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A Novel Assessment of the Temporal and Abiotic Factors Influencing Environmental DNA Derived from Freshwater Biofilms

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Abstract

Environmental management and understanding of ecosystems requires accurate assessment of biodiversity. Environmental DNA (eDNA) offers a non-invasive and objective biodiversity survey method, focusing on the detection of free cellular material. Despite significant advantages over traditional methods many of the dynamics of eDNA within the water column are still unknown such as the persistence and transport of molecules. So far, most eDNA studies have focused on detecting species in water samples. This study focuses on biofilms which have never previously been used to detect eDNA and offer a novel sampling medium for future studies using eDNA. This study aims to detect macrobial eDNA within freshwater biofilms, model the uptake and persistence of eDNA within biofilms over time and determine environmental factors that affect the spatio-temporal persistence of eDNA. DNA of mayfly (*Ephemera danica), Daphnia* (*D. magna*) and European eel (*Anguilla anguilla*) was added to four experimental mesocosms of lotic freshwater, each associated with different land-use types and pH. Biofilms were collected over 43 hours and eDNA quantified using qPCR with CO1 primers. Only mayfly DNA was detected within the biofilm. The quantity of mayfly DNA had a strong negative relationship with time and a positive association with pH, demonstrating that eDNA degraded quickly over time and faster in mesocosms with lower pH. In conclusion, this study builds on the wider knowledge of how eDNA persists in lotic freshwater and provides a new sampling medium for further eDNA studies.

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1.0 Introduction

Accurate methods of monitoring for rare organisms and biodiversity are crucial to effectively manage and understand endangered ecosystems. A challenge of developing accurate surveying methods has been observer bias and disturbance. Environmental DNA offers a non-invasive alternative that relies on DNA inference as opposed to direct encounter.

1.1 Introduction to Environmental DNA – Micro to Macro

Environmental DNA was initially described for microbial studies to understand the diversity of microorganisms many of which are unculturable in laboratory conditions (Ogram *et al.* (1987). The first use of macrobial eDNA was in 2008, extracting mitochondrial DNA from freshwater to identify the presence of invasive American bullfrogs (*Lithobates catesbeianus)* (Ficetola *et al.*, 2008). Since then similar techniques have been used to detect many macro-organisms, such as fish (Dejean *et al.*, 2011; Jerde *et al.*, 2011; Takehara *et al.*, 2012), insects, crustaceans and mammals (Thomsen *et al.*, 2012). In macrobial studies, eDNA is defined as the genetic material isolated directly from a sampled environment without the presence of an organism (Creer *et al.*, 2016; Taberlet *et al.* 2012).

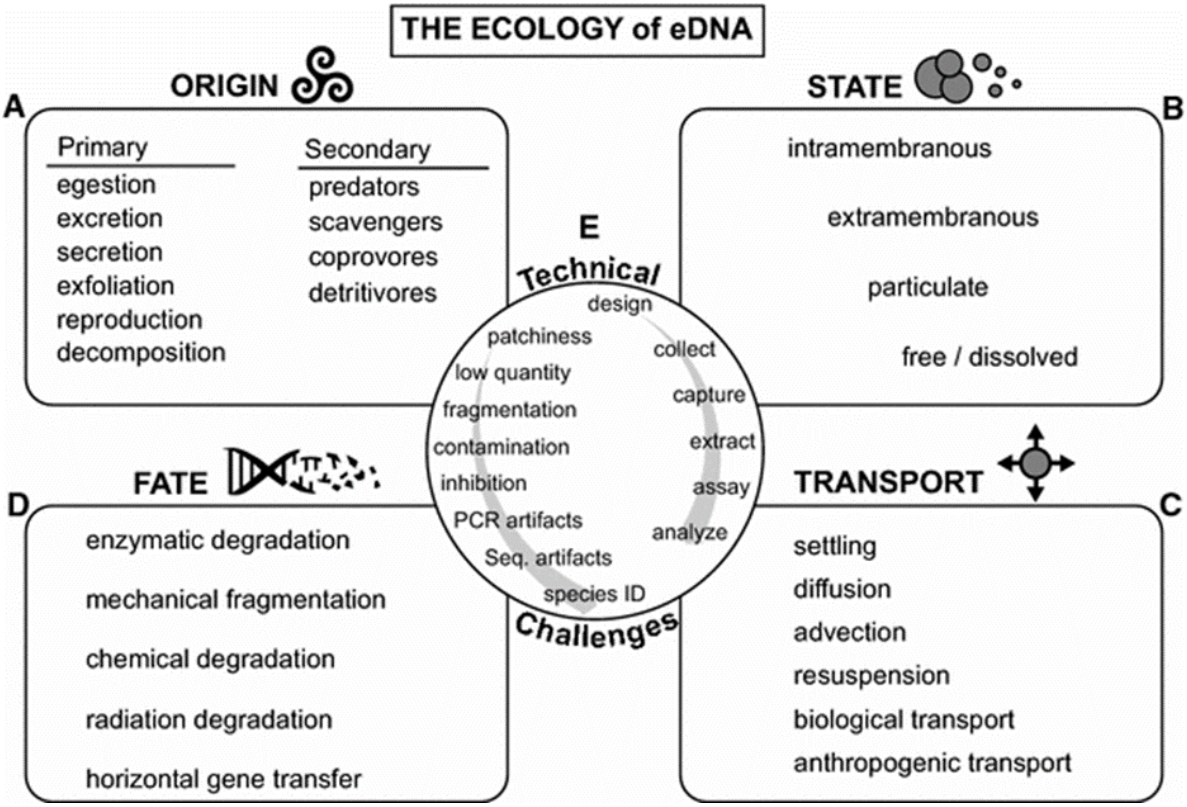


Figure 1: Representation of the important aspects of the ecology of eDNA that require further research split into four sections: Origin (A), State (B), Fate (D) and Transport (C). Central to the graphic (E) are the technical challenges that are common between the four main areas. Taken from Barnes & Turner (2016).

1.2 Ecology of Environmental DNA

The concept that still requires more understanding is how eDNA interacts with its environment in space and time. The major questions about the ecology of eDNA were set out by Barnes and Turner (2016) (Figure 1). The following section further details the most important aspects for the future development and understanding of eDNA: origin, state, fate and transport.

1.2.1 Origin

In macrobial organisms it is understood that eDNA can originate from almost any excretion from the animal, such as mucus, gametes and faeces (Ficetola *et al.,* 2008; Martellini *et al.,* 2005; Anderson *et al.,* 2012). However, it is unknown which excretions are most important in generating a tractable eDNA signal (Barnes & Turner, 2016). It is likely that macrobial organisms shed whole cells that then degrade overtime (Barnes & Turner, 2016). Faeces are likely to be one of the main sources of eDNA from most macrobial organisms in both terrestrial and aquatic environments (Anderson *et al.,* 2012; Thomsen *et al.,* 2012).

Each organism has distinct physiological traits that determine how much DNA is released into the environment, such as body size, metabolic rate and life stage. Dead animals release large quantities of eDNA over time potentially leading to misinterpretation of the actual occurrence of an organism in a location (Foote *et al,* 2012). In the case of rare organisms, the effect of cadavers may be difficult to quantify or rule out. Furthermore, behaviour and seasonality change the concentrations of DNA released (Anderson *et al.,* 2012). For instance, Buxton *et al., (*2017) found that great crested newt eDNA concentrations peaked during adult breeding season and newt larval abundance. Correspondingly, eDNA abundance fell rapidly after the larvae metamorphosed and left the pond.

