eDNA Detection of *Cipangopaludina chinensis* in ditch systems at Pevensey Levels, Sussex

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This version has been amended November 2022 to correct primer and probe concentrations within the Methods section and Appendices as these were incorrect in the original version. The annealing temperature for the *C. chinensis* qPCR has also been amended in the Appendices due to a typo.

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Cipangopaludina chinensis, Chinese mystery snail, eDNA, invasive, invertebrate, snail, qPCR, freshwater, ditches

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Further information

This report can be downloaded from the Natural England Access to Evidence Catalogue: **http://publications.naturalengland.org.uk/**. For information on Natural England publications contact the Natural England Enquiry Service on 0300 060 3900 or e-mail **enquiries@naturalengland.org.uk**.

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2021/2022

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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 aims to make monitoring programmes more efficient and to investigate this they wish to develop DNA and eDNA techniques for the identification of freshwater invertebrate communities and invasive non-native species and to see how this compares with hand identification.

Chinese mystery snail *(Cipangopaludina chinensis)* is a problem invasive non-native species in many parts of the world and was first found within ditches at Pevensey Levels, Sussex in 2018. This project delivers important baseline data on the applications of DNA technologies, specifically the development of species-specific eDNA assays and sampling of ditch systems to survey and monitor the spread of non-native molluscs.

1. Introduction

Biological invasions by invasive non-native species are generally accepted to be one of the greatest threats to biodiversity world-wide. The direct cost to Great Britain's economy has been estimated at £1.9 billion/year (CABI, 2022 in press).

Pevensey Levels in southern England is a large wet grassland complex criss-crossed with freshwater ditches, much of which is designated as a Special Area of Conservation (SAC) and a Ramsar site. Invasive non-native species pose a threat to the site's native biodiversity due to their potential to threaten native species, habitats or whole ecosystems.

Knowledge of species distribution is critical to ecological management and conservation biology. Effective management requires the detection of populations which can sometimes be at low densities and is usually based on visual detection and counting. Building on the approach of using environmental DNA (eDNA) to survey for great crested newts and freshwater fish in the lentic environment, there has been increasing interest in using eDNA techniques to monitor freshwater invertebrate communities and invasive non-native species. One species that Natural England is interested in detecting via DNA and eDNA techniques is *Cipangopaludina chinensis* (Gray, 1834), Chinese mystery snail which is a problem invasive species in many parts of the world (Global Invasive Species Database, 2021) and was first found within ditches at Pevensey Levels, Sussex in September 2018.

Additional survey work undertaken has found a number of smaller juvenile snails suggesting that this is a breeding population (Willing 2021a). Confirmation of breeding was made during DNA extraction from specimens collected in August 2021 (see Figure 1 right hand side) and that they have spread to adjoining ditches distal to the main site. A concern is if *C. chinensis* spreads from this original source then there is the risk of disruption to the diverse and extensive freshwater ditch ecosystems of the Pevensey Levels, a habitat for many rare invertebrate species including rare freshwater molluscs including the Little Whirlpool Ram's-horn Snail *Anisus vorticulus*.

Natural England is intending to employ eDNA monitoring to help determine the likely presence and spread of the snail on Pevensey Levels and the success of any eradication or mitigation operations over subsequent years.

1.1 Cipangopaludina chinensis

C. chinensis[1] the Chinese mystery snail or 'trapdoor snail' is a large freshwater snail native to East Asia. The 'trapdoor' refers to an oval plate (operculum) which seals the aperture of the snail when the snail is fully retracted. Across Asia multiple species of *C. chinensis*

[1] *Cipangopaludina chinensis* is adopted following MolluscaBase (MolluscaBase 2021)

have been recognised and there is considerable confusion on its taxonomy for example *C. fluminalis* is a synonym of, and *C. wingatei* is a subspecies of *C. chinensis* (Lu *et al.* 2014). Two subspecies of *C. chinensis* that are recognised are *C. chinensis chinensis* (Gray, 1834) and *C. chinensis malleata* (Reeve, 1863; also known as *C. chinensis laeta*) (Matthews *et al.* 2017) the latter of which was found to be present at the Pevensey Levels, Sussex during this study.

C. chinensis are an aquatic invasive species due to their ability to avoid predation and out-compete indigenous mollusc species for resources. Additionally, they excrete a large amount of faecal matter which can affect nitrogen and phosphorous cycling in aquatic ecosystems (Olden *et al.* 2013). Females live for around 5 years -males 3-4 years - (Jokinen, 1982, Jokinen, 1992) and give live birth during June to October (Figure 1) with greater than 160 young in a lifetime (Jokinen, 1992). This snail is used throughout China as part of the human diet and are also popular in freshwater aquaria. Both facts could help explain why the species was recently found at the Pevensey Levels either as an accidental or purposeful release.

Figure 1. Images of *C. chinensis.* Left hand side: specimen collected from Pevensey Levels August 2021 prior to DNA extraction (© Helen Rees, ADAS). Middle: specimens removed from Pevensey Levels August 2021 (© Gavin Measures, NE). Right hand side: specimen cut open during DNA extraction to reveal embryos (© Helen Rees, ADAS).

1.2 Environmental DNA

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.

Environmental DNA (eDNA) describes the DNA that can be extracted from an environmental sample for example water, soil or sediment, or air. DNA present within an environmental water sample will originate from the faeces, saliva, urine and skin cells etc. 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. This means that the eDNA from water bodies can be used for the monitoring of aquatic and semi-aquatic populations. 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).

At Pevensey Levels, Sussex, there is a need to define the distribution of *C. chinensis* in order to refine the areas of the ditch system where this species occurs so that mitigation measures can be planned. An effective way of doing this is to use an eDNA based detection assay designed to pick up very small amounts of DNA that has originated from these animals. eDNA techniques have been developed for a number of at risk or invasive species which are otherwise difficult to detect via traditional methods such as survey or trapping, by the qPCR assay of eDNA extracted from water samples.

1.3 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); and *Segmentina nitida* Shining ram's-horn snail which is a rare and declining Section 41 species (NERC Act 2006) and a Ramsar criteria feature of Stodmarsh National Nature Reserve (Rees *et al.* 2021).

The development of an eDNA assay 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). In vitro validation involves the specificity, optimisation, and sensitivity of the assay. The specificity of an eDNA assay should be tested on DNA extracted from the target species and both closely related and co-occurring species (both geographically distinct species and distantly related species). Finally, in situ validation involves surveying sites with known presence/absence of the target species (Goldberg *et al.* 2016). An assay will be successful if the results of the eDNA assay and traditional surveying concur.

1.4 Aims and Objectives

The overall aim of this study was to develop an eDNA assay for the identification of *C. chinensis*. This assay could then be applied to samples taken from ditches located at the Pevensey Levels, Sussex, which could be invaluable for determining both the extent of the snails within the current watercourses and for measuring the success of any eradication or mitigation operation over subsequent years.

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 the most recent traditional survey. 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. Materials and Methods

2.1 Sample Collection

36 ditch water samples were collected by Natural England and ADAS staff at Pevensey Levels between the 10th and 12th August 2021 (Figure 2), detailed methods can be found in Appendix 2A. In addition 32 ditch samples were collected outside the known range of *C. chinensis* (Stodmarsh, Kent and Leicestershire) and were used as controls. These were collected by Natural England and ADAS staff between the 16th January and the 27th November 2020 and 10th January and the 4th February 2021 respectively. Sample information is shown in Tables 4 - 6 (Appendix 1).

