Developing eDNA techniques for the detection of *Segmentina nitida*

In ditch systems at Stodmarsh, Kent

2020/2021

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Developing DNA and eDNA techniques for the detection of *Segmentina nitida* Shining ram's-horn snail and other invertebrates in ditch systems at Stodmarsh, Kent

2020/2021

Natural England Commissioned Report NECR373

Contents

Foreword

DNA based methods offer a significant opportunity to change how we monitor and assess biodiversity. However, for most techniques, there is still much development required before they can be used in routine monitoring. Natural England has been exploring the use of these methods for environmental monitoring for several years, delivering a series of reports which focus on the development of DNA-based methods with potential in a particular area.

Natural England (NE) aims to make monitoring programmes more efficient and to investigate this they wish to develop DNA and eDNA techniques for the identification of *Segmentina nitida* and see how this compares with hand identification. This project delivers important baseline data on the applications of DNA technologies, specifically the development of species-specific eDNA assays and mass DNA sequencing (metabarcoding) of ditch systems to survey and monitor biodiversity.

1. Introduction

Natural England is the Government's advisor for the natural environment. It provides practical advice on how to safeguard England's natural wealth for the benefit of everyone. ADAS is an environmental consultancy which exists to provide ideas, specialist knowledge and solutions to secure our food and enhance the environment.

Natural England has over recent years begun to explore the application of DNA and eDNA based technologies to biodiversity monitoring programmes. There are well over 30,000 different species of invertebrates in the UK (Key *et al.* 2000) and it can take many years to become an expert in species identification meaning that DNA and eDNA techniques could have benefits over traditional identification. One species that Natural England are interested in detecting via these DNA and eDNA techniques is *Segmentina nitida* (O.F.Müller, 1774) Shining ram's-horn snail which is a rare and declining Section 41 species and also a Ramsar criteria feature of Stodmarsh National Nature Reserve (NNR).

1.1 Environmental DNA

Environmental DNA (eDNA) describes the DNA that can be extracted from an environmental sample for example water, soil or sediment, or air. eDNA from water bodies can be used for the monitoring of aquatic and semi-aquatic populations with the DNA present originating from the faeces, saliva, urine and skin cells of animals occupying the water bodies in question. Similarly, the DNA of animals that visit the environment, such as birds and mammals using the water body to drink can also be present. In theory, the presence of a specific animal can be detected anywhere within the water body and not just at its point of origin due to the rapid diffusion of DNA from its source (Rees *et al.* 2014).

1.2 Species-specific Detection

The analysis of water for species-specific eDNA is a technique with application to aquatic organism surveys and conservation projects (Rees *et al.* 2014) and has been successfully used to detect *Potamopyrgus antipodarum* New Zealand mud snail within rivers (Clusa *et al*. 2016). The development of a new eDNA assay for species monitoring requires rigorous validation - *in silico, in vitro* and *in situ* - for meaningful application and interpretation. *In silico* validation involves the design of species-specific primers and checking primer specificity against available DNA sequences (publicly available or custom databases) from closely related and co-occurring species (both geographically distinct species and distantly related species). *In vitro* validation involves the optimisation, specificity, and sensitivity of the assay. Finally, *in situ* validation involves surveying sites with known presence/absence of the target species (Goldberg *et al.* 2016) with the assay being deemed successful if the results of the eDNA assay and traditional surveying concur.

1.3 Metabarcoding

DNA metabarcoding combines two techniques: DNA based identification and high-throughput sequencing (Margulies *et al.* 2005). Using primers that work across a wide range of taxa ('universal' PCR primers), specific target sequences can be amplified, the result being the mass-

amplification of the target of interest from multiple species. Ideally, primers should target a hypervariable region (for high resolution taxonomic discrimination) and additionally, should target short DNA fragments (around 400 bp or less). This allows for the recovery of potentially degraded target DNA which may have been subjected to long term storage - temperature and humidity of storage conditions will affect DNA quality - or that has been taken from hostile sample matrices. Some environments will be more detrimental to DNA quality than others and are therefore described as 'hostile'. DNA is also liable to degradation by factors such as nucleases, UV light, and microbial action.

Universal primers have been developed for a wide range of gene fragments including: nuclear 18S and 28S ribosomal RNA markers (Machida *et al.* 2012a); the mitochondrial 12S rRNA gene (Machida *et al.* 2012b); and the mitochondrial Cytochrome Oxidase I gene (COI). COI has been adopted as the standard 'taxon barcode' for many taxa (Hebert *et al.* 2003) and has been agreed internationally as being the region of the genome (in animals) which allows good discrimination between species (with little variation between individuals of the same species), other more suitable regions are used for plants and fungi. The standard COI target primer set was developed to amplify a 658 bp region (Folmer *et al.* 1994). However, as this fragment was too long for metabarcoding, a modified version of the 'Folmer' reverse primer and a newly designed forward primer were created (Leray *et al.* 2013). Leray also showed them to have a higher amplification success rate than the original 'Folmer' primers. This primer set has been used in many peer reviewed studies and was also the primer set of choice within Natural England report NECR252 (Tang *et al.* 2018) and work commissioned by Natural England in 2019 (in press).

Next generation DNA sequencing methods are used to return large numbers of high quality sequence reads from the amplified target sequences. Sequence data is usually reduced down to a single representative of each species mitochondrial DNA sequence - an operational taxonomic unit (OTU). The individual OTUs can then be compared with existing DNA databases to identify the organisms that they represent.

Metabarcoding has proven an effective technique for community biodiversity assessment across a range of taxa and environments (Deiner *et al.* 2016; Drummond *et al.* 2015; Hajibabaei *et al.* 2011; Murray *et al.* 2012; Valentini *et al.* 2016). When compared with traditional identification methods, metabarcoding can generate comprehensive data sets many times quicker and is therefore a powerful means to study and understand the diversity and distribution of fauna and flora.

1.4 Aims and Objectives

The aim of this study was to develop DNA and eDNA techniques for the identification of *Segmentina nitida* and other aquatic invertebrates. Specifically, this study aims to take samples from ditches located at Stodmarsh NNR subject them to a species-specific eDNA assay for *S. nitida* (to be developed herein) and also to subject them to metabarcoding, to investigate the accuracy of DNA based species identification as compared with traditional identification. DNA barcodes of *S. nitida* specimens from Stodmarsh NNR will also be generated.

The aim of identification by metabarcoding will be to develop techniques for detection of *S. nitida* and other aquatic invertebrates of the open water assemblage W211 (Drake *et al*. 2007) and also contained in the ditch samples down to the species level and in circumstances where this is not possible, to identify down to the genus level. The results of this will be compared to taxonomic identification and species lists generated for the ditch samples by Natural England.

This report details the methodology employed in this study, the results obtained and, discussion of the survey results and comparison between single species assay and metabarcoding results for *S. nitida* including the pros and cons of the assays in detecting the target. All data will be made available for further study and could be used for a training day for Natural England staff on the DNA approaches used.

2. Methods

2.1 Sample Collection

22 ditch water samples were collected by Natural England staff at Stodmarsh NNR between the 16th and 27th November 2020 (Figure 1), detailed methods can be found in Appendix 2A. 10 ditch samples outside the known range of *S. nitida* (Leicestershire) were collected by ADAS staff between the 10th January and the 4th February. Sample information is shown in Tables 4 and 5 (Appendix 1).

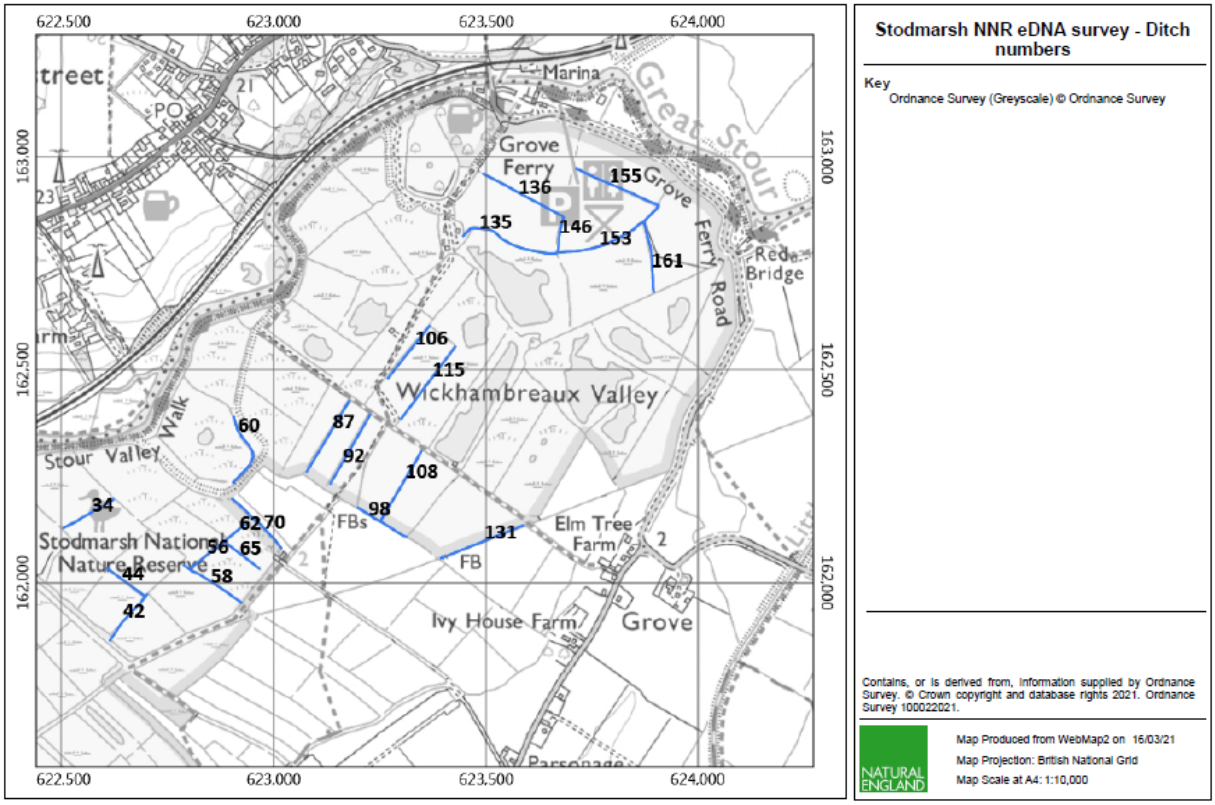

Figure 1 Map of ditch locations within Stodmarsh NNR

2.2 Specimen Collection

Snail and other invertebrate samples were collected from the same 22 ditches at Stodmarsh NNR by Natural England between December and January 2020/2021 (see Appendix 2B). Specimens were identified and preserved in 95% ethanol prior to couriering to ADAS (Figure 2).

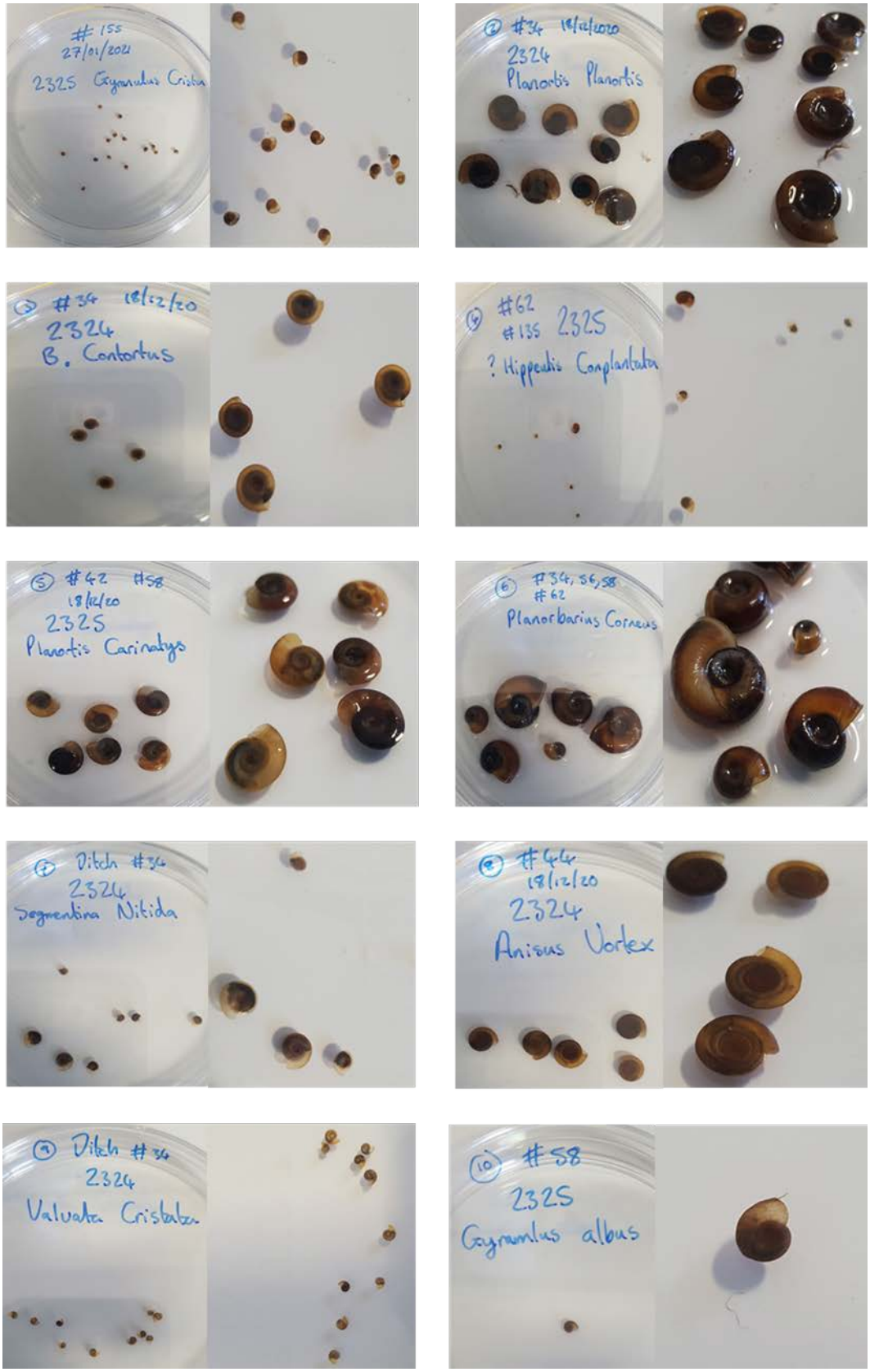

Figure 2 Specimen images taken by © Claire Baker (snail specimens provided by Natural England)

2.3 Manual *S. nitida* Survey

Manual surveys of the 22 ditches was performed between December 2020 and January 2021 (Appendix 2B). The invertebrate sampling protocol was adapted from Drake (2007) for still-water faunas which are usually dominated by adult beetles, bugs and molluscs such as in the assemblage W211 Open water on disturbed mineral sediments. Only pond netting is required for assessing W211, and the effort of this sampling method was standardised by bank-sorting qualitative hauls netted from each of the 22 ditches which had been sampled already for eDNA (see Figure 1). The emphasis was on the free-style netting of suitable looking micro-habitats (e.g. emergent vegetation stands) that are likely to be most productive for this assemblage. Effort was deliberately not divided in proportion to the extent of features nor length of ditch, since species are not distributed in this fashion.

2.4 Laboratory Standards and Specifications

All laboratory activities associated with DNA analysis are subject to errors if quality control is inadequate. Our DNA analysis follows a unidirectional workflow with separate laboratories and staff to act as a physical separation for the different aspects of the analysis work. This greatly reduces the potential for contamination of samples or the PCR amplicons. 'Blank' PCRs (sterile water rather than DNA) are used to monitor for reagent/procedural contamination, and in addition positive control samples are used to increase confidence in the results and identify any cross-contamination issues, should they occur.

2.5 DNA Extraction

Each specimen was individually transferred to a clean, sterile mortar and ground into a fine paste using a pestle and liquid nitrogen. For some snail species, the individual specimens were pooled prior to grinding into a fine paste (see Table 2). After use mortar and pestles were immediately immersed in 10 % bleach for a minimum of 10 minutes and then cleaned in between samples with 10 % Distel (Tristel™), rinsed with dH2O and then autoclaved at 121 ºC for 15-20 minutes.