1.2.2 State

The state of macrobial eDNA is the least explored of the theoretical limitations in the use of eDNA for biodiversity assessment. The term state refers to whether the eDNA captured is within a complete cell, a free-floating organelle or even free DNA strands (Barnes & Turner, 2015). The state of eDNA has a significant impact on the potential methodology used when trying to effectively assess all aspects of eDNA; for example, the choice of filter sizes (Turner *et al.,* 2014).

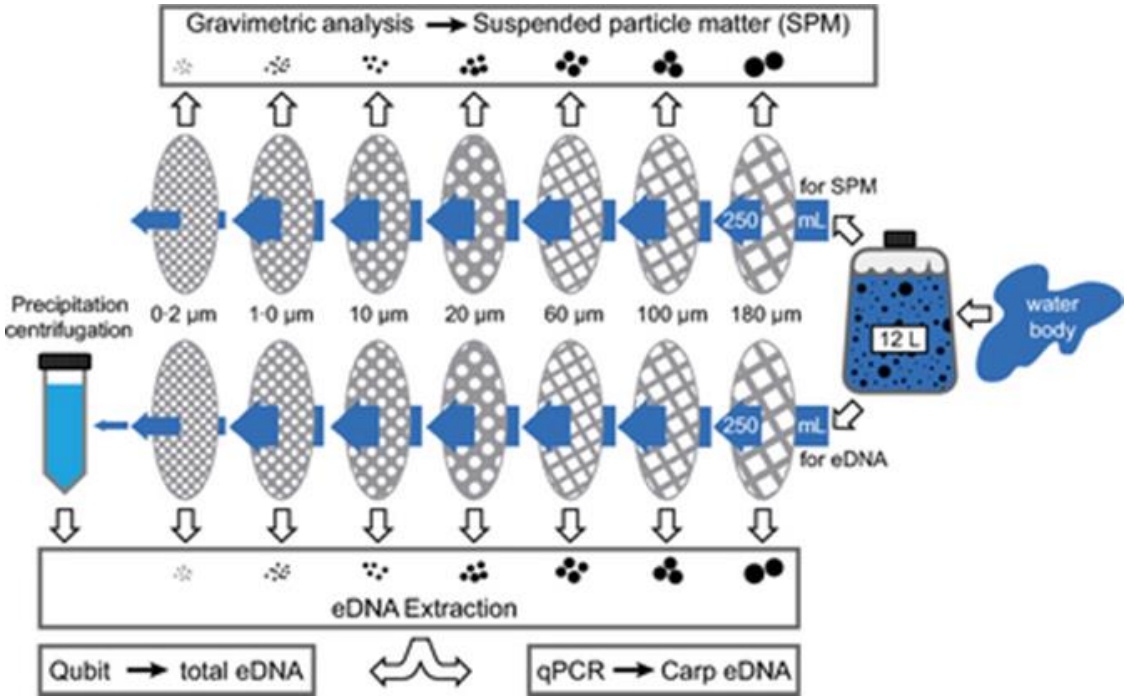


Figure 2: From Turner et al., 2014. Particle size fractionation of 12L of freshwater collected from laboratory controlled tanks containing carp (Cyprinus carpio). The 12L sample was subsampled at 250ml and sequentially filtrated through large to small filters, 180-0.2µm. Ten samples were used for eDNA extraction and analysis and ten were used for gravimetric analysis. 15ml of final eluate (after 0.2µm filter) was subsampled for eDNA analysis.

Turner *et al.,* (2014), demonstrated the importance of state through sequential filtration of water samples targeting carp eDNA (Figure 2). They found that although total eDNA was most abundant at <0.2µm filter size, their target of carp eDNA was most abundant at 1-10µm. This finding suggests that the majority of detectable eDNA is within mitochondria, which in animals are 0.2-1.2µm diameter and 1-8µm in length (Flindt, 2006). On the other hand, Turner *et al.,* (2014), cannot rule out the effect of small cells as nuclear DNA was not part of these experiments.

1.2.3 Persistence

In the context of eDNA, persistence is defined as how long genetic material remains detectable within the environment. In most cases, it is deduced that persistence is affected by a combination of biotic and abiotic factors. However, which factor has the strongest influence is unknown (Foote *et al.,* 2012). Currently, most studies have focused on eDNA persistence in lentic bodies of water and have not recorded potentially important differences in water chemistry, such as pH, that may affect the persistence. There is also evidence that microbial interaction has an important role in degradation of eDNA sources (Torti *et al.,* 2015). Microbial degradation of eDNA is supported by Strickler *et al.* (2015), who found the highest

degradation rate of eDNA in freshwater was when the abiotic factors were likely to promote microbial growth: neutral pH, higher temperatures and moderately strong UV-B.

To explore the effects of microbial interactions with eDNA, there is a need to also understand nutrients that limit microbial growth within aquatic systems and change the ecosystem structure (Glibert, 2012). In the last 100 years humans have had a dramatic effect upon the Earth's nutrient cycles due to the use of fertilizers. Nitrogen is often a limiting factor in many ecosystems and is an essential component of protein synthesis and nucleic acid. Similarly, phosphates are involved in DNA and RNA synthesis and the transfer of energy. If applied incorrectly, nutrients from fertilizers can leach into water bodies and cause uncontrolled algal growth (eutrophication), a major conservation concern (Conley *et al.*, 2009). An important area of research is to know if eDNA persistence is nitrogen dependent as it may affect the uses of eDNA for monitoring species where nitrogen varies between water bodies.

1.2.4 Transport

A working knowledge of transport factors is required to make accurate predictions of organism presence and other ecologically relevant inferences. After DNA is shed from the organism and enters the environment it is subjected to many environmental factors in space and time. Examples of transport include moving water columns in a river transporting fish scales, or wind carrying pollen.

Studies have found that thus far eDNA transport is unpredictable and difficult to model. Most information on the transport of shed organic material was pioneered to test the effects of transgenes from genetically modified crops (Douville *et al.*, 2007). Transport may be roughly split into two types, horizontal and vertical.

Horizontal transport is when the eDNA disperses laterally from point of origin, normally following a prevailing force such as tide or wind. For example, Douville *et al.* (2007) found that a gene from genetically modified crops persisted in a river for 21 to 40 days and could be detected up to 82km downstream, though at a reduced concentration. Similarly, two invertebrate species were detected more than 12km downstream from the lake they were sourced from (Deiner & Altermatt, 2014).

The other form of transport is the settling and resuspension of eDNA particles from water column to substrata. Particle size is a major factor in suspension time. Major sources of eDNA, such as faeces and epithelial cells, are released in particles larger than 1000µm. Turner *et al.* (2014), rarely found suspended particles larger than 60µm, suggesting rapid

breakup or settling. Furthermore, eDNA that does settle in aquatic sediments takes longer to degrade and has a higher concentration than in the water column (Turner *et al.,* 2015).

1.3 Applications of Environmental DNA

Environmental DNA is used for a range of applications for assessing ecosystems and informing conservation management. Mostly, eDNA is used for the biomonitoring of invasive species that could be ecologically and economically damaging. An example of this is the Asian carp (*Hypophthalmichthys molitrix & H. nobilis*) invasion in the United States where eDNA in the water was used to delimit newly invaded frontiers before the Carp were sighted, allowing early intervention measures. (Jerde *et al.,* 2011; Jerde *et al.,* 2013).

On a practical level, the use of eDNA requires less training and taxonomic knowledge, effectively neutralising observer bias that may arise from differing levels of competency (Biggs *et al.,* 2015). Using traditional methods, accurate and skilled surveyors are important for the monitoring of protected species. For example, fish surveys are normally carried out using either seine nets, trawling or snorkelling. However, traditional survey methods are not only limited logistically, but also rely on on-site identification. Molecular techniques employed for detecting eDNA distinguish between species based on DNA as opposed to morphology, which is less subjective and accounts for phenomena such as cryptic species (Bickford *et al.,* 2007). Furthermore, seine and trawling can be destructive and are only suitable for certain seabed types (Thomson *et al.,* 2012). By only taking a small sample of water for later analysis, eDNA effectively removes disturbance which may cause harm to sensitive ecosystems or organisms.