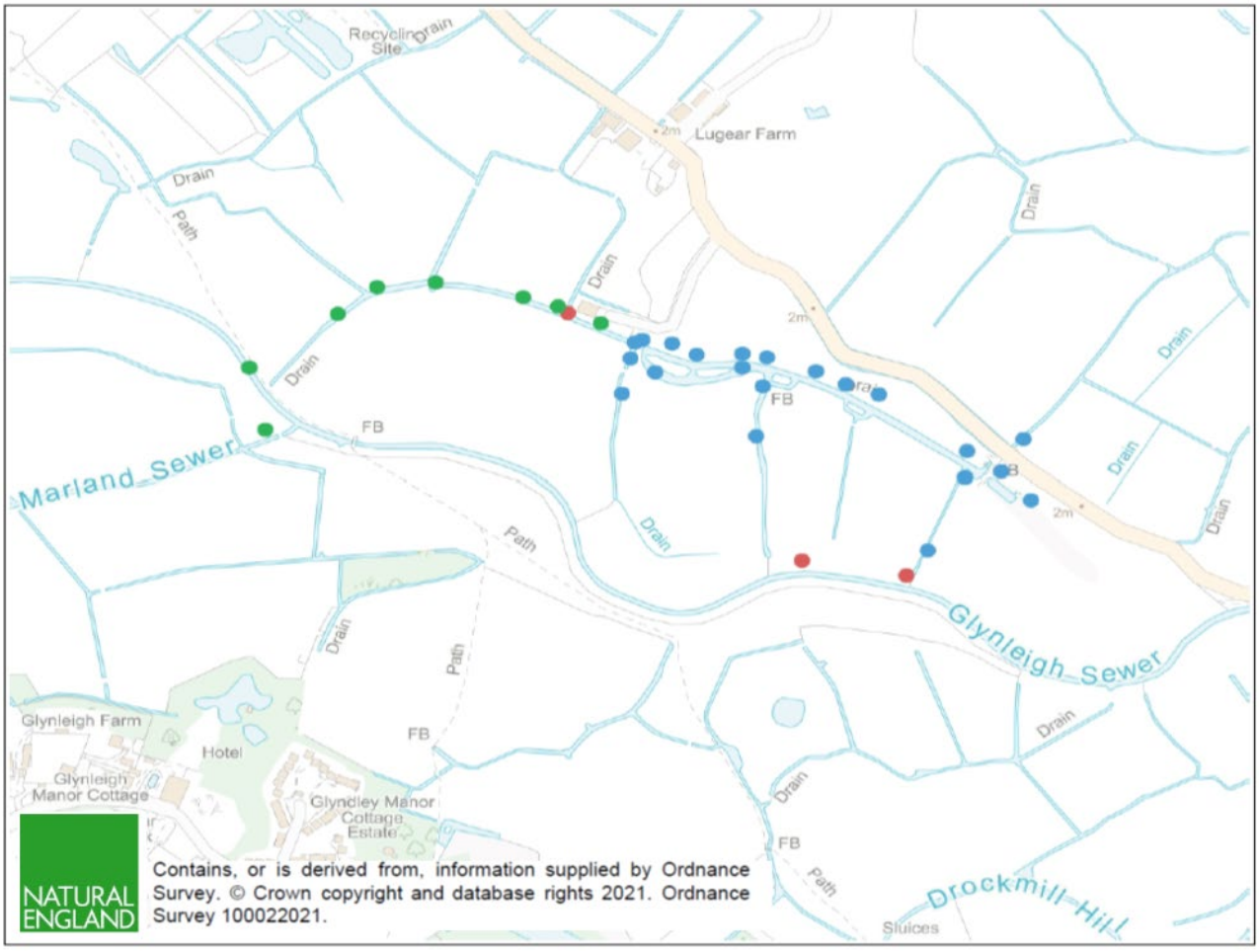

Figure 2. Pevensey Levels sampling locations. Blue dots denote samples collected on 10/08/2021; green dots denote samples collected on 11/08/2021; and red dots those taken on 12/08/2021.

2.2 Specimen Collection

Snails and molluscs were collected from ditches at Pevensey Levels by Natural England in August 2021 in a period of dry weather conditions with no recent rainfall (see Appendix 2B). Specimens were preserved in 95% ethanol prior to couriering to the ADAS

laboratories (Figure 3). The 95% ethanol preservative was replaced after 1 day and 1 week (Ben Price, personal communication).

2.2.1 Ethics Statement

Water samples and snail specimens were collected from several sites at Pevensey Levels and no institutional animal care and use committee (IACUC) or animal welfare protocol was required for their collection as generally invertebrates do not require approval for use (except cephalopods). The field collections did not involve endangered or protected species. None of the collections included herein were from National Parks or otherwise protected wilderness areas. All land was privately owned and was accessed with the permission of the land owners.

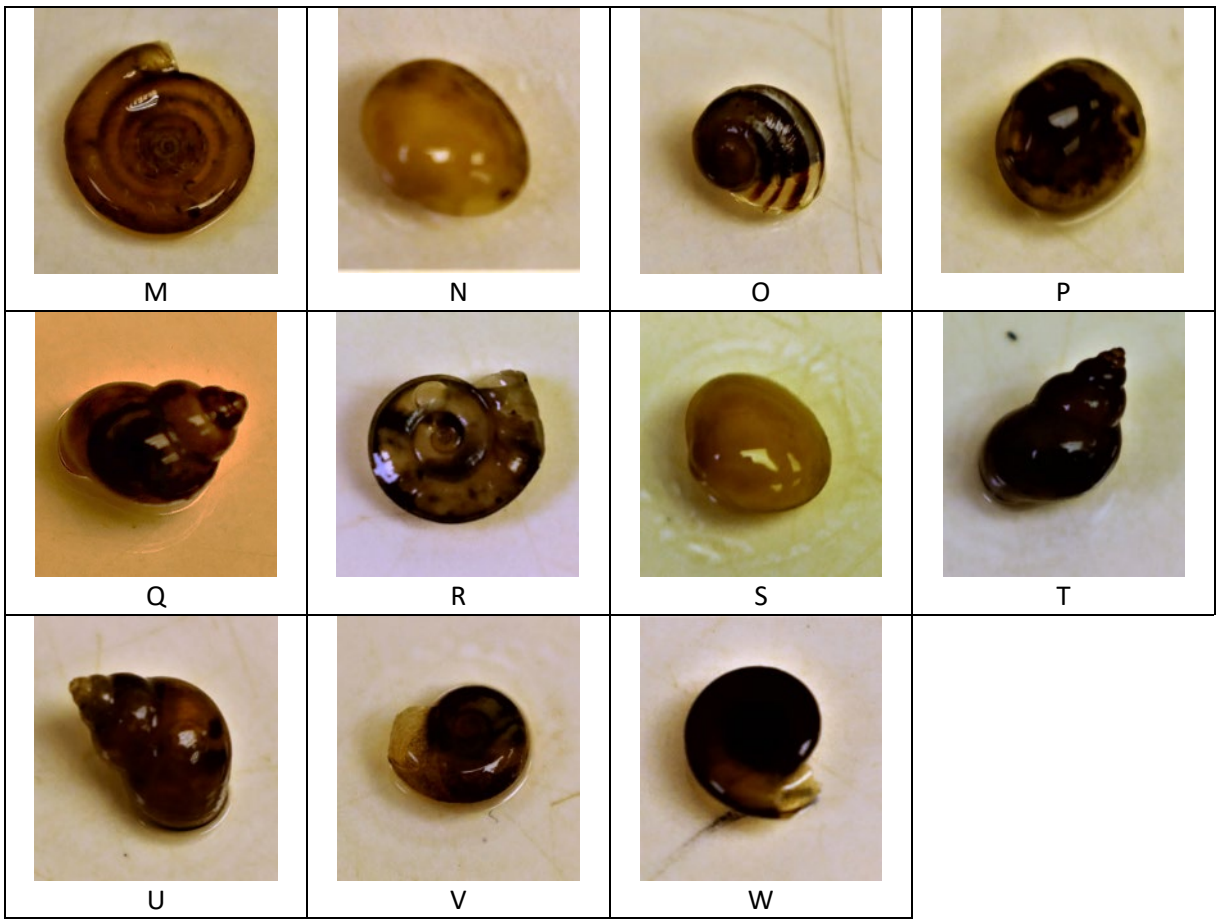

Figure 3. Photos 1-6 *C. chinensis* specimens, Photos A-W images of individual snail specimens isolated from samples taken by Natural England at Pevensey Levels August 2021. Photos taken by © Helen Rees (specimens provided by Natural England).

