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Assessing the Utility of Aqueous eDNA for Invertebrate Biodiversity Assessment in Reens and Ditches.

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Assessing the Utility of Aqueous eDNA for Invertebrate Biodiversity Assessment in Reens and Ditches.

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A thesis submitted to Bangor University in candidature for the degree Master of Science by Research

In Partnership with

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Natural Resources Wales

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Contents

Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards

.---Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir ynwahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid ywsylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael eigyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan yBrifysgol, am gymwysterau deuol cymeradwy.

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Abstract

Observing aquatic invertebrate diversity can provide ecological insights into changing environments. Research into moving waterbodies has primarily focused on rivers, with little exploration into biodiversity within ditches. Ditches often drain into larger ‘reens’, which are artificial structures designed to prevent water-logging in winter and provide livestock with water in summer There has been little investigation into ditches and reens as important habitats for invertebrates, partly due to difficulty surveying them. Often, macroinvertebrate surveys use morphological monitoring methods to understand the area’s biodiversity; however, these methods are often time-consuming, require expert knowledge, and are highly invasive. In this study, an alternative method using environmental DNA (eDNA) was applied to understand the alpha and beta diversity of ditches and reens in St. Brides SSSI, South Wales. Here, the data presented demonstrated that eDNA analysis produces far greater taxonomic information than morphological analysis and that abiotic factors, such as waterbody type and temperature, significantly impact alpha diversity, while the amount of water filtered and salinity influenced beta diversity. These results indicate the importance of utilizing consistent methods during water sample collection. In addition, ditches and reens showed differences in invertebrate diversity despite the waterbodies being connected. We anticipate that the findings from this study can aid ditch and reen management plans to ensure that invertebrate biodiversity is maintained. Furthermore, this study highlights the importance of using invertebrates as indicator species for water quality assessment and displays the benefits of using environmental DNA monitoring in combination with morphological monitoring.

1. Introduction

1.1 Freshwater Ecosystems

Freshwater ecosystems, rich in species diversity and endemism, are essential to sustaining human existence, with human settlements forming preferentially near freshwater (Revenga *et al.*, 2005). However, human interaction causes anthropogenic pressures such as pollution, exploitation, habitat degradation and the introduction of invasive species (Kuntke *et al.*, 2020). At both regional and local levels, freshwater environments are often in poor condition for biodiversity compared to terrestrial habitats (Clarke, 2015). Changes in freshwater ecosystems may not be visible, and declines in biodiversity can occur for long periods without detection (Linke *et al.*, 2018).

One major cause of freshwater species loss has been the simplification and channelization of rivers and their floodplains. In some areas of the United Kingdom, to counteract species loss, drainage schemes in floodplains and low-lying areas have created networks of channels designed to carry water and maintain lower water levels, providing flood control and prevention of erosion or improved navigation (Brookes, 1985, 1986; Keller, 1976). Artificial drainage networks are characterized by larger and smaller channels, referred to here as ditches. Many of the ditches now surround areas of agriculture or urban zones and are managed to maintain specific water levels through regular vegetation management. Despite these anthropogenic pressures, ditches provide an essential freshwater environment for wildlife and have been found to support a wide variety of invertebrate species, including species of conservation interest.

1.2 Freshwater Monitoring Systems

To develop a standardized monitoring system that could provide insight into water quality, the Institute of Freshwater Ecology (IFE) developed a technique for evaluating the biological quality of rivers in the UK (Wright, 1994). The River Invertebrate Prediction and Classification

System (RIVPACS) focuses on benthic macroinvertebrates for biological assessments as macroinvertebrate taxonomy is well known, and a wide variety of species are found in freshwater habitats (Hellawell, 2012; Wright, 1994). RIVPACS, a statistical model software, incorporates the General Quality Assessment classification of river statuses based on Ecological Quality Indices (EQIs) based on two indices (Paisley, Trigg and Walley, 2014). The first index is The Biological Monitoring Working Party (BMWP), which has been used by regulatory authorities in the UK since the 1980s as the basis of their river invertebrate status classification system (Paisley, Trigg and Walley, 2014). The BMWP provides allocated scores to families based on their sensitivity to pollution (Table 1). The more sensitive taxa are to pollution, usually due to Biological Oxygen Demand (BDO), the higher the BMWP score (Paisley, Trigg and Walley, 2014; Aquilina, 2013). The second index incorporated into the EQIs is the average BMWP score per taxon, also known as the Average Score Per Taxon (ASPT). The ASPT was created due to bias experienced when conducting score systems for invertebrate monitoring, as the sample size primarily affects the number of taxa in a sample. To counteract this bias, the ASPT was developed, whereby the BMWP score is divided by the number of contributing taxa (Appendix 1), thus providing an average score (Hawkes, 1998).

Table 1. The Biological Monitoring Working Party (BMWP) and Average Score Per Taxon (ASPT) scoring system.

BMWP score	ASPT score	Quality interpretation
>150	>6	Very good
101-150	>5	Good
51-100	>4	Moderate
16-50	<4	Poor
0-15		Very Poor

In December 2000, the EU Water Framework Directive (WFD) was created as the primary legislative tool to improve polluted inland water surfaces, transitional, coastal and ground waters to prevent a decline in water quality using monitored macroinvertebrates as biological quality elements (European Commission, 2021; Lathouri *et al.*, 2021). The WFD has monitored the status of aquatic ecosystems by characterizing biological communities and physiochemical and hydromorphological conditions using the BMWP and ASPT (Pawlowski *et al.*, 2018). The WFD sets ecological water surface standards for 27 countries globally and is divided by the range of EQI scores into five classes: very good, good, moderate, poor and very poor (European Commission, 2021).

Monitoring invertebrate populations can provide an early warning for ecological change by observing the presence and abundance of species and allowing for the development of mitigations to slow species decline (Schmeller, 2008; Beever, 2006). Biological monitoring has several limitations (practical and theoretical) that need to be considered to allow them to be applied successfully (Beever, 2006). Despite this, those conducting surveys (e.g. government agencies, local councils, water companies, and researchers) often find themselves in a difficult position where data needs to be gathered quickly, at a low cost, and perhaps without clear objectives (Witmer, 2005). Invertebrate studies are often complicated as species of interest are often poorly understood, rare or strongly influenced by human activities (Witmer, 2005).

The first step in an invertebrate survey is sample collection. Many aquatic invertebrate surveys sample relatively small areas or volumes and only collect a small proportion of species in the survey area (Halse *et al.*, 2002). Aquatic macroinvertebrate surveys are often conducted using equipment such as the Surber sampler and kick-nets (Poikane *et al.*, 2016; Brua, Culp and Benoy, 2011; Sharma, Arambam and Sharma, 2009).

1.3 Morphological Macroinvertebrate Monitoring Methods

Kick-net (Figure 1) and U-net sampling are recognized internationally for small, regional, and national bioassessments (Brua, Culp and Benoy, 2011). Both methods involve a net being held downstream while the substrate upstream is disturbed, causing invertebrates to pass into the net. While the methods have been proven effective at obtaining macroinvertebrates, they are highly invasive due to the disturbance of the water substrate and the removal of specimens (Brua, Culp and Benoy, 2011). However, these sampling methods are low-cost, easy to transport, and valuable for surveying various habitats (Carter and Resh, 2001). Conversely, the netting size significantly affects biodiversity assessment, as smaller organisms are often missed by passing through the net or becoming trapped within silt, clogging the net, and larger organisms actively climbing out of the net. In addition, collected invertebrates are often difficult for biologists to identify, and the sample size varies depending on the season due to fluctuations in water levels (Stein, Springer and Kohlmann, 2008; Carter and Resh, 2001). High currents caused by increased water levels can also increase the likelihood of organisms missing the net (Stein, Springer and Kohlmann, 2008).

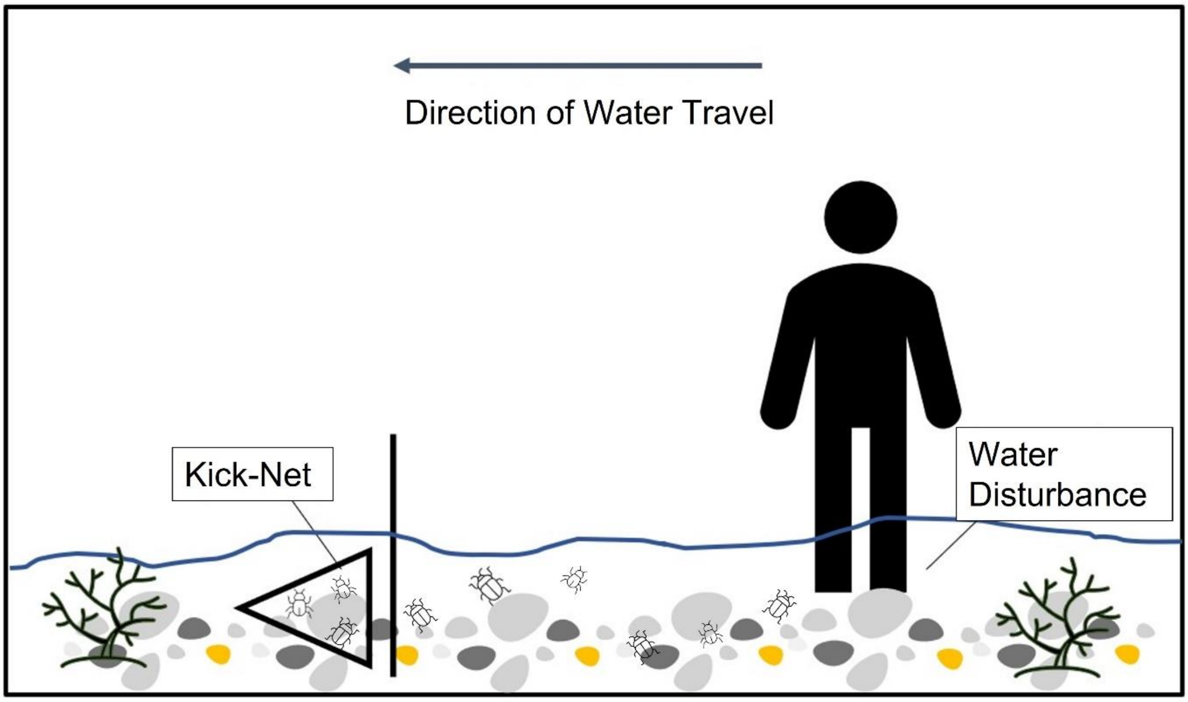

Figure 1. Kick-net sampling is a standard method used for sampling macroinvertebrates. The researcher stands upstream, disturbing the water so that invertebrates are swept downstream into the net.

Surber samplers are nets of a given mesh size fastened around a heavy metal square frame, allowing a known isolated area of the riverbed to be sampled (Surber, 1937). They are used because they are practical and highly suited to varying habitats (Ghani *et al.*, 2016). However, they have a small substrate area and fail to collect macroinvertebrates drifting in the water column above the sample (Ghani *et al.*, 2016). The small size of the sampler also makes it difficult to place it correctly on rough substrates (Al-Shami *et al.*, 2013). Larger specimens can crawl out of the samplers, and others can detect the physical disturbance in the water and avoid the nets altogether (Ghani *et al.*, 2016). Despite this, the quantitative collection from the Surber sampler is reliable in estimating the abundance and diversity of benthic invertebrates (Ghani *et al.*, 2016).

1.4 eDNA Biomonitoring in Freshwater Systems

Environmental DNA (eDNA) metabarcoding is becoming an increasingly valuable and viable tool for ecologists (Taberlet *et al*., 2018). eDNA analysis is a non-invasive molecular species detection method, and eDNA is defined in this paper as DNA deposited from organisms into the environment, which can be collected in samples such as water soil and sediment, without the presence of the organism (Ruppert, Kline and Rahman, 2019; Taberlet *et al*., 2018; Turkelboom *et al*., 2013; Sogin *et al*., 2006). The eDNA can then be extracted, amplified, sequenced, and categorized, allowing for species identification (Ruppert, Kline and Rahman, 2019; Deiner *et al*., 2015). The amount of eDNA an individual produces depends on biomass, age and feeding activity of the organism, physiology, life history, and use of habitat space (Hering *et al*., 2018; Barnes and Turner, 2016; Goldberg, Strickler and Pilliod, 2015).

DNA metabarcoding uses PCR and the deployment of taxonomic-specific oligonucleotide primers, combined with high-throughput sequencing to enable multi-species identification, often with DNA extracted from an environmental sample (Taberlet *et al.*, 2012). However, the definition can also be applied to species identification from bulk samples of entire organisms where they have been isolated prior to analysis (Taberlet *et al.*, 2012; Hajibabaei *et al.*, 2011; Chariton *et al.*, 2010; Creer *et al.*, 2010; Porazinska *et al.*, 2010). Furthermore, metabarcoding has been found to achieve comparable assessment results with morphological studies and offers a powerful alternative, identifying species that would otherwise be unfeasible in morphological studies (Elbrecht *et al.*, 2017).

Environmental DNA metabarcoding combines traditional field-based ecology with in-depth use of molecular methods and advanced computational tools (Ruppert, Kline and Rahman, 2019). While it is still an emerging monitoring method, it can revolutionize modern biodiversity surveys (Ruppert, Kline and Rahman, 2019). eDNA is beneficial for monitoring aquatic taxa due to the DNA shed by an organism into the surrounding environment, which can persist in lotic waters. Understanding the total biodiversity of rivers is fundamental to determining surface water quality (Fernández *et al.*, 2019; Deiner and Altermatt, 2014). Monitoring using eDNA has been compared to traditional monitoring methods, and it has been concluded that DNA metabarcoding and morphological identification give similar correlations with water quality in-stream conditions when linked to the watershed size and shifts in forest composition across various water bodies (Emilson et al., 2017; Fernández et al., 2019). However, inconsistencies remain in the taxonomic composition produced by the two approaches, especially regarding macroinvertebrate and microbial communities (Keck *et al.*, 2022).

eDNA pipelines can facilitate high throughput processing of samples compared to low throughput traditional methods, allowing for greater replication and more geographically and temporally broad surveys (Seymour *et al.*, 2020). eDNA can also better detect ecological signals than morphological methods, presenting higher taxonomic richness due to the improvement of taxon assignment in some groups (e.g. midges, mayflies, caddisflies and black flies) (Fernández *et al.*, 2019). Multi-gene eDNA studies have also been compared against traditional methods revealing interactive networks linked to ecological assessment criteria (Seymour *et al*., 2020). Moreover, eDNA analysis can potentially include a broader range of taxa and indicator groups that would otherwise fail to be included in traditional taxonomic identifications, thus focusing on higher levels of invertebrate biodiversity than morphological identification methods (Seymour *et al*., 2020).

The biomonitoring capabilities of eDNA analysis has meant that it is a powerful tool for conservation, and many studies have focused on detecting invasive species in natural systems. This was first demonstrated by targeting American bullfrogs (*Rana catesbeiana*) in French wetlands (Ficetola *et al.*, 2008). Research has also detected invasive species in transit, such as non-native organisms in the ballast waters of transoceanic ships (Egan *et al.*, 2013; Mahon *et al.*, 2013; Li *et al.*, 2011). Furthermore, similar studies have successfully identified benthic invertebrates and their resting stages within ballast tank sediments (Briski *et al.*, 2011;

Harvey, Hoy and Rodriguez, 2009; Darling and Tepolt, 2008). Conversely, eDNA has been used to locate rare indigenous species; for example, in the UK, the great crested newt (*Triturus cristatus*) has been a focus of many eDNA surveys (Rees *et al.*, 2014). As eDNA analysis is less invasive, it is more applicable than traditional approaches in certain situations and becoming an increasingly used tool (Stein *et al.*, 2014; Mahon *et al.*, 2013).

