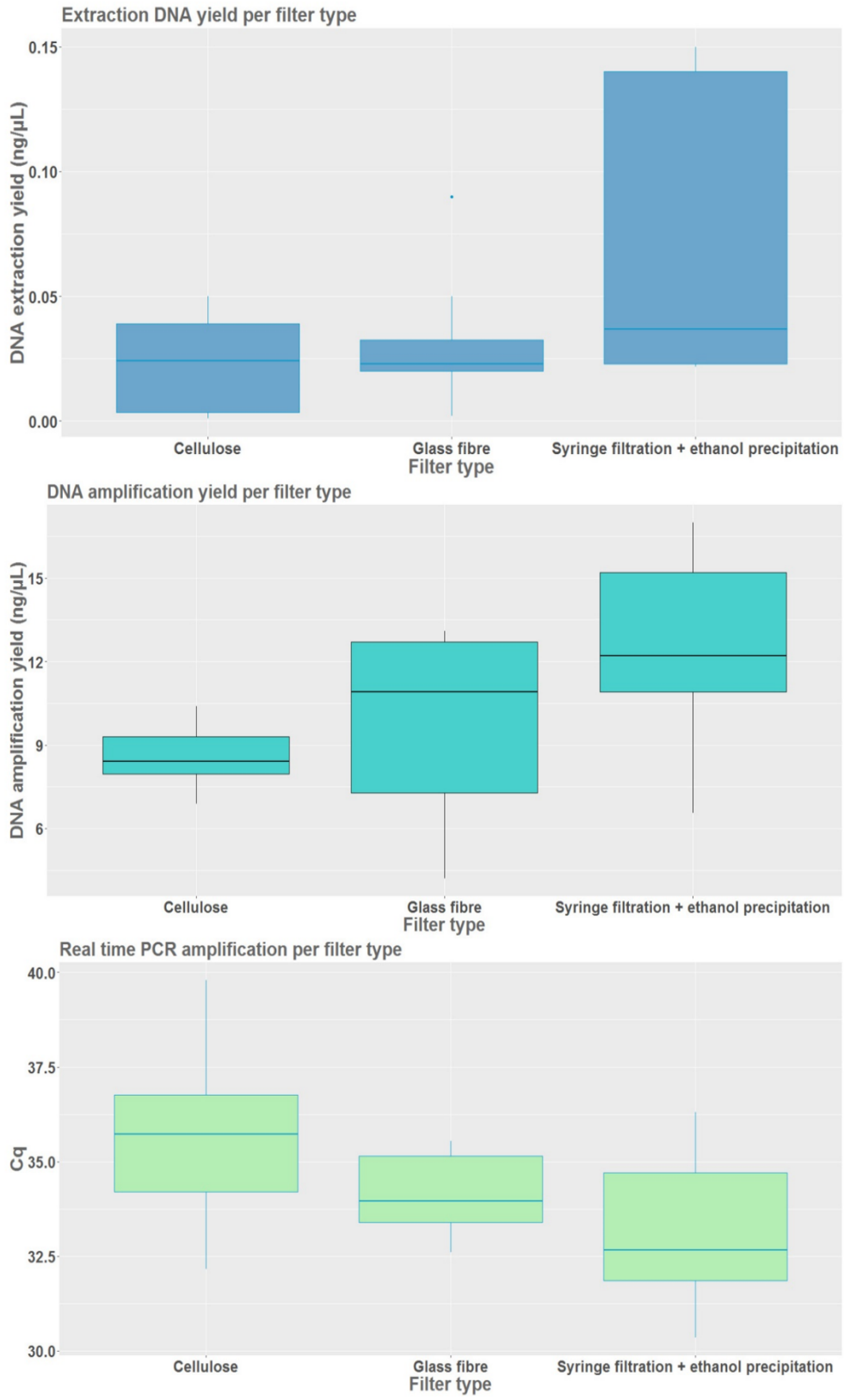


Figure 2. 4- Filter type experiment evaluating eDNA capture yield (ng/µL) and amplification efficiencies. Differences in eDNA capture as well as amplification by PCR and qPCR divided by three categories of filtration type experiment (C- Cellulose nitrate filter, G- Glass fibre filter,

S- Syringe filter with a combination of ethanol- sodium acetate precipitation). Each experimental category is represented by three sampling replicates per three water bodies (9). For the amplification efficiencies the technical duplicates of each sampling replicate was averaged before plotting. The lowest Cq value corresponds to the highest efficiency. The whisker plots represent the standard deviation.

2.3.3 Extraction kit comparison

Thirty-six samples were extracted for the comparison between the filtration types, excluding one negative filtration and four negative extraction controls used for each individual water body, for each of the extraction kit tested. Of these, twelve samples were used for each individual water body as sample triplicates were used for each individual extraction kit. For statistical analysis from DNA capture yield 32 samples were evaluated, for the PCR 34 and for the qPCR 32 samples in total. In a model including both, the experimental groups and water bodies, there were significant differences between extraction kits by the DNA capture yield (Table 2.1, $F(3, 31) = 10.657$, $p = 0.001$) and PCR amplification (Table 2.1, $F(3, 33) = 4.159$, $p= 0.018$) with highest capture and amplification rate of Nexxtec Blood kit (Tukey's Post-hoc test, $p < 0.001$) compared to Nexxtec Tissue and Qiagen, without any significant difference when evaluating amplification with the precise qPCR (Table 2. 1, $F(3, 31) = 0.299$, $p = 0.825$). All Nexxtec kits were generally more efficient with regards to DNA capture in comparison to Qiagen (Figure 2.4). Between the Nexxtec kits the most efficient one appears to be the kit designed for blood samples with much higher efficiency compared to other two, 0.206 ng/ µL higher DNA capture yield on average. The 1 - step Nexxtec DNA Isolation Kit for Blood proved particularly efficient with samples from Cardiff Bay with DNA capture yields of 0.511 ± 0.229 ng/ µL and had on average 4.438 ng/ µL higher amplification rate compared to other Nexxtec kits (Table S. 3).

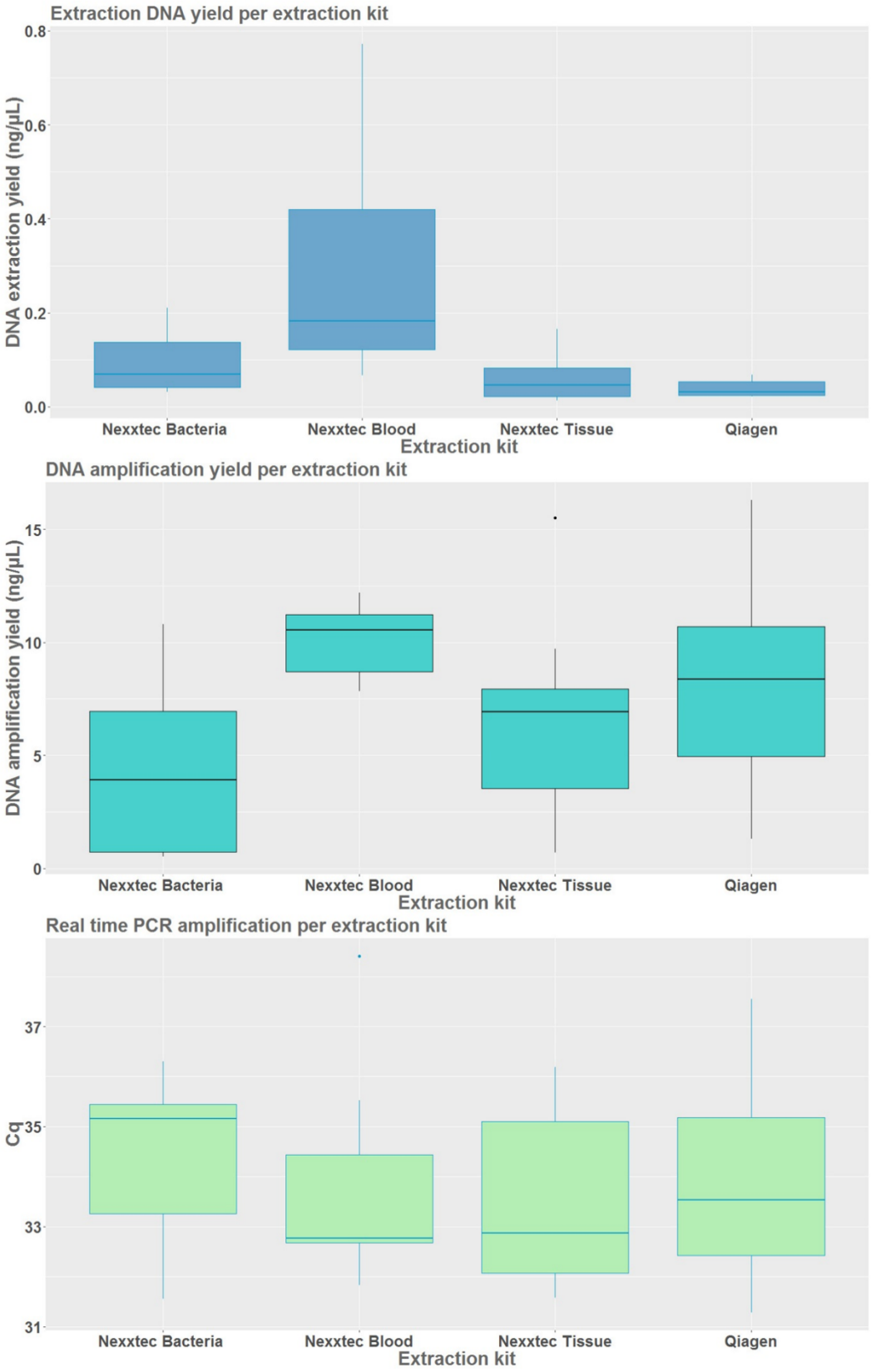


Figure 2. 5- eDNA capture yield (ng/ µL) and amplification efficiencies by extraction kit comparison. The whisker plots represent the standard deviation. Efficiency estimation of DNA

capture extraction efficiency and amplification evaluated by PCR and qPCR, compared between the following extraction kits Nexxtec bacteria, Nexxtec blood, Nexxtec Tissue and Qiagen). Each experimental category is represented by three sampling replicates per three water bodies (9).

2.3.4 Species composition

Sequencing of the cloned PCR products indicated that the three dominant species found in each individual water body were European bullhead (*Cottus gobio*) in the Tawe River, three-spinned stickleback (*Gasterosteus aculeatus*) in the pond and European carp (*Cyprinus carpio*) in Cardiff Bay, irrespective of the sampling technique used (Figure 2. 6, Table S. 4). As 12S-V5 are vertebrate primers, there were also human (*Homo sapiens)*, domestic pig (*Sus scrofa domesticus)* and common mallard (*Anas platyrhynchos*) sequences among the results. From 11 clones in the River Tawe, two were identified as *Anas platyrhynchos* and two remained unidentified, the rest identified as *Cottus gobio.* In the pond, four sequences belonged to *Gasterosteus aculeatus* and three remaining cloning sequences remained unidentified. In Cardiff Bay, five sequences belonged to *Cyprinus carpio*, five to *Homo sapiens,* and one to *Sus scrofa domesticus.*

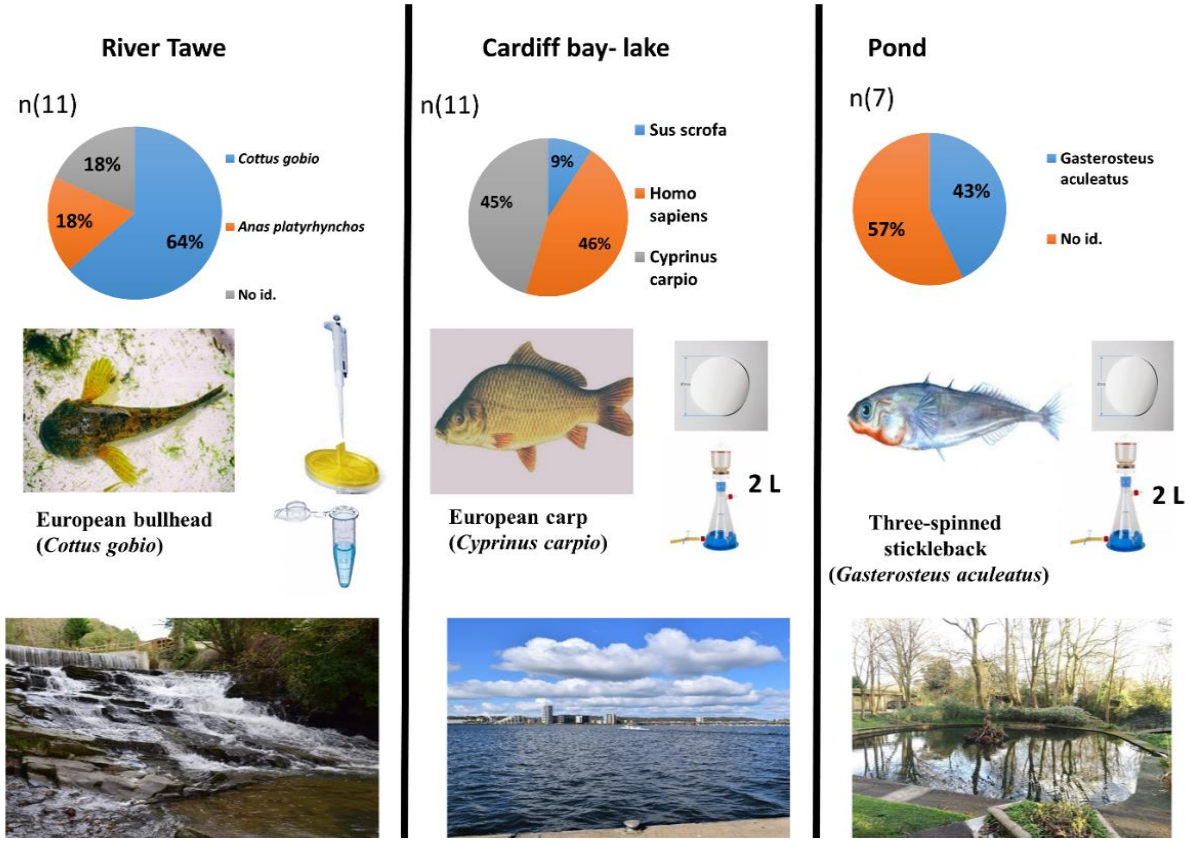


Figure 2. 6- Graphical representation of the most prominent sampling method for each specific water body, river Tawe, Cardiff bay lake and Swansea University pond indicating the most common target fish species. Graphical representation of the most efficient sampling method for all response variables tested for each water body separately based on statistical analysis of capture and amplification efficiencies. Pie charts indicate species proportion from total number (n) of sequenced cloned samples, River Tawe (11), lake Cardiff Bay (11) and Swansea University pond (7). The sequences that were not identified are marked as (No id.). At each water body only one fish species was identified. The 2000 mL filtered water volume and syringe filtering with ethanol- sodium acetate precipitation technique appear to be the most successful.

2.4 Discussion

The results from three different comparisons testing the effects of filtration volume, filter type and extraction procedure, evaluated by DNA capture yield and amplification efficiencies show how important it is to select the appropriate sampling method due to their variable efficiencies. Our results showed no differences in capture yield and amplification success between the three freshwater bodies when all experimental factors were considered, with the exception of (a) eDNA capture rate in relation to filtering volume and extraction kit experiment, with higher

efficiency in pond and Cardiff Bay respectively and (b) higher PCR amplification rate in pond at the extraction kit experiment compared to Cardiff Bay. This study demonstrates that as long as the same eDNA sampling procedure is used in freshwater bodies, species presence and quantitative assessment can be cross- validated and compared across a number of freshwater bodies.

It has been previously shown that a combination of different capture and extraction methods can result in different success rates of eDNA metabarcoding for different target groups (Deiner et al., 2015), using vertebrate primers (Evans et al., 2016, Hänfling et al., 2016, Valentini et al., 2016). Based on our approach, a novel combination of ethanol precipitation with filtration would be the recommended choice as it worked well in lentic and lotic water bodies with a high efficiency, easiness of handling, low cost, low chances of contamination and practicality. The method also appears to be a reliable tool for the eDNA species-specific assessments using species-specific primers, confirming presence/ absence of certain species (Muha et al., 2017), as a tool for cross- amplification validation, as well as a sampling approach to determine community composition based on metabarcoding.

I examined the influence of filtration volume, filter type, filtration method and type of extraction kit on capture yield and amplification efficiencies. DNA capture as well as amplification appeared to be the most responsive towards the changes in filtered water volume as stated elsewhere (Deiner et al., 2015). The efficiency of eDNA capture yield and amplification success rate largely differed between volume groups. It would thus be recommended to filter as much of the water as possible, although the correlation between the size distribution of various particles in the aquatic environment can be a final crucial factor determining selection of filtration between the filter pore size and volume of water (Barnes and Turner, 2016). Size of filtered particles (Barnes and Turner, 2016), contamination and feasibility of the proposed sampling (Ficetola et al., 2016), depending on location and proximity to the laboratory can result in practical limitations in the maximum amount that is possible to filter (Barnes and Turner, 2016). DNA capture efficiency is an important evaluator of sampling technique used as it reflects the presence of the whole DNA within the sample. Another important factor is the number of replicates used for each individual evaluation, as the differences between the sampling triplicates were the most obvious in the DNA capture yield,

where the whole extracted DNA and not just the targeted one was quantified. As so, the highest DNA capture rate was identified in Cardiff Bay at extraction kit experiment, with the lowest amplifications (PCR and qPCR), explained by high non- vertebrate DNA capture. This variability could be due to lack of power and more replicates would be recommended to increase reproducibility.

The most commonly used filter materials in eDNA studies are glass fibre filters (Jerde et al., 2011b, Wilcox et al., 2013, Janosik and Johnston, 2015) and cellulose nitrate filters (Pilliod et al., 2013, Goldberg et al., 2011, Goldberg et al., 2013b) with different pore sizes, where larger pore sizes allow larger filtered water volumes and smaller pore sizes capture more particles but limit volume and speed of filtration (Barnes and Turner, 2016). Here, glass fibre filter resulted in higher efficiency compared to cellulose nitrate filter, a contrasting result to previous ones (Spens et al., 2017). The choice for the material of the filter type used depends as well on the practicality of usage during DNA extraction as filtration materials differ greatly, by the easiness of filter handling and sample preparation for extraction, in our case glass fibre material was preferred. In order to evaluate the efficiency of ethanol precipitation for eDNA capturing, I had used two procedures using same filter materials with same pore sizes. One procedure involved only filtration and resulted in lower DNA capture and amplification efficiency compared to the second procedure with additional ethanol precipitation.

The smallest water volume tested, based only on ethanol precipitation (15 mL) provided solid amplification rates despite small volume. Thus, the newly proposed 100 mL syringe filtration with ethanol precipitation method combines the strength of both techniques: the portability and easiness of the ethanol precipitation while increasing the volume filtered and decreasing contamination risk by minimising filter handling. The proposed syringe filtration method appears to be highly efficient, it is affordable and reliable and it is thus an upgraded method from the efficient one proposed by (Ficetola et al., 2008). High efficiency of syringe filters compared to other filtration techniques has been shown with the use of Sterivex-GP polyethersulfone syringe filters, but it is a more costly alternative to the syringe filters used in this study (Spens et al., 2017). Applying small filtration volumes on rare species, might diminish their detection rate, though successful detection of rare species with 300 mL has been reported (Mächler et al., 2014). An increased number of sampling replicates could compensate small filtered volumes when targeting rare and invasive species.

The extraction kit seems to be the least important factor when it comes to selection of sampling techniques for eDNA capture. On several occasions extraction procedures based on usage of commercial kits resulted in no difference (Djurhuus et al., 2017), whereas in other cases there

has been shown significant variations (Deiner et al., 2015, Djurhuus et al., 2017, Hinlo et al., 2017b). The higher DNA capture efficiency and PCR amplification rates were provided by Nexxtec Blood kit, given its preferences for future usage mainly due to easiness of handling the extraction in a single step before DNA elution which highly minimises risk for the contamination. There was no difference between the extraction kits based on the qPCR assessment.

Species- specific assignment identified one dominant fish species per water body independently of the sampling technique used (Figure 2. 5). The reason for that might be the small sample size for cloning and higher detection rate of the most abundant species in the area (Wharf Angling Club, 2018). In the pond and Cardiff Bay, the most dominant species was expected based on the most common fish species present in each area. For the River Tawe, European bullhead (*Cottus gobio*) is also one of the four most common species found at this location, conducted by working on metabarcoding analysis in 2016 (unpublished). Cardiff Bay is highly associated to human activities and the presence of human DNA is therefore not surprising. Mammal (including human) and avian DNA presence is common in eDNA studies utilising vertebrate primers (Boessenkool et al., 2012, Thomsen et al., 2016) and all our negative filtration, extraction and PCR controls ensured that its origin was not laboratory contamination.

Our study contributes towards the understanding of the role of different sampling and extraction factors on the efficiencies of eDNA capture techniques. Focusing on well-known vertebrate primers, widely used in ecological research (Calvignac-Spencer et al., 2013, Miya et al., 2015, Kelly et al., 2014a, Harper et al., 2018) and to avoid species-specific bias allowed us to compare efficiencies in three different water bodies with distinctive community composition, that can potentially introduce drawbacks assessing eDNA presence/ absence using qPCR, with a preferred species specific assay design. There was no difference between the PCR or qPCR success rate for the two most evident differential factors, the water bodies and volume, whereas filtration type and extraction kit differed greatly. Dissimilarities between capture and extraction techniques between pond, lake and river, highlight the importance of other abiotic aspects affecting eDNA capture efficiency such as acidity, substrate material and hydrological dynamics (Seymour et al., 2018, Jerde et al., 2016, Goldberg et al., 2018), including seasonality (Bista et al., 2017b), which can majorly modify eDNA detection rates in different situations.

In summary, our study indicates that the main source of variation in the eDNA capture and amplification efficiencies is the sampling technique. Our results indicate that a careful sampling plan selecting the most efficient eDNA sampling protocol is essential, and suggest that sampling the largest feasible volume filtered is the optimal. However, a syringe filtration through a 0.45 μm cellulose syringe filter, combined with ethanol- sodium acetate precipitation is an alternative low contamination risk/ high yield method that can be easily used both in lotic and lentic environments with high eDNA yields and decreased sampling efforts.

CHAPTER 3- Impacts of river fragmentation on fish distribution assessed using environmental DNA

Abstract

I have assessed the effects of river fragmentation on fish local distribution by comparing a naturally fragmented and a highly modified river using environmental DNA metabarcoding. For this, I collected water samples upstream and downstream six natural or artificial barriers in both rivers. Shannon- Wiener diversity index indicated a clear division between individual barriers located in individual tributaries in both of the rivers, potentially representing natural differences in local fish communities within a heterogeneous environment. Fish diversity decreased gradually upstream the unmodified river Teifi, whereas in the highly modified river Afan fish diversity differed greatly in between catchments without a clear pattern along the river. Artificial and natural barriers resulted in limited species specific dispersal upstream compared to downstream of the barriers, based on upstream non-migratory species specific absence in Afan river, suggesting upstream distribution limitations, with no identified upstream limitations in Teifi river. Individual non- migratory species specific distribution did not differ between the tributaries, with no apparent decline in abundance upstream the barriers in any of the rivers, as all of the non- migratory species were poorly represented upstream and downstream of the barriers based on total number of sequence reads, excluding Eurasian minnow and European bullhead. Migratory salmonids seemed able to overcome the barrier obstacles during their upstream migration in both of the rivers, with the highest total number of sequence reads found near the headwater tributaries, suggesting no upstream distribution discontinuities for both species, brown trout and Atlantic salmon. The findings suggest eDNA metabarcoding can be a suitable approach for freshwater fish species spatial and temporal dispersal assessment in fragmented rivers.

3.1 Introduction

Dispersal is critical in shaping the composition of fish populations, by affecting their genetics, ecology and long-term evolutionary potential (Radinger and Wolter, 2014). Movement restrictions can reduce gene flow (Horreo et al., 2011) and increase the risk of inbreeding in small populations (Coleman et al., 2018, Murphy et al., 2018), compromising their long term survival. For assessing fish dispersal it is crucial to identify species temporal and spatial diversity, as stream connectivity is needed to allow species movement (Lake et al., 2007). Natural barriers are acting as forces for natural selection, where artificial barriers produce sudden discontinuity patterns, impacting on established populations by isolating them and limiting their life habitats (Rahel, 2013). Both types of barriers can impact fish dispersal by limiting their access to spawning grounds and prey, or simply by restricting their ability to escape from adverse conditions such as high flow or lack of food (Warren Jr and Pardew, 1998, Gehrke et al., 2002). Evaluating the effects of individual barriers on fish dispersal is challenging (Radinger and Wolter, 2014), as river flow determines the temporal variability on passability and permeability of each barrier (Fuller et al., 2015). Thus, understanding the composition of the population community, local species presence, reproductive and seasonal patterns, as well as species specific movement patterns is critical for understanding the degree of disturbance caused (Radinger and Wolter, 2014).

In riverine ecosystems the concept of minimum energy loss involves downstream communities to absorb the upstream surplus of biological production (Vannote et al., 1980), with limited energy flux in fragmented rivers. Changes in functional diversity can reflect disturbance in populations (Maire et al., 2015), therefore assessing changes in fish community composition in time and space can be used to estimate the movement restrictions resulting from natural and artificial barriers in rivers. Fish diversity and genetic variation tend to increase downstream, due to larger niche segregation and the limitations imposed by upstream high flow intensity (Grossman et al., 2010), shaping the distribution of fish species depending on their biology and swimming abilities (Bunt et al., 2012). Patterns of decreased diversity upstream of the barriers have been observed for non- leaping fish, regardless of barrier age, in comparison to its downstream section (Coleman et al., 2018).

New approaches such as eDNA metabarcoding are becoming widely utilised to assess species presence and relative diversity in time and space (Bohmann et al., 2014, Thomsen and Willerslev, 2015, Barnes and Turner, 2016), benefiting towards non- invasive fish diversity

assessment, which could in a combination with traditional assessed diversity techniques, such as electrofishing, net capturing, angling or infrared beaming (Evans et al., 2017b, Bennett et al., 2009, Santos et al., 2008) contribute towards dispersal assessment and dispersal limitations of individual species. Environmental DNA (eDNA) is a non- invasive approach targeting short fragments of DNA extracted from environmental samples which is being increasingly used for the detection of aquatic species (Cristescu and Hebert, 2018, Valentini et al., 2016). The technique can be used for freshwater management purposes, including the detection of rare, cryptic or ephemeral species (Cristescu and Hebert, 2018), assessing barrier limitations towards spread of invasive species (Cowart et al., 2018) or even mitigating disease outbreaks (Bastos Gomes et al., 2017), but has not yet been used to analyse the impact of barriers in fish community structure.

To analyse the role of barriers in fish community structure and connectivity I have compared two rivers with different degrees of artificial modification using eDNA metabarcoding. The main objective was to evaluate how a number of individual natural or artificial barriers affect the distribution of freshwater fish local populations by comparing relative sequence read abundance between the barriers located in different tributaries and upstream and downstream division for each separate barrier.

3.2 Methods

3.2.1 Study site and eDNA water filtration and extraction

I sampled two Welsh small scale rivers, the rivers Teifi and Afan, selected due to their differential levels of artificial modifications, the Teifi being a pristine river with a modest level of artificial barriers, and the Afan being largely modified by a high number of artificial barriers. The rivers were selected for comparison due to their close proximity, similar low altitude, relatively short distances between headwaters and mouths of the river, including high number of small tributaries with known identified barriers and similar resident fish species populations (Cowx et al., 2009, Mortimer, 2012). The river Teifi, is a low impacted river (Dunbar et al., 2010), with little level of human intervention, affected by low population density and sources of pollutants. This Site of Special Scientific Interest (SSSI) is 112 km long in a 1012 km^2 catchment, with high water quality throughout the year, being the most productive salmon and sea trout fisheries ground across England and Wales (Peirson et al., 2001). The river Teifi has five major tributaries, three of them having been sampled in this study. The river Afan is a 17 km long river, which has been highly modified for mining purposes since the early nineteenth century, with a large number of artificial constructions (List of mines in Great Britain, 2009).

With the decline in coal mining industry in 1970s, the salmon and sea trout stocks recovered, with lowest stock assessed in 2015 (Natural Resources Wales, pers. comm), due to historical mine-water pollution, high number of barriers and deforestation (Mortimer, 2012). The lower part of the river Afan is heavily modified and is classified as having moderate ecological potential by the UK Environmental Agency (Mortimer, 2012). Five major tributaries feed the river Afan, four of them sampled here for eDNA.

In both rivers three barriers were selected in the upper part of the catchment and three in the lower. In the river Teifi two barriers were artificial (weirs) and four natural (rock formations and waterfalls) (Figure 3.1) (Table S. 13). In the river Afan five of the barriers selected were artificial (weirs, culverts and rock formations) and the sixth barrier was a natural high waterfall, presumably unpassable for fish moving upstream (Figure 3.2) (Table S. 14). Three replicates of water samples were collected in October 2016 upstream and downstream the barriers at no more than 15 m distance. The selection of barriers was based on close proximity to headwaters at each of the stream tributary for clear assessment of upstream/ downstream individual barrier distribution limitations, with only two barriers in Teifi and one in Afan allocated at the actual river.

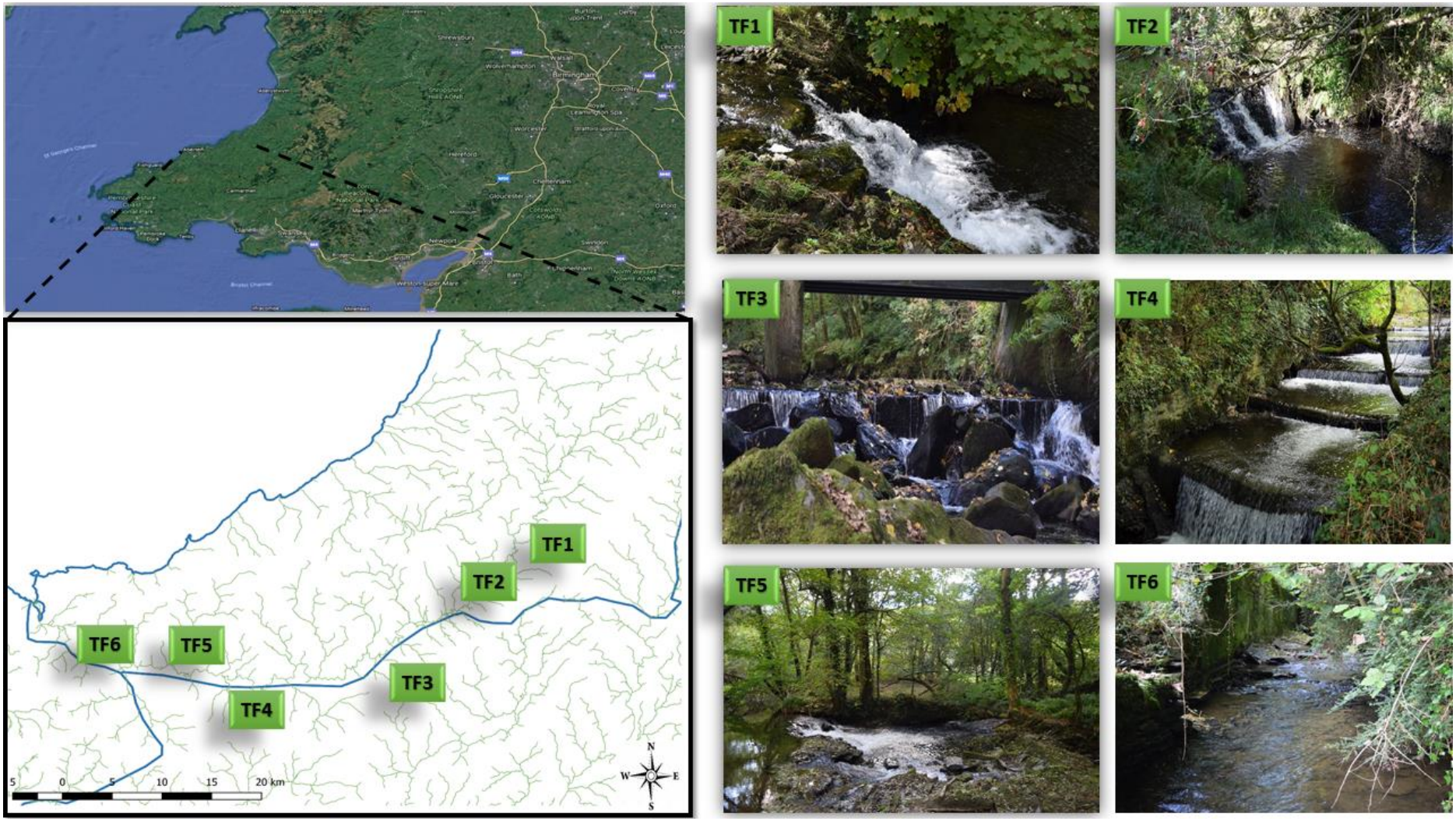


Figure 3. 1- Location of barriers at the river Teifi (Wales) where eDNA sampling was conducted upstream and downstream from each individual barrier, with TF 1 being the most upstream and TF 6 being the most downstream sampling location.