DNA was extracted from ditch samples using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (Appendix 2C and D), with the exception that 720µL of ATL buffer was added to each sample, along with 40µL of PK. Final resuspension was in 50 or 200µL AE buffer for specimens (see Table 2 for volumes) or 200µL for ditch samples. All extractions were quantified using a Qubit 3.0 Fluorometer (Invitrogen) following the manufacturer's instructions then stored at -20 ºC prior to PCR set up (Appendix 2E). DNA was extracted from snail specimens using DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (Appendix 2C and D).

2.6 Specimen Identification PCR

All PCR set up was performed in a clean ‘PCR room’ within a UV sterilisable cabinet and within a separate laboratory to DNA extraction using dedicated equipment and PPE. To ensure the unidirectional workflow DNA extracts are collected from the DNA extraction laboratory and transferred to the PCR set-up laboratory. Laboratory personnel do not return to the DNA extraction laboratory during that same day thus maintaining the unidirectional workflow.

PCRs were performed to confirm the identity of the provided specimens using the mICOIintF/jgHCO2198 primer combination (Table 1, Appendix 2F). These primers amplify a fragment of the Cytochrome c Oxidase subunit I gene (COI) and have been shown to perform well in invertebrate metabarcoding studies (Leray et al. 2013; Geller et al. 2013). After PCR and amplicon clean-up, PCR products were Sanger sequenced and returned sequences identified using BLAST.

S. nitida barcode information was produced using primers specific to the COI sequences of *S. nitida* available through Genbank. This was done in order to prevent any ‘prey’ species from being amplified and resulting in a mixed sequence result.

2.7 Species Specific Primer Design and validation

2.7.1 In silico analysis

In order to design primers specific to *S. nitida* the DNA sequences for the cytochrome oxidase 1 (COI) gene for *S. nitida* and nine other closely related and/or co-occurring snails commonly found at the Stodmarsh NNR were downloaded from Genbank. Sequences were aligned using BioEdit version 7.2.5 (alignment for *S. nitida* shown in Appendix 3). A species-specific quantitative PCR (qPCR) assay for *S.nitida* was designed from COI sequences stored in Genbank (NCBI) using PrimerBLAST with default settings except for targeting a 70-300 bp fragment, and including only base pairs between 70 and 600 in the *S. nitida* consensus sequence as this corresponded to the most variable region on the multi-species alignment. Ten potential primer/probe combinations were generated (Appendix 3). Further analysis using PrimerBLAST of potential primer/probe combinations for cross-species amplification reduced the ten potential species-specific primer/probe combinations down to four (Appendix 3).

2.7.2 In vitro analysis

The four potential primer/probe combinations were tested firstly on DNA extracted from *S. nitida* followed by the other nine closely related and/or co-occurring snail species to test for cross-species reactivity (specificity). PCRs were performed in duplicate using a CFX-Connect real time PCR machine (Bio-Rad) (Appendix 2G).

Once specificity of primer/probe combinations was confirmed, the primer concentrations of two primer/probe combinations (primer/probe combinations 2 and 9) were optimised by independently varying final primer concentrations (the probe was held at a final concentration of 0.1 µmol/L) (Wilcox et al, 2015). The sensitivity of the assay was tested by creating a six-level standard curve dilution series (3x10-1 to 3x10-7 µg/µl). The standard curve was created by quantifying the DNA extracted from *S. nitida* sample 7a on a Qubit Fluorometer (Thermo Fisher Scientific) and diluting the DNA to the desired concentrations using the elution buffer provided in the DNeasy Blood and Tissue kit (Qiagen). 12 replicates of each dilution were run using the optimised primer/probe concentrations to determine the standard curve slope and the limit of detection (LOD) and limit of quantification (LOQ).

2.7.3 In situ analysis

The optimised assay using primer pair 9 was used to determine the presence/absence of *S. nitida* within the 22 ditch samples from Stodmarsh NNR and the 10 ditch samples from outside of the known range of *S. nitida* (Appendix 2G).

2.8 Metabarcoding PCR and Library Preparation

The primer combination used for the first round PCR amplification was mICOIintF/jgHCO2198. Overhang adapter sequences (Table 1, Figure 3) were included at the 5’ end of the primers to ensure compatibility with Illumina index and sequencing adapters (Illumina 2011). PCRs included one negative control (ddH2O in place of DNA); two DNA extraction blanks; a positive control sample (*Allolobophora chlorotica* DNA (earthworm)) and all 22 DNA extracts from ditch samples (Appendix 2H).

Primer Name	Oligonucleotides (5'-3')	%GC	Tm	Reference
SnitCOIF	TGGAATTAGGTACCTCTGGTGT	45	53	-
SnitCOIR	ACCATACCAAACCCTGGTAAAA	41	51	-
mICOIintF (plus adapter)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC	50.8	>75	Leray *et al.* (2013)
jgHCO2198 (plus adapter)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAIACYTCIGGRTGICCRAARAAYCA	47.5	>75	Geller *et al.* (2013)
Index 1	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG	-	-	Illumina (2011)
Index 2	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC	-	-	Illumina (2011)

Table 1 primers used for specimen identification and PCR rounds one and two for metabarcoding

Sequences marked in black are the *S. nitida* COI barcoding primers, sequences marked in green are the first round PCR primer adapter sequences, the remainder in purple are the mICOIintF/jgHCO2190 locus-specific primer sequences. Sequences marked in blue are Illumina overhang adapter sequences, Index 1 and 2 sequences are marked with X's as this sequence is variable for each different sample, those in red are the P5 and P7 sequences. Index 1 (i7) and Index 2 (i5) are examples of the type of primers used with the Index sequence itself being altered for different samples.

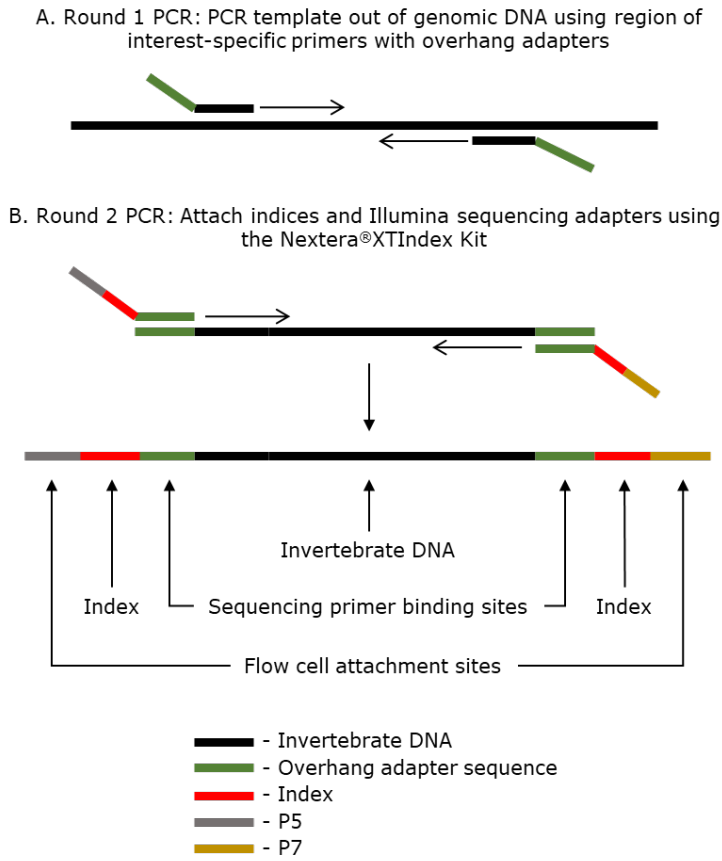

Figure 3 PCR Amplicon workflow

A) Forward and reverse primers complementary to the region of interest including overhanging adapters (see Table 1). B) Subsequent amplification step used to add indices and Illumina sequencing adapters.

The first round PCR amplicons for each sample were pooled and run on a 1.5% agarose gel. Any bands of 400-500bp excised and purified using NucleoSpin® Gel and PCR Clean-up purification columns (Machery-Nagel) according to the manufacturers' instructions (Appendix 2I). The second round of PCR or 'Index' PCR was performed using the Nextera XT index kit v2 Set B (Illumina) to add molecular identification (MID) tags (unique 8-nucleotide sequences) and Illumina MiSeq sequencing adapters to the first round PCR products. In this process a unique Index 1 and Index 2 are added to each first round PCR product on a 96-well plate (Figure 4, Appendix 2J). PCR products were then purified with AMPure XP beads according to the manufacturer's instructions (Appendix 1) and quantified using a Qubit Flourometer and the Qubit dsDNA HS Assay kit (ThermoFisher). Library fragment length distributions were analysed using the Agilent TapeStation 4200 and the Agilent D1000 ScreenTape Assay (Agilent). Libraries were then pooled in equimolar amounts to create one library for Illumina sequencing. Library pool quantification was performed using the KAPA Library Quantification Kit for Illumina (Roche). A Qubit quantification measurement of 2.01 nM was used to adjust the concentration of library pool for sequencing. The amplicon library pool was diluted to 10 pM, spiked with 10 % PhiX Control v3 library (Illumina) and run on the Illumina MiSeq using a MiSeq Reagent Kit v2 500 cycle kit (Illumina), to generate 250-bp paired-end

reads. PhiX DNA is derived from the small, well characterized bacteriophage PhiX genome. It is a concentrated Illumina library (10 nM in 10 µl) that has an average size of 500 bp and consists of balanced base composition at ~45% GC and ~55% AT and serves as an in-run QC for the Illumina sequencing.

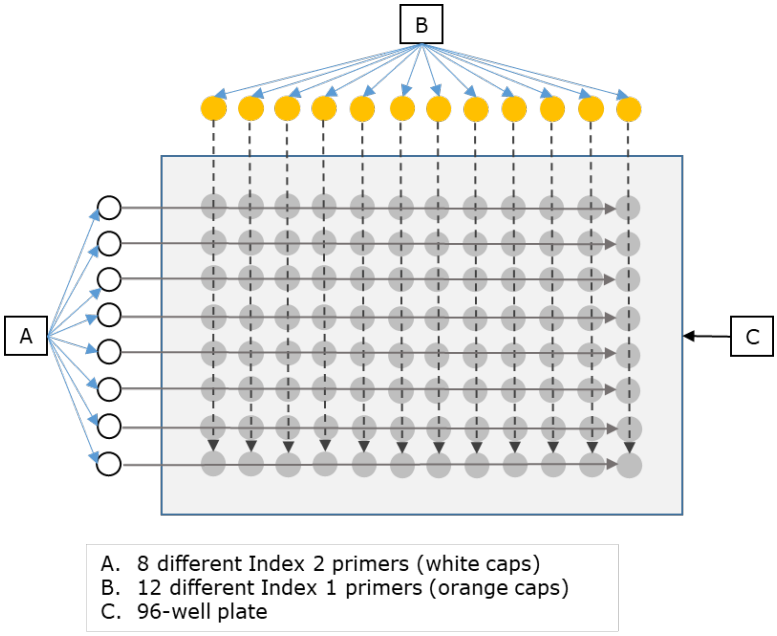

Figure 4 Dual indexing principle for illumina sequencing

Index 2 primers are added across the plate (arrows) and the Index 1 primers are added down the plate (dashed arrows) resulting in 96 separate combinations of primers.

2.9 Bioinformatic Processing

Data provided by the DeepSeq team was subjected to an in-house bioinformatics pipeline to generate taxonomic assignments for each of the Stodmarsh NNR ditch water samples (Appendix 2K). The information generated was then compared to species lists as provided by Natural England.

3. Results

3.1 *S. nitida* Manual Survey

The manual survey for *S. nitida* showed that *S. nitida* was found in 8 out of the 22 ditches (Table 3). All macroinvertebrates recorded at this time are shown in Appendix 1, Table 7.

3.2 DNA Extraction

DNA was extracted from 23 single snail or pooled snail specimens. DNA quantification showed that generally only low concentrations of DNA had been extracted (Table 2). DNA was also extracted in very low concentrations from 22 Stodmarsh NNR ditch samples and 10 ditch samples from Leicestershire (Table 3). Most of the DNA concentrations from ditch samples outside the known range of *S. nitida* were too low to quantify as well as two of the Stodmarsh NNR ditch samples.

Snail ID	Sample ID	DNA (ng/ul)	Eluted volume (ul)	Pooled or individual specimen	Confirmed sequence ID
Gyraulus crista	1	4.68	200	Pool	Yes
Gyraulus crista	1a	29.4	50	Pool	Yes
Planorbis planorbis	2	2.81	200	Individual	Yes
Planorbis planorbis	2a	39.9	50	Individual	Yes
Bathyomphalus contortus	3	1.43	200	Individual	Poor PCR amplification/sequence
Bathyomphalus contortus	3a	10.6	50	Individual	Yes
Hippeutis complanatus	4	3.58	200	Pool	Yes
Hippeutis complanatus	4a	11.8	50	Pool	Yes
Planorbis carinatus	5	3.49	200	Individual	Poor PCR amplification/sequence
Planorbis carinatus	5a	22.4	50	Individual	Yes
Planorbis carinatus	5b	38.6	50	Individual	Yes
Planorbarius corneus	6	9.35	200	Individual	Yes
Planorbarius corneus	6a	29.7	50	Individual	Yes
Segmentina nitida	7	8.52	200	Pool	Yes
Segmentina nitida	7a	29.6	50	Individual	Yes
Segmentina nitida	7b	46.4	50	Individual	Yes
Segmentina nitida	7c	12.5	50	Individual	Yes
Anisus vortex	8	1.39	200	Individual	Poor PCR amplification/sequence
Anisus vortex	8a	33.1	50	Individual	Yes
Valvata cristata	9	1.59	200	Pool	Yes (97%)
Valvata cristata	9a	28	50	Pool	Yes (96%)
Valvata cristata	9b	30.9	50	Pool	Yes (97%)
Gyraulus albus	10	2.42	200	Individual	Yes

Table 2 DNA information per snail species

Where there was poor PCR amplification/sequence we were unable to amplify sufficient target gene to enable us to identify the species.

Sample ID	DNA (ng/µL)	Successful PCR Amplification	Post PCR DNA (ng/µL)	*S. nitida* species-specific qPCR result	*S. nitida* manual survey
1	<1 ng/µL	N/A	N/A	0/12; negative	N/A
2	<1 ng/µL	N/A	N/A	0/12; negative	N/A
3	<1 ng/µL	N/A	N/A	0/12; negative	N/A
4	<1 ng/µL	N/A	N/A	0/12; negative	N/A
5	<1 ng/µL	N/A	N/A	0/12; negative	N/A
D1	<1 ng/µL	N/A	N/A	0/12; negative	N/A
D2	<1 ng/µL	N/A	N/A	0/12; negative	N/A
Top	1.7	N/A	N/A	0/12; negative	N/A
LHS	1.34	N/A	N/A	0/12; negative	N/A
RHS	<1 ng/µL	N/A	N/A	0/12; negative	N/A
34	2.14	Yes	2.72	1*/12; positive	positive
42	2.95	Yes	13.0	0/12; negative	negative
44	1.71	Yes	4.97	0/12; negative	negative
56	6.56	Yes	14.9	12/12; positive	positive
58	11.1	Yes	13.4	0/12; negative	negative
60	3.96	Yes	16.6	0/12; negative	negative
62	2.11	Yes	3.84	0/12; negative	positive
65	3.78	Yes	2.6	9/12; positive	positive
70	0.84	Yes	3.77	0/12; negative	positive
87	18.1	Yes	9.43	12/12; positive	positive
92	4.88	Yes	6.4	6/12; positive	positive
98	4.36	Yes	5.21	3/12; positive	negative
106	1.66	Yes	11.6	0/12; negative	negative
108	2.32	Yes	2.44	0/12; negative	positive
115	2.06	Yes	1.32	4/12; positive	negative
131	1.08	Yes	4.91	0/12; negative	negative
135	1.46	Yes	6.49	0/12; negative	negative
136	<1 ng/µL	No	No product	0/12; negative	negative
146	<1 ng/µL	No	No product	0/12; negative	negative
153	1.89	Yes	5.15	0/12; negative	negative
155	<1 ng/µL	Yes (poor)	<1 ng/µL	0/12; negative	negative
161	1.35	Yes	1.83	0/12; negative	negative
Positive control	5 ng/µl	Yes	0.81	N/A	N/A
Negative control	n/a	No	No product	N/A	N/A

Table 3 Ditch sample information

Samples marked as '<1 ng/µL were samples where the DNA concentration was too low to measure using the Qubit broad range kit. Green highlighted samples were found to be positive for *S. nitida* by both qPCR and manual survey; orange highlighted surveys were found to be positive for *S. nitida* by qPCR but not manual survey; red highlighted samples were found to be positive for *S.nitida* by manual survey but not by qPCR.

