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D2.5 Molecular toolkit: taxon-specific set of primers, protocols and pipelines

This is the 2.0 version of the D2.5 Molecular toolkit: taxon-specific set of primers, protocols and pipelines. This document is a deliverable of the AMBER project which has received funding from the European Union's Horizon 2020 Programme for under Grant Agreement (GA) #689682.

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Executive summary

This is the 2.0 version of the D2.5 Molecular toolkit: taxon-specific set of primers, protocols and pipelines. This document is a deliverable of the AMBER project. This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 689682.

The main focus of WP2 is on the development and implementation of tools for assessing the impact of stream barriers on river connectivity and habitat fragmentation to guide the restoration of river ecosystems across Europe. To gauge the extent of river fragmentation, and assess the success of subsequent restoration efforts, better metrics of connectivity are needed. These should not merely consider fish, but also include other taxa. In this sense, environmental DNA (eDNA) methods can allow a rapid and relatively cheap (compared to traditional, labour intensive field surveys) assessment of stream connectivity. Here we provide a molecular toolkit optimised for the detection of target taxa and communities via PCR and qPCR and/or High-throughput sequencing as part of the AMBER project (T2.5.1; D2.5). The toolkit has been developed to minimize cross-contamination and improve species detection.

eDNA-based metabarcoding is used to simultaneously detect multiple taxa, combining the advantages of DNA barcoding and new sensitive methods of DNA extraction from water samples, being the preferred method to assess the effects of loss of stream connectivity. Different markers for eDNA metbarcoding have been tested and validated, and the best working primers have been selected to be tested in the field, ready for their use in the AMBER case studies.

The overall workflow for eDNA metabarcoding consists of:

(1). Sampling and filtration: In the context of routine monitoring, on-site filtration using an enclosed filter (operated with a pump or manually with a syringe) was found to be the preferred option as it will reduce handling and potential contamination. In running waters, filtering of c. 1 L of water from multiple locations along the river banks was found to be satisfactory.

(2). DNA extraction: There is a wide range of DNA extraction kits available but we recommend PowerSoil and PowerWater as they seem more effective in removing inhibitors during the extraction process.

(3). Library preparation and Sequencing: PCR amplification/s of extracted DNA using the taxa-specific selected primers and subsequent sequencing of the amplicons (selected Platform Illumina MiSeq).

(4). Data analysis: Bioinformatic data-processing including paired-end sequence assemblage, error trimming, sequence sorting, data de-noising, chimera removal, clustering into MOTUs and taxonomical assignment.

Species-specific and taxon-specific eDNA assays are considered the best approach when targeting particular species which occur at very low densities (rare) or are difficult to distinguish from similar species, as it provides higher sensitivity and discrimination power over a multi-species approach. Taxon-specific primer sets for the detection of native, indicator and exotic fish and invertebrate species from European waters have been designed and validated in silico, in vitro and in the field as part of D2.5.

The application of the toolkit developed within T2.5.1 and described in this document will be used in the AMBER case studies for assessing connectivity. It will help to map the spatial distribution of aquatic biota in relation to the distribution of barriers at test catchments (T2.5.2), and in combination with results derived from models developed in T2.1, will help to identify barrier impacts on multiple taxa.

The aim of this deliverable is to produce practical guidance for the application of eDNA in studies of stream connectivity in human-altered freshwater ecosystems, including the effect of barriers on community composition. This will provide an essential management tool for barrier management.

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1. Introduction

The Water Framework Directive (WFD, 2000) was launched by the EU with the main goal of ensuring that the different types of water-bodies attain a good ecological status. Most of the studies on the consequences of river alterations have focused on individual species, particularly those which are emblematic or have a high socio-economic interest (e.g. salmonids), or those that have become pests as a consequence of stream modifications (e.g. sea weeds, Téllez *et al.* 2008). In contrast, there are few studies on the consequences of stream modifications at the community level. Community studies are expensive and difficult to carry out, mainly due to sampling and logistic constraints (e.g. sampling different species/habitats requires different techniques). Yet, there is a pressing need to understand and predict the consequences of human modifications of freshwater ecosystems, and to get a better grasp of how human-altered environments shape species interactions across taxa. Environmental DNA (eDNA) is considered to be more sensitive, cost-effective and faster than traditional survey methods. Compared to traditional survey methods, eDNA does not impact on species or their habitats, requires no taxonomical knowledge, and is generally less time consuming. Combined with metagenomics, eDNA is rapidly becoming the tool of choice for the early detection of multiple taxa, including aquatic invasive species (Lodge *et al.* 2012; Takahara, Minamoto & Doi 2013; Deiner *et al.* 2017), as well as for the study of current and past community dynamics (Pedersen *et al.* 2015). eDNA represents a mixture of heterogenous genetic material of different qualities (i.e. at various decay stages), including chromosomes, plasmids contained in cells, as well as extracellular DNA fragments, free in the environment or adsorbed onto particles (Siuda & Chrost 2000). Environmental DNA from fish is detectable between 7 and 25 days in freshwater (Dejean *et al.* 2011; Thomsen *et al.* 2012a) but only 7 days for marine fish (Thomsen *et al.* 2012b).

Until recently, most of the applications of eDNA have been for monitoring the presence of one or several target species (Bohmann *et al.* 2014) but the potential of eDNA for the study of stream connectivity remains largely unexplored. Experimental designs specifically directed at measuring and understanding the impacts of loss of stream connectivity on species and communities are needed in order to fully understand the effects of barriers. Species-specific and taxon-specific eDNA assays are considered the best approach when targeting particular species which occur at very low densities (rare) or are difficult to distinguish from similar species, as it provides higher sensitivity and discrimination power over a multi-species approach. On the other hand, eDNA meta-barcoding holds much potential for holistic biodiversity assessment and routine freshwater monitoring (Kelly *et al.* 2014; Creer *et al.* 2016; Deiner *et al.* 2017). The community approach provided by eDNA meta-barcoding is most appropriate for the assessment of the effects of loss of stream connectivity, as whole communities can be surveyed, fluctuations in species richness can be detected, and information on presence/absence and relative abundance of multiple species can be obtained.

We have optimised and validated a number of eDNA-based meta-barcoding protocols for freshwater fish, invertebrates and macrophytes. These protocols are ready to be used in the AMBER case studies and can help to map the spatial distribution of aquatic biota in relation to the distribution of barriers at test catchments (T2.5.2), and in combination with results derived from models developed in T2.1, to identify barrier impacts on multiple taxa. In addition to eDNA meta-barcoding protocols, species-specific markers have also been developed for the detection of a number of indicator salmonid species (Clusa *et al.* 2017a), as well as highly invasive invertebrates in European rivers (Clusa *et al.* 2017b; Robinson *et al.* under revision). We have also developed workflows for planning, sampling and analysis of eDNA data, and provided practical guidance to be applied in the AMBER case studies.

The aim of this deliverable is to produce practical guidance for the application of eDNA in studies of stream connectivity in human-altered freshwater ecosystems, including the effect of barriers on community composition of native and aquatic invasive species.

2. Workflows. Practical Guidance on the use of eDNA in AMBER Case Studies

The workflows presented in this section (Figure 1 and 2) provide practical guidance for the application of eDNA in the field, and represent a decision tool as to when an eDNA approach might be used as a replacement or complement of more traditional survey methods. This guide provides advice on the type of samples to be collected, the downstream technique to be applied and the laboratory protocols to be adopted (including eDNA extraction, eDNA markers, sequencing platform) in the AMBER case studies. A summary of the methodology applied in the AMBER case studies is provided in section 3.

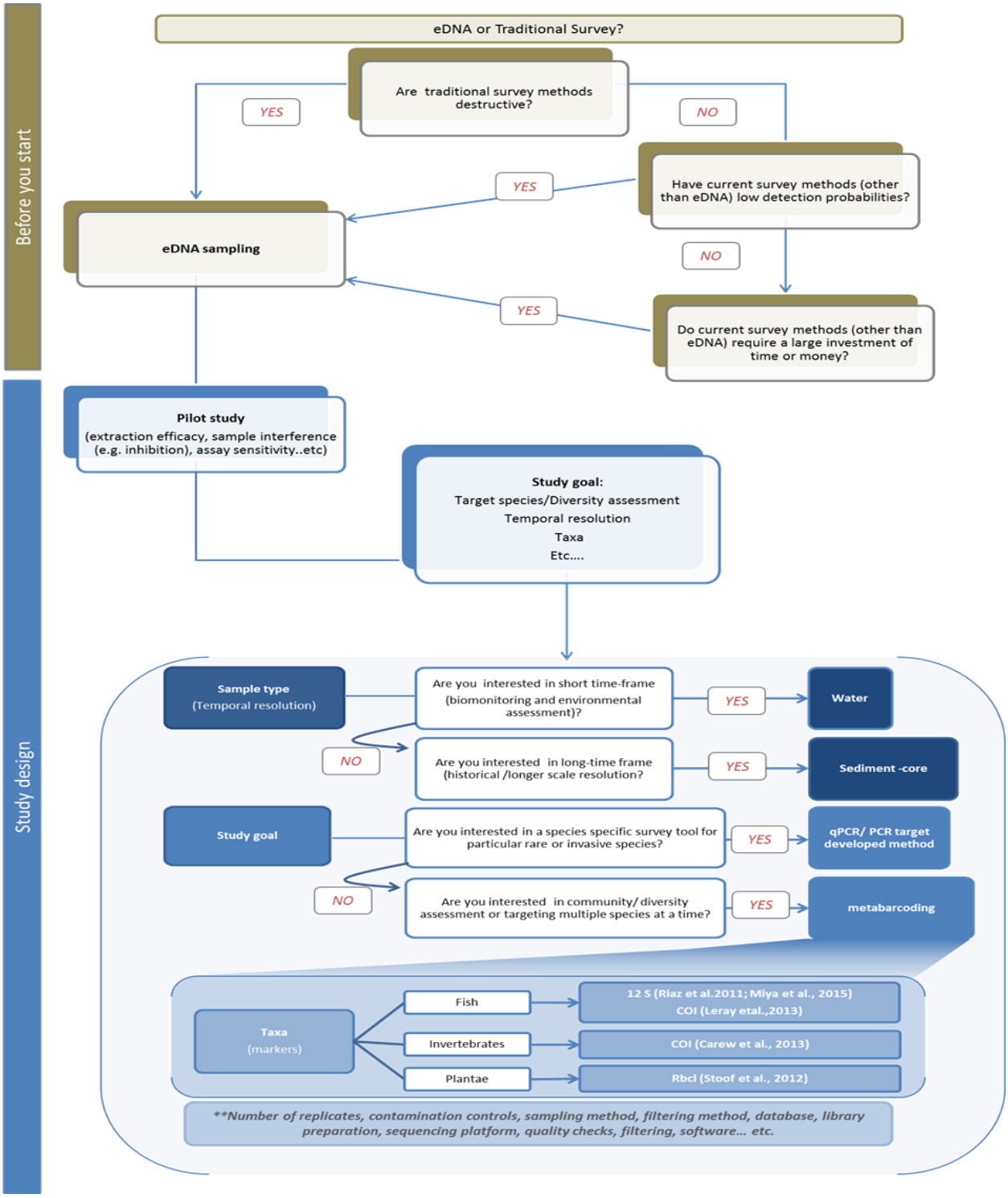

Figure 1. Decision flow chart for adopting an eDNA approach over traditional survey methods (before work begins) and guidance for eDNA experimental design (study design).