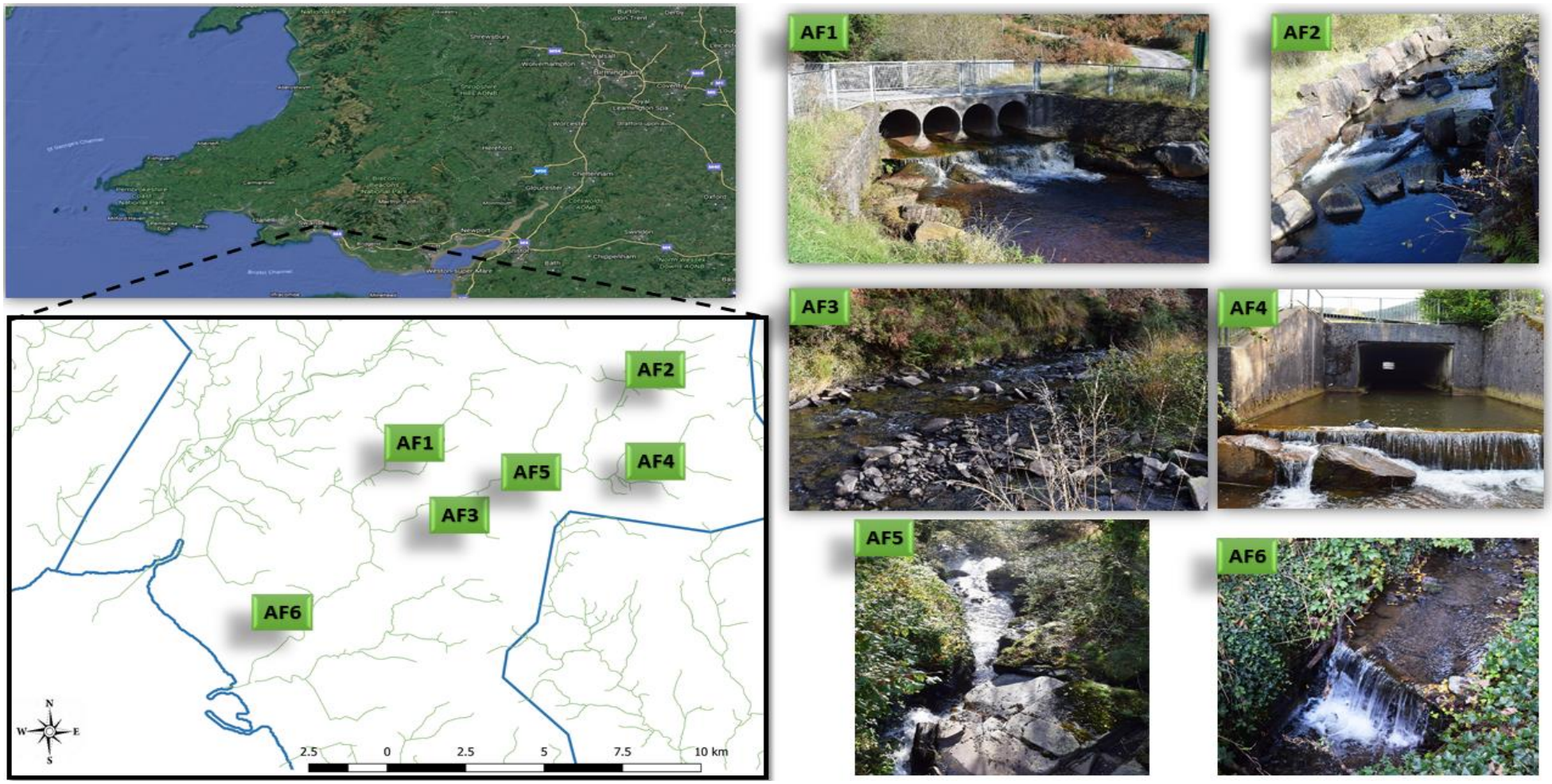


Figure 3. 2- Location of barriers at the river Afan (Wales) where eDNA sampling was conducted upstream and downstream from each individual barrier, with AF 2 being the most upstream and AF 6 being the most downstream sampling location.

Abiotic parameters in the river Teifi River on the day of collection consisted of low flow of 14.89 $m^3\ s^{-1}$, with no precipitation a week before. In the river Afan the water was collected twelve days later, with a water flow of 1.53 $m^3\ s^{-1}$. Three replicates of 1 litre of water were obtained from each sampling point. Water was collected using Nalgene polyethylene bottles treated with 10 % bleach and rinsed with sterile distilled water beforehand. The bottles were refrigerated and transported to the laboratory for filtration within 4 hours of collection. The water was filtered through a filter funnel attached to a collection bottle and connected to the electronic vacuum pump, using Advantec GA55 Borosilicate Glass Fibre Filters with 0.6 µm pore size (47 mm). To minimize the risk of cross-contamination, disposable nitrile gloves were used, filter funnel and handling tweezers were cleaned with a 10 % bleach solution and rinsed with 99 % molecular grade ethanol and sterile nuclease-free water afterwards. No more than two filters were used per 1 L of the water and stored separately per each sampling triplicate in 1.5 mL vials at - 20 °C until the DNA extraction a week later. Negative control samples were filtered using sterile nuclease-free water between filtering samples from different sampling stations for both river sampling events.

The extractions and pre-PCR handling of eDNA water samples was carried out in a fume hood dedicated to eDNA analyses only. For eDNA extraction, the Qiagen DNeasy Blood & Tissue DNA extraction kit (Qiagen GmbH, Hilden, Germany) was used following the manufacturer's protocol for dried blood spots, with last step reduced to 50 µL of elution volume. Filter and extraction negative controls were extracted using sterile nuclease- free water for each of the individual sampling events, following same procedure as for the environmental samples.

3.2.2 Amplicon validation, PCR preparation and high- throughput sequencing

Vertebrate primers (Kelly et al., 2014a, Port et al., 2016) targeting 144 bp of the 12s rRNA mtDNA gene were used (Riaz et al., 2011a). The primers 12S-V5-F (5'- ACTGGGATTAGATACCCC- 3') and 12S-V5-R (3'- TAGAACAGGCTCCTCTAG- 5') were initially tested *in silico*, and validated by amplification and Sanger sequencing of six fish species, including sea trout (*Salmo trutta*), and Atlantic salmon (*Salmo salar*). For this, DNA was extracted from fin clips using same extraction kit as for the eDNA samples.

The PCR amplification was performed with 12.5 µL Bioline BioMix Red PCR Mastermix (2X), 3 µL template, 1.5 µL of each primer (10 µM), adding sterile nuclease- free water to final total volume of 30 µl. The thermal cycle profile after an initial 10 min 95 °C was as follows: denaturation at 95 ºC for 30 s; annealing at 52 ºC for 30 s; and extension at 72 ºC for 30 s with

the final extension at the same temperature for 5 min, using 35 annealing cycles in total. For the evaluation of eDNA samples 10 additional cycles were used, adding a PCR negative control to all PCR runs. The products were visualised on a 2 % agarose gel stained with 2 µL of GelRed™ nucleic acid gel stain.

High- throughput sequencing using Illumina MiSeq (Illumina, San Diego, CA, USA) was used for all the samples from both rivers following a 16S Metagenomic Sequencing Library Preparation protocol by Illumina (Illumina, 2013), using a 2-step PCR approach. The sampling triplicates were normalised and pooled to create 4 nM pooled libraries. All extraction and PCR amplification efficiencies were measured by Qubit™ 4 Fluorometer (ThermoFisher Scientific, UK) applying the high-sensitivity assay (Life Technologies, Carlsbad, CA, USA) for extraction efficiencies and broad assay for PCR efficiencies. The paired-end sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) at the Institute of Life Sciences, College of Medicine, Swansea University.

3.2.3 Data processing and bioinformatics analysis

Mothur v1.39.5 (Schloss et al., 2009) was used for the analysis of high- throughput sequences produced by MiSeq Illumina. After de-multiplexing the reads from individual sampling triplicates, the removal of poor quality reads (minimum quality score of Q= 20) together with primer and sample tags was done using Trimmomatic (Bolger et al., 2014). Combining reads were paired-ended, with ambiguous bases removed before further work. Only fish sequences of 12S- V5 target region were aligned to a custom reference database. Removal of chimeras and noise was conducted through UCHIME (Edgar et al., 2011), following the singleton removals. Negative filtration and extraction control samples were also analysed. The clustering was performed at 97 % using the opticlust algorithm. Trimmed sequence reads were taxonomically assigned using BLASTn to a 12S rRNA vertebrate DNA reference database (RefSeq), downloaded from NCBI taxonomy database (Pruitt et al., 2006). A minimum score of S' > 109 and > 92.3 % sequence identity of the top hit for each cluster were used. Sequence files containing raw reads have been deposited to GenBank and can be allocated through the following accession number: PRJNA514035.

3.2.4 Statistical analysis

Fish diversity, presence and species specific abundance based on total number of sequence reads were evaluated for assessing differences between tributaries and changes between upstream/ downstream at each individual barriers, by comparing a) individual barriers (tributaries) (TF 1- 6 in Teifi and AF 1- 6 in Afan River) and b) upstream/ downstream of

individual barriers (TF 1- 6 upstream/ downstream; AF 1- 6 upstream/ downstream division), for each of the rivers. For comparisons among individual barriers (tributaries), upstream and downstream triplicates for each individual barrier were merged for the analysis, resulting in six sampling replicates representing one individual barrier. Fish diversity assessment in both Teifi and Afan was based on the Shannon- Wiener index (H') (Pielou, 1966), which was calculated for each sampling site. Differences in species diversity based on Shannon- Wiener Index were assessed using two predictors, individual barriers and upstream/ downstream, including interaction between them, using linear model (LMs). For the evaluation of fish presence/ absence, a binary logistic regression was used, assessing presence among individual barriers (tributaries), and upstream/ downstream of each individual barrier, evaluated individually for each river, applying a model with three predictors, species, individual barriers and upstream/ downstream, including interaction between them. For presence, only samples positive for at least two sampling duplicates were considered, whereas absence of a species was only considered when all replicates were negative. Species specific total number of sequence reads was evaluated using LMs, based on two predictors, individual barriers (tributaries), and upstream/ downstream of individual barriers and their interaction. For post-hoc analyses, the 'eemeans' package was used (Lenth, 2016) based on Tukey's contrasts, used for pairwise assessment comparing species specific total number of sequence reads changes between individual obstacles, at both rivers individually. Sampling triplicates were applied independently for LMs. 'Vegan' package (Dixon, 2003) was used for all Shannon- Wiener index diversity measures. Models were compared based on AIC criteria using the 'mass' package. All statistical analyses were done using R, version 3.3.2.

3.3 Results

3.3.1 Qualitative eDNA species assignment

A total of 72 eDNA samples were sequenced. After merging paired- end reads, quality filtering, de-replications and removal of chimeras and singletons, 303911 and 53804 sequences were left to be assigned to taxa for the rivers Teifi and Afan, respectively. No amplification occurred in the eDNA filtration and extraction negative controls, but appeared in PCR negative controls, with known species occurring from laboratory contamination, being removed from further analysis. Illumina sequencing produced single peaks at 144 bp, as expected. In the river Teifi, 31% of sequences belonged to non- fish representatives, of which 58% belonged to mammals and 42% to birds. In the river Afan, 29% of the sequences were assigned to species other than fish, of which 43% belonged to mammals and 57% to birds. All sequences were identified up

to species level, representing 9 and 10 species in total for both, Afan and Teifi, respectively: Eurasian minnow (*Phoxinus phoxinus*), stone loach (*Barbatula barbatula*), European bullhead (*Cottus gobio*), three-spined stickleback (*Gasterosteus aculeatus*), grayling (*Thymallus thymallus*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), European eel (*Anguilla anguilla*), and lamprey (*Lampetra spp*.) (only in Teifi). Misleading sequence assignments from fish species not known to UK freshwaters were removed from the dataset. Sequences belonging to *Oncorhynchus masau, Oncorhynchus kisutch, Cottus rheanus* and *Anguilla japonica* mitochondrion genome references were miss-assigned and thus manually curated to known target species, rainbow trout (*Oncorhynchus mykiss*), European bullhead (*Cottus gobio*) and European eel (*Anguilla anguilla*) accordingly, all being included in the analysis.

3.3.2 eDNA fish connectivity assessment

3.3.2.1 River Teifi

Species richness was compared among individual barriers (tributaries) and between upstream/ downstream of individual barriers using the Shannon- Wiener Index. Ten species were identified in the river Teifi, for which the diversity differed significantly among individual barriers (tributaries) (H', F (df= 5, N=24) = 3.823, $p = 0.01$), with fish diversity at the two highest natural barriers, TF 1 and TF 3, being the lowest compared to the sampling location closest to the river mouth, TF 6, which had the highest diversity (Tukey's Post-hoc test, $p < 0.045$, $p = 0.024$), respectively. There were no significant differences in fish diversity between upstream and downstream sampling locations of each individual barrier (H', F (df= 5, N=24) = 2.431, $p = 0.06$). Lowest Shannon- Wiener diversity index was found upstream the most upstream river barrier (TF 1), with an average H'= 0.82, and the highest located below the most downstream barrier (TF 6), with an average H'= 1.17.

Presence/ absence, assessed with binary logistic regression, differed between species (Table 3.1, χ^2 (df=9, N=100) = 49.624, $p < 0.001$) and the individual barriers with corresponding tributaries (Table 3.1, χ^2 (df=5, N=95) = 32.89, $p = 0.005$), with *Lampetra spp*., *B. barbatula*, *G. aculeatus* eDNA not found around TF 3, TF 6 and TF 1, respectively. These three non-migratory fish species, together with *T. thymallus* were not identified upstream several barriers, TF 1, 2, 3, 5 and 6 (Fig 3.1), despite no significant differences found between upstream/ downstream of each individual barrier (Table 3.1, χ^2 (df=5, N=90) = 22.402, $p = 0.072$).

Species specific diversity distribution assessment applying linear models, based on total number of sequence reads indicated statistically significant differences between individual barriers (tributaries) for one species only, *A. anguilla* (Table 3.2, F (N= 30) = 10.491, df= 5, p < 0.001). *A. Anguilla* density differed significantly between TF 6 with all other barriers, with the highest total number of sequence reads at TF 6 (Table 3.3, Tukey's Post-hoc test, p < 0.001) (Fig 3.1). None of the barriers posed a limitation for upstream dispersion (Table 3.2), based on total number of sequence reads found upstream for majority of species. The most abundant species with similar density patterns at majority of sampling stations were *S. trutta*, *S. salar*, *C. gobio* and *P. phoxinus* (Figure 3.2). In general, eDNA from all the species was found at majority of sampling stations, with a similar average abundance, at both upstream and downstream from the barriers sampling locations, with the exception of *Lampetra spp*., that was in majority detected downstream of the barriers (Figure 3. 2).

Table 3. 1- Species specific presence/ absence evaluation using binary logistic regression by applying the following model, Presence/ absence = Species * Individual barrier * Upstream/ downstream, evaluated individually for both Teifi and Afan rivers.

Factors	**Df**	**Deviance**	**Residual df**	**Residual deviance**	**< Chi square**	**AIC**
River Teifi						
Presence/ absence= Species * Individual barrier * Upstream/ downstream						220
Species	9	96.11	109	54.24	**< 0.001**	
Individual barrier	5	14.027	104	40.214	**0.015**	
Upstream/ downstream	1	2.412	118	150.35	0.120	
Species x Upstream/ downstream	9	7.722	95	32.492	0.562	
Individual barrier x Upstream/ downstream	5	10.09	90	22.402	0.072	
Species x Individual barrier	45	22.402	45	0	0.998	

Factors	Df	Deviance	Residual df	Residual deviance	< Chi square	AIC
Species x Individual barrier x Upstream/ downstream	45	0	0	0	1	
Presence/ absence= Species * Individual barrier						133.86
Species	9	75.176	100	49.624	**< 0.001**	
Individual barrier	5	16.726	95	32.89	**0.005**	
Species x Individual barrier	45	19.035	50	13.86	0.999	
River Afan						
Presence/ absence= Species * Individual barrier * Upstream/ downstream						
Species	9	97.19	109	53.8	**< 0.001**	
Individual barrier	5	0.984	104	52.817	0.963	
Upstream/ downstream	1	0.342	118	150.997	0.558	
Species x Upstream/ downstream	9	4.2	95	48.617	0.897	
Individual barrier x Upstream/ downstream	5	16.04	90	32.603	**0.006**	
Species x Individual barrier	45	32.603	45	0	0.915	
Species x Individual barrier x Upstream/ downstream	45	0	0	0	1	

Table 3. 2- Linear models assessing species specific total number of sequence reads variations by two predictors, Individual barrier (tributary) and Upstream/ downstream division including interactions between them. Linear models were applied for all ten species individually, found in Teifi river.

Predictor	**F**	**N**	**df**	**p**	**AIC**
		Anguilla anguilla			
N. of sequence reads= Individual barrier * upstream/ downstream					478.62
Individual barrier	12.623	30	5	< **0.001**	
Upstream/ downstream	2.2781	29	1	0.144	
Individual barrier x upstream/ downstream	1.963	24	5	0.120	
N. of sequence reads= Individual barrier					481
Individual barrier	10.491	30	5	< **0.001**	
		Cottus gobio			
Individual barrier	1.609	30	5	0.195	
Upstream/ downstream	0.05	29	1	0.823	
Individual barrier x upstream/ downstream	1.511	24	5	0.221	
		Phoxinus phoxinus			
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	1.308	30	5	0.293	
upstream/ downstream	0.0001	29	1	0.991	
Individual barrier x upstream/ downstream	1.227	24	5	0.306	
		Gasterosteus aculeatus			

Predictor	F	N	df	p	AIC
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	0.96	30	5	0.45	
upstream/ downstream	1.02	29	1	0.32	
Individual barrier x upstream/ downstream	1.24	24	5	0.31	
Barbatula barbatula					
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	1.459	30	5	0.241	
upstream/ downstream	0.48	29	1	0.492	
Individual barrier x upstream/ downstream	0.41	24	5	0.835	
Oncorhynchus mykiss					
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	0.829	30	5	0.541	
upstream/ downstream	0.271	29	1	0.607	
Individual barrier x upstream/ downstream	0.643	24	5	0.668	
Salmo salar					
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	0.401	30	5	0.843	
upstream/ downstream	0.805	29	1	0.805	
Individual barrier x upstream/ downstream	0.448	24	5	0.448	
Salmo trutta					

Predictor	F	N	df	p	AIC
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	0.80	30	5	0.555	
upstream/ downstream	0.023	29	1	0.878	
Individual barrier x upstream/ downstream	0.484	24	5	0.784	
Thymallus thymallus					
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	1.411	30	5	0.255	
upstream/ downstream	0.235	29	1	0.632	
Individual barrier x upstream/ downstream	0.941	24	5	0.472	
Lampetra spp.					
N. of sequence reads= Individual barrier * upstream/ downstream					
Individual barrier	1.94	30	5	0.123	
upstream/ downstream	1.90	29	1	0.180	
Individual barrier x upstream/ downstream	0.819	24	5	0.548	

Table 3. 3- Tukey's post- hoc analysis for species specific total number of sequence reads comparison between individual obstacles (tributaries), based on the single pairwise assessment in Teifi river for each species individually. Only statistically significant differences are reported.

Predictor	Variability	SE	df	t ratio	p
Anguilla anguilla					
Individual barrier	TF1- TF6	0.022	24	- 5.94	< **0.001**

	TF3- TF6	0.022	24	-	5.639	< **0.001**
	TF4- TF6	0.022	24	-	5.599	< **0.001**
	TF5- TF6	0.022	24	-	5.028	< **0.001**

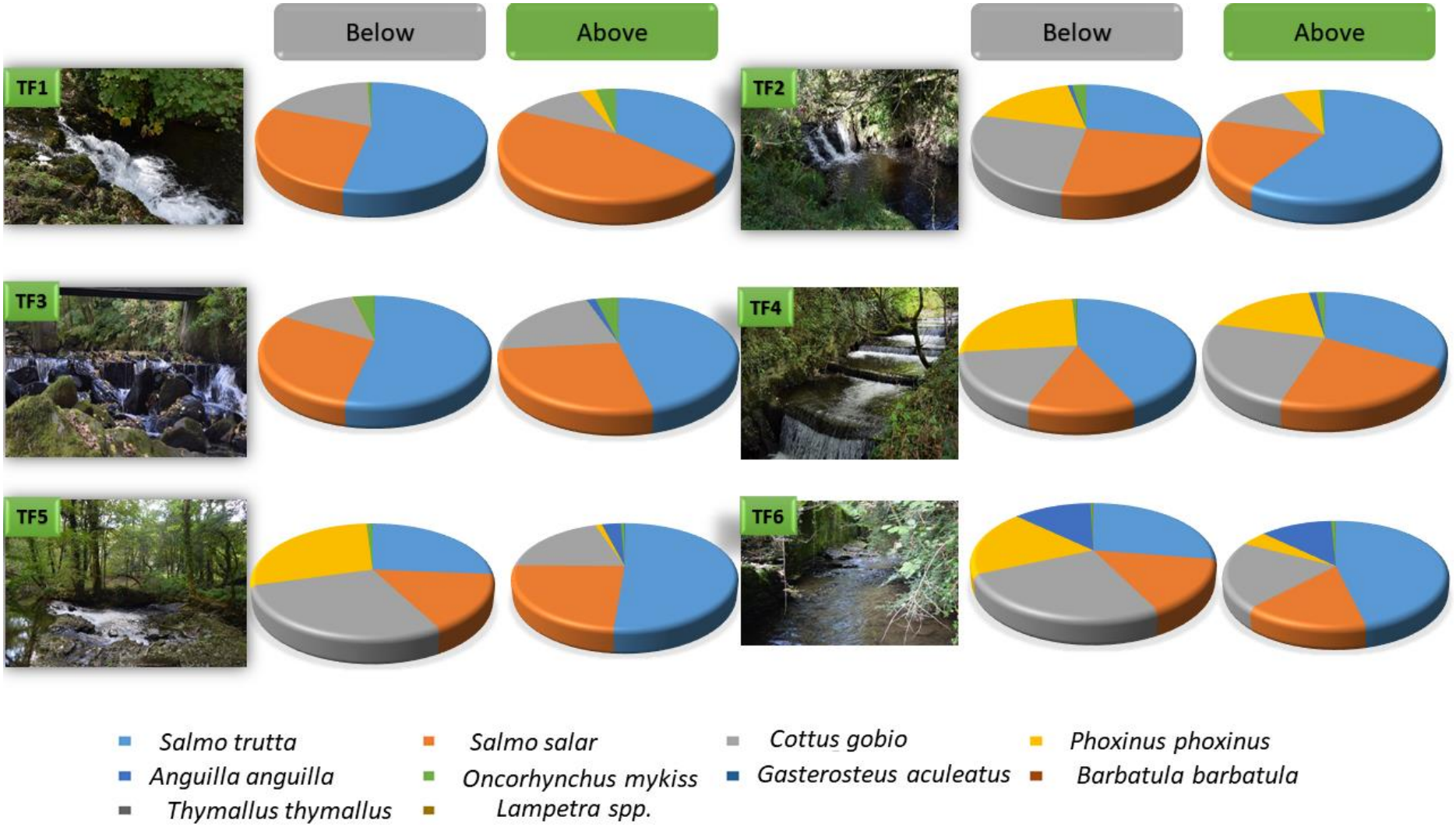


Figure 3. 1- Species distribution assessed by total number of sequence reads at each particular sampling location (upstream/ downstream the six barriers) in Teifi river.

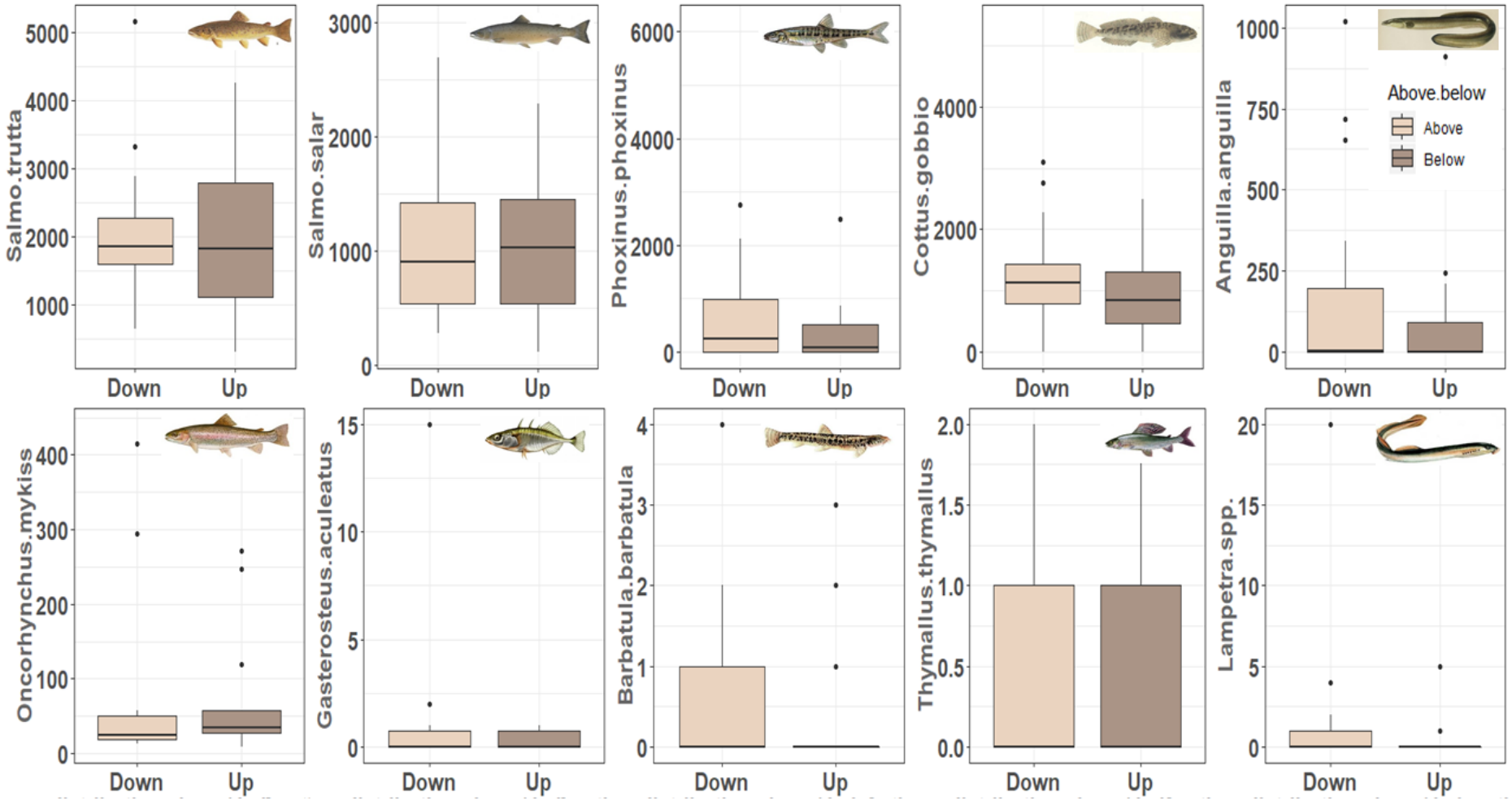


Figure 3. 2- Fish species upstream (left boxplot) and downstream (right boxplot) the barriers in the river Teifi represented for all ten fish species, *Salmo trutta*, *Salmo salar*, *Phoxinus phoxinus*, *Cottus gobio*, *Anguilla anguilla*, *Onchorhynchus mykiss*, *Gasterosteus aculeatus*, *Barbatula barbatula*, *Thymallus thymallus* and *Lampetra spp.*, based on total number of sequence reads.

3.3.2.2 River Afan

Linear model, assessing fish diversity based on Shannon- Wiener Index in Afan river, indicated significant differences between the barriers (tributaries) (H', $F(5, N= 24) = 4.55$, $p = 0.004$) with the highest diversity at sampling locations AF 2, 4 and 6. AF 1 (culvert), had much lower diversity compared to other three AF 2 (rock formation), AF 4 (culvert), and AF 6 (weir) (Tukey's Post-hoc test, $p = 0.003$, $p= 0.002$, $p = 0.003$), respectively (Figure 3. 3). The highest average diversities were identified at the stations closest to the river mouth and at the most upstream sampled barrier, with an average of 1.043 (AF 6) and 1.275 (AF 2) based on Shannon-Wiener diversity index. The fish diversity based on Shannon- Wiener Index did not differ between upstream/ downstream distribution of individual barriers (H', $F(5, N= 24) = 0.624$, $p = 0.682$).

Presence/ absence, assessed with binary logistic regression, differed between species (Table 3.1, $\chi^2(9, N=109) = 53.8$, $p < 0.001$) and upstream/ downstream individual barriers division (Table 3.1, $\chi^2(5, N=90) = 32.603$, $p = 0.006$), but did not differ between individual tributaries (barriers) (Table 3.1, $\chi^2(5, N=104) = 52.817$, $p = 0.963$). Several non- leaping fish were not detected upstream particular barriers, i.e. *B. barbatula* (AF 1, 2 and 6), *T. thymallus* (AF 4) and *G. aculeatus* (AF 1, 2 and 3).

Linear species specific models, assessing total number of sequence reads of particular species, did not differ significantly between individual barriers, neither between upstream/ downstream individual barrier distribution (Table 3.4). The two most representative non- migratory fish species are the *P. phoxinus* and *C. gobio (*Table 3.4, Figure 3. 4), with the least representative three species, *B. barbatula*, *T. thymallus* and *G. aculeatus*, who had low number of reads at all sampling stations, with an average of 0.013 % in comparison to other six dominant species. The list of most abundant species in the river Afan follows the same pattern as in the Teifi (Figure 3. 4).

Table 3. 4- Linear models assessing species specific total number of reads variations by two predictors, Individual barrier (tributary) and Upstream/ downstream division including interactions between them. Linear species specific models were applied for all ten species found in Afan river.

Predictor	**F**	**N**	**df**	**p**
	Anguilla anguilla			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	0.510	30	5	0.765
Upstream/ downstream	0.782	29	1	0.385
Individual barrier x upstream/ downstream	1.346	24	5	0.279
	Cottus gobio			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	2.347	30	5	0.071
Upstream/ downstream	4.074	29	1	0.054
Individual barrier x upstream/ downstream	0.957	24	5	0.463
	Phoxinus phoxinus			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	1.389	30	5	0.263
upstream/ downstream	2.785	29	1	0.108
Individual barrier x upstream/ downstream	0.727	24	5	0.609
	Gasterosteus aculeatus			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	1.077	30	5	0.397
upstream/ downstream	0.03	29	1	0.854
Individual barrier x upstream/ downstream	1.07	24	5	0.399

Predictor	F	N	df	p
	Barbatula barbatula			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	0.885	30	5	0.503
upstream/ downstream	0.885	29	1	0.355
Individual barrier x upstream/ downstream	0.755	24	5	0.590
	Oncorhynchus mykiss			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	0.905	30	5	0.493
upstream/ downstream	1.370	29	1	0.253
Individual barrier x upstream/ downstream	1.7840	24	5	0.154
	Salmo salar			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	2.43	30	5	0.063
upstream/ downstream	2.468	29	1	0.129
Individual barrier x upstream/ downstream	1.932	24	5	0.126
	Salmo trutta			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	1.082	30	5	0.395
upstream/ downstream	2.2.13	29	1	0.149
Individual barrier x upstream/ downstream	1.319	24	5	0.289
	Thymallus thymallus			
N. of sequence reads= Individual barrier * upstream/ downstream				
Individual barrier	1.19	30	5	0.343

Predictor	F	N	df	p
upstream/ downstream	0.606	29	1	0.606
Individual barrier x upstream/ downstream	1.300	24	5	0.300

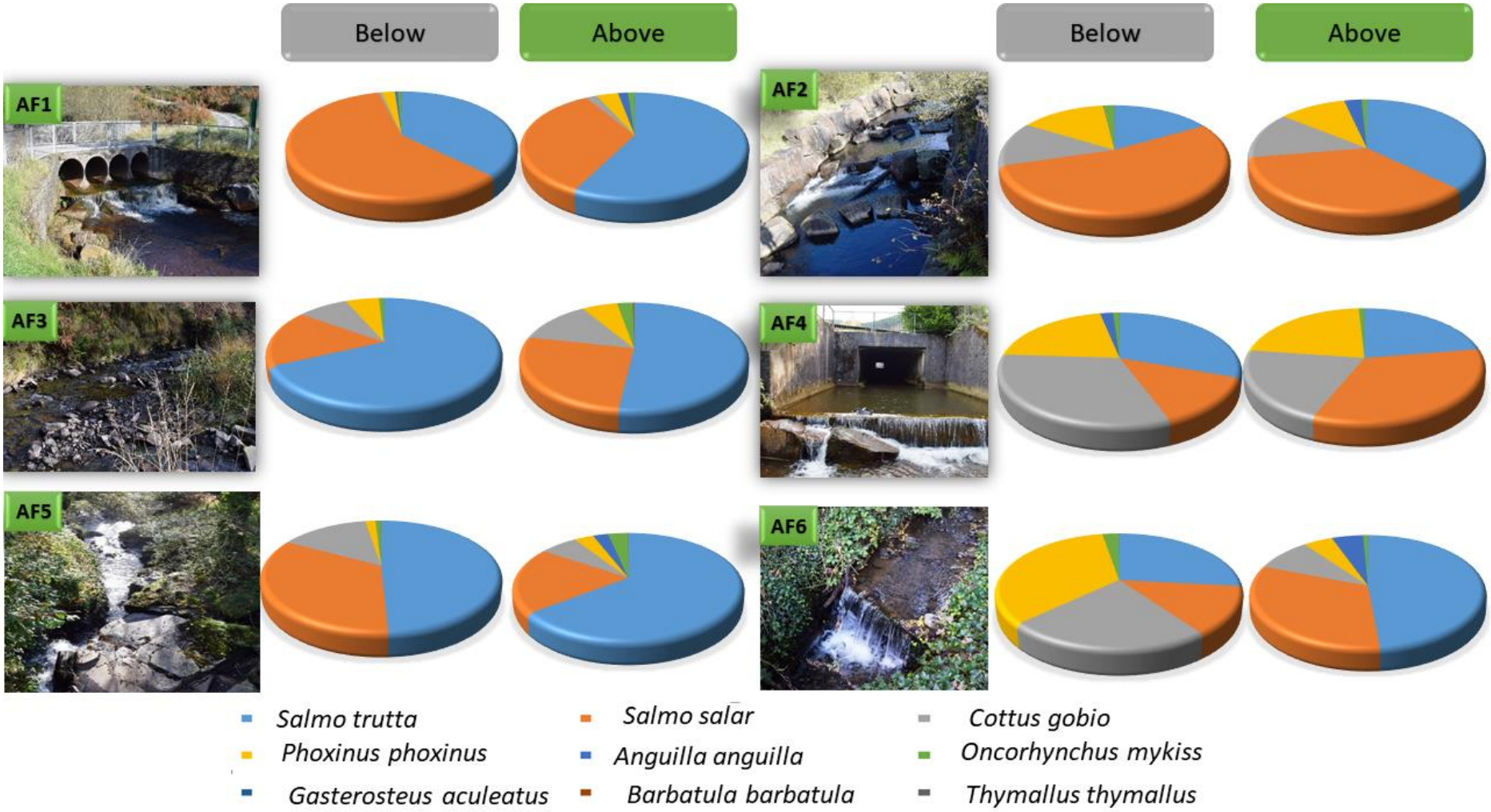


Figure 3. 3- Species distribution assessed by total number of sequence reads at each particular sampling location (upstream and downstream the six barriers) in Afan river.