1.5 Study Site

This study focused on the Gwent Levels (Figure 2), a lowland area between Cardiff and Chepstow, UK, where a network of drainage ditches has been maintained since the roman ages (Living Levels, 2021; Countryside Council Wales, 1991). The Levels are an example of one of the most extensive areas of reclaimed wet pasture in the UK and are rich in biodiversity, including in aquatic invertebrates (CCW, 1991). The Levels have provided habitat for nationally rare or notable species such as the water beetles *Halipus mucronatus* and *Hydrophilus piceus*. The area is also crucial in Wales for its snail and dragonfly populations, including *Physa heterostropha* and *Brachytron pratense* (CCW, 1991). St. Brides SSSI is one in a series of SSSIs located in the Gwent Levels consisting of 5700 hectares (Countryside Council Wales, 2008). The entire landscape is artificial and formed from a 2,000-year history of land reclamation from the sea (Living Levels, 2021). The land is below sea level; however, extensive sea defences prevent submersion (Living Levels, 2021).

Ditches are artificial bodies of water primarily draining excess water and groundwater seepage from agricultural lands in winter while providing livestock with drinking water in warmer months (Verdonschot, 2012). Field ditches are relatively small in width and depth and have varying water volumes and vegetation cover around the edges of agricultural fields. Field ditches feed into more extensive reens, usually between 2-8 m wide and up to 1 m deep, which are generally well maintained. Both ditch types are found in temperate and boreal zones in the Northern Hemisphere in almost all low-lying or wetland areas (Herzon and Helenius, 2008).

With little water movement, field ditches and reens have intensive organic and inorganic matter exchange with the surrounding terrestrial matrix (Herzon and Helenius, 2008). As a result, they must be regularly managed, mowing aquatic vegetation to avoid accumulated sediment (Twisk, Noordervliet and ter Keurs, 2000; Beltman, 1984). Without such maintenance, complete territorialization would occur (Verdonschot, Keizer-vlek and Verdonschot, 2011). The ditches are drained by gravity, pumps, and other water controls (i.e., sluices) to control water levels. Water levels are kept high in summer for fencing and providing livestock water. In winter, however, structures that maintain high water levels are removed to allow floodwater to drain into the estuary when the tide goes out. Many reens and ditches on the Gwent Levels are periodically cleared as part of management regimes to prevent silting (CCW, 2008).

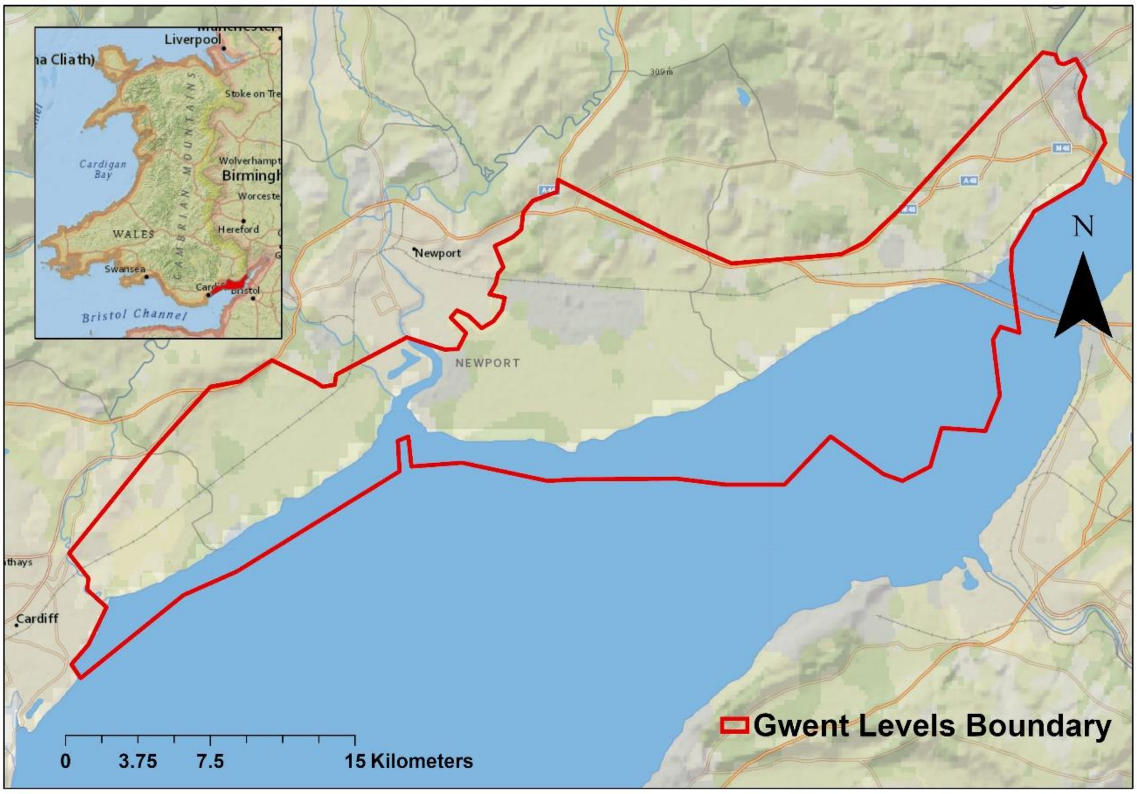

Figure 2. The Gwent Levels comprise eight SSSIs on the south coast of Wales, stretching from the east of Cardiff to Caldicot. Sources: National Geographic, Esri, Garmin, HERE, UNEP-WCMC, USGS, NASA, ESA, METI, NRCAN, GEBCO, NOAA, increment P Corp.

1.6 Study Aims

While eDNA is used more frequently to explore aquatic invertebrate biodiversity, it is often not utilized in monitoring schemes and water quality surveys. Here, we explore how alpha (α) and beta (β) diversity differ between ditches and reens in the Gwent Levels while providing a comprehensive insight into the community composition of St. Brides SSSI using eDNA. We investigate how a range of physiochemical parameters and land use impacts diversity, measured via eDNA analysis. In addition, we identify critical methodological considerations and the relative inputs from terrestrial and aquatic systems when sampling from ditches and reens. Finally, we compare how eDNA biomonitoring in the Gwent Levels compares to traditional morphological monitoring techniques through species analysis of the previous surveys.

2. Materials and Methods

2.1 Sample Collection

Water samples consisting of 26 reen samples and five ditch samples were selected across St. Brides SSSI, South Wales (Figure 3) from December 1st – 6^{th,} 2021. Only five ditch sites were selected due to high organic content clogging the filters after filtering so it was thought little DNA could be extracted from the samples. The amount of water filtered at the ditch per replicate varied significantly, with FD2 and FD3 filtering an average of 10ml, compared to FD5, which filtered an average of 867ml. Each EA9.1 replicate also filtered an average of 10ml, compared to IDB16.1 and IDB16.2, which filtered the full 1000ml. However, most reens filtered ~ 200ml per replicate. The sites were selected to capture both urban and rural environments. Three ecological replicates were taken at each site, and three negative controls were taken in the field on the last day. Each water sample was collected using sterilized 1-litre bottles. The water was pumped through a Sterivex filter using a Geopump™ Peristaltic Pumps until the filter for each replicate became clogged before adding 1ml of Qiagen lysis buffer ATL. The Sterivex filter was then sealed with a Leur Lock Syringe Cap and stored at 4˚C until extraction.

Water depth was measured at each site along with the following water physiochemical measurements: depth (cm), sample amount (ml), waterbody type, pH, RDO concentration (mg/L), salinity (PSU), turbidity (NTU), temperature (°C). Physiochemical measurements were taken last to prevent contamination using an Aqua Troll 500, while depth was measured with a measuring stick.

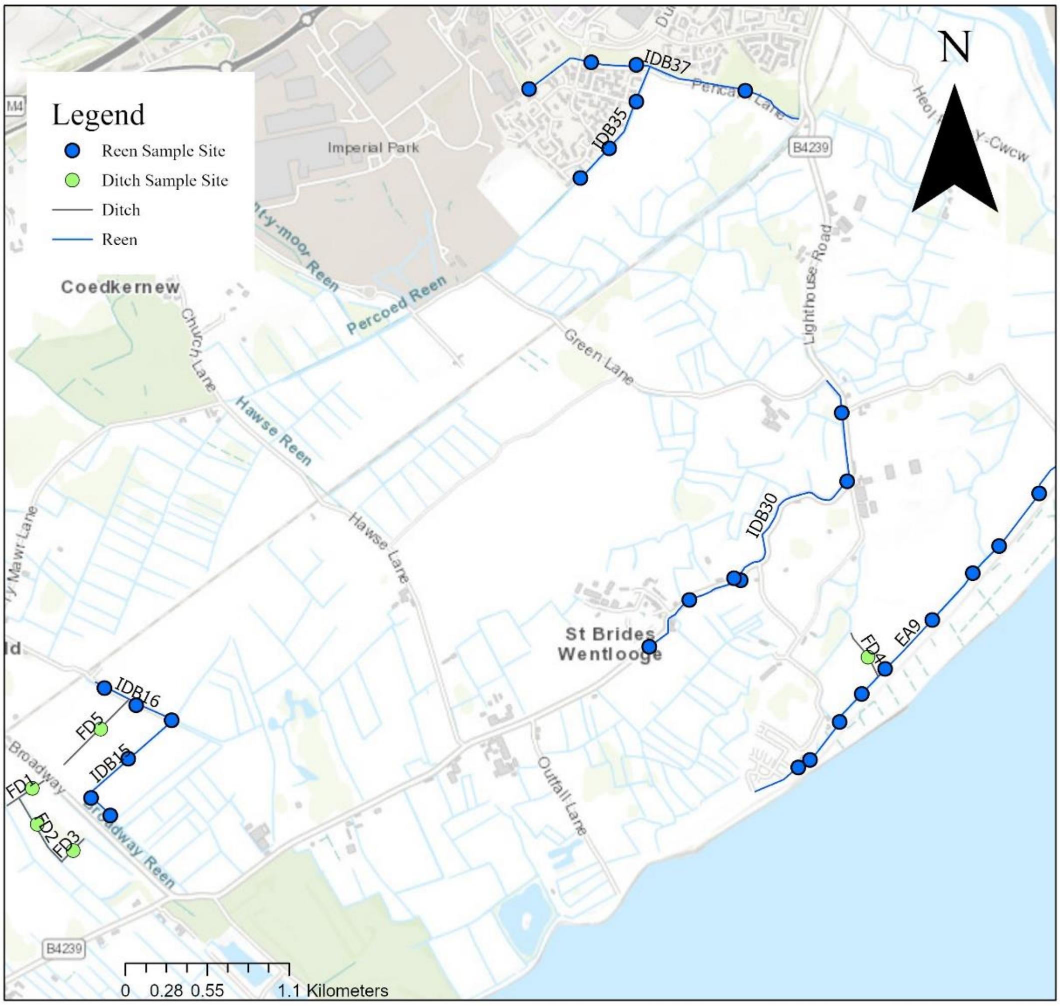

Figure 3. A total of 33 sites were selected in the St. Brides SSSI on various reens and ditches covering both urban and rural sites to create sample diversity. Sources: Esri, UK, Esri, HERE, Garmin, GeoTechnologies, Inc., USGS, METI/NASA.

2.2 DNA Extraction and Sequencing

DNA extractions and PCR (Polymerase Chain Reaction) reactions took place in the Molecular Ecology and Evolution laboratories at Bangor University. The extractions were conducted using the Spens *et al*. (2016) Capsule Methodology protocol and the Qiagen DNeasy Blood and Tissue Kit. Three negative controls from the field were extracted, and a negative PCR control was added to each PCR plate, along with a positive control. COI primers m1COIintF (5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′) and jgHCO2198 (5′-TAIACYTCIGGRTGICCRAARAAYCA-3′) with universal tails were used (Leray *et al.*, 2013). Each sample library was amplified using the primer set in triplicates and a Qiagen Mastermix kit using an identical library build protocol as in Brennan *et al.* (2019). Specifically, a 2-step PCR approach was used with a 25 µL total volume consisting of 12.5 µL Qiagen multiplex PCR Mastermix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 10.5 µL PCR grade water and 1 µL template DNA. A Thermal Cycler was used for the following COI PCR Protocol; 95ºC 15 mins, then 35 cycles of 94ºC 30s; 54ºC 90s; 72ºC 1min; 72ºC 10 min. The triplicates were pooled, and the products from the first round of PCR were purified using 9 µL of Agencourt AMPure XP beads. The second round PCR protocol consisted of 12.5 µL of the Qiagen multiple PCR Master Mix, 1 µL of Round 2 primer index i5-i7, 6.5 µL of PCR water and 5 µL of purified Round 1 PCR product. The PCR machine was run for 10min at 95˚C, ten cycles of 30s at 98˚C, 30s at 65˚C and 30s at 72˚C. AMPure XP purification was performed using 12 µL of the Agencourt AMPure XP to 20 µL of the Round 2 PCR product. The PCR round 2 products were quantified using a Qubit dsDNA Broad Range kit, and the equimolar concentrations for the library were pooled before being cleaned using Agencourt AMPure XP beads. Each amplicon library was normalised to 4ng/l by diluting with PCR water and then 1 µL of each normalised amplicon was added to the final pool. The final library was loaded at 12 pM with 10% Phi-X spike-in, and sequenced using an Illumina MiSeq® Reagent Kit v2 (500 cycle) in accordance with the manufacturer's instructions at the Centre for Environmental Technology (CEB), Bangor, Wales.

2.3 Bioinformatics

After obtaining the raw demultiplexed read data, the DADA2 (Callahan *et al.*, 2016) pipeline was run using the statistical software R (R Core Team, 2022), with the default parameters unless specified. By default in the DADA2 pipeline, forward reads with higher than 2 "expected errors" were discarded after trimming and reverse reads with higher than 5 "expected errors" were discarded. Filtering included trimming reads at the first instance of a quality score less than or equal to 2. Basic Local Alignment Search Tool (BLAST) was used for taxonomic assignment in conjunction with the MIDORI COI reference database (Leray *et al.*, 2018; Altschul *et al.*, 1990). Any Amplicon Sequence Variants (ASVs) which did not reach at least 70% percentage identity and query cover were removed from the analysis. Samples with a read depth lower than 1,000 were removed based on the rarefaction curves, and ASVs with a read depth of less than 0.05% of the total reads in a sample were also removed. The proportion of reads per sample were calculated by dividing ASV read depth by total sample read depth. For invertebrate analyses, the following phyla were included: Arthropoda, Gastrotricha, Platyhelminthes, Annelida, Rotifera, Mollusca, Cnidaria, Nematoda, Tardigrada, Porifera, Placozoa, Onychophora, Nemertea, Echinodermata and Bryozoa to obtain a comprehensive overview of invertebrate biodiversity. The greatest impact on read depth was the removal of non-metazoans (Figure 4).