*qPCR was repeated for this sample to confirm positivity and 3/12 replicates were found to be positive for *S nitida*.

3.3 Specimen Identification

Specimens were confirmed as the taxonomically identified species (Table 3) by Sanger sequencing of PCR products with identities of at least 98% over the length of the sequence. The exception to this was *Valvata cristata* (samples 9, 9a, and 9b) where the sequences only showed 96 or 97% identity to those on the NCBI database with the sequences also being found to be similarly close to those of *Valvata relicta* which is a species not found in the UK and is therefore unlikely to be the correct species. The sequences were manually checked versus their respective chromatograms to resolve any errors in base calling prior to identification via BLAST searches, however, no improvements were made.

3.4 *S. nitida* PCR

A qPCR-based assay was developed based on primers designed against the *S. nitida* COI sequences deposited in Genbank (EF012178.1 and LC429396.1). Of 10 potential primer/probe combinations, two were found to be specific to *S. nitida*; primer/probe combinations 2 and 9 (Appendix 3B), amplicons were sequenced to confirm species identity. The primer concentrations and amplification conditions were optimised and during this process it became clear that primer/probe combination 9 was superior to primer/probe combination 2 due to far higher relative fluorescence units (RFU) and lower quantification cycle (Cq) values (the number of cycles required for the fluorescent signal to exceed the background fluorescence) (Figure 5). Optimal primer concentrations were found to be 0.2 µmol/L for the forward primer and 0.4 µmol/L for the reverse, probe concentration was held at 0.1 µmol/L.

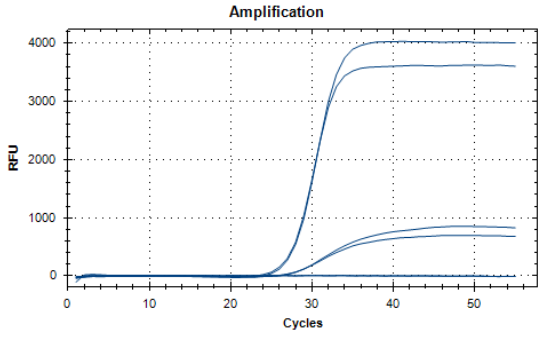

Figure 5 Optimal primer concentrations

qPCR results showing the optimal primer concentrations in terms of RFU and Cq values. Higher plots show the results of primer/probe combination 9 (RFU ~3800; Cq 25.68) and the lower plots, primer/probe combination 2 (RFU ~700; Cq 26.13). A negative control with no amplification is also shown.

The limit of detection (LOD) and limit of quantification (LOQ) of primer/probe combination 9 were found to be $3x10^{-4}$ ng/µL and $3x10^{-5}$ ng/µL respectively. The LOD and LOQ have various definitions in the eDNA literature, here LOD is defined as the lowest standard concentration at which 95% of technical replicates amplify and LOQ is the lowest standard concentration for which the coefficient of variation (CV; equal to the standard deviation quantity divided by the mean quantity of a group of replicates) value is <35% (Klymus *et al*. 2019).

All Stodmarsh NNR ditch water samples and ditch samples from outside the known range of *S. nitida* were subjected to the optimised assay, with seven ditches from Stodmarsh NNR being positive for *S. nitida* DNA. All remaining ditches were negative for *S. nitida* DNA, therefore other than those ditches where *S. nitida* was found by manual survey, *S. nitida* is likely to be absent from these ditches (Table 3).

3.5 Metabarcoding PCR and Library Preparation

Of the 22 DNA samples from ditches all but two were successfully amplified (even after several attempts) (Figure 6). The two samples that did not amplify corresponded to two DNA samples whose concentration were too low to quantify (Table 2) and were not processed any further. Additionally, these ditches were the first to be sampled and filtering the water was found to be especially difficult on that day which could also help to explain the low DNA concentrations obtained. Extraction blanks and PCR negative controls were negative for amplification. Positive control DNA (worm, *Alloloborphora chlorotica*) also successfully amplified. Successful indexing PCR amplification (and bands of the correct size) was confirmed using the Tapestation system (Figure 7).

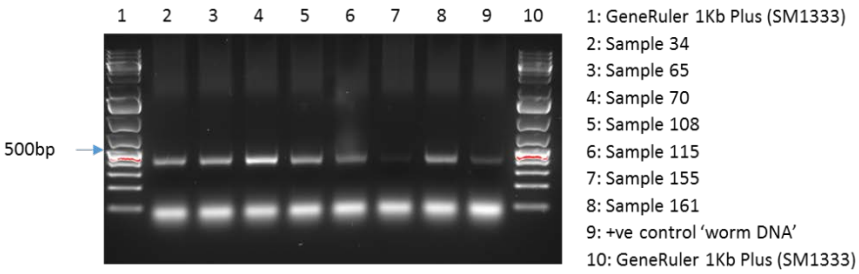

Figure 6 Example first round PCR results

Successful amplification of 7 of the DNA extracts: 5 µL PCR product loaded per well; 3 µL 1 Kb Ladder loaded. Negative controls were also preformed and were negative for amplification run but are not shown on this gel.

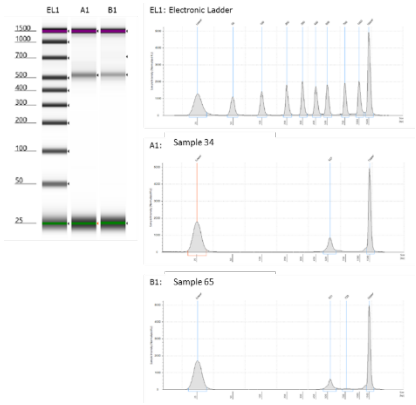

Figure 7 Example tapestation readout
Successful amplification of two first round PCR products subjected to indexing PCR using AmpliTaq Gold illustrating correct band size (~530 bp).

3.6 Bioinformatics and Data Analysis

A total of 4.7M raw reads (2.3M read pairs) assigned to sample barcodes were returned from sequencing. A further 290K raw reads were attributable to control samples and finally 81M raw reads could not be assigned to a barcode. The mean number of raw read pairs per sample barcode was 59K, ranging from 12K reads for ditch 34 to 270K for ditch 106. After bioinformatics processing to convert paired end reads to a single merged read, trimming these, and identifying those that contained the target specific primer site around 48% of the non-control raw read pairs went onto taxonomic assignment (range 48.8% to 66.3%).

Overall, a total of 38K sequences were assigned a taxonomic identification which represented 29 species. After taxonomic assignment the data was compared to information as supplied by Natural England (Appendix 1 Table 8).

4. Discussion

4.1 Overview

This work was undertaken to determine the applicability of both single-species qPCR assay and metabarcoding methodology to monitor the presence of *S. nitida* and other invertebrates (the latter by metabarcoding only) during ditch water sampling of Stodmarsh NNR. The project was undertaken, to apply the currently available methods (qPCR and metabarcoding) and the associated DNA sequence reference databases that could be used, to uncover any gaps in this approach and highlight areas of research and development effort that may make this approach more applicable and viable for use by Natural England and others. Wherever possible this project followed previous examples of similar qPCR and recommended validation of qPCR assays (Ficetola et al. 2008; Thalinger et al. 2021) and metabarcoding work that had been published in peer reviewed articles (for example, Deiner et al. 2016; Drummond et al. 2015; Hajibabaei et al. 2011; Murray et al. 2012; Serrana et al. 2019; Valentini et al. 2016; Yu et al. 2012) with additional information being found within Natural England commissioned report NECR252 (Tang et al. 2018) and work commissioned by Natural England in 2019 (in press).

4.2 Single-species Assay

A species-specific real-time PCR assay targeting a 167bp fragment of the COI region of *S. nitida* was developed and optimised for determining the presence of *S. nitida* based on the detection of eDNA in ditch water samples. The COI region has been targeted and sequenced for a wide range of organisms (DNA barcoding, Hebert et al. 2003) making it an ideal target for this kind of assay. eDNA-based assays require that closely related, co-occurring species whose DNA may be present in the environmental samples are considered when designing the assay to ensure specificity to the target of interest (Goldberg et al, 2016). The qPCR assay designed herein successfully distinguished between *S. nitida* and the morphologically similar *H. complanatus* in that no cross-amplification was detected either in silico or in vitro for this and several other snail species present in the Stodmarsh NNR ditch systems.

Positive amplification of *S. nitida* DNA was found in five ditches known to contain *S.nitida* and also in two ditches where *S. nitida* was not found by the manual survey and there are no historic records either. The amplification ‘score’ for these two samples was 3 or 4 out of 12 replicates which is unlikely to be a false positive. The laboratories used for this study are unidirectional so that high DNA containing samples i.e. *S. nitida* control specimens are not processed within the eDNA extraction laboratory used to process the ditch water samples, nor are PCRs set up within either of these laboratories and different staff were involved in the different steps of the procedure. Thus it is unlikely that these results were due to a contamination event. The finding of these two additional eDNA positive ditches could therefore suggest further survey would be worthwhile to confirm the presence of *S. nitida.*

It is also the case that three ditches where *S. nitida* were found were negative by the qPCR assay. In terms of the volumes of water filtered, although there was considerable variation between ditches, the eDNA positive ditches had a similar range of volumes of water filtered (200mL to 400mL) when compared with these potential false negatives (200mL to 500mL). For all three of these ditches, the concentration of DNA extracted from the filters was low (0.84 ng/µL to 2.32 ng/µl), when compare with the concentration of DNA extracted from the filters where eDNA results were positive (2.14ng/µL to 18.1ng/µl). It could therefore be the case that the concentration of DNA extracted from the filters was too low to allow for positive amplification and that if more DNA had been extracted from these samples this may have resulted in a positive detection.

The concentration of eDNA at any one time will depend on the rate of productions by the species and how long it persists in the environment (Dejean et al. 2011). eDNA released into the environment will depend on many factors including: the number of individuals present, their physiology and metabolism; and temperature (Treguier et al. 2014). eDNA is broken down in the environment by a range of biotic and abiotic factors including: high temperatures, UV, and extracellular enzymes (Dejean et al. 2011; Treguier et al. 2014; Barnes et al. 2014; Pilliod et al. 2014). During manual survey, *S. nitida* was reported in these ditches as relative abundance 'rare' (ditch 70) to 'occasional' (ditches 62 and 108). Ditch 70 had recently been sensitively reprofiled, following a survey for *S. nitida* when it was reported as occasional in this ditch (Cousins, 2019). Suitable habitat was affected by this ditch management and thus snail numbers reduced to lower abundance, also affecting the eDNA detection. It could also be the case that they 'shed' less DNA into their environment meaning that if a much larger volume of water was sampled these may then become eDNA positive. The samples were collected in December, so there could have been a dilution effect due to autumnal rainfall, although these ditches were sampled prior to the main rainfall events of winter 2020/21.

A further issue that could be explored is the time of year that the samples are taken in relation to the ecology and life cycle of the snail. It is possible that at different times of year *S. nitida* may 'shed' larger amounts of DNA into their environment, thus making detection of low amounts of their DNA more likely. It is known that for example great crested newts shed different amounts of DNA into their environments depending on their body condition, life stage, and whether they are breeding or not (Buxton et al. 2017). This is also known to be the case for crayfish where it has been found that the eDNA detection is more efficient when water bodies contain a large population of young crayfish (Treguier et al, 2014), this is likely to be due to frequent moulting which could enhance the release of DNA into the water. Additional experiments to explore the mechanisms of *S. nitida* DNA release and sampling methods (time of year, volume of water sampled) could therefore improve detection rates in line with manual surveys.

The study has shown that qPCR screening tools can allow managers to identify target species in samples without the need for taxonomic expertise. It is relatively inexpensive (although broadly similar to a manual survey) and can be completed within a few days of receiving the samples given co-ordination and pre-planning with the testing laboratory. The qPCR assay did detect DNA where no animals were found which could therefore

enhance management decisions when it comes to planning ditch management at Stodmarsh NNR and other sites where *S. nitida* is present.

4.3 Metabarcoding

In this study, we were not able to detect *S. nitida* nor other macroinvertebrates of interest using the metabarcoding methodology. There are several reasons as to why this may have occurred which are discussed below.

During the bioinformatics the majority of the returned sequences were discarded due to low sequence identity (<95%) with the Animalia COI database used for the bioinformatics. Of these discarded sequences those with the highest read counts were run through the BLAST database to see if they had identity to species not within the Animalia COI database used in the bioinformatic pipeline. The majority had low sequence identity (<80%) to anything in the BLAST database suggesting that the species present within the samples have not yet been COI barcoded (sequenced) and/or that they were likely to be nontarget taxa. The primers used here are known to amplify fungi, algae and bacterial sequences in addition to the invertebrate sequences that they were designed for (Mioduchowska et al. 2018; Leese et al. 2020). In a recently published study utilising an alternative set of universal COI primers, a new reverse primer was designed to have increased specificity towards benthic invertebrate taxa in order to try and improve benthic invertebrate detection from eDNA samples (Leese et al. 2020). The study showed that the newly designed primer avoided the normal non-target amplification bias thus improving detection of the benthic invertebrates of interest. It is therefore possible that if the Stodmarsh NNR samples were analysed using this alternative primer set with the improved reverse primer that the metabarcoding results would also improve the detection of the invertebrates of interest. This would still be dependent on there being a high enough concentration of DNA from target species within the water samples but it could go some way to relieving primer bias towards fungi, algae and bacteria.

It is possible that if alternative or multiple primer sets had been used for the metabarcoding different results would have been generated. Variations in results have been found both within PCR replicates due to the stochastic nature of the PCR reactions (Yamamoto et al. 2017) and between different primer sets. Instead of designing and trialling new PCR primers, which was beyond the scope of this project, primers that had previously been described (and are in widespread use) were used to generate the COI PCR amplicons from each sample (Leray et al. 2013). In the first round PCR the aim was to capture as much as possible of the sequence diversity within the samples. In order to do this the primers are degenerate, that is, they contain variations at some of the nucleotide positions within the primer sequence (Table 1). Degenerate primers can be more difficult to use because there will inevitably be some nucleotides that are mismatched upon primer binding to the target sequences. A modification of the jgHCO2198 forward primer as described by Geller et al. (2013), was to use the nucleotide ‘Inosine’ at three positions within the primer. Inosine is useful in that it can base pair with

any natural base, resulting in a more stable primer/target duplex, and hence a more efficient PCR.

Considering the PCR amplification reactions themselves, PCRs were carried out using an ‘environmental mastermix’ containing the polymerase Amplitaq Gold (a standard Taq polymerase as opposed to a high fidelity Taq polymerase). This mastermix/enzyme was chosen as it has been developed to have good tolerance to PCR inhibitors such as may be co-extracted from environmental samples, which ordinarily may have inhibited the PCR. Higher fidelity enzymes can also be used but are more difficult to use where target DNA concentration may be low and where degenerate primers are employed. There is therefore a trade-off in getting the PCR to work effectively with the choice between a high fidelity polymerase which should be highly accurate (but may not be as sensitive as a lower fidelity enzyme) and a lower fidelity enzyme (such as amplitaq gold) which may be better at generating the DNA product to start with. Previous experience with this enzyme in metabarcoding experiments suggested that the enzyme fidelity was good enough to retrieve good quality sequence data additionally due to the inclusion of inosine bases within the degenerate primers used a standard Taq polymerase had to be used as high fidelity polymerases are not able to ‘read’ past these inosine bases resulting in a lack of amplification.

It is possible that *S. nitida* and other species of interest were missed by the initial metabarcoding PCR step. There are several reasons why this could be the case: 1) DNA from certain species may be misrepresented in the sample, either by coming from invertebrates that are much smaller in size than others within the sample pool, or being present in lower abundance than the dominant species both scenarios contributing to differences in starting biomass. 2) DNA may have been inefficiently extracted from different species, again this could contribute to a low DNA target number at the start. 3) Perhaps the biggest source of bias may be in the primers that are used in the initial PCR which may have missed some of these species, the primers used may simply not work efficiently for some invertebrate species although *S. nitida* DNA was amplified with an unmodified version of the metabarcoding primers for species identification purposes. DNA extracted from community samples may be subject to potential amplification bias where different species’ DNA is in competition to bind to the universal primers which can prevent the capture of all species present in a given sample as more common template DNAs are likely to be amplified (Kelly et al. 2014). This in turn can mean that for very large individuals, high abundance species can prevent the detection of low abundance species resulting in ‘species masking’ (Brandon-Mong et al. 2015; Kelly et al. 2014). Metabarcoding may therefore be less capable of identifying the DNA of less abundant species within a community than a species-specific qPCR as could be the case in this study.