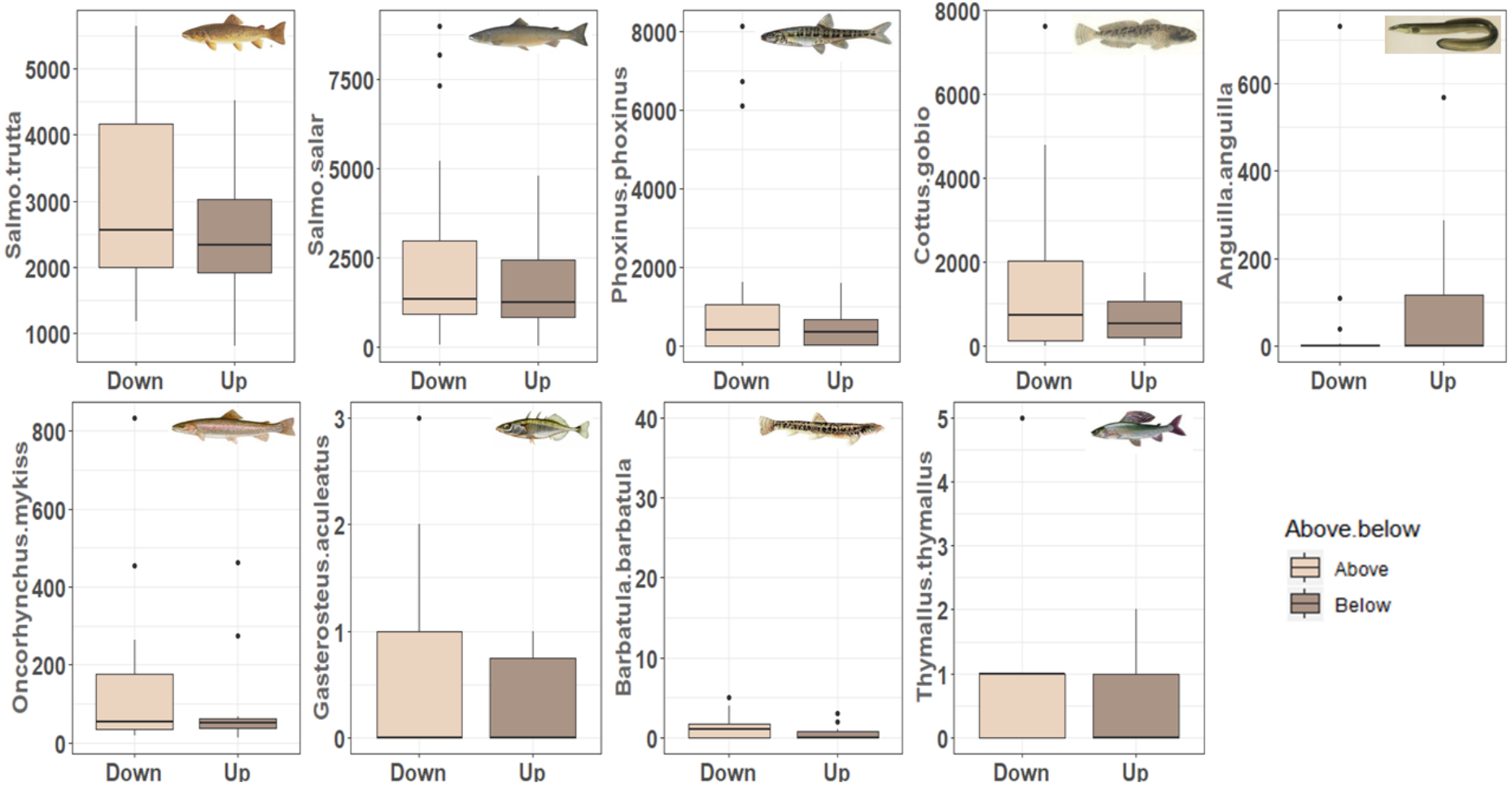


Figure 3. 4- Fish species upstream (left boxplot) and downstream (right boxplot) the barriers in the river Afan represented for all ten fish species found in both of the rivers, *Salmo trutta*, *Salmo salar*, *Phoxinus phoxinus*, *Cottus gobio*, *Anguilla anguilla*, *Onchorhynchus mykiss*, *Gasterosteus aculeatus*, *Barbatula barbatula* and *Thymallus thymallus*, based on total number of sequence reads.

3.4 Discussion

Freshwater fish distribution was assessed in two distinctively fragmented rivers. Diversity indices and species specific models identified differences in species composition between the individual barriers located in different tributaries, and also between upstream/ downstream individual barriers segregation, indicating limited upstream dispersal, particularly for non- migratory species, in both rivers. This fragmentation could potentially result in decreasing species diversity on a long run as well as decreased gene flow, particularly in headwaters (Junker et al., 2012). The difference in diversity of fish assemblages along the river corridor between individual tributaries, can be explained by the effects of spatially heterogeneous landscapes and processes (Altermatt, 2013), increasing fish populations structural heterogeneity. In both rivers, the sampling occurred at least two km distance between individual barriers, located in different tributaries, which may have shaped the composition of fish assemblages in the local communities (Altermatt, 2013). For example, in the river Teifi, where the highest diversity was found downstream closest to the mouth of the river compared to the lowest at the most upstream sampling point located above the most upstream barrier, the natural pattern of fish assemblage coincides with the river continuum concept (Vannote et al., 1980), and can be difficult to separate the pattern from the river habitat fragmentation effects. In the river Afan, the diversity distribution did not follow a continuous pattern with decreasing diversity from the mouth of the river all the way up to the highest located sampling points, but represented a patchy diversity distribution between tributaries, which might be acting as refugial habitats for species being isolated by dams and other barriers (Aparicio et al., 2000). Increasing fragmentation may make fish populations vulnerable to decline, particularly in the case of catastrophic events (Aparicio et al., 2000), as it happened in the river Afan due to pollution and overfishing (Winstone et al., 1985). In both rivers, the four most represented species, *S. trutta*, *S. salar*, *C. gobio* and *P. phoxinus*, had a similar species distribution pattern in all tributaries.

At several sampling upstream locations, species specific eDNA was not detected at all, particularly for the demersal- benthic species *B. barbatula, T. thymalus* and *G. aculeatus,* limited in their upstream dispersal in both rivers by several barriers, including significant absence in Afan river, and known to be affected by slope in artificially modified rivers (Junker et al., 2012, Santoul et al., 2005, McLaughlin et al.,

2006). Non- migratory non- leaping fish, with known lower swimming capacities compared to long- distance migrators (Tudorache et al., 2008), had a limited upstream dispersion at a higher number of barriers in the river Afan compared to the Teifi, with no sequences found upstream. The swimming capacities of the three most common non- migratory fish species found in both rivers, the *P. phoxinus*, *C. gobio* and *B. barbatula*, had been previously defined as one of the lowest (Tudorache et al., 2008, Holthe et al., 2005), which explains their higher abundance downstream in both of the rivers. The artificial culverts in the Afan posed a high limitation for the dispersal of some rare species. One of the culverts accounted for the lowest average number of reads upstream, and the second mainly displayed eDNA from the two most abundant salmonids (*S. trutta* and *S. salar*) suggesting this is a type of barrier difficult to overcome for non- migratory species (Nislow et al., 2011). For some species, upstream decrease in dispersal is a natural occurrence, as for *A. anguilla*, for which population abundance tends to reduce in numbers upstream from the sea (White and Knights, 1997), explaining the distribution patterns found here. In general, none of the migratory fish were negatively affected by upstream dispersal in both rivers, with eDNA found at both upstream and downstream sampling locations, implying their capacity to overcome the barriers at high water discharge. Non- migratory species tended to decrease upstream, where salmonids were the most represented species.

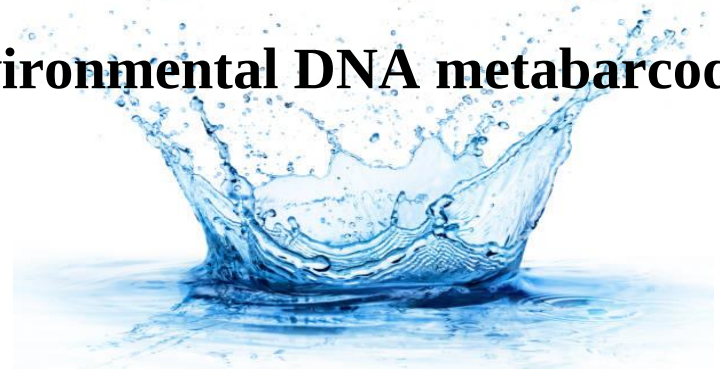
The two salmonid species, *S. salar* and *S. trutta*, were apparently not affected by fragmentation, with the highest total number of sequence reads detected at the most upstream sampling locations. *S. salar* eDNA was more abundant only in the Afan's most upstream tributaries (AF 1, 2 and 4) compared to *S. trutta*, showing the importance of spawning grounds in the river headwater for *S. salar* (Bardonnet and Baglinière, 2000). In the Afan, there were local differences between the more abundant *C. gobio* and *P. phoxinus* (AF 2, 4 and 6), with less abundant *S. trutta*, which might indicate interspecific competition for food and habitat preferences, including known *C. gobio* and *P. phoxinus* predation over salmonids eggs (Palm et al., 2009, Holthe et al., 2005). Barriers limiting upstream dispersal of non- salmonid species at the most upstream localities in both rivers, can increase salmonids local population survival rate. *S. trutta*'s eDNA had doubled on the average in both rivers, compared to *S. salar*, which could be the result of both, juvenile and adult fish being present throughout the year, which increases their chance for upstream dispersion, when the flow is high

enough to overcome the barriers. *S. salar* and *S. trutta* spawning season coincided with the sampling, increasing eDNA abundance by accounting for all specimens' life stages, including sperm and eggs release.

Downstream eDNA abundance may not reflect local presence of specific species accurately, as it can be biased by eDNA transported from upstream localities. Yet, eDNA detection rate tends to diminish further downstream from the source (Balasingham et al., 2017a), being no longer detectable after 48 h in river, and also accounting for difference in seasonal detection rates (Deiner and Altermatt, 2014). The river Afan had for 10-fold lower water velocity at the time of the sampling compared to Teifi, which could have affected not only fish dispersal (Warren Jr and Pardew, 1998), but also eDNA detection rates at each individual barrier, as eDNA transport distance is related to flow discharge (Wilcox et al., 2016). The persistence of eDNA is also influenced by environmental dynamics (Dejean et al., 2011), which affects DNA degradation, influencing detection rates between rivers.

This study shows the potential of eDNA combined with metabarcoding usage to assess fish dispersal in relation to particular barriers (Maire et al., 2015). For a better interpretation of the limitations on fish dispersal imposed by barriers, it would be necessary to expand the sampling on a temporal scale, accounting for differences in flow dynamics, migratory species reproductive cycles, and eDNA differences in detection rates throughout the year.

CHAPTER 4- Assessment of fish connectivity after weir removal by environmental DNA metabarcoding



Abstract

Environmental DNA (eDNA) is becoming widely used for assessing the spatial and temporal distribution of freshwater fish, and can also be used to evaluate connectivity in fragmented rivers. Here I used eDNA metabarcoding to analyse fish community changes following the removal of a river obstacle. Nine fish species were identified and their change in abundance was assessed one year after barrier removal. There was a temporal increase in detectability of species presence, particular for rare species representatives and also an increase in non- migratory fish species abundance year after removal. There were no effects detected associated to weir removal based on assessment of fish diversity, as all nine species eDNA was detected before removal at both, upstream and downstream sampling locations. Five non- migratory species total number of sequence reads increased in time, with Eurasian minnow, *Phoxinus phoxinus,* highest increase (31.3%) at both upstream and downstream sampling locations following removal. The total number of sequence reads of Atlantic salmon and brown trout increased after weir removal, based on 15.17% and 20% higher sequence reads count, respectively. Seven fish species have been equally identified by previous surveillance in comparison to our eDNA metabarcoding, including similar levels of species specific density proportions. Detected increase of both, migratory and non- migratory fish in time, suggests potential effects of weir removal towards fish population dispersion, and proved eDNA metabarcoding tool as a useful indicator for river restoration measures.

4.1 Introduction

Artificial barriers are one of the major causes for fish habitat fragmentation, dispersal in rivers (Kemp and O'Hanley, 2010), contributing to the creation of discontinuity patterns in fish population structure by limiting fish movements (Morita and Yamamoto, 2002). Concerns regarding the ecological impacts of barriers in Europe and North America have led to an increase in removal plans (O'Hanley, 2011, Gardner et al., 2013, Birnie-Gauvin et al., 2017, Garcia De Leaniz, 2008). Removal of barriers can reduce sediment retention (Doyle et al., 2005), increase general biodiversity (Bednarek, 2001) and gene flow (Wofford et al., 2005), reduce levels of infectious diseases (Garcia De Leaniz, 2008), but most crucially increase migratory as well as non- migratory fish dispersal (Fullerton et al., 2010). Assessing the consequences of barrier removal requires the adoption of appropriate ecological indicators (Jackson et al., 2000). As dam removal projects increase (Birnie-Gauvin et al., 2018, Schiermeier, 2018), it is important to critically evaluate their effects by applying measures which assess changes in fish connectivity in time and space. For fish populations to remain sustainable, a minimum degree of interconnectivity between physical habitats is needed to ensure successful recruitment from spawning (Burger et al., 2015). Thus, river fragmentation not only affects migratory species but also dispersal of non-migratory species (Ovidio and Philippart, 2002).

Changes in the spatial and seasonal distribution of fish following barrier removal have traditionally been assessed using fish trapping (Birnie-Gauvin et al., 2018), nest counts (Lasne et al., 2015), electrofishing (Rolls et al., 2014) and telemetry (Kemp and O'Hanley, 2010), which can be time consuming, limited by species detection thresholds at low abundance or rare species representatives, may be biased by species preference sampling technique, potentially harming fish (Kruse et al., 1998, Bertrand et al., 2006, Bacheler et al., 2017). Therefore, a non-invasive, efficient tool is required for detecting species presence and abundance to improve monitoring of barrier removal projects.

Environmental DNA (eDNA) has been developed recently (Jerde et al., 2011a, Ficetola et al., 2015b) for species detection by collecting and amplifying genetic material from environmental samples. The ability to detect species through eDNA water samples has proved a useful tool for the detection of spatial and seasonal

distribution patterns (Handley et al., 2018, Yamamoto et al., 2016, Sigsgaard et al., 2017). Environmental DNA metabarcoding takes advantage of next generation sequencing (NGS) techniques, to simultaneously detect several taxa targeting short fragments of mtDNA (Valentini et al., 2016). Sequences can then be assigned to molecular operational taxonomic units (MOTU) and more accurately to species level using a referenced databases (Ryberg, 2015). It is currently one of the most accurate and reliable methods to assign species in communities (Ji et al., 2013, Hänfling et al., 2016). eDNA metabarcoding has been used as an ecological indicator reflecting presence or absence of fish species, as well as relative abundance fluctuations based on comparison between total number of sequence reads corresponding to individual species (Dale and Beyeler, 2001), a parameter used for the diversity assessment, with previous measures of success (Lacoursière-Roussel et al., 2016a, Yamanaka and Minamoto, 2016).

The River Lugg in England was fragmented by a series of weirs built in the 1980s (Symondson, 2010), that affect fish migrations. Atlantic salmon and sea trout spawn in the lower sections of the River Lugg, but their distribution is restricted by weirs and in -channel structures (Kemble, 2013). I used eDNA metabarcoding to analyse the spatial and temporal changes in the fish community of the river Lugg before and after a single 1.85 m high weir was removed in 2016. The main aim was to assess whether fish connectivity was impacted by stream fragmentation and whether eDNA metabarcoding could be used as a metric for restoration of connectivity, using the River Lugg as a case study.

4.2 Material and methods

4.2.1 Study site and water collection

The river Lugg is one of the two main tributaries within the lower Wye catchment (Jarvie et al., 2005) and a Site of Special Scientific Interest (SSSI) with a total length of 101 km (Wye and Usk foundation, 2015). This water body has been classified as "good ecological status" by the Water Framework directive (Wye and Usk foundation, 2015) according to the Environmental Agency latest assessment in 2014. Information on water level at the time of sampling was collected from a nearby monitoring station at Byton (riverlevels.uk). The studied Kingsland weir was 1.85 m high and removed in order to improve fish connectivity in stream. Sampling points were located close to Kingsland, upstream from Hereford, UK, upstream and downstream the weir to be

removed (latitude 52°15'12.0"N, longitude 2°48'15.9"W). The total distance between the most upstream (latitude 52°15’16.053” N, longitude 2°48’27.001” W) and downstream (latitude 52°15’3.474” N, longitude 2°47’57.184” W) sampling point was 750 m. Water depth based on a monthly average at the time of sampling before barrier removal was 0.48 m and a year after removal in May 2017 was 0.35 m. Water samples were collected before and a year after the barrier removal (5th of October 2016), in the end of May 2016, and at the beginning of June 2017 from six sampling points, three above and three below the weir (Figure 4. 1). Three sample replicates were collected at each sampling station, collected 30 cm under the water surface, including additional three blanks upstream and downstream from the weir. An eDNA sampling method combining water filtration with ethanol precipitation was implemented using closed syringe Minisart® cellulose syringe filters (Sartorius, Germany) with 0.45 µm pore size. In total 100 mL of water were collected and filtered on site by pushing by hand through 50 mL syringes at an approximate flow rate of 50 mL per 30 s, including two blanks below and above the barrier. Individual syringes were used for each of the sampling points to avoid cross-contamination between sites. Disposable nitrile gloves were used while collecting water samples and filtering with syringes. The syringe filters were kept in separate sterile bags and transferred in a cooling bag to the laboratory, where the ethanol precipitation was conducted on a same day as sample collections. A mixture of 1350 µL absolute ethanol and 150 µL of sodium acetate was passed through the filters which were then centrifuged at 5000 g for 45 min at 6 ^{0}C and stored in 1,5 mL vials at -20 °C until the DNA extraction one week later. Three control blanks were used in laboratory with only the mixture and sterile water being pushed through the syringe.

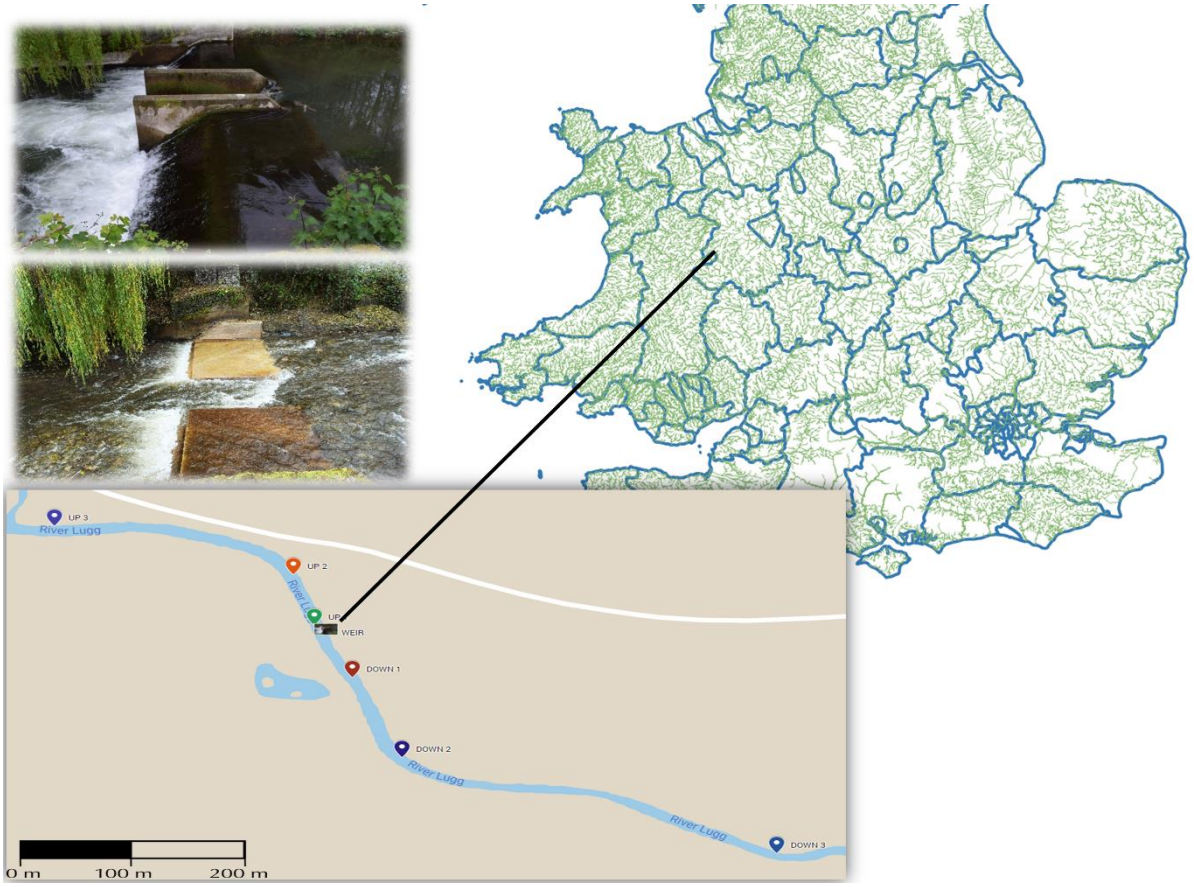


Figure 4. 1-The studied Kingsland weir, removed in October 2016, with water sample collected from 3 sampling point above (UP 1-3) and three below (DOWN 1- 3) in May, before and a year after the removal.

4.2.2 Sample processing

eDNA was extracted using the Nexxtec 1-step DNA Isolation Kit for Tissues & Cells (Nexttec™ Biotechnologie GmbH, Germany), following the manufacturer's guidance, with the elution volume reduced to 50 µL in the last step reduced. Water filtration and extraction negative controls were additionally extracted using sterile nuclease- free water for each of the individual sampling events, following same procedure of ethanol precipitation as for all the environmental samples.

Extractions and pre-PCR handling of eDNA water samples were carried out in a fume hood dedicated to eDNA analyses only, bleached and exposed to UV light for 45 min beforehand. DNA extraction efficiency as well as the efficiency of conventional PCR fish amplification was measured by Qubit™ 4 Fluorometer (Thermo Fisher Scientific Inc.) applying the high-sensitivity assay (Life Technologies, Carlsbad, CA, USA).

4.2.3 Amplicon validation and PCR preparation

Vertebrate specific primers were used targeting a 144 bp fragment of the 12s rRNA mtDNA gene (hereafter 12S-V5) (Riaz et al., 2011a), previously used in several barcoding and metabarcoding studies (Kelly et al., 2014a, Harper et al., 2017, Port et al., 2016). The primers were tested *in silico* using ecoPCR (Boyer et al., 2016) based on a list of known Welsh species with reference sequences obtained from NCBI. The primers were further validated by amplification and Sanger sequencing of three fish species: sea trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). For these, DNA was extracted from muscle or fin tissue using the Qiagen DNeasy Blood & Tissue DNA extraction kit (Qiagen GmbH, Hilden, Germany). With each PCR amplification, a negative PCR without DNA template was added as a negative PCR control. The amplification reaction was performed with 12.5 µL Bioline BioMix Red PCR Mastermix (2X), 3 µL template, 1.5 µL of each primer (10 µM), adding sterile nuclease- free water to final total volume of 30 µl. The products were visualised on 2 % agarose gel stained with 2 µL of GelRed™ nucleic acid gel stain.

4.2.4 High- throughput sequencing conditions

Samples from the Lugg River were prepared for high throughput sequencing with Illumina MiSeq. The library preparation followed the 16S Metagenomic Sequencing Library Preparation protocol by Illumina (Illumina, 2013). A 2-step PCR approach was used. First PCR amplification of the target template using universal primers with Illumina adapters, and then a second PCR to tag the samples using Illumina Kit. The first PCR was carried out with 35 cycles on a 12 µl reaction volume containing 6 µL of Kapa HiFi HotStart ReadMix (KAPA Biosystems, Wilmington, MA, USA), 1 µL of each primer (5µM), 1 µL of sterile nuclease-free water and 3 µL of template. The cycling profile had an initial 3 min step at 95 °C followed by denaturation at 95 °C for 30 s; annealing at 52 °C for 30 s following the 72 ^{0}C for 30 s with the final extension at the same temperature for 5 min. After the confirmation of PCR efficiency on 2 % agarose gel, PCR products were cleaned up using Agencourt AMPure XP beads (BioLabs, New England, UK). The second PCR was done on a volume of 25 µL, including 12 µL Kapa HiFi HotStart ReadMix, 2.5 µL each Nextera XT Index primers, 5 µL of sterile nuclease-free water and 3 µL of template eDNA, following the same cycling profile than the first one but only for 12 cycles. For the second PCR, a clean-

up step in a total volume of 45 µL of AMPure XP beads was used. Libraries were diluted down to 4 nM in 10 nM Tris. The paired-end sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) at the Institute of Life Sciences, College of Medicine, Swansea University. PCR amplification efficiency was measured by Qubit 1.0 fluorometer (Thermo Fisher Scientific Inc., UK) applying the high-sensitivity assay (Life Technologies, Carlsbad, CA, USA).

4.2.5 Bioinformatic analysis

Mothur v1.39.5 (Schloss et al., 2009) was used for the analysis of high- throughput fish vertebrate sequences produced by MiSeq Illumina. I used Trimmomatics (Bolger et al., 2014) at the start by checking and eliminating low quality sequence reads (minimum quality score of Q= 20) and errors produced through PCR and sequencing, with additional removal of primers and sample tags. After paired- end combining of reads, the sequences were sifted based on size selection of the target amplicons, to eliminate potential spurious sequences. Sequences were aligned using a custom reference database containing RefSeq Welsh local fish species. For chimera removals the UCHIME (Edgar et al., 2011) was used applying chimera.uchime command. Negative filtration and extraction control samples were additionally analysed. A 3% level of clustering was used for dereplication due to appropriateness of MOTUs segregation, sequence similarities between each other based on a common output of the most representative sequence in a group. Taxonomical assignment was conducted with BLASTn from the command line with our RefSeq database from NCBI consisting of all the vertebrate reference sequences from the whole database. Only sequences with a minimum of 100 sequence reads across 20 samples for each cluster were used for subsequent analyses. The minimum bit score of useful MOTUs was 125, including the lowest 91.3% sequence identity match. MOTUs belonging to same species were merged before further statistical analysis. Sequence files containing raw reads have been deposited to GenBank/ under accession numbers: PRJNA514271.

4.2.6 Statistical analysis

Alpha and beta fish diversity based on Shannon- Wiener index (H'), presence/ absence and species relative read abundance based on total number of sequence reads were used to assess fish patterns. Species richness was calculated per each sampling replicate, using Shannon-Wiener index (Pielou, 1966). Species diversity based on

Shannon- Wiener Index was assessed on a temporal scale (before and after weir removal) and also between upstream/ downstream barrier sampling locations, including interactions between both predictors, using a linear model. For fish presence/ absence evaluation, binary logistic regression was used, using species and upstream/ downstream before after removal division (four groups) as predictors, including the interaction between them. Two or more positive detections (out of three replicates) were considered as evidence of presence. For confirmation of absences only samples with no sequences found in all triplicates were used. The only exception was *A. anguilla*, detected in only one sampling replicate before weir removal at both upstream and downstream sampling locations, accounting for presence, as the number of sequence reads was proportionally high compared to other species (249 and 284 number of sequence reads, upstream and downstream from the weir). Species specific abundance based on species specific total number of reads was evaluated using linear models (LMs) evaluating their distribution in time (before and after removal) and space (upstream/ downstream of the weir). For this, sampling triplicates from three sampling locations downstream of the (removed) barrier and three sampling triplicates from three upstream sampling locations were merged. The 'Vegan' package (Oksanen et al. 2010) was used for the diversity, presence and species distribution estimates. Three sampling replicates were used for all statistical analysis. All statistical analyses were done in R, version 3.3.2 (Team, 2013).

4.3 Results

4.3.1 *In silico* and *in vitro* validation of vertebrate primer

Thirty- seven fish species, known to be present in Welsh rivers were used for *in silico* bioinformatic validation, accounting for 1131 reference sequences, with twenty-one species being identified to species level, 78 % of which could be identified to genus level (Table S. 8), allowing for 0 mismatches. All nine identified species in our results have been identified *in silico* to species level. All three fish species used for *in vitro* testing were successfully amplified, and confirmed by Sanger sequencing.

4.3.2 Qualitative eDNA MOTUs species assignment

In total 36 samples were extracted from both sampling events in the Lugg River, with additional 3 filtration and 3 extraction negative controls for each of the sampling event. A single library preparation was used, with Illumina MiSeq recovered sequences

producing a peak at 144 bp, as expected, with 5.82 x 10^5 total number of sequence reads. Non-fish amplification of the 12S-V5 primers accounted for 18.52% (1.07 x 10^5 sequences) belonging to other vertebrates, most of them being human (*Homo sapiens*) (23.12%) and chicken (*Gallus gallus domesticus*) (11.147%). In total 4.749 x 10^5 (81.47%) fish sequences were recovered out of all sequences where 107 MOTUs were identified with a total of 3.379 x 10^5 sequences (71.15%) corresponding to Lugg river resident fish species. The rest of fish sequences, the 1.805% belonged to fish species from known laboratory contamination, tropical and North Atlantic marine species representatives that were used in laboratory and could not belong to Lugg river, which were removed from further analysis, also found in negative controls. The rest of fish MOTUs (27%), were also removed from further statistical analysis, which belonged to species not previously identified in Welsh rivers. All sequences were identified up to species level except for lampreys (*Lampetra spp*.). Nine fish species were detected, belonging to the following seven families: *Anguillidae*, *Nemacheilidae*, *Cottidae*, *Gasterosteidae*, *Petromyzontidae*, *Salmonidae* and *Cyprinidae*, identified as Eurasian minnow (*Phoxinus phoxinus*), stone loach (*Barbatula barbatula*), European bullhead (*Cottus gobio*), three-spined stickleback (*Gasterosteus aculeatus*), grayling (*Thymallus thymallus*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), European eel (*Anguilla anguilla*) and lamprey (*Lampetra spp*.). A survey of the river Lugg between 2011- 2015 using electrofishing and fry netting identified nine species, seven of which coincided with our eDNA findings, *P. phoxinus, C. gobio, S. trutta, S. salar, B. barbatula, G. aculeatus* and *T. thymallus* (Table S. 9), accounting for the highest densities of *P. phoxinus* and *B. barbatula* at both, electrofishing and eDNA surveys (Capps, 2017). For the comparison of eDNA survey with electrofishing and fry netting, only two sampling locations at Bodenham and Marden were selected, as these were the closest two sampling stations with fry netting and electrofishing, allocated 10 -13 miles away from our sampling locations.

4.3.3 Quantitative eDNA fish diversity and dispersal assessment

Shannon-Wiener (H') diversity index was used for species richness assessment (Figure 4.2) evaluating the impacts of weir removal on fish diversity in time, including downstream/ upstream temporal division (before and a year after barrier removal), identifying no difference between sampling events ($F (df = 1, N= 32) = 0.0003$, $p= 0.985$), neither between downstream/ upstream division ($F (df = 1, N= 32) = 1.239$, $p=$

0.274), or interaction between them (F (df = 1, N = 32) = 1.985, p = 0.985). Fish diversity based on Shannon- Wiener index between upstream and downstream from the removed weir has been equally distributed a year after the removal with an average downstream H'= 0.87, and upstream H'= 0.88 (Figure 4.2). Presence/ absence assessment accounting for species and upstream/ downstream temporal division, was assessed using binary logistic regression model with a significant difference found between species (Table 4.1, χ^2 (df = 8, N= 84) = 37.353, p < 0.001) and temporal division, with increased presence in majority of sampling replicates after removal for *T. thymallus*, *G. aculeatus* and *B. barbatula* (Table 4.1, χ^2 (df = 1, N=92) = 66.765, p = 0.003). There was no interaction of downstream/ upstream with sampling time (Table 4.1, χ^2 (df = 1, N= 82) = 31.630, p = 0.614), indicating no impact of weir removal on presence/ absence of species based on current sampling effort. The four most abundant species *P. phoxinus*, *C. gobio*, *S. trutta* and *S. salar* eDNA were detected in all sampling replicates, excluding one replicate without *S. trutta* and *C. gobio* (before removal downstream sampling replicates).

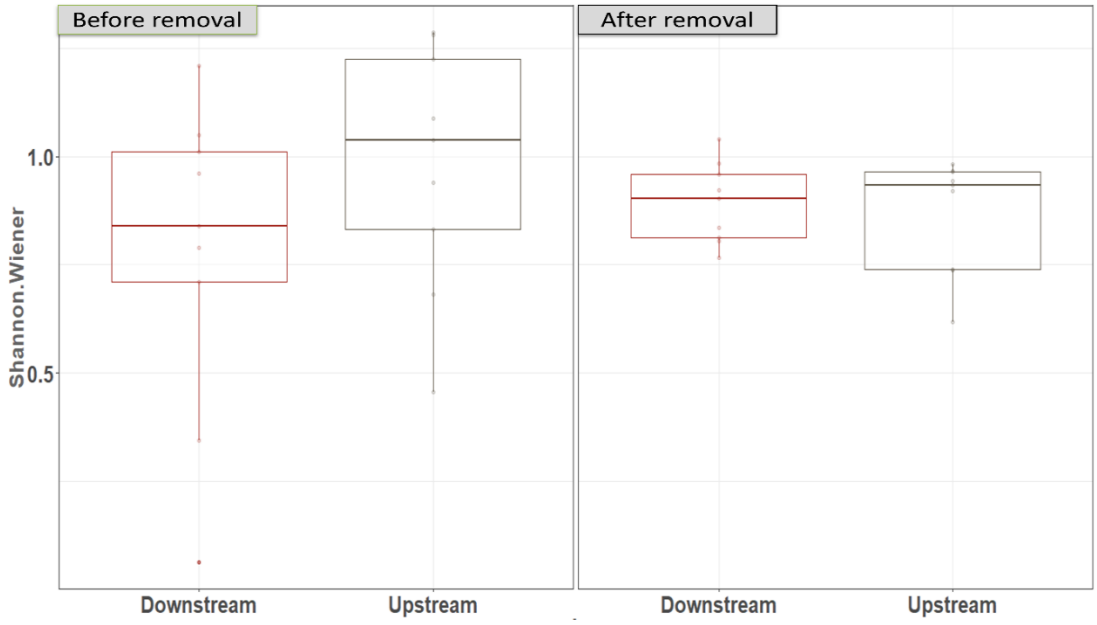


Figure 4. 2- Species richness calculated between the two sampling events divided by downstream/ upstream division applying Shannon- Wiener index.