One aspect that eDNA does not outperform traditional methods in is population estimates. Amplification of eDNA is population density dependent which would indicate it may be possible to estimate population size. Although, population estimates have been attempted in several systems the results are inconclusive. A possible confounding factor of estimating populations is that eDNA excretion rates are different for individual species and degradation rates change between locations (Foote *et al.,* 2012; Kelly *et al.,* 2014). For instance, eDNA was used to detect pilot whales to confirm rare sightings, but as excretion rates and cell degradation was unknown there was a chance of eDNA detection from a dead individual or transportation of eDNA over a significant distance (Foote *et al.,* 2012).

1.4 Molecular Detection – Quantitative Polymerase Chain Reaction

Quantitative PCR (qPCR) is a method for quantifying concentration of DNA in a sample by measuring the accumulation of a PCR product. Quantitative PCR is performed using dual labelled hydrolysis probes or fluorogenic dyes (e.g. SYBR Green) which attach to the primer site. When the probe is broken down during the extension phase it produces light that is measured by a camera. The concentration of DNA is then calculated using the standard curve of samples with a known concentration of the target DNA. Alternatively, a flurogenic dye is used, commonly SYBR green, which fluoresces when bound to double stranded DNA. However, SYBR Green is often not appropriate for eDNA studies as signal is also produced for primer dimers and non-specific PCR products (Bustin *et al.,* 2009), likely to be present when extracting DNA directly from the environment.

Quantitative PCR is fast and reproducible without the necessary post-PCR processes of other techniques, reducing likelihood of cross contamination (Heid *et al.,* 1996). In the study of eDNA, qPCR has been essential for methodological studies on degradation and in attempts to understand the relationship between eDNA concentration and biomass (e.g. Dejean *et al.,* 2011; Pilliod *et al.,* 2013; Takahara *et al.,* 2012).

1.5 Sampling Substrates

Extraction of eDNA can be achieved from a variety of mediums including soil, ice cores, fresh water, salt water and stomach contents (See reviews: Taberlet *et al.,* 2012; Thomson & Willerslev, 2015). The main differences between sampling medium is the length of time taken for eDNA to degrade. For instance, eDNA in the water column degrades quickly over time, generally between hours and a couple of weeks (Barnes *et al.,* 2014). On the other hand, eDNA in permafrost can last more than 10,000 years (Willerslev *et al.,* 2003). Slower degradation leads to accumulation of eDNA. For instance, Turner (2015) found that eDNA accumulated in aquatic sediments and was at a much higher concentration when compared to eDNA detected in lentic water. The difference in eDNA degradation times mean that samples deriving from different sources can be used to answer various ecological questions. Aquatic sediments could give a more accurate representation of a water bodies biodiversity but a less up to date record of species currently within that system.

1.5.1 Biofilm

The above discoveries on the ecology of eDNA raise the question of appropriate sampling mediums. For instance, before the eDNA can enter the aquatic sediment it must pass through

the biofilm. Biofilm is a generalised term for the community matrix of algae, archaea and other microorganisms that coat all exposed substrata in aquatic environments (Costerton *et al.*, 1987). The surface is sticky and may be a possible medium for eDNA accumulation and detection. Conversely, many of the microorganisms in biofilms may degrade eDNA over time and hydraulic action of the water may also contribute to degradation. Biofilms may create an eDNA medium that has a high concentration whilst giving an accurate picture of current biodiversity.

1.6 Aims and Objectives

1.6.1 Aim

The aim of this study is to identify the uptake, accumulation and degradation of eDNA from three target species, mayfly (*Ephemera danica*), European eel (*Anguilla anguilla*) and *Daphnia* (*Daphnia magna*) within freshwater biofilms and to improve understanding of the transport of macrobial eDNA within lotic freshwater.

1.6.2 Objectives

1. Extract and amplify (qPCR) mitochondrial DNA (CO1) eDNA in biofilms from a semi-natural river system.

2. Identify environmental factors important to the persistence of eDNA within the biofilms

2.0 Methods

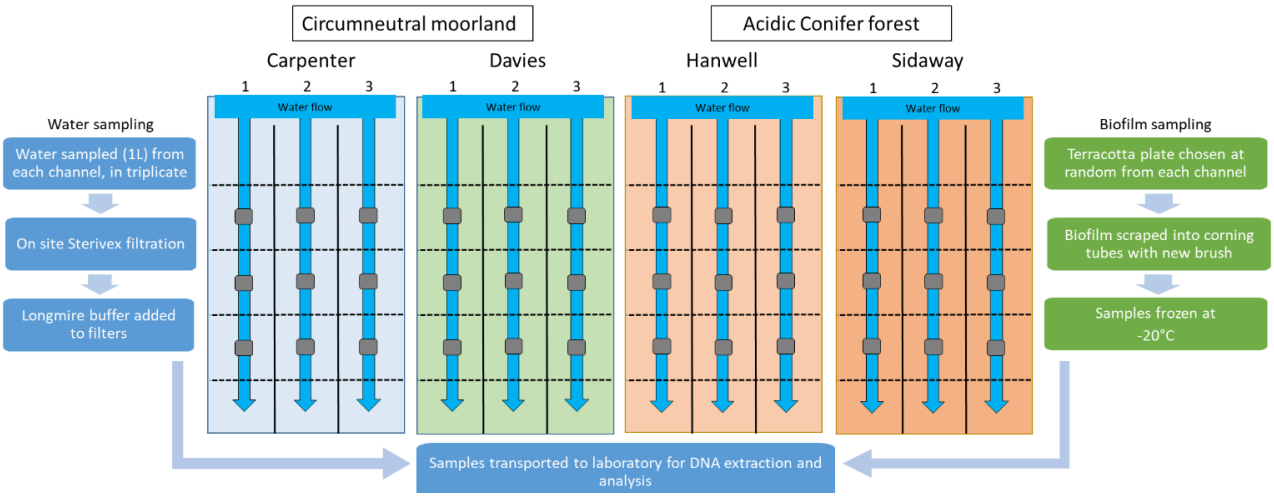


Figure 3: Schematic overview of the study design including sampling work flow for the biofilm eDNA sampling and a sister study that analysed eDNA in water samples. Mesocosms are depicted with their associated names above them. The dotted lines represent 1m channel sections (20m in total for each) in which a single terracotta tile (small brown boxes) was placed for biofilm accumulation. Background colors (blue, green, orange, red) correspond to the natural to acidic gradient of the mesocosms.

2.1 Experimental System

Biofilm sampling took place upstream of the Llyn Brianne reservoir located in the central Welsh uplands (UK; 52°07'57.4"N 3°45'07.8"W) The area has a temperate climate annually ranging between 0-16°C with 1900mm of rainfall (Durance *et al.,* 2007). The location is split into four experimental stream mesocosms named Davies, Carpenter, Hanwell and Sidaway after the land owners (Figure 3). The mesocosms are fed by a natural river in the Tywi catchment (Ormerod & Durance, 2009). The mesocosms covered three types of land. Davies and Carpenter covered circumneutral (soil pH between 6.5 and 7.5) moorland and both Hanwell and Sidaway were covered by acid water conifer forests. Sidaway had recently been logged. Each land type differed in water chemistry and species assemblage. The circumneutral sites (Davies & Carpenter) had an average pH of 6.8-7.2 and the conifer forest sites (Sidaway & Hanwell) had an average pH of 5.3-5.8 (Ormerod & Durance, 2009). The mesocosms are environmentally typical representative of the Tywi catchment and are representative of upland Welsh systems (Ormerod & Durance, 2009; Kowalik *et al.,* 2007; Ormerod *et al.,* 1987). Each mesocosm was made up of 3 channels (20mx20cmx20cm) which were isolated from the river system and a mechanical pump run by a petrol generator circulated water an average of 2m per second for 43 hours (Figure 3).During the experiment the each mesocosm had a total volume of 800L. Total dissolved nitrogen (TDN) and pH were recorded at every time point for each site. Temperature was taken every 15 minutes during the experiment using data loggers (model 650MDS, YSI Inc, USA).