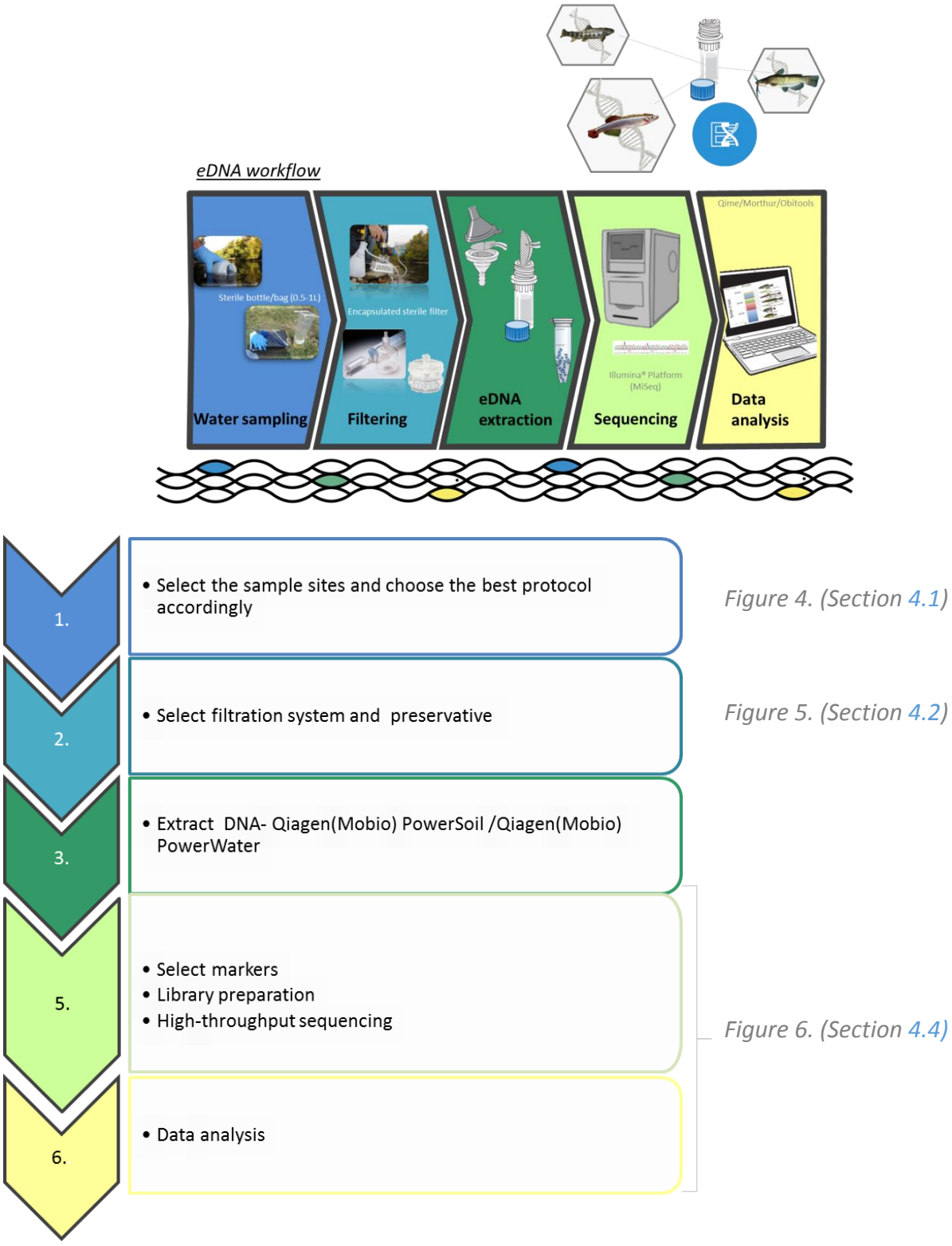

Figure 4. (Section 4.1)

Figure 5. (Section 4.2)

Figure 6. (Section 4.4)

Figure 2. eDNA workflow summary diagram. (Summary diagrams for each of the stages that are part of the eDNA workflow are provided at the beginning of each subsection within section 4 (Protocol Development and Optimization).

3. eDNA TOOLKIT.

AMBER case studies---Brief summary of protocols, markers and pipelines to be applied.

Goals

1. To assess stream connectivity (mapping the spatial distribution of biota in relation to barriers)

2. To assess the impact of barriers on biodiversity change and community structure

Number of samples/sampling sites. This will vary depending on the case study. It is recommended that at least 10 points are sampled, distributed both upstream and downstream of the study barriers. In all cases, three replicates per sampling point are required. The recommended volume of water to be collected is 1L per site, made up of 5 x 200 ml pooled subsamples collected within 100 m of each sampling point. Filtration controls are required at each sampling point; these can be obtained by filtrating distilled ultrapure water and will help to detect any potential contamination during sampling.

Sampling kit

-1L Sterile bags (Whirl-Pak® stand -up Sample Bag or sterile bottle).

-50 ml disposable syringes (Thermo Scientific National Sci.)

- Filter holder + filter

-Filter caps

-Preservative (Ethanol)

-re-sealable plastic back

-nitrile gloves

Sample collection Protocol

1. Fill up the Whirlpak bag (or sterile bottle) with water by holding it into the stream.
2. Draw up 50 ml of water into the large syringe.
3. Attach the syringe to the filter inlet.
4. Press the plunger and push the water through the filter.
5. Remove the filter from the syringe, and repeat steps 2 to 4 until a 1 litre of water has been filtered.

*If the filter is clogged (e.g. if the water is turbid and there is a lot of suspended sediments) change the filter and use a new one. Make a note and specify which volume has passed through each filter.

6. Remove the filter from the syringe, fill the syringes with air. Re-attach the syringe to the filter system and push the air through to expel the water from the filter. Repeat this step until the filter seems dry.
7. Take a sterile syringe previously filled with ethanol and pass it through the filter. Stop when the solution starts to come through the filter.
8. Seal the outlet and inlet with the caps
9. Place the capped filters into the re-sealable bag and note down geo-location (GPS) and date.
10. Report any biotic and abiotic variables likely to affect eDNA quality and concentration, such as water temperature, pH, flow rate, and river turbidity.

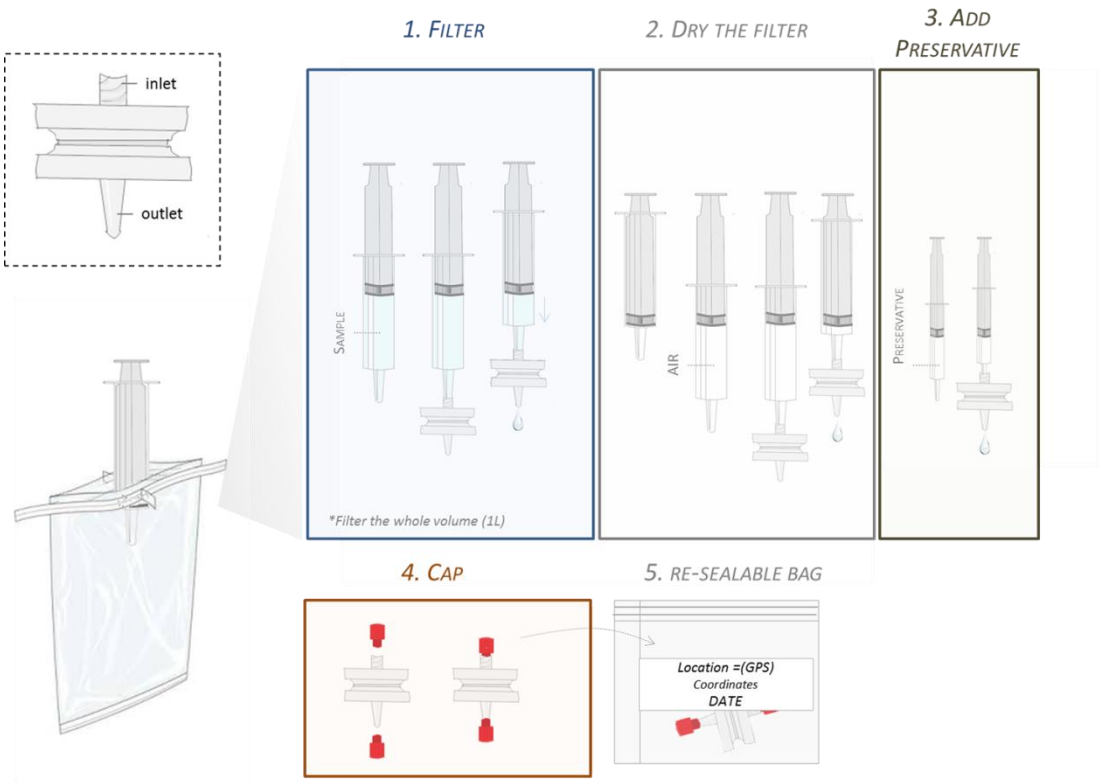

Figure 3. Schematic sampling protocol

Filtration and eDNA extraction

- Filtration: Enclose Filter on-site filtration

Filter Holder (Polycarbonate Filter Holder for 25 mm [#COL PLAMER-ref. WZ-29550-42])
Filter (Millipore polyethersulfone, Hydrophilic, 0.45 µm, 25 mm [#Millipore – ref. HPMP02500])

- Extraction: PowerWater or Power Soil DNA Isolation Kit (QIAGEN laboratories).

Metabarcoding markers

Fish (12S)

Universal Vertebrate primers (Riaz *et al.* 2011):

Vertebrate 1F: ACTGGGATTAGATACCCC

Vertebrate 1R: TAGAACAGGCTCCTCTAG

Invertebrates (COI)

Universal macroinvertebrate primers (Carew *et al.* 2013)

HCO2198/ 912 TAAACTTCAGGGTGACCAAAAAATCA

COI LCO1490 GGTCAACAAATCATAAAGATATTGG

Macrophytes and diatoms (rbcL)

Universal plants primers rbcL (Stoof-Leichsenring *et al.* 2012, Bell *et al.* 2017)
rbcL2_F: TGGCAGCATTYCGAGTAACTC
rbcLa R: CTTCTGCTACAAATAAGAATCGATCTC

Diat_rbcL_705F AACAGGTGAAGTTAAAGGTTCATAYTT
Diat_rbcL_808R TGT AACCCATAACTAAATCGATCAT

Library preparation

2-STEP amplicon library prep method (Illumina)

- **Amplicon PCR (1st stage PCR**) primers with overhang adaptors (specified by Illumina) amplify eDNA samples. PCRs are carried out in triplicate and pooled prior to sequencing to minimise bias.
- **Quantify using ZAG** (high resolution capillary electrophoresis) * Size verification (Optional).
- **PCR clean-up**-this step uses AMPure XP beads to purify the 16s V3 and V4 amplicon away from free primers and primer dimer species.
- **Index PCR (2nd stage PCR)**- this step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index kit.
- **PCR Clean-up 2**-This step uses AMPure XP bead to clean up the final library before quantification.
- **Validate library (Optional)**
- Q**uantify the library-** qubit dsDNA/ qPCR method
- **Library Normalization and pooling-** calculate DNA concentration in nM based on the size of DNA amplicons, dilute and pool.
- **Library Denaturing and Miseq sample loading-** pooled libraries are denatured with NaOH, diluted with hybridisation buffer and then hat denatured before MiSeq sequencing. Recommend adding 5% of PhiX genomic library.