In light of the above reasons it is probable that a combination of these resulted in the non-detection of *S.nitida* and other macroinvertebrates of interest to Natural England.

5 Recommendations

1. Additional experiments to explore the mechanisms of *S. nitida* DNA release and also sampling methods (time of year, volume of water sampled).
2. As there are few COI sequences for *S. nitida* and other macroinvertebrates of interest within publicly available sequence databases, it would be worth catching/trapping individuals before taxonomically identifying them and Sanger sequencing their COI genes using the 'Folmer' barcoding primers and subsequently submitting these to the Genbank/BOLD databases to supplement the existing data. This would ideally need to be carried out on multiple individuals of each species.
3. For metabarcoding, it is recommended that the issue of 'species-masking' is investigated.
    A. Although the primers used in this study successfully amplified the DNA from a number of species it was still the case that the species of interest were not detected via metabarcoding despite the fact that the primers should work. It could be investigated whether the primer design could be improved specifically for UK species or for specific genus/families which are underrepresented or not seen in the metabarcoding data.
    B. Alternative primer sets could also be trialled to see if their use allows for the detection of *S. nitida* and other macroinvertebrates of interest including using the recently published newly designed reverse primer (Leese *et al.* 2020) to reduce non-target amplification.
    C. Likewise, it could be investigated how species size and/or abundance effects metabarcoding outputs via the creation of 'mock' samples containing known biomass and or numbers of different species (see for example Braukmann *et al*. 2019).

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Appendices

Appendix 1. Sample/Specimen Information

Sample ID	Collection date/time	Sampler	What 3 words Location	Volume filtered	Sample Condition	Site conditions
1	31/01/21	BM	Unguarded-Grove-Decanter	420ml	Low turbidity	Overcast, 1°C
2	31/01/21	BM	Chap-City-Decanter	90ml	Low turbidity	Overcast, 1°C
3	31/01/21	BM	Cassettes-Legroom-Housing	60ml	Low turbidity	Overcast, 1°C
4	31/01/21	BM	Rattled-Games-Foresight	60ml	Low turbidity	Overcast, 1°C
5	31/01/21	BM	Observers-Rises-Stoppage	200ml	Low turbidity	Overcast, 1°C
D1	10/01/21; 10.00	HR	Happily-Zealous-Mixes	240ml	Low turbidity	Overcast, 1°C
D2	10/01/21; 10.10	HR	Processor-Symphonic-Winded	240ml	Low turbidity	Overcast, 1°C
Top	04/02/21; 13.05	HR	Repayment-Trail-Occupiers	85ml	Low turbidity	Sunny, light breeze 6°C

LHS	04/02/21: 12.55	HR	Mush-Arrive-Squad	130ml	Medium turbidity	Sunny, light breeze, 6°C
RHS	04/02/21: 12.50	HR	Smokers-Sprouted-Copiers	65ml	Medium turbidity	Sunny, light breeze, 6°C

Table 4 Leicestershire ditch samples

Sample ID	Collection date/time	Sampler	GPS Location	Volume filtered	Sample Condition	Site conditions
34	23/114/2020; 13.00	KO/PW	51.315018-1.192531	250ml	Low turbidity	Sunny, 8 degrees
42	23/11/2020; 14.00	KO/PW	51.313210-1.193633	450ml	Low turbidity	Sunny, 8 degrees
44	23/11/2020; 13.30	KO/PW	51.313603-1.193356	250ml	Low turbidity	Sunny, 8 degrees
56	23/11/2020; 11.30	KO/PW	51.313864-1.196120	400ml	Low turbidity	Sunny, 7 degrees
58	23/11/2020; 10.55	KO/PW	51.313686-1.196216	300ml	Low turbidity	Sunny, 7 degrees
60	17/11/2020; 14.00	KO/PW	51.315713-1.197350	62/1: 200ml; 62/2: 150ml	Low turbidity	Sunny spells, 14 degrees
62	17/11/2020; 14.30	KO/PW	51.314608-1.197528	62/1: 200ml; 62/2: 150ml	Low turbidity	Sunny spells, 14 degrees
65	23/11/2020; 14.50	KO/PW	51.313703-1.198258	65/1: 70ml, 65/2: 70ml, 65/3: 70ml	High turbidity	Cloud cover, 7 degrees
70	17/11/2020; 13.10	KO/PW	51.314726-1.197878	500ml	Low turbidity	Sunny, 14 degrees
87	18/11/2020; 11.55	KC/KO	51.316071-1.200108	350ml	Low turbidity	Sunny, windy
92	18/11/2020; 10.58	KC/KO	51.316634-1.201841	92/1: 170ml; 92/2: 150ml	Low turbidity	Sunny, breezy, warm
98	18/11/2020; 14.00	KC/KO	51.314733-1.201146	98/1: 100ml; 98/2: 100ml	Medium turbidity	Windy, overcast

106	27/11/2020; 13.00	KO/PW	51.317576-1.202240	400ml	Low turbidity	Overcast, 6 degrees
108	18/11/2020; 13.00	KC/KO	51.314511-1.202374	108/1: 100ml; 108/2: 100ml	Medium turbidity	Clear, breezy
115	27/11/2020; 11.45	KO/PW	51.316764-1.203014	220ml	Low turbidity	Overcast, 6 degrees
131	27/11/2020; 10.30	KO/PW	51.314365-1.206790	500ml	Low turbidity	Overcast, 6 degrees
135	17/11/2020; 10.20	KO/PW	51.320395-1.207171	300ml	Low turbidity	Sunny, 12 degrees
136	16/11/2020; 10.55	KC/PW	51.321417-1.207495	136/1: 320ml; 136/2: 180ml	Low turbidity	Overcast, dry, 12 degrees
146	16/11/2020; 13.05	KC/PW	51.320626-1.20948	146/1:220ml; 146/2: 200ml	Low turbidity	Overcast, dry, 12 degrees
153	27/11/2020; 14.30	KO/PW	51.320135-1.209509	350ml	Low turbidity	Overcast, 7 degrees
155	27/11/2020; 14.00	KO/PW	51.321650-1.209372	250ml	Low turbidity	Overcast, 6 degrees
161	17/11/2020; 11.30	KO/PW	51.320258-1.211856	500ml	Low turbidity	Sunny spells, 12 degrees

Table 5 Stodmarsh National Nature Reserve ditch samples

 Ditch 34	 Ditch 42
 Ditch 44	 Ditch 56
 Ditch 58	 Ditch 60

Ditch 62

Ditch 65

Ditch 70

Ditch 87

Ditch 92

Ditch 98

Ditch 106

Ditch 108

Ditch 115

Ditch 131

Ditch 135

Ditch 136

 Ditch 146	 Ditch 153
 Ditch 155	 Ditch 161

Table 6 Stodmarsh National Nature Reserve ditch photos, Nov-Dec 2020, © Ken Obbard (Natural England)

SPECIES	Order	Ditch no.	Grid Ref	Date	Sampled by	Relative Abundance
Crangonyx pseudogracilis	*Amphipoda*	34	TR 22569 62165	18/12/20	DJB	Common
Glossiphonia heteroclita	*Annelida*	34	TR 22569 62165	18/12/20	DJB	Rare
Helobdella stagnalis	*Annelida*	34	TR 22569 62165	18/12/20	DJB	Rare
Colymbetes fuscus	*Coleoptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Rhantus frontalis	*Coleoptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Ceratopogonidae	*Diptera*	34	TR 22569 62165	18/12/20	DJB	Occasional
Chaoborus sp.	*Diptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Dixidae	*Diptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	34	TR 22569 62165	18/12/20	DJB	Occasional
Hesperocorixa linnaei	*Hemiptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Hesperocorixa sahlbergi	*Hemiptera*	34	TR 22569 62165	18/12/20	DJB	Rare
Notonecta glauca	*Hemiptera*	34	TR 22569 62165	18/12/20	DJB	Frequent

Asellus aquaticus	*Isopoda*	34	TR 22569 62165	18/12/20	DJB	Abundant
Acroloxus lacustris	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional
Bathyomphalus contortus	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Common
Bithynia leachii	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Frequent
Bithynia tentaculata	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Frequent
Lymnaea balthica (peregra)	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Common
Lymnaea palustris/fusca	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional
Lymnaea stagnalis	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional
Physa fontinalis	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Common
Planorbarius corneus	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Rare
Planorbis planorbis	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Common
Segmentina nitida	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional
Sphaerium nucleus	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional

Valvata cristata	*Mollusca*	34	TR 22569 62165	18/12/20	DJB	Occasional
Coenagrion puella	*Odonata*	34	TR 22569 62165	18/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	42	TR 22650 61921	18/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	42	TR 22650 61921	18/12/20	DJB	Rare
Erpobdella testacea	*Annelida*	42	TR 22650 61921	18/12/20	DJB	Rare
Noterus clavicornis	*Coleoptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Rhantus frontalis	*Coleoptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Eurycercus lamellatus	*Cladocera*	42	TR 22650 61921	18/12/20	DJB	Occasional
Simocephalus sp.	*Diplostraca*	42	TR 22650 61921	18/12/20	DJB	Occasional
Ceratopogonidae	*Diptera*	42	TR 22650 61921	18/12/20	DJB	Occasional
Dixidae	*Diptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	42	TR 22650 61921	18/12/20	DJB	Common
Corixa punctata	*Hemiptera*	42	TR 22650 61921	18/12/20	DJB	Rare

Ilyocoris cimicoides	*Hemiptera*	42	TR 22650 61921	18/12/20	DJB	Frequent
Notonecta glauca	*Hemiptera*	42	TR 22650 61921	18/12/20	DJB	Frequent
Notonecta marmorea viridis	*Hemiptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Plea minutissima (leachi)	*Hemiptera*	42	TR 22650 61921	18/12/20	DJB	Frequent
Asellus aquaticus	*Isopoda*	42	TR 22650 61921	18/12/20	DJB	Common
Anisus vortex	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Abundant
Bathyomphalus contortus	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Frequent
Bithynia leachii	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Occasional
Bithynia tentaculata	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Occasional
Lymnaea balthica (peregra)	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Abundant
Lymnaea palustris/fusca	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Rare
Lymnaea stagnalis	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Abundant

Pisidia	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Rare
Planorbarius corneus	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Rare
Planorbis carinatus	*Mollusca*	42	TR 22650 61921	18/12/20	DJB	Frequent
Coenagrion puella	*Odonata*	42	TR 22650 61921	18/12/20	DJB	Frequent
Coenagrion pulchellum	*Odonata*	42	TR 22650 61921	18/12/20	DJB	Occasional
Agrypnia pagetana	*Trichoptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	42	TR 22650 61921	18/12/20	DJB	Occasional
Plectrocnemia spp.	*Trichoptera*	42	TR 22650 61921	18/12/20	DJB	Rare
Dendrocoelum lacteum	*Turbellaria*	42	TR 22650 61921	18/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	42	TR 22650 61921	18/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	44	TR 22648 62002	18/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	44	TR 22648 62002	18/12/20	DJB	Rare
Piscicola geometra	*Annelida*	44	TR 22648 62002	18/12/20	DJB	Rare

Laccophilus minutus	*Coleoptera*	44	TR 22648 62002	18/12/20	DJB	Rare
Noterus clavicornis	*Coleoptera*	44	TR 22648 62002	18/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	44	TR 22648 62002	18/12/20	DJB	Common
Ilyocoris cimicoides	*Hemiptera*	44	TR 22648 62002	18/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	44	TR 22648 62002	18/12/20	DJB	Frequent
Notonecta marmorea viridis	*Hemiptera*	44	TR 22648 62002	18/12/20	DJB	Rare
Sigara dorsalis	*Hemiptera*	44	TR 22648 62002	18/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	44	TR 22648 62002	18/12/20	DJB	Common
Cataclysta lemnata	*Lepidoptera*	44	TR 22648 62002	18/12/20	DJB	Rare
Acroloxus lacustris	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Occasional
Anisus vortex	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Common
Bathyomphalus contortus	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Common
Bithynia leachii	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Frequent

Bithynia tentaculata	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Frequent
Lymnaea balthica (peregra)	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Common
Lymnaea palustris/fusca	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Frequent
Physa fontinalis	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Frequent
Pisidia	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Rare
Planorbarius corneus	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Rare
Planorbis carinatus	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Rare
Planorbis planorbis	*Mollusca*	44	TR 22648 62002	18/12/20	DJB	Common
Brachytron pratense	*Odonata*	44	TR 22648 62002	18/12/20	DJB	Rare
Coenagrion puella	*Odonata*	44	TR 22648 62002	18/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	44	TR 22648 62002	18/12/20	DJB	Rare
Erythromma najas	*Odonata*	44	TR 22648 62002	18/12/20	DJB	Rare
Ischnura elegans	*Odonata*	44	TR 22648 62002	18/12/20	DJB	Rare

Agrypnia pagetana	*Trichoptera*	44	TR 22648 62002	18/12/20	DJB	Occasional
Limnephilus marmoratus	*Trichoptera*	44	TR 22648 62002	18/12/20	DJB	Occasional
Dendrocoelum lacteum	*Turbellaria*	44	TR 22648 62002	18/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	44	TR 22648 62002	18/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	56	TR 22851 62073	18/12/20	DJB	Frequent
Erpobdella octaculata	*Annelida*	56	TR 22851 62073	18/12/20	DJB	Occasional
Theromyzon tessulatum	*Annelida*	56	TR 22851 62073	18/12/20	DJB	Rare
Dytiscidae	*Coleoptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Laccophilus minutus	*Coleoptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Noterus clavicornis	*Coleoptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Noterus crassicornis	*Coleoptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Ceratopogonidae	*Diptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Chaoborus sp.	*Diptera*	56	TR 22851 62073	18/12/20	DJB	Rare

Dixella attica/autumnalis	*Diptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	56	TR 22851 62073	18/12/20	DJB	Occasional
Corixa punctata	*Hemiptera*	56	TR 22851 62073	18/12/20	DJB	Occasional
Hesperocorixa linnaei	*Hemiptera*	56	TR 22851 62073	18/12/20	DJB	Occasional
Hesperocorixa sahlbergi	*Hemiptera*	56	TR 22851 62073	18/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	56	TR 22851 62073	18/12/20	DJB	Frequent
Notonecta glauca	*Hemiptera*	56	TR 22851 62073	18/12/20	DJB	Frequent
Asellus aquaticus	*Isopoda*	56	TR 22851 62073	18/12/20	DJB	Frequent
Acroloxus lacustris	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Anisus vortex	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Frequent
Bathyomphalus contortus	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Common
Bithynia leachii	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Bithynia tentaculata	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Frequent

Hippeutis complanata	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Common
Pisidia	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Planorbarius corneus	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Planorbis planorbis	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Common
Segmentina nitida	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Occasional
Sphaerium nucleus	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Rare
Valvata cristata	*Mollusca*	56	TR 22851 62073	18/12/20	DJB	Occasional
Coenagrion puella	*Odonata*	56	TR 22851 62073	18/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	56	TR 22851 62073	18/12/20	DJB	Rare
Limnephilidae	*Trichoptera*	56	TR 22851 62073	18/12/20	DJB	Rare

Dugesia polychroa	*Turbellaria*	56	TR 22851 62073	18/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	58	TR 22863 61997	18/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	58	TR 22863 61997	18/12/20	DJB	Occasional
Piscicola geometra	*Annelida*	58	TR 22863 61997	18/12/20	DJB	Rare
Berosus affinis	*Coleoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Dytiscidae	*Coleoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Hydroporus discretus	*Coleoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Laccophilus minutus	*Coleoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Noterus clavicornis	*Coleoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Dixella attica/autumnalis	*Diptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Orthocladiinae	*Diptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	58	TR 22863 61997	18/12/20	DJB	Common
Corixa punctata	*Hemiptera*	58	TR 22863 61997	18/12/20	DJB	Rare

Ilyocoris cimicoides	*Hemiptera*	58	TR 22863 61997	18/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	58	TR 22863 61997	18/12/20	DJB	Frequent
Notonecta marmorea viridis	*Hemiptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Plea minutissima (leachi)	*Hemiptera*	58	TR 22863 61997	18/12/20	DJB	Frequent
Asellus aquaticus	*Isopoda*	58	TR 22863 61997	18/12/20	DJB	Frequent
Anisus vortex	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Abundant
Bathyomphalus contortus	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Common
Bithynia leachii	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Occasional
Bithynia tentaculata	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Frequent
Gyraulus albus	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Common