2.2 Experimental Targets

Ephemera danica, *Anguilla anguilla* and *Daphnia magna* were chosen as eDNA targets within the biofilm samples. *E. danica* is a species of mayfly commonly found in freshwater lakes and rivers throughout Europe and the British Isles. *Ephemera danica* was chosen as it is used in a wide range of studies, such as the bioaccumulation of pesticides (Södergren & Svensson, 1973). *Ephemera danica* were collected near Galsbury, UK and kept in microcosms (~100 Individuals/L) at Bangor University two weeks prior to the experiment.

Daphnia magna (water fleas) are used for a variety of ecotoxicology studies and as model organisms (e.g. Biesinger *et al.*,1972). *Daphnia magna* were clones bred in Bangor University (UK) from an individual sourced from Birmingham University (UK). Environmental DNA cultures for *E. danica and D. magna* were collected by sieving

individuals from the water using a 250µm sieve. Collected eDNA water cultures were then stored in sterilized plastic containers for transport to the experiment site.

Anguilla anguilla, is a temperate eel species and is used in this experiment as an example of a larger fish species that is not found in the experimental area. *Anguilla anguilla* water (exposed to Ultraviolet light cleaning treatment before collection) was provided by Cynrig Fish culture unit in the Brecon beacons (UK).

2.3 Experimental set up

In each of the four experimental mesocosms 1L of *E. danica* water and 2L of *A. anguilla* and *D. magna* water were added. Additionally, dissolved organic carbon (DOC) in the form of sucrose solution (>99%) was added to one of the three channels in each mesocosm to stimulate microbial activity and simulate high energy sites (Bernhardt & Likens, 2002).

A Qubit (2.0) fluorometer (Life Technologies, Carlsbad, USA) was used to quantify the eDNA concentration for each species resulting in 5.45 ng/µl (5.45E6 ng/L) for *D. magna*, 7.33 ng/µl (7.33E6 ng/L) for *E. danica* and 1.75 ng/µl (1.5E6 ng/L) for *A. anguilla* (Seymour *et al.,* 2017, in review). The final concentration of DNA when added to the mesocosm resulted in the following dilutions; 1:400 for *A. anguilla* (4375 ng/L) and *D. magna (*18600 ng/L), *E. danica* was diluted 1:800 (9162.5 ng/L). The described concentrations are five orders of magnitude greater than those found in natural rivers (Pilliod *et al.,* 2013; Mächler *et al.,* 2014).

2.4 Sampling

Two weeks prior to the experiment, unglazed terracotta tiles (15cm x 15cm x 5cm) were added at one metre intervals to the experimental streams to allow biofilm to accumulate. Biofilm samples were collected over a period of 43 hours. One sample of biofilm was taken an hour prior to the start of the experiment as a negative control (time point -1). At time point 0, eDNA was added and after 10 minutes a biofilm sample was harvested. Subsequent samples were harvested at 1, 3, 7, 19, 29 and 43 hours after collection of time point 0. At each time point one tile was removed from each channel and the biofilm was removed using a new toothbrush into 50ml corning tubes. A total of 84 samples were collected with an additional 12 collected at time point 120. Samples were stored at -20°C. At each time point, pH, temperature and total dissolved nitrogen were recorded.

All equipment was treated with 10% bleach solution and rinsed in clean water before and after collection of eDNA. Bleach was used to fragment DNA contamination of target species in re-used bottles preventing unwanted amplification in PCR reaction. Collected samples were stored at -20 degrees at Bangor University (Wales, UK) for DNA extraction and analysis.

2.5 DNA extraction and qPCR analyses

Extractions and qPCR were performed in a designated PCR free eDNA laboratory within Bangor University. All extractions and qPCR were carried out in a fume hood that was decontaminated with UV and cleaned with disinfectant wipes, water and bleach water solution after each laboratory session. Pre-extraction, samples were centrifuged at 20,000rpm for 20 minutes and excess water was removed. Extraction of eDNA from the biofilms was carried out using a MoBio PowerMax Soil Isolation kit (Hilden, Germany). The standard protocol was carried out except the final solution (C6) was reduced from 5ml to 500µl to ensure maximum concentration of the final product and to prevent false negatives. Due to similar conditions in the circumneutral moorland sites and limited extraction kits, only 10 samples from Carpenter up to time point 29 were extracted.

Quantitative PCR of extracted eDNA was carried out in triplicate with hydrolysis probes. The primer chosen for *E. danica, D. magna and A. anguilla* quantification was Cytochrome oxidase 1 (CO1) designed by Primerdesign Ltd (Southampton, UK) (Table 4). Reactions were 20µl containing 0.8 µl primer/probe mix (300nM), 9.5µl (2X) PrecisionPLUS Mastermix (Primer Design Ltd.), 2 µl DNA and 7.1 µl DNAse free water. Reactions were run on a QuantStudio™ Flex 6 Real-Time PCR System (Applied Biosystems, USA) set to 2 min at 95 °C, followed by 40 cycles of 10s at 95 °C and 60s at 60 °C. Every qPCR plate had a five step dilution series in triplicate of the appropriate control DNA (*D. magna* 6500 copies/reaction to 0.65 copies/reaction, *E. danica* 4000 copies/reaction to 0.40 copies/reaction, *A. anguilla* 1500 copies/reaction to 0.15 copies/reaction). Mean Ct values were generated from the dilution series and plotted against log gene copy number generating a standard curve and linear line of best fit to assess amplification efficiency, y-intercept and R^2 value.

A OneStep™ PCR Inhibitor Removal Kit (Zymo Research Corp was carried out on 7 randomly selected samples across the 4 mesocosms to identify if inhibition was causing

eDNA within the samples to not amplify. The inhibitor test did not change the results of the qPCR showing that inhibition was either not important or affected the results consistently.

Table 1: Sequences of primers (Sense and antiSense) and probes used for quantitative PCR of target species: E. danica, D. magna and A. anguilla.

Target Species	Primer/Probe	SensePrimer
Ephemera danica	Sense	5'TCGGAATGATCTCTCATATTATCAGTC3'
	AntiSense	ACCTAAGACACCAATAGCTAATATAGC
	Probe	TCCCAAAGGCTTCCTTCTTCCCTCTTTCG
Daphnia magna	Sense	CTTCCTCCTGCTTTAACACTTCTT
	AntiSense	GGGCGATTCCTGCTGCTAA
	Probe	ACAGTTCAACCTGTTCCTGCTCCTCTTTCT
Anguilla anguilla	Sense	GCAGGTATTTCATCAATTCTAGGG
	AntiSense	GAGTAGTAAAACGGCGGTTACTAA
	Probe	ACCGCCTGCAATTACACAGTACCA

2.6 Statistical analyses

Statistical analyses and graphical representations were explored using R (3.3.2) (R Core Team, 2016). Firstly, a linear model was run with quantity (copy numbers) as the response variable and TDN, pH, time and two-way interactions as explanatory variables. However, the data violated the assumptions of heterogeneity, and was over dispersed. Therefore, a negative binomial error distribution was fitted to account for the assumption violations (Zuur *et al.*, 2009). The model was fitted with the same response and explanatory variables as the linear model. The number of explanatory variables was then reduced using Akaike information criterion (AIC) for backwards model selection. The final fitted model included: Time and pH as explanatory factors. Due to violation of heterogeneity, TDN was removed from the final model, although the marked change of TDN over time may warrant further investigation in another study. TDN was low in all the streams except for Sidaway where at the commencement of the experiment it was extremely high (~0.7mg/L) and then reduced to a level similar to the other mesocosms within 19 hours (T19) (Figure 5). The addition of DOC was found to have no significant effect on eDNA quantity when tested using mixed affects model with DOC as a mixed effect and was subsequently discounted in further analyses (Seymour *et al.*, 2017).