  Controls and blanks are always required, including **sample controls, **negative controls** and **blanks** (filtration blanks, extraction blanks and PCR blanks), even when no bands can be detected on agarose gels.*

Analytical pipeline

QIIME CAPORASO, J. Gregory, *et al.* QIIME allows analysis of high-throughput community sequencing data. Nature methods, 2010, vol. 7, no 5, p. 335-336.

**(OBItools and Mothur also used for comparative purposes)*

4. PROTOCOL DEVELOPMENT AND OPTIMIZATION

4.1. Sampling

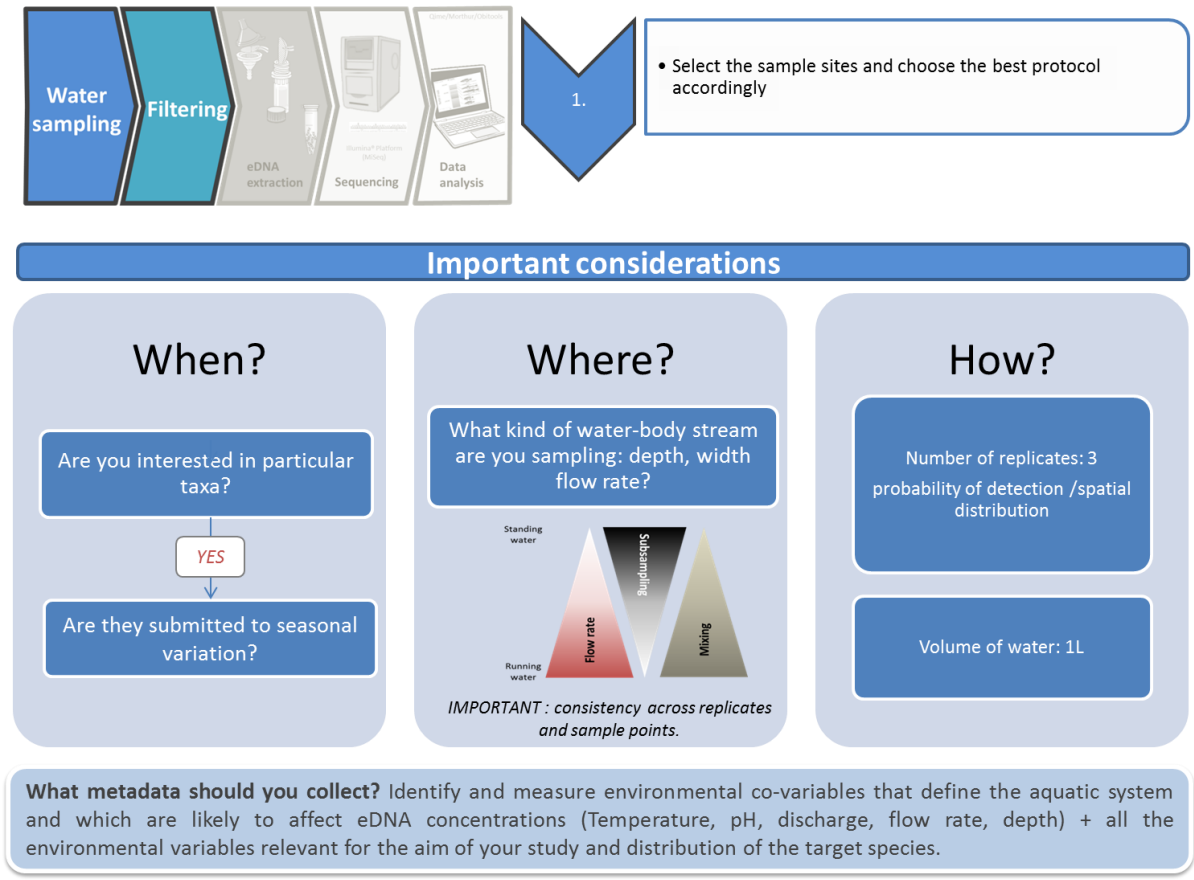

Figure 4. Considerations for water sampling.

Experimental design requires careful planning of timing, spatial arrangements, number of replicates, volume and filtration system (Figure 4).

The timing of the sampling is important as the abundance and occurrence of some species can change seasonally, which should be considered when scheduling the sampling, particularly for migratory species. Environmental covariates should also be considered, since the probability of detection can also change seasonally due to variation in eDNA dilution and degradability (Bista *et al.* 2017; Buxton *et al.* 2018). Thus, timing will be dependent on latitude and water-body local dynamics.

The spatial arrangement of the sampling is system dependent. Little is known about eDNA dynamics in lotic systems (flowing water). The best location is dependent on the species distribution in the water column and the level of mixing of the water.

Number of replicates: We recommend a minimum of three replicates at each sample site, in order to assess detection probabilities and control for sampling variability.

Volume: 1L is a reasonable volume which is commonly used. There is a trade-off between detection probability and efficiency. Although smaller volumes have given good results, this may reduce detection probability. On the other hand, higher volumes may increase detection probability but can be very time consuming. The sample volume is also dependent on water turbidity. Samples collected from turbid streams with abundant tannins and organic matter may clog the filter before the target water volume (1L) is filtered using the proposed filter (filter pore diameter 0.22 µm). Filters with larger pore diameters (0.45 to 1 µm) or pre-filtering could help reduce filter clogging in such cases.

Below we detail the different volumes tested within the context of the AMBER project, as well as the protocols used to sample fish and macroinvertebrates and validate in vitro the primers used on eDNA water samples.

4.1.1. Water sampling for species detection (UNIOVI/SU)

Three different methods have been tested

1. 1L-1.5L of water per replicate (at least three replicates per sample) collected in sterile and hermetic bottles at each sampling point. Water can be stored frozen until filtration or immediately filtrated after collection. All material must be cleaned with bleach between samples, following instructions by Goldberg *et al.* (2016).
2. Samples of 15 mL, 100mL, 250mL, 1L and 2L water samples collected in triplicate at each sampling station and filtered in the lab following the methods described in 4.2.1. Results comparing sampling strategies indicated that 250 ml represented an optimal sampling volume in terms of yield/sampling time, however higher volumes may increase detection probability although this can be more time consuming. Three to nine replicates were collected per site and two ultrapure water blanks were taken at the beginning and end of the sampling period.
3. For qPCR detection of invertebrates using eDNA 15 mL water samples were taken. Immediately after collection, a standard method of preserving and extracting eDNA was applied by the addition of 33 mL of absolute ethanol and 1.5 mL of 3M sodium acetate to samples and subsequent storage at -20^{o}C for a minimum of 24 hours before DNA extraction (Ficetola *et al.* 2008).

4.1.2. Fish tissue (UNIOVI)

Fish scales were obtained from anglers in Spain through collaboration with the Rioseco Council (Asturias).

4.1.3. Macroinvertebrate sampling (UNIOVI)

The protocol for macroinvertebrate sampling followed the guidelines provided in the EU Water Framework Directive 2000/60/CE (UNE-EN ISO 10870:2012) and Water quality (UNE-EN 16150:2012). At each sampling point, a 100 m representative reach of the river was sampled. The section was characterized based on habitat types, and sampling units were distributed following the habitat representation. Sampling was carried with a Surber net to trap benthic macroinvertebrates, and these were stored in 70% ethanol. The use of 70% ethanol is recommended over 96-100% ethanol as it maintains specimens more flexible and easier to classify based on morphological traits. Classification was performed shortly after sample collection, samples were then transferred to 96% ethanol for long term preservation. This preservation protocol has been successfully used in previous barcoding studies (Macher *et al.* 2016; Radulovici *et al.* 2009) with ethanol preservation being recommend when molecular methods such as DNA barcoding are integrated into bio-monitoring programs (Stein *et al.* 2013). At least two sampling replicates must be collected per sampling site.

Although invertebrates (other than cephalopods) do not require sampling permit from the University Research Ethics Committee, researchers were encouraged to carry out an ethical self-screening before sampling, to ensure that no more invertebrates than those strictly required for the barcoding survey were collected.

4.2. Filtration and extraction

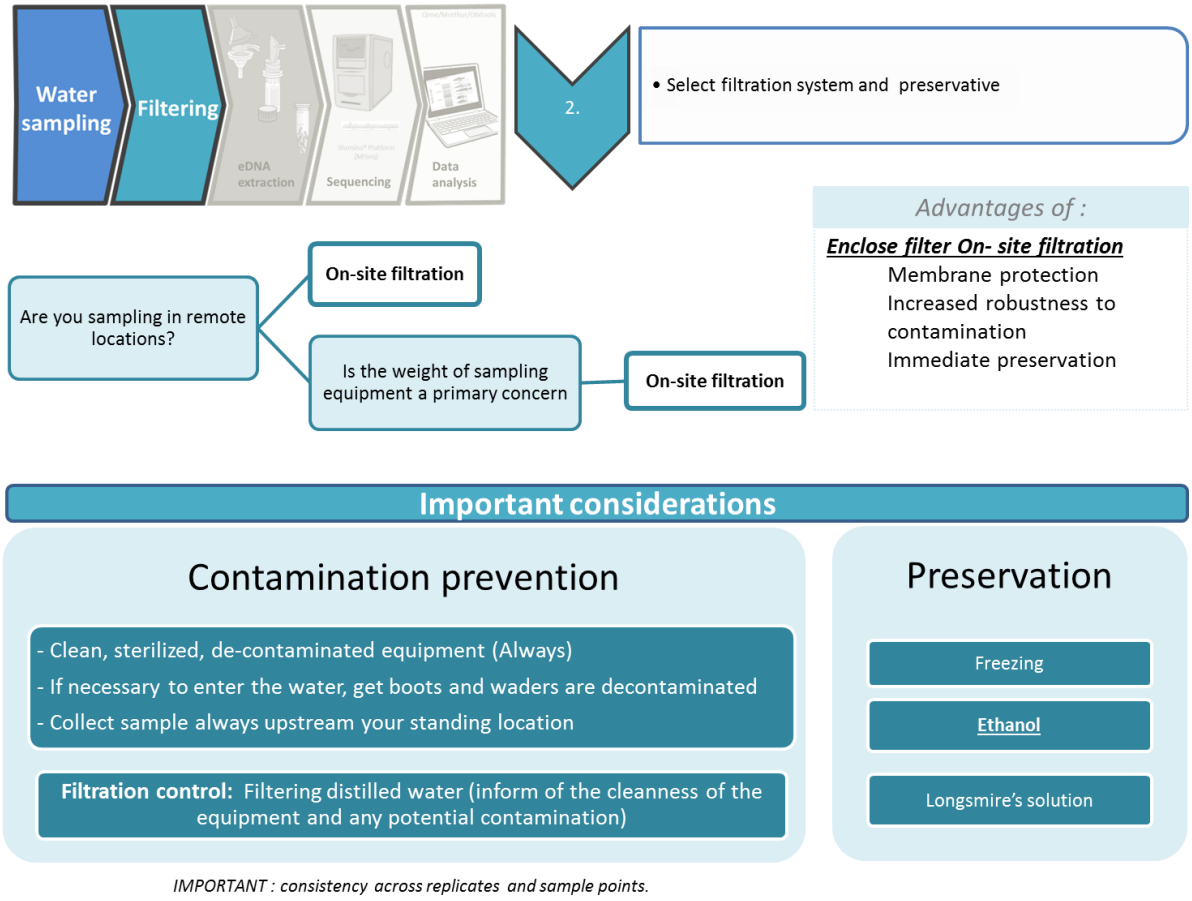

Figure 5. Considerations for water filtration.