2.3 Laboratory Standard 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.

2.4 DNA Extraction and Quantification

DNA was extracted from the ditch water samples using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (Appendix 2C), with the exception that 720µL of ATL buffer was added to each sample, along with 40µL of PK. 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).

Each snail specimen was individually transferred to a clean, sterile petri dish and a photographic record made (Figure 3). For larger specimens a fresh sterile scalpel blade was used to remove a piece of tissue from each specimen and mash the tissue into a paste before placing into a sterile 1.5 mL Eppendorf tube. For smaller specimens the whole organism was transferred into a sterile 1.5 mL Eppendorf tube and using a fresh sterile Eppendorf pestle was ground into a paste. DNA was extracted from snail specimens using DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (Appendix 2D). 200µL ATL and 20µL PK buffer was added to each specimen and incubated for 1-2 hours at 56°C until completely digested. Final resuspension was in 100 or 200µL AE buffer for specimens (see Table 1 for volumes).

DNA extraction from specimens taken during Natural England project NECR373 (Stodmarsh, Kent) were extracted as above except that 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 dH_2O and then autoclaved at 121 ºC for 15-20 minutes.

Extracted DNA was quantified using the Qubit® dsDNA BR assay kit and Qubit 3.0 fluorimeter.

2.5 Specimen Identification PCR

All PCR set up was performed in a clean 'PCR room' within a UV sterilisable cabinet 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 mlCOIintF/jgHCO2198 primer combination (Appendix 2G). These primers amplify a fragment of the Cytochrome 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.

2.6 Species Specific Primer Design and Validation

2.6.1 In silico Analysis

To design primers specific to *C. chinensis* the DNA sequences for the cytochrome oxidase 1 (COI) gene for *C. chinensis* and other closely related and/or co-occurring snails commonly found at the Pevensey Levels and the wider United Kingdom were downloaded from BOLD (accessed 8th-10th September 2021). This list of species comprised the 54 species of freshwater snails known to occur in the UK, both native and non-native

(Rowson *et al.* 2021). Sequences were aligned using BioEdit version 7.2.5 (alignment for *C. chinensis* shown in Appendix 3). A species-specific quantitative PCR (qPCR) assay for *C. chinensis* was designed from COI sequences stored in NCBI using PrimerBLAST with default settings. Ten potential primer/probe combinations were generated (Appendix 3, Table 9). 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).

2.6.2 In vitro Analysis

Four potential primer/probe combinations (4, 6, 8, and 9) were tested firstly on DNA extracted from *C. chinensis* followed by sixteen other 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).

The specificity of the four primer/probe combinations was confirmed, however only the most promising primer/probe combination (primer/probe combination 8) was optimised by independently varying final primer and probe concentrations (Wilcox et al, 2015). Additionally, once primer/probe combination 8 had been selected and optimised this was repeated with 12 replicates per *C. chinensis* or closely related/co-occurring species.

The sensitivity of the assay was tested by creating a seven-level standard curve dilution series. The standard curve was created by quantifying the DNA extracted from *C. chinensis* samples 2b, 4, and 6 on a Qubit Fluorometer (Thermo Fisher Scientific) and diluting the DNA to the desired concentrations (1ng/µl to $1x10^{-6}$ ng/µl) using the elution buffer provided in the DNeasy Blood and Tissue kit (Qiagen). Twelve replicates of each dilution were run using the optimised primer/probe concentration 8 to determine the standard curve slope and the limit of detection (LOD) and limit of quantification (LOQ). When the concentration of the DNA is high the cycle threshold (cycle at which amplification begins) will be lower than when the DNA concentration is low i.e. the higher the concentration of DNA the quicker amplification will commence (Figure 6). The cycle thresholds for each concentration of DNA were plotted on a base-10 semi-logarithmic graph versus the dilution factor and the data was fitted to a straight line (Figure 7) to give the PCR efficiency and R^2 values.

2.6.3 In situ Analysis

The optimised assay using primer pair 8 was used to determine the presence/absence of *C. chinensis* within the 36 ditch samples from the Pevensey Levels and the 30 ditch samples from outside of the known range of *C. chinensis* (Appendix 2H).

All samples were also tested for the presence of inhibitors that may interfere with the sensitive detection of *C. chinensis* eDNA by adding a known fragment of DNA (inhibition control) to the eDNA extract and performing a PCR specific to the inhibition control DNA. The eDNA sample is considered to contain inhibitors if the results of this PCR are outside of acceptable limits when compared with a similar reaction not containing the eDNA sample.

2.7 Coaster Analysis

COASTER: Confidence Assessment Tool for eDNA qPCR Results (Harper *et al.* 2021) was used here to verify the results of the qPCR assay developed for *C. chinensis* and to understand the confidence in the qPCR assay. Default settings were used except that: the number of plates which was set to 13; the Cq value for the limit of detection (set at 48.35); and the calculate LOD setting was set to zero for 'no' as a value was supplied.

COASTER was developed to meet Natural England's need to understand and interpret species-specific qPCR assay results. It enables a confidence level assessment to be made (high, medium, low) for the level of assay development and therefore determine how confident you can be in the accuracy of the results and whether target species DNA was present or not. The COASTER output report provides an interpretation of the results as either positive, negative, inconclusive, or tentative and then aids the user in interpreting tentative results as a true or false positive and includes whether further steps are required to confirm the results for example the collection of further samples.

3. Results

3.1 *C. chinensis* Manual Survey

The manual survey for *C. chinensis* carried out in March 2021 showed that *C. chinensis* was found at four sites within the Pevensey Levels (Figure 4). During August 2021 a further manual survey showed that *C. chinensis* had spread from site 12 up to site 15 (Figure 4). A close-up image of the site shows sites 8 to 13 re-numbered as 1-9 again illustrating the spread of *C. chinensis* (Figure 5).

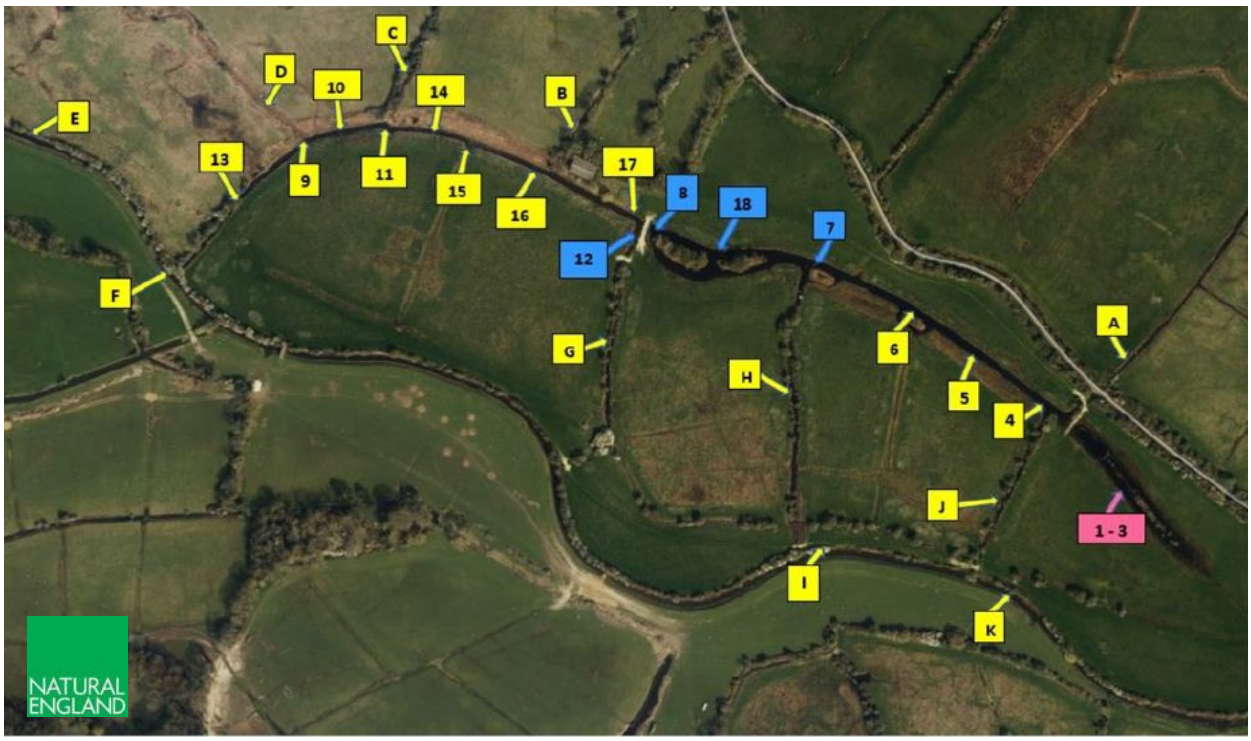

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Figure 4. Satellite image of ditch system at the Pevensey Levels showing sample locations in March 2021. Blue labels (7,8,12,18) show where *C. chinensis* was found and yellow where *C. chinensis* was not found. Pink labels (1-3) show sites with *Anisus vorticulus.*