Table 4. 1- Species presence/ absence evaluation using binary logistic regression at spatial and temporal scales to assess barrier removal effects, based on species, sampling time and downstream/ upstream division, including the interactions between them. AIC test was used for model comparison.

Factors	**Df**	**Deviance**	**Residual df**	**Residual deviance**	**< Chi**	**AIC**
Presence/ absence= Species * Sampling time* Downstream/ upstream						94.64
Sampling time	1	9.371	91	64.137	**0.002**	
Downstream/ upstream	1	2.042	92	73.508	0.152	
Species	8	32.254	83	31.883	**< 0.001**	
Sampling time x Species	8	5.910	66	24.641	0.657	
Downstream/ upstream x Sampling time	1	0.254	82	31.630	0.614	
Downstream/ upstream x Species	8	1.080	74	30.55	0.997	
Downstream/ upstream x Sampling time x Species	7	0	59	24.641	1	
Presence/ absence= Species * Sampling time						68.95
Sampling time	1	8.784	92	66.765	**0.003**	
Species	8	29.412	84	37.353	**< 0.001**	
Sampling time x Species	8	4.394	76	32.958	0.819	

Species specific linear models assessing total number of sequence reads accounting for time and upstream/ downstream distribution, including the interaction between them, showed statistically significant differences in time, between before and after weir removal for five species *P. phoxinus*, *C. gobio*, *B. barbatula*, *G. aculeatus* and *T. thymallus* (Table 4.2, F (df = 1, N= 34) =73.93, < 0.001, F (df = 1, N= 34) = 12.488, p = 0.001, F (df = 1, N= 34) =11.8, p = 0.001, F (df = 1, N= 34) = 6.4, p = 0.016, F (df = 1, N= 34) = 18, p = 0.001), respectively. There was no difference for any of the species solely on upstream/ downstream division and neither for an interaction effect between time and upstream/ downstream division based on total number of sequence reads (Table 4.2). *P. phoxinus, C. gobio, B. barbatula, G. aculeatus* and *T. thymallus* total number of sequence reads increased in time, with 31.3 % increase of *P. phoxinus*, with two of the least representative species, *B. barbatula* and *T. thymallus* increase related to increased detectability in the majority of the sampling replicates after removal (Figure 4.3). The distribution heatmap had shown highly clustered similarities of sampling replicates between the two sampling events, before and a year after barrier removal (Figure 4.4). There was no distinguishable clustering separation between upstream/ downstream sampling replicates for each of the sampling events, with a smoother diversity pattern a year after removal (Figure 4.4). The major proportion from all nine fish species found represents two non-migratory species, the *P. phoxinus* with an average total number of sequence reads of 61.62%, followed by the *C. gobio* with 26.33%, followed by the two salmonids, *S. trutta* with 5.2% and *S. salar* with 1.7% (Figure 4.3).

Table 4. 2- Linear models of species total number of sequence reads variations, assessed by two predictors and interactions between them, sampling time * upstream/ downstream division. Linear models were applied for all nine species. AIC test was used for model comparison.

Factors	**Df**	**Sum sq**	**Mean sq**	**F value**	**p**	**N**	**AIC**
***Salmo salar* (total number of reads) = Time * Upstream/ downstream**						*32*	
Time	1	33063	33063	0.16	0.691		

Factors	**Df**	**Sum sq**	**Mean sq**	**F value**	**p**	**N**	**AIC**
Upstream/ downstream	1	1406	1406	0.006	0.934		
Time x Upstream/ downstream	1	59292	59292	0.160	0.691		
***Salmo trutta* (total number of reads) = Time * Upstream/ downstream**						**32**	
Time	1	52247	52247	0.46	0.502		
Upstream/ downstream	1	2809	2809	0.002	0.96		
Time x Upstream/ downstream	1	83741	83741	0.073	0.787		
***Phoxinus phoxinus* (total number of reads) = Time * Upstream/ downstream**							
						32	681
Time	1	6.06 x 10^8	6.06 x 10^8	73.82	< **0.001**		
Upstream/ downstream	1	1.9 x 10^6	1.9 x 10^6	0.236	0.632		
Time x Upstream/ downstream	1	1.4 x 10^7	1.4 x 10^7	1.71	0.199		
***Phoxinus phoxinus* (total number of reads) = Time**						34	679
Time	1	06 x 10^8	06 x 10^8	73.93	< **0.001**		
***Cottus gobio* (total number of reads) = Time * Upstream/ downstream**						32	646
Time	1	3.9 x 10^7	3.9 x 10^7	12.31	**0.001**		
Upstream/ downstream	1	1.7 x 10^6	1.7 x 10^6	0.558	0.46		

Factors	**Df**	**Sum sq**	**Mean sq**	**F value**	**p**	**N**	**AIC**
Time x Upstream/ downstream	1	3.07 x 10^6	3.07 x 10^6	0.967	0.332		
***Cottus gobio* (total number of reads) = Time**						34	644
Time	1	3.9 x 10^7	3.9 x 10^7	12.488	**0.001**		
***Anguilla anguilla* (total number of reads) = Time * Upstream/ downstream**						32	
Time	1	10336	10336	0.847	0.364		
Upstream/ downstream	1	3803	3802	0.311	0.5808		
Time x Upstream/ downstream	1	5378	5377	0.44	0.511		
***Barbatula barbatula* (total number of reads) = Time * Upstream/ downstream**						32	
							385
Time	1	25175	25175	11.208	**0.002**		
Upstream/ downstream	1	455	455	0.202	0.655		
Time x Upstream/ downstream	1	187	185	0.083	0.77		
***Barbatula barbatula* (total number of reads) = Time**						34	382
Time	1	25175	25175	11.8	**0.001**		
***Gasterosteus aculeatus* (total number of reads) = Time * Upstream/ downstream**						32	248
Time	1	306	306	6.143	**0.018**		
Upstream/ downstream	1	26	26	0.535	0.46		
Time x Upstream/ downstream	1	4.69	4.69	0.094	0.76		

Factors	**Df**	**Sum sq**	**Mean sq**	**F value**	**p**	**N**	**AIC**
***Gasterosteus aculeatus* (total number of reads) = Time**						34	245
Time	1	306	306	6.4	**0.016**		
***Thymallus thymallus* (total number of reads) = Time * Upstream/ downstream**						32	83
Time	1	9	9	17.51	**< 0.001**		
Upstream/ downstream	1	0.44	0.44	0.864	0.359		
Time x Upstream/ downstream	1	0.11	0.11	0.21	0.645		
***Thymallus thymallus* (total number of reads) = Time**						34	81
Time	1	9	9	18	**< 0.001**		
***Lampetra spp.* (total number of reads) = Time * Upstream/ downstream**						32	
Time	1	30	30	1.515	0.227		
Upstream/ downstream	1	17.36	17.36	0.869	0.35		
Time x Upstream/ downstream	1	0.03	0.03	0.001	0.97		

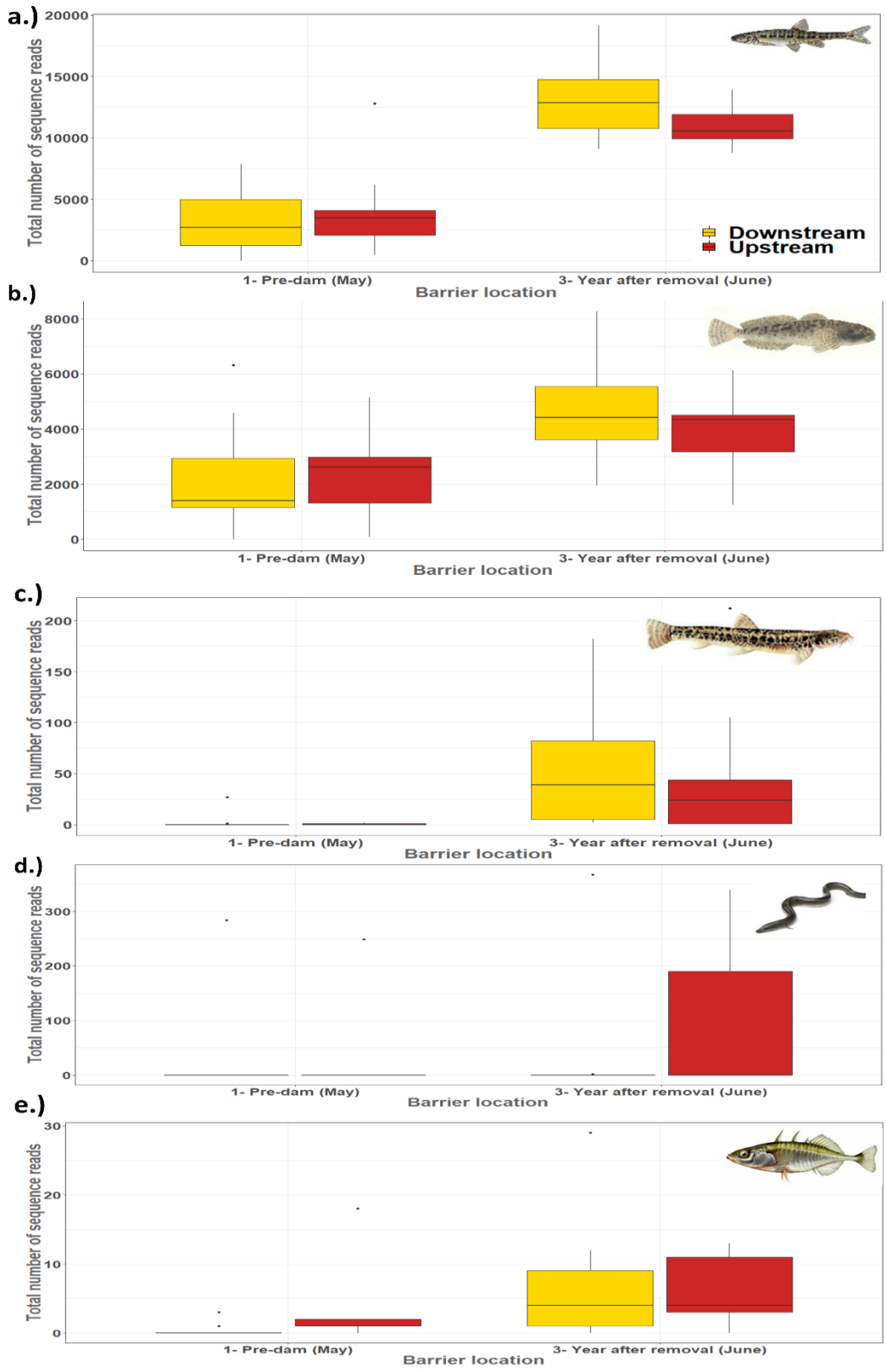


Figure 4. 3- Fish temporal distribution of the non- migratory species before (left) and after (right) weir removal, including European eel (a.) *P. phoxinus*, b.) *C. gobio*, c.) *B. barbatula, d.) A. anguilla,* e.) *G. aculeatus*, f.) *T. thymallus* with downstream distribution on the left boxplot and upstream on the right boxplot.

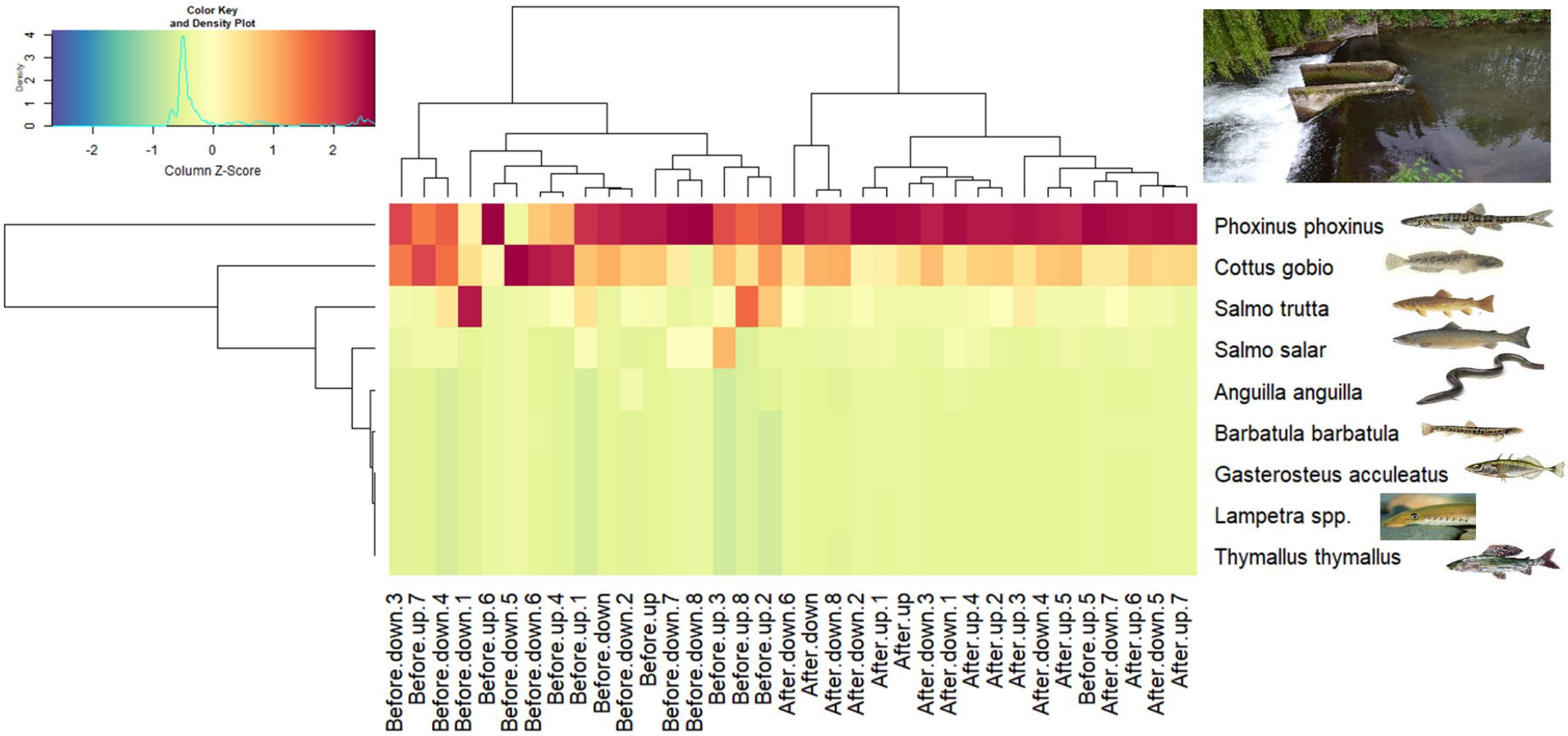


Figure 4. 4- Distribution heatmap based on total number of sequence reads of Lugg river fish species, represented from the most abundant one on the top to the least one at the bottom, clustered by similarity measure of sampling triplicates merged by before/ after weir removal, up/ down the weir. All sampling triplicates are represented on X- axis clustered together by similarities, with fish species found on Y- axis.

4.4 Discussion

In this study, I had assessed how barrier removal affects fish distribution by using eDNA metabarcoding approach. eDNA method has been used as an ecological indicator reflecting presence or absence of fish species, as well as fluctuations (Dale and Beyeler, 2001) with total number of sequence reads, a parameter used for the diversity assessment. The method appeared useful for fish dispersion in fragmented river in time. The fish traditional electrofishing surveillance from years 2011- 2015 (Capps, 2017) detected nine fish species as did our metabarcoding approach, with seven equal species being detected by both assessments, majority of them belonging to non- migratory species, most of them being equally represented comparing density per catch with total number of sequence reads in both studies. Previous studies assessing both, traditional surveillance together with eDNA assessment have concluded similar findings in favour of eDNA precision and findings of elusive species (Civade et al., 2016, Pilliod et al., 2013, Evans et al., 2016, Nakagawa et al., 2018). The eDNA metabarcoding approach detected two additional migratory species, the European eel (*Anguilla anguilla*) and lamprey (*Lampetra spp*.), which were not detected with previous surveillance. This study indicates that it is possible to simultaneously detect *A. anguilla*, *Lampetra spp.* and the rest of the fish using same surveillance method, namely eDNA metabarcoding, requiring less effort compared to traditional methods and reducing the cost of monitoring, which normally required separate approach for anguilliform fish (Jolley et al., 2012, Moser et al., 2007). For an accurate correlation between observed species richness and eDNA metabarcoding, simultaneous studies should be used (Olds et al., 2016), avoiding annual and seasonal fish species density fluctuations.

Barrier removal had no evident effect on fish diversity or species specific dynamics in time and space. The species richness index indicated that there were no differences in diversity between downstream/ upstream sampling stations before and after weir removal. All nine species' eDNA was detected at least in two sampling replicates in either upstream or downstream the barrier for each consecutive sampling year, with the exception of *A. anguilla*, being detected in high numbers in one of the sampling replicates. For characterisation of diversity on such a wide temporal scale, when abiotic conditions, such as water temperature, flow rate and transport dynamics can change and influence eDNA dynamics (Deiner et al., 2016a, Takahara et al., 2012), it

is advisable to simultaneously monitor the crucial parameters influencing the eDNA ecology (Barnes and Turner, 2016), such as acidity, substrate material and hydrological dynamics and seasonality while conducting eDNA surveys. Total number of sequence reads of five non- migratory fish species increased in time, *P. phoxinus*, *C. gobio*, *B. barbatula*, *G. aculeatus* and *T. thymallus*, at all sampling stations upstream and downstream of the removed weir. Patterns of non- migratory fish increase over the whole open river corridor after a year of barrier removal at both upstream and downstream sampling locations, indicates a clear increase of non- migratory species density in the sampled corridor. As the non- migratory fish species were not detected in higher numbers downstream compared to upstream before weir removal, there is no direct relation towards weir dispersal limitations. This trend might indicate a lack of sampling further downstream the weir, currently with less than 500 m distance, which would contribute to effects of weir removal, potentially identifying rarely detected species downstream. eDNA long downstream persistence of detection previously evaluated (Deiner and Altermatt, 2014, Shogren et al., 2017a), suggests increased sampling on longitudinal scale for weir removal effects estimates. All nine species were present before the weir removal, albeit some at low abundance, with less than 10 sequence reads in some of the replicates. The eDNA metabarcoding definition of presence/ absence calls for caution in the interpretation, in particular for rare species represented by a low number of reads which can be more affected by detectability fluctuations related to sampling effort, amplification and sequencing errors (Goldberg et al., 2016, Ficetola et al., 2015a). As a measure of quality, stringent filters can be applied during the bioinformatics analysis, eliminating MOTUs with a certain reduced number of sequence reads resulting in unreliable presence/ absence assessment (Guardiola et al., 2015) or a more conservative approach can be taking by removing singletons (Bakker et al., 2017). The occurrence of false positives can also be controlled by applying cumulative relative frequency of contaminant reads in control libraries as a minimum detection threshold (Evans et al., 2017a).

Two species, *Leuciscus leuciscus* and *Squalius cephalus*, had been previously detected in the proximity of the sampling locations during electrofishing surveys (Capps, 2017) but were not found in the eDNA metabarcoding, despite *S. cephalus* high densities (Capps, 2017). Both species detectability had been confirmed *in silico* using 12S-V5 primers, thus, either species were not present at the time, or there were represented by a low number of individuals, making them difficult to detect. This could be related to

the method used here, which was based on 100 mL syringe filtration with ethanol-sodium acetate precipitation and resulted in low detectability of the five least representative species. It is possible that filtration of higher volumes would have resulted in higher efficiency at detecting rare species (Valentini et al., 2016, Civade et al., 2016). Additionally, a higher number of sampling replicates could be used, resulting in higher rates of detectability and replicability, reducing as well the likelihood of false negatives (Ficetola et al., 2015a). Increasing the number of PCR replicates of each of the sampling triplicate, pooling them together before library preparation or sequencing each individually, would be another control measure to reduce levels of false negatives (Thomsen et al., 2016). The case of *A. anguilla* atypical relative reads abundance representation, being detected in high numbers in one of the sampling replicates and none in other two, could be associated to the sampling method, suggesting that a minimum 1 L of water may need to be filtered for an accurate detection (Mächler et al., 2015). This potential increase in water filtration volume could contribute to a higher and more accurate detectability of the four rare species presence, *A. anguilla*, *B. barbatula*, *T. thymallus* and *Lampetra spp*., which had only one confirmed presence below the barrier before removal accounting for all three downstream sampling locations.

The fish assemblages did not differ on a longitudinal scale but its abundance increased in time. It can be difficult to define patterns of distribution on small scale, which were separated between each other for merely 70- 200 m, for each consecutive sampling event, indicating a need for wider sampling range for clearer indications of restoration practice estimates. Also, eDNA detection largely depends on its concentration, a result of each individual specimen as well as species production rates, environmental conditions and their residence times (Pilliod et al., 2014, Furlan et al., 2016). A number of parameters can influence detection rates of eDNA in rivers, such as stream velocity (Jane et al., 2015) or inconsistent transport in flowing waters by different eDNA retention to benthic substrate (Shogren et al., 2017b), making it difficult to compare spatial distribution patterns on a temporal scale. The correlation between the organismal behaviour, seasonal patterns or certain activity with the eDNA detection rates has also been reported (de Souza et al., 2016).

The detection of salmonids upstream the barrier indicated that the weir have not been a dispersal limitation for these species. Slightly higher species richness upstream the weir before its removal, majorly contributed by higher total number of sequence reads

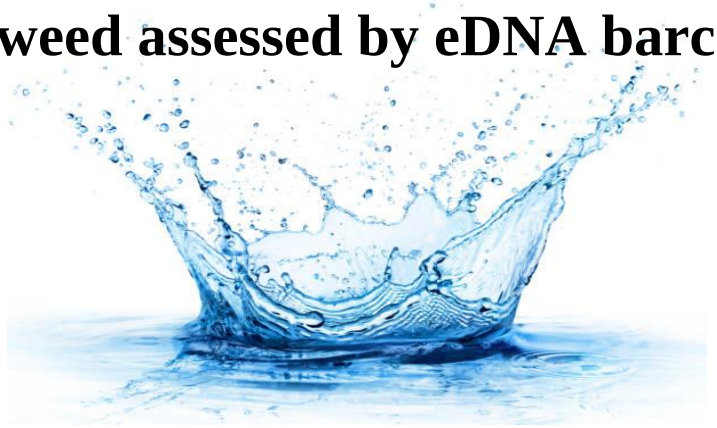
of *S. trutta*. The presence of both salmonid species upstream the weir indicates no upstream dispersal limitation of both, *S. salar* and *S. trutta*, capable of overcoming 1.85 m height barrier at low flows, with even higher barriers threshold identified (Timm et al., 2016). *S. salar* eDNA detection at the time of sampling, not coinciding with their spawning period, reflects juvenile or parr eDNA being detected during both sampling events, predicting adults' capabilities of upstream dispersion at the time of spawning before barrier removal (McCormick et al., 1998).

Three species of lampreys are known to be present in Lugg river, the sea lamprey (*Petromyzon marinus*), brook lamprey (*Lampetra planeri*) and river lamprey (*Lampetra fluviatilis*) (Capps, 2017), where I was able to distinguish between the lampreys only up to the genus level using 12S – V5 primer, *Lampetra spp.*. The presence of *Lampetra's spp*. eDNA above and below the barrier even before the removal indicates that the movement of these threatened species which migrates during the night (Kemp et al., 2011), had not been negatively affected by the removed barrier. The highest increase of the most abundant non- migratory fish, *P. phoxinus* can be associated to other ecological parameters, such as good water quality. *P. phoxinus* is sensitive to pollution, and disappears early with the onset of environmental degradation (Oberdorff et al., 2001). Increase of the *P. phoxinus* may be indicative a good water quality, suggesting that eDNA metabarcoding could be used for determining environmental quality parameters. The detection of European eel presence, classified as critically endangered species (IUCN Red list, 2018), in the river Lugg is of vital importance, as this species is facing many threats on their migration, including damage from hydropower turbines (Calles et al., 2013), being the target of a number of restoration programmes across Europe (Dekker and Beaulaton, 2015). eDNA metabarcoding could thus be used as a non-intrusive tool for their detection and dispersal monitoring.

Clear patterns of non- migratory fish increase over the whole open river corridor after a year of barrier removal, approves the restoration practice and contributes to increase gene and species diversity (Yamamoto et al., 2004). By default, barrier removal is a disturbance (Stanley and Doyle, 2003) and as such relaying on ecological effect of the removal on a small longitudinal scale is not optimal, thus a continuous monitoring several meters further downstream and upstream from the removed barrier would be beneficial, depending on the extent of the removal process and barrier type to assess its full effects. Full recovery of the ecosystem to pre- barrier conditions may not

happen and so partial recovery accounting for sensitivity of the organisms, type of barrier and watershed characteristics (Stanley and Doyle, 2003) need to be accounted for the success of the restoration management. It has been evaluated that restoration projects with most superficial evaluation strategies, result in most positive success of restoration (Morandi et al., 2014), thus I call for an extended repeatable evaluation of species assemblages in the area of interest before restoration measures take places using eDNA metabarcoding, for better conclusions about restoration success measures. For the assessment of barrier removals, eDNA metabarcoding comes handy and proves to be a reliable indicator for the river ecological recovery state, including pre-screening of potential barrier removal needs.

CHAPTER 5- Seasonal and spatial dispersion of invasive and native Codium green seaweed assessed by eDNA barcoding



Abstract

Codium fragile, known invasive seaweed, has spread widely during the last century, impacting local seaweed communities through competition and disturbance. Early detection of *C. fragile* can help on its control and management. I used eDNA barcoding to investigate the spatial distribution, abundance and coexistence of the invasive and native *Codium* species (*Codium vermilara*, *Codium tomentosum* and *Codium decorticatum*) in the Cantabrian sea. I designed species specific barcodes targeting short fragments of the rbcL gene for the invasive Codium species, and the elongation factor Tu (tufA) gene for the native species, to assess their spatial and seasonal distributions using quantitative real-time PCR in samples collected during summer, autumn and winter. I found seasonal differences in the presence of the invasive and two of the native species, but did not detect *C. decorticatum* at any point. Species distribution patterns produced with eDNA barcoding coincided with the known distribution based on previous conventional sampling, with a seasonal alternance of *C. fragile* and *C. vermilara*, and an obvious dominance of the non-native *C. fragile* in ports, which tend to be hotspots for invasive species. The results demonstrate the utility of using eDNA for early detection and monitoring of invasive seaweed. Regular monitoring of ports and adjacent areas using eDNA should help to assess the potential expansion of invasive *Codium* and the need for management interventions to avoid the displacement of native seaweed.

5.1 Introduction

The invasive seaweed *Codium fragile* is regarded as one of the four most damaging seaweed invaders (Provan et al., 2005), displacing local seaweed communities by its opportunistic physiological adaptations (Scheibling and Gagnon, 2006) and changing the structure of faunal assemblages (Drouin et al., 2011). *C. fragile* is normally introduced to new localities as a fouling organism on ships' hulls (Carlton and Scanlon, 1985, Drouin and McKindsey, 2007), and can be easily spread by currents before getting established on the coast (Carlton and Scanlon, 1985). Ports are known hotspots for invasive species (Drake and Lodge, 2004), and can potentially host more dense populations of invasive *C. fragile* in comparison to natural locations without artificial structures, which facilitate their growth (Bulleri and Airoldi, 2005). The invasive green seaweed *Codium fragile ssp. fragile* (Suringar) Hariot (hereafter *C. fragile*) has become established on the intertidal shores of the Cantabrian Sea (Northwestern Spain), coexisting with native *C. tomentosum Stackhouse, C. vermilara (Ollivi) Delle Chiaje* and *C. decorticatum (Woodward) Howe* (Skukan et al., 2017, Juanes et al., 2008, Martínez-Gil et al., 2007*),* with *C. fragile* being the only present subspecies identified in the area (Rojo et al., 2014). A temporal niche differentiation and a different life cycle strategy have been identified between the native *C. tomentosum* with the invasive *C. fragile* (García et al., 2018a), with higher abundance of invasive species in summer period compared to native *C. tomentosum*. There is a known spatio-temporal gradient, *C. tomentosum* species being predominantly found on the Western coast throughout the year, *C. fragile* distributed towards the East coast of Cantabrian Bay with highest densities found in summer (Cires Rodríguez and Rico Ordás, 2007, Rojo et al., 2014, García et al., 2018a), and few sightings of *C. vermilara* along the bay with increased presence in the winter (Rojo et al., 2014). Recruitment of *C. fragile* in the Bay of Biscay relies on newcomers rather than on established populations' vegetative regeneration (García et al., 2018b), implying that higher densities of invasive seaweed are likely found in ports.

Cryptic invasion of morphologically similar invasive and native species (Provan et al., 2008), is defined as the most possible cause for previously unrecognised *C. fragile* out-competition over native *Codium spp.* (García et al., 2018b). Due to *C. fragile* broad physiological adaptations and preference for higher reproductive temperatures (Hanisak, 1979), new potential niches for its settlement are proliferating under the

current climatic conditions (Zanolla and Andreakis, 2016). Spatio- temporal information of native and invasive *Codium spp.* is crucial for evaluating whether patterns of competitive displacement or coexistence take place in Cantabrian Sea, where rising sea- surface temperatures has favoured the spread of warm-water non-indigenous species for the past three decades (Díez et al., 2012).

Until now, the spatial and seasonal distribution of seaweed has relied on traditional methods (García et al., 2018b), which highlighted important limitations of *Codium spp.* taxonomical assessment based on phenotypic traits (Zanolla and Andreakis, 2016), and the difficulties imposed by tide induced sampling variations (Rojo et al., 2014) and evaluation of spatial spread due to their multiple reproductive patterns (Schmidt and Scheibling, 2005). A more rapid and accurate detection tool is therefore needed to monitor and/or control the distribution of invasive seaweed.

Early detections allow rapid response to eradicate or limit the spread of aquatic invasive species (AIS) (Jerde et al., 2011b). Environmental DNA (eDNA), a non-invasive technique, can detect species presence from genetic material in the surrounding sampling environment (Thomsen and Willerslev, 2015) and is increasingly being used for detection of AIS (Dejean et al., 2012, Piaggio et al., 2014, Takahara et al., 2013). It is an accurate technique used for presence-absence as well as relative abundance estimates, providing comparable estimates to traditional sampling techniques (Dejean et al., 2012, Valentini et al., 2016). eDNA has proved useful for the detection of aquatic invertebrates (Mächler et al., 2014, Deiner et al., 2016a) and vertebrates (Sigsgaard et al., 2016, Takahara et al., 2013, Piaggio et al., 2014), but the information on the aquatic plants and algae is still limited. Only a few studies have addressed the detectability of aquatic plants or algae with eDNA (Scriver et al., 2015, Keller et al., 2017, Fujiwara et al., 2016, Zimmermann et al., 2015), due to the limited availability of reference databases (Cristescu, 2014) and the lineage-specific barcodes (Zanolla and Andreakis, 2016). To be useful for detecting seaweed, eDNA barcodes need to be specific (Verbruggen et al., 2010) and have a suitable resolution across multiple regions (Zanolla and Andreakis, 2016) for targeting taxa within their suspected introduced range (Geller et al., 2010). Given the increase in invasion rates worldwide (Ruiz et al., 1997), the use of eDNA has the potential to revolutionise the detection of cryptic invasive seaweed, which has been rarely assessed until now.

Early detection of *Codium spp.* spatial and temporal variations is essential to assess the potential of non-native warm– temperate seaweeds to replace native cold-temperate species (Fernández, 2016) in the Bay of Biscay. I evaluated the extent of seasonal and spatial variation of the intertidal green seaweed, to identify whether there are temporal and/ or seasonal overlaps, or niche separations between invasive and native *Codium spp*. I also investigated whether invasive species presence is higher in ports in comparison to natural coastal locations, to identify potential areas for targeted containment management.

5.2 Methods

5.2.1 Study sites

Water samples were collected in July, October and December 2017 at four different stations in Asturias (N. Spain) including a sandy beach with few rock formations, Concha de Artedo (latitude 43°34'01.7"N, longitude 6°11'29.5"W), the small port of Cudillero (latitude 43°34'02.1"N, longitude 6°09'04.1"W), the rocky cliff Cabo de Peñas (latitude 43°37'31.3"N, longitude 5°53'48.5"W) and the large international port of Gijón (latitude 43°33'18.3"N, longitude 5°41'25.9"W) (Figure 5. 1a). The sampling covered 40.26 km of coast. Samples for Cabo de Peñas were not available for July. Average water temperatures in all three sampling months (July, October, December) were 21.9 ºC, 20.6 ºC and 15.8 ºC in Gijón and 21.5 ºC, 20.2 ºC and 15.6 ºC in Cudillero. I recorded seawater temperature in situ at Concha de Artedo and Cabo de Peñas using two Hobo Temperature Logger (Onset Computer Corporation, Pocasset, MA, USA) permanently fixed to the substratum at an average height 1 m above mean sea level, with measured 22.2 ºC maximum summer seawater temperature (SST) at Concha de Artedo and 21.7 ºC at Cabo de Peñas, and 12.4 ºC and 12.0 ºC minimum winter temperatures at both stations respectively. There was a difference of 0.4 – 0.5 ºC between W and E measurements on average monthly SST.