For comparisons with traditional sampling methods and to provide National Resources Wales with a species list, the accuracy of the taxonomic assignment was more important. ASVs were searched against the NCBI Nucleotide database using BLAST with a $1e^{-10}$ e-value threshold. Taxonomic assignments were performed using the “Assign-Taxonomy-with-BLAST” python script (Sevigny, 2018) using default parameters except for a 70% length cut-off to remove potential PCR amplification errors and sample cross contaminations (Macher *et al.*, 2023; Jacot *et al.*, 2021; Corse *et al.*, 2017; Ransome *et al.*, 2017; Leray and Knowlton, 2015) and a 99% species-level assignment threshold to increase identification accuracy when producing a species list. Invertebrate species were then manually checked to ensure they were UK species.

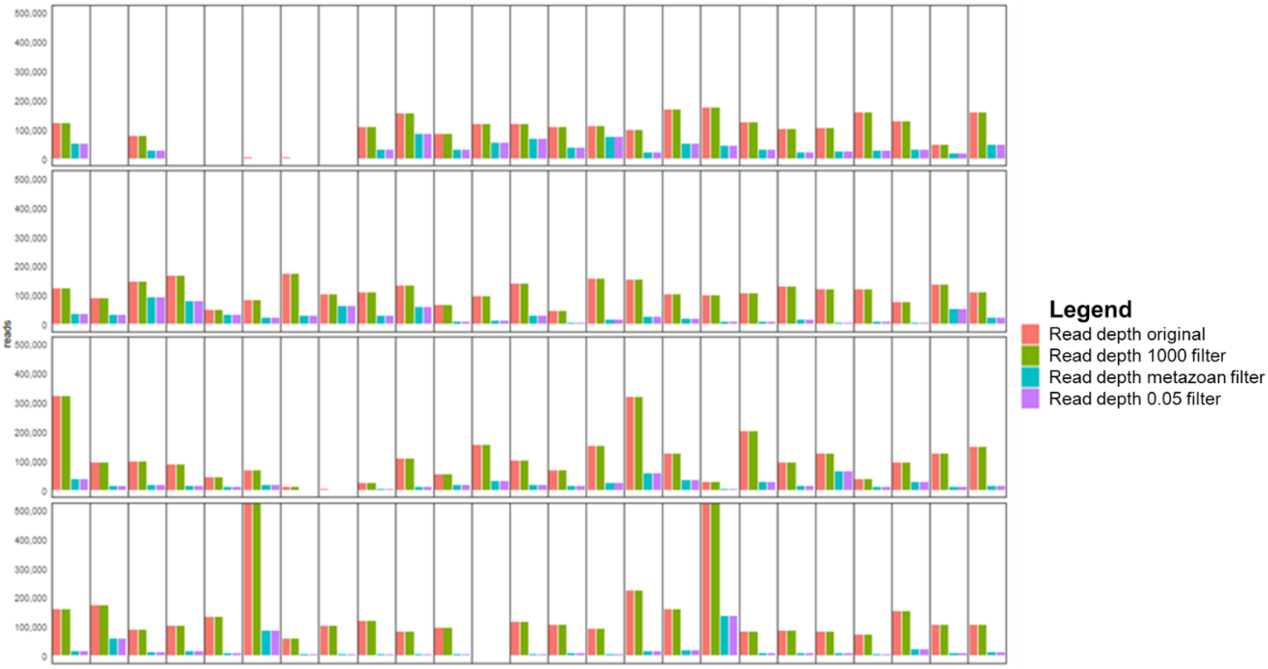

Figure 4. The raw ASVs underwent a series of filtration steps in R studio using the DADA2 pipeline presented on the x-axis, while the number of reads can be viewed on the y-axis. The first step was removing all sample sites with less than 1000 reads. Secondly, only metazoan ASVs remained before those with a depth of less than 0.05 were removed.

2.4 Statistical Analysis

Analyses were conducted in R (2022) with scripts provided by Dr William Perry at Cardiff University. All plots were created using the package ggplot2 (Hadley, 2016), apart from the Venn diagram, which was created using the package VennDiagram (Hanbo, 2022). A PERMANOVA (Permutational Analysis of Variance) was used to explore β-diversity, using the adonis2 function in the package vegan (Oksanen *et al.*, 2022), the Bray-Curtis method and 999 permutations. The relationship between the number of ASVs and environmental factors was assessed using a linear model. Furthermore, α diversity was calculated by observing the number of ASVs. The full model contained the following fixed effects: waterbody type, depth (cm), pH (pH), dissolved oxygen concentration (mg/L), salinity (PSU), turbidity (NTU), temperature (°C) and land use and the amount of water filtered. After a step function using automatic backward elimination, the final model contained water body type, pH, and salinity. Finally, a sparse partial least squares (sPLS) analysis was conducted between 12,278,512 reads and all of the physiochemical variables and then plotted on a heatmap using the package mixOmics (Rohart *et al.*, 2017).

2.5 Methods of previous surveys within St. Brides SSSI

In 2011 Boyce conducted an aquatic macroinvertebrate survey on the Gwent Levels to develop a monitoring strategy designed to aid NRW's commitments under the Common Standards Monitoring programme. The survey was conducted in June and concentrated on collecting aquatic beetles, bugs and snails using Kick-net sampling before the organisms were sorted on a polyethene sheet (Boyce, 2012). The specimens were later organised into the taxonomic groups: Coleoptera, Hemiptera and Mollusca before being identified at the species level (Boyce, 2012). The reens: EA9, IDB15, IDB16, IDB30, IDB35, IDB37 in both the 2011 survey and this eDNA survey.

Graham and Hammond's (2022) survey, which focused on terrestrial and aquatic macroinvertebrates, was conducted in August 2020 using nets and 24-hour bottle traps. The taxa were identified at the species level, either in the field or the laboratory. While this study focused on the Gwent levels, one site, Fair Orchard Farm, was based in St. Brides SSSI.

2.6 Ecological Quality Indices Assessment

Water quality assessment was conducted once the sequences were assigned to the family level using the Biological Monitoring Working Party score (BMWP) and the Average Score Per Taxa (ASPT). The BMWP scores were calculated using the BMWP scoring system (Appendix II), in which the score equals the sum of macroinvertebrate families' tolerance scores in each sample (Mandaville, 2002). If a higher BMWP were calculated, it would reflect a better quality of water in the ditches and reens (Aquilina, 2013). The ASPT can then be calculated to reduce bias as it is the average tolerance score of all macroinvertebrate families within the sample site. The ASPT score ranged from 0 to 10 and was calculated by dividing the BMWP score by the number of families present (Sor *et al.*, 2021).

3. Results

3.1 Pipeline Output

A total of 12.2 million paired reads passed through the DADA2 (2016) filtering thresholds. After filtering for metazoan hits only, with a 70% BLAST percentage identity query cover, 2.2 million reads remained, meaning 81% of the reads were of non-metazoan origin. In total, the negative controls had a read depth of <800 and so were removed from the analysis as our baseline was set at a minimum of 1000 reads (Appendix I). To obtain the invertebrate data, the following phyla were included; Arthropoda, Gastrotricha, Platyhelminthes, Annelida, Rotifera, Mollusca, Cnidaria, Nematoda, Tardigrada, Porifera, Placozoa, Onychophora, Nemertea, Echinodermata and Bryozoa. Regarding Class assignment, Insecta (28%) contained the most significant percentage of ASVs, followed by Citellata (15%). In total, 379 species were assigned (Appendix II), including *Dicrotendipes lobiger*, a new species to the Gwent Levels, and *Chaetogaster diastrophus*, a new species in the UK.

3.2 Invertebrate Diversity

The PERMANOVA results (Figure 5) for invertebrates showed that the amount of water filtered ($F_{1,31}$ =1.34, Sum sq = 0.50, p = 0.05) and temperature ($F_{1,31}$ = 1.40, Sum sq = 0.52, p = 0.04) were the only abiotic variables that had a significant effect on beta diversity (Figure 6). The other variables, pH ($F_{1,31}$ = 1.21, Sum Sq = 0.45, p = 0.1), depth ($F_{1,\,31}$ = 0.97, Sum Sq = 0.36, p = 0.55), waterbody (F1, 31 = 1.1, Sum Sq = 0.41, p = 0.25), rugged dissolved oxygen ($F_{1,31}$ = 1.26, Sum Sq = 0.47, p = 0.1), salinity ($F_{1,31}$ = 1.09, Sum Sq = 0.40, p = 0.28), turbidity ($F_{1,31}$

= 1.16, Sum Sq = 0.43, p = 0.17) and land use ($F_{1,31}$ = 0.94, Sum Sq = 0.35, p = 0.61) had no significant effect on the β diversity of invertebrates.

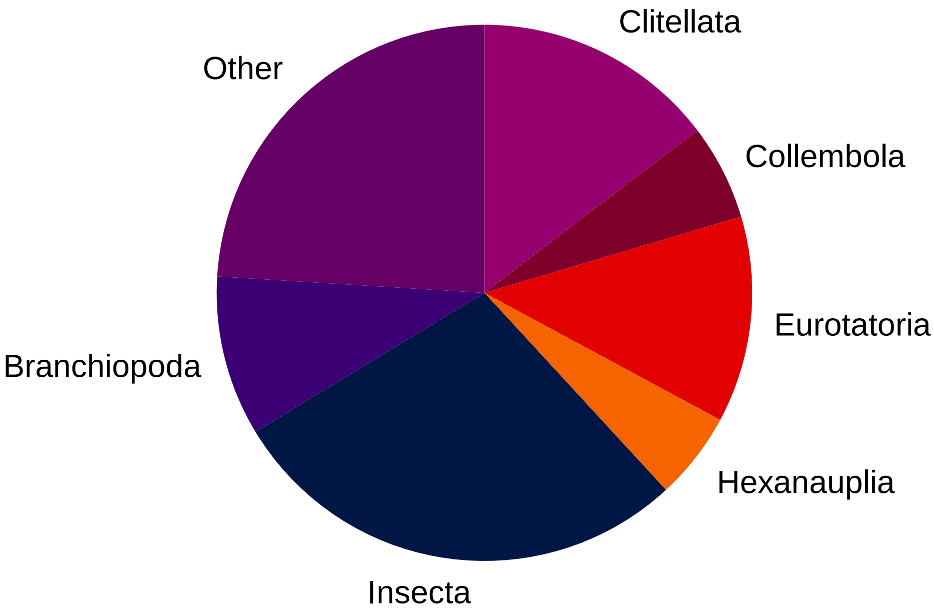

Figure 5. A pie chart regarding the division of Class. Insecta was the largest class (28%), followed by Citellata (15%) and Eurotatoria (12%). Branchipodada (10%), Collembola (6%) and Hexanaupalia (5%) all obtained above 5% of assignations compared to the Others (24%), which included; Hydrozoa (4.4%), Malacostraca (4.4%), Polychaeta (4.4%), Arachnida (4%), Gastropoda (3.8%), Bivalvia (1.2%), Chromadorea (1%), Ostracoda (0.9%), Catenulida (0.4%), Diplopoda (0.2%), Rhabditophora (0.2%), Trematoda (0.2%), Cestoda (0.1%), Chilopoda (0.1%), Demospongiae (0.1%), Enoplea (0.1%) and Phylactolaemata (0.1%).

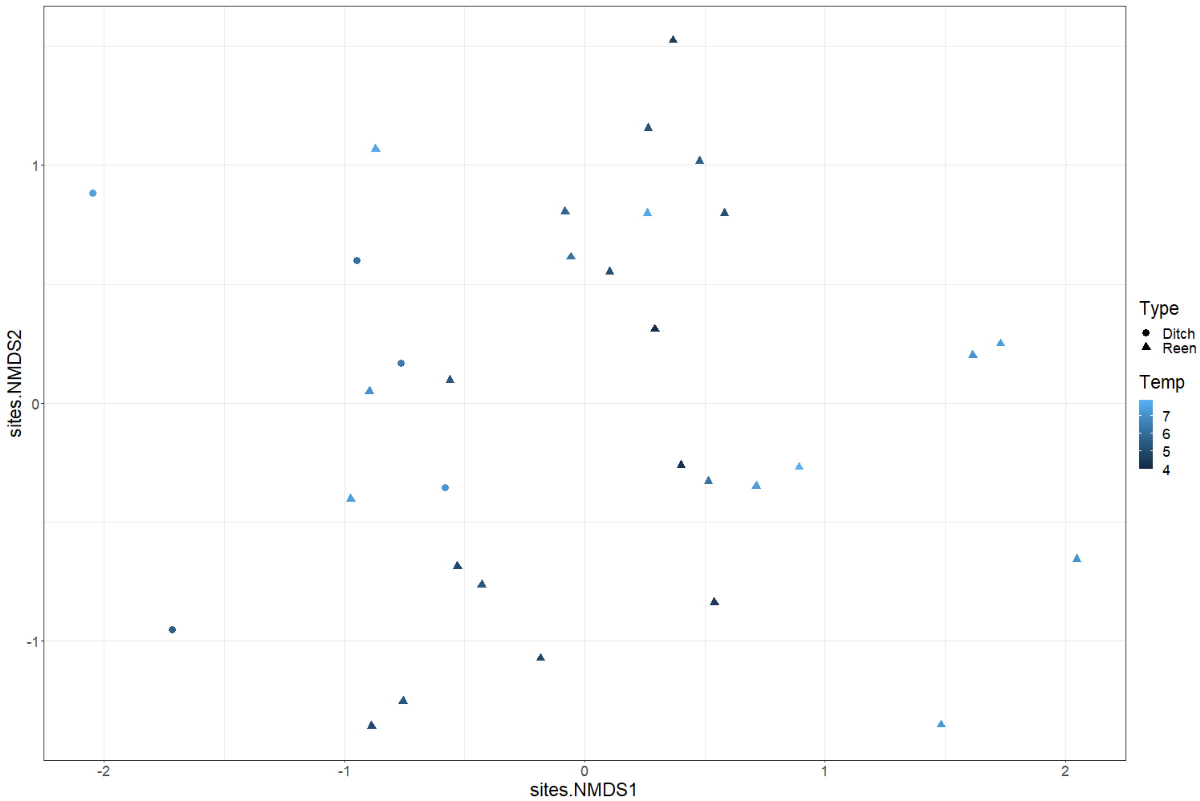

Figure 6. The NMDS plots explore the similarities between different variables and invertebrate diversity. Both waterbody type and temperature influenced β diversity. The NMDS plot compares the two different waterbody types, ditches and reens and indicates the impact temperature has on invertebrate diversity, with the colder sites being dark blue.

In total, there were 1,045 unique invertebrate ASVs with a greater number of unique invertebrate ASVs in reens, with 613, compared to 272 in ditches (Figure 7) with a 15% species overlap. However, when exploring the α diversity (Figure 8), there is the implication that the ditches have a higher ASV count when the factor of more reens sampled is removed.