APGB Aerial photography © Bluesky International Ltd/Getmapping PLC. PGA Aerial Photography licensed to Natural England for PGA, through Next Perspectives™. Permitted use: Natural England

Figure 5. Close up of area of ditch system at the Pevensey Levels where *C. chinensis* was shown to have spread by August 2021. Pink labels (1,2,3,9) show where *C. chinensis* was found to be present and yellow (4,5,6,7,8) where *C. chinensis* was not present.

3.2 DNA Extraction

DNA was extracted from individual snail specimens and the DNA quantified (Table 1 and 2). DNA was extracted in low concentrations from the 36 Pevensey Levels ditch water samples, the 22 Stodmarsh NNR ditch samples, and the 10 ditch samples from Leicestershire (Table 3 and 4). Most of the DNA concentrations from ditch samples outside the known range of *C. chinensis* were too low to quantify as well as two of the Stodmarsh NNR ditch samples. The DNA in these samples was too low to measure using the Qubit Fluorometer either due to poor DNA extraction from the filter or a lack of DNA within the water sample prior to filtration.

Table 1. DNA information per snail specimen collected from Pevensey Levels.

Sample ID	Elution Volume	DNA concentration (ng/ul)	Snail ID from DNA	Photograph identification matched (using Rowson *et al.* 2021)
1	200ul	14.3	*C. chinensis*	Yes
2	200ul	24.1	*C. chinensis*	Yes
3	200ul	1.51	*Sequencing not successful*	N/A
4	200ul	16.4	*C. chinensis laeta*	Yes
5	200ul	40.6	*C. chinensis laeta*	Yes
6	200ul	5.0	*C. chinensis laeta*	Yes
2b	200ul	27.4	*C. chinensis laeta*	Likely (newborn)
A	100ul	54.0	***Planorbis carinatus***	Likely
B	100ul	59.0	***Sphaerium nucleus***	Likely
C *	100ul	10.7	***Bithynia tentaculata***	Yes
D *	100ul	55.0	***Stagnicola palustris***	Likely
E *	200ul	17.6	*Manacha cantiana*	Yes (land snail)
F	200ul	39.0	*C. chinensis laeta*	No
G	200ul	28.0	***Planorbis corneus***	Likely

Sample ID	Elution Volume	DNA concentration (ng/ul)	Snail ID from DNA	Photograph identification matched (using Rowson *et al.* 2021)
H	100ul	25.0	PCR amplification unsuccessful/no sequence	N/A
I	100ul	27.1	Sequencing not successful, looks mixed	N/A
J	100ul	12.4	***Anisus vortex***	Likely
K	100ul	3.91	Sequencing not successful	N/A
L *	100ul	31.3	***Bithynia tentaculata***	Yes
M	100ul	35.6	*C. chinensis laeta*	No, looks like a ramshorn snail
N	100ul	118.0	***Sphaerium nucleus***	Likely
O *	100ul	150.0	*Helix aspersa*	Possibly, juvenile (land snail)
P	100ul	4.88	PCR amplification unsuccessful/no sequence	N/A
Q	100ul	36.1	***Bithynia tentaculata***	Yes
R	100ul	3.78	*Psectrotanypus varius*	No, Chironimidae (non-biting midge)
S *	100ul	54.0	***Sphaerium nucleus***	Likely
T	100ul	90.0	Sequencing not successful	N/A
U	100ul	45.1	***Bithynia tentaculata***	Yes
V	100ul	44.6	***Planorbis carinatus***	Yes
W	100ul	42.3	***Planorbis carinatus***	Yes

* Denotes those samples used for primer/probe specificity testing. Native snails highlighted in bold were confirmed in the ditch by manual surveys (Willing 2021a).

Table 2. DNA information per snail species collected during Natural England project NERC373 at Stodmarsh NNR, Kent.

Snail ID	Sample ID	Eluted volume (ul)	DNA (ng/ul)	Pooled or individual	Confirmed sequence ID
Gyraulus crista	1	200	4.68	Pool	Yes
Planorbis planorbis	2	200	2.81	Individual	Yes
Bathyomphalus contortus	3a	50	10.6	Individual	Yes
Hippeutis complanatus	4	200	3.58	Pool	Yes
Planorbis carinatus	5b	50	38.6	Individual	Yes
Planorbarius corneus	6a	50	29.7	Individual	Yes
Segmentina nitida	7	200	8.52	Pool	Yes
Anisus vortex	8a	50	33.1	Individual	Yes
Valvata cristata	9b	50	30.9	Pool	Yes
Gyraulus albus	10	200	2.42	Individual	Yes

3.3 Specimen Identification

Specimens were identified by Sanger sequencing with identities of at least 98% over the length of the sequence (Table 1). The exception to this was *Valvata cristata* where the sequences only showed 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 C. chinensis PCR

A qPCR-based assay was developed based on primers designed against the *C. chinensis* COI sequences found within BOLD. Of 10 potential primer/probe combinations (Appendix 3, Table 9), 4 were shown *in silico* to amplify only *C. chinensis* and *C. cathayensis* (primer/probe combinations 4, 6, 8, and 9). Of these 4 primer/probe combinations, all were found to be specific to *C. chinensis* (we were unable to source *C. cathayensis* for testing),

however, primer/probe combination 8 was the most consistent and reproducible so was chosen for optimisation and further use. Positive amplicons were sequenced to confirm species identity.

Optimal primer concentrations for primer/probe combination 8 were: 0.2 µmol/L for the forward primer, 0.8 µmol/L for the reverse, and 0.5 µmol/L for the probe.

The limit of detection (LOD) and limit of quantification (LOQ) of primer/probe combination 8 were both found to be $1x10^{-4}$ ng/µL (Figure 6 and 7). 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). The PCR efficiency was 95% indicating that the assay works close to optimally (100% is optimal, with 90-110% considered to be the optimised assay range) with an R^2 value of 0.834 (the ideal being over 0.985) which indicates that there may have been pipetting errors or the assay range may be inefficient (Rodgers-Broadway and Karteris, 2015).