5.2.2 *Ex- situ* optimisation

An *ex-situ* optimisation was designed focusing on *C. tomentosum* to validate primer efficiency based on eDNA copy number with species density. An experimental set was built consisting of six treatments and a control group containing only marine water. The experimental groups consisted of six pre- sterilised glass bottles with 1 L of marine water to which different densities (5, 10, 20, 40, 80, 160 g) of *C. tomentosum* were

added. Individual specimens were collected at Cabo de Peñas in October 2017 and brought in a cooling bag back to the laboratory. The specimens were morphologically identified following Provan et al. (2008), gently dried with a towel and weighted on a scale before being added to 1 L water bottles (Figure 5. 1b). The weights were in geometric order to test for a correlation between eDNA quantity assessed by qPCR (Ct values) and species biomass. The marine water for the experiment was collected at a location with no known presence of *C. tomentosum.* Water temperature was kept between 16- 17.5 ºC. *C. tomentosum* specimens were kept in bottles for 36 hours and removed afterwards. The water from the bottles was filtered using the same eDNA filtering procedure as described below for each bottle separately. The negative filtration control using sterile nuclease-free water was filtered first, followed by filtration of marine water only and then the rest of the bottles containing *C. tomentosum* in order of concentration, starting by the lowest. The DNA was extracted using the same protocol as for the collected eDNA water samples from field described below, including an additional negative extraction control, with extractions being stored at -20 ºC.

5.2.3 Environmental DNA collection, filtration and extraction

Three replicates of water samples (1 L of each) were collected with sterile bottles approximately 30 cm under the surface at all sampling sites at consistent sampling points for each of the three sampling periods (Figure 1a). All four sites were sampled either on the same day or in two consecutive days. Nitrile gloves were used while collecting the water. A cooling bag was used for the transportation to the laboratory where filtration took place immediately after returning from the field. The filtering station in the laboratory was based in a room especially dedicated to environmental DNA sample handling. All the recommended steps for contamination- free eDNA work were carried out following Goldberg et al. (2016). A filter funnel was used for vacuum filtering in a combination with sterile Supor1-200 Membrane Disc Filter (Pall Corporation, US) with 0.2 µm pore size. Water flow was 70 kPA. For each of the sampling replicates one or maximum two filters were used and stored together in a separate tube from other replicates at – 20 ºC until the next day when DNA extraction was processed. A negative control sample was filtered using sterile nuclease-free water between filtering samples from different sampling locations. DNA was extracted on the following day of filtrations using the PowerWater® DNA Isolation Kit Sample

(Qiagen GmbH, Hilden, Germany) following the manufacturer's recommendations with a modified last step of 50 µL for DNA elution. The DNA extraction took place in a pressurised fume hood dedicated solely to eDNA handling. Sampling triplicates were extracted individually, including all five negative filtration controls with an additional negative control extraction samples for each of the sampling seasonal periods. DNA extractions were stored at – 20 ºC before further processing.

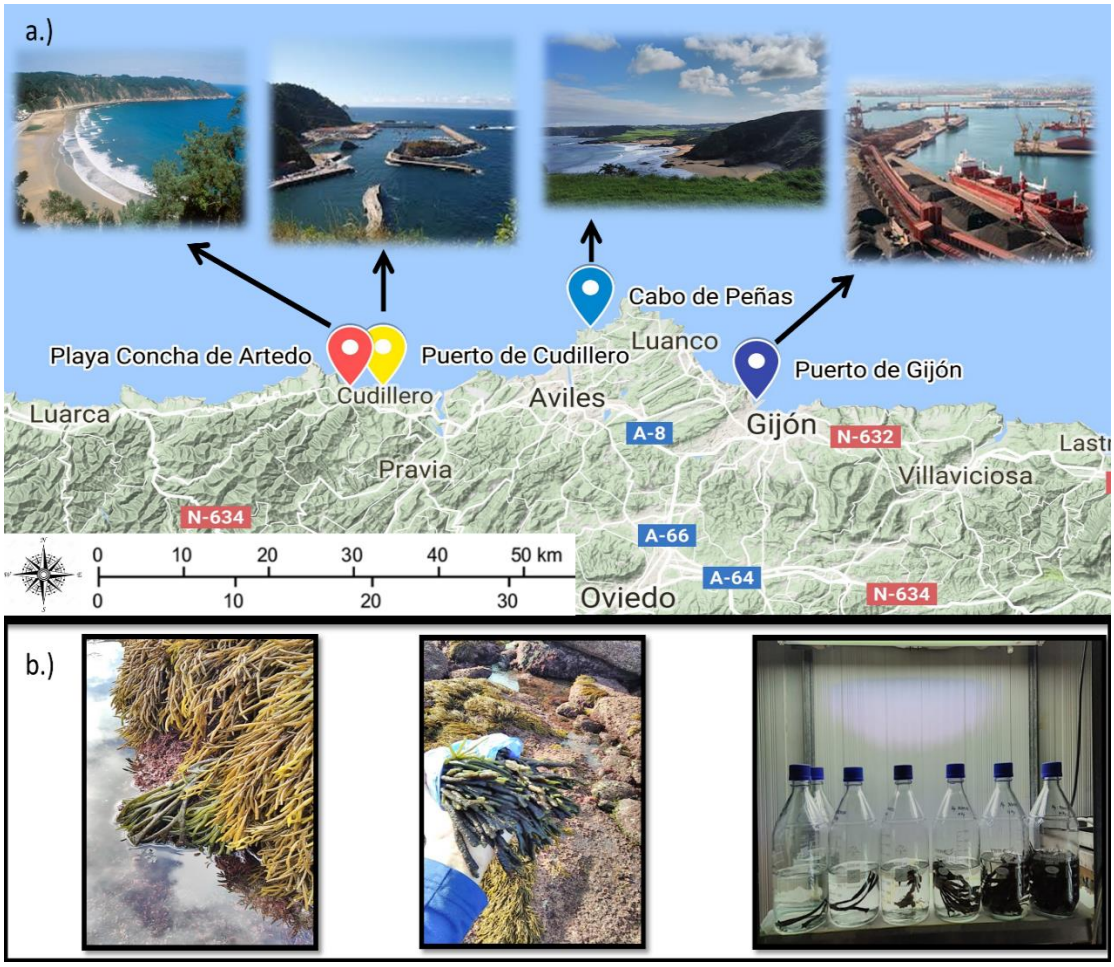


Figure 5. 1- (a.) DNA sampling locations from East to West: Concha de Artedo, small port of Cudillero, rocky intertidal platform Cabo de Peñas and international port of Gijón; (b.) Collection of *C. tomentosum* specimens and lay out of the eDNA mesocosm experiment. The selected images of natural localities and ex- situ experiment belong to authors and the images of ports were collected from the google marked with permission for reuse and modifications.

5.2.4 Primer design and validation

I developed specific barcoding primers (rbcL and tufA genes) for the assessment of the invasive species *C. fragile* in coexistence with native *Codium spp.* in the

Cantabrian Sea during three different seasons at four different sampling stations. I targeted 364 bp of the rbcL gene chloroplast subunit for the invasive *C. fragile* based on reference nucleotide sequences from GenBank, as this gene has previously been used for species identification (Verbruggen et al., 2007). For the three native species *C. tomentosum*, *C. vermilara* and *C. decorticatum*, 211 bp, 180 bp and 249 bp short fragments of plastid elongation factor Tu (tufA) gene were targeted to design species specific markers (Table 5. 1). The plastid tufA and rbcL markers are some of the most widely applied markers to taxonomically separate the green algae group (Saunders and Kucera, 2010, Škaloud et al., 2012). In order to avoid species cross- amplification, two different plastid regions were chosen. To test the species specificity of the primers they were firstly tested *in silico* using Primer – BLAST (Ye et al., 2012) and afterwards used to amplify and cross-amplify tissue samples of the individual species before being used on eDNA samples for PCR and qPCR. Cross- species amplifications were tested on each individual species amplifying it with all four primer pairs. *C. decorticatum* primers could not be tested on this species as no specimens were found along the Asturian coast at the time of the research. Extraction mixtures contained several specimens of each individual species to account for intra-species variability. Tissues were extracted using GeneMATRIX Plant and Fungi Purification Kit (GeneMATRIX purification Kit, Roboklon GmbH, Berlin, Germany). A 100- fold dilution of an initial 1 ng/ µL of each tissue was used for cross- amplifications in order to mimic eDNA detection levels in the environment. All specimens of *C. fragile* collected in the Bay of Biscay region were identified based on sequencing as the invasive subspecies *C. fragile ssp. fragile* (Rojo et al., 2014), confirming the primer specificity for the subspecies. Oligo Analyser 3.1 tool (Integrated DNA Technologies, US) was used for primer check on hairpins and primer dimers. To estimate the detection sensitivity of each specific primer pair, tenfold serial dilutions, starting from 1 ng/ µL down to 1: 10 000 000, were used and limits of detection were defined by PCR amplification. The last visible band on gel was defined as the detection limit for each species. Additionally, the same tenfold dilution was applied for defining the qPCR standard curve

Table 5. 1- Species-specific PCR primers used for amplification of targeted chloroplast rbcL and tufA region, annealing temperature, gel electrophoresis detection limit, and specific PCR and qPCR running conditions.

Target species	Primer	Sequence (5'- 3')	Amplicon size (bp)	Annealing PCR (T ºC)	Gel electrophoresis detection limit (ng/ mL)	Melt peak (ºC)	Annealing PCR (T ºC)
C. fragile ssp. fragile	C. fragRBCL F	ACATTCTTGCAGCTTTTCGT	364	58	0.01	82	65
	C. fragRBCL R	TTCATCCCATGAGGTGGTC					
C. tomentosum	C. tomCDS F	AACCAGCTTCTATTTTACCCCA	211	56	10	79.5	65
	C. tomCDS R	TCCATTTGAATACGATCTCCCG					
C. vermilara	C. verCDS F	CGCCATTTTCAAGCACAGGTA	180	57	0.0001	78	65
	C. verCDS R	AATTCGATCTCCCGGCATTAC					
C. decorticatum	C. decorCDS F	TACAGGAAGGGGTACGGTTG	249	57	/	/	65
	C. decorCDS R	TGTCGATGAGGCATAATAGAAGC					

*bp- base pair.

5.2.5 PCR amplification

PCR and qPCR were optimised to avoid cross- species amplification for each specific primer pair. PCR conditions were as follows, 7 min at 95 °C, followed by 10 touchdown cycles of 95 °C for 30 s, 58 C - 68°C for 30 s, 72°C for 30 s, with additional 15 cycles of 95°C for 30 s, 58°C for 30s, 72°C for 30 s, and a final extension step at the 72 °C for 5 min. For *C. vermilara*, *C. tomentosum*, *C. fragile* and *C. decorticatum* the annealing temperature were 57, 56, 58 and 57°C, respectively (Table 5. 1). The amplification reaction for the PCR included 1X Colorless GoTaq® Buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 50 pmol of each primer, 0.5 U of DNA Taq polymerase (Promega), 0.2 µg/ µL BSA and 3 µl of eDNA with nuclease-free water added up to total volume of 20 µl. The same PCR conditions were used for both, tissue and eDNA samples, with the only difference in the number of annealing cycles, 25 for tissue and 40 cycles for eDNA. For positive controls, tissues were diluted down to 0.1 ng/ µL including tested 10x and 100x fold dilutions to define primer efficiency on eDNA dilution level. PCR products were visualised on 2% agarose gel with added 2 µl of SimplySafe™. All PCR products were directly sequenced using Sanger sequencing at Macrogen Europe (Spain). Sequences were confirmed for each specific species by BLAST. Negative filtration and extraction samples were amplified using the same procedures.

For the quantification of each individual species from the eDNA samples real- time PCR (qPCR) was performed using SYBR Green technology (Bio-Rad, US). The reaction mixture contained 1x SsoAdvanced™ Universal SYBR® Green Supermix, 25 pmol of forward and reverse primer and 3 µl of extracted DNA with additional nuclease free water to the final volume of 20 µl with all amplifications run out on a 96- well reaction plate (Bio- Rad, US) including triplicates of negative control PCR where nuclease-free water was added instead of the template, as well as triplicates of positive controls added to each run. All species specific amplifications were run on separate plates. All eDNA samples were run in triplicate. Additional cross- species assessment was evaluated through qPCR with all four primers tested on all three different tissues. The qPCR conditions were as follows, 10 min at 95 °C, followed by 10 s at 95 °C and 30 s and 65 °C, in 35 cycles total for all four species. A melting curve was included at the end of qPCR run within a range of 60 °C to 95 °C. Data were analysed with Bio-Rad CFX Manager (Bio- Rad, US).

5.2.6 eDNA absolute quantification

In order to compare the seasonal and spatial distribution between the three species, absolute quantification based on differences in eDNA copies was performed, calibrated by each specific qPCR run efficiency. Absolute quantification determines the input copy number by correlating PCR signal to a standard curve (Schmittgen and Livak, 2008). Each individual species' copy number estimate was determined by the exact copy concentration of the target gene correlated to Ct values according to the standard curve (Lee et al., 2006) as used previously in eDNA studies (Dougherty et al., 2016, Renshaw et al., 2015), by firstly calculating the number of copies per each individual species specific targeted DNA length, using Avogadro's number (6.022x 10^{23} molecules/ mole) and a general assumption that the average weight of a base pair (bp) is 650 Daltons as calculated by Whelan et al. (2003), following:

DNA (copy number) = (6.02 X 10^{23} (copy/ mol) * DNA concentration (ng/ µL)) / (DNA length (bp) X 650 (g/ mol/ bp))

The DNA copy number was used for calculation of the initial concentration given for the standard curve. Each standard curve was performed by a linear regression of the plotted standards. The slope of each standard curve determines qPCR efficiency (E), calculated by the following equation (Lee et al. (2006):

$E = 10^{-1/\text{slope}} - 1$

From the copy number of each standard I quantified each sample by relating Ct values to the standard curve (Yu et al., 2005). Each specific sample quantification was performed as in (Gallup, 2011):

Absolute copy number (eDNA copies) = $E^{(\text{Standard curve intercept- Sample average Ct value})}$

All eDNA copy numbers were estimated per microliter of filtered water (eDNA copies/ µL).

5.2.7 Statistical analysis

I modelled presence/ absence data and species density in relation to season, sampling site and artificial/ natural locations applying four different models. The two ports (Gijon, Cudillero) and two natural locations (Concha de Artedo, Cabo de Peñas) were grouped together by artificial/ natural categories to see if there is any difference between the origin of sampling localities. For presence/ absence data, I employed a

binary logistic regression within two models, firstly assessing interactions between species, location and sampling season, and secondly the interactions between species, sampling season and type of location (natural/ artificial). At least two positive detections (out of three sampling replicates) were considered sufficient as evidence of presence. To model abundance, I used a linear model with a Gaussian error distribution to investigate variation in eDNA copies/ µL as function of species, location and sampling season in first model and species, sampling season and natural/ artificial location in the second model, including their interactions. For the post-hoc analysis, the 'lsmeans' package was used (Lenth, 2016) based on Tukey contrasts. The qPCR triplicates of each of the three sampling replicates were averaged before statistical analysis. In case one of the sampling triplicates did not amplify and the other two did, the amplification of sampling triplicates was repeated for confirmation, with at least two sampling replicates used for further statistical analysis. For estimation of efficiency in species specific models, as well for comparison of abundance among species the eDNA copies/ µL was used. For the *ex-situ* optimisation, a simple correlation between the *C. tomentosum* and eDNA copy number (based on Ct values) and seaweed density was calculated. All statistical analyses were done with the R, version 3.3.2, with 'dplyr' and 'ggplot2' package used for data representation.

5.3 Results

In total 132 eDNA qPCR technical triplicates, 11 filtering and 3 extraction negative controls were used for qPCR quantification. In seven of the samples not all three sampling replicates produced species specific positive confirmations, five targeting *C. tomentosum* and two targeting *C. fragile,* thus sampling duplicates were used for further analysis. Triplicates of 21 eDNA samples, 2 filtrations and one extraction negative controls from *ex-situ* optimisation were additionally processed for individual assessment based on correlation between *C. tomentosum* eDNA copies/ µL and species density (g/ L). There was no *in silico* possible cross- contamination with the three native species, tested with the PRIMER BLAST tool on NCBI page (Johnson et al., 2008). No cross- amplification was produced either in PCR or in qPCR for any of the three species tested with all four primer sets, using dilution series of the three target species *C. tomentosum, C. fragile* and *C. vermilara*.

Negative controls produced no amplification in any cases. Both controls from the ex-situ experiment, the marine water and nuclease- free water did not amplify during PCR and qPCR tested with all four primer pairs. All positive controls confirmed the target species by accurate alignment to sequences from target species, using BLAST and BioEdit (Hall, 1999). In total, 4 individual forward and reverse sequences for all three primer sets on *C. vermilara*, *C. fragile* and *C. tomentosum* were used for measures of primers' efficiencies as positive controls on species' tissue extractions. In total, 81 eDNA samples were sequenced for each species separately, 30 for *C. tomentosum*, 29 for *C. vermilara* and 22 for *C. fragile*, confirmed by 98- 100% similarity rate in BLAST, with 9 unique sequences added to the Genbank under the nucleotide accession numbers (MK503248- MK503252, MK503325- MK503328, MK507407- MK507412). *C. decorticatum* did not amplify in any of the qPCR triplicates of 132 eDNA samples and was not considered for further analysis.

For qPCR cross- amplification, no melt peaks were observed using cross- referenced primers on species specific target samples, confirming the specificity of the primers. Melt peaks of the three target species *C. fragile*, *C. tomentosum* and *C. vermilara* were at 82 ºC, 79.5 ºC and 78 ºC respectively (Table 5. 1, Figure S. 4). For the invasive *C. fragile*, the qPCR quality run resulted in R^2 = 0.97 based on the standard curve approach, with an efficiency of 99% and a slope of -3.345. For the native *C. tomentosum*, the qPCR run resulted in R^2 = 0. 991, efficiency of 99.9% and a slope of -3.325. For the native *C. vermilara* the qPCR runs resulted in R^2 = 0.998 with an efficiency of 96.3% and a slope of -3.414. The relative fluorescence unit threshold for all qPCR runs was set up at 300 RFU (Figure S. 4). Melt peaks under the threshold were not considered for further analysis.

5.3.1 *C. tomentosum* ex-situ optimisation

C. tomentosum eDNA density variation based on Ct values (eDNA copies/ µL) amplified until the biomass threshold of 80 g/ L (Figure 5. 2), which was the upper limit of detection by qPCR. The eDNA qPCR concentration was linearly correlated to the actual specimens' biomass up to 20 g/ L, reaching a plateau between 20 g/ L and 40 g/ L, with an average of 26.610 ± 0.861 Ct values ($1.083 \times 10^6 \pm 6.4 \times 10^5$ eDNA copies/ µL). The lowest and highest *C. tomentosum* eDNA densities measured in the field were 4.930×10^2 up to 5.812×10^6 eDNA copies/ µL, which would correspond

to an approximate density of 1.504 up to 47.66 g/ L when compared to the *ex-situ* optimisation.

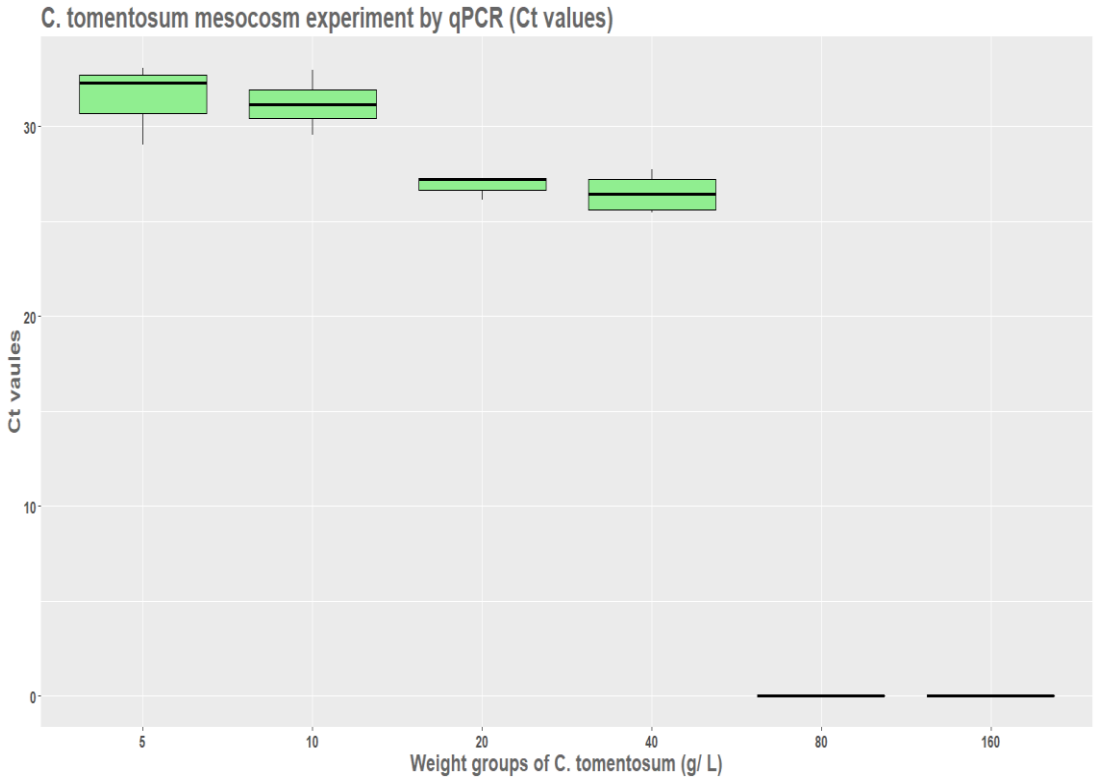


Figure 5. 2- eDNA density (Ct values) correlated to *C. tomentosum* actual biomass (g/ L) in the *ex-situ* optimisation collected from Cabo de Peñas sampling point.

5.3.2 Species specific seasonal and spatial evaluation

I evaluated *C. fragile*, *C. tomentosum* and *C. vermilara* seasonal and spatial representation individually by qPCR quantification (Figure 5. 3). Overall the most predominant two species were *C. fragile* and *C. tomentosum*, the latter accounting for the highest abundance of all the species, with an average of 6.079 x 10^5 eDNA copies/ µL in the two Western sampling points and 2.201 x 10^5 eDNA copies/ µL at the Eastern sampling side. *C. fragile* was predominantly found on the East with an average of 5.629 x 10^5 eDNA copies/ µL and a more even distribution between the three localities with species occurrence (± 6.653 x 10^4 eDNA copies/ µL), without spatially predominant patterns of *C. vermilara* eDNA presence (Figure 5. 3). There was an obvious temporal gradient of *C. fragile* where the highest eDNA density was found in summer month, decreasing through autumn, with the lowest representation in the winter (Figure 5. 3). I did not found *C. fragile* at Concha de Artedo, the most Western sampling point, whereas the highest eDNA presence was found at both ports, Cudillero

with an average of 32.956 ± 1.78 Ct values corresponding to 4.780 x 10^5 ± 4.945 x 10^5 eDNA copies/ µL, and Gijon with 32.733 ± 2.348 Ct values, corresponding to 7.929 x 10^5 ± 6.323 x 10^5 eDNA copies/ µL. Despite highest average summer density of *C. fragile*, the absolute highest single eDNA detection was measured in October in the port of Gijon with 3.192 x 10^6 eDNA copies/ µL. The only locality where I found eDNA of *C. fragile* over all seasons is at port of Cudillero, whereas in the port of Gijon I only detected it in the Autumn sampling. *C. tomentosum* eDNA presence was detected at all four stations, with higher rate coverage in summer and winter periods and a slight prevalence towards the West Coast (Figure 5. 3). *C. tomentosum* exhibit the overall highest presence in summer and winter compared to other two species, whereas *C. fragile* maintained its eDNA detection rate density over summer and autumn with a decline in winter period (Figure 5. 3). The highest *C. tomentosum* eDNA copies/ µL was detected in July at Concha de Artedo with 4.922 x 10^6 ± 9.515 x 10^5 copies/ µL (24.814 ± 0.288 Ct value). eDNA from *C. vermilara* had been also found at all four stations with the highest representation in winter periods, where on the average the eDNA copy number was for 11.390% higher compared to autumn period (Figure 5. 3). In the summer I only detected it at port of Cudillero with 32.023 ± 1.113 corresponding to 5.082 x 10^3 ± 3.380 x 10^3 eDNA copies/ µL.

Seasonal and spatial presence of species indicated high variation between species (Table 5. 2, χ^2 (N= 88) = 87.978, df= 2, $p < 0.001$), location (Table 5. 2, χ^2 (N = 83) = 15.727, df= 3, $p < 0.001$) and sampling season (Table 5. 2, χ^2 (N= 86) = 24.752, df= 2, $p < 0.001$), with a significant interaction of species and location (Table 5. 2, χ^2 = 8.997, df= 5, $p < 0.001$). The model focusing on species seasonal presence between natural and artificial environment identified a higher overall presence of all species at the two artificial ports (Table 2, χ^2 (N= 85) = 56.906, df= 1, $p = 0.011$). A density dependence model accounting for differences among species, location and season, including their interactions, shows significant differences in abundance between species (Table 5.3, F (N = 62) = 12.468, df = 2, $p < 0.001$) due *C. tomentosum* high and *C. vermilara* lower abundance (Tukey's Post-hoc test, $p = 0.001$) and sampling seasons (Table 5.3, F (N= 60) = 3.409, df = 2, $p = 0.042$), based on eDNA copies/ µL. Significant density dependence interactions were identified among species and sampling season (Table 5.3, F (N= 55) = 3.617, df = 4, $p = 0.013$), in particular between low *C. vermilara* density in October and December compared to high *C. fragile* density

in October and also *C. tomentosum* higher winter densities compared to *C. fragile* (Tukey's Post-hoc test, $p < 0.011$), and also between sampling season and location ($F (N= 44) = 3.309$, $df = 4$, $p = 0.019$), mainly due to low seasonal representation of species at Concha de Artedo compared to other localities at all sampled seasons (Tukey's Post-hoc test, $p < 0.006$). The second density dependence model assessed an interaction between artificial/ natural segregation of specific species in seasons and two significantly different relations were identified, the species specific density change within season and the artificial/ natural segregation with seasonal changes (Table 5.3, $F (N = 55) = 3.403$, $df = 4$, $p = 0.015$; $F (N = 51) = 3.939$, $df=2$, $p= 0.025$) respectively, with an average higher eDNA copies/ µL found at the two artificial ports compared to the natural locations, particularly in autumn.

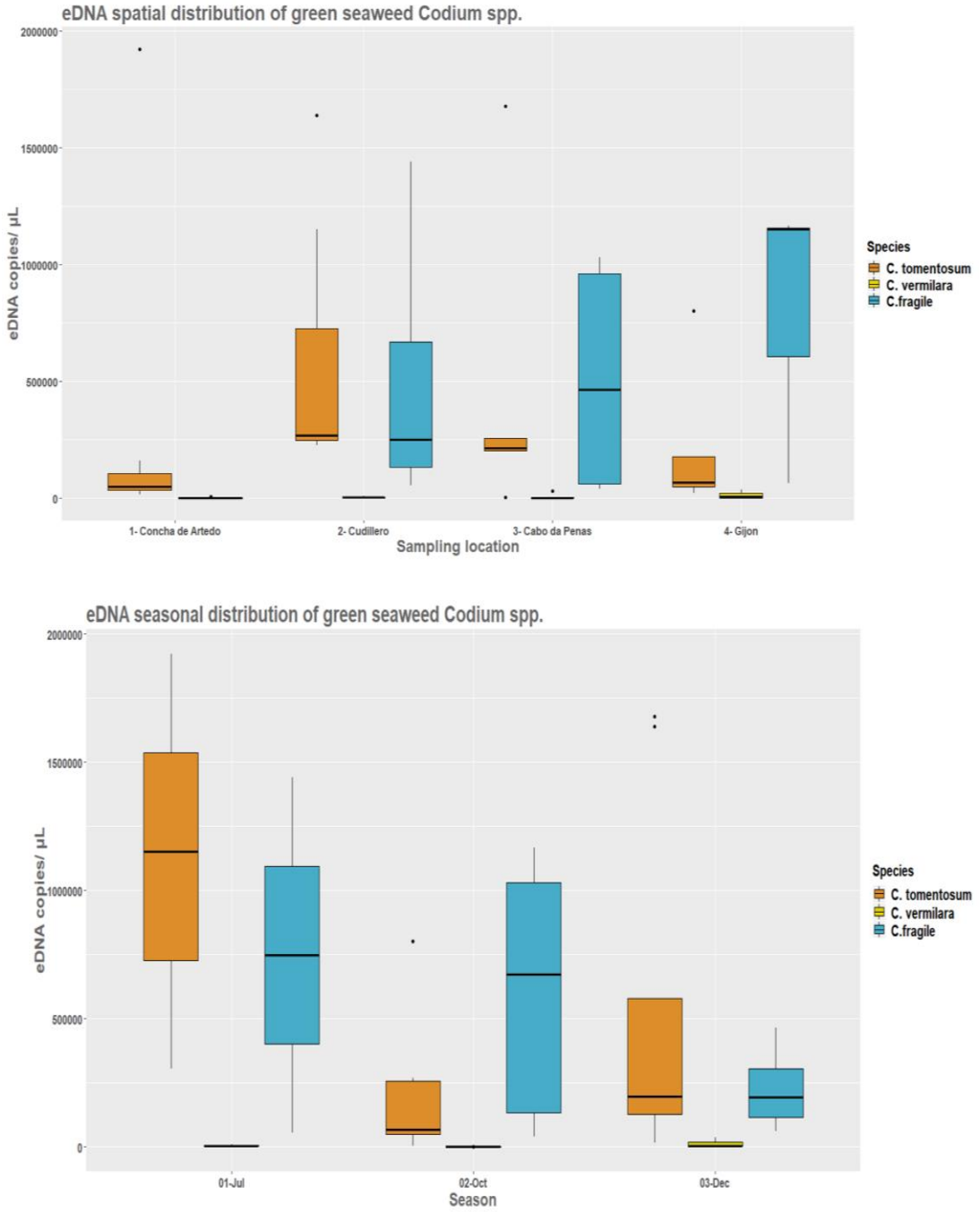


Figure 5. 3- Spatial (upper graph) and seasonal (lower graph) density variation (eDNA copies/ µL) of all three species, *C. fragile*, *C. tomentosum* and *C. vermilara*. For spatial variation representation, all four sampling stations, Concha de Artedo, Cudillero, Cabo de Peñas (excluding sampling in July) and Gijón, are presented in separate groups where samples were merged from all three sampling events conducted in July, September and December. For seasonal variation, the sampling stations are merged together, with separate sampling events groups.

Table 5. 2- Evaluation of seasonal and spatial patterns of all three species using binary logistic regression for species presence/ absence assessment, identified with two models, first one based on species, sampling season and location, and second one based on species, sampling season and artificial/ natural categories, including interactions between them. All sampling locations, Concha de Artedo, Cudillero, Cabo de Peñas and Gijón, were included in the analysis.

Predictors	**Deviance**	**Df**	**Residual df**	**Residual deviance**	**< Chi**	**AIC**
Presence/ absence= Species * Sampling season * Location						74.137
Species	20.908	2	88	87.978	**< 0.001**	
Sampling season	24.752	2	86	63.225	**< 0.001**	
Location	47.798	3	83	15.727	**< 0.001**	
Species x Sampling season	0.078	4	79	15.727	0.9889	
Sampling season x Location	0	4	67	6.730	1	
Species x Location	8.997	5	73	6.730	**< 0.001**	
Species x Sampling season x Location	0	4	57	6.730	1	
Presence/ absence= Species * Sampling season * Artificial/ natural						78.912
Species	20.907	2	88	87.978	**< 0.001**	
Sampling season	24.752	2	86	63.225	**< 0.001**	
Artificial/ natural	6.318	1	85	56.906	**0.011**	

Predictors	Deviance	Df	Residual df	Residual deviance	< Chi	AIC
Species x Sampling season	8.001	4	81	48.903	0.091	
Species x Artificial/ natural	3.151	2	79	45.752	0.206	
Sampling season x Artificial/ natural	2.839	2	77	42.912	0.241	
Species x Sampling season x Artificial/ natural	0	4	73	42.912	1	

Table 5. 3- Evaluation of seasonal and spatial patterns of all three species using linear models for species abundance estimation by eDNA copies/ µL. The first linear model (Species x Sampling season x Location) includes all three species, together with sampling season, location and interaction terms between them, and the second model (Species x Artificial/ natural x Sampling season) evaluates additional difference between the artificial/ natural species specific seasonal distribution. All sampling locations, Concha de Artedo, Cudillero, Cabo de Peñas and Gijón, were included in the analysis.

Predictors	**F**	**Residual df**	**df**	**p**	**AIC**
eDNA copies/ µL= Species * Sampling season * Location					1872.7
Species	12.468	62	2	**< 0.001**	
Sampling season	3.409	60	2	**0.042**	
Location	0.303	57	3	0.822	
Species x Sampling season	3.617	53	4	**0.013**	
Sampling season x Location	3.309	44	4	**0.019**	

Predictors	**F**	**Residual df**	**df**	**p**	**AIC**
Species x Location	0.350	48	5	0.878	
Species x Sampling season x Location	0.673	40	4	0.614	
eDNA copies/ µL= Species * Sampling season * Artificial/ natural					1869.9
Species	12.088	62	2	< **0.001**	
Artificial/ natural	0.115	59	1	0.735	
Sampling season	3.272	60	2	**0.046**	
Species x Artificial/ natural	0.103	53	2	0.902	
Species x Sampling season	3.403	55	4	**0.015**	
Sampling season x Artificial/ natural	3.939	51	2	**0.025**	
Species x Artificial/ natural x Sampling season	0.045	49	2	0.955	

5.4 Discussion

I used an environmental DNA (eDNA) approach to assess the spatio-temporal variation of a non-native algal species in relation to two of the closest native species, using eDNA absolute quantification approach in the Bay of Biscay at three different seasons and at four locations along an environmental longitudinal gradient. Our results largely confirmed those from more traditional surveillance methods, indicating that eDNA barcoding is an efficient and effective way of monitoring invasive and native green seaweed species seasonal and spatial patterns (Skukan et al., 2017, García et al., 2018b). High *C. fragile* eDNA densities in both ports and a novel detection of species at Cabo de Peñas confirms further spread of invasive species in between the recipient ports. The additional *ex- situ* optimisation of *C. tomentosum* contributed towards relative density assessment in the field. eDNA density assessments using ex- situ optimisation have been previously used as an estimate of relative abundance correlated with eDNA (Wilcox et al., 2016, Takahara et al., 2012, Doi et al., 2015), finding it as the most suitable measure for general biomass/ density species specific assessment. I found no *C. decorticatum* in our eDNA sampling, confirming previous studies along the coast (García et al., 2018b), despite having been occasionally reported (Cires Rodríguez and Rico Ordás, 2007). Tide induced sampling had been one of the potential causes proposed for the species absence during sampling events (Rojo et al., 2014), but our study clearly indicates absence of species at the sampling stations at the time of sampling. Confirmation of *C. decorticatum* absence at all sampling events as well as absence of *C. fragile* at the most Western sampling point reflects on the usefulness of eDNA as a tool for both, presence as well as absence estimates for spatial species distribution control.