One of the most critical aspects of eDNA sampling is to prevent contamination and ensure the accuracy of the results (Figure 5).

Filtration system: Filtration can be done either on-site or in the lab. We recommend on–site filtration, either with a pump or manually (syringe) using encapsulated filters for routine monitoring, as these reduce the risk of contamination. The fact that the filter is encapsulated limits handling, protects the membrane from other external contamination, and allows immediate preservation, being the preferred option for standardized routine monitoring (Spens *et al.* 2016). Although on site filtration is the preferred method, particularly when sampling remote locations, laboratory filtration under sterile conditions may be recommended when the laboratory is relatively close and/or when the number of samples is too high to be handled in the field. This was the case in our pilot optimization study when different volumes of water, filters and processing methods were tested.

Sample/Filter Preservation: Filters can be preserved by freezing, cold storage (Jerde *et al.* 2011; Santas *et al.* 2013),or by immersion in ethanol (Goldberg *et al.* 2011; Goldberg et al 2013) or in Longmire's solution (Renshaw *et al.* 2015; Spens *et al.* 2016; Williams *et al.* 2016). Of these, ethanol is widely available, inexpensive and can be used straight away, and is therefore our recommended choice. Below we summarize the different collection and processing methods for eDNA samples.

4.2.1. Water filtration (UNIOVI/SU)

Two separate areas were used for the whole process, one for pre-PCR and another one for PCR and post-PCR. Filtration of water samples was performed in the pre-PCR room, where there are no positive DNA controls, nor tissue samples.

At UNOVI water samples were vacuum filtered using the Supor®-200 Membrane Filter (Pall Corporation) with 0.2µm pore size and a reusable filter holder. The filter holder was dismantled, sprayed with 10% bleach, cleaned with detergent and 10% bleach, rinsed with distilled water and sterilized by 20 minutes under UV light between two consecutive samples. To ensure the cleaning process was correct, one sample with 1L distilled water was filtrated between two problem samples and included in all eDNA analyses to confirm that contamination did not occur in the filtration or extraction process. Filters were stored individually within 15ml tubes at -20°C until DNA extraction.

At SU five different water volumes, three different filter materials and five different filtration techniques were tested to determine the best method. Decontamination and check points between samples were carried out as described above. A summary of the collection and processing methods used for eDNA samples is provided in Table 1.

Table 1. Comparison of collection and processing of eDNA samples.

Source	Volume sampled	Filter type	Preservation method	Precipitation method	Extraction method used
(Ficetola *et al.* 2008)	15 mL	No filter used—entire sample centrifuged.	Samples combined with 1.5 mL sodium acetate (3 M) and 33 mL absolute ethanol, then stored at -20°C.	Centrifuged the mixture (5000g, 35 min, 6°C) and discarded the supernatant (Valiere and Taberlet, 2000).	DNA Purification kit from Blood protocol (QIAamp DNA Mini Kit).
Mixed	100 mL	Minisart® syringe cellulose acetate filters with 0.45 µm pore size (Sartorius).	Whole filters were stored at -20°C.	Filters were washed by the some method as used for 15mL- 15mL of ultrapure water, 2mL sodium acetate, 33 mL absolute ethanol. Afterwards they were centrifuged (5000g, 35 min, 6°C).	DNA Purification kit from Blood protocol (QIAamp DNA Mini Kit).
Mixed	100 mL	Minisart® syringe cellulose acetate filters with 0.45 µm pore size (Sartorius).	Whole filters were stored at -20°C.	Firstly, filters washed with 1350 µL of absolute ethanol + 150 mL of sodium acetate.	DNA Purification kit from Blood protocol (QIAamp DNA Mini Kit).
(Evans *et al.* 2016)	250 mL	Cellulose Nitrate Membrane Circle, 0.45 µm pore size, 47 mm (Whatman)	Filters stored in 1.5 mL vial completely emerged by absolute ethanol, then stored at -20°C.	Following the purification protocol by Nexxtec & Qiagen.	DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit) & Nexttec™ 1- Step Tissue & Cells.
(Goldberg *et al.* 2011)	1L	Cellulose Nitrate Membrane Circle, 0.45 µm pore size, 47 mm (Whatman)	Filters stored in 1.5 mL vial completely emerged by absolute ethanol, then stored at -20°C.	Following the purification protocol by Qiagen.	DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit).
(Goldberg *et al.* 2011)	2L	Cellulose Nitrate Membrane Circle, 0.45 µm pore size, 47 mm (Whatman).	Filters stored in 1.5 mL vial completely emerged by absolute ethanol, then stored at -20°C.	Following the purification protocol by Qiagen.	DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit.)
(Jerde *et al.* 2011)	1L	Advantec Grade GA55 Borosilicate Glass Fiber Filters, 47mm diameter, 0.6 µm pore size.	No preservation buffer used, stored at -20°C in 1.5 mL vial.	Following the purification protocol by Qiagen.	DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit).
(Evans *et al.* 2016)	45L	peristaltic pump (nominal flow of 1.67 L.min -1) with a filtration capsule (Envirochek HV 1 µm, Pall Corporation, Ann Arbor, MI, USA)	capsule was filled with a preservation buffer (Tris–HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1% with pH 7.5–8), and stored at 4°C in the dark.	Following the purification protocol by Qiagen	Qiagen blood and tissue
(Evans *et al.* 2017)	250ml	1.2-lm pore size Isopore TM polycarbonate membrane filters (EMD Millipore Corporation, Billerica, MA, USA	Filters were placed in 2-mL tubes with 700uL of CTAB buffer and stored at -20°C until further processing.	DNA was precipitated in isopropanol and salt	DNA extractions followed a CTAB protocol where chloroform dissolves filters

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(Hänfling *et al.* 2016)	2l	0.45-lm cellulose nitrate membrane filters and pads (47 mm diameter; Whatman, GE Healthcare, UK)	-	-	PowerWater DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, USA)
(Lim *et al.* 2016)	630ml	not filtered, collected using Van Dorn horizontal water samplers	Water was poured into 50 ml tubes containing 33 ml of absolute ethanol and 1.5 ml of 3 M sodium acetate. Samplers were kept on ice until arrival at the laboratory, where they were stored at −80°C	-	CTAB extraction
(Valentini *et al.* 2016)	600ml/100l	filtration capsule (Envirochek HV 1um; Pall Corporation, Ann Arbor, MI,USA)	Filtration capsules were transported at 4°C to the extraction room and then stored at -20°C.	Following the purification protocol by Qiagen	modified Qiagen blood and tissue
(Shaw *et al.* 2016)	1l	47 mm, 0.45µm pore size nitrocellulose membrane filters (Merck Millipore, Billerica, Massachusetts)	-	-	MoBio PowerSoil DNA Isolation kit

4.2.2. DNA extraction

Different DNA extraction kits have been used for extraction and purification of eDNA from water samples (e.g. Hänfling *et al.* 2016; Lim *et al.* 2016; Valentini *et al.* 2016; Shaw *et al.* 2016). Of these, MoBio (now QIAgen) PowerSoil and PowerWater appear more effective at removing inhibitors during the extraction process, although other extraction kits can be used followed by clean-up steps for inhibitor removal.

Below we detail the different extraction protocols used for eDNA extraction and purification from water samples, as well as the protocols used to extract DNA from fish tissue (scales) and macroinvertebrates used to validate in vitro the primers used on eDNA water samples.

DNA extraction from water samples (UNIOVI/SU)

DNA extraction was carried out with the PowerWater® DNA Isolation Kit (QIAGEN laboratories) following manufacturer's instructions. The eDNA extraction was done in a separate laboratory unit inside a PCR laminar flow cabinet treated with ultraviolet light, where no tissue samples had ever been handled. The process was done using filter tips, to avoid contamination of the extraction kit and between samples.

To recover precipitated DNA in 15 ml samples, tubes were centrifuged to create a DNA pellet. The supernatant was discarded and the remaining pellet was air-dried before being subjected to DNA extraction. eDNA extraction was performed using Qiagen® DNeasy Blood and Tissue Kit (Qiagen, UK), following the manufacturer's instructions, apart from a reduction in the elution volume from a single elution step of 200 µl to two elution steps of 50 µl to maximise DNA yield.

DNA extraction from tissue samples (UNIOVI)

Scales and muscle

DNA was extracted from two scales, or approx. 1mm^3 of muscle tissue per individual following a Chelex resin-based protocol (Estoup *et al*. 1996).

Macroinvertebrates

DNA was extracted from each individual using a specific kit for mollusc and arthropods, the E.Z.N.A.® Mollusc DNA Kit from Omega Bio-tek. Prior to perform the DNA extraction, the tissue was excised from the thorax to prevent contamination with DNA from the gut content.

4.3. Design of specific primers (UNIOVI/SU)

Barcoding and Metabarcoding primer design

Programs and packages such as PrimerMiner (Elbrecht and Leese, 2017a); ecoPCR (Ficetola *et al.* 2010) and, ecoPRIMERS (Riaz *et al.* 2011) can be used to assist with primer design for eDNA barcoding and metabarcoding as they offer the ability to scan a large databases, selecting highly conserved primers among a training set of sequences and testing an amplified region for its capacity to discriminate among taxa. These programs are used 'in silico' to test the efficacy of available primers as well as creating project-specific primers using publicly available sequences. The primers can then be tested in vivo on mock communities to check for primer bias. A list of vertebrate, fish and invertebrate primers for eDNA-metabarcoding including mitochondria RNA genes 12S and 16S, and mitochondrial protein coding COI obtained either by ecoPRiMERs and/or used in barcoding and meta-barcoding studies targeting those taxa are listed in 5.1and 0.

We selected the best working primers to be tested in the field (WP4) (section 3)

Species-specific and family specific primer design

Primers may be designed from specific software such as PRIMER BLAST or PRISE, but may be designed manually as well. In the latter case, the 16S rRNA or COI (cytochrome oxidase subunit I) genes were chosen as target regions for the design of the primers based on the abundance of reference nucleotide sequences from GenBank (www.ncbi.nlm.nih.gov/). Sequences of these genes (either individual 16S DNA and COI sequences or complete mitochondrial genomes) for the target species and other species of a wide range of aquatic taxa were downloaded from GenBank and aligned with the ClustalW application included in BioEdit (Thompson *et al.* 1994). Polymorphisms were analyzed with the DNASP software (Rozas *et al.* 2003). The different haplotypes were visualized employing the BioEdit Sequence Alignment Editor software (Hall, 1999). Within the 600 nucleotide amplicon obtained with the universal primers designed by Palumbi *et al.* (1991) or Geller *et al.* (2013), we searched for regions conserved within each of the target species (identical in all sequences of that species) but different in the rest of species. These regions were used to design the sets of specific primers.

The specificity of the new primers was validated in silico by running comparisons across GenBank's international databases using the National Center for Biotechnology Information's BLASTn program (NCBI, http://www.ncbi.nlm.nih.gov/). We focused this analysis on highly similar sequences (Megablast) with voucher specimens and defined our parameters for short input sequences.