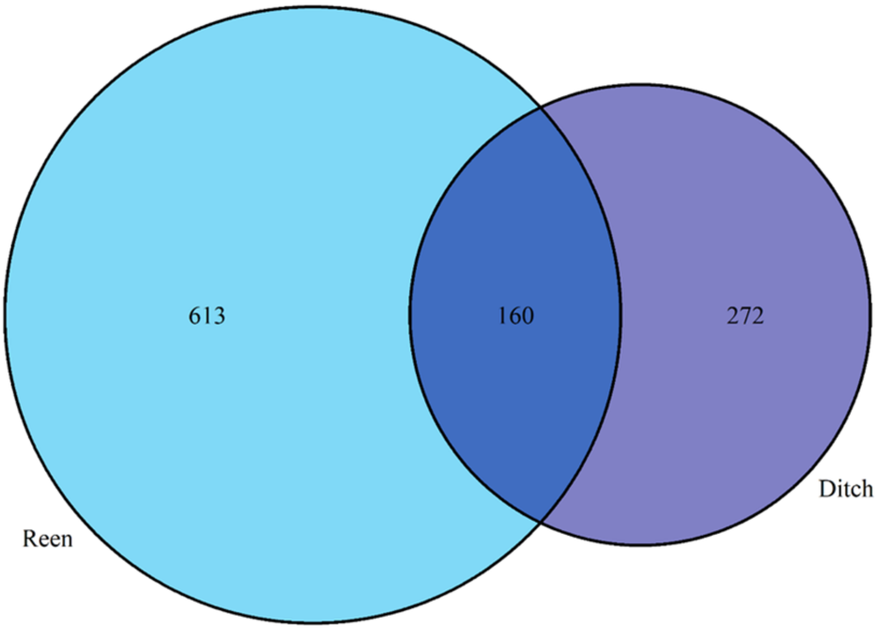

Figure 7. A Venn Diagram exploring the relationship of ASVs between ditches and reens. The diagram size is relative to the number of A.S.V.s, with the specific count appearing in the centre. There was only a 15% overlap, which consisted of 160 unique ASVs between ditches and reens.

The average amount of water sampled in ditches was 215 ml compared to 275 ml for reens. Field Ditch 5 (FD5) saw an average of 867 ml filtered, whereas the other ditches combined had an average of 37 ml. Reens IDB15 and IDB16 filtered the highest water quantity; however, that does not appear to have impacted the ASVs per site.

After a step function, the waterbody type, pH, and salinity remained in the final linear model. Alpha diversity was calculated using the number of observed ASVs. Both waterbody type ($F_{1,28}$ = 8.70, Sum Sq = 6035.0, p = 0.01) and salinity ($F_{1,28}$ = 4.05, Sum Sq = 2816.3, p = 0.05) had a significant effect on ASV count, but pH did not ($F_{1,28}$ = 0.43, Sum Sq = 295.4, p = 0.52).

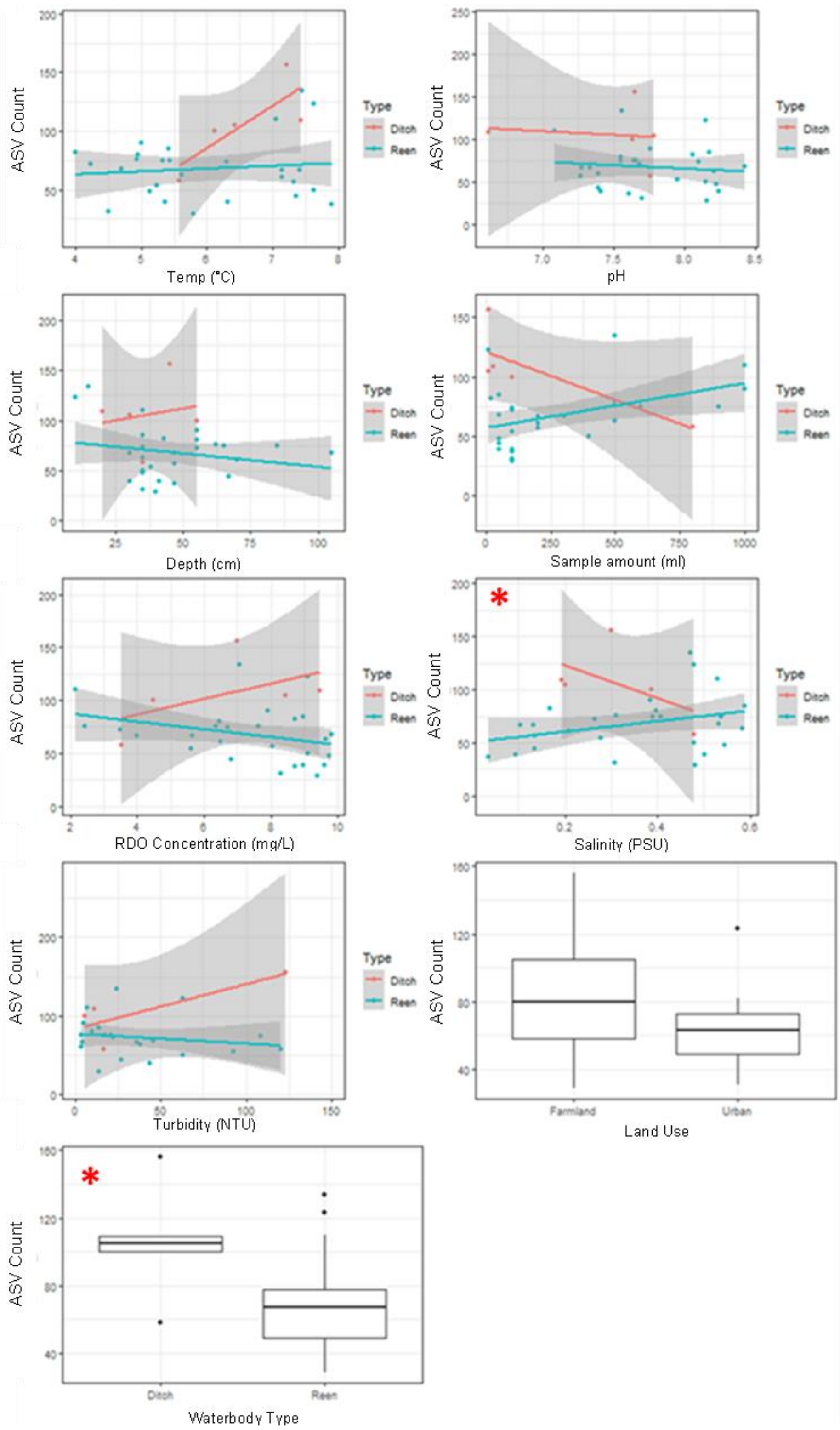

Figure 8. The α-diversity results presenting that water body type ($F_{1,28}$ = 8.70, Sum Sq = 6035.0, p = 0.01) and salinity ($F_{1,28}$ = 4.05, Sum Sq = 2816.3, p = 0.05) had a significant effect on ASV count. The results indicate that, on average, the ditches had a higher ASV count than reens, although the species present in ditches appear less resilient to salinity as there is a negative correlation.

3.3 Comparison of Previous Surveys

Surveys using morphological identification methods have previously been conducted on St. Brides SSSI as the area is a nationally significant assemblage of plants and invertebrate features (Murton, Hunt and Rodgers, 2019; Boyce, 2012). When comparing the eDNA survey results from this study with previous surveys of St. Brides, we see an increase in species detection.

No statistical analysis could be conducted with the previous surveys due to seasonality and location differences. However, when comparing the reens that were used in the eDNA study in the Boyce survey, it was apparent that the eDNA survey detected a far greater number of species than morphological methods (Figure 9). Furthermore, there was minimal overlap between species detected in both surveys.

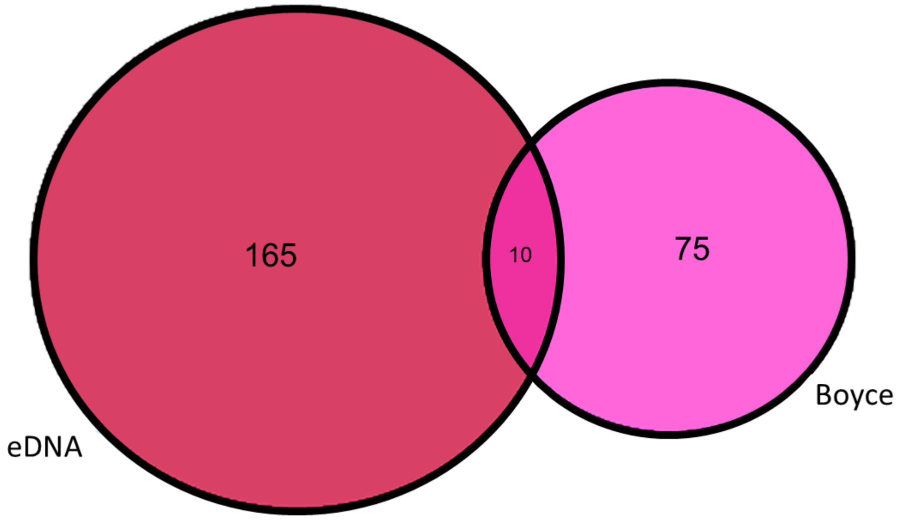

Figure 9. A comparison of two invertebrate sampling methods. Boyce's survey was conducted in 2011 using kick-net sampling. The eDNA survey was conducted in 2021 using molecular techniques. The results implied eDNA was a far more sensitive survey method, although further assessment is required to understand why there is not more of an overlap between species detected.

Despite the combination of methods, Graham and Hammond's survey recorded the least number of species (Figure 10). However, as the survey was conducted in summer and was in the same area, although not the same ditches as this study, no statistical analysis could be made. In total, 28 aquatic macro-invertebrate taxa were identified at Fair Orchard Farm.

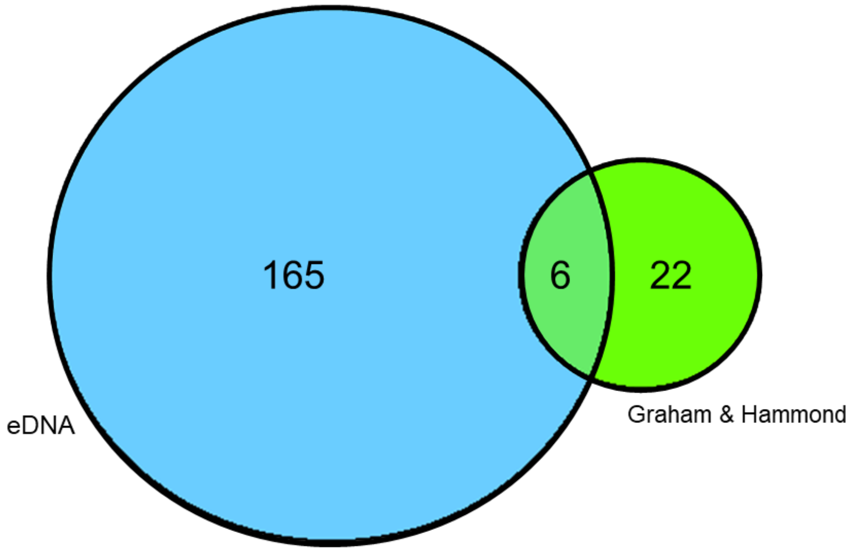

Figure 10. A comparison of Graham and Hammond's morphological identification using kick net and bottle sampling and a survey using eDNA. Graham & Hammond (2022) were able to identify 28 species of invertebrates, with six of those also being detected in the eDNA survey. However, eDNA alone was able to detect 165 species in the reens in St Brides.

Despite all morphological surveys occurring within St. Brides SSSI, different ditches and reens were studied than in this survey. Furthermore, seasonality and site will have influenced the results, so a manual comparison, rather than a statistical analysis, was conducted regarding the morphological survey results and the results from this study.

3.4 Ecological Quality Indices

In total, 31 different families, using a 99% BLAST identity filtering threshold belonging to the Orders: Ephemeroptera, Plecoptera, Trichoptera, Odonata, Hemiptera, Coleoptera, Diptera and Hirudinida were identified and used in the BMWP calculations (Table 2). The field ditches FD2, and FD3 had 'moderate' water quality according to the quality interpretation, while FD1 and IDB35 were both 'good' quality. The rest of the field ditches and reens were all awarded 'very good' quality, with IDB16 having the highest ASPT score, with 8. While FD2 and FD3 had the lowest ASPT scores, with 4.6 each, FD1 had the lowest BMWP score; however, three ditches surrounding the same field had the lowest BMWP and ASPT scores.

Table 2. The results of the water quality analysis according to the WFD guidelines using the Orders sequenced from the eDNA analysis.

Site	BMWP score	ASPT score	Quality interpretation
FD1	20	5	Good
FD2	23	4.6	Moderate
FD3	28	4.6	Moderate
FD4	74	6.1	Very good
FD5	29	7.3	Very good
EA9	101	6.3	Very good
IDB15	76	6.9	Very good
IDB16	72	8	Very good
IDB30	100	6.6	Very good
IDB35	89	5.9	Good
IDB37	84	6	Very good

4. Discussion

Following the results of this study, it is apparent that analysing eDNA in reens and ditches can provide valuable insights into invertebrate biodiversity. There were clear differences in the factors affecting diversity, with β diversity significantly impacted by the amount of water sampled and temperature. Furthermore, α diversity was influenced by salinity and waterbody type. The difference in impact by waterbody type can be seen in the ASVs detected. There was notable turnover in the assigned species to those environments, suggesting different community compositions between the ditches and reens. Furthermore, the species associated with the ASVs could be used to calculate the B.M.W.P. and A.S.P.T., suggesting that the water quality of St. Brides is at least "moderate", with reens having better quality and higher α diversity.

4.1 Water Quality Effect on Biodiversity

Using the invertebrate metabarcoding data to assess water quality has indicated that the field ditches were poorer in quality overall when compared to reens. Three of the five ditches in the same area had the lowest BMWP and ASPT scores (≤ 5). Furthermore, no families with a score of 10 were present in ditches FD1, FD2 or FD3, indicating some pollution. The most common family detected in both ditches and reens was Chrysomelidae, in the Order Coleoptera, which scored 5, followed by Asellidae, which scored three. Two sites detected the *Hydrophilus piceus*, the Great Silver Water Beetle, which is classed as Near Threatened by the IUCN (NBN Atlas, 2021). It has a restricted British distribution, whereas, in Wales, modern records only originate from three 10km squares around the Gwent Levels. Also detected in the Coleoptera family is the declining Nationally Rare species *Choragus sheppardi,* which was detected in one of the field ditches (FD2) in this survey.

Ditches displayed poorer water quality using the Ecological Quality Indices, potentially due to a lack of management. The reens are maintained by NRW, who dredge the water on a seven-year cycle to prevent overgrowth (Boyce, 2012), compared to ditches, whose management is more sporadic and dependent on landowners, which often results in neglect. Reen and ditch management have been linked to species diversity. Removing sediments and vegetation is required to allow for drainage, which can significantly impact vegetation and invertebrate fauna (Shaw *et al.*, 2015; Milsom *et al.*, 2004; Twisk, Noordervliet and ter Keurs, 2000). In this survey and previous surveys on the Gwent Levels, duckweed species have been allowed to grow undisturbed in ditches indicating eutrophication (Graham and Hammond, 2022; Whitehead, 2022; Boyce, 2012).

Furthermore, dense mats of duckweed suppress the growth of submerged beds of aquatic macrophytes, which are essential niches for aquatic invertebrates (Boyce, 2012). The eDNA survey conducted in this paper did not determine the cause of dominant duckweed; however, studies have shown that high levels of nitrogen and phosphorus from over-fertilization have caused a vegetation shift (Janse and Van Puijenbroek, 1998). In ditches, the plants have moved from mainly submerged aquatic vegetation to eutrophication levels of duckweed (Janse and Van Puijenbroek, 1998). Management, especially of connected ditches, may allow for faster recolonization, while other processes, such as preventing the use of agro-chemicals close to banks, further benefit macroinvertebrates (Shaw *et al.*, 2015; Leng, Musters and de Snoo, 2009; Manhoudt, Visser and de Snoo, 2007).