Planorbis carinatus	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	58	TR 22863 61997	18/12/20	DJB	Common
Anax imperator	*Odonata*	58	TR 22863 61997	18/12/20	DJB	Rare
Coenagrion puella	*Odonata*	58	TR 22863 61997	18/12/20	DJB	Occasional
Coenagrion pulchellum	*Odonata*	58	TR 22863 61997	18/12/20	DJB	Occasional
Ischnura elegans	*Odonata*	58	TR 22863 61997	18/12/20	DJB	Rare
Agrypnia pagetana	*Trichoptera*	58	TR 22863 61997	18/12/20	DJB	Rare
Limnephilus politus	*Trichoptera*	58	TR 22863 61997	18/12/20	DJB	Occasional
Dugesia polychroa	*Turbellaria*	58	TR 22863 61997	18/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	60	TR 22922 62351	17/12/20	DJB	Common
Oligochaeta	*Annelida*	60	TR 22922 62351	17/12/20	DJB	Rare
Theromyzon tessulatum	*Annelida*	60	TR 22922 62351	17/12/20	DJB	Rare
Dytiscidae	*Coleoptera*	60	TR 22922 62351	17/12/20	DJB	Occasional

Gyrinus marinus	*Coleoptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Peltodytes caesus	*Coleoptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Orthocladiinae	*Diptera*	60	TR 22922 62351	17/12/20	DJB	Occasional
Tipulidae	*Diptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	60	TR 22922 62351	17/12/20	DJB	Common
Corixa punctata	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Frequent
Hesperocorixa linnaei	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Notonecta glauca	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Occasional
Notonecta marmorea viridis	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Plea minutissima (leachi)	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Frequent
Sigara dorsalis	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Sigara falleni	*Hemiptera*	60	TR 22922 62351	17/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	60	TR 22922 62351	17/12/20	DJB	Abundant

Anisus vortex	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Common
Bithynia leachii	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Common
Bithynia tentaculata	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Common
Lymnaea balthica (peregra)	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Abundant
Lymnaea palustris/fusca	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Occasional
Lymnaea stagnalis	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Rare
Pisidia	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Rare
Planorbarius corneus	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Rare
Planorbis carinatus	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Rare
Planorbis planorbis	*Mollusca*	60	TR 22922 62351	17/12/20	DJB	Occasional
Coenagrion puella	*Odonata*	60	TR 22922 62351	17/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	60	TR 22922 62351	17/12/20	DJB	Rare
Limnephilus affinis/incisus	*Trichoptera*	60	TR 22922 62351	17/12/20	DJB	Occasional

Dugesia polychroa	*Turbellaria*	60	TR 22922 62351	17/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	62	TR 22922 62130	17/12/20	DJB	Occasional
Eisinella tetraedra	*Annelida*	62	TR 22922 62130	17/12/20	DJB	Rare
Erpobdella octaculata	*Annelida*	62	TR 22922 62130	17/12/20	DJB	Rare
Glossiphonia heteroclita	*Annelida*	62	TR 22922 62130	17/12/20	DJB	Rare
Hemiclepsis marginata	*Annelida*	62	TR 22922 62130	17/12/20	DJB	Rare
Berosus affinis	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Demetrias imperialis	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Hydrophilus piceus	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Limnoxenus niger	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Peltodytes caesus	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Rhantus grapii	*Coleoptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	62	TR 22922 62130	17/12/20	DJB	Frequent

Corixa punctata	*Hemiptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Hesperocorixa linnaei	*Hemiptera*	62	TR 22922 62130	17/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	62	TR 22922 62130	17/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	62	TR 22922 62130	17/12/20	DJB	Frequent
Asellus aquaticus	*Isopoda*	62	TR 22922 62130	17/12/20	DJB	Frequent
Acroloxus lacustris	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Occasional
Anisus vortex	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Bathyomphalus contortus	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Bithynia leachii	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Bithynia tentaculata	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Hippeutis complanata	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Rare

Lymnaea stagnalis	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Planorbarius corneus	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Rare
Planorbis carinatus	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Segmentina nitida	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Occasional
Sphaerium nucleus	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Rare
Valvata cristata	*Mollusca*	62	TR 22922 62130	17/12/20	DJB	Frequent
Coenagrion puella	*Odonata*	62	TR 22922 62130	17/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	62	TR 22922 62130	17/12/20	DJB	Rare
Dendrocoelum lacteum	*Turbellaria*	62	TR 22922 62130	17/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	62	TR 22922 62130	17/12/20	DJB	Occasional
Erpobdella octaculata	*Annelida*	65	TR 22932 62063	17/12/20	DJB	Occasional

Colymbetes fuscus	*Coleoptera*	65	TR 22932 62063	17/12/20	DJB	Rare
Hesperocorixa linnaei	*Hemiptera*	65	TR 22932 62063	17/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	65	TR 22932 62063	17/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	65	TR 22932 62063	17/12/20	DJB	Frequent
Asellus aquaticus	*Isopoda*	65	TR 22932 62063	17/12/20	DJB	Abundant
Acroloxus lacustris	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Occasional
Anisus vortex	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Occasional
Bathyomphalus contortus	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Common
Bithynia tentaculata	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Frequent
Lymnaea balthica (peregra)	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Rare
Lymnaea stagnalis	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Occasional

Planorbarius corneus	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Rare
Planorbis planorbis	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Common
Segmentina nitida	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Occasional
Valvata cristata	*Mollusca*	65	TR 22932 62063	17/12/20	DJB	Rare
Brachytron pratense	*Odonata*	65	TR 22932 62063	17/12/20	DJB	Rare
Coenagrion puella	*Odonata*	65	TR 22932 62063	17/12/20	DJB	Rare
Agrypnia pagetana	*Trichoptera*	65	TR 22932 62063	17/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	65	TR 22932 62063	17/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	70	TR 22978 62130	17/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Occasional
Helobdella stagnalis	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Rare
Hemiclepsis marginata	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Rare
Oligochaeta	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Occasional

Piscicola geometra	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Rare
Theromyzon tessulatum	*Annelida*	70	TR 22978 62130	17/12/20	DJB	Rare
Agabus/Ilybus	*Coleoptera*	70	TR 22978 62130	17/12/20	DJB	not recorded
Gyrinus marinus	*Coleoptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Hyphydrus ovatus	*Coleoptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Eurycercus lamellatus	*Cladocera*	70	TR 22978 62130	17/12/20	DJB	Occasional
Chironomini	*Diptera*	70	TR 22978 62130	17/12/20	DJB	not recorded
Orthocladiinae	*Diptera*	70	TR 22978 62130	17/12/20	DJB	not recorded
Tanypodinae	*Diptera*	70	TR 22978 62130	17/12/20	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	70	TR 22978 62130	17/12/20	DJB	Abundant
Hesperocorixa linnaei	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Frequent

Plea minutissima (leachi)	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Common
Ranatra linearis	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Sigara dorsalis	*Hemiptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	70	TR 22978 62130	17/12/20	DJB	Abundant
Cataclysta lemnata	*Lepidoptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Acroloxus lacustris	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Bathyomphalus contortus	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Common
Bithynia leachii	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Bithynia tentaculata	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Frequent
Hippeutis complanata	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Common
Lymnaea palustris/fusca	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Common
Lymnaea stagnalis	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Rare

Physa fontinalis	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Common
Pisidia	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Planorbarius corneus	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Planorbis planorbis	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Segmentina nitida	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Rare
Valvata cristata	*Mollusca*	70	TR 22978 62130	17/12/20	DJB	Occasional
Anax imperator	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Brachytron pratense	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Coenagrion puella	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Erythromma najas	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Ischnura elegans	*Odonata*	70	TR 22978 62130	17/12/20	DJB	Rare
Agrypnia pagetana	*Trichoptera*	70	TR 22978 62130	17/12/20	DJB	Rare

Limnephilus marmoratus	*Trichoptera*	70	TR 22978 62130	17/12/20	DJB	Occasional
Plectrocnemia spp.	*Trichoptera*	70	TR 22978 62130	17/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	87	TR 23140 62365	27/01/21	DJB	Frequent
Glossiphonia heteroclita	*Annelida*	87	TR 23140 62365	27/01/21	DJB	Rare
Oligochaeta	*Annelida*	87	TR 23140 62365	27/01/21	DJB	Rare
Cymbiodyta marginellus	*Coleoptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Enochrus testaceus	*Coleoptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Simocephalus sp.	*Diplostraca*	87	TR 23140 62365	27/01/21	DJB	Occasional
Eurycercus lamellatus	*Cladocera*	87	TR 23140 62365	27/01/21	DJB	Occasional
Chironomini	*Diptera*	87	TR 23140 62365	27/01/21	DJB	Occasional
Tabanidae	*Diptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Dolichipodidae	*Diptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	87	TR 23140 62365	27/01/21	DJB	Frequent

Ilyocoris cimicoides	*Hemiptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Asellus aquaticus	*Isopoda*	87	TR 23140 62365	27/01/21	DJB	Common
Bathyomphalus contortus	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Frequent
Bithynia leachii	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Occasional
Bithynia tentaculata	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Occasional
Hippeutis complanata	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Rare
Physa fontinalis	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Rare
Pisidia	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Occasional
Planorbarius corneus	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Rare
Planorbis planorbis	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Frequent
Segmentina nitida	*Mollusca*	87	TR 23140 62365	27/01/21	DJB	Frequent

Coenagrion puella	*Odonata*	87	TR 23140 62365	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	87	TR 23140 62365	27/01/21	DJB	Rare
Dendrocoelum lacteum	*Turbellaria*	87	TR 23140 62365	27/01/21	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	92	TR 23180 62323	27/01/21	DJB	Frequent
Oligochaeta	*Annelida*	92	TR 23180 62323	27/01/21	DJB	Rare
Enochrus testaceus	*Coleoptera*	92	TR 23180 62323	27/01/21	DJB	Rare
Hydroporus pubescens	*Coleoptera*	92	TR 23180 62323	27/01/21	DJB	Rare
Odacantha melanura	*Coleoptera*	92	TR 23180 62323	27/01/21	DJB	Rare
Culiseta sp.	*Diptera*	92	TR 23180 62323	27/01/21	DJB	Frequent
Dixella attica/autumnalis	*Diptera*	92	TR 23180 62323	27/01/21	DJB	Occasional
Limoniidae (Ormosia)	*Diptera*	92	TR 23180 62323	27/01/21	DJB	not recorded
Tanypodinae	*Diptera*	92	TR 23180 62323	27/01/21	DJB	Rare
Tipulidae	*Diptera*	92	TR 23180 62323	27/01/21	DJB	Rare

Asellus aquaticus	*Isopoda*	92	TR 23180 62323	27/01/21	DJB	Frequent
Acroloxus lacustris	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Occasional
Bathyomphalus contortus	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Frequent
Bithynia leachii	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Rare
Bithynia tentaculata	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Occasional
Lymnaea palustris/fusca	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Occasional
Physa fontinalis	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Frequent
Planorbis planorbis	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Occasional
Segmentina nitida	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	Frequent
Sphaerium corneum	*Mollusca*	92	TR 23180 62323	27/01/21	DJB	not recorded
Limnephilus marmoratus	*Trichoptera*	92	TR 23180 62323	27/01/21	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	98	TR 23224 62163	22/12/20	DJB	Frequent
Oligochaeta	*Annelida*	98	TR 23224 62163	22/12/20	DJB	Rare

Anacaena limbata	*Coleoptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Haliplus lineatocollis	*Coleoptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Hydroporus pubescens	*Coleoptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Noterus clavicornis	*Coleoptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Eurycercus lamellatus	*Cladocera*	98	TR 23224 62163	22/12/20	DJB	Frequent
Simocephalus sp.	*Diplostraca*	98	TR 23224 62163	22/12/20	DJB	Frequent
Chironomini	*Diptera*	98	TR 23224 62163	22/12/20	DJB	Occasional
Dixella attica/autumnalis	*Diptera*	98	TR 23224 62163	22/12/20	DJB	Occasional
Limoniidae (Ormosia)	*Diptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Notonecta glauca	*Hemiptera*	98	TR 23224 62163	22/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	98	TR 23224 62163	22/12/20	DJB	Common
Anisus vortex	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional

Bathyomphalus contortus	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Common
Bithynia leachii	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Frequent
Bithynia tentaculata	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Lymnaea balthica (peregra)	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Lymnaea palustris/fusca	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Physa fontinalis	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Rare
Pisidia	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Planorbis carinatus	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Planorbis planorbis	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Valvata cristata	*Mollusca*	98	TR 23224 62163	22/12/20	DJB	Occasional
Coenagrion pulchellum	*Odonata*	98	TR 23224 62163	22/12/20	DJB	Rare
Glyphotaelius pellucidus	*Trichoptera*	98	TR 23224 62163	22/12/20	DJB	not recorded
Limnephilidae	*Trichoptera*	98	TR 23224 62163	22/12/20	DJB	Frequent

Limnephilus marmoratus	*Trichoptera*	98	TR 23224 62163	22/12/20	DJB	Frequent
Dugesia polychroa	*Turbellaria*	98	TR 23224 62163	22/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	106	TR 23325 62545	27/01/21	DJB	Abundant
Erpobdella octaculata	*Annelida*	106	TR 23325 62545	27/01/21	DJB	Occasional
Piscicola geometra	*Annelida*	106	TR 23325 62545	27/01/21	DJB	Occasional
Theromyzon tessulatum	*Annelida*	106	TR 23325 62545	27/01/21	DJB	Rare
Berosus affinis	*Coleoptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Gyrinus marinus	*Coleoptera*	106	TR 23325 62545	27/01/21	DJB	Occasional
Gyrinus paykulli	*Coleoptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Haliplus obliquus	*Coleoptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Chironomini	*Diptera*	106	TR 23325 62545	27/01/21	DJB	Frequent
Tanypodinae	*Diptera*	106	TR 23325 62545	27/01/21	DJB	Frequent
Cloeon dipterum	*Ephemeroptera*	106	TR 23325 62545	27/01/21	DJB	Common

Corixa dentipes	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Corixa panzeri	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Frequent
Corixa punctata	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Cymatia coleoptrata	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Occasional
Notonecta glauca	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Occasional
Sigara sp.	*Hemiptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Asellus aquaticus	*Isopoda*	106	TR 23325 62545	27/01/21	DJB	Common
Sialis lutaria	*Megaloptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Anisus vortex	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Rare
Bithynia leachii	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Rare
Bithynia tentaculata	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Frequent
Lymnaea balthica (peregra)	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Frequent

Physa fontinalis	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Abundant
Pisidia	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Occasional
Planorbis carinatus	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Occasional
Planorbis planorbis	*Mollusca*	106	TR 23325 62545	27/01/21	DJB	Rare
Erythromma najas	*Odonata*	106	TR 23325 62545	27/01/21	DJB	Rare
Agrypnia pagetana	*Trichoptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Holocentropus dubius	*Trichoptera*	106	TR 23325 62545	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	106	TR 23325 62545	27/01/21	DJB	Frequent
Dendrocoelum lacteum	*Turbellaria*	106	TR 23325 62545	27/01/21	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	108	TR 23306 62242	22/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	108	TR 23306 62242	22/12/20	DJB	Occasional
Piscicola geometra	*Annelida*	108	TR 23306 62242	22/12/20	DJB	Rare
Berosus affinis	*Coleoptera*	108	TR 23306 62242	22/12/20	DJB	Rare

Rhantus suturalis	*Coleoptera*	108	TR 23306 62242	22/12/20	DJB	Rare
Eurycercus lamellatus	*Cladocera*	108	TR 23306 62242	22/12/20	DJB	Occasional
Chaoborus sp.	*Diptera*	108	TR 23306 62242	22/12/20	DJB	Occasional
Chironomidae	*Diptera*	108	TR 23306 62242	22/12/20	DJB	Occasional
Tipulidae	*Diptera*	108	TR 23306 62242	22/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	108	TR 23306 62242	22/12/20	DJB	Common
Hesperocorixa linnaei	*Hemiptera*	108	TR 23306 62242	22/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	108	TR 23306 62242	22/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	108	TR 23306 62242	22/12/20	DJB	Occasional
Asellus aquaticus	*Isopoda*	108	TR 23306 62242	22/12/20	DJB	Frequent
Acroloxus lacustris	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Anisus vortex	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Bathyomphalus contortus	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent

Bithynia leachii	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Bithynia tentaculata	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Lymnaea stagnalis	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Rare
Physa fontinalis	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Planorbarius corneus	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Occasional
Planorbis carinatus	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Occasional
Segmentina nitida	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Occasional
Sphaerium corneum	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Rare
Valvata cristata	*Mollusca*	108	TR 23306 62242	22/12/20	DJB	Occasional
Anax imperator	*Odonata*	108	TR 23306 62242	22/12/20	DJB	Rare
Coenagrion puella	*Odonata*	108	TR 23306 62242	22/12/20	DJB	Rare
Agrypnia pagetana	*Trichoptera*	108	TR 23306 62242	22/12/20	DJB	Occasional

Limnephilus marmoratus	*Trichoptera*	108	TR 23306 62242	22/12/20	DJB	Frequent
Dendrocoelum lacteum	*Turbellaria*	108	TR 23306 62242	22/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	108	TR 23306 62242	22/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	115	TR 23369 62472	27/01/21	DJB	Common
Glossiphonia heteroclita	*Annelida*	115	TR 23369 62472	27/01/21	DJB	Rare
Hemiclepsis marginata	*Annelida*	115	TR 23369 62472	27/01/21	DJB	Rare
Oligochaeta	*Annelida*	115	TR 23369 62472	27/01/21	DJB	Rare
Argyroneta aquatica	Spider	115	TR 23369 62472	27/01/21	DJB	Rare
Agabus bipustulatus	*Coleoptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Dytiscidae	*Coleoptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Simocephalus sp.	*Diplostraca*	115	TR 23369 62472	27/01/21	DJB	Rare
Chaoborus sp.	*Diptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Chironomini	*Diptera*	115	TR 23369 62472	27/01/21	DJB	Occasional

Coquilletidia richardii	*Diptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	115	TR 23369 62472	27/01/21	DJB	Common
Corixa panzeri	*Hemiptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Corixa punctata	*Hemiptera*	115	TR 23369 62472	27/01/21	DJB	Occasional
Hesperocorixa linnaei	*Hemiptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Notonecta glauca	*Hemiptera*	115	TR 23369 62472	27/01/21	DJB	Occasional
Sigara distincta	*Hemiptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Asellus aquaticus	*Isopoda*	115	TR 23369 62472	27/01/21	DJB	Common
Acroloxus lacustris	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Occasional
Anisus vortex	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Frequent
Bathyomphalus contortus	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Bithynia leachii	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Occasional
Bithynia tentaculata	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Frequent

Lymnaea balthica (peregra)	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Common
Lymnaea palustris/fusca	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Lymnaea stagnalis	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Physa fontinalis	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Common
Pisidia	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Planorbarius corneus	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Planorbis carinatus	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Frequent
Planorbis planorbis	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Sphaerium nucleus	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Rare
Valvata cristata	*Mollusca*	115	TR 23369 62472	27/01/21	DJB	Frequent
Coenagrion puella	*Odonata*	115	TR 23369 62472	27/01/21	DJB	Rare
Erythromma najas	*Odonata*	115	TR 23369 62472	27/01/21	DJB	Rare
Ischnura elegans	*Odonata*	115	TR 23369 62472	27/01/21	DJB	Rare

Holocentropus dubius	*Trichoptera*	115	TR 23369 62472	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	115	TR 23369 62472	27/01/21	DJB	Frequent
Dendrocoelum lacteum	*Turbellaria*	115	TR 23369 62472	27/01/21	DJB	Rare
Dugesia polychroa	*Turbellaria*	115	TR 23369 62472	27/01/21	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	131	TR 23516 62109	22/12/20	DJB	Common
Erpobdella testacea	*Annelida*	131	TR 23516 62109	22/12/20	DJB	Rare
Glossiphonia complanata	*Annelida*	131	TR 23516 62109	22/12/20	DJB	Rare
Dytiscidae	*Coleoptera*	131	TR 23516 62109	22/12/20	DJB	Rare
Hygrobia hermanni	*Coleoptera*	131	TR 23516 62109	22/12/20	DJB	Occasional
Hyphydrus ovatus	*Coleoptera*	131	TR 23516 62109	22/12/20	DJB	Rare
Chironomini	*Diptera*	131	TR 23516 62109	22/12/20	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	131	TR 23516 62109	22/12/20	DJB	Occasional
Corixa dentipes	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Rare

Corixa punctata	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Frequent
Plea minutissima (leachi)	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Frequent
Ranatra linearis	*Hemiptera*	131	TR 23516 62109	22/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	131	TR 23516 62109	22/12/20	DJB	Common
Anisus vortex	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Bathyomphalus contortus	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Common
Bithynia leachii	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Bithynia tentaculata	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Rare
Lymnaea stagnalis	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Physa fontinalis	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional

Pisidia	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Planorbarius corneus	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Planorbis carinatus	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Common
Sphaerium nucleus	*Mollusca*	131	TR 23516 62109	22/12/20	DJB	Occasional
Coenagrion puella	*Odonata*	131	TR 23516 62109	22/12/20	DJB	Rare
Coenagrion pulchellum	*Odonata*	131	TR 23516 62109	22/12/20	DJB	Occasional
Agrypnia pagetana	*Trichoptera*	131	TR 23516 62109	22/12/20	DJB	Occasional
Limnephilus marmoratus	*Trichoptera*	131	TR 23516 62109	22/12/20	DJB	Frequent
Dendrocoelum lacteum	*Turbellaria*	131	TR 23516 62109	22/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	131	TR 23516 62109	22/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	135	TR 23568 62793	22/12/20	DJB	Occasional
Erpobdella octaculata	*Annelida*	135	TR 23568 62793	22/12/20	DJB	Rare

Erpobdella testacea	*Annelida*	135	TR 23568 62793	22/12/20	DJB	Rare
Anacaena limbata	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Berosus affinis	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Haliplus ruficollis	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Hygrotus impressopunctatus	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Laccophilus minutus	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Occasional
Liopterus haemorrhoidalis	*Coleoptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Simocephalus sp.	*Diplostraca*	135	TR 23568 62793	22/12/20	DJB	Occasional
Ceratopogonidae	*Diptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Cloeon dipterum	*Ephemeroptera*	135	TR 23568 62793	22/12/20	DJB	Occasional
Corixa punctata	*Hemiptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	135	TR 23568 62793	22/12/20	DJB	Frequent
Notonecta glauca	*Hemiptera*	135	TR 23568 62793	22/12/20	DJB	Frequent

Notonecta marmorea viridis	*Hemiptera*	135	TR 23568 62793	22/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	135	TR 23568 62793	22/12/20	DJB	Common
Anisus vortex	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Frequent
Bathyomphalus contortus	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Occasional
Bithynia tentaculata	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Occasional
Hippeutis complanata	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Frequent
Lymnaea palustris/fusca	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Rare
Lymnaea stagnalis	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Occasional
Physa fontinalis	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Frequent
Planorbis carinatus	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Frequent
Sphaerium nucleus	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Rare

Valvata cristata	*Mollusca*	135	TR 23568 62793	22/12/20	DJB	Occasional
Aeshna isoceles	*Odonata*	135	TR 23568 62793	22/12/20	DJB	Rare
Coenagrion puella	*Odonata*	135	TR 23568 62793	22/12/20	DJB	Rare
Dendrocoelum lacteum	*Turbellaria*	135	TR 23568 62793	22/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	135	TR 23568 62793	22/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	136	TR 23583 62913	15/12/20	DJB	Common
Erpobdella octaculata	*Annelida*	136	TR 23583 62913	15/12/20	DJB	Rare
Piscicola geometra	*Annelida*	136	TR 23583 62913	15/12/20	DJB	Rare
Gyrinus aeratus	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Gyrinus marinus	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Occasional
Gyrinus paykulli	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Gyrinus substriatus	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Hydrophilus piceus	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare

Hydroporus palustris	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Laccophilus minutus	*Coleoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Chironomini	*Diptera*	136	TR 23583 62913	15/12/20	DJB	Occasional
Tanypodinae	*Diptera*	136	TR 23583 62913	15/12/20	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	136	TR 23583 62913	15/12/20	DJB	Occasional
Corixa punctata	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Hesperocorixa linnaei	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Frequent
Notonecta glauca	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Frequent
Notonecta marmorea viridis	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Occasional
Ranatra linearis	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Sigara dorsalis	*Hemiptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Asellus aquaticus	*Isopoda*	136	TR 23583 62913	15/12/20	DJB	Frequent

Bathyomphalus contortus	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Rare
Bithynia leachii	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Common
Bithynia tentaculata	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Common
Gyraulus crista	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Rare
Lymnaea balthica (peregra)	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Common
Physa fontinalis	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	136	TR 23583 62913	15/12/20	DJB	Frequent
Anax imperator	*Odonata*	136	TR 23583 62913	15/12/20	DJB	Occasional
Coenagrion puella	*Odonata*	136	TR 23583 62913	15/12/20	DJB	Rare
Erythromma najas	*Odonata*	136	TR 23583 62913	15/12/20	DJB	Rare
Ischnura elegans	*Odonata*	136	TR 23583 62913	15/12/20	DJB	Occasional
Sympetrum striolatum	*Odonata*	136	TR 23583 62913	15/12/20	DJB	Rare
Agrypnia pagetana	*Trichoptera*	136	TR 23583 62913	15/12/20	DJB	Rare

Limnephilus marmoratus	*Trichoptera*	136	TR 23583 62913	15/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	136	TR 23583 62913	15/12/20	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	146	TR 23674 62820	27/01/21	DJB	Occasional
Erpobdella octaculata	*Annelida*	146	TR 23674 62820	27/01/21	DJB	Rare
Piscicola geometra	*Annelida*	146	TR 23674 62820	27/01/21	DJB	Occasional
Gyrinus marinus	*Coleoptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Haliplus flavicollis agg	*Coleoptera*	146	TR 23674 62820	27/01/21	DJB	not recorded
Haliplus obliquus	*Coleoptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Laccophilus minutus	*Coleoptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Simocephalus sp.	*Diplostraca*	146	TR 23674 62820	27/01/21	DJB	Rare
Ceratopogonidae	*Diptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Orthocladiinae	*Diptera*	146	TR 23674 62820	27/01/21	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	146	TR 23674 62820	27/01/21	DJB	Common

Corixa panzeri	*Hemiptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Corixa punctata	*Hemiptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Notonecta glauca	*Hemiptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Notonecta marmorea viridis	*Hemiptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Sigara falleni	*Hemiptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Asellus aquaticus	*Isopoda*	146	TR 23674 62820	27/01/21	DJB	Frequent
Bithynia tentaculata	*Mollusca*	146	TR 23674 62820	27/01/21	DJB	Occasional
Lymnaea balthica (peregra)	*Mollusca*	146	TR 23674 62820	27/01/21	DJB	Frequent
Physa fontinalis	*Mollusca*	146	TR 23674 62820	27/01/21	DJB	Common
Planorbis planorbis	*Mollusca*	146	TR 23674 62820	27/01/21	DJB	Rare
Coenagrion puella	*Odonata*	146	TR 23674 62820	27/01/21	DJB	Rare
Erythromma najas	*Odonata*	146	TR 23674 62820	27/01/21	DJB	Occasional
Ischnura elegans	*Odonata*	146	TR 23674 62820	27/01/21	DJB	Occasional

Agrypnia pagetana	*Trichoptera*	146	TR 23674 62820	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	146	TR 23674 62820	27/01/21	DJB	Occasional
Dugesia polychroa	*Turbellaria*	146	TR 23674 62820	27/01/21	DJB	Rare
Crangonyx pseudogracilis	*Amphipoda*	153	TR 23802 62806	27/01/21	DJB	Common
Oligochaeta	*Annelida*	153	TR 23802 62806	27/01/21	DJB	Rare
Berosus affinis	*Coleoptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Gyrinus marinus	*Coleoptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Gyrinus substriatus	*Coleoptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Daphnia sp.	*Cladocera*	153	TR 23802 62806	27/01/21	DJB	Rare
Chironomidae	*Diptera*	153	TR 23802 62806	27/01/21	DJB	Frequent
Orthocladiinae	*Diptera*	153	TR 23802 62806	27/01/21	DJB	not recorded
Tanypodinae	*Diptera*	153	TR 23802 62806	27/01/21	DJB	not recorded
Caenis horaria	*Ephemeroptera*	153	TR 23802 62806	27/01/21	DJB	Rare

Cloeon dipterum	*Ephemeroptera*	153	TR 23802 62806	27/01/21	DJB	Abundant
Corixa punctata	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Cymatia coleoptrata	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Common
Hesperocorixa linnaei	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Occasional
Notonecta glauca	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Occasional
Plea minutissima (leachi)	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Common
Sigara dorsalis	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Sigara falleni	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Rare
Sigara fossarum	*Hemiptera*	153	TR 23802 62806	27/01/21	DJB	Occasional
Asellus aquaticus	*Isopoda*	153	TR 23802 62806	27/01/21	DJB	Common
Bithynia tentaculata	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Frequent
Lymnaea balthica (peregra)	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Frequent

Lymnaea palustris/fusca	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Rare
Physa fontinalis	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Common
Planorbis carinatus	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Occasional
Planorbis planorbis	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Occasional
Sphaerium nucleus	*Mollusca*	153	TR 23802 62806	27/01/21	DJB	Rare
Coenagrion puella	*Odonata*	153	TR 23802 62806	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	153	TR 23802 62806	27/01/21	DJB	Occasional
Dugesia polychroa	*Turbellaria*	153	TR 23802 62806	27/01/21	DJB	Rare
Polycelis tenuis	*Turbellaria*	153	TR 23802 62806	27/01/21	DJB	Rare
Piscicola geometra	*Annelida*	155	TR 23810 62930	27/01/21	DJB	Occasional
Berosus affinis	*Coleoptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Hyphydrus ovatus	*Coleoptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Porhydrus lineatus	*Coleoptera*	155	TR 23810 62930	27/01/21	DJB	Rare

Simocephalus sp.	*Diplostraca*	155	TR 23810 62930	27/01/21	DJB	Occasional
Ceratopogonidae	*Diptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Chironomidae	*Diptera*	155	TR 23810 62930	27/01/21	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	155	TR 23810 62930	27/01/21	DJB	Frequent
Caenis robusta	*Ephemeroptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Callicorixa praeusta	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Corixa punctata	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Occasional
Cymatia rogenhoferi	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Ilyocoris cimicoides	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Notonecta glauca	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Notonecta marmorea viridis	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Paracorixa concinna	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare
Plea minutissima (leachi)	*Hemiptera*	155	TR 23810 62930	27/01/21	DJB	Rare

Asellus aquaticus	*Isopoda*	155	TR 23810 62930	27/01/21	DJB	Frequent
Sialis lutaria	*Megaloptera*	155	TR 23810 62930	27/01/21	DJB	not recorded
Bithynia tentaculata	*Mollusca*	155	TR 23810 62930	27/01/21	DJB	Rare
Gyraulus crista	*Mollusca*	155	TR 23810 62930	27/01/21	DJB	Abundant
Lymnaea balthica (peregra)	*Mollusca*	155	TR 23810 62930	27/01/21	DJB	Common
Aeshna isoceles	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Rare
Anax imperator	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Rare
Coenagrion puella	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Rare
Ischnura elegans	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Occasional
Orthetrum cancellatum	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Rare
Sympetrum striolatum	*Odonata*	155	TR 23810 62930	27/01/21	DJB	Rare
Limnephilus marmoratus	*Trichoptera*	155	TR 23810 62930	27/01/21	DJB	Occasional
Crangonyx pseudogracilis	*Amphipoda*	161	TR 23889 62771	16/12/20	DJB	Common

Theromyzon tessulatum	*Annelida*	161	TR 23889 62771	16/12/20	DJB	Rare
Berosus affinis	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Dytiscidae	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Haliplus lineatocollis	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Haliplus obliquus	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Hydroporus angustatus	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Laccophilus minutus	*Coleoptera*	161	TR 23889 62771	16/12/20	DJB	Rare
Eurycercus lamellatus	*Cladocera*	161	TR 23889 62771	16/12/20	DJB	Rare
Simocephalus sp.	*Diplostraca*	161	TR 23889 62771	16/12/20	DJB	Rare
Chironomidae	*Diptera*	161	TR 23889 62771	16/12/20	DJB	Occasional
Cloeon dipterum	*Ephemeroptera*	161	TR 23889 62771	16/12/20	DJB	Occasional
Cymatia coleoptrata	*Hemiptera*	161	TR 23889 62771	16/12/20	DJB	Occasional
Hesperocorixa linnaei	*Hemiptera*	161	TR 23889 62771	16/12/20	DJB	Rare

Ilyocoris cimicoides	*Hemiptera*	161	TR 23889 62771	16/12/20	DJB	Occasional
Notonecta glauca	*Hemiptera*	161	TR 23889 62771	16/12/20	DJB	Frequent
Plea minutissima (leachi)	*Hemiptera*	161	TR 23889 62771	16/12/20	DJB	Occasional
Asellus aquaticus	*Isopoda*	161	TR 23889 62771	16/12/20	DJB	Frequent
Anisus vortex	*Mollusca*	161	TR 23889 62771	16/12/20	DJB	Frequent
Bithynia tentaculata	*Mollusca*	161	TR 23889 62771	16/12/20	DJB	Frequent
Planorbis carinatus	*Mollusca*	161	TR 23889 62771	16/12/20	DJB	Frequent
Planorbis planorbis	*Mollusca*	161	TR 23889 62771	16/12/20	DJB	Frequent
Sphaerium nucleus	*Mollusca*	161	TR 23889 62771	16/12/20	DJB	not recorded
Coenagrion puella	*Odonata*	161	TR 23889 62771	16/12/20	DJB	Rare
Dendrocoelum lacteum	*Turbellaria*	161	TR 23889 62771	16/12/20	DJB	Rare
Dugesia polychroa	*Turbellaria*	161	TR 23889 62771	16/12/20	DJB	Rare

Table 7 Invertebrate species list as sampled by Dan Bennett (Dec 2020). The abundance attribution made by D. Bennett is qualitative.