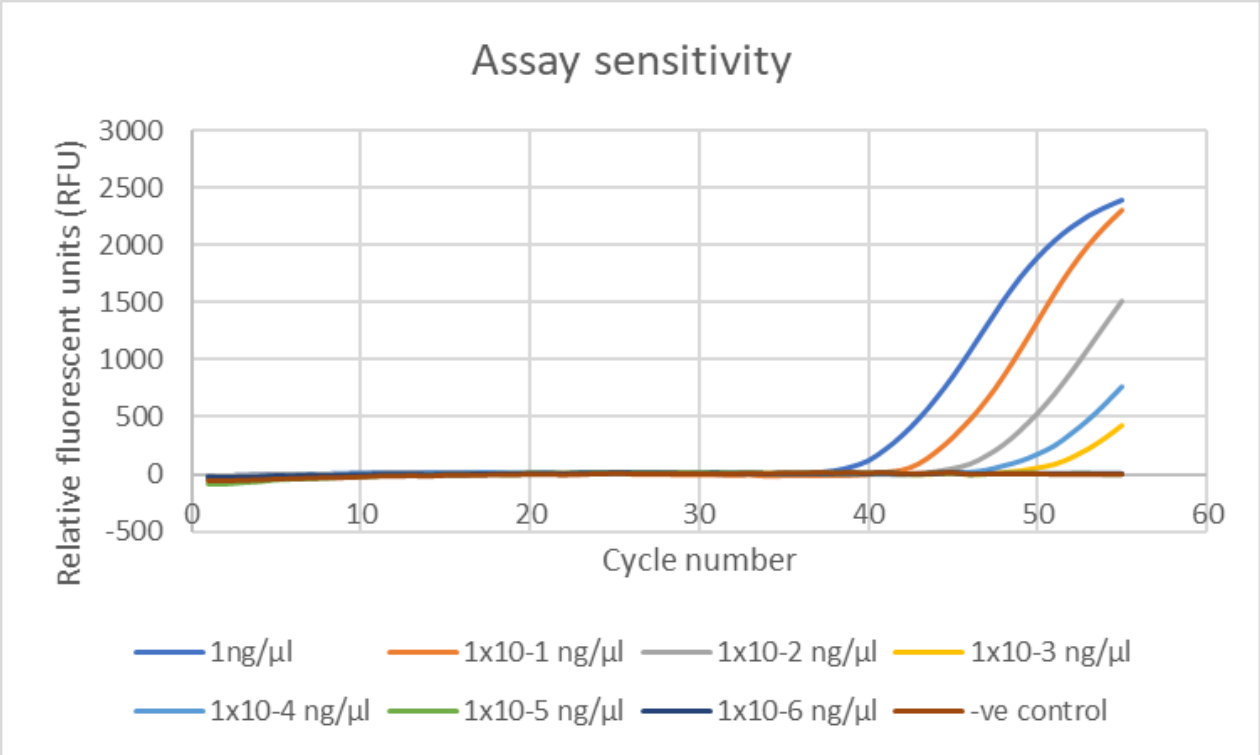

Figure 6. Real-time PCR result showing a seven-level standard curve for *C. chinensis* DNA. Average values from each set of 12 replicates are shown i.e. one line per DNA dilution. Higher concentrations of DNA commence amplification before lower concentrations.

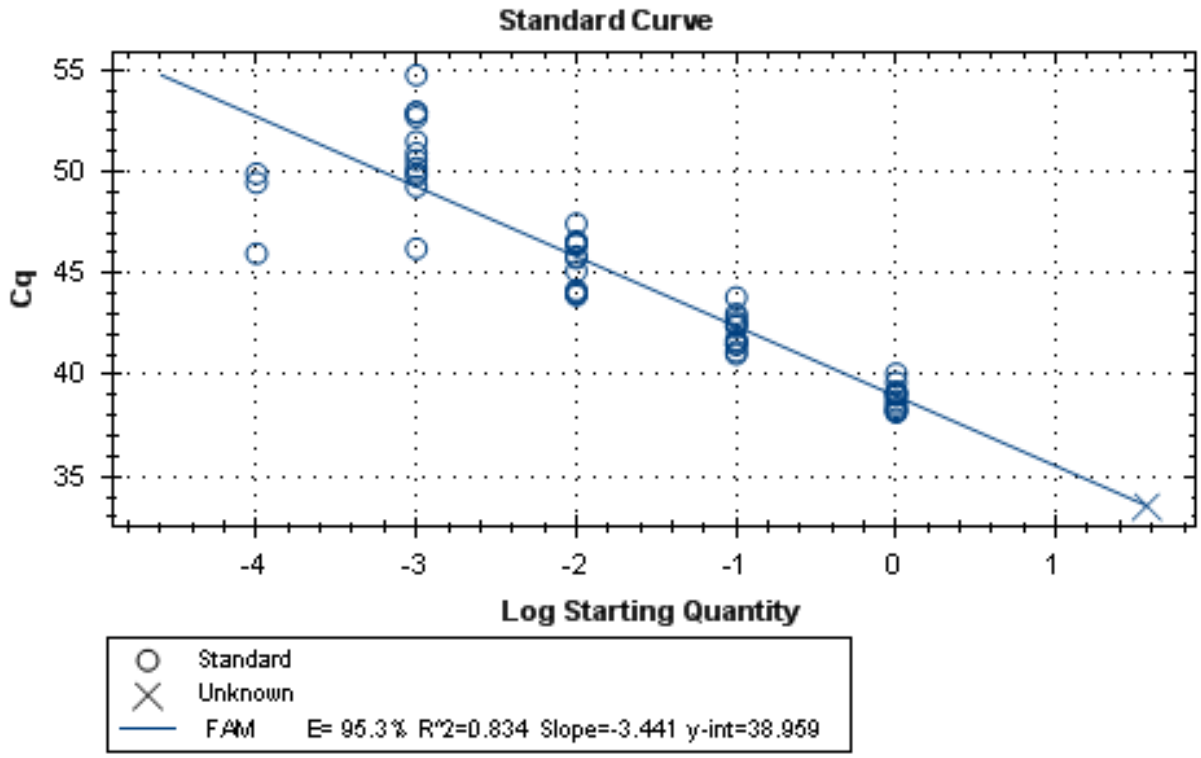

Figure 7. Standard curve obtained from seven-level DNA dilution series. Semi-logarithmic graph of the cycle threshold for each dilution of DNA versus dilution factor.

All Pevensey Levels ditch water samples and ditch samples from outside the known range of *C. chinensis* were subjected to the optimised assay, with 16 water samples from the Pevensey Levels being positive for *C. chinensis* DNA (Figure 8). All remaining samples were negative for *C. chinensis* DNA, therefore other than those ditches where *C. chinensis* was found by manual survey, *C. chinensis* is likely to be absent from these ditches (Tables 3 and 4).