4.2 Abiotic Factors Influencing Biodiversity

4.2.1 Water Filtration Quantity

The amount of water sampled was a significant variable in this study regarding the β diversity, although it was not significant regarding α diversity in this study. The least amount of water filtered was obtained from the ditches with an average of 203ml, compared to 350ml for reens. Mächler *et al.* (2016) studied water filtration effects on the detection rate for three macroinvertebrate species belonging to the order Mollusca, Ephemeroptera and Amphipoda using volumes ranging from 250 to 2000ml. They concluded that an increase in the volume of extracted DNA screened and primer performance was more important in reducing the false negative detections of some species, although increasing sample volume was also beneficial. Only one species used had a positive relationship between increased sample volume and detection (Mächler *et al.*, 2016).

Similarly, Peixoto *et al*. (2021) investigated the importance of eDNA capture methods' regarding detecting species in aquatic environments in Portugal. The study covered the usual range of applications in eDNA monitoring, considering both targeted detection of ubiquitous species and the overall characterization of amphibian community composition using qPCR, High Throughput Sequencing and two PCR replication thresholds (stringent and relaxed). They found that the filtration method influenced eDNA recovery and species detection, with filtering methods being more effective than precipitation, implying this was associated with the amount of water filtered (Peixoto *et al.*, 2021). Water filtering quantities are among the first biases experienced when using water eDNA methods (Mächler *et al.*, 2016). A lack of uniform methodology adds to the issue of filtration amounts, while there is little evidence on the optimal amount of extracted eDNA to reduce the likelihood of detecting false negatives (Mächler *et al.*, 2016; Deiner and Altermatt, 2014).

4.2.3 Temperature

In this study, temperature ($p = 0.05$) significantly impacted β diversity, although it did not affect α diversity. The ditches had an average temperature of 6.55°C, while the reens had an average temperature of 6.03°C. The temperature of lotic environments has been considered important in influencing the life histories of aquatic organisms (Elliott, 1987a, 1987b; Vannote and Sweeney, 1980; Brittain, 1975). Aquatic invertebrate thermal histories cause responses at the organismic, population and community levels of organization, establishing ecological and evolutionary time scales (Ward and Stanford, 1982). Moon (1940) explored the impact of temperature when researching the movements of freshwater invertebrates in lake Windermere and found it could influence the movement of invertebrates. Furthermore, they found that while low temperatures (5˚C) limited the amount of movement, they did not completely inhibit invertebrate activity (Moon, 1940). Further research is required to explore temperature impact on seasonal changes and its influence on invertebrate diversity in ditches and reens.

Strickler *et al.* (2015), who studied the effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms, found DNA degradation rates were lowest under cold temperatures (5˚C), low UV-B levels, and alkaline conditions. Higher degradation rates were associated with higher temperatures, neutral pH, and moderately high UV-B, creating favourable microbial growth environments. Moreover, they found that temperature positively affected degradation rates in its own right, increasing as the temperature increased (Strickler, Fremier and Goldberg, 2015). However, in a natural system such as that studied here, with a temperature range of (4.2 °C – 7.9°C), results were robust and showed no evidence of eDNA degradation impacting diversity.

Global climate change may further impact invertebrate biodiversity. Although this was not considered in this study, there is scope for long-term monitoring of the Gwent Levels to understand how seasonal temperature changes will impact diversity. Species react differently to changes in the environment. While some invertebrates may become locally extinct, others may expand their ranges (Davey *et al.*, 2013; Parmesan and Yohe, 2003). Flying insects have seen a dramatic decrease of 75% in biomass over a three-decade period in Germany (Hallmann *et al.*, 2017). Furthermore, there are increasing reports of other Orders experiencing declines, with a potential consequence of changing ecosystem connectivity and function (Hallmann *et al.*, 2019; Powney *et al.*, 2019; van Strien *et al.*, 2019; Dirzo *et al.*, 2014; Schuch, Wesche and Schaefer, 2012; Shortall *et al.*, 2009; Gaston and Fuller, 2007; Conrad *et al.*, 2006). This could be due to streams and rivers being among the most sensitive ecosystems regarding climate change (Durance and Ormerod, 2009). Their temperatures closely track air temperature, particularly in headwaters, with streams warming in response to climate change (Durance and Ormerod, 2007; Caissie, 2006). Over the last few decades, 50 southern English streams have increased by a mean of 2.1-2.9ºC in winter and 1.1-1.5ºC in summer (Durance and Ormerod, 2009).

4.2.4 pH

The pH of water is influenced by the natural conditions of the environment (Baaloudj *et al.*, 2020). In this study, pH was found not to impact significantly α and β-diversity for invertebrates, with the ditch pH ranging between 6.6 and 7.8 compared to reens, which ranged from 7 to 8.4.

Berezina (2001) found that the highest species diversity for freshwater invertebrates occurred between pH 4.09-8.65, with a decrease in species diversity below 4 and above 9 under experimental conditions. Petrin, Laudon and Malmqvist (2007) investigated macroinvertebrate response to a low pH gradient in rivers across Sweden. They found that Plecoptera richness did not change with varying pH levels in the north or south Sweden. However, while Ephemeroptera richness was susceptible to changes in pH in both regions, Trichoptera decreased with the increasing pH in the north, and in southern Sweden, they increased with increasing pH. The results support the hypothesis that stream invertebrates can tolerate low pH through exaptation or adaptation; however, the degree of which depends on the taxa (Petrin, Laudon and Malmqvist, 2007). Berezina (2001) further supported this, demonstrating that invertebrate survival depends on the tolerance limits of individuals, and their communities are formed depending on relationships between the pH of the aquatic environment and individual adaptability. This further implies that the pH range within this study was not enough to cause a significant impact on α and β-diversity in freshwater invertebrates.

pH significantly impacted eDNA capture and degradation in a controlled laboratory microcosm experiment (Kagzi *et al.*, 2022). While some research found that eDNA persists for several weeks in water, other studies have demonstrated that even small (<100 bp) eDNA fragments can degrade to undetectable levels within hours or days after an organism's removal (Thomsen *et al.*, 2012; Kagzi *et al.*, 2022; Strickler, Fremier and Goldberg, 2015). Several studies have indicated that more acidic conditions (pH<5) have a significant impact on degradation in both the field and the laboratory (Goldberg, Strickler and Fremier, 2018; Seymour *et al.*, 2018; Strickler, Fremier and Goldberg, 2015). However, evidence has also suggested that pH only significantly influenced the degradation rate when combined with other abiotic variables, such as temperature and UV-B (Lance *et al.*, 2017; Strickler, Fremier and Goldberg, 2015).

4.2.5 Salinity

While salinity was not found to impact β diversity ($p=0.28$) significantly, it was a significant variable for α diversity ($p=0.05$). The Water Framework Directive requires monitoring salinity to determine the waterbody's ecological condition (Pickwell *et al.*, 2022; European Commission, 2000). Salinization is a globally important stressor in freshwater ecosystems and is defined as the total concentration of dissolved inorganic ions in water or soil (Cañedo-Argüelles *et al.*, 2013; Williams and Sherwood, 1994). Natural salinization occurs from salts transported by seawater evaporation, catchment weather and sea spray (Williams and Sherwood, 1994). Salinisation can also be impacted by anthropogenic effects through water harvesting, road de-icing, mining activities and changes to vegetation leading to water table movement (Cañedo-Argüelles *et al.*, 2013).

Bray *et al.* (2018) investigated how macroinvertebrate communities from high-salinity sites impacted low-salinity sites along a salinity gradient in experimentally manipulated streams. At the community level, it was expected that salinity and biological interactions between sensitive and tolerant organisms would influence the community composition, reduce diversity and potentially homogenize communities (Bray *et al.*, 2018). This declining pattern with salinity was seen by Ephemeroptera abundance, regarding both the salinity effects and in response to interactions with more tolerant taxa. Salt-sensitivity appeared to increase in Plecoptera and EPT taxa in the presence of tolerant taxa, with little salinity impact in salt-sensitive treatments (Bray *et al.*, 2018). Overall, Bray *et al.* (2018) found that at higher salinities, direct effects of salinity dominated community responses, and this resulted in reduced abundance and altered community composition, with almost a complete loss of Ephemeroptera and a reduction in Trichoptera.

In this study, when exploring the α diversity, ditches declined in species count regarding salinity levels, thus supporting Bray *et al.*'s (2018) findings. However, reens presented a positive correlation with increasing salinity levels, implying that the taxa present in the reens are more tolerable to salinization stressors. One explanation for the increase in reen diversity is whether the communities consisted of salt-tolerant taxa. Bray *et al.* (2018) found that interspecific interactions between salt tolerance and salt-sensitive taxa became more critical as the sensitivity to the toxicant increased. This may be due to more salt-tolerant predators; therefore, there are greater predator impacts with increased salinity (Bray *et al.*, 2018; Kefford, 1998).

4.2.6 Dissolved Oxygen

Dissolved oxygen (DO) concentration is a key factor in determining macroinvertebrate assemblage composition, despite being non-significant in this survey (Williams, 1996). Macroinvertebrates in ditches face both predictable and unpredictable variations in DO concentration, with low oxygen concentration viewed as an important environmental filter (Verdonschot and Verdonschot, 2014). Several factors could influence the DO concentrations, including salinity, time of day, algal bloom and depth (Natural Resources Wales, 2022). Previous research in laboratory experiments has shown increased mortality, decreased growth rate, and prolonged development time under oxygen stress (Kolar and Rahel, 1993; Moore and Burn, 1968).

Dissolved oxygen levels can be impacted by temperature, and cold water can hold more dissolved oxygen than warm water, leading to frequent higher DO levels in winter (U.S. Geological Survey, 2022). The DO ranges recorded in this study are between 2.1 mg/L and 9.8 mg/L. Hypoxia occurs when DO concentrations fall below 2-3 mg/L (US Environmental Protection Agency, 2015). Therefore, this suggests that DO is not a significant variable for α and β diversity as the DO levels in most ditches and reens were acceptable for invertebrates. Two sites (IDB16.2 and IDB30.1) had a DO concentration <3mg/L, while the other sites in the same reens had concentrations >3 mg/L, therefore the site did not impact the overall significance of α and β diversity.

4.2.7 Turbidity

There is little research to suggest turbidity directly impacts freshwater invertebrate communities, which was also found not to have a significant influence within this study. Kefford *et al*. (2007) investigated freshwater invertebrates' response to the gradient of salinity and turbidity in Australia. They found one test species, *Micronecta annae* (Hemiptera: Corixidae), preferred relatively high turbidity (>200 NTU), but only from one of two locations. However, another species, *Austrochiltonia subtenuis* (Amphipoda: Hyalellidae), showed the opposite, responding to low turbidity (<200 NTU). The evidence found was weak, potentially because turbidity levels are not directly harmful to invertebrates, which corroborates what we found in this study (Kefford *et al.*, 2007).

4.2.8 Depth

While this study did not find depth to be a significant factor, evidence suggests that water depth is more likely to influence invertebrate community structure throughout the seasons. In winter, water levels in the ditches and reens in St. Brides SSSI are lower than in summer, allowing floodplains to be drained. This could reduce diversity in winter, as aquatic invertebrates may not be able to travel as freely to different reens due to movement barriers, such as sluice gates separating the waterbodies. Although this has not been explored with invertebrates, Katano *et al.* (2003) found this true for fish. Furthermore, research has shown that water depth and shade can have a small impact on invertebrate communities in ditches (Shaw *et al.*, 2015). While in this study, ditches had a positive correlation with depth regarding α diversity, and reens had a non-significant correlation regarding α diversity and a slightly negative non-significant correlation relating to β diversity. However, Shaw *et al.* (2015) found increased biodiversity within invertebrate communities in deeper sites. They suggested that shade and water depth were important environmental factors but admitted that their study's significance was minor (Shaw *et al.*, 2015).

4.2.9 Waterbody Type & Land use

There was no significant difference in waterbody type on β diversity; however, there was on α ($p = 0.01$) diversity implying no difference in the species composition within the community was observed. However, it suggests a greater variety of species between the ditches and reens. One potential explanation is that more plants are in the ditches due to a lack of maintenance. Clare and Edwards (1983) found that most macroinvertebrates collected from ditch samples were within the water column and on plants rather than in the benthos. Furthermore, Herzon and Helenius (2008) found that terrestrial vegetation on the banks of the ditches was essential for many invertebrate communities, with a specific host and nectar plants providing food and overwintering sites. The ditches in this study had overhanging plants, providing food and shelter and may significantly impact the variety of species that can inhabit that waterbody type. Freshwater ecosystems are directly influenced and or/ indirectly by human activities, so it was essential to understand if there were any anthropogenic impacts in this study (Juvigny-Khenafou *et al.*, 2021). Reens IDB37, IDB35, and half of IDB30 were categorized as urban, while the other reens and ditches were rural. There was no significant difference in biodiversity when comparing invertebrate α and β diversity between ditches and reens in urban and rural environments, potentially due to minimal urban sites where samples were taken compared to rural. However, other research on lotic ecosystems has found that urbanisation negatively affects the diversity of freshwater macroinvertebrates (Gál *et al.*, 2019). Conversely, Vermonden *et al.* (2009) found that urban drainage ditches can obtain the same levels of macroinvertebrate biodiversity as those in rural areas. Further research is needed to understand if the linkage between reens means land use has minimal impact on invertebrate biodiversity.

4.2.10 Seasonality

The study presented here was conducted in December, which could impact species detection, especially in groups such as Ephemeroptera, where a single generation overwinters in the nymphal stage (Clifford, 1982). Musters *et al.* (2019) used a small, well-connected network of drainage ditches to measure the spatiotemporal β diversity of a freshwater macrofaunal metacommunity in the temporal climate zone in the Netherlands from May to November 2011 and 2012. They found no temporal patterns between the years and months (Musters *et al.*, 2019). Conversely, Zizka, Geiger and Leese (2020) used DNA metabarcoding of stream invertebrates in Western Germany to explore spatial-temporal variation, where variation had distinct seasonal effects on their OTU composition at the near-natural river in spring (April/May 2017/2018) and autumn/ winter (September 2016/2017 and November/December 2016/2017). They found variations in taxa response, with taxa that are highly abundant in spring, almost absent, or present only in eggs or overwintering in autumn, and vice versa (Zizka, Geiger and Leese, 2020). Aggregated at the order level, a higher number of reads were assigned to Ephemeroptera in spring than autumn, compared to Plecoptera had a higher number of reads assigned in autumn. However, ecological status assessment remained consistent throughout the seasons and comparable to other assessments where morphological identification was used (Zizka, Geiger and Leese, 2020).

Conversely, Rehinholdt Jenson *et al*. (2021) used eDNA metabarcoding to explore seasonal turnover in the community composition of stream macroinvertebrates in Denmark and found that Plecoptera were most abundant in spring sampling as they are more abundant during the spring season. Šporka *et al.* (2006) explored streams in central Europe to understand the influence of seasonal variation on the bioassessment of streams using macroinvertebrates. They found an increase in Trichoptera and Ephemeroptera abundance when comparing October sampling to spring sampling, a trend not seen in either of the studies by Rehinholdt Jenson *et al.* (2021) or Zizka, Geiger and Leese (2020) (Šporka *et al.*, 2006). They presented significant differences in communities in spring and autumn, with a considerable increase in taxa detected when sampling in both seasons (Reinholdt Jensen *et al.*, 2021).