Specific primers for qPCR were designed using Primer3 software, tested in silico using Beacon Primer Designer (ver. 2.1, PREMIER Biosoft), and checked for cross-amplification using NCBI Primer-BLAST (Ye, McGinnis & Madden 2006).

The different primers developed for detection of salmonids, invasive fish, native and invasive invertebrates and macroinvertebrate families in European freshwaters using PCR, as well as the primers for quantification of trout DNA using Real Time PCR are listed in section 5.2. and 5.3.1.

4.4. Metabarcoding- NGS analysis from river water eDNA. Illumina platform.

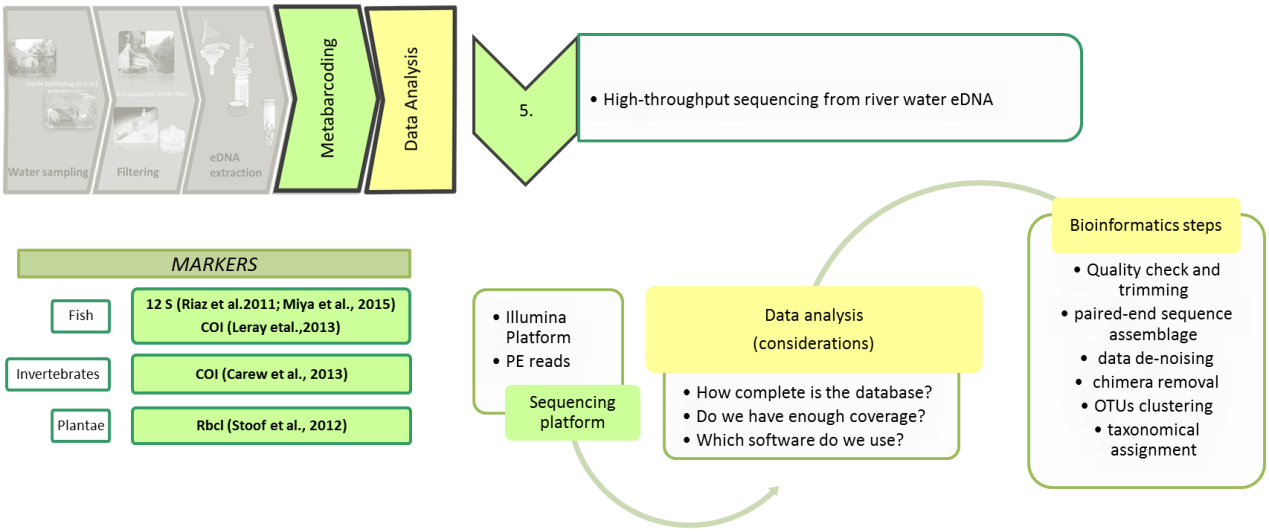

Figure 6. High-throughput sequencing: summary and important considerations.

4.4.1. PCR amplification

18S rDNA amplicons

PCR amplifications were undertaken on an Eppendorf Mastercycler (Eppendorf, Germany) in a total volume of 35 µl using 18 µl of AmpliTaq Gold® 360 PCR Master Mix (Life Technologies, USA), 5 µl of AmpliTaq PCR Enhancer (Life Technologies, USA), 2 µl of BSA, 1 µl of each primer and 3 µl of template DNA. Reaction cycling conditions were: 95°C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and a final extension of 72 °C for 8 min. Negative and positive controls were included in any instance.

The amplification success was visually assessed on 1.5% agarose gel. For samples, where no visible bands detected, additional PCRs were run adjusting DNA concentration.

COI amplicons

PCR amplifications were undertaken on an Eppendorf Mastercycler (Eppendorf, Germany) in a total volume of 53 µl using 25 µl of MyTaq™Red Mix (Bioline, USA), 2 µl of each primer and 3 µl of template DNA. Reaction cycling conditions were: 95°C for 1 min, followed by 35 cycles of 95 °C for 15 s, 46 °C for 15 s, 72 °C for 10 s, and a final extension of 72 °C for 3 min. Negative and positive controls were included in any instance. The amplification success was visually assessed on 1.5% agarose gel. For samples, where no visible bands detected, additional PCRs were run adjusting DNA concentration.

16S rRNA amplicons

Reaction conditions for the first PCR amplification consisted of an initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s, then a final elongation at 72°C for 5 min, using 12.5 ng genomic DNA, 0.2 µM of primers and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, London, UK) in a total volume of 25 µl. Negative and positive controls were included in all cases.

The amplification success was visually assessed on 1.5% agarose gel. For samples where no visible bands detected, additional PCRs were run adjusting DNA concentration.

4.4.2. Library preparation and sequencing

18S and COI amplicons were purified using the AMPureTM XP system (Agencourt, USA) and quantified using the QuBit BR dsDNA kit (Invitrogen, USA), diluted to a concentration of 3 ng/µl and sent to New Zealand

Genomics Limited (University of Auckland) for library preparation and sequencing. Sequencing adapters and sample-specific indices were added to each amplicon via a second round of PCR using the Nextera™ Index kit (Illumina™). Amplicons were pooled into a single library and paired-end sequences (2 × 250) generated on a MiSeq instrument using the TruSeqTM SBS kit (Illumina™). Sequence data were automatically demultiplexed using MiSeq Reporter (v2) (Illumina, http://www.illumina.com/systems/miseq/software/miseq-reporter.html), and forward and reverse reads assigned to samples.

16S amplicons were purified with Agencourt Ampure XP beads (Beckman Coulter) then used as template for the second PCR reaction to add indexed sequencing adaptors to each library (Nextera XT Indices, Illumina). The reaction conditions used were the same as before, but using eight cycles, and a total reaction volume of 50 µl. The final product (420 bp) was verified and size selected from a 2 % agarose gel, and purified with Ampure XP beads. All samples were quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, UK) and pooled in equal concentrations, before sequencing using an Illumina MiSeq at Swansea University (300 bp PE reads).

4.5. Metabarcoding-NGS analysis from river water eDNA. PGM (Ion Torrent) platform.

4.5.1. PCR amplification

18S gene amplicons

The amplification reaction was performed in a total volume of 20 µl, including Green GoTaq® Buffer 1X, 2.5mM $MgCl_2$, 0.25mM dNTPS, 20 pmol of each primer, 200 µg/mL of BSA, 2µl of template DNA or 4 µl of DNA extracted from water samples in case of metabarcoding, and 0.65 U of DNA Taq polymerase (Promega). PCR conditions were 95◦C for 3min, followed by 30 cycles at 94◦C for 30 s, 55◦C for 30 s and 72◦C for 90 s, and a last step of elongation at 72 ◦C for 8 minutes.

COI gene amplicons

The amplification reaction was performed in a total volume of 20µl, including Green GoTaq® Buffer 1X, 2.5mM $MgCl_2$, 0.25mM dNTPS, 20 pmol of each primer, 200 µg/mL of BSA, 2µl of template DNA or 4 µl of DNA extracted from water samples in case of metabarcoding, and 0.65 U of DNA Taq polymerase (Promega). PCR conditions were 95◦C for 1 min, followed by 35 cycles at 95◦C for 15 s, 46◦C for 15 s and 72◦C for 10 s, and a last step of elongation at 72 ◦C for 3 minutes.

4.5.2. Library preparation and sequencing

Amplicons were purified and quantified using the QuBit BR dsDNA kit (Invitrogen, USA) and the Bioanalyser 2100 (Agilent Technologies, USA) diluted to a concentration of 26pmol and sequencing at University of Oviedo Sequencing Facilities. Amplicons were pooled into a single library on an Ion PGM instrument using the Ion PGM™ Hi-Q™ View Sequencing Kit on an Ion 318™ Chip Kit v2 BC. Sequence data were automatically de-multiplexed using Ion Torrent Server (ThermoFisherScientific) https://www.thermofisher.com/order/catalog/product/4483643.

4.6. PIPELINES

4.6.1. Barcoding analysis of river invertebrates

Pipeline summary

The invertebrate sequences produced from Sanger methodology (ABI Prism 3100) using the universal primers described in 0 were edited with Sequence Scanner Software v1.0 (Applied Biosystems).

Each sequence was assigned to a species by comparison with public DNA databases (GenBank, https://www.ncbi.nlm.nih.gov/genbank/) using the BLAST tool including in the NCBI webpage (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the following settings: best match with minimum E-value 1e-50, 99% identity, 99% coverage.

Pipeline results

Macroinvertebrates were taxonomically identified down to a Family level, as recommended by EU Water Framework Directive 2000/60/CE. The results revealed that the COI-coding DNA region was able to assign 87.8% of the samples to the correct family as identified *de visu* by taxonomic experts. The 18S rDNA region assigned correctly 83.8% of the same samples. Failures to assign were due to PCR failure (4.1% and 5.4% samples for COI and 18S, respectively), assignment to upper taxonomic levels such as Order, Class, Kingdom (8.1% and 10.8% for COI and 18S respectively).

Assignment down to a species level was achieved for 83.9% and 67.7% macroinvertebrate samples employing COI and 18S amplified regions, respectively.

4.6.2. Metabarcoding - NGS analysis from river water eDNA

Standardization of bioinformatics in a pipeline can ensure quality and reproducibility of findings; however, some level of customization is required across studies. Customization is needed to compensate for advances in sequencing technology, software workflows and the question being addressed. Reaching an absolute consensus for the approaches and software used is not necessary as these will always be in flux (Deiner *et al.* 2017). Therefore, several bioinformatics pipelines (e.g. QIIME, OBItools, mothur) can be tested following the same steps involving paired-end sequence assemblage, data de-noising, chimera removal, clustering into OTUs and taxonomical assignment (Figure 6).

We give below details of the application and results on macroinvertebrate metabarcodes using 18S and COI markers obtained from two different pipelines (Illumina MiSeq platform & VSearch tool and PGM Ion Torrent platform & QIIME tool).

4.6.2.1. Pipeline 1: Illumina MiSeq platform & VSearch tool

Pipeline summary

1. PCR amplification of the target region using universal primers modified to include IlluminaTM overhang adaptors
2. Purification, quantification and dilution of amplicons to the equimolar concentration as required for sequencing the multiplexed samples.
3. Preparation the sequencing library by adding sequencing adapters and sample-specific indices to each amplicon.
4. Generation of paired-end sequences on MiSeq Illumina® platform.

5. De-multiplexing and quality check of the sequence data.

6. Bioinformatic analysis of metabarcoding data using VSEARCH tool (Rognes 2015), involving paired-end sequence assemblage, data denoising, chimera removal, clustering into OTUs and taxonomical assignment

Quality check

For Quality Check (QC) analysis, three different tools were tested: SolexaQA++, fastQC and fastQscreen. A summary of each of these tools is provided below.

I) SolexaQA++

Website: http://solexaqa.sourceforge.net/

As each sequence generates 7 files for each read direction, there are 14 files per sample (including the 'Undetermined' sequences). For a given read a default number of 10000 sequences are taken per file, and analysed with the programme. Different kinds of outputs are generated: Heatmap, Quality, Cumulative, either as a PDF or a png file, along with the appropriate text file that made it. Data are reported in terms of error probabilities, rather than the NGS equivalent of Phred scores that some other tools use.