The east side tendency of *C. fragile* eDNA confirmed previous findings (Cires Rodríguez and Rico Ordás, 2007). Our results were also concordant with the previous surveillance at most western point of Concha de Artedo where in summer sampling events the majority of the specimens belonged to *C. tomentosum* with a small representation of *C. vermilara* and no confirmed presence of *C. fragile* (Rojo et al., 2014).

C. fragile are reproductively more successful in warmer waters with maximum growth at 24 ºC (Hanisak, 1979) compared to the two native ones with lower temperature preferences (Yang et al., 1997). This could explain the higher densities of *C. fragile*

on the East side of Cantabrian coast due to higher summer temperatures modifying seaweed assemblages (Díez et al., 2012). Our results confirm seasonal variation of species coverage at same locations as defined previously (García et al., 2018b). *C. vermilara's* optimum growth occurs at 18 µmol/ mol of photon irradiance (Yang et al., 1997), averaged quarter and half of the averaged photon irradiance of other five *Codium spp*., making it an ideal candidate species to shifts it's reproductive cycle towards colder seasons. *C. fragile* becomes a dominant canopy- forming species once established as dense meadows in new environments (Scheibling and Gagnon, 2006) and could force *C. vermilara* to shift towards winter growth preferences. Similar co-existing acclimatisation of two native and invasive kelp species in same environment have been previously evidenced, where habitat preferences were identified through specific gene expression in correlation to temperature shifts (Henkel and Hofmann, 2008). The results show that *C. fragile* was the predominant species during autumn sampling, whereas previously it had been predominantly found in the summer period (Rojo et al., 2014). Colder spring and summer temperatures in the year of the eDNA sampling, with additional warmer temperatures in autumn (only 1 °C degree difference from summer sampling), could have postponed *C. fragile* reproductive season towards autumn and the corresponding increase in release of gametes (Bohmann et al., 2014) might be correlated to the eDNA density increase in that particular Autumn. With the increasing temperatures along the N Spanish coast (Gómez-Gesteira et al., 2008), a range shift in the relative abundance of seaweed species (Duarte et al., 2013, Voerman et al., 2013) and the potential increase of *C. fragile* towards the West could be expected.

A high eDNA density of invasive *C. fragile* was detected in both ports, with potential displacement of the native species. Colonisation of *C. fragile* subspecies on artificial marine structures is a regular occurrence around the globe (Bulleri and Airoldi, 2005, Trowbridge, 1995, Campbell, 1999), where artificial structures facilitate its spread. eDNA based methods could be used for invasive green seaweed monitoring, by integration with port baseline surveys (David et al., 2013) for ballast water management or implementation within Marine Strategy Framework Directive (Directive, 2008, Borja et al., 2010). Despite the apparent non-competitive status of *C. fragile* in the Cantabrian Sea due to their clear seasonal reproductive segregation with native species (García et al., 2018b), there is no potential reduction in its introduction

rates, which depends on multiple vectors (Boudouresque and Verlaque, 2010) such as shipping routes through ports.

Early detection of seaweed species in the aquatic environment can significantly improve aquatic invasive species management and potential eradication (Jerde et al., 2013), with more efficient monitoring and containment of its spread (Tréguier et al., 2014), predicting its dispersal through spatial distribution models (Muha et al., 2017), or influencing management and policy decisions (Kelly et al., 2014b). As I have demonstrated here, eDNA can be used to assess the spatial and seasonal distribution patterns of invasive and native green seaweed algae species quickly and relatively cheaply, estimating invasive species patterns, such as competition or potential co-existence, representing an ideal tool for their routine monitoring.

CHAPTER 6- Using environmental DNA to improve Species Distribution Models for freshwater invaders

Abstract

Species Distribution Models (SDMs) have been reported as a useful tool for the risk assessment and modelling of the pathways of dispersal of freshwater invasive alien species. Environmental DNA (eDNA) is a novel tool that can help detect invasive alien species at their early stage of introduction. SDMs rely on presence and absence of the species in the study area to infer the predictors affecting species distributions. Presence is verified once a species is detected, but confirmation of absence can be problematic because this depends both on the detectability of the species and the sampling strategy. eDNA is a technique that presents high detectability and can effectively differentiate between presence or absence of specific species or entire communities by using a barcoding or metabarcoding approach. However, a number of potential bias can be introduced during (i) sampling, (ii) amplification, (iii) sequencing, or (iv) through the usage of bioinformatics pipelines. Therefore, it is important to report and conduct the field and laboratory procedures in a consistent way, by (i) introducing eDNA independent observations, (ii) amplifying and sequencing control samples, (iii) achieving quality sequence reads by appropriate clean-up steps, (iv) controlling primer amplification preferences, (v) introducing PCR-free sequence capturing, (vi) estimating primer detection capabilities through controlled experiments and/or (vii) post-hoc introduction of 'site occupancy-detection models'. With eDNA methodology becoming increasingly routine, its use I strongly recommend to retrieve species distributional data for SDMs.

6. 1 Introduction

Current policies on aquatic invasive species (AIS) depend on the availability and quality of data used for their risk assessment (Groom et al., 2017). Species Distribution Models (SDMs) use available data of invasive species and are one of the most widely used tools for risk assessment, predicting species distribution and pathways of dispersal (Jiménez-Valverde et al., 2011).

This methodology relates the distribution data of the AIS (e.g., presence and absence records) in the study area with a set of independent spatially explicit variables to explain and predict the range expansion of the species. However, there are limitations on these approaches because of two main reasons: i) confirmed absences are desirable but scarce in available databases, and ii) independent data for evaluation is normally not available. The consideration of absences has been reported to provide more accurate predictions of the actual distribution of IAS (Václavík and Meentemeyer, 2009). Therefore, there is a need for tools that allow the recording of presence and absence and a faster compilation of independent data to test spatially explicit models. Efficient spatial monitoring of invasive species vectors of introduction, further dispersal as well as initial detection of newly present species, are crucial for species management as are prevention, control and eradication.

In the recent years, a new environmental molecular tool has been developed-environmental DNA (eDNA). eDNA refers to DNA which can be extracted from environmental samples without separation of specific organisms from the environment (Taberlet et al., 2012). eDNA contains both cellular as well as extracellular DNA from all kinds of organisms. It is subject to high levels of degradation but can be preserved in nature from few weeks up to hundreds of thousands of years (Thomsen and Willerslev, 2015). The ability to detect species through eDNA water samples is relatively novel and has proved as a useful tool for the detection of aquatic IAS (Nathan et al., 2014, Dejean et al., 2012, Goldberg et al., 2013a). It can be applied for the detection of a number of specific IAS (barcoding), or detecting multiple IAS as part of whole communities (metabarcoding). New revolutionary techniques for eDNA are being developed on a daily basis with the aim to provide a number of useful information such as, presence or absence of the species (Ficetola et al., 2008), density assessments (Moyer et al., 2014), population dynamics (Sigsgaard et al., 2016), sex

(Nichols and Spong, 2017), hybridisation process between subspecies, (Uchii and Minamoto, 2016, Gorički et al., 2017), spatial representativeness (Civade et al., 2016, Bista et al., 2017a) and ability to amplify whole mitochondrial genome (Deiner et al., 2017d). A wide range of eDNA detection possibilities is currently limited. Knowing what are the limitations of eDNA methods is key to successful estimation of species presence (or absence) and estimations of their biological characteristics.

6. 2 Approach

Nowadays, useful information on AIS within SDMs is in the detection of presence and absence of the species (Ficetola et al., 2008). In this chapter, I discuss the range of possibilities and limitations with regard to reporting AIS presence or absence using eDNA in freshwater ecosystems in order to obtain additional and more accurate distribution data to be used in the SDMs.

6.2.1 Potential applications

eDNA has thus far been mainly used in the early detection and monitoring of invasive species, contributing to the increase of IAS presence records. The use of eDNA techniques could facilitate a more effective method for recording IAS absence than do regular monitoring surveys or possibly may aid in the compilation of independent data similar to the approach used for proving (non)successful eradications (Dejean et al., 2012). Currently, eDNA research is focusing its effort on the species detection efficiencies based on the competence of sampling, amplification and sequencing techniques. I have implemented a detailed review based on the potential for the future application of eDNA tool by identifying the proportion of positive detections of AIS within individual research (Table 6. 1). The review proves how useful the tool can be dealing with AIS detection. A recent increase in presented eDNA research conducted on invasive species is only the tip of the iceberg of what can be achieved for conservation and AIS management. There is however a number of limitations that should be remembered before applying eDNA data to retrieve distribution data for SDMs.

Table 6. 1- eDNA studies targeting freshwater invasive alien species, including description of water sampling and filtration techniques, DNA loci, barcoding or metabarcoding as well as the proportion of positive detections.

Taxon	**Target freshwater AIS**	**Sampling technique; filtration or precipitation procedure**	**DNA loci**	**eDNA amplification/ sequencing method**	**Proportion of positive detections (%)**	**Reference**
Insects	Tiger mosquito, *Aedes albopictus* Asian bush mosquito, *Aedes japonicus japonicas;* *Aedes koreicus*	Collection of 3x 15 ml; Ethanol precipitation (EP) (15mL of water + 1.5 mL of sodium acetate 3 M and 33 ml absolute ethanol) Precipitation of DNA by centrifuge (5500*g*, 35 min, 6°C) (Ficetola et al., 2008)	Ribosomal internal transcribed spacer 1 (ITS 1) Cytochrome oxidase subunit I (COI)	Quantitative real-time PCR (qPCR) + DNA metabarcoding	100% cPCR; 80% DNA metabarcoding	Schneider et al. (2016)

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
Macrophytes	Brazilian waterweed, *Egeria densa*	EP –centrifuge by (20 min at 5350*g)* (Ficetola et al., 2008)	trnL– trnF	qPCR	Detected in all the ponds where it was observed.	Fujiwara et al. (2016)
Reptiles	Burmese python, *Python bivittatus*	EP –centrifuge by (20 min at 5350*g)* (Ficetola et al., 2008)	Cyt b gene	Conventional PCR (cPCR)	100% (detected in the 5 sites where it has been observed)	Piaggio et al. (2014)
Amphibians	American bullfrog, *Lithobates catesbeianus*	EP- (Ficetola et al., 2008)	Cyt b gene	cPCR	77.5% by eDNA, 14.3% by traditional methods (eDNA method indicated bullfrog occurrence in 38 out of 49 ponds.	Dejean et al. (2012)
	Chinese giant salamander, *Andrias davidianus*	One 4-L container of surface water sample was collected per site; Glass fibre filter (0.7 μm)	mt NADH-1	Real-time TaqMan® PCR	Detected in 9 over 37 sites.	

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	African clawed frog, *Xenopus laevis*	20 water samples of 40 ml per site; EP by Ficetola et al. (2008)	12s *rRNA*	qPCR	Mean: 83%	Secondi et al. (2016)
	American bullfrog, *Lithobates catesbeianus*	One 250mL water sample per tank; polycarbonate filters (1.2 μm)	12s *rRNA*	DNA metabarcoding	10/12 tanks	(Dejean et al., 2012)
Crustaceans	Red swamp crayfish, *Procambarus clarkii*	Twenty 40 ml water samples per pond; EP Ficetola et al. (2008)	COI	qPCR	eDNA 73%, trapping 65%	Tréguier et al. (2014)
	Signal crayfish, *Pacifastacus leniusculus* Rusty crayfish, *Orconectes rusticus*	Five to ten water samples of 250 ml per site; cellulose nitrate filters (1.2 μm)	COI	qPCR	Weak relationships between eDNA copy number for *P. leniusculus* and relative abundance as catch per unit effort (CPUE)	Larson et al. (2017)

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	Rusty crayfish, *Orconectes rusticus*	Ten 250mL surface water samples per site; cellulose nitrate or polycarbonate track-etch filters (1.2 μm)	COI	qPCR	Detection probability 95% at moderate-high abundance	Dougherty et al. (2016)
Mollusc	New Zealand mudsnails, *Potamopyrgus antipodarum*	Three 4L water samples per site; mixed cellulose ester membranes (0.45 μm)	COI	qPCR	Species detected in all 3 water samples from the first site and in 2 of 3 in the second site.	Goldberg et al. (2013a)
Fish	Bluegill sunfish, *Lepomis macrochirus*	1 L water sample from the surface of each pond; cellulose acetate filter (3.0 μm)	COI	qPCR	Species found in 19 over 70 ponds, with traditional methods only 8 over 70 ponds.	Takahara et al. (2013)
	Common carp, *Cyprinus carpio*	Six 2L water samples per site; glass fibre filters (1.2 μm)	12S rRNA	qPCR	No significant correlation between	Hinlo et al. (2017a)

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	Redfin perch, *Perca fluviatilis*				catch per unit effort (CPUE) and DNA	
	Oriental weatherloach, *Misgurnus anguillicaudatus*				Positive correlation between CPUE and DNA	
					Positive correlation between CPUE and DNA	
	Common carp, *Cyprinus carpio*	One 250mL water sample per tank; polycarbonate membrane filters (1.2 μm)	12s + 16s rRNA	DNA metabarcoding	NA	Evans et al. (2016)
	Eastern mosquitofish, *Gambusia holbrooki*				3/12 tanks	

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	Common carp, *Cyprinus carpio*	One 50 mL water sample per tank; polycarbonate filter (0.2 μm)	COI	Multiplex qPCR	NA	Eichmiller et al. (2016a)
	Silver carp, *Hypophthalmichthys molitrix*	2L water sample; glass fibre filter (1.5 μm)	mtDNA D-loop	cPCR	Consistent with the traditional surveys	Jerde et al. (2013)
	Bighead carp, *Hypophthalmichthys nobilis*					
	Common carp, *Cyprinus carpio*	36x 2L samples in three lakes; cellulose nitrate filter (0.45 μm)	CytB + 12S	eDNA metabarcoding	NA	Hänfling et al. (2016)

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	Rainbow trout, *Oncorhynchus mykiss* Minnow, *Phoxinus phoxinus* Brown trout, *Salmo trutta* Pike, *Esox Lucius*					
	Common carp, *Cyprinus carpio*	One 500 mL water sample per tank; glass fibre filter (0.7 μm)	mtDNA D-loop	qPCR	NA	Uchii and Minamoto (2016)

Taxon	Target freshwater AIS	Sampling technique; filtration or precipitation procedure	DNA loci	eDNA amplification/ sequencing method	Proportion of positive detections (%)	Reference
	Pike, *Esox lucius*	Ten 1L water samples; nitrocellulose mixed ester membrane (0.45–1.5 μm)	COI	qPCR	90% success rate	Dunker et al. (2016)
	Northern snakehead, *Channa argus*	211 water samples in 7 locations; glass microfiber filters (1.5 μm)	16S	ddPCR	NA	Simmons et al. (2015)
	Ruffe, *Gymnocephalus cernua*	2-L water samples from 24 locations; glass microfiber filters (1.5 μm)	COI	qPCR	Consistently higher success rate compared to conventional sampling	(Tucker et al., 2016)
	Round Goby, *Neogobius melanostomus*	500 mL water samples; glass microfiber filters (1.2 μm)	COI	eDNA metabarcoding	Out of 82 fish species - eDNA methods detected 86.2% and 72.0 % in two rivers.	(Balasingham et al., 2017b)

6.2.2 Current limitations

Freshwater ecosystems, lentic and lotic, provide excellent study area for defining the wide range of detection possibilities of eDNA techniques as well as the limitations. Small-scale freshwater lentic bodies provide an excellent opportunity to study eDNA characteristics related to degradation, which can affect successful detectability of species. Recent studies have tried to underline degradation rates in correlation to abiotic factors, such as, (i) most effective water stratum for eDNA detection (Moyer et al., 2014), (ii) pH, UV-B (Strickler et al., 2015), (iii) effects of temperature on eDNA degradation (Eichmiller et al., 2016a, Strickler et al., 2015) and (iv) temporal effects (Dejean et al., 2011). Freshwater lotic bodies can provide important information due to their longitudinal downstream dynamics, such as, (i) eDNA persistence in the environment (Wilcox et al., 2016, Jerde et al., 2016), (ii) residence time of eDNA (Jerde et al., 2016) and (iii) the ecology of eDNA (Barnes and Turner, 2016). In case of newly introduced AIS, measures of low abundances present another limitation (Jerde et al., 2011b) which is highly important when discerning between presence and absence records. Some of the reported examples are applied to non-invasive species, but the reason why I focus on AIS is that time, i.e. rapid response, is key to management, so that an identified AIS can be eradicated/ controlled before any negative ecosystem impact occurs. Since eDNA can assist in more rapid detection and early response to AIS invasions than traditional sampling, this technology most greatly benefits identification of invasive species.

All the limitations of eDNA that are currently being studied are crucial for AIS assessment. When monitoring, especially in a new environment, it is fundamental to detect it at extremely low abundances and report negative or positive presence. False positives and negatives are essentially relevant for their use within SDM and cannot be misjudged, whether they are products of sampling bias or metabarcoding bioinformatics pipeline. The distribution patterns and biology of the eDNA is another important factor influencing the accuracy of information which is relevant for the distribution of IAS within the models. The accuracy that we can obtain through eDNA highly depends on the strategies followed during the fieldwork and through laboratory protocols. In order to more accurately state the proportion of the positive (or negative) detections, independent observations (Steel et al., 2013) would need to become an essential part of eDNA studies to overcome the bias of false positives or negatives. An

increased eDNA sampling effort based on a temporary scale would provide a more accurate proportion of positive (negative) detections and should be replaced by research proposed on a single sampling events (Simmons et al., 2015, Hänfling et al., 2016, Fujiwara et al., 2016). Independent observations would need to become a necessary procedure especially when dealing with estimations of newly introduced species (Jerde et al., 2011b) or dealing with the estimations of successful eradication measures (Dunker et al., 2016).

To avoid bias due to inconsistent use of eDNA tools a minimum information based on field and laboratory procedures should always be reported and presented in a consistent manner as presented by (Goldberg et al., 2016). Pioneers in eDNA research (Ficetola et al., 2016) highly recommend following general requirements such as, precautionary approach to avoid contamination, respecting a general practice of obtaining control samples, extraction blanks, as well as incorporating PCR positive and negative controls. In cases of individual species assessment, parallel mesocosm experiments are highly recommended in order to be able to estimate the limitations of detectability for each individual primer set. Another method to assess limitations of primer detections is assessing detectability of the species 'in time' after its removal from the controlled environment. When working on multiple species assessment using a metabarcoding approach, it is recommended, to sequence the control samples, compare the sequencing control outputs with the actual samples, and if none of the last achieve high quality sequence reads by appropriate clean up steps; removal of singletons, chimeras, as well as including a record of removed sequences (Deiner et al., 2017b). Bias due to universal primer preferential amplifications of species can alter the relative abundance of individual species eDNA (Deiner et al., 2017b). A PCR-free method, namely sequence capturing offers promising solutions in order to avoid amplification bias (Shokralla et al., 2016).

In terms of AIS certainty of existence in a non-native environment, false- positive and false- negative are crucial points for management and environmental policies (Moyer et al., 2014, Lahoz-Monfort et al., 2016). Even low rate false- positives pose a bias towards species specific occupancy (Lahoz-Monfort et al., 2016). Errors produced during PCR and sequencing are main source of bias for false- positives whereas false-negatives normally appear due to bias during sampling. Sampling and PCR replicates are key to avoid obtaining false presence and absence and should be routinely

corrected with the appropriate statistical tools referred to 'site occupancy-detection modelling' (SODM) (Lahoz-Monfort et al., 2016). The SODM model shows precise estimation of the probability for the site occupancy, including overall probability of detection at sites where the species is present. The model provides unbiased estimation of occupancy when properly applied using large amount of initial data, even with a smaller number of replications. Researchers (Ficetola et al., 2016) adopting SODM as part of their eDNA pipeline, give advice to avoid referring to single occurrences within one sample as reliable ones. Precautionary measures should be taken up before coming to conclusions that non- detection of species corresponds to species absence, and in converse that detections directly relies to species presence (Roussel et al., 2015) simply due to eDNA characteristics, such as potential longevity. In order to overcome the frontiers of eDNA techniques and to make it generally applicable within the SDM the above consistency is pivotal within the immense growing body of eDNA literature.

6.3 Combination of eDNA and SDMs

The method appears to be highly efficient on bony fish and amphibians with successful spatial representativeness in lotic and lentic systems (Civade et al., 2016). It has been shown that the eDNA samples are able to overcome spatial autocorrelation biases (Deiner et al., 2016b) which are normally a result of conventional biodiversity assessments. eDNA seasonal diversity at the ecosystem scales (Bista et al., 2017a) are key for more holistic understanding of the successful invasions of species within SDMs.

There are many possibilities of using eDNA for SDMs but currently one of the most important novel uses is a more precise sampling of absences which is sometimes difficult or impossible to obtain (Nezer et al. 2016). As commented, the information regarding species existence in certain system measured through eDNA can be susceptible to certain bias, due to eDNA characteristics. However, there exist approaches within the spatial modelling that might be applied to deal with the uncertainties from eDNA results. For instance, Dudík et al. (2006) presented the di-bias approach, which gives a higher weight in the models to those localities where presences or absences are more reliable. In the same way, those localities where eDNA is less reliable can receive a lower weight in the models, such weighting might correspond with the reported detection rates (Table 6. 1). Therefore, there are

possibilities from the SDMs to deal with the potential bias arising from using eDNA as a sampling technique which encourage its use despite current relative limitations. The ability to cope with the limitations and strength of the combination of these distinct research fields will benefit from the collaboration between molecular ecologists and modellers contributing to the evolution of two scientific disciplines (Coccia and Wang, 2016). Other disciplines apart from invasion ecology (e.g., biogeography or spatial ecology) might also benefit from future development of molecular ecology tools as a sampling technique. Thus, I highly recommend involving eDNA analysis into spatial models to predict future invasions and many other ecological processes. Spatial representativeness of invasive alien species within the SDMs is key to understanding the ecology behind their successful dispersal and the management of invasions.

CHAPTER 7- General discussion

Globally, this work has assessed some of the uses and limitations of eDNA detection as a tool to analyse spatial and seasonal dispersion of aquatic species, as well as identifying distribution discontinuities and invasive species hotspots. The individual chapters contribute with novel eDNA collection methods for sampling different types of freshwater bodies, and show their application to assess the dispersal limitations of fish in lotic environments and for the early detection and distribution analyses of AIS. This thesis proves that spatial distribution of species and long term monitoring can be successfully assessed by analysing eDNA using PCR, qPCR and metabarcoding.

Here, I have shown the wide applicability of the information obtained from eDNA, only recently recognised (Civade et al., 2016, O'Donnell et al., 2017, Stoeckle et al., 2017), such as seasonal distribution, for example, to identify spatial patterns of seaweed distribution.

7.1 eDNA presence/ absence assessment

About a decade ago, eDNA detection methods were introduced (Ficetola et al., 2008), transforming aquatic species detection, majorly contributing towards the spatial identification of rare, endangered and recently introduced nonindigenous potentially invasive species (Jerde et al., 2011b). Defining presence and also absence of particular aquatic species is important for biodiversity assessment, for protecting refugia of critically endangered species and for early detection, monitoring and containment of AIS, including the evaluation of eradication attempts (Jetz et al., 2012, Simmons et al., 2015, Hayes et al., 2005). I have optimised and employed methods for aquatic species presence/ absence identification using eDNA barcoding and metabarcoding

approaches. Applying eDNA metabarcoding for presence/ absence assessment in lotic communities contributes towards understanding of fish dispersal limitations in fragmented rivers and the effects of barrier removal (Bracken et al., 2018, Strobel et al., 2017, Cowart et al., 2018, Yamanaka and Minamoto, 2016).

It is important to understand that lack of species detection using eDNA does not necessarily indicate physical absence, due to the strong relatedness with sampling effort. eDNA metabarcoding definition of presence, requires caution in particular at interpreting rare sequence reads and their corresponding species assignations, potentially caused by amplification and sequencing errors (Goldberg et al., 2016) produced with high- throughput sequencing, including potential lack of detection due to primer preferences (Ji et al., 2013). To avoid misinterpretation of presence/ absence as a result of sequencing errors, I used three technical PCR replicates of each of the sampling triplicates, an estimated sufficient number of replicates used even for highly degraded ancient DNA (Ficetola et al., 2015a), including conventional control over targeted species probe coverage using *in silico* PCR test (Ficetola et al., 2010). Still, the interpretation of the results based on fish species with low relative abundance must be interpreted with caution.

From my studies, a general advice would be towards the usage of species specific assessment tools for estimations of presence/ absence of rare and recently introduced invasive species, due to its higher accuracy (Simmons et al., 2015), avoiding eDNA metabarcoding as a single tool for this type of assessment. When AIS or rare species of particular interest are found by metabarcoding, a control applying species specific probes would be needed for the final confirmation of their presence and further density related estimates. Using occupancy models (Hunter et al., 2015) for the prediction of detection probabilities may be useful when applying presence/ absence eDNA estimations.

7.2 eDNA density, relative abundance assessment

It has been recently suggested that eDNA might accurately represent density of species, based on an eDNA density- actual species density linear relation (Pilliod et al., 2014, Lacoursière-Roussel et al., 2016b, Dyer and Roderique, 2017). Here, I detected a linear correlation between *C. tomentosum* seaweed biomass and eDNA detection rate, and also defined upper detection limits (usually an underestimated

parameter) (Hunter et al., 2015). The quantitative PCR (qPCR) method proved to be sensitive for the estimation of the relationship between eDNA detection rates and organisms density, representing an ideal approximation towards intraspecific comparability, potentially closer to what ddPCR can offer (Doi et al., 2015).

eDNA metabarcoding has proved highly beneficial in fish community assessment using relative sequence read abundance as a measure for comparison, as sequence read counts can vary considerably among species (Porazinska et al., 2010). I have excluded inclusion of mock communities as a control measure for eDNA metabarcoding, representing a control approximation measure of density- sequence read dependence and also, *in vitro* primer control for species present in mock community. Combined with the use of mock communities for the comparative analysis, it is highly valuable to quantify probe efficiency and species specificity (Elbrecht and Leese, 2015), with its use recommend within each NGS run. My study confirms the utility of eDNA metabarcoding information based on relative fish species abundance for spatial comparative analyses. Thus, species- specific eDNA barcoding approach using qPCR and eDNA metabarcoding can be used for active and passive surveillance, respectively (Simmons et al., 2015), as confirmed by my results.

Conclusive remarks comparing species- specific and community-based approach are that eDNA barcoding qPCR approach is more appropriate when interested in density assessment, whereas eDNA metabarcoding is more relevant for non- target approach, general screening of biodiversity, evaluating patterns of targeted community. The difference between the targeted and community based approach, the so called active and passive surveillance (Simmons et al., 2015), each defines priorities including limitations thus, I highly recommend initially well planned experiments based on type of interest, to end up with the most reasonable, cost and time efficient research or surveillance. It is also important to keep in mind that a number of parameters needs to be accounted for when using eDNA density as an approximation of species densities, due to multiple environmental, ecological and behavioural parameters impacting eDNA detection rate, as for instance the reproductive cycle, where egg- bearing female

resulted in higher eDNA detection compared to males (Dunn et al., 2017), despite the same densities of both.

7.3 eDNA limitations and thresholds

The comparison between the three types of freshwater bodies highlights the importance of procedure standardisation, as it revealed important differences among eDNA capture procedures resulting in very different yields. With the increase in research related to eDNA, the importance of rigorous sampling methods and reporting guidelines has been highlighted for the quality control and comparability of results (Goldberg et al., 2016). However, the eDNA research field is still young and developing. Standardisation is key for its implementation from a policy and management perspective, and from a more theoretical point of view, defining eDNA detection thresholds and capabilities is crucial to fully understand its dynamics. For instance, there is a disproportionate eDNA information on vertebrates (Port et al., 2016, Maruyama et al., 2014, Kelly et al., 2014a), while data on plants and algae in aquatic environment is scarce and very recent (Gantz et al., 2018, Kuzmina et al., 2018, Alsos et al., 2018).

PCR inhibition can be another limitation for species detection, thus positive controls containing tissue of targeted species are a necessity to control for this type of inhibition, which can be resolved by a number of approaches such as applying inhibitor removal kit or sample dilution, which can result in no detection due to over diluting the sample (Goldberg et al., 2016) or, as here, by adding bovine serum album to PCR mix (Wilson, 1997).

7.4 eDNA as a dispersal assessment tool

Inferring the spatial distribution of aquatic species is a difficult task, with eDNA becoming a reliable source of information about dispersal, as presented throughout this thesis, but still needing further research for assessing thresholds and limitations of eDNA detection. Understanding eDNA degradation in time and space is needed to improve species dispersion predictions. Here, comparing an artificially modified river with a mostly pristine one, revealed the importance of temporal sampling and to account for differences in river flow for better assessment of discontinuities in river when targeting eDNA. Defining the limits of detection of species specific eDNA as a

result of actual species presence at certain location, was the most important limitation of my thesis, as this represent a crucial factor for spatial distribution assessment. For instance, in rivers an important challenge for estimation of the actual species density from eDNA densities, is that higher eDNA density at specific location does not necessarily mean actual higher species density at that location, as eDNA could be transported from upstream localities (Deiner et al., 2017a). I found this particularly limiting when assessing fish species with low relative abundance. Also, as reflected by the seaweed analyses, using abiotic factors, such as currents, can be used as an additional source of information combined with eDNA for predicting the spread of AIS in the marine environment, particularly for species with a juvenile and/ or adult pelagic life form (O'Donnell et al., 2017).

Comparing species relative abundance on seasonal and temporal scales presents another limitation, as eDNA may vary with different water temperature, light and ultraviolet radiation (UV) (Klymus et al., 2015, Pilliod et al., 2014). Most important, about the eDNA limitations is that eDNA is composed of molecules which can potentially reflect differences in specimens and species shedding rates, environments and seasons (Klymus et al., 2015, Sassoubre et al., 2016, Turner et al., 2015). Thus, defining eDNA thresholds, both for eDNA detection limits and provision of information, is currently one of the closest approximations to actual density estimates, which I have applied as a dispersion assessment tool.

7.5 eDNA in Species Distribution Models (SDMs)

Spatial distribution models (SDMs) and other ecological modelling approaches could potentially use eDNA information, such as in spatial distribution predictions of AIS, decision support systems in ports, river restorations or marine coastal zone management (Valentini et al., 2016, Lejzerowicz et al., 2015, Aylagas et al., 2016). This is because spatial distribution models rely on presence and absence of the species in the study area to infer the predictors affecting species distributions, for which absences are particularly difficult to obtain, and could be obtained more easily from rigorous eDNA analyses than with traditional surveillance (Goodwin et al., 2017, Bohmann et al., 2014). Thus, eDNA can contribute towards improvements of AIS distribution models (Muha et al., 2017), to avoid biases derived from the identification of species presence and absence.

7.6 Management and policy implementation guidelines

Detection of eDNA can majorly contribute towards advancing conservation actions, prioritising preferential grounds for management actions, such as early detection, mitigation, restoration, protection and eradication (Jerde et al., 2011b, Rees et al., 2014, Jerde et al., 2013), by assessing aquatic species presence and distribution. In order to complement eDNA studies with the conventional surveys (Deiner et al., 2017a), it is crucial to standardise its handling approach to become useful for long term monitoring, comparison between years and localities, applying same protocol and primers of choice. First chapter, the three water bodies comparison highlights the importance of procedure standardisation, as it has showed differences between eDNA capture procedures, reflecting changes in eDNA capture yield. With the accelerating number of research related to eDNA, a well prepared sampling plan based on minimum reporting guidelines (Goldberg et al., 2016) needs to be accounted for quality control, easier interpretation and comparability of results.

However, eDNA research field is young and it is still developing. Standardisation is key from policy and management perspective for actual implementation, but from the research point of view, defining eDNA detection threshold capabilities is crucial to fully understand its dynamics. For instance, there is a disproportionate eDNA information coverage with vertebrates being the most represented (Port et al., 2016, Maruyama et al., 2014, Kelly et al., 2014a), but still lacking on plants and algae in aquatic environment, with only recent applications (Gantz et al., 2018, Kuzmina et al., 2018, Alsos et al., 2018). The most beneficial part of this PhD in relation to standardisation of procedures is the outcome of three water bodies studies, emphasising no difference between water bodies and target species, and also providing evidence of correlation between seaweed biomass and eDNA density.

Despite eDNA usefulness, its use in management remains restricted (Barnes and Turner, 2016). Using eDNA as a tool to assess aquatic species distribution, with its high sensitivity and relatively low cost, is bridging the gap between research and management, benefiting public agencies by reduction of public funds designated for surveillance. Environmental laws are the result of policy- driven data obtained through surveillance (Kelly et al., 2014b), which require substantial efforts, continuously adopting improved practices, updated lately in policies. By providing standardised

eDNA protocols, the environmental agencies would build a trust towards eDNA techniques, increasing eDNA usage for national environmental surveillance. This thesis reveals the utility of targeting eDNA for spatial and seasonal dispersal assessment of aquatic species, suggesting future applications for eDNA. The future of eDNA full potential is yet to be discovered.

Concluding remarks

- By assessing the efficiency of different eDNA capture techniques, I found no major differences on eDNA capture yield or amplification rates between filtering or extraction methods among freshwater bodies, but increasing water volume had a significantly positive effect on eDNA capture and amplification efficiency.

- Although highest eDNA capture rates were obtained using 2 L of filtered water, using 100 mL syringe filtration in combination with ethanol precipitation proved to be more practical and increased quantitative PCR amplification efficiency by 6.4 %.