3.0 Results

3.1 Environmental Factors

Mean temperatures were 15.289°C (±1.797) for Carpenter, 14.724°C (±1.522) for Davies, 14.470°C (±1.871) for Hanwell and 16.162°C (±2.572) for Sidaway. All temperatures of

mesocosms were similar between all sites (Supplementary figure 1). TDN was similar for Hanwell (0.174mg/L±0.030), Carpenter (0.146 mg/L ±0.030) and Davies (0.137 mg/L ±0.030) (Figure 4, A). Sidaway TDN was more than two-fold greater than the other three mesocosms (0.485 mg/L ±0.200) (Figure 4, A). Mean pH was lowest in Sidaway 5.352 (±0.050) followed by Hanwell 5.900 (±0.072) which had a slightly higher pH (Figure 4, B). Carpenter (6.732 ±0.025) and Davies (6.817 ±0.036) had similar pH (Figure 4, B). All pH for the mesocosms were normal for the Llyn Brianne area.

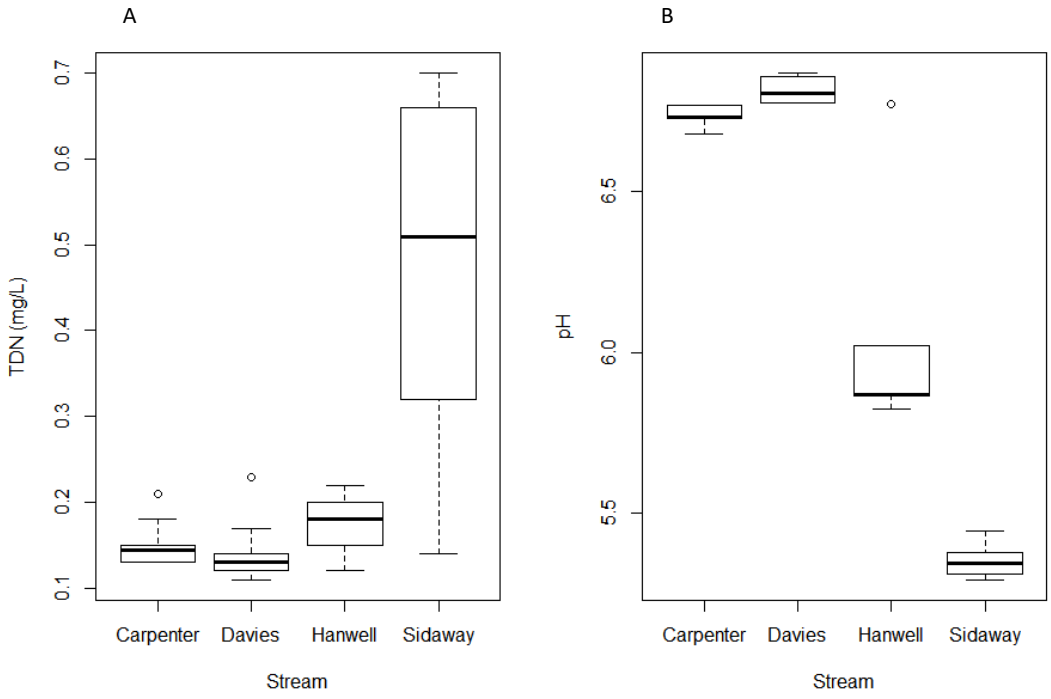


Figure 4: Mean Total Dissolved Nitrogen (mg/L) (A) and pH (B) in four experimental mesocosms: Carpenter, Davies, Hanwell and Sidaway.

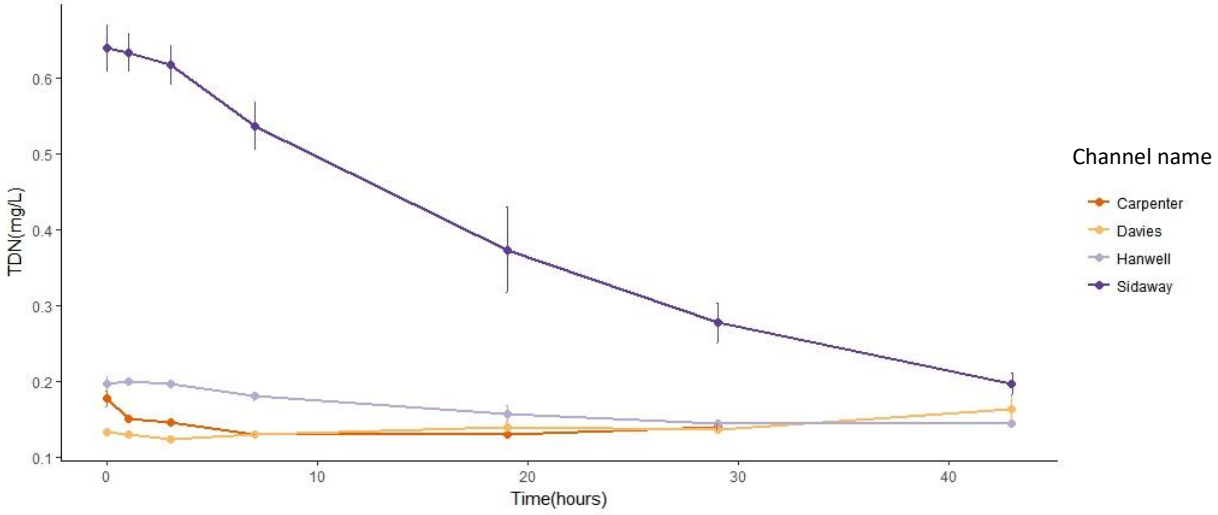


Figure 5: Mean Total Dissolved Nitrogen (mg/L) over time for four experimental mesocosms: Carpenter (orange), Davies (yellow), Hanwell (grey) and Sidaway (purple). Error bars represent standard error.

3.2 Quantitative PCR

Amplification from qPCR analysis was successful for *E. danica*. However, *A. anguilla* and *D. magna* failed to amplify. The quantity of amplified *E. danica* eDNA was calculated as copy numbers. Initial mean quantity at time point 0 were 1.440(±1.209) at Carpenter, 5.555(±5.668) at Davies, 1.015(±1.156) at Hanwell and none for Sidaway. The concentration of eDNA fell over time and was undetectable in all streams by time point 43 (Figure 6). No amplification was recorded at the -1 time points, confirming no contamination of target species or their prior presence in the experimental streams.

3.3 Results of statistical analyses

Three data points in Davies with comparatively high quantity which did not reflect the overall trends in the data and over inflated statistical findings. A General Linear Model (GLM) with a negative binomial error distribution fitted to the quantity data showed a strong negative relationship to time ($p<0.001$, SE=0.007) and strong positive relationship to higher pH ($p<0.001$, SE=0.166), summarised in Table 2 and graphically represented in Figure 6.