4.3 eDNA as a Tool for Assessing Water Quality

Using eDNA as an identification tool can potentially reduce processing times, labour intensity, and time spent on sample analysis (Kuntke *et al.*, 2020). Environmental DNA-derived data allows for the inclusion of a much more comprehensive range of taxa and indicator groups that would otherwise be ignored due to the limitations of traditional taxonomic identification (Seymour *et al.*, 2020). Elbrecht *et al*. (2017) compared DNA metabarcoding with a morphology-based protocol and found that eDNA identified more than twice the number of taxa. Traditional morpho-taxonomic methods and eDNA face limitations regarding the quality and completeness of sequence databases or identification keys used for taxonomic assignment (Seymour *et al.*, 2020). With traditional methods such as kick-net sampling, taxonomic assignment is often limited to family or general-level assignments and can often oversimplify or omit groups such as Rotifers, Oligochaeta, or Chironomidae, which are critical environmental indicators (Seymour *et al.*, 2020; Elbrecht *et al.*, 2017; Furse *et al.*, 2009).

When comparing the eDNA survey results from this study with previous surveys of St. Brides, we see an increase in species detection. This is due to one of the benefits of eDNA being that it can detect both aquatic and terrestrial macroinvertebrates. The previous surveys on the Gwent Levels have been conducted using traditional morphological-taxonomic methods and, therefore, due to time or expertise constraints, have either focused on specifically terrestrial or aquatic macroinvertebrates (Graham and Hammond, 2022; Boyce, 2012)

While some of the reens in this study were used in Boyce’s survey, they are incomparable due to seasonality. Boyce and Graham, and Hammond conducted the traditional surveys in summer, when invertebrates are more active, compared to the eDNA survey, which was conducted in winter. Temperature both directly and indirectly influences aquatic invertebrate life histories, with colder temperatures reducing the amount of energy available for growth and reproduction (Hart, 1985). However, despite the eDNA sampling being conducted in winter, the species detection was far greater. Further investigation is required to understand how seasonality effects invertebrate detection in water to understand whether surveys can be conducted throughout the year.

5. Conclusion

Monitoring invertebrates is essential for understanding how an aquatic community is functioning as often declines in biodiversity go unnoticed as they are not always visible. As invertebrates are sensitive to ecological changes, they are useful biological indicators; however, many previous invertebrate surveys in the Gwent Levels have used morphological identification methods. This study analysing eDNA has provided a more comprehensive taxonomic database for invertebrates in ditches and reens without the results being impacted by environmental degradation from elements such as pH and temperature. However, some abiotic variables significantly impacted the invertebrate community, such as waterbody type and salinity, indicating an interaction between the two variables and is vital in understanding the α diversity of communities in these wetland channels, potentially influenced by marine inputs. Regarding β diversity, the amount of water filtered has a significant impact meaning there is further scope to develop more consistent filtering methods. Furthermore, temperature also impacts β diversity, which demonstrates there could be an interaction between the amount of water filtered and temperature; however, this is unlikely.

Further research is required to analyse more eDNA samples from ditches and explore seasonality as a variable. Furthermore, exploration is required to understand if there is any correlation between salinity and waterbody type and the impact it has on α diversity. Nevertheless, using aqueous eDNA analysis has proven effective in understanding biodiversity in St. Brides SSSI, with the results being applicable to aid future management plans in the protection of invertebrates.

6. References

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7. Appendices

Appendix I.

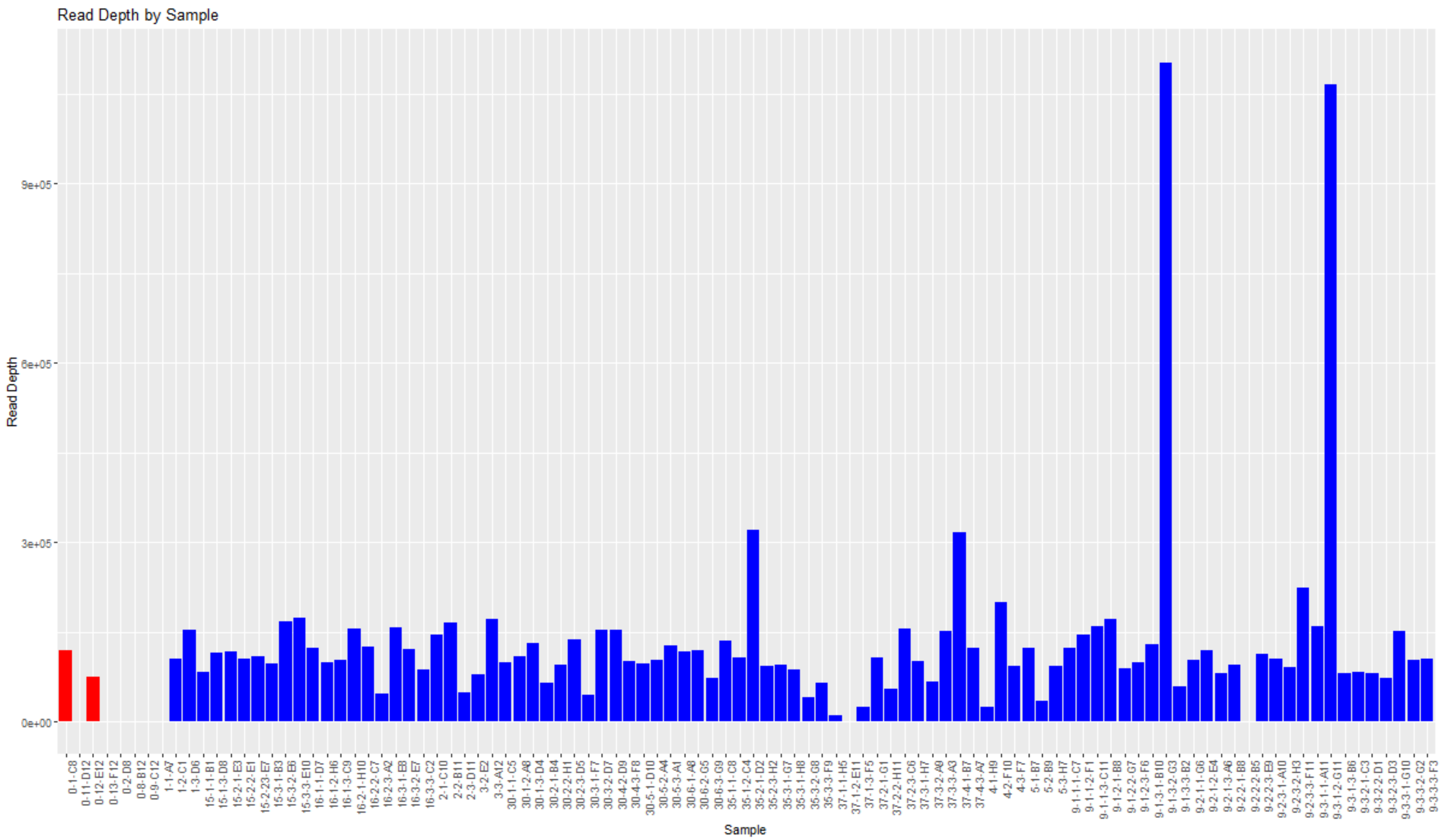

Figure 21. Sample read depths. The samples starting with a 0 code are controls. Those highlighted in red are positive controls, while the negative control read depths were so minimal, they did not appear on the Y axis.

Appendix II. Complete Species List at 99% filtration identity level.

Phylum	Class	Order	Family	Genus	Species
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Allolobophora	*Allolobophora chlorotica*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Aporrectodea	*Aporrectodea icterica complex sp. L1 DP-2018*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Aporrectodea	*Aporrectodea longa*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Aporrectodea	*Aporrectodea rosea*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Bimastos	*Bimastos rubidus*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Eiseniella	*Eiseniella sp. BIOUG32056-F01*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Eiseniella	*Eiseniella tetraedra*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Lumbricus	*Lumbricus castaneus*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Lumbricus	*Lumbricus festivus*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Lumbricus	*Lumbricus terrestris*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Murchieona	*Murchieona minuscula*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Octolasion	*Octolasion cyaneum*
Annelida	Clitellata	Crassiclitellata	Lumbricidae	Satchellius	*Satchellius mammalis*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	Cernosvitoviella	*Cernosvitoviella minor*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	Chamaedrilus	*Chamaedrilus cognettii*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	Cognettia	*Cognettia pseudosphagnetorum*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	Fridericia	*Fridericia striata*
Annelida	Clitellata	Enchytraeida	Enchytraeidae	Globulidrilus	*Globulidrilus riparius*
Annelida	Clitellata	Hirudinida	Erpobdellidae	Erpobdella	*Erpobdella testacea*
Annelida	Clitellata	Lumbriculida	Lumbriculidae	Lumbriculus	*Lumbriculus variegatus*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Annelida	Clitellata	Lumbriculida	Lumbriculidae	Stylodrilus	*Stylodrilus heringianus*
Annelida	Clitellata	Rhynchobdellida	Glossiphoniidae	Glossiphonia	*Glossiphonia complanata*
Annelida	Clitellata	Rhynchobdellida	Glossiphoniidae	Helobdella	*Helobdella stagnalis*
Annelida	Clitellata	Tubificida	Naididae	Aulodrilus	*Aulodrilus pluriseta*
Annelida	Clitellata	Tubificida	Naididae	Chaetogaster	*Chaetogaster cf. diastrophus MK-2019*
Annelida	Clitellata	Tubificida	Naididae	Chaetogaster	*Chaetogaster diastrophus*
Annelida	Clitellata	Tubificida	Naididae	Dero	*Dero digitata*
Annelida	Clitellata	Tubificida	Naididae	Dero	*Dero obtusa*
Annelida	Clitellata	Tubificida	Naididae	Ilyodrilus	*Ilyodrilus templetoni*
Annelida	Clitellata	Tubificida	Naididae	Limnodrilus	*Limnodrilus claparedianus*
Annelida	Clitellata	Tubificida	Naididae	Limnodrilus	*Limnodrilus hoffmeisteri*
Annelida	Clitellata	Tubificida	Naididae	Nais	*Nais communis*
Annelida	Clitellata	Tubificida	Naididae	Nais	*Nais communis/variabilis complex sp. A2*
Annelida	Clitellata	Tubificida	Naididae	Nais	*Nais communis/variabilis complex sp. A3*
Annelida	Clitellata	Tubificida	Naididae	Potamothrix	*Potamothrix bavaricus*
Annelida	Clitellata	Tubificida	Naididae	Potamothrix	*Potamothrix hammoniensis*
Annelida	Clitellata	Tubificida	Naididae	Potamothrix	*Potamothrix heuscheri*
Annelida	Clitellata	Tubificida	Naididae	Rhyacodrilus	*Rhyacodrilus falciformis*
Annelida	Clitellata	Tubificida	Naididae	Spirosperma	*Spirosperma ferox*
Annelida	Clitellata	Tubificida	Naididae	Stylaria	*Stylaria lacustris*
Annelida	Clitellata	Tubificida	Naididae	Tubifex	*Tubifex tubifex*
Annelida	Clitellata	unknown_order	unknown_family	unknown_genus	*Oligochaeta sp. 1 RV-2016*
Annelida	Polychaeta	unknown_order	Capitellidae	Dasybranchus	*Dasybranchus sp. DH1*
Arthropoda	Arachnida	Araneae	Anyphaenidae	Anyphaena	*Anyphaena accentuata*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Arachnida	Araneae	Clubionidae	Clubiona	*Clubiona phragmitis*
Arthropoda	Arachnida	Araneae	Linyphiidae	Gnathonarium	*Gnathonarium dentatum*
Arthropoda	Arachnida	Araneae	Lycosidae	Pardosa	*Pardosa amentata*
Arthropoda	Arachnida	Araneae	Pisauridae	Pisaura	*Pisaura mirabilis*
Arthropoda	Arachnida	Araneae	Theridiidae	Anelosimus	*Anelosimus vittatus*
Arthropoda	Arachnida	Araneae	Theridiidae	Paidiscura	*Paidiscura pallens*
Arthropoda	Arachnida	Araneae	Theridiidae	Theridion	*Theridion varians*
Arthropoda	Arachnida	Opiliones	Leiobunidae	Leiobunum	*Leiobunum blackwalli*
Arthropoda	Arachnida	Opiliones	Phalangiidae	Oligolophus	*Oligolophus tridens*
Arthropoda	Arachnida	Opiliones	Phalangiidae	Paroligolophus	*Paroligolophus agrestis*
Arthropoda	Arachnida	Opiliones	Sabaconidae	Sabacon	*Sabacon viscayanus*
Arthropoda	Arachnida	Sarcoptiformes	Acaridae	Tyrophagus	*Tyrophagus curvipenis*
Arthropoda	Arachnida	Sarcoptiformes	Acaridae	Tyrophagus	*Tyrophagus fanetzhangorum*
Arthropoda	Arachnida	Sarcoptiformes	Camisiidae	Platynothrus	*Platynothrus peltifer*
Arthropoda	Arachnida	Sarcoptiformes	Damaeidae	unknown_genus	*Damaeidae sp. AMUEnv005*
Arthropoda	Arachnida	Trombidiformes	Eriophyidae	Abacarus	*Abacarus hystrix*
Arthropoda	Arachnida	Trombidiformes	Eriophyidae	Aculodes	*Aculodes mckenziei*
Arthropoda	Arachnida	Trombidiformes	Penthaleidae	unknown_genus	*Penthaleidae sp. Q091*
Arthropoda	Arachnida	Trombidiformes	Pygmephoridae	Elattoma	*Elattoma abeskoun*
Arthropoda	Arachnida	Trombidiformes	Tydeidae	Tydeus	*Tydeus sp. BMOC 17-0901-48*
Arthropoda	Arachnida	unknown_order	unknown_family	unknown_genus	*Arachnida sp. BOLD:ACM9770*
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia curvirostris*
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia longispina*
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia pulex*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Simocephalus	*Simocephalus vetulus*
Arthropoda	Branchiopoda	Diplostraca	Eurycercidae	Eurycercus	*Eurycercus lamellatus*
Arthropoda	Chilopoda	Geophilomorpha	Himantariidae	Stigmatogaster	*Stigmatogaster subterranea*
Arthropoda	Collembola	Entomobryomorpha	Entomobryidae	Lepidocyrtus	*Lepidocyrtus cyaneus*
Arthropoda	Collembola	Entomobryomorpha	Entomobryidae	Lepidocyrtus	*Lepidocyrtus lanuginosus*
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Desoria	*Desoria trispinata*
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Isotoma	*Isotoma viridis*
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Isotomurus	*Isotomurus palustris*
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Isotomurus	*Isotomurus unifasciatus*
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Parisotoma	*Parisotoma aff. notabilis L0*
Arthropoda	Collembola	Entomobryomorpha	Tomoceridae	Tomocerus	*Tomocerus minor*
Arthropoda	Collembola	Neelipleona	Neelidae	Megalothorax	*Megalothorax minimus*
Arthropoda	Collembola	Poduromorpha	Neanuridae	Neanura	*Neanura muscorum*
Arthropoda	Collembola	Poduromorpha	Poduridae	Podura	*Podura aquatica*
Arthropoda	Collembola	Symphypleona	Dicyrtomidae	Dicyrtomina	*Dicyrtomina saundersi*
Arthropoda	Collembola	Symphypleona	Dicyrtomidae	unknown_genus	*Dicyrtomidae sp. BOLD:ACL8646*
Arthropoda	Collembola	Symphypleona	Katiannidae	Sminthurinus	*Sminthurinus aureus*
Arthropoda	Collembola	Symphypleona	Katiannidae	Sminthurinus	*Sminthurinus elegans*
Arthropoda	Collembola	Symphypleona	Sminthuridae	Sminthurus	*Sminthurus viridis*
Arthropoda	Collembola	Symphypleona	Sminthurididae	Sminthurides	*Sminthurides aquaticus*
Arthropoda	Diplopoda	Julida	Julidae	Ophyiulus	*Ophyiulus pilosus*
Arthropoda	Hexanauplia	Cyclopoida	Cyclopidae	Acanthocyclops	*Acanthocyclops vernalis*
Arthropoda	Hexanauplia	Cyclopoida	Cyclopidae	Cyclops	*Cyclops abyssorum*
Arthropoda	Hexanauplia	Cyclopoida	Cyclopidae	Eucyclops	*Eucyclops cf. serrulatus BOLD:AAZ6402*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Hexanauplia	Harpacticoida	Canthocamptidae	Canthocamptus	*Canthocamptus staphylinus*
Arthropoda	Insecta	Coleoptera	Anobiidae	Anobium	*Anobium punctatum*
Arthropoda	Insecta	Coleoptera	Anthribidae	Choragus	*Choragus sheppardi*
Arthropoda	Insecta	Coleoptera	Cantharidae	Cantharis	*Cantharis rustica*
Arthropoda	Insecta	Coleoptera	Cantharidae	Crudosilis	*Crudosilis ruficollis*
Arthropoda	Insecta	Coleoptera	Carabidae	Bembidion	*Bembidion biguttatum*
Arthropoda	Insecta	Coleoptera	Carabidae	Carabus	*Carabus problematicus*
Arthropoda	Insecta	Coleoptera	Carabidae	Leistus	*Leistus fulvibarbis*
Arthropoda	Insecta	Coleoptera	Carabidae	Leistus	*Leistus spinibarbis*
Arthropoda	Insecta	Coleoptera	Carabidae	Nebria	*Nebria brevicollis*
Arthropoda	Insecta	Coleoptera	Chrysomelidae	Donacia	*Donacia clavipes*
Arthropoda	Insecta	Coleoptera	Chrysomelidae	Donacia	*Donacia semicuprea*
Arthropoda	Insecta	Coleoptera	Chrysomelidae	Donacia	*Donacia simplex*
Arthropoda	Insecta	Coleoptera	Chrysomelidae	Luperus	*Luperus longicornis*
Arthropoda	Insecta	Coleoptera	Chrysomelidae	Plateumaris	*Plateumaris sericea*
Arthropoda	Insecta	Coleoptera	Coccinellidae	Adalia	*Adalia decempunctata*
Arthropoda	Insecta	Coleoptera	Coccinellidae	Tytthaspis	*Tytthaspis sedecimpunctata*
Arthropoda	Insecta	Coleoptera	Curculionidae	Hylastes	*Hylastes cunicularius*
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	*Agabus bipustulatus*
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	*Agabus guttatus*
Arthropoda	Insecta	Coleoptera	Dytiscidae	Agabus	*Agabus sturmii*
Arthropoda	Insecta	Coleoptera	Dytiscidae	Hydroporus	*Hydroporus palustris*
Arthropoda	Insecta	Coleoptera	Erirhinidae	Stenopelmus	*Stenopelmus rufinasus*
Arthropoda	Insecta	Coleoptera	Helophoridae	Helophorus	*Helophorus aequalis*
Phylum	**Class**	**Order**	**Family**	**Genus**	***Species***