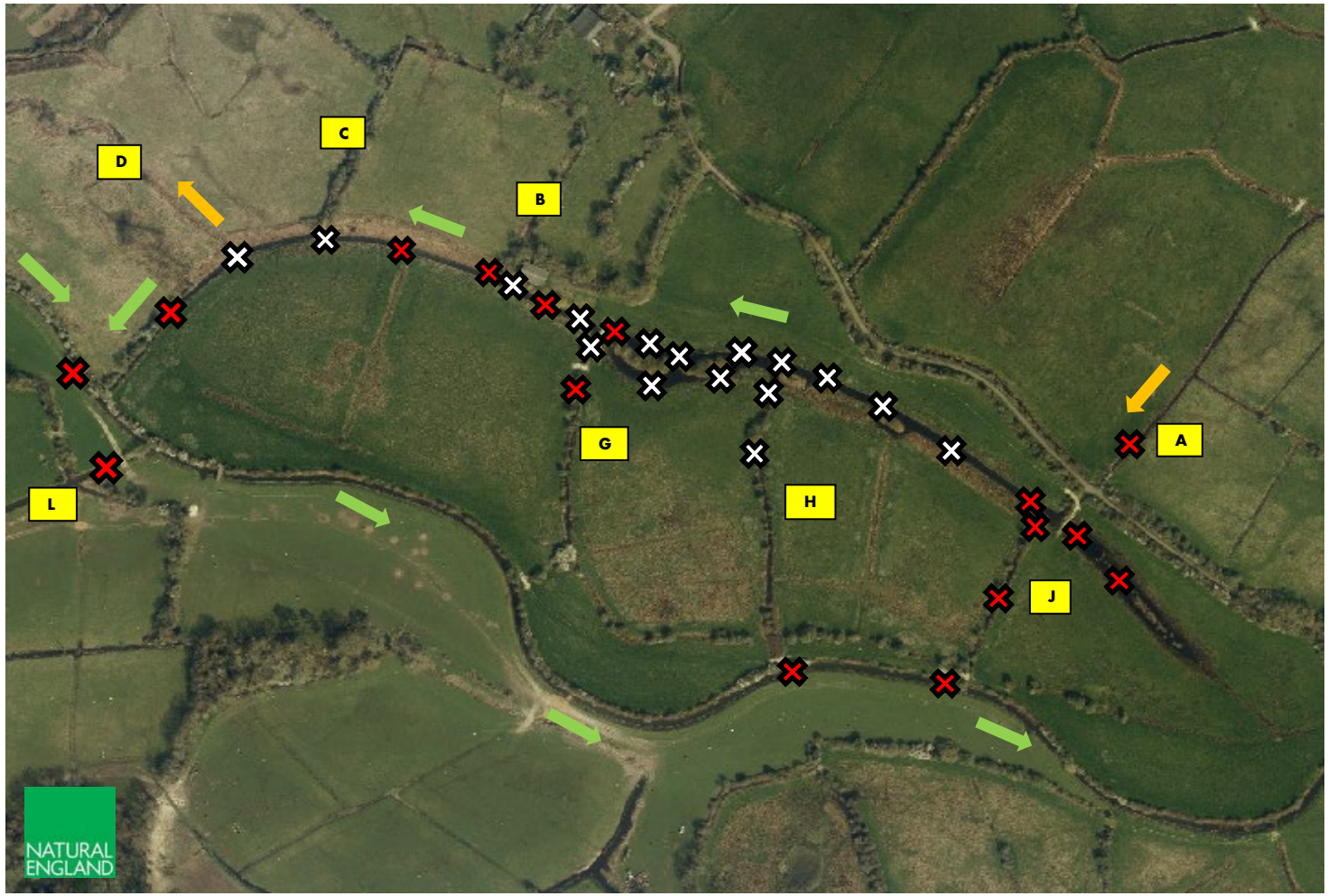

Figure 8. Satellite image of ditch system at Pevensey Levels showing *C. chinensis* DNA results. Red and White crosses mark the eDNA sampling sites and whether negative or positive for *C. chinensis* DNA. Green arrows show direction of flow of main ditch; Orange arrows show direction of flow of side ditches. Side ditches labelled – yellow boxes.

Table 3. Pevensey Levels ditch water sample information.

Sample ID	Volume of water filtered (ml)	DNA (ng/µL)	*C. chinensis* species-specific qPCR result	COASTER results	2020-2021 Manual Survey result
EASTERN SECTION DITCH					
1-3 pool - 1	230	3.71	0/12	Negative	Not found
1-3 pool - 2	150; 150	0.88	0/12	Negative	Not found
4	130	2.74	0/12	Negative	Not found
5	250	2.78	4/12	Positive	Not found
6	260	4.68	11/12	Positive	Not found
6-7	400	4.12	11/12	Positive	Present
7	100; 100	5.74	12/12	Positive	Present
8	500	5.29	0/12	Negative	Present
8-18 lower side	500	13.50	12/12	Positive	Present
8-18 upper side	200	21.40	12/12	Positive	Present
18	300	2.88	12/12	Positive	Present
18-7 lower	400	13.30	12/12	Positive	Present
18-7 upper *	250	23.30	1/12, 3/12, 4/12	Tentative, Positive, Positive	Present
12	120; 100	4.61	11/12	Positive	Present
WESTERN SECTION DITCH					
17-16	170; 170	21.80	0/12	Negative	Present
16	150; 150	11.20	0/12	Negative	Present
End of barn at oak tree* (near 16)	150	4.66	1/12, 0/12, 2/12	Tentative, Negative, Positive	Present

Sample ID	Volume of water filtered (ml)	DNA (ng/µL)	*C. chinensis* species-specific qPCR result	COASTER results	2020-2021 Manual Survey result
15	90; 80	8.88	0/12	Tentative	Present
11 opposite ditch C*	250	4.48	1/12, 3/12, 2/12	Tentative, Positive, Positive	Not found
9 opposite ditch D *	300	3.01	1/12, 4/12, 4/12	Tentative, Positive, Positive	Not found
13 opposite pond in field	140; 140	10.90	0/12	Negative, Negative, Negative	Not found
SIDE DITCHES					
4-J (between 4 and J)	350	2.77	0/12	Negative	Not found
Ditch J	265	1.49	0/12	Negative	Not found
Ditch A	230	4.95	0/12	Negative	Not found
Ditch G lower *	70; 80	5.41	2/12, 4/12, 5/12	Positive, Positive, Positive	Present
Ditch G upper above culvert	290	7.08	0/12	Negative	Not found
Ditch H lower section (near 7)	250	2.18	4/12	Positive	Present
Ditch H higher section *	280	15.60	2/12, 2/12, 3/12	Positive, Positive, Positive	Not found
WIDER DITCH NETWORK					
Down Sewer Main	300	1.37	0/12	Negative	N/S
Down Sewer * small ditch opposite	250	5.41	0/12	Negative	N/S
Horse Eye Sewer	250	2.11	0/12	Negative	N/S
Rickney Sewer	120; 150	2.54	0/12	Negative	N/S

Sample ID	Volume of water filtered (ml)	DNA (ng/µL)	*C. chinensis* species-specific qPCR result	COASTER results	2020-2021 Manual Survey result
Site L (Marland Sewer)	260	2.89	0/12	Negative	N/S
GLYNLEIGH LEVEL SEWER					
Main sewer F	400	1.02	0/12	Negative	Not found
Main sewer K	300	2.01	0/12	Negative	Not found
Main sewer I	300	3.58	0/12	Negative	Not found

* Samples that were low positives on first qPCR amplification and repeated twice more to confirm positivity. N/S – not surveyed

Table 4. Stodmarsh NNR and Leicestershire ditch water sample information.

Sample ID	DNA (ng/µL)	*C. chinensis* species-specific qPCR result	COASTER results
1	<1 ng/µL	0/12	Negative
2	<1 ng/µL	0/12	Negative
3	<1 ng/µL	0/12	Negative
4	<1 ng/µL	0/12	Negative
5	<1 ng/µL	0/12	Negative
D1	<1 ng/µL	0/12	Negative
D2	<1 ng/µL	0/12	Negative
Top	1.7	0/12	Negative
LHS	1.34	0/12	Negative
RHS	<1 ng/µL	0/12	Negative
34	2.14	0/12	Negative
42	2.95	0/12	Negative
44	1.71	0/12	Negative
58	11.1	0/12	Negative
60	3.96	0/12	Negative
62	2.11	0/12	Negative
65	3.78	0/12	Negative
70	0.84	0/12	Negative
92	4.88	0/12	Negative
98	4.36	0/12	Negative
106	1.66	0/12	Negative
108	2.32	0/12	Negative

Sample ID	DNA (ng/µL)	*C. chinensis* species-specific qPCR result	COASTER results
115	2.06	0/12	Negative
131	1.08	0/12	Negative
135	1.46	0/12	Negative
136	<1 ng/µL	0/12	Inconclusive
146	<1 ng/µL	0/12	Inconclusive
153	1.89	0/12	Negative
155	<1 ng/µL	0/12	Negative
161	1.35	0/12	Negative

3.5 Coaster

The results of the Coaster analysis agree with the eDNA analysis results in that all samples classed as positive by qPCR analysis were found to be positive or tentative by Coaster analysis. Those that were tentative were 1/12 positive amplifications as the Coaster default settings require 2/12 positive replicates to call a sample positive. All qPCR negatives were recorded as negative by Coaster except samples 136 and 146 from Stodmarsh NNR which were called as indeterminate due to complete inhibition of the inhibition control test carried out on all the samples. The *C. chinensis* qPCR was negative in these two samples, however, Coaster assumes that a sample is indeterminate if the inhibition control qPCR results are not within certain limits.

4. Discussion

4.1 Overview

This work was undertaken to develop a single-species qPCR assay to monitor the presence of *C. chinensis* during ditch water sampling of the Pevensey Levels, Sussex. Wherever possible this project followed previous examples of similar qPCR and recommended validation of qPCR assays that have been published in peer reviewed articles (Ficetola *et al.* 2008; Thalinger *et al.* 2021). Additional information was found within Natural England commissioned report number 373 (Rees *et al.* 2021).