Table 2: Results of a Generalised linear model with a negative binomial error distribution with the explanatory variable of quantity and response variables of time and pH. Described are the P-values, z-values, Standard error and Estimates.

	Estimate	Standard error	z-value	P-value
Intercept	-6.634	1.085	-6.117	<0.001
Time	-0.0247	0.007	-3.548	<0.001
pH	1.052	0.166	6.343	<0.001

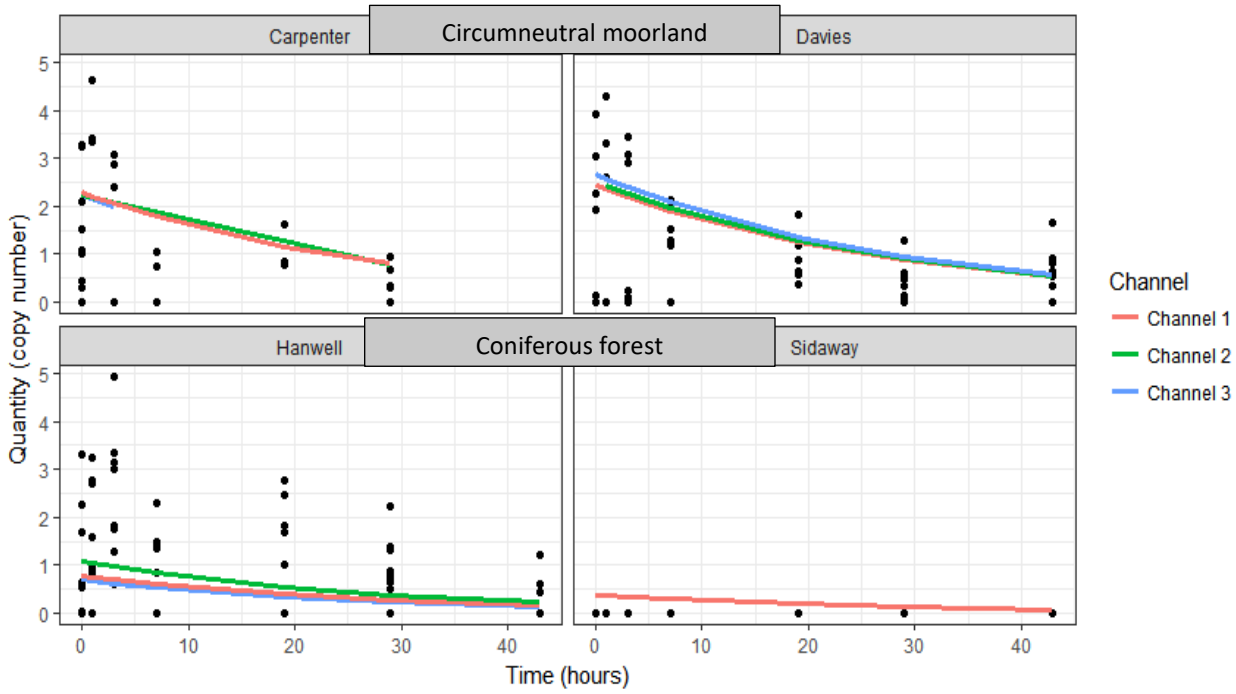


Figure 6: Quantity of DNA (E. danica) plotted against time (hours) in four experimental mesocosm: Carpenter, Davies, Hanwell and Sidaway. Each graph has fitted curves from a general linear model with a negative binomial distribution with the response variable quantity and explanatory variables pH and time. The fitted curves are split into three representing the channels in each mesocosm. Channel 1 is red, channel 2 (green) and channel 3 (blue).

4.0 Discussion

This thesis builds on what is known about the persistence dynamics of eDNA within a lotic freshwater context and explores the concepts of other mediums through which eDNA can be detected. Firstly, the experiment shows that eDNA can be detected within biofilms, however, only the eDNA of *E. danica* could be reliably amplified and quantified out of the target species. Secondly, the eDNA detected within the biofilm degraded quickly over time. Models showed that persistence was also affected by the pH level, with lower concentrations and faster degradation of eDNA in more acidic mesocosms compared to circumneutral mesocosms.

4.1 Temporal Dynamics

Biofilms are a unique structure that occupies a position between sediment and the water column. Therefore, it was hypothesised that biofilm may act similarly to sediment, leading to accumulation of eDNA. Alongside this experiment, a sister study undertook a similar experiment with the same methodology except using water (500ml) as opposed to biofilms for detecting environmental DNA. The degradation of eDNA within the biofilms acted identically to the water column study starting at a high concentration and degrading quickly over time (Supplementary figure 2) (Seymour *et al.*, 2017, in review). The most marked difference between the two studies was that the concentration of eDNA was much higher in the water column study. Furthermore, the other target species, *A. anguilla and D. magna*, were also quantified as opposed to only *E.danica* from the biofilm.

The low eDNA concentration in biofilm may be a sign that much of the eDNA is diluted and broken down in the water column before being transported to the biofilm. Furthermore, any eDNA degraded in the mesocosm that reaches a size of 0.2 µm or less is likely to then stay suspended in the water column, reducing the quantity of settling eDNA (Isao *et al.*, 1990).

4.2 Environmental interactions

4.2.1 Effect of pH

A factor that showed important relevance to the degradation of environmental DNA was pH. The streams with a lower pH were found to have a lower concentration of eDNA when compared to those with a higher pH which was also mirrored in the water based study. In the biofilm, the most extreme example was Sidaway which failed to amplify any DNA at any time point. Low pH sites were likely associated with a low concentration of eDNA due to acidity catalysing the hydrolytic process. Strickler *et al.* (2015) found a similar negative relationship between concentration and low pH. The fastest sites for degradation had neutral pH, moderate UV and higher temperatures that combined to give more favourable conditions to the microorganisms consuming eDNA. This thesis suggests that the pH of Hanwell and Sidaway were low enough to accelerate chemical hydrolysis but the mesocosms were still close enough to neutral to not inhibit the effects of exonuclease producing bacteria consuming eDNA.

In the future, studies and conservation efforts must take pH into account to mitigate the risk of false negatives. Generally, pH is easy to evaluate. This study and its sister study have provided evidence of the importance of pH in eDNA, monitoring pH should be an important

factor in future lotic freshwater biodiversity monitoring schemes. It will also be important to examine if the relationship between eDNA concentration and pH is the same in different species or types of water body, i.e. oceans, lakes and rivers. Furthermore, it may be found that species accustomed to lower pH levels and are adapted to that environment have slower eDNA degradation rates due to cells with higher tolerance. An issue with this experiment is that the target organisms were from neutral or near neutral pH environments, the sudden change upon entering the water may have artificially sped up the degradation process. Another option to understand what effect pH has on eDNA detection may be to study an organism that covers a range of habitats with differing pH and then record if at the different sites eDNA can be detected at the same concentrations.

4.2.2 Interactions of eDNA with biofilms and nutrient availability

Ecosystem dynamics in an aquatic system are heavily based on nutrient availability, even helping to define different environments (Smith *et al.,* 1998). For instance, the classification of temperate streams uses nutrients such as phosphorus levels and total nitrogen when identifying stream trophic state (Dodds *et al.,* 1997). Similarly, biofilms have been found to be reliant on nutrient availability causing changes in growth rates and community structure. For instance, biofilm growth increases with the availability of salmon carcases that seasonally arrive for spawning (Wipfli *et al.,* 1998). Therefore, it is likely a similar phenomenon of biofilm microorganisms consuming eDNA may have occurred in this experiment; due to the introduction of nutrients in the form of eDNA.