Heatmap: This shows the quality on a cycle by cycle basis for each of the 28 tiles in the MiSeq run as a heatmap using a black body radiation scale. They are virtual tiles that are captured by the camera for analysis. Good data is white, bad data goes through orange to black. As an additional check, the tile fraction (calculated as the number of reads in each tile over the total number of reads) is shown next to the tile name.

Quality: The same data as above are shown here, but as a graphical chart on a cycle by cycle basis. The qualities for individual tiles are shown as thin lines and the average by a red dot. Plot shows distribution of the longest contiguous sequence where the base quality is better than the value chosen for analysis. The default value is 0.05, approximately equal to a Phred score of ~13. *Cumulative*: A line graph showing the cumulative frequency of trimmed read lengths, with the perfect result shown as a dotted line. This graph allows you to see what fraction of your data is at a given length or longer.

II) fastQC

Website: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

The analysis in FastQC is performed by a modular set of analysis (Modules: Per Base Sequence Quality, Per Sequence Quality Scores, Per Base Sequence Content, Per Base GC Content, Per Sequence GC Content, Per Base N Content, Sequence Length Distribution, Duplicate Sequences, Overrepresented Sequences, Overrepresented Kmers) and the output report shows a summary of the modules. Giving a quick impression of whether the data has any problems of which you should be aware before doing any further analysis. A HTML based permanent report is generated.

III) fastQscreen

Website: http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/

The sequences are screened against a set of sequences for checking the level of any potential contamination. Currently, this means screening against *E. coli*, PhiX, yeast, Illumina adapters and cloning vectors. A text file and a graphical png file for each sequence file is generated.

Bioinformatics pipeline

All sequence reads were assessed for quality and trimmed with Phred quality score threshold 30 applied.

The pair-end reads from each sample were merged and filtered (discarding all reads with more than 1 error per assembled read and reads too long and too short compared to the expected amplicon length) and dereplicated into unique sequences.

Singletons were then removed and chimeras were identified and removed in the de-novo mode, using UCHIME algorithm (Edgar *et al*. 2011).

The sequences were clustered at 97% identity threshold.

The resulting OTUs were taxonomically assigned against the Protist Ribosomal 2 (PR2) database (18S) and BOLD database (COI), using RDP classifier requiring at least 80% confidence score (Wang *et al*. 2007).

Pipeline-1 results

Following the pipeline described above (molecular and bioinformatics), the COI Metabarcode OTU profile matched well the biota profile obtained from macroinvertebrate sampling and *de visu* taxonomic assignment, with a higher abundance of Insecta larvae followed by Gastropoda and Clitellata (Annelida). The 18S Metabarcode provided an invertebrate profile strongly biased to Nematoda and Annelida with relatively fewer Insecta OTUs, unlike the *de visu* dataset.

4.6.2.2. Pipeline-2: PGM Ion Torrent platform & QIIME tool

Pipeline summary

1. PCR amplification of the target region using universal primers modified to include PGM sequencing adapters and sample-specific indices
2. Purification, quantification and dilution of amplicons to the equimolar concertation as required for sequencing the multiplexed samples.
3. Template preparation using the Ion PGM™ Hi-Q™ View OT2 Kit.
4. Sequencing on an Ion 318™ Chip Kit v2 BC using the Ion PGM™ Hi-Q™ View Sequencing Kit
5. Generate paired-end sequences on Ion-PGM platform.
6. De-multiplexing and quality check of the sequence data.
7. Bioinformatic analysis of Metabarcoding data using QIIME tool (Caporaso et al 2010), involving quality and length trimming and blast taxonomical assignment.

Quality check

For Quality Check (QC) analysis fastQC was used.

Bioinformatics pipeline

All sequence reads were trimmed to remove primers and adaptors, and filtered for a mean quality score > 20 and length >200bp.

The sequences were taxonomically assigned against NCBI GenBank database using BLAST tool requiring 90% identity and an E-value 10^{-50}

Pipeline-2 results

From Pipeline-2, the Metabarcode profile obtained from COI gene matched much better the real composition of river macroinvertebrate communities (sampled conventionally) than the Metabarcode obtained from 18S rDNA, as it was determined when applying Pipeline-1.

The results obtained from Pipeline-2 matched better the macroinvertebrate community inventoried from conventional sampling than those obtained from Pipeline-1. Especially for the COI Metabarcode, taxonomic assignments at family level were more accurate (coincident between *de visu* and Metabarcoding methods), and more assignments were done at a species level.

4.7. Targeted approach -PCR protocols

GENERAL: PCR conditions on eDNA samples

The PCR reactions were prepared in the pre-PCR room inside a PCR cabinet treated with ultraviolet light. Once every sample was ready, closed and inside the PCR machine, the positive control was added in the post-PCR room and put into the machine, to avoid any contact between tubes with samples and with positive control. In every step, negative controls were added to ensure the samples were contamination free.

Salmonid-specific marker (UNIOVI). Published in Clusa *et al.* (2017)

First PCR:

The amplification reaction was performed in a total volume of 20 µl, including Green GoTaq®Buffer 1X, 2.5mM $MgCl_2$, 0.25mM dNTPS, 1µM of each primer (section 5.2.1), 4 µl of template DNA, 200ng/µl of BSA (bovine serum albumin) and 0.65 U of DNA Taq polymerase (Promega). PCR conditions were the following: an initial denaturation step at 95ºC for 5 minutes, 50 cycles at 94ºC for 1 minute, annealing at 55ºC for 1 minute and elongation at 72ºC for 1 minute. A final step of elongation was set at 72ºC for 10 minutes. Both negative control with only distilled water and positive control with *S. salar* DNA from tissue were included.

The product of the first PCR was used as template for the nested-PCR, that amplifies a smaller fragment of the 16S rRNA gene with the Salmonidae specific primers.

Nested PCR:

The nested PCR amplification with the pair of Salmonidae-specific primers was performed in a total volume of 20 µl, including Green GoTaq® Buffer 1X, 2 mM $MgCl_2$, 0.25mM dNTPS, 1µM of each primer (section 5.2.1), 200ng/µl of BSA, 0.5µl of PCR product from the previous 16S amplification as template and 0.65 U of DNA Taq polymerase (Promega). When the first PCR product was too concentrated (>50ng/µl), 1/10 or 1/50 dilutions were performed prior the Nested-PCR. The PCR conditions were the following: an initial denaturation step at 95ºC for 5 minutes, 35 cycles at 94ºC for 30 seconds, annealing at 68ºC for 30 seconds and elongation at 72ºC for 30 seconds. A final step of elongation was set at 72ºC for 10 minutes.

In nested PCR two negative and two positive controls were included, one negative with only distilled water and another negative using as template the PCR product from the negative control in the first PCR. The same was done with the positive controls.

PCR products were visualized in 2% agarose gels with 2.5µl of SimplySafe™.

Taxon-specific markers for invasive fish in European freshwaters (UNIOVI)

The amplification reaction with the specific markers was performed in a total volume of 20µl, including Green GoTaq® Buffer 1X, $MgCl_2$, 0.25mM dNTPS, 1µM of each primer (Table 6; section 5.2.2), 6µl of

template DNA, 200ng/µl of BSA and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95ºC for 5min, 45 cycles at 94ºC for 30s, annealing at the temperature of choice (Table 2) for 30s and elongation at 72ºC for 30s and a final step of elongation at 72ºC for 10 min.

Table 2. PCR amplification conditions for the taxon-specific primers developed for detection of invasive fish. Detection limit is given as taxon's DNA concentration in water samples.

Taxon	Primers	Annealing Temperature	[Mg^{2+}]	Detection limit
Gambusia sp (G. holbrooki and *G. affinis)*	Ga-16S-F Ga-16S-R	68°C	1mM	0.89 pg/ml
Micropterus salmoides	MiSa-16S-F MiSa-16S-R	68°C	2mM	5.7 pg/ml
Ameiurus sp (A. melas and *A. nebulosus)*	Am-16S-F Am-16S-R	65°C	1mM	140 pg/ml
Pseudorasbora parva	PsPa-16S-F PsPa-16S-R	58°C	2,5mM	100 pg/ml
Lepomis gibbosus	LeGi-16S-F LeGi-16S-R	68°C	1mM	0.89 pg/ml
Carassius sp (C. auratus, C. gibelio, C.carassius)	Ca-16S-F Ca-16S-R	58°C	2mM	100 pg/ml

Taxon-specific markers for detection of invasive invertebrates in European freshwaters (UNIOVI)

The amplification reaction with the specific markers was performed in a total volume of 20µl, including Green GoTaq® Buffer 1X, $MgCl_2$, 0.25mM dNTPS, 1µM of each primer (Table 9; section 5.3.2), 6µl of template DNA, 200ng/µl of BSA and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95ºC for 5min, 45 cycles at 94ºC for 30s, annealing at the temperature of choice (Table 3) for 30s and elongation at 72ºC for 30s and a final step of elongation at 72ºC for 10min. The protocol for the PCR is the same for all the taxa, except for *Potamopyrgus* sp, where the different steps of each cycle is 1 minute long, described in Clusa *et al.* (2016).

Table 3. PCR amplification conditions for the taxon-specific primers developed for invasive invertebrate species. Detection limit is given as taxon's DNA concentration in water samples.

Taxon	Primers	Annealing Temperature	[Mg^{2+}]	Detection limit
Potamopyrgus sp (P. antipodarum, P. estuarinus) Clusa *et al.* 2016	16SPA-R 16SAr	60°C	2.5mM	860 pg/ml
Corbicula sp (C. fluminea, C. fluminalis)	CoFl-16S-F CoFl-16S-R	55°C	2 mM	375 pg/ml
Melanoides tuberculata	MeTu-16S-F MeTu-16S-R	58°C	2 mM	3000 pg/ml
Sinanodonta woodiana	SiWo-COI-F SiWo-COI-R	68°C	0.5 mM	202 pg/ml
Mytilopsis leucophaeata	MyLe-COI-F MyLe-COI-R	66°C	1 mM	760 pg/ml

Native freshwater macroinvertebrates (UNIOVI).

The reaction was performed in a total volume of 20 µl, including Green GoTaq®Buffer 1X, 1 OR 1.5 mM $MgCl_2$ (see Table 4), 0.25mM dNTPS, 1µM of each primer (Table 8; section 5.3.1), 4 µl of template DNA, 200ng/µl of BSA (bovine serum albumin) and 0.5 U of DNA Taq polymerase (Promega). PCR conditions were

the following: an initial denaturation step at 95ºC for 10 minutes, 55 cycles at 95ºC for 20 seconds in *A. pallipes* or 10 seconds for *M. margaritifera*, annealing at 65ºC for 20 seconds for *A. pallipes* or at 60ºC for 10 seconds in the case of *M. margaritifera*, and elongation at 72ºC for 30 seconds in *A. pallipes* or 20 seconds for *M. margaritifera*, and a final step of elongation was set at 72ºC for 10 minutes (Table 4). Both negative control with only distilled water and positive control with DNA from tissue were included in the PCR.

Table 4. PCR amplification conditions for the taxon-specific primers developed for native freshwater invertebrates. Detection limit is given as taxon's DNA concentration in water samples; Temp=Temperature.