4.2 Single-species Assay

A species-specific real-time PCR assay targeting a 204bp fragment of the COI region of *C. chinensis* was developed and optimised for determining the presence of *C. chinensis* based on the detection of eDNA in ditch water samples. COI region is the chosen target for sequencing identification 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 designed in this study successfully distinguished between *C. chinensis* and other co-occurring species in that no cross-amplification was detected either *in silico* or *in vitro*. The only exception to this was *C. cathayensis* a very closely related species found to cross-amplify during *in silico* testing which would be likely to cross-amplify if tested with this assay. *C. chinensis* and *C. cathayensis* are distinguishable via various anatomical characteristics (Lu *et al.* 2014) but have very similar COI sequences which prevented the development of species-specific primers. We were unable to source DNA from this species to test our qPCR assay *in vitro*, however, as the species is not present in the UK this will not affect the utility of the qPCR assay designed. If *C. cathayensis* was introduced into the UK this assay may be suitable for its detection and any positive amplifications could be confirmed by sequencing and comparing to those of *C. chinensis* and *C. cathayensis* that are accessible through online databases.

The 2021 manual surveys carried out by Natural England (Willing 2021a, b) confirmed the continued presence of recruiting *C. chinensis (sites 12, 8, 18, 7)* but surprisingly found that it does not seem to have colonised additional areas of the eastern section of the ditch since 2019 (beyond site 7). The eDNA results however, showed that the snail has now colonised to site 5 but not to site 4.

Positive amplification of *C. chinensis* was found in 16 of the ditch samples, 11 of which were from samples taken within the known area of *C. chinensis* colonisation. The remaining 5 samples were from ditches where *C. chinensis* was not found during manual survey and where there are also no historic records (the species being recently introduced). The amplification ‘score’ for these 5 samples ranged from 1/12 to 11/12 with those scoring ≤3/12 being re-tested twice more to confirm their positivity. It should be noted that for great crested newts in the UK the sanctioned eDNA test (Biggs *et al.*2014) considers anything ≥1/12 to be a positive and that the low scores found in this study are likely to be positives. The low PCR scores are likely to represent extremely low levels of eDNA (possibly from very small populations of animals) which are around the limit of detection of the PCR test. When very low concentrations of DNA are present in a sample, stochastic or random sampling effects can occur in the early cycles of the PCR amplification. At limiting numbers of target DNA molecules in the sample, the PCR primers may not consistently bind to the DNA molecules present in the amplification reaction. This can manifest as a fluctuation of results between replicate analyses i.e. as seen here with replicate analyses of samples varying between 1/12 and 4/12 (Table 4). Stochastic effects cannot be avoided when very small amounts of DNA are present therefore the use of an

‘enhanced interrogation’ (Butler & Hill 2010) as carried out here involving replicate testing and increasing the assay sensitivity by using a high number of PCR cycles allows us to limit the impact of stochastic variation when interpreting the results. When samples scoring ≤ 3/12 were re-tested, 5 out of the 6 samples scoring were positive for *C. chinensis* DNA by both qPCR and Coaster analysis and the sixth was found to be positive once by qPCR and Coaster analysis and negative once by qPCR and Coaster analysis upon re-testing. The finding of the qPCR positive samples at sites where no *C. chinensis* were found during manual survey should be confirmed by further survey of the site.

Ditch sample site 8 was taken in an area known to be colonised by *C. chinensis* and was found to be negative by qPCR. This was not expected as the sample was taken on the opposite side of the ditch from a highly positive (12/12) sample. Less water was filtered for ditch sample 8 as particulates within the water sample made it impossible to filter a larger volume of water. However, as other samples with lower amounts of filtered water also gave positive qPCR results this does not fully explain why this sample failed to amplify. The presence of *C. chinensis* at site 8 could have been lower than on the opposite bank resulting in a lower concentration of DNA that was below the limit of detection. The concentration of eDNA in a water body will depend on the rate of production versus how long it persists in the environment (Dejean *et al.* 2011). The rate of eDNA production for a species will depend on many factors including: the number of individuals present, their physiology and metabolism; and temperature (Treguier *et al.* 2014). eDNA degradation is affected 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). It is possible that the *C. chinensis* favour certain parts of the ditches in addition to their preference for the marginal areas during the summer months (favouring the deeper more central areas outside of this time) and sample 8 may just not have been taken close enough to any *C. chinensis* that were present in and around this sampling site.

Ditch samples 15, 16, and 17-16 were all found to be negative by qPCR analysis, however, during manual survey in August 2021 *C. chinensis* was found at site 15 (site 3 on Figure 5). At these sites a thorough search was performed and only small numbers (1 or 2) of *C. chinensis* were found compared with manual survey of the eastern side of the ditch where many specimens were found with less survey effort (Measures, G, personal communication). This suggests that although there has been spread to the Western side of the ditch their abundance is still low compared with the Eastern side of the ditch. Where abundance is low, eDNA concentrations are often also low which could help to explain why these samples were found to be qPCR negative. The complication with these results is that further downstream samples 9 and 11 were found to be positive by qPCR albeit at very low positivity scores (1/12 at both). It is unlikely that the slow flow of the water in the ditch from East to West (site 4 to 9) caused the low qPCR positivity in samples 9 and 11, as if this was the case you would expect 15, 16 and 17-16 to also have low positivity. Coaster analysis found samples 9 and 11 to be tentative positives and suggested that sequencing or further assay validation be performed. However, when the qPCR analysis of these sites was repeated twice more positivity ranged between 2/12 to 4/12 with Coaster analysis scoring these samples as positive i.e. the species DNA was present. It is possible that the stochastic nature of PCR at these low levels of target eDNA could explain

the negative results seen for samples 15, 16 and 17-16 and we would recommend that further samples are taken, and repeated analysis performed whenever the number of positive replicates is ≤ 3/12.

It is unknown whether the time of year that the samples are taken in relation to the ecology and life cycle of the snail play a part in the detection of *C. chinensis*. *C. chinensis* may 'shed' larger amounts of DNA into their environment at certain times of year. It seems likely that higher amounts of DNA will be shed into the environment during May to August when young are born, it is known that for example great crested newts shed different amounts of DNA 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 that when water bodies contain a large population of young crayfish that eDNA detection is more efficient (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 *C. chinensis* 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. The qPCR assay for *C. chinensis* developed in this study detected DNA where no animals were found. This could therefore enhance management decisions when it comes to planning ditch management at the Pevensey Levels for example in the placement of coffer dams to reduce the spread of *C. chinensis* within the ditch system. Coffer dams were introduced at three sites to prevent further spread at Pevensey Levels prior to the completion of the eDNA study and the results of the eDNA analysis suggest that two of these may not be beyond the current range of *C. chinensis*.

5. Recommendations for further work

- Confirmation of the presence of *C. chinensis* by eDNA analysis at sample sites 8, 15, 16, and 17-16. Several water samples should be taken in and around these sites with greater sampling effort to confirm the known presence of *C. chinensis*.
- Manual survey should be conducted at sample sites 9, 10 (approximate site of coffer dam) and 11, and side ditches G and H to confirm presence/absence of *C. chinensis*. This should be carried out during peak activity of the snails during their breeding season to maximise the potential of finding their presence. This manual survey could be carried out in conjunction with eDNA analysis with greater sampling effort to inform or pinpoint where the manual survey effort should be.
- Further survey of site 4 is recommended to ensure that *C. chinensis* has not yet reached this point again during snail peak activity. It may be necessary to block off sites 1-3 and 4 so that *C. chinensis* cannot spread into sites 1-3 which contains *Anisus vorticulus* and *Segmentina nitida*, the former being a rare water snail listed as vulnerable in the latest UK status review (Seddon *et al.* 2014) and latter of which has been included in the UK Biodiversity Action Plan as a priority species following a rapid decline in numbers.

- Further investigate habitat suitability and water flows/depth of the side ditches (B, C, D) and consider installation of additional coffer dams to limit the spread of *C. chinensis*. The implications of their long-term use will need to be considered as there is a concern over the possibility of the site flooding in the winter and whether water levels can be managed long-term.

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Appendices

Appendix 1. Sample/Specimen Information

Table 5. Pevensey Levels ditch water samples.