This study found a notable pattern of high nitrogen availability in Sidaway coupled with an absence of eDNA signal. Sidaway is an area used for agro-forestry which may explain the initial high concentration and rapid reduction of TDN. Studies have found riparian vegetation is important for preventing nitrates and other nutrients from reaching waterbodies (Lowrance *et al.,* 1984). Within an aquatic environment higher levels of dissolved nitrates will cause algae to grow rapidly in higher volumes, potentially creating a eutrophic environment (Conley *et al.,* 2009). Therefore, if biofilms are responsible for the degradation of settling eDNA higher growth rates may also increase the rate of degradation.

The effects of other nutrients may not be discerned in the experimental mesocosms as the systems are fed by upland streams and represent an oligotrophic system. To date, eDNA studies have neglected nutrients as a potential link to eDNA loss and only a few have linked degradation with the growth of microorganisms (e.g. Strickler *et al.,* 2015).

4.3 Implications for lotic Environmental DNA studies

Despite the economic and ecological importance of lotic freshwater systems research into applying eDNA techniques has been limited. The experiment was designed to represent a realistic system. Some factors were controlled, such as water flow and eDNA quantities, whereas dynamic factors such as water chemistry and temperature were the same as the natural system. The purpose of allowing the system to show its own variability is that the interactions of a real-world scenario are mostly too complex to control. Therefore, this study creates an applicable understanding of eDNA detection within biofilm.

Due to the very low concentrations of eDNA found in biofilms when compared to the water based study, biofilms may not be an effective sampling medium in some instances. However, biofilm could be effective for counteracting detection bias that may exist against benthic species where eDNA may not diffuse through water column as effectively as pelagic organisms. Similarly, biofilms may give a signal more localised to species in one area compared to signals from water carried from further upstream.

Now that the goal of retrieving eDNA from biofilms has been achieved, further multispecies analyses in live systems can be implemented to explore species and system differences. Due to the nature of biofilms as a multispecies complex, different biofilms may produce results depending on what is present in the matrix. Furthermore, this data suggests that water chemistry such as pH should be taken into account with the understanding that lower pH will negatively affect eDNA persistence, increasing the chance of false negative results.

The experiment conducted was not appropriate for the broader understanding of nutrient content and eDNA persistence as the study site did not cover a large enough range of concentrations. Therefore, a future study should be conducted comparing streams across a spectrum of nutrient concentrations and ratios. The follow up experiment is important as the findings will not only have implications for the use of biofilms as an eDNA sampling medium but could inform how future eDNA studies are conducted depending on a sampling sites' nutrient content.

4.5 Conclusion

To our knowledge biofilms have never previously been used for detecting eDNA of a target species in any environment. When combined with the sister study focusing on water, these data begin to close the loop in understanding how eDNA is transported through the water column and highlights the importance of environmental factors in eDNA degradation. Using

biofilms for eDNA detection offers a useful alternative to other sampling mediums and presents unique opportunities for conservation management and future ecological research.

*5.0 R*eferences

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-madsen, H., Kjaer, K. H., Orlando, L., Gilbert, M.T.P., Willerslev, E., 2012. Meta-barcoding of "dirt" DNA from soil reflects vertebrate biodiversity. Molecular Ecology, 21(8), 1966–1979.

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science and Technology, 48(3).

Barnes, M.A. and Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics, 17(1), 1-17.

Bernhardt, E.S. and Likens, G.E., 2002. Dissolved organic carbon enrichment alters nitrogen dynamics in a forest stream. Ecology, *83*(6), 1689-1700.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P. and Williams, P., 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biological Conservation, 183, 19-28.

Biesinger, K.E. and Christensen, G.M., 1972. Effects of various metals on survival, growth, reproduction, and metabolism of Daphnia magna. Journal of the Fisheries Board of Canada, 29(12),1691-1700.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. and Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends in ecology & evolution, 22(3), 148-155.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. and Vandesompele, J., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry, 55(4), 611-622.

Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A., 2017. Seasonal variation in environmental DNA in relation to population size and environmental factors. Scientific Reports, 7(46294).

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Karl, E., Karl, E., Lancelot, C., Gene, E. and Gene, E., 2009. Controlling eutrophication: nitrogen and phosphorus. Science, 123, 1014-1015.

Costerton, J., Cheng, K., & Geesey, G., 1987. Bacterial biofilms in nature and disease. In Microbiology. 47, 435-464

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Potter, C. and Bik, H.M., 2016. The ecologist's field guide to sequence-based identification of biodiversity. Methods in Ecology and Evolution. 7(9), 1008-1018

Deiner, K. and Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One, 9(2).

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. and Miaud, C., 2011. Persistence of environmental DNA in freshwater ecosystems. PloS one, 6(8).

Dodds, W.K., Jones, J.R. and Welch, E.B., 1998. Suggested classification of stream trophic state: distributions of temperate stream types by chlorophyll, total nitrogen, and phosphorus. Water Research, 32(5), 1455-1462.

Douville, M., Gagné, F., Blaise, C. and Andre, C., 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn cry1Ab gene from an aquatic environment. Ecotoxicology and environmental safety, 66(2), 195-203.

Durance, I. and Ormerod, S.J., 2007. Climate change effects on upland stream macroinvertebrates over a 25-year period. Global change biology, 13(5), 942-957.

Ficetola, G.F., Miaud, C., Pompanon, F. and Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biology letters, 4(4), 423-425.

Flindt, R., 2006. Comparative Data. Amazing Numbers in Biology, 247-258.

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A., Salling, A.B., Galatius, A., Orlando, L. and Gilbert, M.T.P., 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PloS one, 7(8).

Glibert, P.M., 2012. Ecological stoichiometry and its implications for aquatic ecosystem sustainability. Current Opinion in Environmental Sustainability, 4(3), 272-277.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M., 1996. Real time quantitative PCR. Genome research, 6(10), 986-994.

Isao, K., Hara, S., Terauchi, K., & Kogure, K. 1990. Role of sub-micrometre particles in the ocean. Nature, 345(6272), 242–244.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. and Lodge, D.M., 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. Conservation Letters, 4(2), 150-157

Jerde, C.L., Chadderton, W.L., Mahon, A.R., Renshaw, M.A., Corush, J., Budny, M.L., Mysorekar, S. and Lodge, D.M., 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. Canadian Journal of Fisheries and Aquatic Sciences, 70(4), 522-526.

Kelly, R.P., Port, J.A., Yamahara, K.M. and Crowder, L.B., 2014. Using environmental DNA to census marine fishes in a large mesocosm. PloS one, 9(1).

Kowalik, R.A., Cooper, D.M., Evans, C.D. and Ormerod, S.J., 2007. Acidic episodes retard the biological recovery of upland British streams from chronic acidification. Global Change Biology, 13(11), 2439-2452.

Lowrance, R., Todd, R., Fail, J., Hendrickson, O., Leonard, R., & Asmussen, L. 1984. Riparian Forests as Nutrient Filters in Agricultural Watersheds. BioScience, 34(6), 374–377.

Mächler, E., Deiner, K., Steinmann, P. and Altermatt, F., 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshwater Science, 33(4), 1174-1183.

Martellini, A., Payment, P., & Villemur, R. 2005. Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. Water Research, 39(4), 541–548.

Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., Colbourne, J. K., Wilgar, G., Carvalho, G.R., de Bruyn, M., Edwards, F., Emmett, B. A., Bik, H. M., Creer, S. 2017. Acidity promotes degradation of multi-species eDNA in lotic mesocosms. In review for Nature Communications Biology.

Ogram, A., Sayler, G.S. and Barkay, T., 1987. The extraction and purification of microbial DNA from sediments. Journal of microbiological methods, 7(2), 57-66.

Ormerod, S. J. & Durance, I., 2009. Restoration and recovery from acidification in upland Welsh streams over 25 years. Journal of Applied Ecology. 46**,** 164–174.