Arthropoda	Insecta	Coleoptera	Hydrophilidae	Hydrophilus	*Hydrophilus piceus*
Arthropoda	Insecta	Coleoptera	Staphylinidae	Lathrobium	*Lathrobium brunnipes*
Arthropoda	Insecta	Coleoptera	Staphylinidae	Lathrobium	*Lathrobium fulvipenne*
Arthropoda	Insecta	Coleoptera	Staphylinidae	Lesteva	*Lesteva pubescens*
Arthropoda	Insecta	Coleoptera	Staphylinidae	Ocypus	*Ocypus aeneocephalus*
Arthropoda	Insecta	Coleoptera	Staphylinidae	Ocypus	*Ocypus olens*
Arthropoda	Insecta	Coleoptera	Tenebrionidae	Lagria	*Lagria hirta*
Arthropoda	Insecta	Diptera	Agromyzidae	Chromatomyia	*Chromatomyia milii*
Arthropoda	Insecta	Diptera	Bibionidae	Bibio	*Bibio marci*
Arthropoda	Insecta	Diptera	Bibionidae	Dilophus	*Dilophus febrilis*
Arthropoda	Insecta	Diptera	Calliphoridae	Calliphora	*Calliphora vicina*
Arthropoda	Insecta	Diptera	Ceratopogonidae	Brachypogon	*Brachypogon nitidulus*
Arthropoda	Insecta	Diptera	Ceratopogonidae	Culicoides	*Culicoides chiopterus*
Arthropoda	Insecta	Diptera	Ceratopogonidae	Culicoides	*Culicoides impunctatus*
Arthropoda	Insecta	Diptera	Ceratopogonidae	Forcipomyia	*Forcipomyia aristolochiae*
Arthropoda	Insecta	Diptera	Ceratopogonidae	Forcipomyia	*Forcipomyia sp. 2ES*
Arthropoda	Insecta	Diptera	Chaoboridae	Chaoborus	*Chaoborus flavicans*
Arthropoda	Insecta	Diptera	Chironomidae	Camptocladius	*Camptocladius stercorarius*
Arthropoda	Insecta	Diptera	Chironomidae	Chaetocladius	*Chaetocladius dissipatus*
Arthropoda	Insecta	Diptera	Chironomidae	Chaetocladius	*Chaetocladius melaleucus*
Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	*Chironomus muratensis*
Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura sp. 4ES*
Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus cf. curtus ATNA376-09*
Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sylvestris*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Diptera	Chironomidae	Dicrotendipes	*Dicrotendipes pulsus*
Arthropoda	Insecta	Diptera	Chironomidae	Eukiefferiella	*Eukiefferiella claripennis*
Arthropoda	Insecta	Diptera	Chironomidae	Eukiefferiella	*Eukiefferiella ilkleyensis*
Arthropoda	Insecta	Diptera	Chironomidae	Eukiefferiella	*Eukiefferiella minor*
Arthropoda	Insecta	Diptera	Chironomidae	Glyptotendipes	*Glyptotendipes nr. paripes CH152*
Arthropoda	Insecta	Diptera	Chironomidae	Heterotanytarsus	*Heterotanytarsus apicalis*
Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes minimus*
Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes pentaplastus*
Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes sp. 14ES*
Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra pallidula*
Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra roseiventris*
Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius dentifer*
Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius frigidus*
Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius schnelli*
Arthropoda	Insecta	Diptera	Chironomidae	Parochlus	*Parochlus kiefferi*
Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius platypus*
Arthropoda	Insecta	Diptera	Chironomidae	Pseudorthocladius	*Pseudorthocladius filiformis*
Arthropoda	Insecta	Diptera	Chironomidae	Pseudorthocladius	*Pseudorthocladius pilosipennis*
Arthropoda	Insecta	Diptera	Chironomidae	Rheocricotopus	*Rheocricotopus atripes*
Arthropoda	Insecta	Diptera	Chironomidae	Smittia	*Smittia sp. F190*
Arthropoda	Insecta	Diptera	Chironomidae	Stempellinella	*Stempellinella brevis*
Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus buchonius*
Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus sylvaticus*
Arthropoda	Insecta	Diptera	Chironomidae	Trissopelopia	*Trissopelopia longimana*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Diptera	Chironomidae	unknown_genus	*Chironomidae sp. DL-2020*
Arthropoda	Insecta	Diptera	Chironomidae	unknown_genus	*Chironomidae sp. RAK1*
Arthropoda	Insecta	Diptera	Culicidae	Coquillettidia	*Coquillettidia richiardii*
Arthropoda	Insecta	Diptera	Culicidae	Culiseta	*Culiseta annulata*
Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila suzukii*
Arthropoda	Insecta	Diptera	Ephydridae	Hydrellia	*Hydrellia maura*
Arthropoda	Insecta	Diptera	Ephydridae	Notiphila	*Notiphila dorsata*
Arthropoda	Insecta	Diptera	Ephydridae	Scatella	*Scatella paludum*
Arthropoda	Insecta	Diptera	Limoniidae	Dicranomyia	*Dicranomyia modesta*
Arthropoda	Insecta	Diptera	Lonchopteridae	Lonchoptera	*Lonchoptera lutea*
Arthropoda	Insecta	Diptera	Mycetophilidae	Mycetophila	*Mycetophila lunata*
Arthropoda	Insecta	Diptera	Polleniidae	Pollenia	*Pollenia labialis*
Arthropoda	Insecta	Diptera	Psychodidae	Psychoda	*Psychoda phalaenoides*
Arthropoda	Insecta	Diptera	Ptychopteridae	Ptychoptera	*Ptychoptera contaminata*
Arthropoda	Insecta	Diptera	Rhagionidae	Rhagio	*Rhagio tringarius*
Arthropoda	Insecta	Diptera	Scathophagidae	Scathophaga	*Scathophaga sp. BIOUG02375-A02*
Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium armoricanum*
Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium aureum*
Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium velutinum*
Arthropoda	Insecta	Diptera	Syrphidae	Eristalinus	*Eristalinus sepulchralis*
Arthropoda	Insecta	Diptera	Syrphidae	Eristalis	*Eristalis pertinax*
Arthropoda	Insecta	Diptera	Syrphidae	Helophilus	*Helophilus pendulus*
Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula oleracea*
Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula paludosa*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Diptera	unknown_family	unknown_genus	*Diptera sp. RAK1*
Arthropoda	Insecta	Ephemeroptera	Baetidae	Baetis	*Baetis rhodani*
Arthropoda	Insecta	Ephemeroptera	Baetidae	Cloeon	*Cloeon dipterum*
Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Rhithrogena	*Rhithrogena semicolorata*
Arthropoda	Insecta	Hemiptera	Aphididae	Amphorophora	*Amphorophora rubi*
Arthropoda	Insecta	Hemiptera	Aphididae	Aulacorthum	*Aulacorthum solani*
Arthropoda	Insecta	Hemiptera	Aphididae	Ceruraphis	*Ceruraphis eriophori*
Arthropoda	Insecta	Hemiptera	Aphididae	Elatobium	*Elatobium abietinum*
Arthropoda	Insecta	Hemiptera	Aphididae	Hyalopterus	*Hyalopterus pruni*
Arthropoda	Insecta	Hemiptera	Aphididae	Metopolophium	*Metopolophium dirhodum*
Arthropoda	Insecta	Hemiptera	Aphididae	Periphyllus	*Periphyllus hirticornis*
Arthropoda	Insecta	Hemiptera	Aphididae	Periphyllus	*Periphyllus testudinaceus*
Arthropoda	Insecta	Hemiptera	Aphididae	Rhopalosiphum	*Rhopalosiphum enigmae*
Arthropoda	Insecta	Hemiptera	Aphididae	Rhopalosiphum	*Rhopalosiphum padi*
Arthropoda	Insecta	Hemiptera	Aphididae	Schizaphis	*Schizaphis graminum*
Arthropoda	Insecta	Hemiptera	Aphididae	Tuberolachnus	*Tuberolachnus salignus*
Arthropoda	Insecta	Hemiptera	Aphrophoridae	Neophilaenus	*Neophilaenus lineatus*
Arthropoda	Insecta	Hemiptera	Cicadellidae	Aphrodes	*Aphrodes makarovi*
Arthropoda	Insecta	Hemiptera	Cicadellidae	Fagocyba	*Fagocyba douglasi*
Arthropoda	Insecta	Hemiptera	Cicadellidae	Iassus	*Iassus lanio*
Arthropoda	Insecta	Hemiptera	Cicadellidae	Idiocerus	*Idiocerus herrichii*
Arthropoda	Insecta	Hemiptera	Cicadellidae	Ribautiana	*Ribautiana debilis*
Arthropoda	Insecta	Hemiptera	Coccidae	Pulvinaria	*Pulvinaria idesiae*
Arthropoda	Insecta	Hemiptera	Corixidae	Hesperocorixa	*Hesperocorixa linnaei*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Hemiptera	Corixidae	Hesperocorixa	*Hesperocorixa sahlbergi*
Arthropoda	Insecta	Hemiptera	Gerridae	Gerris	*Gerris lacustris*
Arthropoda	Insecta	Hemiptera	Hydrometridae	Hydrometra	*Hydrometra stagnorum*
Arthropoda	Insecta	Hemiptera	Nepidae	Nepa	*Nepa cinerea*
Arthropoda	Insecta	Hemiptera	Notonectidae	Notonecta	*Notonecta glauca*
Arthropoda	Insecta	Hemiptera	Pemphigidae	Thecabius	*Thecabius affinis*
Arthropoda	Insecta	Hemiptera	Psyllidae	Cacopsylla	*Cacopsylla melanoneura*
Arthropoda	Insecta	Hemiptera	Psyllidae	Cacopsylla	*Cacopsylla sp. SO-2015*
Arthropoda	Insecta	Hemiptera	Psyllidae	Psylla	*Psylla alni*
Arthropoda	Insecta	Hemiptera	Triozidae	Trioza	*Trioza urticae*
Arthropoda	Insecta	Hemiptera	Veliidae	Velia	*Velia caprai*
Arthropoda	Insecta	Hymenoptera	Formicidae	Formica	*Formica fusca*
Arthropoda	Insecta	Hymenoptera	Formicidae	Lasius	*Lasius flavus*
Arthropoda	Insecta	Hymenoptera	Formicidae	Lasius	*Lasius niger*
Arthropoda	Insecta	Hymenoptera	Formicidae	Myrmica	*Myrmica ruginodis*
Arthropoda	Insecta	Hymenoptera	Tenthredinidae	Euura	*Euura imperfecta*
Arthropoda	Insecta	Hymenoptera	Tenthredinidae	Pristiphora	*Pristiphora nigella*
Arthropoda	Insecta	Lepidoptera	Crambidae	Cataclysta	*Cataclysta lemnata*
Arthropoda	Insecta	Lepidoptera	Crambidae	Chrysoteuchia	*Chrysoteuchia culmella*
Arthropoda	Insecta	Lepidoptera	Crambidae	Donacaula	*Donacaula forficella*
Arthropoda	Insecta	Lepidoptera	Depressariidae	Depressaria	*Depressaria ultimella*
Arthropoda	Insecta	Lepidoptera	Elachistidae	Spuleria	*Spuleria flavicaput*
Arthropoda	Insecta	Lepidoptera	Erebidae	Diaphora	*Diaphora mendica*
Arthropoda	Insecta	Lepidoptera	Gracillariidae	Cameraria	*Cameraria ohridella*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Lepidoptera	Hepialidae	Phymatopus	*Phymatopus hecta*
Arthropoda	Insecta	Lepidoptera	Lasiocampidae	Euthrix	*Euthrix potatoria*
Arthropoda	Insecta	Lepidoptera	Nepticulidae	Stigmella	*Stigmella ulmivora*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Apamea	*Apamea crenata*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Cerapteryx	*Cerapteryx graminis*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Noctua	*Noctua fimbriata*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Noctua	*Noctua pronuba*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Phlogophora	*Phlogophora meticulosa*
Arthropoda	Insecta	Lepidoptera	Noctuidae	Xestia	*Xestia xanthographa*
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Pararge	*Pararge aegeria*
Arthropoda	Insecta	Lepidoptera	Tortricidae	Ancylis	*Ancylis achatana*
Arthropoda	Insecta	Lepidoptera	Tortricidae	Epinotia	*Epinotia nisella*
Arthropoda	Insecta	Lepidoptera	Tortricidae	Gypsonoma	*Gypsonoma dealbana*
Arthropoda	Insecta	Mecoptera	Panorpidae	Panorpa	*Panorpa germanica*
Arthropoda	Insecta	Neuroptera	Hemerobiidae	Hemerobius	*Hemerobius lutescens*
Arthropoda	Insecta	Odonata	Aeshnidae	Anax	*Anax imperator*
Arthropoda	Insecta	Odonata	Coenagrionidae	Pyrrhosoma	*Pyrrhosoma nymphula*
Arthropoda	Insecta	Orthoptera	Acrididae	Chorthippus	*Chorthippus binotatus*
Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra hippopus*
Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra inermis*
Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra nigra*
Arthropoda	Insecta	Psocoptera	Trichopsocidae	Trichopsocus	*Trichopsocus sp. KY322*
Arthropoda	Insecta	Thysanoptera	Thripidae	Anaphothrips	*Anaphothrips obscurus*
Arthropoda	Insecta	Thysanoptera	Thripidae	Aptinothrips	*Aptinothrips rufus*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Arthropoda	Insecta	Trichoptera	Beraeidae	Beraea	*Beraea pullata*
Arthropoda	Insecta	Trichoptera	Hydroptilidae	Agraylea	*Agraylea multipunctata*
Arthropoda	Insecta	Trichoptera	Limnephilidae	Glyphotaelius	*Glyphotaelius pellucidus*
Arthropoda	Insecta	Trichoptera	Limnephilidae	Halesus	*Halesus radiatus*
Arthropoda	Malacostraca	Amphipoda	Crangonyctidae	Crangonyx	*Crangonyx pseudogracilis*
Arthropoda	Malacostraca	Isopoda	Asellidae	Asellus	*Asellus aquaticus*
Arthropoda	Malacostraca	Isopoda	Oniscidae	Oniscus	*Oniscus asellus*
Arthropoda	Malacostraca	Isopoda	Philosciidae	Philoscia	*Philoscia muscorum*
Arthropoda	Malacostraca	Isopoda	Porcellionidae	Porcellio	*Porcellio scaber*
Arthropoda	Ostracoda	Podocopida	Candonidae	Candona	*Candona candida*
Arthropoda	Ostracoda	Podocopida	Candonidae	Candona	*Candona neglecta*
Arthropoda	Ostracoda	Podocopida	Candonidae	Candonopsis	*Candonopsis kingsleii*
Arthropoda	Ostracoda	Podocopida	Cyprididae	Eucypris	*Eucypris virens*
Arthropoda	unknown_class	unknown_order	unknown_family	unknown_genus	*Maxillopoda sp. BOLD:ACW5478*
Arthropoda	unknown_class	unknown_order	unknown_family	unknown_genus	*Maxillopoda sp. BOLD:ACW5664*
Bryozoa	Phylactolaemata	unknown_order	Lophopodidae	Lophopus	*Lophopus crystallinus*
Bryozoa	Phylactolaemata	unknown_order	Plumatellidae	Plumatella	*Plumatella fungosa*
Chordata	Actinopteri	Cypriniformes	Leuciscidae	Rutilus	*Rutilus rutilus*
Chordata	Actinopteri	Cypriniformes	Leuciscidae	Scardinius	*Scardinius erythrophthalmus*
Chordata	Actinopteri	Esociformes	Esocidae	Esox	*Esox lucius*
Chordata	Actinopteri	Perciformes	Gasterosteidae	Gasterosteus	*Gasterosteus aculeatus*
Chordata	Amphibia	Anura	Ranidae	Rana	*Rana temporaria*
Chordata	Amphibia	Caudata	Salamandridae	Lissotriton	*Lissotriton vulgaris*
Chordata	Aves	Anseriformes	Anatidae	Anas	*Anas platyrhynchos*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Chordata	Aves	Anseriformes	Anatidae	Cygnus	*Cygnus olor*
Chordata	Aves	Charadriiformes	Scolopacidae	Tringa	*Tringa totanus*
Chordata	Aves	Columbiformes	Columbidae	Columba	*Columba oenas*
Chordata	Aves	Columbiformes	Columbidae	Columba	*Columba palumbus*
Chordata	Aves	Gruiformes	Rallidae	Fulica	*Fulica atra*
Chordata	Aves	Gruiformes	Rallidae	Rallus	*Rallus aquaticus*
Chordata	Aves	Passeriformes	Corvidae	Coloeus	*Coloeus monedula*
Chordata	Aves	Passeriformes	Corvidae	Garrulus	*Garrulus glandarius*
Chordata	Aves	Passeriformes	Corvidae	Pica	*Pica pica*
Chordata	Aves	Passeriformes	Fringillidae	Chloris	*Chloris chloris*
Chordata	Aves	Passeriformes	Fringillidae	Fringilla	*Fringilla coelebs*
Chordata	Aves	Passeriformes	Fringillidae	Linaria	*Linaria cannabina*
Chordata	Aves	Passeriformes	Fringillidae	Pyrrhula	*Pyrrhula pyrrhula*
Chordata	Aves	Passeriformes	Sylviidae	Sylvia	*Sylvia atricapilla*
Chordata	Aves	Passeriformes	Turdidae	Turdus	*Turdus iliacus*
Chordata	Aves	Passeriformes	Turdidae	Turdus	*Turdus philomelos*
Chordata	Aves	Passeriformes	Turdidae	Turdus	*Turdus pilaris*
Chordata	Aves	Passeriformes	Turdidae	Turdus	*Turdus viscivorus*
Chordata	Mammalia	Artiodactyla	Bovidae	Bos	*Bos taurus*
Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	*Ovis aries*
Chordata	Mammalia	Carnivora	Canidae	Canis	*Canis lupus*
Chordata	Mammalia	Eulipotyphla	Soricidae	Sorex	*Sorex araneus*
Chordata	Mammalia	Eulipotyphla	Soricidae	Sorex	*Sorex minutus*
Chordata	Mammalia	Perissodactyla	Equidae	Equus	*Equus caballus*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Chordata	Mammalia	Primates	Hominidae	Homo	*Homo sapiens*
Chordata	Mammalia	Rodentia	Cricetidae	Microtus	*Microtus agrestis*
Chordata	Mammalia	Rodentia	Gliridae	Muscardinus	*Muscardinus avellanarius*
Chordata	Mammalia	Rodentia	Muridae	Mus	*Mus musculus*
Chordata	Mammalia	Rodentia	Muridae	Rattus	*Rattus norvegicus*
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra circumcincta*
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra oligactis*
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra viridissima*
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra vulgaris*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus aff. persimilis MK-2019*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus aff. subtilis 4 MK-2019*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus borealis*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus jaceki*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Heterolepidoderma	*Heterolepidoderma ocellatum*
Gastrotricha	unknown_class	Chaetonotida	Chaetonotidae	Polymerurus	*Polymerurus rhomboides*
Gastrotricha	unknown_class	Chaetonotida	Dasydytidae	Stylochaeta	*Stylochaeta scirtetica*
Mollusca	Bivalvia	Galeommatida	Montacutidae	Kurtiella	*Kurtiella bidentata*
Mollusca	Bivalvia	Venerida	Sphaeriidae	Pisidium	*Pisidium obtusale*
Mollusca	Bivalvia	Venerida	Sphaeriidae	Pisidium	*Pisidium subtruncatum*
Mollusca	Bivalvia	Venerida	Sphaeriidae	Sphaerium	*Sphaerium corneum*
Mollusca	Bivalvia	Venerida	Sphaeriidae	Sphaerium	*Sphaerium nucleus*
Mollusca	Gastropoda	Littorinimorpha	Bithyniidae	Bithynia	*Bithynia tentaculata*
Mollusca	Gastropoda	Stylommatophora	Agriolimacidae	Deroceras	*Deroceras invadens*
Mollusca	Gastropoda	Stylommatophora	Agriolimacidae	Deroceras	*Deroceras laeve*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Mollusca	Gastropoda	Stylommatophora	Arionidae	Arion	*Arion hortensis*
Mollusca	Gastropoda	Stylommatophora	Arionidae	Arion	*Arion intermedius*
Mollusca	Gastropoda	Stylommatophora	Arionidae	Arion	*Arion subfuscus*
Mollusca	Gastropoda	Stylommatophora	Helicidae	Cepaea	*Cepaea nemoralis*
Mollusca	Gastropoda	Stylommatophora	Hygromiidae	Monacha	*Monacha cantiana*
Mollusca	Gastropoda	Stylommatophora	Hygromiidae	Zenobiellina	*Zenobiellina subrufescens*
Mollusca	Gastropoda	Stylommatophora	Succineidae	Succinea	*Succinea putris*
Mollusca	Gastropoda	Stylommatophora	Vitrinidae	Vitrina	*Vitrina pellucida*
Mollusca	Gastropoda	unknown_order	Acroloxidae	Acroloxus	*Acroloxus lacustris*
Mollusca	Gastropoda	unknown_order	Lymnaeidae	Ampullaceana	*Ampullaceana balthica*
Mollusca	Gastropoda	unknown_order	Physidae	Aplexa	*Aplexa hypnorum*
Mollusca	Gastropoda	unknown_order	Physidae	Physa	*Physa fontinalis*
Mollusca	Gastropoda	unknown_order	Physidae	Physella	*Physella ancillaria*
Mollusca	Gastropoda	unknown_order	Planorbidae	Anisus	*Anisus cf. vortex P2333*
Mollusca	Gastropoda	unknown_order	Planorbidae	Hippeutis	*Hippeutis complanatus*
Mollusca	Gastropoda	unknown_order	Planorbidae	Planorbis	*Planorbis planorbis*
Nematoda	unknown_class	unknown_order	unknown_family	unknown_genus	*unidentified nematode*
Nemertea	unknown_class	unknown_order	unknown_family	unknown_genus	*Nemertean sp. NT000047*
Platyhelminthes	Catenulida	unknown_order	Stenostomidae	Stenostomum	*Stenostomum cf. simplex AW-2018*
Platyhelminthes	Rhabditophora	Macrostomida	Microstomidae	Microstomum	*Microstomum lineare*
Platyhelminthes	Rhabditophora	Tricladida	Dugesiidae	Schmidtea	*Schmidtea polychroa*
Rotifera	Eurotatoria	Adinetida	Adinetidae	Adineta	*Adineta sp. FR.5*
Rotifera	Eurotatoria	Adinetida	Adinetidae	Adineta	*Adineta vaga*
Rotifera	Eurotatoria	Adinetida	Adinetidae	Adineta	*Adineta vaga complex sp. B JFF-2016*
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**