Species	Classification	Ditch Number	Read Count	Found in manual search	Previously found in the UK (NBN atlas, accessed 08/04/2021)
Chaetonotus aff. *euhystrix*	Chaetonotida	34	11	No	Yes
Eucyclops cf. *serrulatus*	Copepoda	42	2156	No	No known occurrence
Simocephalus vetulus	Phyllopoda	42	13	*Simocephalus* sp. recorded	Yes
Chaetonotus aff. *euhystrix*	Chaetonotida	44	9	No	Yes
Cygnus olor	Anseriformes	44	7	No but likely to be present	Yes
Eucyclops cf. *serrulatus*	Copepoda	56	4091	No	No known occurrence
Chaetonotus aff. *euhystrix*	Chaetonotida	56	1906	No	Yes
Ochthebius minimus	Coleoptera	56	1540	No	Yes
Chaetonotus jaceki	Chaetonotida	56	307	No	No known occurrence
Chaetonotus aff. *maximus*	Chaetonotida	56	80	No	No known occurrence
Chaetogaster disatrophus	Annelida	58	89	No	No known occurrence

Eucyclops cf. *serrulatus*	Copepoda	58	7	No	No known occurrence
Chaetonotus aff. *euhystrix*	Chaetonotida	58	6	No	Yes
Simocephalus vetulus	Crusacea	58	5	*Simocephalus* sp. Recorded in other ditches	Yes
Brachionus calyciflorus	Rotifera	60	28	No	No known occurrence
Chydoridae sp.	Phyllopoda	60	7	No	Members of the genus are known to occur in the UK
Eucyclops cf. *serrulatus*	Copepoda	60	6	No	No known occurrence
Chaetonotus persimilis	Chaetonotida	60	5	No	No known occurrence
Chaetonotus aff. *euhystrix*	Chaetonotida	62	736	No	Yes
Chaetonotus borealis	Chaetonotida	62	10	No	No known occurrence
Cygnus olor	Anseriformes	65	4929	No but likely to be present	Yes
Thermocyclops crassus	Copepoda	70	1362	No	Yes
Nais sp.	Annelida	70	757	No	Yes
Eucyclops cf. *serrulatus*	Copepoda	70	55	No	No known occurrence

Homo sapiens	Primates	70	13	No	Yes
Chaetonotus persimilis	Chaetonotida	87	32	No	No known occurrence
Rallus aquaticus aquaticus	Gruiformes	87	9	No but likely to be present	Yes
Turdus iliacus iliacus	Passeriformes	87	6	No but likely to be present	Yes
Eucyclops cf. *serrulatus*	Copepoda	87	5	No	No known occurrence
Emberiza citrinella citrinella	Passeriformes	87	5	No but likely to be present	Yes
Chaetonotus persimilis	Chaetonotida	92	305	No	No known occurrence
Chaetonotus gelidus	Chaetonotida	92	227	No	No known occurrence
Chaetonotus aff. *maximus*	Chaetonotida	92	157	No	No known occurrence
Chaetonotus aff. *subtilis*	Chaetonotida	92	71	No	No known occurrence
Eiseniella sp.	Annelida	92	15	No	Member of the genus are known to occur in the UK
Chaetonotus jaceki	Chaetonotida	92	10	No	No known occurrence
Eucyclops cf. *serrulatus*	Copepoda	98	260	No	No known occurrence

Chaetonotus aff. *euhystrix*	Chaetonotida	98	106	No	Yes
Chaetogaster disatrophus	Annelida	98	75	No	No known occurrence
Rallus aquaticus aquaticus	Gruiformes	98	55	No but likely to be present	Yes
Simocephalus vetulus	Crustacea	98	10	*Simocephalus* sp. recorded	Yes
Chaetonotus persimilis	Chaetonotida	98	10	No	No known occurrence
Chaetonotus jaceki	Chaetonotida	98	10	No	No known occurrence
Polymerurus rhomboides	Chaetonotida	98	5	No	No known occurrence
Chaetogaster disatrophus	Annelida	106	9287	No	No known occurrence
Polyarthra dolichoptera	Rotifera	106	10	No	Yes
Chaetonotus aff. *euhystrix*	Chaetonotida	108	146	No	Yes
Thermocyclops crassus	Copepoda	108	36	No	Yes
Chaetonotus jaceki	Chaetonotida	108	22	No	No known occurrence
Asellus aquaticus	Isopoda	108	6	Yes	Yes

Chaetonotus borealis	Chaetonotida	108	5	No	No known occurrence
Maxillopoda sp.	Maxillopoda	115	98	No	No known occurrence
Chaetonotus aff. *euhystrix*	Chaetonotida	115	22	No	Yes
Eucyclops cf. *serrulatus*	Copepoda	115	6	No	No known occurrence
Eucyclops cf. *serrulatus*	Copepoda	131	339	No	No known occurrence
Chaetonotus aff. *euhystrix*	Chaetonotida	135	30	No	Yes
Maxillopoda sp	Maxillopoda	135	15	No	No known occurrence
Eucyclops cf. *serrulatus*	Copepoda	1353	15	No	No known occurrence
Cygnus olor	Anseriformes	153	329	No but likely to be present	Yes
Eucyclops cf. *serrulatus*	Cyclopoida	153	34	No	No known occurrence
Chaetonotus jaceki	Chaetonotida	153	17	No	No known occurrence
Acanthocyclops americanus	Copepoda	153	5	No	Members of the genus known to occur in the UK
Podocopida sp.	Crusteacea	155	61	No	Members of the genus are

					known to occur in the UK
Anas platyrhynchos	Anseriformes	155	5	No but likely to be present	Yes
Homo sapiens	Primates	155	5	n/a	Yes
Eucyclops cf. *serrulatus*	Copepoda	161	544	No	No known occurrence
Simocephalus vetulus	Phyllopoda	161	85	*Simocephalus* sp. recorded	Yes
Chydoridae sp.	Phyllopoda	161	59	No	Members of the genus are known to occur in the UK
Chaetonotus borealis	Chaetonotida	161	31	No	No known occurrence

Table 8 Species list per ditch as found by metabarcoding

Appendix 2. Detailed Materials and Methods

A. Sampling Methodology – Collection of Ditch Water Samples

1. Collect 20 samples of 30 mL of water from along the ditch using a sampling ladle and empty each sample into a sterile Whirl-Pak bag.
2. Close the bag securely using the top tabs (fold over several times and bend tabs over) and shake the Whirl-Pak bag for 10 seconds.
3. Draw up water into the 50 ml syringe and then attach the syringe to the filter unit. Filter the water through the column. Keep repeating this process to pass up to 500 mL through the filter. Differences in pond turbidity could clog the filter up at a far lower volume. In this case use more than one filter at each site.
4. Once 500 ml water has been filtered (or as much as possible, record volume on sample record sheet), flush any excess water out of the filter unit by drawing up air into the syringe, attaching to the filter unit and pushing the air through forcing water out of the filter. This will not remove all the water, so tap the residual water out onto some tissue.
5. Using a 10ml syringe draw up 2 ml 95% ethanol preservation solution, attach the syringe to the filter unit hold upside-down and push into the filter until it starts to come out of the top.
6. Screw caps onto each end of the filter unit and label the filter unit accordingly with a permanent marker.

B. Sampling Methodology – Collection of Invertebrate Samples

1. Using a Freshwater Biological Association design, rectangular frame pond net (20-25 cm x 19-22 cm x 30 cm deep) with a 1 mm mesh, each ditch was pond netted three times from the bankside. The surveyor netted the vegetation by making short jabbing thrusts into dense emergent and raft-forming plants, making occasional longer strokes into submerged plants and over bare substrate in deeper water. The surveyor selected patches of vegetation that exhibit the greatest small-scale mosaic structure, since these patches yield more specimens. Netting stopped after 1 to 3 minutes when the net begins to fill to the point where it becomes difficult to push and is usually a quarter to a third full of plant material (about 2 to 3 litres by volume). Careful manipulation slowed the rate at which algae and duckweed were caught while probing more productive structures. Bottom sediment was avoided since it clogs the net and contains almost no species that contribute to the analysis.
2. Bank sorting was carried out for each haul for 10 minutes each, giving 30 minutes of sorting for each ditch. The sample was tipped onto a white plastic sheet and up to 30 secs are spent spreading out the material into a thin layer. The more thinly spread the material is, the greater the chances of seeing animals. Fast crawling beetles, bugs and dragonfly larvae are collected or identified (if recognisable) before they escape during the spreading-out process. The sheet is then scanned for other animals. This cannot be hurried since it relies on the animals recovering from their shock and they can often remain still for some time. After a few minutes, the debris can be turned over and poked about, when more animals will be found. The pool of water that forms in the centre of the sheet allows weakly swimming animals to escape and be seen. Species identified in the field were not collected but recorded in situ and released back to the ditch. Fine flexible forceps were used for picking up animals, collecting one or two individuals of each different taxa encountered and placing them in ethanol (70%) for identification in the lab if required. Note that in some cases the

whole sample for a ditch was taken back to the lab for sorting due to inclement conditions in the field.

3. After sorting and picking over on the plastic sheet, the material from the sheet was tipped into a large bucket with some ditch water, combining the catch from the three hauls taken from a particular ditch. In order to find any weakly swimming animals and molluscs two final operations were carried out. Part of the debris was put into a large, strong, white tray with 1-2cm of water so that feeble animals can swim free, and were collected, and the tray emptied back into the bucket.
4. Finally, the plant material was swished about in the bucket of water, the larger pieces removed, and most of the water decanted, and then the heavy residue was tipped into a large, strong, white tray with around 1 cm of water. By tilting the tray slowly back and forth, carefully pouring off excess water from one corner, the molluscs were left stranded in a pile because they sink, enabling identification in the field and/or collection for preservation where required.
5. Specimens of *Segmentina nitida* and any similar species (ram's-horn shaped) if encountered such as *Hippeutis complanatus, Anisus vortex, Anisus spirorbis, Planorbis planorbis, Planorbis carinatus, Bathyomphalus contortus and Gyraulus sp*. were collected for the DNA analysis and development of primers for the single species assay. These were separated into sterile tubes, containing 95% ethanol, with one species only per tube, approximately five individuals for each species (not separated by ditch at this point) and couriered to ADAS.

C.DNA Extraction from Sterivex Filters

1. Remove the preservative solution from the filter by pushing air through the filter with a syringe.
2. Add 720µL of pre-warmed ATL buffer and 40µL PK from the DNeasy Blood and Tissue kit to the filter via the top of the unit before sealing the unit with a cap.
3. Place the filter unit into a 50ml falcon tube and place at 56°C in a water bath for 1 hour, vortexing the tube along the length of the filter unit every 10 minutes.
4. An extra 1.5 mL tube was set up to act as an extraction blank for every set of extractions performed. Therefore, add 360 µL of buffer ATL into a 1.5 mL microfuge tube and perform the DNA extraction as per steps below. Label this tube as extraction blank (EB).
5. After incubation, remove the ATL/PK mix from the filter unit into the 50ml falcon tube by pushing air through the filter with a syringe.
6. Wash the filter unit through with 400µL molecular biology grade ethanol and add to the 50ml falcon tube, mix by vortexing.
7. Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube.
8. Centrifuge at ≥ 6000 xg (8000 rpm) for 1 min. Discard the flow-through and collection tube.
9. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1.
10. Centrifuge for 1 min at ≥6000 xg. Discard the flow-through and collection tube.
11. Place the spin column in a new 2 mL collection tube, add 500 µL Buffer AW2.
12. Centrifuge for 3 min at 20,000 xg (14,000 rpm). Discard the flow-through and collection tube.
13. Transfer the spin column to a new pre-labelled 1.5 mL microcentrifuge tube.
14. Elute the DNA by adding 200 µL Buffer AE to the centre of the spin column membrane. Incubate for 1 min at room temperature (15–25°C).
15. Centrifuge for 1 min at ≥6000 xg.

D.DNA Extraction from Snail Specimens

1. Add 360µL of buffer ALT from the DNeasy Blood and Tissue kit to the ground up specimen/s.
2. An extra 1.5 mL tube must be set up to act as an extraction blank for every set of extractions performed. Therefore, add 360 µL of buffer ATL into a 1.5 mL microfuge tube and perform the DNA extraction as per steps below. Label this tube as extraction blank (EB).
3. Add 20 µL of proteinase K and 200 µL buffer AL. Mix thoroughly by vortexing. Heat at 56ºC for 10 min.
4. Add 200 µL of 100% ethanol. Mix thoroughly by vortexing.
5. Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube.
6. Centrifuge at ≥ 6000 xg (8000 rpm) for 1 min. Discard the flow-through and collection tube.
7. Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1.
8. Centrifuge for 1 min at ≥6000 xg. Discard the flow-through and collection tube.
9. Place the spin column in a new 2 mL collection tube, add 500 µL Buffer AW2.
10. Centrifuge for 3 min at 20,000 xg (14,000 rpm). Discard the flow-through and collection tube.
11. Transfer the spin column to a new pre-labelled 1.5 mL microcentrifuge tube.
12. Elute the DNA by adding 200 µL Buffer AE to the centre of the spin column membrane. Incubate for 1 min at room temperature (15–25°C).
13. Centrifuge for 1 min at ≥6000 xg.

E.DNA Quantification

DNA extracts were quantified using the Qubit® dsDNA BR assay kit and Qubit 3.0 fluorimeter as follows:

1. The Qubit® working solution was prepared by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer.
2. Make up two standards by adding 190 µL Qubit® working solution into each of two tubes before adding 10 µL of each Qubit® standard to the appropriate tube. Mix by vortexing.
3. For each extract make up a tube with a final volume of 200 µL containing 1-20 µL extract and 180-199 µL Qubit® working solution.
4. Allow all tubes to incubate for two minutes before reading the standards and extracts on the Qubit® 3.0 fluorimeter.

F. Specimen Identification PCR

PCRs were set up in a total volume of 25 µL consisting of:

a. 2 µL of extracted template DNA,
b. 2.5 µL of each primer (0.4 µmol/L),
c. 12.5 µL of Itaq (BioRad) Sybr Green mastermix
d. 5.5 µL ddH2O.

Each sample was run in duplicate on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 1 minute at 95^0C; followed by 35 cycles with a melting temperature of 95°C for 1 minute; an annealing temperature of 40^0C and a final extension step at 72^0C for 90 seconds before holding at 4^0C until collection of PCR products for analysis.

G. Single-Species PCR

PCRs were set up in a total volume of 25 µL consisting of:

a. 3 µL of extracted template DNA,
b. 1 µL of each primer/probe (0.2 µmol/L forward primer; 0.4 µmol/L reverse primer; 0.1 µmol/L probe),
c. 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase),
d. 6.5 µL ddH2O.

Each sample was run as 12 replicates on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 5 minutes at 56.3^{0}C then 10 minutes at 95°C; followed by 55 cycles with a melting temperature of 95°C for 30 seconds and an annealing temperature of 59.6^{0}C for 1 minute before holding at 4^{0}C until collection of PCR products for analysis.