Ormerod, S. J., Wade, K. R. & Gee, A. S., 1987, Macro-floral assemblages in upland Welsh streams in relation to acidity, and their importance to invertebrates. Freshwater Biology. 18, 545–557

Pilliod, D.S., Goldberg, C.S., Arkle, R.S. and Waits, L.P., 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences, 70(8), 1123-1130.

Södergren, A., & Svensson, B., 1973. Uptake and accumulation of DDT and PCB by *Ephemera danica* (*Ephemeroptera*) in continuous-flow systems. Bulletin of Environmental Contamination and Toxicology, 9(6), 345–350.

Smith, V. H., Tilman, G. D., & Nekola, J. C., 1998. Eutrophication: Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. In Environmental Pollution, 100, 179–196).

Strickler, K.M., Fremier, A.K. and Goldberg, C.S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation, 183, 85-92.

Taberlet, P., Coissac, E., Hajibabaei, M. and Rieseberg, L.H., 2012. Environmental DNA. Molecular Ecology, 21(8), 1789-1793.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. and Kawabata, Z.I., 2012. Estimation of fish biomass using environmental DNA. PloS one, 7(4).

Thomsen, P., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. and Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular ecology, 21(11), 2565-2573.

Thomsen, P.F. and Willerslev, E., 2015. Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation, 183, 4-18.

Torti, A., Lever, M.A. and Jørgensen, B.B., 2015. Origin, dynamics, and implications of extracellular DNA pools in marine sediments. Marine genomics, 24, 185-196.

Turner, C. R., Uy, K. L., & Everhart, R. C., 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biological Conservation, 183, 93–102.

Turner, C.R., Barnes, M.A., Xu, C.C., Jones, S.E., Jerde, C.L. and Lodge, D.M., 2014. Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods in Ecology and Evolution, 5(7), 676-684.

Willerslev, E., Davison, J., Moora, M., Zobel, M., Coissac, E., Edwards, M.E., Lorenzen, E.D., Vestergård, M., Gussarova, G., Haile, J. and Craine, J., 2014. Fifty thousand years of Arctic vegetation and megafaunal diet. Nature, 506(7486), 47-51.

Willerslev, E., Hansen, A.J. and Poinar, H.N., 2004. Isolation of nucleic acids and cultures from fossil ice and permafrost. Trends in Ecology & Evolution, 19(3), 141-147.

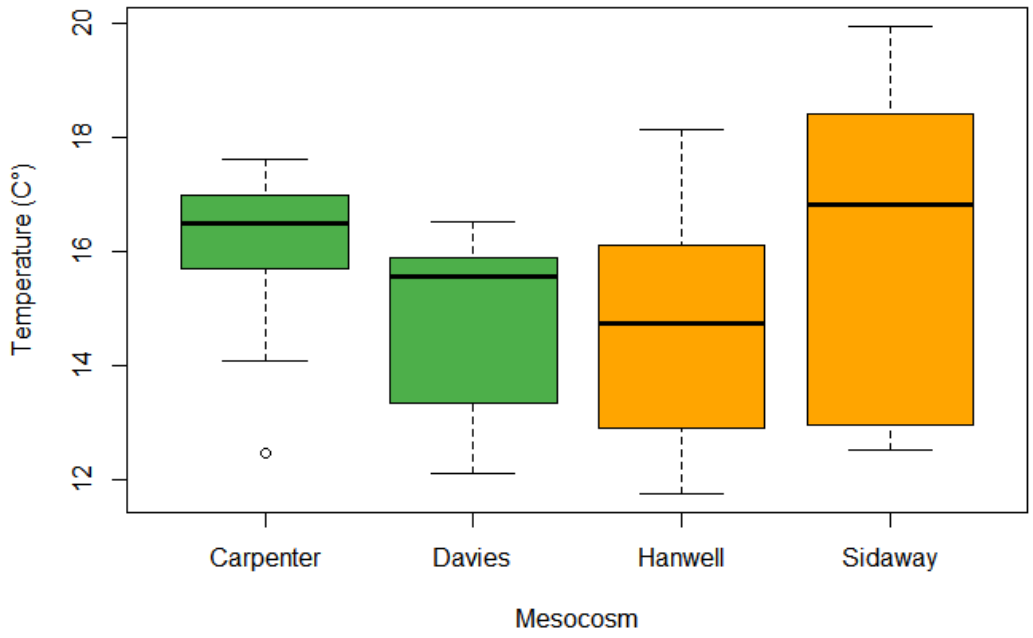
Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D.A. and Cooper, A., 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science, 300(5620), 791-795.

Wipfli, M.S., Hudson, J. and Caouette, J., 1998. Influence of salmon carcasses on stream productivity: response of biofilm and benthic macroinvertebrates in southeastern Alaska, USA. Canadian Journal of Fisheries and Aquatic Sciences, *55*(6), 1503-1511.

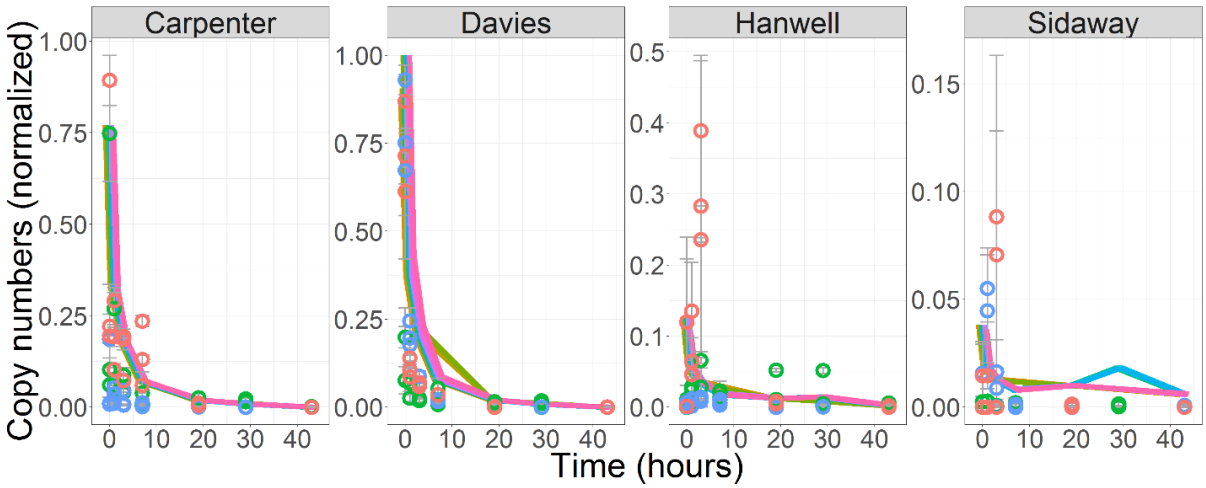
R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. & Smith, G. M. Mixed effects models and extensions in ecology with R. (Springer, 2009).

Supplementary Material



Supplementary Figure 1: Mean Temperature (C°) in four experimental mesocosms: Carpenter, Davies, Hanwell and Sidaway. Mesocosms covering circumneutral moorland coloured green. Mesocosms covering coniferous forest are coloured orange.



Supplementary Figure 2: From Seymour et al., (2017, in review). Results of a quantitative PCR analysis of eDNA extracted from 1L water samples. Quantity (x-axis) as normalized copy numbers relative to time (y-axis) in hours with each point representing mean quantity values (n=3) for each time point at the respective experimental stream (separate panels). Whisker bars show the standard deviation. Lines are the fitted values from a generalized linear mixed effects model. Colours represent the target species (D. magna, E. danica, A. anguilla) for each stream replicate.