Taxon	Primers	Denaturation		Annealing		Elongation		$[Mg^{2+}]$	Detection limit
		time	*Temp.*	*time*	*Temp.*	*time*	*Temp.*		
Austropotamobius pallipes	ApalFCOI1	20 s	95°C	20 s	65°C	30 s	72 °C	1 mM	26 µg/ml
	ApalRCOI1								
Margaritifera margaritifera	MarMa_16S1.1	10 s	95°C	10 s	60°C	20 s	72 °C	1.5 mM	39.8 µg/ml
	MarMa_16S1.2								

4.7.1. Targeted approach-qPCR Protocols.

Quantification of trout DNA from water samples (UNIOVI). Published in Fernandez *et al.* (2017)

Quantitative PCR on eDNA was performed using 7900HT Fast Real-Time PCR System (Life Technologies, Inc., Applied Biosystems). Amplification reaction mixtures for *S. trutta* included: 10µl of TaqMan Environmental Master Mix 2·0 (Life Technologies, Inc., Applied Biosystems), 0.4µl of each primer (final concentration of 0·2 µM), and 0.4 µl TaqMan probe (final concentration of 0·2µM), and DNA template (6µl of eDNA extracted from water samples), up to a final 20µl volume. Amplification reaction mixtures for *O. mykiss* included: 10µl of TaqMan Environmental Master Mix 2·0 (Life Technologies, Inc., Applied Biosystems), 0.6µl of Forward primer (final concentration of 0·3µM), 1.2µl of Reverse Primer (final concentration of 0·6µM) and 0.5 µl of TaqMan probe (final concentration of 0·25µM) and DNA template (6µl of eDNA extracted from water samples), also up to a final 20µl reaction volume. Primer and probes sequences are provided in Table 7 (section 5.2.3) Indications from Gustavson *et al*. (2015) and Wilcox *et al*. (2015), respectively, were followed. The qPCR was run at 95◦C for 10 min, for activation of the hot-start polymerase, followed by 35 cycles at 95◦ C for 15s and 60◦ C for 1min.

PCR amplicons were generated with the two primer sets from tissue DNA in a total volume of 20µl, including Green GoTaq® Buffer 1X, $MgCl_2$, 0.25mM dNTPS, 0.25µM of each primer, 4µl of template DNA and 0.65 U of DNA Taq polymerase (Promega). PCR conditions were 95◦C for 5min, followed by 35 cycles at 94◦C for 30 s, 57◦C for 30 s and 72◦C for 30 s, and a final step of elongation at 72 ◦C for 10 minutes.

The PCR amplicons obtained were quantified by fluorimetry using Qubit® dsDNA BR Assay Kit. The amount of DNA was transformed into molecules per µl, calculated from the known base composition of the amplicon sequence. A standard curve was constructed including a serial dilution (from $2.34x10^9$ to $2.34x10^3$ molecules/µL for *O. mykiss* and from $6.3x10^9$ to $6.3x10^3$ molecules/µL for *S. trutta*), and used as reference for DNA molecules quantification in water samples.

Identification of invasive macro-invertebrates (SU)

For invertebrate samples, amplifications were carried out in triplicate in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, UK), in 10 µl reactions consisting of 5 µl SsoFast™ EvaGreen® Supermix (Bio-Rad, UK), 0.25 µl each specific primer (Table 10; section 5.3.3), 3.5 µl HPLC water and 2 µl extracted DNA. Amplifications were carried out in triplicate with 15 min of denaturation at 95 °C, followed by 40 cycles of 95 °C for 10 s and 61.5 °C for 30 s. A HRM step was applied to the end of RT-qPCR reactions, ranging

from 55 ºC to 95 ºC in 0.1 ºC increments to assess the consistency of amplicon melt temperature (tm) for both crayfish species. Limit of detection (LOD) and limit of quantification (LOQ) were determined through running a dilution series ranging from 5 ng/µl to 5 x 10^{-7} ng/µl, using DNA pools for every species. Two positive controls per species were added to each plate once all the eDNA samples were loaded and sealed to prevent false positives in the eDNA samples. Two amplification negative controls consisting of HPLC water and two extraction negative controls were also added in the same well location on each plate test for contamination in eDNA samples.

4.7.2. RFLP protocols for salmonid-specific markers (UNIOVI)

This protocol identifies five salmonids present in Europe directly from water samples (Clusa *et al.* 2017). The PCR product amplified with the nested PCR described above is digested with FastDigest enzymes (Thermo Scientific). The digestion reaction was performed in a total volume of 15 µl, including 5 µl of PCR product (approximately 100ng of DNA), 1.5µl of Green Buffer 10X, 0.3µl of Enzyme and 8.2µl H_2O. The incubation time was 10 minutes at 37°C for the *HindIII*, *SchI* and *VspI* enzymes and 10 minutes at 65°C for *TaaI* and *Tru1I*. In Table 5 the diagnostic bands for each species is shown. Bands can be perfectly differentiated in a 2% agarose gel with 2.5 µl of SimplySafe™ running during 1 hour at 80V.

Table 5. Restriction patterns obtained with the enzymes considered for the five salmonid species. Restriction enzyme and its FastDigest code; restriction target; species; pattern of restriction fragments (*Bands*) obtained for each species; restriction fragments obtained for the rest of species. The fragments highlighted in bold are diagnostic to identify each species.

Enzyme	FastDigest	Restriction Site	Species detected	Bands	Rest of species
HindIII	FD0504	AAGCTT	*Salvelinus namaycush*	**231** and **146** bp	377 bp
VspI	FD0914	ATTAAT	*Salvelinus fontinalis*	**222** and **155** bp	377 bp
SchI	FD1374	GAGTC$(N)_5$	*Salmo salar*	**272** and **103** bp	374 y 3 bp
TaaI	FD1364	ACNGT	*Salmo trutta*	**205** and **172** bp	377 bp
Tru1I	FD0984	TTAA	*Oncorhynchus mykiss*	155, 156 and **66** bp	222, 150 and 5 bp

5. MARKERS

5.1. Universal primers

5.1.1. Primers for eDNA-metabarcoding.

Given below are primers 16S, 18S and COI used for Metabarcoding-NGS analysis from river water eDNA in sections 4.4 and 4.5

Target region: eukaryotic V4 region of the nuclear small subunit ribosomal DNA (18S rRNA) gene

Universal primers Uni18SF and Uni18SR (Zhan *et al*. 2013), modified to include IlluminaTM overhang adaptors

Uni18SF: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGGGCAAKYCTGGTGCCAGC

Uni18SR: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GRCGGTA-TCTRATCGYCTT

Target region: Mitochondrial cytochrome oxidase subunit I coding region.

Universal primers COI NexF- mlCOIintF and NexR-jgHCO2198 (Leray *et al*. 2013), modified to include IlluminaTM overhang adaptors

COI NexF- mlCOIintF: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGWACWGGWTGAACWGTWTAYCCYCC

NexR-jgHCO2198: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TAIACYTCIGGRTGICCRAARAAYCA

Target region: Mitochondrial 16S rRNA region **(12S-V5)*.

Universal primers Vertebrate1 (Riaz *et al.* 2011):

Vertebrate 1F: ACTGGGATTAGATACCCC

Vertebrate 1R: TAGAACAGGCTCCTCTAG

Target region: Mitochondrial cytochrome oxidase subunit I coding region.

Universal macro-invertebrate primers (Carew *et al.* 2013)

HCO2198: TAAACTTCAGGGTGACCAAAAAATCA

COI LCO1490: GGTCAACAAATCATAAAGATATTGG

Target region: chloroplastidic marker rbcL

Universal plants primers rbcL2 (Palmieri *et al.* 2009) and rbcLa-R (Kress and Erickson, 2007) (Bell *et al.* 2017)

rbcL2_F: TGGCAGCATTYCGAGTAACTC

rbcLa R: CTTCTGCTACAAATAAGAATCGATCTC

Target region: chloroplastidic marker rbcL

Universal plants primers rbcL (Stoof-Leichsenring etal., 2012)

Diat_rbcL_705F AACAGGTGAAGTTAAAGGTTCATAYTT

Diat_rbcL_808R TGT AACCCATAACTAAATCGATCAT

Target region: Mitochondrial 12S rRNA region.

Universal primers Vertebrate1 (Riaz *et al.* 2011):

Vertebrate 2F: TAGAACAGGCTCCTCTAG

Vertebrate 2R: TTAGATACCCCACTATGC

Target region: Mitochondrial 16S rRNA region.

Universal primers Vertebrate1 (Riaz *et al.* 2011):

Vertebrate 3F: CTCCGGTCTGAACTCAGA

Vertebrate 3R: GATGTTGGATCAGGACAT

Target region: Mitochondrial 12S rRNA region.

Universal primers Teleo (Valentini *et al.* 2016):

Teleo_F: ACACCGCCCGTCACTCT

Teleo_R: CTTCCGGTACACTTACCATG

Teleo_blk: ACCCTCCTCAAGTATACTTCAAAGGAC

Target region: eukaryotic Vb1 region mitochondrial 16S rRNA region.

Universal primes with Illumina adaptors:

16s_Vb1_Illumina_F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTGGGATTAGATACCCC

16s_Vb1_Illumina_R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGAACAGGCTCCTCTAG

Target region: Mitochondrial 16s rRNA region

Amplicon region: 250bp

Targeted to vertebrates (Vences *et al.* 2016)

Vert-16S-eDNA-F1:, GACGAGAAGACCCYdTGGAGCTT

Vert-16S-eDNA-R1: GATCCAACATCGAGGTCGTAA

Target region: Mitochondrial cytB region

Expected amplicon region: 460

vertebrate primers (Kocher *et al.* 1989 cited in Hänfling *et al.* 2016)

CytB_L14841: AAAAACCACCGTTGTTATTCAACTA

CytB_H1514 :GCDCCTCARAATGAYATTTGTCCTCAGCDCCTCARAATGAYATTTGTCCTCA

Target region: Mitochondrial 16s rRNA region

Expected amplicon region: 115

Universal metzoa primers (O'Donnell *et al.* 2017)

16s_Metazoa_fwd: GTTACYYTAGGGATAACAGCG

16s_Metazoa_rev: CCGGTCTGAACTCAGATCAYGT

Target region: Mitochondrial 16s rRNA region

Expected amplicon region: 100bp

Fish specific (Shaw *et al.* 2016, modified from Deagle *et al.* 2009)

16S fish-specific F: GGTCGCCCCAACCRAAG

16S fish-specific R: CGAGAAGACCCTWTGGAGCTTIAG

Target region: Mitochondrial 12s rRNA region

Expected amplicon region: 200bp

Targeted to fish (Miya *et al.* 2015)

MiFish-U_F: GTCGGTAAAACTCGTGCCAGC

MiFish-U_R: CATAGTGGGGTATCTAATCCCAGTTTG

Target region: Mitochondrial cytB region

Expected amplicon region: 413

(Burgener and Hübner, 1998, cited in Evans *et al.* 2016)

L14912: AAAAACCACCGTTGTTATTCAACT

H15149c: GCCCCTCAGAATGATATTTGTCCTC

Target region: Mitochondrial 12s rRNA region

Expected amplicon region: 241

(Evans *et al.* 2016)

Am12sf: AGCCACCGCGGTTATACG

Am12sr: CAAGTCCTTTGGGTTTTAAGC

Target region: Mitochondrial 16s rRNA region

Expected amplicon region: 330

(Evans *et al.* 2016)

Ac16sf: CCTTTTGCATCATGATTTAGC

Ac16sr: CAGGTGGCTGCTTTTAGGC

5.1.2. Primers for freshwater vertebrate and invertebrate barcoding

Target region: eukaryotic V4 region of the nuclear small subunit ribosomal DNA (18S rRNA) gene.