- The removal of a small weir had direct effect on fish diversity and species specific total number of sequence reads, as species specific eDNA of all nine species, was detected before the removal at both, upstream and downstream sampling locations, but total number of sequence reads of both, migratory and non- migratory species did increase in time suggesting improved fish pathways at the following monitored river corridor. This research also suggests that a pre-screening of the negative impacts of obstacles should be beneficial for targeting removals.
- The migratory and non- migratory fish species abundance increased with time, suggesting that eDNA is a sensitive method to study the temporal variation of fish species.
- Migratory salmonids were able to overcome thirteen assessed barriers during their upstream migration in three different rivers, in contrast to non-leaping species, for which some of the obstacles were limiting.

- The Shannon- Wiener diversity index indicated a clear effect of individual barriers and their corresponding tributaries in fish community structuring when comparing a modified and a pristine river, with gradual upstream diversity decrease in the unmodified river Teifi, in contrast to the highly modified river Afan where fish diversity differed greatly between tributaries without a clear pattern along the river.
- eDNA metabarcoding proved to be a useful indicator for river restoration measures and for freshwater fish species spatial and temporal dispersal assessment in fragmented rivers.
- Seasonal differences in the presence of the invasive and two of the native *Codium spp*. seaweed species were found, using an eDNA barcoding approach, which has proved useful for early detection and monitoring of invasive seaweed.
- eDNA detection using barcoding and metabarcoding identified distribution patterns which coincided with the known distribution based on previous surveillance, accounting for equal number of identified species and concordant levels of species specific density proportions.
- Spatial representativeness of invasive alien species using Spatial Distribution models including eDNA retrieved data is highly recommended.

APPENDICES

Appendix 1- Supplementary figures and tables

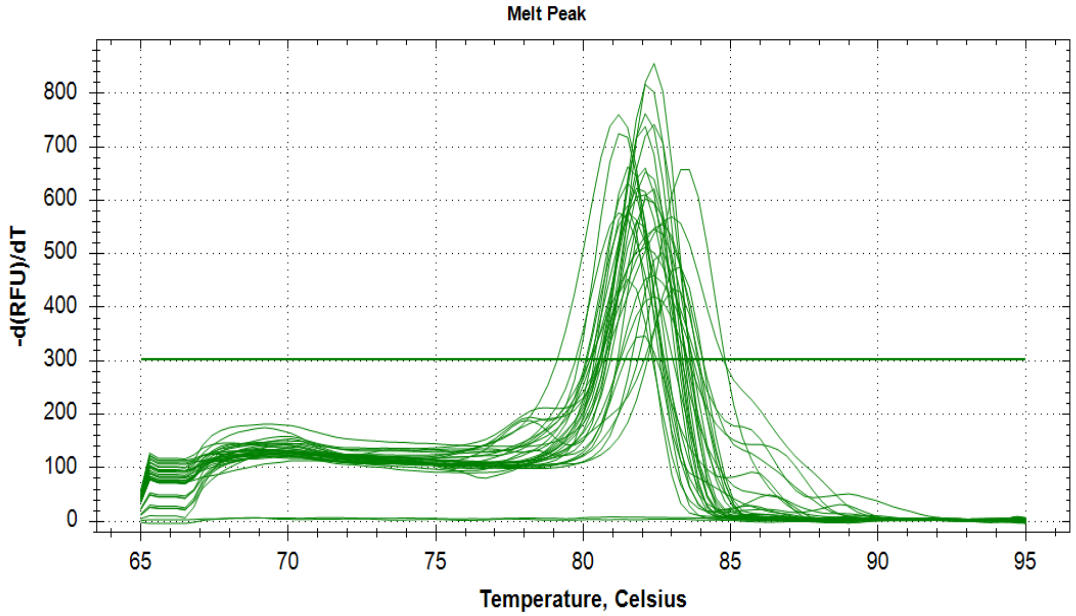


Figure S. 1- qPCR melt curve plots of all three experiments volume, filter type and extraction kit carrier out in Tawe river.

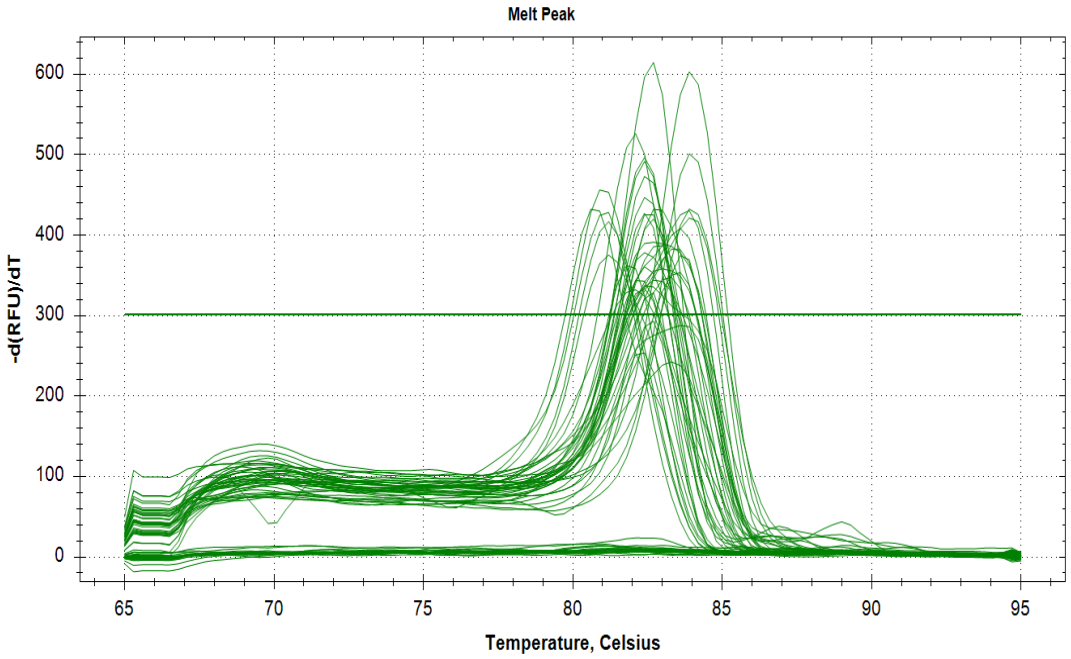


Figure S. 2- qPCR melt curve plots of all three experiments volume, filter type and extraction kit carrier out in Cardiff Bay.

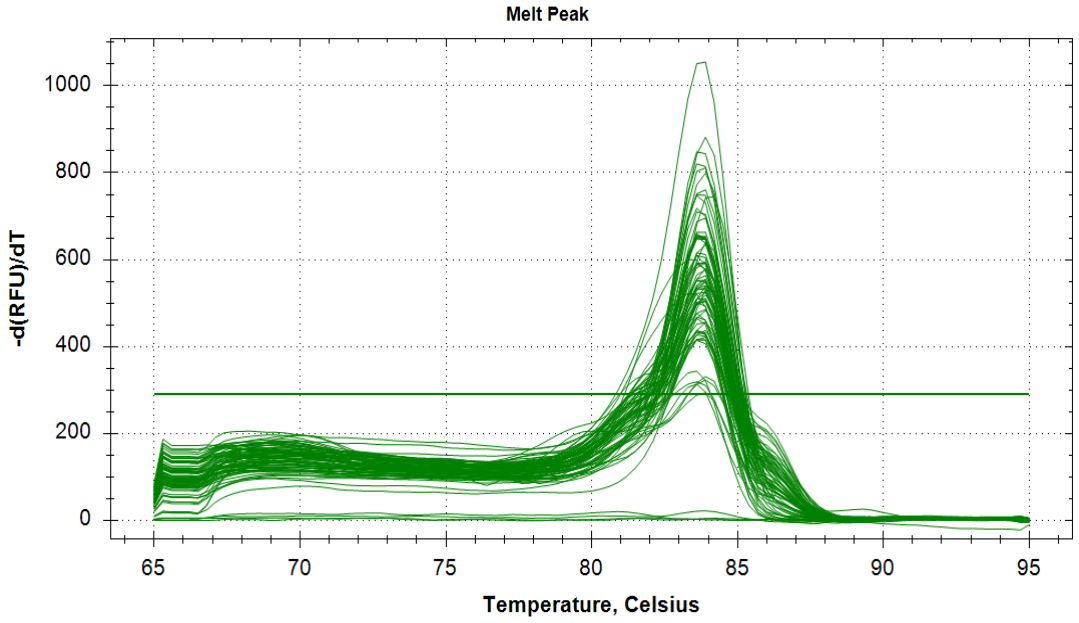


Figure S. 3- qPCR melt curve plots of all three experiments volume, filter type and extraction kit carrier out in Swansea University pond.

Table S. 1- Comparison of average water filtering efficiencies by eDNA capture and amplification efficiencies with reported mean and standard deviation for each individual response DNA capture yield (ng/ µL), PCR (ng/ µL) and qPCR (Cq), including gel electrophoresis confirmation.

Filtering volumes design						
Water body (n. of samples per water body)	**Number of samples**	**Categories tested**	**DNA capture yield (ng/ µL)**	**cPCR (ng/ µL)**	**qPCR (Cq)**	**Gel Electrophoresis (number of positive confirmations /total)**
Cardiff Bay (15)	45	15 mL	0.027 ± 0.009	12.738 ± 4.203	32.978 ± 1.896	9/9
		100 mL	0.044 ± 0.045	8.813 ± 3.383	34.194 ± 1.236	7/9
Pond (15)		250 mL	0.040 ± 0.019	8.156 ± 4.797	33.960 ± 1.983	7/9
Tawe (15)		1000 mL	0.087 ± 0.131	13.386 ± 1.793	33.683 ± 1.893	9/9
		2000 mL	0.406 ± 0.497	15.111 ± 2.473	31.242 ± 0.699	9/9

*The 15 mL volume within the volume experiment is based solely on ethanol- sodium acetate precipitation.

Water bodies (Cardiff Bay, Tawe river and Pond) including number of sampling replicates per water body (15), total number of samples (45) and categories tested (15, 100, 250, 1000 and 2000 mL) are stated. Only duplicates of positive qPCR amplifications were considered for statistical

analysis. The independent variables in volume experiments are Qiagen extraction kit and glass fibre filter. Number of positive confirmations in gel electrophoresis is included.

Table S. 2- Comparison of methods for eDNA capture and amplification efficiencies for filter type experiment with reported mean and standard deviation for each individual response DNA capture yield (ng/ µL), PCR (ng/ µL) and qPCR (Cq), including confirmation through gel electrophoresis.

Filter type						
Water body (n. of samples per water body)	**Number of samples**	**Categories tested**	**DNA capture yield (ng/µL)**	**cPCR (ng/µL)**	**qPCR (Cq)**	**Gel electrophoresis (number of positive conformations/ total)**
Cardiff Bay (9)	27	Cellulose nitrate	0.023 ±0.019	8.645 ± 1.207	35.626 ±2.341	8/9
Pond (9)		Glass fibre filter	0.022 ± 0.013	9.280 ± 3.293	34.115 ±1.157	9/9
Tawe (9)		Syringe filtration + precipitation	0.070 ±0.058	12.593 ± 3.455	33.253 ±1.925	9/9

Water bodies (Cardiff Bay, Tawe river and Pond) including number of sampling replicates per water body (9), total number of samples (27) and categories tested (cellulose nitrate, glass fibre filter and syringe filtration + ethanol – sodium acetate precipitation) are stated. The independent variables in filter type experiments are 100 mL of water filtered and Qiagen extraction kit. Number of positive confirmations in gel electrophoresis is included.

Table S. 3- Comparison of extraction kits for eDNA capture and amplification efficiencies for extraction kit experiment with reported mean and standard deviation for each individual response DNA capture yield (ng/ µL), PCR (ng/ µL) and qPCR (Cq), including gel electrophoresis confirmation.

Extraction kit						
Water body (n. of samples per water body)	**Number of samples**	**Categories tested**	**DNA capture yield (ng/µL)**	**cPCR (ng/µL)**	**qPCR (Cq)**	**Gel electrophoresis (number of positive conformations/ total)**
Cardiff Bay (12) **Pond (12)** **Tawe (12)**	36	Nexxtec Blood	0.284 ±0.232	10.080 ±1.603	33.929 ±2.045	8/9
		Nexxtec Bacteria	0.095 ±0.068	4.784 ±4.133	34.392 ±1.841	8/9
		Nexxtec Tissue	0.061 ±0.051	6.607 ±4.721	33.551 ±1.848	6/9
		Qiagen	0.039 ±0.018	8.156 ±4.797	33.949 ±1.975	7/9

Water bodies (Cardiff Bay, Tawe River and Pond) with the number of sampling replicates per water body (12), total number of samples (36) and categories tested (Nexxtec Blood, Nexxtec Bacteria, Nexxtec Tissue and Qiagen) are stated. The independent variables in extraction kit experiments are 250 mL of water filtered and Glass fibre filter. Number of positive confirmations in gel electrophoresis is included.

Table S. 4- Sanger sequence identification of species in each of the water bodies pond, lake and river defined by capture and extraction technique.

Species	**Experiment**	**Volume (mL)**	**Filter type**	**Pore size (µm)**	**Extraction kit**	**Technique**	**Water body**
Cyprinus carpio	Volume	2000	Glass fibre	0.6	Qiagen	Filtration	Lake
Cyprinus carpio	Filter type	100	Cellulose	0.45	Qiagen	Filtration	Lake
Cyprinus carpio	Extraction kit	250	Glass fibre	0.6	Nexxtec Blood	Filtration	Lake
Cyprinus carpio	Extraction kit	250	Glass fibre	0.6	Nexxtec Blood	Filtration	Lake
Cyprinus carpio	Volume	100	Glass fibre	0.6	Qiagen	Filtration	Lake
Homo sapiens	Volume	100	Glass fibre	0.6	Qiagen	Filtration	Lake
Homo sapiens	Volume	2000	Glass fibre	0.6	Qiagen	Filtration	Lake
Homo sapiens	Filter type	100	Cellulose	0.45	Qiagen	Filtration	Lake
Homo sapiens	Extraction kit	250	Glass fibre	0.6	Qiagen	Filtration	Lake
Homo sapiens	Extraction kit	250	Glass fibre	0.6	Qiagen	Filtration	Lake
Sus scrofa domesticus	Volume	100	Glass fibre	0.6	Qiagen	Filtration	Lake
Anas platyrhynchos	Extraction kit	250	Glass fibre	0.6	Nexxtec Tissue	Filtration	River

Species	**Experiment**	**Volume (mL)**	**Filter type**	**Pore size (µm)**	**Extraction kit**	**Technique**	**Water body**
Anas platyrhynchos	Volume	250	Glass fibre	0.6	Qiagen	Filtration	River
No identification	Volume	250	Glass fibre	0.6	Qiagen	Filtration	River
No identification	Extraction kit	250	Glass fibre	0.6	Nexxtec Tissue	Filtration	River
Cottus gobio	Extraction kit	250	Glass fibre	0.6	Nexxtec Tissue	Filtration	River
Cottus gobio	Filter type	100	Cellulose	0.45	Qiagen	Ethanol precipitation	River
Cottus gobio	Filter type	100	Cellulose	0.45	Qiagen	Ethanol precipitation	River
Cottus gobio	Volume	250	Glass fibre	0.6	Qiagen	Filtration	River
Cottus gobio	Volume	1000	Glass fibre	0.6	Qiagen	Filtration	River
Cottus gobio	Volume	1000	Glass fibre	0.6	Qiagen	Filtration	River
Cottus gobio	Filter type	100	Cellulose	0.45	Qiagen	Ethanol precipitation	River
Gasterosteus aculeatus	Volume	15			Qiagen	Ethanol precipitation	Pond
Gasterosteus aculeatus	Extraction kit	250	Glass fibre	0.6	Nexxtec Tissue	Filtration	Pond
Gasterosteus aculeatus	Volume	1000	Glass fibre	0.6	Qiagen	Filtration	Pond

Species	Experiment	Volume (mL)	Filter type	Pore size (µm)	Extraction kit	Technique	Water body
No identification	Volume	1000	Glass fibre	0.6	Qiagen	Filtration	Pond
No identification	Volume	15			Qiagen	Ethanol precipitation	Pond
No identification	Extraction kit	250	Glass fibre	0.6	Nexxtec Tissue	Filtration	Pond
No identification	Filter type	100	Cellulose	0.45	Qiagen	Ethanol precipitation	Pond

Table S. 5- Data for filtration volume experiment from Chapter 2.

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
A1 15	15	Cardiff Bay	Q	G	0.0268	11.8	31.8
A2 15	15	Cardiff Bay	Q	G	0.0396	12.4	32.62
A3 15	15	Cardiff Bay	Q	G	0.0344	13.9	31.57
A1 G	100	Cardiff Bay	Q	G	0	12.7	33.6
A2 G	100	Cardiff Bay	Q	G	0.09	7.64	33.59
A3 G	100	Cardiff Bay	Q	G	0.131	5.08	NA
A1 250	250	Cardiff Bay	Q	G	0.026	7	31.28
A2 250	250	Cardiff Bay	Q	G	0.068	4.96	32.33
A3 250	250	Cardiff Bay	Q	G	0.056	1.3	32.93
A1 1L	1000	Cardiff Bay	Q	G	0.03	11.3	NA
A2 1L	1000	Cardiff Bay	Q	G	0.0232	14.4	31.92
A3 1L	1000	Cardiff Bay	Q	G	0.0236	12.4	35.64
A1 2L	2000	Cardiff Bay	Q	G	0.0288	14.4	NA
A2 2L	2000	Cardiff Bay	Q	G	0.062	14.6	NA
A3 2L	2000	Cardiff Bay	Q	G	0.08	14.8	32.39
P1 15	15	Pond	Q	G	0.0224	12.6	33.84

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
P2 15	15	Pond	Q	G	0.02	9.48	29.47
P3 15	15	Pond	Q	G	0.03	7.96	33.85
P1 100	100	Pond	Q	G	0.024	6.52	35.05
P2 100	100	Pond	Q	G	0.02	10.9	35.54
P3 100	100	Pond	Q	G	0.022	13.1	34.3
P1 250	250	Pond	Q	G	0.032	16.3	32.43
P2 250	250	Pond	Q	G	0.0216	8.36	35.18
P3 250	250	Pond	Q	G	0	10.7	35.73
P1 1L	1000	Pond	Q	G	0.428	NA	31.4
P2 1L	1000	Pond	Q	G	0.024	NA	34.15
P3 1L	1000	Pond	Q	G	0.0504	NA	36.42
P1 2L	2000	Pond	Q	G	0.323	11.1	31.38
P2 2L	2000	Pond	Q	G	0.672	14.4	31.23
P3 2L	2000	Pond	Q	G	0.9	19.2	30.27
T1 15	15	Tawe	Q	G	0.024	22.9	33.59
T2 15	15	Tawe	Q	G	0.01	11.3	33.88
T3 15	15	Tawe	Q	G	0.036	12.3	36.18
T1 100	100	Tawe	Q	G	0.002	4.2	35.5

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
T2 100	100	Tawe	Q	G	0.02	11.9	32.78
T3 100	100	Tawe	Q	G	0.046	7.28	32.6
T1 250	250	Tawe	Q	G	0	8.44	34.68
T2 250	250	Tawe	Q	G	0.0232	3.04	33.53
T3 250	250	Tawe	Q	G	0.0516	13.3	37.55
T1 1L	1000	Tawe	Q	G	0.0964	11.2	32.41
T2 1L	1000	Tawe	Q	G	0.0744	15.4	NA
T3 1L	1000	Tawe	Q	G	0.0292	15.4	33.84
T1 2L	2000	Tawe	Q	G	0.0776	16.5	NA
T2 2L	2000	Tawe	Q	G	0.259	18	31.33
T3 2L	2000	Tawe	Q	G	0.572	13	30.85

Table S. 6- Filter type experiment data from Chapter 2.

Test Name	Volume	Water body	Extraction kit	Filter type	DNA yield	PCR	qPCR
A2 C	100	Cardiff_Bay	Q	Cellulose	0.026	6.88	32.16
A1 C	100	Cardiff_Bay	Q	Cellulose	0.038	8	33.6
A3 C	100	Cardiff_Bay	Q	Cellulose	0	NA	
A1 100	100	Cardiff_Bay	Q	Glass fibre	0.0267	12.7	33.6
A2 100	100	Cardiff_Bay	Q	Glass fibre	0.09	7.64	33.59
A3 100	100	Cardiff_Bay	Q	Glass fibre	0	12.7	35.3
A3 S	100	Cardiff_Bay	Q	Syringe filtration	0.0228	11	32.28

Test Name	Volume	Water body	Extraction kit	Filter type	DNA yield	PCR	qPCR
A1 S	100	Cardiff_Bay	Q	Syringe filtration	0.0228	15.2	32.66
A2 S	100	Cardiff_Bay	Q	Syringe filtration	0.14	17	31.86
P1 C	100	Pond	Q	Cellulose	0.001	8.8	35.19
P2 C	100	Pond	Q	Cellulose	0.002	8.04	39.8
P3 C	100	Pond	Q	Cellulose	0.0416	10.2	36.86
P2 100	100	Pond	Q	Glass fibre	0.02	10.9	35.54
P3 100	100	Pond	Q	Glass fibre	0.022	13.1	34.3
P1 100	100	Pond	Q	Glass fibre	0.024	6.52	35.05
P3 S	100	Pond	Q	Syringe filtration	0.0612	6.56	34.7
P1 S	100	Pond	Q	Syringe filtration	0.144	16.1	31.74
P2 S	100	Pond	Q	Syringe filtration	0.15	10.9	30.34
T1 C	100	Tawe	Q	Cellulose	0.004	10.4	36.26
T3 C	100	Tawe	Q	Cellulose	0.0224	9	36.73
T2 C	100	Tawe	Q	Cellulose	0.05	7.84	34.41
T1 100	100	Tawe	Q	Glass fibre	0.002	4.2	35.46
T2 100	100	Tawe	Q	Glass fibre	0.02	11.9	32.78
T3 100	100	Tawe	Q	Glass fibre	0.05	7.28	32.6
T3 S	100	Tawe	Q	Syringe filtration	0.0216	14.9	36.3
T2 S	100	Tawe	Q	Syringe filtration	0.0272	9.48	34.9
T1 S	100	Tawe	Q	Syringe filtration	0.0368	12.2	34.5

Table S. 7- Data for extraction kit experiment from Chapter 2.

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
A1 NG	250	Cardiff_Bay	Nexxtec Bacteria	G	0.211	0.724	NA
A2 NG	250	Cardiff_Bay	Nexxtec Bacteria	G	0.127	6.96	31.56
A3 NG	250	Cardiff_Bay	Nexxtec Bacteria	G	0.0908	0.58	NA

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
P1 NG	250	Pond	Nexxtec Bacteria	G	0.0312	3.92	NA
P2 NG	250	Pond	Nexxtec Bacteria	G	0.0484	0.516	35.48
P3 NG	250	Pond	Nexxtec Bacteria	G	0.0424	2.64	34.96
T1 NG	250	Tawe	Nexxtec Bacteria	G	0.168	10.8	3.27E+01
T2 NG	250	Tawe	Nexxtec Bacteria	G	0.038	6.12	35.35
T3 NG	250	Tawe	Nexxtec Bacteria	G	NA	10.8	36.3
A1 NB	250	Cardiff_Bay	Nexxtec Blood	G	0.42	8.12	38.41
A2 NB	250	Cardiff_Bay	Nexxtec Blood	G	0.343	11.3	34.44
A3 NB	250	Cardiff_Bay	Nexxtec Blood	G	0.772	7.84	31.83
P1 NB	250	Pond	Nexxtec Blood	G	0.128	10.5	32.77
P2 NB	250	Pond	Nexxtec Blood	G	0.122	11.2	32.68
P3 NB	250	Pond	Nexxtec Blood	G	0.436	10.6	34.34
T1 NB	250	Tawe	Nexxtec Blood	G	0.183	8.88	32.76
T2 NB	250	Tawe	Nexxtec Blood	G	0.0868	12.2	32.61
T3 NB	250	Tawe	Nexxtec Blood	G	0.0672	NA	35.52
A1 NT	250	Cardiff_Bay	Nexxtec Tissue	G	NA	NA	NA
A2 NT	250	Cardiff_Bay	Nexxtec Tissue	G	0.166	0.704	32.1
A3 NT	250	Cardiff_Bay	Nexxtec Tissue	G	0.0788	1.53	31.58
P1 NT	250	Pond	Nexxtec Tissue	G	0.0488	15.5	33.03
P2 NT	250	Pond	Nexxtec Tissue	G	0.0448	6.8	32.72
P3 NT	250	Pond	Nexxtec Tissue	G	0.0924	7.04	31.99
T1 NT	250	Tawe	Nexxtec Tissue	G	0.0132	9.72	3.48E+01
T2 NT	250	Tawe	Nexxtec Tissue	G	0.0218	7.36	36.19

Test Name	Volume	Water body	Extraction kit	Filter type	eDNA yield	PCR	qPCR
T3 NT	250	Tawe	Nexxtec Tissue	G	0.0208	4.2	36
A1 250	250	Cardiff_Bay	Qiagen	G	0.026	7	31.28
A2 250	250	Cardiff_Bay	Qiagen	G	0.068	4.96	32.33
A3 250	250	Cardiff_Bay	Qiagen	G	0.056	1.3	32.93
P1 250	250	Pond	Qiagen	G	0.032	16.3	32.43
P2 250	250	Pond	Qiagen	G	0.0216	8.36	35.18
P3 250	250	Pond	Qiagen	G	NA	10.7	35.73
T1 250	250	Tawe	Qiagen	G	NA	8.44	34.68
T2 250	250	Tawe	Qiagen	G	0.0232	3.04	33.53
T3 250	250	Tawe	Qiagen	G	0.0516	13.3	37.55

Table S. 8- *In silico* 12S-V5 primer specificity test evaluating 37 species known to be present in Welsh rivers using ecoPCR allowing 0 mismatches.

Rank	**Number of identified taxon**	**Total number of taxon**	**Percentage of identification**
subspecies	0	8	0
family	9	9	100
phylum	1	1	100
subfamily	3	5	60
infraclass	1	1	100
subphylum	1	1	100
species	21	37	56.76
genus	18	23	78.26
superkingdom	1	1	100
superclass	1	1	100
class	1	1	100
kingdom	1	1	100
superfamily	1	1	100
infraorder	2	2	100
superorder	1	1	100
order	7	7	100

suborder	3	3	100
subclass	1	1	100

Table S. 9- A list of species found in Lugg river based on electrofishing in 2013 (density per 100 m^2) compared to list of species found by applying eDNA metabarcoding, listed from the most abundant species to the least.

Species name	**Similarity**	**Abundance estimate from the most to the least abundant one by eDNA metabarcoding**	**Abundance estimate from the most to the least abundant one by fry survey in 2015 and electrofishing in 2013**
Phoxinus phoxinus	☑	1	1
Cottus gobio	☑	2	2
Salmo trutta	☑	3	4
Salmo salar	☑	4	5
Barbatula barbatula	☑	5	6
Gasterosteus aculeatus	☑	6	9
Thymallus thymallus	☑	7	3
Anguilla anguilla		8	
Lampetra spp.		9	
Leuciscus leuciscus			7
Squalius cephalus			8

Table S. 10- Sampling locations, obstacles, below, above the barriers and total number of sequence reads for each of the represented species in Teifi river, Chapter 3.

Sample	**Obstacle**	**Below. above**	***Anguilla anguilla***	***Cottus gobio***	***Phoxinus phoxinus***	***Gasterosteus aculeatus***	***Barbatula barbatula***	***Oncorhynchus mykiss***	***Salmo salar***	***Salmo trutta***	***Thymallus thymallus***	***Lampetra spp.***
A1 DT	A	Down	0	1390	2	0	2	20	446	1710	1	1
A2 DT	A	Down	0	298	2	0	1	20	1176	1647	1	0
A3 DT	A	Down	1	6	1	0	0	18	931	1579	0	0
A1 UT	A	Up	0	389	4	0	0	271	1477	1114	0	0
A2 UT	A	Up	0	247	256	0	0	15	581	316	0	0
A3 UT	A	Up	0	443	3	0	1	36	2249	2005	0	0
B1DT	B	Down	2	1102	417	0	0	415	2179	1532	2	0
B2DT	B	Down	0	2271	988	0	0	13	1328	687	0	1
B3DT	B	Down	151	2037	2109	0	1	57	1541	3322	1	0

B1UT	B	Up	2	3	2	0	0	50	1014	4260	2	0
B2UT	B	Up	0	1153	196	0	0	35	1365	1656	0	0
B3UT	B	Up	1	746	674	1	0	26	444	2361	1	0
C1DT	C	Down	2	798	2	0	1	52	839	1964	0	0
C2DT	C	Down	1	459	5	0	0	14	587	1082	0	0
C3DT	C	Down	0	7	5	0	1	294	1462	2168	0	0
C1UT	C	Up	210	557	4	0	0	441	356	3931	0	0
C2UT	C	Up	0	1655	1	1	0	58	409	539	2	0
C3UT	C	Up	0	976	4	1	1	25	3012	1962	0	0
D1DT	D	Down	1	1012	2749	0	4	56	875	2042	2	0
D2DT	D	Down	2	1106	318	1	0	42	397	2893	1	2

D3DT	D	Down	1	782	1306	2	1	26	874	2310	0	1
D1UT	D	Up	0	2500	856	0	0	31	2286	961	1	0
D2UT	D	Up	244	2141	2499	1	3	247	1053	3175	1	1
D3UT	D	Up	2	857	594	0	0	34	1357	3073	0	0
ED1T	E	Down	2	1170	182	0	0	48	2689	5159	0	0
ED2T	E	Down	212	1313	6	15	0	21	277	1747	0	0
ED3T	E	Down	341	1345	6	1	0	23	1018	1644	0	4
E1UT	E	Up	1	5399	6197	0	2	119	1242	1644	1	0
E2UT	E	Up	1	12	1	0	0	56	1715	2379	0	0
E3UT	E	Up	1	754	0	0	0	33	532	1697	0	0
F1DT	F	Down	655	1460	1836	0	0	18	1943	645	0	0

F2DT	F	Down	1020	2767	966	0	0	16	529	2039	0	4
F3DT	F	Down	719	3096	760	1	0	28	431	2561	0	20
F1UT	F	Up	911	1052	276	0	0	25	788	502	1	0
F2UT	F	Up	174	831	66	0	0	9	118	1151	1	0
F3UT	F	Up	121	1358	105	1	0	35	661	2934	0	5

Table S. 11- Sampling locations, obstacles, below, above the barriers and total number of sequence reads for each of the represented species in Afan river, Chapter 3.

Sample	**Obstacle**	**Below/ above**	***Salmo trutta***	***Salmo salar***	***Cottus gobio***	***Phoxinus phoxinus***	***Anguilla anguilla***	***Barbatula barbatula***	***Thymallus thymallus***	***Gasterosteus aculeatus***	***Oncorhynchus mykiss***
A1DA	A	Down	4563	7316	16	8	1	1	1	1	92
A2DA	A	Down	1995	5207	2	3	108	0	0	0	27
A3DA	A	Down	1773	540	101	494	0	2	0	0	18
A1UA	A	Up	1897	781	8	2	1	0	2	0	59
A2UA	A	Up	958	2065	2	2	0	0	1	0	13
A3UA	A	Up	2317	220	162	335	153	0	0	0	31
B1DA	B	Down	1811	1729	915	907	1	1	1	0	834
B2DA	B	Down	4300	9000	131	310	39	1	5	0	212

B3DA	B	Down	4734	8195	7625	8126	4	1	1	3	92
B1UA	B	Up	2120	2580	1338	690	567	0	0	0	52
B2UA	B	Up	2919	1289	514	140	0	0	0	0	51
B3UA	B	Up	2675	3458	936	1391	1	0	2	0	68
C1DA	C	Down	4404	960	11	1	0	0	1	0	42
C2DA	C	Down	3631	621	558	3	0	1	0	0	55
C3DA	C	Down	2181	961	724	837	0	1	0	1	29
C1UA	C	Up	3797	1059	1187	457	1	40	1	0	45
C2UA	C	Up	4525	1079	5	449	0	1	0	0	41
C3UA	C	Up	2599	3452	1499	339	1	0	0	0	463

D1DA	D	Down	5643	62	3917	6	731	4	1	0	53
D2DA	D	Down	1991	2176	2273	1087	2	0	1	0	40
D3DA	D	Down	2947	2535	4778	6097	0	5	0	1	264
D1UA	D	Up	812	3071	1104	1317	2	3	0	1	66
D2UA	D	Up	2005	1221	714	840	0	0	0	0	36
D3UA	D	Up	1006	1478	1755	1578	0	0	0	1	40
ED1A	E	Down	3423	3115	1261	5	1	2	1	0	54
ED2A	E	Down	2032	927	738	6	0	4	1	0	55
ED3A	E	Down	1793	928	130	232	0	0	0	0	20
E1UA	E	Up	2350	938	304	3	0	1	0	0	274

E2UA	E	Up	3227	32	226	0	286	2	2	0	63
E3UA	E	Up	1688	1271	183	367	3	0	0	1	37
F1DA	F	Down	1184	881	413	1608	1	1	0	1	206
F2DA	F	Down	2144	1206	1027	794	1	0	0	0	33
F3DA	F	Down	3749	1474	4623	6711	2	0	1	2	454
F1UA	F	Up	4078	4805	543	4	710	0	1	1	49
F2UA	F	Up	1974	554	484	187	202	0	1	0	34
F3UA	F	Up	3069	714	689	661	0	0	1	1	51

Table S. 12- Lugg river data for Chapter 4 representing sampling points, time of sampling activities, upstream and downstream removed weir locations, distance (m) and total number of sequence reads recovered for each individual species.