H. Metabarcoding Round 1 PCR

PCRs were set up in a total volume of 25 µL consisting of:

a. 3 µL of extracted template DNA,
b. 1 µL of each primer (0.4 µmol/L),
c. 12.5 µL of TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase),
d. 7.5 µL ddH2O.

A touchdown PCR (Don *et al.* (1991)) was used to amplify the invertebrate DNA extracted from sub-samples and included: an initial incubation for 5 minutes at 95^{0}C; then 17 cycles (denaturation at 95^{0}C for 30 seconds, annealing temperature for 30 seconds, and extension at 72^{0}C for 60 seconds) where the annealing temperature is reduced by 1^{0}C each cycle from 62^{0}C down to 47^{0}C; followed by 30 cycles at an annealing temperature of 46^{0}C and a final extension step at 72^{0}C for 30 seconds before holding at 4^{0}C until collection of PCR products for analysis.

I. DNA Purification

AMPure XP PCR Purification

1. Add 1.8 µL AMPure XP per 1.0 µL of PCR product and mix thoroughly by pipette mixing.
2. Incubate at room temperature for five minutes to allow DNA fragments to bind to the paramagnetic beads.
3. Separate the beads from the solution using a magnetic plate by waiting for the solution to clear before aspirating and discarding the solution leaving ~5 µL behind so as not to disturb the separated magnetic beads.
4. Wash beads twice with 200 µL of 70% Ethanol to remove contaminants, aspirating and discarding the solution for each wash (tubes remain on the magnetic plate throughout).
5. Remove the tubes from the magnetic plate and add 40 µL of elution buffer to elute purified DNA fragments from beads. Mix ten times by pipette mixing and incubate for two minutes.
6. Place the tubes back onto the magnetic plate and leave for one minute to separate the beads from the solution.
7. Transfer the eluate to a fresh tube.

Nucleospin® Gel and PCR Cleanup

1. If using small volumes (< 30 µL) adjust the volume of the reaction mixture to 50-100 µL with ultrapure water.

2. Mix one volume of PCR product with two volumes of Buffer NTI.
3. Place a NucleoSpin® Gel and PCR clean-up column into a collection tube and load onto the spin column.
4. Wash the silica membrane by adding 700 µL Buffer NT3 to the column and centrifuge for 30 seconds and 11,000 xg.
5. Discard the flow-through and place the column back into the collection tube before repeating this wash step.
6. Dry the silica membrane for one minute at 11,000 xg to remove Buffer NT3 completely.
7. Elute the DNA by placing the column into a fresh 1.5 mL microcentrifuge tube and add 20 µL Buffer NE and incubate at room temperature for one minute before centrifuging for one minute at 11,000 xg.

J. Sequence Library Preparation

Illumina sequencing requires that sequences are able to physically attach to the high throughput sequencer. In order to achieve this, adapter sequences are added to the target amplicons (Table 2.2, Figure 2.2) thus allowing them to attach to the complementary adapters on the sequencer.

Sequencing libraries were prepared according to Illumina's '16S rRNA Sequencing Protocol'. Briefly, this requires:

1. Purification of the target specific PCR amplicons (including overhang adapters) was performed with Nucleospin® Gel and PCR cleanup columns (as above).
2. Second round PCRs (indexing PCRs using the Nextera XT index Kit v2 Set B kit) were set up in a total volume of 50 µL consisting of:
    a. 5 µL of first round PCR amplicon,
    b. 5 µL of each primer,
    c. 25 µL of Taqman Environmental Mastermix 2.0 (containing AmpliTaq GOLD DNA polymerase,
    d. 10 µL ddH_2O.
3. The indexing PCR included:
    a. an initial incubation for 3 minutes at 95°C,
    b. 12 cycles of 95^{0}C for 30 seconds,
    c. 55^{0}C for 30 seconds,
    d. 72^{0}C for 30 seconds,
    e. a final extension step at 72^{0}C for five minutes,
    f. hold at 4^{0}C until collection of PCR products.
4. The second round PCR products were then quantified using a Qubit 3.0 Fluorometer (see above).
5. Indexed PCR products were normalized by diluting to 2 nM using 10 mM Tris pH 8.5 before pooling (in equimolar amounts) of 5 µL aliquots of each to create a single pooled library for one Illumina MiSeq run.
6. The pooled library was then denatured with NaOH and diluted with hybridization buffer.
7. A PhiX library was also prepared in the same fashion.
8. The amplicon library pool was diluted to 10 pM, spiked with 10 % PhiX.
9. The combined library was then heat denatured at 96^{0}C for 2 minutes, inverted to mix and placed in an ice-water bath for 5 minutes. This heat denaturation step was performed immediately before loading the combined library into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell.
10. The library was run on the Illumina MiSeq using a MiSeq Reagent Kit v2 500 cycle kit, to generate 250-bp paired-end reads.

K Bioinformatics

Data processing was performed on an Intel i7 PC running Ubuntu Linux 18.04.1 LTS. The program FLASH 1.2.11 (Fast Length Adjustment of SHort reads, Magnoc and Salzberg 2011) was used to convert paired end reads (R1 and R2 in the MiSeq platform) to a single merged read, using a minimum overlap length of 10 nucleotides (the default) and a maximum of 150 nucleotides to calculate the alignment. Reads were trimmed reading from the 5' end using trimmomatic 0.38 (Bolger, Lohse and Usadel 2014) to truncate the sequence if the average phred score of a 5nt sliding window dropped below 30. Those reads that matched the template specific primers at the 5' and 3' ends (maximum error rate of 0.1% within target specific primer site i.e. 2 bp variants allowed) and had a target region of >120bp were then pulled out of the data using Cutadapt 2.8 (Martin 2011). Degeneracy within the primer sequences was accounted for when identifying primer sequences within the dataset. Data was next converted from fastq to fasta format using seqtk-1.3 (r106) (github).

Before taxonomic assignment standard Linux tools were used to identify 100% identical reads and condense them down to a single read to minimise time-consuming repetitive BLAST searches, however a record of the frequency of replicate sequences was maintained. Any reads with less than 5 replicates were excluded from the BLAST search.

A custom arthropod BLAST database was created from the National Centre for Biotechnology Information (NCBI) database using the search terms 'animalia', 'COI', and 'cytochrome oxidase 1' before downloading the records in FASTA format. From a total of 6.22M sequences downloaded from NCBI, 3.54M sequences were included in the final database after for example duplicates were removed.

BLAST searching was performed using the "megablast" program which is optimised to identify alignments in highly similar sequences and returned the top hit for each query sequence in a custom tabulated format. An e-value of 1e-15 was set; higher values such a 1 or 10 return a larger list of more low-scoring hits, and actual e-values returned were in the order of 1e-150 for a full length alignment.

A custom perl script filtered the BLAST output, identifying hits sharing an accession number and passing a set of criteria covering the percentage similarity between the query sequence and the database sequence (typically 95%), and having a query alignment length difference less than 6 bp. Note that ≥ 99% similarity indicates an approximately three-base difference between query and reference sequences because the maximum sequence length subjected to taxonomic assignment are around 300 bp. Read counts for each sequence passing the similarity and query alignment length filters were pooled based on accession number to generate a final frequency count for each accession. Taxonomic assignments were then compared to data provided by Natural England.

Appendix 3. *Segmentina nitida* sequence information

A. *S. nitida* Sequence Alignment

```
AY577519.1      ------------------------------------------------------------ 0
LC429396.1      ACCTTATATTTGATTTTTGGTGTTTGATGTGGTTTAGTTGGTACTGGTTTATCTCTATTA 60
EF012178.1      ------------------------------GTTTAGTCGGTACTGGTTTATCTCTATTA 29

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      ATTCGTTTGGAATTAGGTACCTCTGGTGTATTAATAGATGAACATTTTTATAATGTTATT 120
EF012178.1      ATTCGTTTGGAATTAGGTACCTCTGGTGTATTAATAGATGAACATTTTTATAATGTTATT 89

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      GTTACTGCACATGCTTTTATTATAATTTTTTTTATAGTTATACCAATAATAATTGGTGGT 180
EF012178.1      GTTACTGCACATGCTTTTATTATAATTTTTTTTATAGTTATACCAATAATAATTGGTGGT 149

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      TTTGGTAATTGAATAATTCCACTTTTAATTGGGGCTCCGGATATATCATTTCCTCGTATA 240
EF012178.1      TTTGGTAATTGAATAATTCCACTTTTAATTGGGGCTCCGGATATATCATTTCCTCGTATA 209

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      AATAACATATCATTCTGGTTACTACCACCATCTTTTATCCTTTTATTGATTTCTTCTATA 300
EF012178.1      AATAACATATCATTCTGGTTACTACCACCATCTTTTATCCTTTTATTGATTTCTTCTATA 269

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      GTTGAAGGAGGTGTTGGTACTGGGTGAACTGTTTATCCCCCCTTAAGCGGTCCTATTGCA 360
EF012178.1      GTTGAAGGAGGTGTTGGTACTGGGTGAACTGTTTATCCCCCCTTAAGCGGTCCTATTGCA 329

AY577519.1      ------------------------------------------------------------ 0
LC429396.1      CATGGTGGTGCATCAGTTGATTTAGCTATTTTTTCATTACACTTGGCCGGTATATCTTCT 420
EF012178.1      CATGGTGGTGCATCAGTTGATTTAGCTATTTTTTCATTACACTTGGCCGGTATATCTTCT 389
```

```
AY577519.1      ----------------------------------------------------------0
LC429396.1      ATTTTAGGTGCTATTAATTTTATTACCACTGTAATAAACATGCGGGCTCCAGGTATTACT480
EF012178.1      ATTTTAGGTGCTATTAATTTTATTACCACTGTAATAAACATGCGGGCTCCAGGTATTACT449

AY577519.1      ----------------------------------------------------------0
LC429396.1      ATGGAACGATTATCTTTATTTGTCTGGTCTGTATTAATTACAGCATTTTTGTTACTATTA540
EF012178.1      ATGGAACGATTATCTTTATTTGTCTGGTCTGTATTAATTACAGCATTTTTGTTACTATTA509

AY577519.1      ----------------------------------------------------------0
LC429396.1      TCATTACCAGTTTTAGCTGGTGCCATTACAATATTATTAACGGATCGTAATTTTAATACT600
EF012178.1      TCATTACCAGTTTTAGCTGGTGCCATTACAATATTATTAACGGATCGTAATTTTAATACT569

AY577519.1      ----------------------------------------------------------0
LC429396.1      AGTTTCTTTGATCCAGCAGGTGGTGGTGATCCTATCTTATA----------------------641
EF012178.1      AGTTTCTTTGATCCAGCAGGTGGTGGTGATCCTATCTTATATCAACATTTATTTTGATTT629

AY577519.1      -----------------------------------------------------CTCTCATATT10
LC429396.1      ----------------------------------------------------------641
EF012178.1      TTTGGTCATCCAGAAGTATATATTTTAATTTTACCAGGGTTTGGTATGGTTTCACATATT689

AY577519.1      TTAAGTAATTTTGTTTCTAAACCTGCTTTTGGAACATTAGGAATAATTTATGCAATAGTT70
LC429396.1      ----------------------------------------------------------641
EF012178.1      TTAAGTAATTTTGTTTCAAAACCAGCTTTTGGTACTTTAGGAATAATTTATGCTATAGTT749
```

Figure 8 *S. nitida* COI sequence alignment

Alignment shows the location of the primers used to generate A. COI barcode sequence - SnitCOIF/SnitCOIR (yellow) and B. short COI sequence for specimen identification and metabarcoding - mICOIintF/jgHCO2198 (green).

B. Potential Primer/Probe Combinations

Primer Pair	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	Probe Sequence (5' to 3')	Product Length	Cross-species amplification
1	GGAGGTGTTGGTACTGGGTG	GTAATACCTGGAGCCCGCAT	GCGGTCCTATTGCACATGGTGGT	173	Yes
2	AGGAGGTGTTGGTACTGGGT	CCTGGAGCCCGCATGTTTAT	GCGGTCCTATTGCACATGGTGGT	168	No
3	CATGCGGGCTCCAGGTATTA	CCACCACCTGCTGGATCAAA	ACCAGTTTTAGCTGGTGCCATTACAA	168	Yes
4	ATAAACATGCGGGCTCCAGG	TAGGATCACCACCACCTGCT	ACCAGTTTTAGCTGGTGCCATTACAA	181	No
5	CCTATTGCACATGGTGGTGC	ACCACCTGCTGGATCAAAGA	ACATGCGGGCTCCAGGTATTACT	273	Yes
6	TGCGGGCTCCAGGTATTACT	ATAGGATCACCACCACCTGC	ACCAGTTTTAGCTGGTGCCATTACAA	175	Yes
7	GAAGGAGGTGTTGGTACTGGG	AGTAATACCTGGAGCCCGCA	GCGGTCCTATTGCACATGGTGGT	177	Yes
8	GCGGTCCTATTGCACATGGT	CACCACCTGCTGGATCAAAG	ACATGCGGGCTCCAGGTATTACT	279	Yes
9	CCACTTTTAATTGGGGCTCCG	CCATGTGCAATAGGACCGCT	TGAAGGAGGTGTTGGTACTGGGTG	167	No
10	GAGGTGTTGGTACTGGGTGA	CCTGGAGCCCGCATGTTTA	GCGGTCCTATTGCACATGGTGGT	166	No

Table 9 Ten potential primer/probe combinations for S. nitida species-specific PCR

C.Potential Primer/Probe Positions on *S. nitida* Sequence Alignment

```
AY577519.1
------------------------------------------------------------        0

LC429396.1    TTTGGTAATTGAATAATTCCACTTTTAATTGGGGCTCCGGATATATCATTTCCTCGTATA 240

EF012178.1    TTTGGTAATTGAATAATTCCACTTTTAATTGGGGCTCCGGATATATCATTTCCTCGTATA 209


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    AATAACATATCATTCTGGTTACTACCACCATCTTTTATCCTTTTATTGATTTCTTCTATA 300

EF012178.1    AATAACATATCATTCTGGTTACTACCACCATCTTTTATCCTTTTATTGATTTCTTCTATA 269


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    GTTGAAGGAGGTGTTGGTACTGGGTGAACTGTTTATCCCCCCTTAAGCGGTCCTATTGCA 360

EF012178.1    GTTGAAGGAGGTGTTGGTACTGGGTGAACTGTTTATCCCCCCTTAAGCGGTCCTATTGCA 329


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    CATGGTGGTGCATCAGTTGATTTAGCTATTTTTTCATTACACTTGGCCGGTATATCTTCT 420

EF012178.1    CATGGTGGTGCATCAGTTGATTTAGCTATTTTTTCATTACACTTGGCCGGTATATCTTCT 389


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    ATTTTAGGTGCTATTAATTTTATTACCACTGTAATAAACATGCGGGCTCCAGGTATTACT 480

EF012178.1    ATTTTAGGTGCTATTAATTTTATTACCACTGTAATAAACATGCGGGCTCCAGGTATTACT 449


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    ATGGAACGATTATCTTTATTTGTCTGGTCTGTATTAATTACAGCATTTTTGTTACTATTA 540

EF012178.1    ATGGAACGATTATCTTTATTTGTCTGGTCTGTATTAATTACAGCATTTTTGTTACTATTA 509


AY577519.1    ------------------------------------------------------------ 0

LC429396.1    TCATTACCAGTTTTAGCTGGTGCCATTACAATATTATTAACGGATCGTAATTTTAATACT 600

EF012178.1    TCATTACCAGTTTTAGCTGGTGCCATTACAATATTATTAACGGATCGTAATTTTAATACT 569


AY577519.1    ------------------------------------------------------------ 0
```

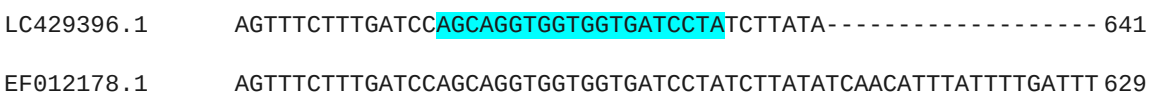

Figure 9 *S. nitida* sequence alignment showing the positions of the four potential species-specific primer/probe combinations.

Primer pair 2 and its probe shown in green; primer pair 4 and its probe shown in blue; primer pair 9 and its probe shown in red; and primer pair 10 and its probe shown in grey. Only the relevant part of the alignment is shown.

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