Expected amplicon size: 400-600bp.

Universal primers Uni18SF and Uni18SR (Zhan *et al*. 2013)

Forward primer: Uni18S-F AGGGCAAKYCTGGTGCCAGC

Reverse primer: Uni18S-R GRCGGTATCTRATCGYCTT

Target region: Mitochondrial cytochrome oxidase subunit I coding region.

Expected amplicon size: 313bp.

Universal primers mlCOIintF (Leray *et al*. 2013) and jgHCO2198 (Geller *et al*. 2013)

Forward primer: mlCOIintF-GGWACWGGWTGAACWGTWTAYCCYCC

Reverse primer: jgHCO2198-TAIACYTCIGGRTGICCRAARAAYCA

5.2. Specific markers for native and invasive freshwater fish in European waters

5.2.1. Primers for the detection of salmonids using PCR (Clusa *et al.* 2017)

The protocol is designed for nested PCR for higher sensitivity. For this, two primer pairs are employed in two consecutive PCR, the second one on the amplicons obtained from the first PCR. The second PCR can be applied alone, directly on tissue or environmental DNA, but the nested protocol is more sensitive.

First PCR:

Target amplicon: a 567bp fragment within the 16S rRNA gene

Forward primer: 16S-new-F 5'-GCCTGCCCTGTGACTATGG-3'

Reverse 16S-Br universal primer (Palumbi *et al.* 1991) 5'-CCGGTCTGAACTCAGATCACGT-3'

Second PCR: Salmonidae-specific primers

Target amplicon: amplify 377bp fragment within the previous amplicon.

Forward primer: 16S-F-Salm 5'-AAGACCTGTATGAATGGCATC-3'

Reverse primer: 16S-R-Salm 5'-TCGATAGGGACTCTGGGAGA-3'

5.2.2. Primers for invasive fish in European freshwaters using PCR

Target DNA region: Mitochondrial regions 16S rDNA

Table 6. Taxon-specific primers for fish invasive in Europe. Scientific and common names, primer names, primer sequences, expected amplicon size (in base pairs, bp).

Taxon	Common name	Primer	Sequence (5'-3')	Amplicon size
Gambusia sp	Eastern (*G. holbrooki*) and western (*G. affinis*) mosquitofishes	Ga-16S-F	GRAACCAACTGACCCCTGCTT	117bp
		Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	
Micropterus salmoides	Largemouth black bass	MiSa-16S-F	WCATCCCRAAACAAAGGGCY	142 bp
		MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG	
Ameiurus sp	Black (*A. melas*) and brown (*A. nebulosus*) bullhead catfishes	Am-16S-F	CGTCAAGAACYCAGTTRAACT	134 bp
		Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA	
Pseudorasbora parva	Topmouth gudgeon	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	192 bp
		PsPa-16S-R	TTCGTTGATCGACTATGTGT	
Lepomis gibbosus	Pumpkinseed	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	113 bp
		LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA	
Carassius sp	Goldfish (*C. auratus*), crucian carp (*C. carassius*), Prussian carp (*C. gibelio*)	Ca-16S-F	TRAAAACTTTGTGGRAYATGAGA	101bp
		Ca-16S-R	CTCTCTTAGYTTTAGGAAATTYT	

5.2.3. Primers for quantification of trout DNA using Real Time PCR (qPCR).

The protocol is based on quantitative PCR (RT-qPCR) to detect and quantify eDNA from two trout species present in Europe, with high sensitivity. One is native, *Salmo trutta,* and one exotic introduced in Europe, *Oncorhynchus mykiss*. Two specific TaqMan primers and probes are employed.

Table 7. Taxon-specific primers and Taqman probes for Brown trout (*Salmo trutta*) and Rainbow trout (*Oncorhynchus mykiss)* in Europe. Scientific names, primer and probe sequences and expected amplicon size (in base pairs, bp).

Taxon	Primer	TaqMan primers	Probe	Amplicon size
Salmo trutta	F	TTTTGTTTGGGCCGTGTTAGT	6FAM-5'ACCGCCGTCCTCT-3'	61 bp
	R	TGCTAAAACAGGGAGGGAGAGT		
Oncorhynchus mykiss	F	AGTCTCTCCCTGTATATCGTC	6FAM-5'-CCAACAACTCTTTAACCATC-3' - MGBNFQ MGBNFQ	102 bp
	R	GATTTAGTTCATGAAGTTGCGTGAGTA		

- Molecular marker f*or Salmo trutta* is based on a 61bp fragment of the mitochondrial cytochrome oxidase I gene (COI: Cytochrome Oxidase Subunit I) from Gustavson *et al*. (2015).
- Molecular marker f*or Oncorhynchus mykiss* is based on a 102bp fragment within the NADH gene (Wilcox *et al*. 2015*)*.

Both molecular markers were tested first on control DNAs from both species and in a mixture of such DNAs, to discard possible co-amplification or interferences between them. If other salmonid species co-occur in the region where this method is going to be employed, the markers should be tested in DNA mixtures of these salmonids, in order to check possible co-amplification or interferences prior to use the method on eDNA from water samples.

5.3. Specific markers for invertebrate species in European waters

5.3.1. Primers for detection of native invertebrate species in European waters using PCR

Target DNA regions: Mitochondrial regions 16SrDNA, Cytochrome oxidase subunit I gene (COI).

Table 8. Species-specific primers for European native freshwater invertebrates. Scientific and common names, target DNA region, primer names, primer sequences, expected amplicon size (in base pairs, bp).

Taxon	Common name	DNA region	Primer	Sequence (5'-3')	Amplicon size
Austropotamobius pallipes **(UNIOVI)**	Atlantic stream crayfish and white-clawed crayfish	COI	ApalFCOI1	GTT GGG ACA GGG TGA ACT GT	324 bp
			ApalRCOI1	AAC CGG GTC TCC TCC CC	
Margaritiferea margaritifera (Stoeckle *et al*. 2015. Aquatic Conserv: Mar. Freshw. Ecosyst. 26: 1120–1129 (2016)	Freshwater pearl mussel	16S rDNA	MarMa_16S1.1	CAA CCC TGG AAC CGC TAA AG	132 bp
			MarMa_16S1.2	GGC TGC GCT CAT GTG AAT TA	

5.3.2. Primers for detection of invasive invertebrate species using PCR

Target DNA regions: Mitochondrial regions 16SrDNA, Cytochrome oxidase subunit I gene (COI).

Table 9. Taxon-specific primers for invasive freshwater invertebrates. Scientific and common names, target DNA region, primer names, primer sequences, expected amplicon size (in base pairs, bp).

Taxon	Common name	DNA region	Primer	Sequence (5'-3')	Amplicon size
Potamopyrgus sp (P. antipodarum, P. estuarinus)	New Zealand mudsnails	16S rDNA Clusa *et al.* 2016	16SPA-R	TCAAAGATTTTGGATCATAGCT	380 bp
			16SAr	CGCCTGTTTATCAAAAACAT	
Corbicula sp (C. fluminea, C. fluminalis)	Asian freshwater clams	16S rDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	165 bp
			CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	
Melanoides tuberculata	Red-rimmed melania	16S rDNA	MeTu-16S-F	GGTCTRACGAAAGCAATACT	230 bp
			MeTu-16S-R	GCTTTGCTKGATCTAAAYYT	
Sinanodonta woodiana	Chinese pond mussel	COI	SiWo-COI-F	GGGTCAGCCMGGRAGGCTTTTA	258 bp
			SiWo-COI-R	TGTTCACCCTGTACCAACRCCC	
Mytilopsis leucophaeata	Conrad's false mussel	COI	MyLe-COI-F	GGTTGTAACAACGCACGGTTTAG	193 bp
			MyLe-COI-R	CACCTTCTCTGAAAGCCGAGC	
Pacifastacus leniusculus	Signal crayfish	16S rRNA (Robinson *et al.* submitted)	16S_seq1 F	TGACCGTGCAAAGGTAGCAT	376 bp
			16S_seq1 R	CATCGAGGTCGCAAACTTTTT	
Dikerogammarus villosus	Killer shrimp	16S rRNA(Müller, Schramm & Seitz 2002)	LR-J-Gam	ATTTTAATTCAACATCGAGGTTGC	322 bp
			LR-N-Gam	TTTAACGGCTGCGGTATTTTGAC	

5.3.3. Primers for the detection of native and invasive invertebrates using qPCR

Target DNA regions: Mitochondrial regions 16SrDNA, 12S rDNA and Cytochrome oxidase subunit I gene (COI).

Table 10. Taxon-specific primers for invasive freshwater invertebrates. Scientific and common names, target DNA region, primer names, primer sequences, expected amplicon size (in base pairs, bp).

Taxon	Common name	DNA region	Primer	Sequence (5'-3')	Amplicon size
Pacifastacus leniusculus	Signal crayfish	16S rDNA (Robinson *et al.* submitted)	ApalPlen_16S F	AGTTACTTTAGGGATAACAGCGT	84
Austropotamobius pallipes	White-clawed crayfish		ApalPlen_16S R	CTTTTAATTCAACATCGAGGTCG	
Pacifastacus leniusculus	Signal crayfish	12S rDNA	12S F	AGTTACTTTAGGGATAACAGCGT	94
			12S R	TTACAATTAAATCCTCCTTCATAGC	
Eriocheir sinensis	Chinese mitten crab	COI	COI F	TCTTATGCTAGGAGCCCCAGA	150
Eriocheir japónica	Japanese mitten crab		COI R	GCTGCTAAAGGTGGATAGACAGT	
Eriocheir sinensis	Chinese mitten crab	12S rDNA	12S F	GCCAATAAGCAAGGTAAAATGGGT	155
			12S R	AATGAAAGCGACGGGCGATA	

5.3.4. Specific markers for the detection of macroinvertebrate families using PCR

Three macroinvertebrates families were targeted to be detected in environmental samples, since they are part of the preferred families consumed by salmonids.

- **Rhyacophilidae marker:** from Koester *et al.* 2013. It amplyfies a 315 region of 18S gene.

As there is only one genus in Europe, the genus-specific primer can be used for evidencing the presence of the whole family.

Forward primer: 5'-CTCAAAGCGGGCTAAGTT-3'

Reverse primer: 5'-CCACCGGGTTAAAATAATG-3'

- **Baetidae marker (UNIOVI):**

For this family, we have an in-silico design of a combination of two forward primers, and as an alternative, another degenerated primer. Any of these two alternative forward primers, together with the same reverse primer amplify a 320 bp region of the 28S gene.

Forward primers: 5'-GTCGGGACGCACTAGACCCG-3'

5'-GACGGGATCTGCTAGACCCG-3'

Forward degenerated primer: 5'-GWCGGGAYSYRCTAGACCCG-3'.

Reverse primer: 5'-GGCGCCAGCTATCCTGAGGG-3'

- **Simuliidae marker (UNIOVI):**

The following primer pair amplifies a 300bp fragment of the 18S rDNA region.

Forward primer: 5’-AACGTGATACCTGATCTGAAAGGATTGGG-3’.

Reverse primer: 5’-AGGTTCACCTACGGAAACCTTGTTACG-3’

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