Sampling point	**Time**	**up down**	**Distance**	***S. salar***	***S. trutta***	***P. phoxinus***	***C. gobio***	***A. anguilla***	***B. barbatula***	***G. aculeatus***	***T. thymallus***	***Lampetra***
Down-3	Pre-dam	Downstream	-479	14	20	2061	1146	0	0	0	0	0
Down-3	Pre-dam	Downstream	-479	62	4454	1136	1565	0	0	0	0	0
Down-3	Pre-dam	Downstream	-479	7	388	2707	1148	284	0	0	0	0
Down-2	Pre-dam	Downstream	-180	515	986	7862	6319	0	1	3	1	20
Down-2	Pre-dam	Downstream	-180	756	2172	4990	4583	0	0	0	2	0
Down-2	Pre-dam	Downstream	-180	1	0	5	1408	0	0	1	0	6
Down-1	Pre-dam	Downstream	-67	8	257	1263	2943	0	27	0	0	0
Down-1	Pre-dam	Downstream	-67	616	10	4445	1075	0	0	0	0	7
Down-1	Pre-dam	Downstream	-67	648	13	5714	0	0	0	0	0	1
Up-1	Pre-dam	Upstream	8	17	654	6148	2617	0	1	0	0	0
Up-1	Pre-dam	Upstream	8	365	781	2046	1065	0	1	1	0	0
Up-1	Pre-dam	Upstream	8	362	2203	3868	2878	249	0	1	0	0
Up-2	Pre-dam	Upstream	78	2124	733	3484	2037	0	1	2	0	9
Up-2	Pre-dam	Upstream	78	12	564	1570	2979	0	0	1	0	2
Up-2	Pre-dam	Upstream	78	8	802	12797	3341	0	2	1	0	0
Up-3	Pre-dam	Upstream	270	1	3	471	79	0	0	0	0	0
Up-3	Pre-dam	Upstream	270	546	555	4108	5142	0	0	18	0	0
Up-3	Pre-dam	Upstream	270	35	2714	2777	1311	0	0	2	0	10
Down-3	Year after	Downstream	-479	738	912	15033	7816	0	136	4	0	0
Down-3	Year after	Downstream	-479	1084	775	10772	3246	367	82	12	2	0
Down-3	Year after	Downstream	-479	270	902	9064	1936	2	25	0	1	0
Down-2	Year after	Downstream	-180	330	713	9348	4413	0	39	5	2	0

Down-2	Year after	Downstream	-180	136	1139	12942	5541	0	2	2	0	0
Down-2	Year after	Downstream	-180	9	963	11433	3923	0	2	1	2	0
Down-1	Year after	Downstream	-67	138	2362	19140	5476	0	5	9	0	0
Down-1	Year after	Downstream	-67	556	2225	12867	3613	0	182	1	2	5
Down-1	Year after	Downstream	-67	642	1345	14758	8267	0	72	29	2	12
Up-1	Year after	Upstream	8	10	642	9927	3176	0	36	1	0	1
Up-1	Year after	Upstream	8	638	583	9652	2240	244	212	13	1	3
Up-1	Year after	Upstream	8	331	1512	10583	4469	0	16	8	2	1
Up-2	Year after	Upstream	78	49	3721	13929	4501	0	1	11	2	0
Up-2	Year after	Upstream	78	850	1087	10057	4125	0	1	0	1	0
Up-2	Year after	Upstream	78	757	1388	13223	6132	1	44	3	2	0
Up-3	Year after	Upstream	270	624	873	11920	4721	0	105	3	0	0
Up-3	Year after	Upstream	270	11	166	11850	4348	339	24	4	1	0
Up-3	Year after	Upstream	270	15	337	8767	1255	190	1	29	1	0

Table S. 13- Information on barriers in Teifi river.

Barrier name	ID Name	Latitude	Longitude	Natural/ artificial	Type of barrier	Lower/ Upper river	Comments from field
TF 1	Nant Rhysgog (Brefi)	52.171929	-3.9374849	Natural	Waterfall	Upper	Small tributary close to main stream
TF 2	Nant Clywenog	52.141579	-4.0058	Natural	Waterfall	Upper	Main stream
TF 3	Nant Hust	52.044381	-4.1375789	Natural	Rock formation	Upper	Small tributary
TF 4	Siedi	52.015677	-4.3463653	Artificial	Successive number of weirs	Lower	Small tributary close to main stream
TF 5	Afon Ceri	52.056747	-4.4707626	Artificial	Weir made of rocks	Lower	Main stream
TF 6	Nant Arberth	52.062904	-4.6024129	Artificial	Weir	Lower	In close proximity to main stream

Table S. 14- Information on barriers in Afan River.

Barrier name	ID Name	Latitude	Longitude	Natural/ artificial	Type of barrier	Lower/ Upper river	Connection with Afan river
AF 1		51.6617	-3.71345	Artificial	Culvert	Upper	Larger tributary
AF 2	Afon Corrwg	51.68526	-3.61321	Artificial	Rock formations	Upper	Afon Corrwg tributary
AF 3	Cynonville	51.64104	-3.70925	Artificial	Weir	Lower	Small tributary close to main stream
AF 4	Blaengwynfi	51.65866	-3.60884	Artificial	Culvert	Upper	Main stream
AF 5	Abercregan	51.6548	-3.6669	Natural	Waterfall	Lower	Small tributary close to main stream
AF 6	Nant Cwn clais	51.61042	-3.77207	Artificial	Weir	Lower	Small tributary close to main stream

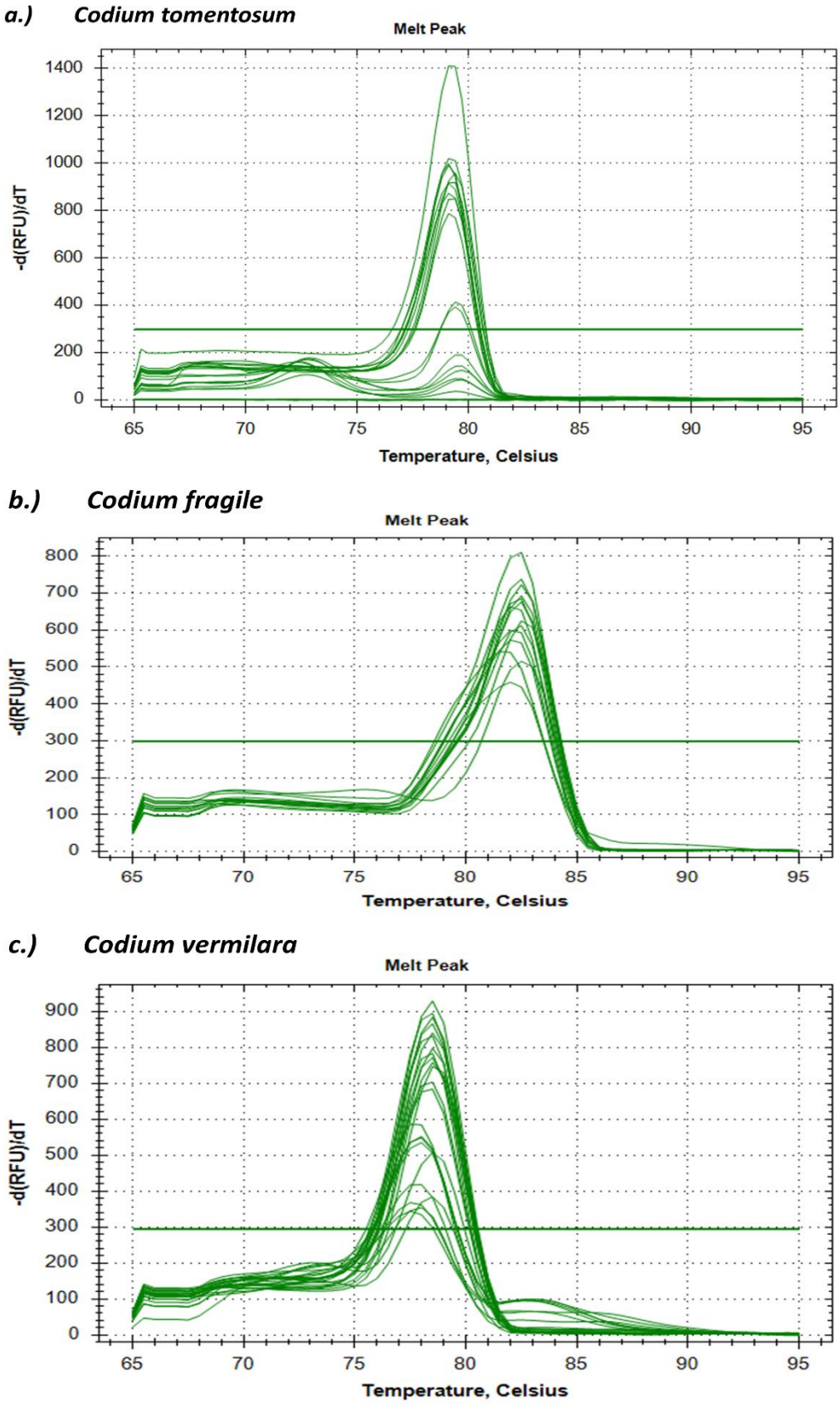


Figure S. 4- qPCR melt peak temperatures for all three species a.) *C. tomentosum*, b.) *C. fragile, c.) C. vermilara*).

Table S. 15- Data for *Codium spp*. seasonal and spatial distribution assessment, Chapter 5.

Sampling point	Season	Location	Species	eDNA/ uL
CA1	01-Jul	Concha de Artedo	*C. tomentosum*	1922416
CA2	02-Oct	Concha de Artedo	*C. tomentosum*	39684.63
CA2	02-Oct	Concha de Artedo	*C. tomentosum*	49692.95
CA2	02-Oct	Concha de Artedo	*C. tomentosum*	53239.39
CA3	03-Dec	Concha de Artedo	*C. tomentosum*	159446.1
CA3	03-Dec	Concha de Artedo	*C. tomentosum*	16379.3
CA3	03-Dec	Concha de Artedo	*C. tomentosum*	25073.77
CU1	01-Jul	Cudillero	*C. tomentosum*	1149953
CU1	01-Jul	Cudillero	*C. tomentosum*	305656
CU2	02-Oct	Cudillero	*C. tomentosum*	268938.1
CU2	02-Oct	Cudillero	*C. tomentosum*	260128.4
CU2	02-Oct	Cudillero	*C. tomentosum*	234794.5
CU3	03-Dec	Cudillero	*C. tomentosum*	1640433
CU3	03-Dec	Cudillero	*C. tomentosum*	226710.8
CP2	02-Oct	Cabo da Penas	*C. tomentosum*	202690
CP2	02-Oct	Cabo da Penas	*C. tomentosum*	2930.44
CP2	02-Oct	Cabo da Penas	*C. tomentosum*	256268.3
CP3	03-Dec	Cabo da Penas	*C. tomentosum*	214412.3
CP3	03-Dec	Cabo da Penas	*C. tomentosum*	1679858
G1	01-Jul	Gijon	*C. tomentosum*	
G2	02-Oct	Gijon	*C. tomentosum*	65908.17
G2	02-Oct	Gijon	*C. tomentosum*	47147.13
G2	02-Oct	Gijon	*C. tomentosum*	22336.43
G2	02-Oct	Gijon	*C. tomentosum*	800615
G3	03-Dec	Gijon	*C. tomentosum*	178942.2
CA1	01-Jul	Concha de Artedo	*C. vermilara*	
CA2	02-Oct	Concha de Artedo	*C. vermilara*	135.56
CA2	02-Oct	Concha de Artedo	*C. vermilara*	1567.83
CA2	02-Oct	Concha de Artedo	*C. vermilara*	110.34
CA3	03-Dec	Concha de Artedo	*C. vermilara*	388.11
CA3	03-Dec	Concha de Artedo	*C. vermilara*	6619.92
CA3	03-Dec	Concha de Artedo	*C. vermilara*	395.36
CU1	01-Jul	Cudillero	*C. vermilara*	2081.93
CU1	01-Jul	Cudillero	*C. vermilara*	8753.14
CU1	01-Jul	Cudillero	*C. vermilara*	4468.27
CU2	02-Oct	Cudillero	*C. vermilara*	665.64
CU2	02-Oct	Cudillero	*C. vermilara*	431.95
CU2	02-Oct	Cudillero	*C. vermilara*	755.7
CU3	03-Dec	Cudillero	*C. vermilara*	3828.51
CU3	03-Dec	Cudillero	*C. vermilara*	4655.08
CU3	03-Dec	Cudillero	*C. vermilara*	4685.09
CP2	02-Oct	Cabo da Penas	*C. vermilara*	1198.86

Sampling point	Season	Location	Species	eDNA/ uL
CP2	02-Oct	Cabo da Penas	*C. vermilara*	75.82
CP2	02-Oct	Cabo da Penas	*C. vermilara*	368.87
CP3	03-Dec	Cabo da Penas	*C. vermilara*	31691.2
CP3	03-Dec	Cabo da Penas	*C. vermilara*	4131.76
CP1	01-Jul	Cabo da Penas	*C. vermilara*	
CP1	01-Jul	Cabo da Penas	*C. vermilara*	
CP1	01-Jul	Cabo da Penas	*C. vermilara*	
G1	01-Jul	Gijon	*C. vermilara*	
G1	01-Jul	Gijon	*C. vermilara*	
G1	01-Jul	Gijon	*C. vermilara*	
G2	02-Oct	Gijon	*C. vermilara*	328.7
G2	02-Oct	Gijon	*C. vermilara*	36.7
G2	02-Oct	Gijon	*C. vermilara*	350.7
G3	03-Dec	Gijon	*C. vermilara*	25621.7
G3	03-Dec	Gijon	*C. vermilara*	11811
G3	03-Dec	Gijon	*C. vermilara*	36433.7
CA1	01-Jul	Concha de Artedo	*C.fragile*	
CA1	01-Jul	Concha de Artedo	*C.fragile*	
CA1	01-Jul	Concha de Artedo	*C.fragile*	
CA2	02-Oct	Concha de Artedo	*C.fragile*	
CA2	02-Oct	Concha de Artedo	*C.fragile*	
CA2	02-Oct	Concha de Artedo	*C.fragile*	
CA3	03-Dec	Concha de Artedo	*C.fragile*	
CA3	03-Dec	Concha de Artedo	*C.fragile*	
CA3	03-Dec	Concha de Artedo	*C.fragile*	
CU1	01-Jul	Cudillero	*C.fragile*	1439625
CU2	02-Oct	Cudillero	*C.fragile*	672077.7
CU1	01-Jul	Cudillero	*C.fragile*	53199.11
CU2	02-Oct	Cudillero	*C.fragile*	133067.9
CU2	02-Oct	Cudillero	*C.fragile*	665175.9
CU3	03-Dec	Cudillero	*C.fragile*	133553.8
CU3	03-Dec	Cudillero	*C.fragile*	250007
CP2	02-Oct	Cabo da Penas	*C.fragile*	962706.9
CP2	02-Oct	Cabo da Penas	*C.fragile*	1029995
CP2	02-Oct	Cabo da Penas	*C.fragile*	40338.99
CP3	03-Dec	Cabo da Penas	*C.fragile*	
CP3	03-Dec	Cabo da Penas	*C.fragile*	
CP3	03-Dec	Cabo da Penas	*C.fragile*	60549.1
CP3	03-Dec	Cabo da Penas	*C.fragile*	
CP3	03-Dec	Cabo da Penas	*C.fragile*	464526.5
G1	01-Jul	Gijon	*C.fragile*	
G1	01-Jul	Gijon	*C.fragile*	
G1	01-Jul	Gijon	*C.fragile*	
G2	02-Oct	Gijon	*C.fragile*	1166408
G2	02-Oct	Gijon	*C.fragile*	62206.77

Sampling point	Season	Location	Species	eDNA/ uL
G2	02-Oct	Gijon	*C.fragile*	1150190
G3	03-Dec	Gijon	*C.fragile*	
G3	03-Dec	Gijon	*C.fragile*	
G3	03-Dec	Gijon	*C.fragile*	

Table S. 16- *C. tomentosum* data for ex- situ optimisation.

Sample name	Ct values	Weight
CT1	33.07	5
CT1	29.04	5
CT1	32.3	5
CT2	32.97	10
CT2	31.56	10
CT2	30.74	10
CT2	29.56	10
CT3	27.22	20
CT3	26.11	20
CT3	27.27	20
CT4	26.83	40
CT4	27.37	40
CT4	25.45	40
CT4	27.72	40
CT4	25.43	40
CT4	26.09	40
CT5	0	80
CT5	0	80
CT5	0	80
CT6	0	160
CT6	0	160

Appendix 2- Developing innovative methods to face aquatic invasions in Europe: the Aquainvad- ED project

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Short Communication

Developing innovative methods to face aquatic invasions in Europe: the Aquainvad-ED project

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This study was first presented at the 19th International Conference on Aquatic Invasive Species held in Winnipeg, Canada, April 10–14, 2016 (http://www.icais.org/html/previous19.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Aquatic Invasive Alien Species (AIAS) are increasing due to the synergistic effects of climate change and habitat destruction. AIAS can heavily impact biodiversity and human health, causing a loss of ecosystem services; therefore, their control and management have now become a priority, particularly in the light of the new EU regulation 1143/2014 on invasive alien species. The main research goal of the Innovative Training Network Marie Skłodowska-Curie Aquainvad-ED is to exploit the application of innovative tools and the power of citizen science for early detection, control and management of AIAS. Eight early stage researchers are involved in Aquainvad-ED, engaged in four main research themes: (1) development and application of novel methods for early detection of AIAS; (2) identification of vectors of introduction and pathways of dispersal; (3) impacts of freshwater and marine invaders; and (4) risk assessment and control of AIAS. In order to develop multidisciplinary approaches to address these issues, the fellows are working within an international consortium (UK, Spain, Italy) composed of scientists and conservation practitioners from three universities (Swansea University, Universidad de Oviedo, Università degli Studi di Firenze), one technological institute (AZTI), two governmental agencies (Natural Resources Wales and Cardiff Harbour Authority), one NGO (Wye & Usk Foundation) and five SMEs working in fundamental and applied aspects of AIAS (Neoalgae, Natural Applications, NEMO, Ecohydros, and Itinera C.E.R.T.A).

Key words: alien species, invasive, early detection, control, pathway

Introduction

Globally, as a result of the advancing breakdown of biogeographic barriers, the introduction of alien invasive species is greatly contributing to biodiversity decline, ecosystem homogenization, and loss of ecosystem services, heavily impacting human health and economic activities (Kettunen et al. 2009; Simberloff et al. 2013; Jeschke et al. 2014; Mazza et al. 2014; Roy et al. 2016). The problems posed by aquatic invasive alien species (AIAS) are particularly dramatic due to the synergistic effects of climate change and habitat destruction. Aquatic ecosystems, especially freshwater ones, are vulnerable to biological invasions due to the strong affinity of humans to water (e.g. alteration, exploitation, utilization) and the intrinsic dispersal ability of aquatic species compared to terrestrial ones (Gherardi et al. 2009; Strayer 2010; Havel et al. 2015; Tricarico et al. 2016).

Over the last centuries, aquatic ecosystems in Europe have been colonized by highly invasive alien species. A total of 1,369 alien species have been reported in the European seas (Katsanevakis et al. 2013), mostly introduced through the Suez Canal (with an increase since 1990s) that was recently enlarged, facilitating the arrival of new species (Zenetos et al. 2012; Galil et al. 2015). There are 756 alien species in European fresh waters (Nunes et al. 2015), introduced mainly through aquaculture, the pet/aquarium trade (an emerging important pathway since 2000s: Maceda-Veiga et al. 2013; Mazza et al. 2015) and through sport fishing (Nunes et al. 2015). In both aquatic realms, many notable invaders are present, causing substantial damage to invaded ecosystems (Katsanevakis et al. 2013; Nunes et al. 2015).

The management of AIAS has become a priority, particularly in the light of the new EU regulation 1143/2014 (EU 2014) on the prevention and management of the introduction and spread of invasive alien species (art. 25). More than half (57%) of invasive alien species included in the EU concern list are freshwater species (Implementing Regulation EU 2016/1141 of 13 July 2016). Successful management of AIAS requires several steps: early detection, identification of introduction routes and dispersal pathways, and development of efficient control measures (CBD 2002). Public awareness and stakeholder involvement are also critical for preventing new introductions and for mitigating the impact of existing ones (CBD 2002).

Several projects (e.g. DAISIE, IMPASSE, COST Action TD1209 Alien Challenge) and initiatives (e.g. Essl et al. 2015; Latombe et al. 2016; Lucy et al. 2016) have addressed and are addressing different issues concerning invasive alien species in Europe and worldwide in order to harmonize terminology and optimize actions (databases, pathways, monitoring process, fostering collaboration). In this context, the project Marie Sklodowska Curie 2014 ITN (Innovative Training Network) H2020 Aquainvad-ED (AQUAtic INVaders: Early Detection, Control and Management; 2015–2019; http://www.aquainvad-ed.com/) was developed to tackle AIAS in Europe and to harmonize with the Marine Strategy Framework Directive (2008) and the Water Framework Directive (2000). ITN projects bring together universities, research centres and companies from different European countries to train a new generation of researchers. The funding boosts scientific excellence and business innovation, and enhances researchers' career prospects through developing their skills in entrepreneurship, creativity and innovation. The main research goal of Aquainvad-ED is to exploit novel tools combined with the power of crowd data sourcing (citizen science) to develop innovative methods for early detection, control and management of AIAS.

The project

In order to develop multidisciplinary approaches to address AIAS issues, Aquainvad-ED involves an international consortium of three European countries (UK, Spain, Italy), composed by scientists and professionals from three universities (Swansea University, project leader, Universidad de Oviedo, Università degli Studi di Firenze); one technological institute (AZTI); two governmental agencies (Natural Resources Wales and Cardiff Harbour Authority); one NGO (Wye & Usk Foundation) and five SMEs (Small and medium-sized enterprises) working in fundamental and applied aspects of AIAS (Neoalgae, Natural Applications, NEMO srl, Ecohydros and Itinera C.E.R.T.A scarl). Together, the Aquainvad-ED network offers a multidisciplinary approach (genetics, behaviour, ecology, citizen science, risk assessment) and the expertise of academic and non-academic partners to the assessment and management of biological invasions in aquatic habitats, through the enhancement of unique skills (e.g. technical, research and analytical competences), knowledge-sharing and capacity building.

The specific goals of the project are: i) developing, optimising and trialling innovative methods for early detection of freshwater and marine invaders; ii) identifying ecological and demographic factors determining AIAS establishment and spread; iii) recommending novel procedures for AIAS control which are applicable to natural and managed aquatic systems (e.g. rivers, estuaries, artificial reservoirs);

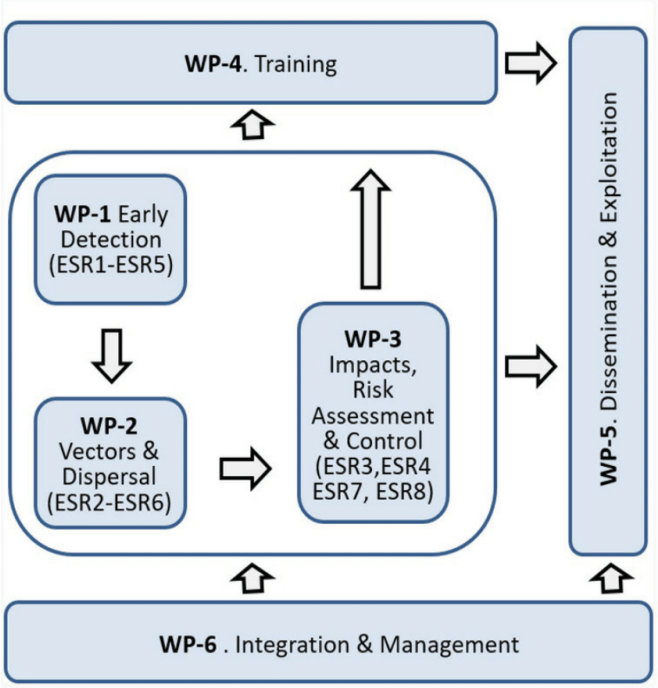

Figure 1. The six Aquainvad-ED Work Packages (WPs) along with the corresponding Early Stage Researchers (ESRs).

iv) integrating information on location, dispersion and control measures into management plans in order to prevent further AIAS introductions and dispersal in Europe, and v) raising public awareness about introduction routes and dispersal pathways, as well as about ecological and socio-economic impacts caused by AIAS.

In order to accomplish these goals, Aquainvad-ED is structured into six complementary Work Packages (WPs; Figure 1): WP1, dealing with the development and application of novel methods for early detection for AIAS; WP2, focussing on identification of introduction vectors and dispersal pathways; WP3, concerning the assessment of impacts of selected freshwater and marine invaders, as well as risk assessment and control of AIAS; WP4 on organizing training activities devoted for the recruited fellows; WP5 on dissemination and exploitation; and WP6 dedicated to the integration and management of the whole project.

The Fellows

Eight Early Stage Researchers (ESRs) are involved in Aquainvad-ED, and are dedicated to four projects linked to WP1, WP2 and WP3 (Figure 1): (1) development and application of novel methods for early detection AIAS; (2) identification of introduction vectors and dispersal pathways; (3) impacts of aquatic invaders; and (4) risk assessment and control of AIAS. Each ESR has academic and non-academic supervisors, and two planned secondments within the consortium partners in order to acquire multidisciplinary and multi-sectorial skills. WPs 4, 5 and 6 are dealing with training activities, dissemination and project management, involving the supervisors coordinated by Swansea University (WPS 4, 6) and by Wye & Usk Foundation (WP5).

Development and application of novel methods for early detection AIAS

Teja Muha (Swansea University, UK) and Anaïs Rey (AZTI, Spain) are developing molecular methods based on metabarcoding, able to detect the overall community, and qPCR, suitable for detecting specific species in freshwater [for detection of the killer shrimp *Dikerogammarus villosus* (Sowinsky, 1894), the zebra mussel *Dreissena polymorpha* (Pallas, 1771), alien macrophytes and fish] and marine environments (for detection of invaders as required by the "Ballast Water Convention"). To achieve this, laboratory and

field calibration of molecular methods are applied to a range of freshwater and marine systems in the UK, Spain and Italy. As part of a citizen science programme, a smartphone app (AquaInvaders) is being used to promote citizen science programs for the early detection of AIAS.

Identification of vectors of introduction and pathways of dispersal

Marta Rodríguez-Rey (Swansea University, UK) and Sabine Rech (Universidad de Oviedo, Spain) are working on the identification of physical and ecological constraints for the survival of AIAS. Rodríguez-Rey is mainly addressing the different role of natural vs. anthropic variables in the dispersal of non-native invasive species, as well as the social perception towards alien species. Rech is focussing on floating objects and marine litter as potential vectors of AIAS (Rech et al. 2016a). The fellows will compile an inventory of AIAS arriving to selected freshwater and marine systems in the three project countries. They will estimate optimal conditions, potential floating vectors, and high-risk activities and source and sink areas for invasion and dispersal of AIAS, before mapping the main routes of introduction and dispersal, based on floating and stranded samples of rafting biota (Rech et al. 2016b), traffic research, meta-barcoding profiles (deriving from Teja and Anaïs), fouling experiments, using eDNA and experiments under controlled conditions. In this way, it will be possible to develop recommendations and guidelines for identifying vectors of introduction and pathways of spread of key aquatic invaders.

Impacts of aquatic invaders

To quantify current ecological and socio-economic impacts and the relationships among invaders, Matteo Rolla (Swansea University, UK) and Phillip J. Haubrock (NEMO srl, Italy) are studying selected freshwater invaders [such as *D. villosus*, *D. polymorpha*, the red swamp crayfish *Procambarus clarkii* (Girard, 1852), the channel catfish *Ictalurus punctatus* (Rafinesque, 1818), the bullfrog *Lithobates catesbeianus* (Shaw, 1802); Haubrock et al. 2016a] through laboratory and field experiments (Haubrock et al. 2016b; Rolla et al. 2016). Moreover, they are assessing the ecosystem services affected by these AIAS in order to quantify the economic costs. These fellows will develop guidelines for estimating current and future AIAS impacts in aquatic environments under a range of future climate and environmental scenarios.

Risk assessment and control of AIAS

To accomplish the last step of AIAS management, Iva Johović (Università degli Studi di Firenze, Italy) and Roberta Skukan (Neoalgae, Spain) are modelling and assessing the risk of invasion for a range of freshwater (e.g. *P. clarkii*, *L. catesbeianus*) and marine invaders [the invasive seaweeds *Codium* spp., *Sargassum muticum* (Yendo) Fensholt, 1955, and *Undaria pinnatifida,* Harvey (Suringar), 1873], respectively (Haubrock et al. 2016a). They are also testing different control techniques and mitigation measures to prevent the spread of selected AIAS (Johović et al. 2016). In order to assess the risk of marine invasion, molecular species identifications and biogeography data will also be integrated in Roberta's research as a valuable tool for effective management strategies (Skukan et al. 2016a). Citizen science programs as a useful tool for early detections and prevention of algae invasions will be also implemented (i.e. Skukan et al. 2016b). Their final aim will be to develop guidelines for mitigating biological and socio-economic impacts caused by freshwater and marine invaders, as well as predictive models for the identification of vulnerable areas under current and future climate change.

Network and training activities

Aquainvad-ED partners meet annually to review progress and provide an update on project status. They also engage in specific training activities to enable ESRs to develop new skills. For example, in December 2015, they attended the Inaugural Training Event on Entrepreneurship Skills at Swansea (UK), and the Rivers Trust Spring Conference at Hay-on-Wye (UK) in May 2016, where they also participated in a training event on Citizen Science and Communication. In April 2017, they attended a training workshop in Spain on early detection methods for aquatic invaders, and in spring 2018 they will attend a training workshop on strategies and methods for AIAS management in Italy.

Conclusion

As introductions of alien species in Europe increases, new legislation requires more efficient management tools for AIAS. The Aquainvad-ED project will contribute to this task, not only through the production of science-based guidelines and deliverables, but also through the training of a new generation of multidisciplinary researchers who will be able to face biological invasions from different perspectives. The project will benefit from the outputs of the previous

cited projects and initiatives, and will surely establish a collaboration with the recently developed INVASIVESNET network (http://www.invasivesnet.org) (ET is part of COST Action Alien Challenge and INVASIVESNET).

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Appendix 3 – R scripts

Chapter 2

Example – eDNA yield for Volume experiment; same model for PCR and qPCR, different dependent variable

*lm_volume<- lm(Original_sample~ factor(Volume)*factor(Water_body), data = three_water_bodies_total_Volume)*

par(mfrow=c(2,2))

plot(lm_volume)

par(mfrow=c(1,1))

summary(lm_volume)

anova(lm_volume, test = "F")

Chapter 3

#Shannon- Wiener

*Shannon_mod <-lm(Shannon.Wiener~ factor(Time)*factor(up.down), data=Lugg)*

par(mfrow=c(2,2))

plot(Shannon_mod)

par(mfrow=c(1,1))

summary(Shannon_mod)

anova(Shannon_mod, test="F")

Presence/ absence

*GLM_presence_absence_lugg<- glm(Presence.absence~factor(up.down)*factor(Species)*factor(Time), data = Lugg_species, family= binomial(link="logit"))*

par(mfrow=c(2,2))

plot(GLM_presence_absence_lugg)

par(mfrow=c(1,1))

summary(GLM_presence_absence_lugg)

anova(GLM_presence_absence_lugg, test = "Chi")

Total number of sequence reads calculated for each individual species

*Salmo_salar<- lm(Salmo.salar._rel.ab~factor(Time)*factor(up.down), data = Lugg_species)*

par(mfrow=c(2,2))

plot(Salmo_salar_RRA)

par(mfrow=c(1,1))

summary(Salmo_salar_RRA)

anova(Salmo_salar_RRA, test = "F")

Chapter 4

#shannon

*Shannon_mod <-lm(Shannon~ factor(Obstacle)*factor(Below.above), data=Teifi_river)*

par(mfrow=c(2,2))

plot(Shannon_mod)

par(mfrow=c(1,1))

summary(Shannon_mod)

anova(Shannon_mod, test = "F")

#presence, absence

*Presence_Teifi<- glm(Presence..absence ~factor(Below.above)*factor(name.of.species)*factor(Obstacle), data = Teifi_river, family= binomial(link="logit"))*

par(mfrow=c(2,2))

plot(Presence_Teifi)

par(mfrow=c(1,1))

summary(Presence_Teifi)

anova(Presence_Teifi, test = "Chisq")

LM for each specific species (reduced model with Obstacle only)

*lm_fixed_species<- lm(Anguilla.anguilla~factor(Obstacle.)*factor(Below.above.), data=Teifi_river)*

par(mfrow=c(2,2))

plot(lm_fixed_species)

par(mfrow=c(1,1))

summary(lm_fixed_species)

anova(lm_fixed_species, test = "F")

#posthoc

leastsquare = lsmeans(lm_fixed_species,

pairwise ~ Obstacle,

adjust="tukey")

leastsquare$contrasts

Chapter 5

Presence/ absence

*glm_Codium_all_species<- glm(presence.absence ~factor(Species)*factor(Sampling.season)*factor(Location), data = Codium, family= binomial(link="logit"))*

par(mfrow=c(2,2))

plot(glm_Codium_all_species)

par(mfrow=c(1,1))

summary(glm_Codium_all_species)

anova(glm_Codium_all_species, test = "Chisq")

Codium abundance

*Codium_all_species<- lm(eDNA.technical.average ~ factor(Species)*factor(Sampling.season)* factor(Location), data =Codium)*

par(mfrow=c(2,2))

plot(Codium_all_species)

par(mfrow=c(1,1))

summary(Codium_all_species)

anova(Codium_all_species, test = "F")

One-way comparison

leastsquare = lsmeans(Codium_all_species,

pairwise ~ factor(Sampling.season),

adjust="tukey")

leastsquare$contrasts

Two-way comparison

leastsquare2 = lsmeans(glm_Codium_all_species,pairwise ~ Sampling.season:Species,

adjust="tukey")

leastsquare2$contrasts

GLOSSARY

Barcoding	Term refers to taxonomic identification of species based on single specimen sequencing using diagnostic barcoding markers.
DNA amplification	Production of multiple copies of a sequence of DNA.
Environmental DNA	DNA captured from an environmental sample without the need for target organism isolation.
In silico	Produced by means of computer simulation.
Limit of detection	Lowest quantity or concentration of a component that can be reliably detected with a given analytical method.
Macro-organism environmental DNA	Environmental DNA originating from animals and higher plants.
Metabarcoding	Taxonomic identification of a number of target group of species extracted from a mixed sample (community DNA or eDNA), PCR-amplified and sequenced on a high-throughput platform.
Molecular Operational Taxonomic Unit	Identified group through use of cluster algorithms and a predefined percentage sequence similarity.
Next generation sequencing	Sequencing techniques that allow for simultaneous analysis of millions of sequences compared to the Sanger sequencing method of processing one sequence at a time.
PCR inhibition	Any factor which inhibits the amplification of nucleic acids through the PCR (polymerase chain reaction).
Primer, probe	Short strand of RNA or DNA that is used as a starting point for DNA synthesis.

*Glossary update from (Deiner et al., 2017a)

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