Rotifera	Eurotatoria	Philodinida	Habrotrochidae	Habrotrocha	*Habrotrocha elusa*
Rotifera	Eurotatoria	Philodinida	Habrotrochidae	Habrotrocha	*Habrotrocha ligula*
Rotifera	Eurotatoria	Philodinida	Philodinidae	Macrotrachela	*Macrotrachela quadricornifera*
Rotifera	Eurotatoria	Philodinida	Philodinidae	Philodina	*Philodina citrina*
Rotifera	Eurotatoria	Philodinida	Philodinidae	Philodina	*Philodina sp. A459_PR6*
Rotifera	Eurotatoria	Ploima	Asplanchnidae	Asplanchna	*Asplanchna sieboldii*
Rotifera	Eurotatoria	Ploima	Brachionidae	Brachionus	*Brachionus calyciflorus*
Rotifera	Eurotatoria	Ploima	Brachionidae	Euchlanis	*Euchlanis dilatata*
Rotifera	Eurotatoria	Ploima	Brachionidae	Mytilina	*Mytilina mucronata*
Rotifera	Eurotatoria	Ploima	Epiphanidae	Epiphanes	*Epiphanes senta*
Rotifera	Eurotatoria	Ploima	Gastropidae	Ascomorpha	*Ascomorpha ecaudis*
Rotifera	Eurotatoria	Ploima	Lecanidae	Lecane	*Lecane closterocerca*
Rotifera	Eurotatoria	Ploima	Proalidae	Proales	*Proales daphnicola*
Rotifera	Eurotatoria	Ploima	Synchaetidae	Synchaeta	*Synchaeta cf. tremula/oblonga UO-2012*
Rotifera	Eurotatoria	Ploima	Synchaetidae	Synchaeta	*Synchaeta pectinata*
Rotifera	Eurotatoria	unknown_order	unknown_family	Rotaria	*Rotaria macroceros*
Rotifera	Eurotatoria	unknown_order	unknown_family	Rotaria	*Rotaria magnacalcarata*
Rotifera	Eurotatoria	unknown_order	unknown_family	Rotaria	*Rotaria rotatoria*
Rotifera	Eurotatoria	unknown_order	unknown_family	Rotaria	*Rotaria socialis*
Rotifera	Eurotatoria	unknown_order	unknown_family	Rotaria	*Rotaria sp. RotS1*

7.1 Appendix i. Biological Monitoring Working Party (BMWP) Average Score per Taxon (ASPT) Scoring System

Taxonomic Class	Taxonomic Families	Score
Ephemeroptera	Ephemeridae	10
	Heptagoniidae	10
	Leptophlebiidae	10
	Pothamanthidae	10
	Siphonurridae	10
Plecoptera	Capniidae	10
	Chloroperlidae	10
	Leuctridae	10
	Perlidae	10
	Taeniopteterygidae	10
Hemiptera	Aphelochereididae	10
Trichoptera	Beraecidae	10
	Brachycentridae	10
	Goeridae	10
	Lepidostomatidae	10
	Leptoceridae	10
	Mollanidae	10
	Odontoceridae	10
	Phyrgancineidae	10
	Sericostomatidae	10
Ephemeroptera	Caenidae	7
Plecoptera	Nemouridae	7
Trichoptera	Rhyacophilidae	7
	Polycentropodidae	7
	Limnepphilidae	7
Mollusca	Neritidae	6

	Viviparidae	6
	Ancylidae	6
	Unionidae	6
Trichoptera	Hydroptilidae	6
Crustacea	Corophiidae	6
	Gammaridae	6
	Paleamonidae	6
Polychaeta	Nereidae	6
	Nephthyidae	6
Odonata	Plaqthycnemididae	6
	Coenagriidae	6
Hemiptera	Mesovelidae	5
	Hydrometridae	5
	Gerridae	5
	Nepidae	5
	Naucoridae	5
	Notonectidae	5
	Pletidae	5
Coleoptera	Chrysomelidae	5
	Corixidae	5
	Curculionidae	5
	Dryopidae	5
	Dytiscidae	5
	Eliminthidae	5
	Gyrinidae	5
	Haliplidae	5
	Helobidae	5
	Hydrophilidae	5
	Hygrobiidae	5
Phyrgancineidae	Hydropsychidae	5

Diptera	Tipulidae	5
	Simuliidae	5
Planaria	Planariidae	5
	Dendrocoelidae	5
Ephemeroptera	Baetidae	4
Megaloptera	Sialidae	4
Hirudinida	Piscicolidae	4
Mollusca	Valvatidae	3
	Hygrobiidae	3
	Lymnaeitidae	3
	Physidae	3
	Planorbidae	3
	Sphaeriidae	3
Hirudinida	Erpobdellidae	3
	Glossiphonidae	3
	Hirudidae	3
Others	Alderfly (meglaoptera, Sialidae)	4
	Shrimps (Caridea)	6
	Hoglice (Asellidae)	3
	Blackfly (Simuliidae)	5
	Cranefly (Tipulidae)	5
	Worms	1