Sample ID	GPS Location	Sampler	Collection date/time	Volume filtered	Turbidity of sample	Site conditions
EASTERN SECTION DITCH						
1-3 pool - 1	TQ 61255 07046	HR	10/08/2021 13.30	230ml	medium	sunny/breeze
1-3 pool - 2	TQ 61283 07016	HR	10/08/2021 13.50	1-150ml 2-150ml	high	sunny/breeze
4	TQ 61223 07068	GM	10/08/2021 13.25	130ml	high	sunny/breeze
5	TQ 61140 07129	DC	10/08/2021 14.00	250ml	low	sunny/breeze
6	TQ 61109 07140	GM	10/08/2021 15.00	260ml	medium	sunny/breeze
6-7#	TQ 61081 07154	HR	10/08/2021 15.30	400ml	low	sunny/breeze
7	TQ 61035 07169	DC	10/08/2021 11.00	1-100ml 2-100ml	high	sunny/breeze
8	TQ 60918 07188	GM	10/08/2021 09.30	500ml	low	cloudy/breeze
8-18 lower side	TQ 60930 07153	HR	10/08/2021 10.15	500ml	medium	cloudy/breeze
8-18 upper side	TQ 60946 07184	GM	10/08/2021 10.10	200ml	medium, difficult access	cloudy/breeze
18	TQ 60969 07172	DC	10/08/2021 10.25	300ml	medium	sunny/breeze
18-7 lower*	TQ 61012 07158	HR	10/08/2021 10.45	400ml	low	cloudy/breeze
18-7 upper	TQ 61012 07173	GM	10/08/2021 10.45	250ml	medium	sunny/breeze
12	TQ 60911 07185	HR	10/08/2021 09.30	1-118ml 2-100ml	medium	cloudy/breeze
WESTERN SECTION DITCH						
17-16	TQ60879 07206	HS	11/08/2021 09.25	1-170ml 2-170ml	medium, slight colouration	cloudy
16	TQ60839 07224	GM	11/08/2021 09.55	1-150ml 2-150ml	low	cloudy/sunny

Sample ID	GPS Location	Sampler	Collection date/time	Volume filtered	Turbidity of sample	Site conditions
End of barn at oak tree* (near 6)	TQ60848 07217/ 259	GM	12/08/2021 12.45	150ml	med, lots of dick weed	sunny intervals, warm
15	TQ60806 07234	HS	11/08/2021 10.20	1- 90ml 2- 80ml	high, pink colouration	sunny intervals, warm
11 opposite ditch C	TQ60724 07250/ 244	HS	11/08/2021 10.50	250ml	low	sunny, warm
9 opposite ditch D	TQ60669 07245	HS	11/08/2021 11.25	300ml	low	sunny, some cloud
13 opposite pond in field	TQ60632 07216	GM	11/08/2021 11.35	1-140ml 2-140ml	medium, ditch scrubbed over, reeds, difficult access, some colouration	sunny, some cloud
SIDE DITCHES						
4-J (between 4 and J)	TQ 61221 07040	GM	10/08/2021 14.50	350ml	low	sunny/breeze
Ditch J	TQ 61186 06962	HR	10/08/2021 15.00	265ml	medium	sunny/breeze
Ditch A	TQ 61435 06975	DC	10/08/2021 14.10	230ml	medium	sunny/breeze
Ditch G lower	TQ 60907 07168	GM	10/08/2021 12.10	1-70ml 2-80ml	high, very fine sediment	sunny/breeze
Ditch G upper above culvert	TQ 60899 07130	DC	10/08/2021 12.15	290ml	medium	sunny/breeze
Ditch H lower section (near 7)	TQ 61031 07138	GM	10/08/2021 11.40	250ml	high	sunny/breeze
Ditch H higher section	TQ 61025 07084	HR	10/08/2021 11.50	280ml	medium	sunny/breeze very vegetated
WIDER DITCH NETWORK						
Down Sewer Main	TQ62291 07329	GM	12/08/2021 09.20	300ml	low, ditch recently cleared of vegetation	cloudy
Down Sewer - small ditch opposite	TQ62350 07281	GM	12/08/2021 08.50	250ml	slight colouration, algae, floating pennywort present, medium	cloudy, warm
Horse Eye Sewer	TQ62868 07974	GM	12/08/2021 10.30	250ml	medium, slight colouration	floating pennywort, algae, medium

Sample ID	GPS Location	Sampler	Collection date/time	Volume filtered	Turbidity of sample	Site conditions
Rickney Sewer	TQ62794 07932	GM	12/08/2021 09.55	1-120ml 2-150ml	high, algae	floating pennywort dominant, cloudy
Site L (Marland Sewer)	TQ60564 07091/ 247	GM	11/08/2021 12.20	260ml	low, some colouration	sunny
GLYNLEIGH LEVEL SEWER						
Main sewer F	TQ60549 07158/ 248	HS	11/08/2021 12.20	400ml	low, clear	sunny
Main sewer K	TQ61166 06935/ 257	GM	12/08/2021 12.00	300ml	low, tall reed fringe, difficult access	cloudy, overcast
Main sewer I	TQ61068 069581/ 258	GM	12/08/2021 12.20	300ml	low, some colouration, difficult access, scrub, reed fringed	sunny intervals, warm

**C. chinensis* specimens found, [#]*C. chinensis* shell found

Table 6 Leicestershire ditch water samples.

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 7. Stodmarsh National Nature Reserve ditch water 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
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
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

Sample ID	Collection date/time	Sampler	GPS Location	Volume filtered	Sample Condition	Site conditions
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 8. Pevensey Levels ditch photos, August 2021 © Helen Rees and Gavin Measures (ADAS, NE respectively).

 Pool – Site 1-3	 Site 4 (Ditch J)
 Site 4 looking to site 5	 Site 6
 Site 7	 Site 8

Site 8-18 upper side

Site 8-18 lower side

Site 10

Site 11 (opposite site C)

Site 12 (looking to site 17)

Site 13 (opposite pond in field)

Site 16

End of barn at oak tree (between 16 and 17)

Site 17-16

Site 18 looking to lower side between two islands

Site 18-7 (lower side)

Site 18-7 (lower side looking to upper side)

Ditch A

Main Sewer F

Ditch G (lower section) looking to site 12

Ditch G (upper section above culvert)

Ditch H (lower section near site 7)

Ditch H (upper section)

Main sewer I

Ditch J (upper)

Site 4-J (between site 4 and J)

Main sewer K

Site L

Main Down Sewer

Small site opposite main Down Sewer

Rickney Sewer

Horse Eye Sewer

Appendix 2. Detailed Materials and Methods

A. Sampling Methodology – Collection of Water Samples

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.

B. Sampling Methodology – Collection of Invertebrate Specimens

At each survey location in the infested ditch, search for the snail entailed:

1. Initial visual searches of ditch margins for evidence of the snails which appear to favour shallow poached edges.
2. For shallow and deeper water sediments (the snails are on the bottom) a framed survey net fitted with a 2mm mesh (Figure 9). This allowed rapid exit of ditch sediments, but retention of even the smallest newly born *C. chinensis.*
3. Sediment samples were washed through a large (30 cm diameter) course 2-tier (10mm / 2mm) sieve-nest, again to allow large samples to be processed quickly and with the retention of even juvenile *C. chinensis.*

4. All captured *C. chinensis* were retained and stored in 95% ethanol for later laboratory analysis.

Figure 9. 10mm / 2mm grade 30cm sieve nest and 2.5mm mesh net for sampling for *C. chinensis* (© Dr Martin Willing)

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. DNA Purification

Nucleospin® Gel and PCR Cleanup (Machery-Nagel)

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.

G. Specimen Identification PCR

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

a. 3 µL of extracted template DNA at $1x10^{-3}$ ng/µl,
b. 2.5 µL of each primer (0.4 µmol/L),
c. 12.5 µL of Itaq (BioRad) Sybr Green mastermix
d. 4.5 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 95ºC; followed by 35 cycles with a melting temperature of 95°C for 1 minute; an annealing temperature of 40ºC and a final extension step at 72ºC for 90 seconds before holding at 4ºC until collection of PCR products for analysis. The primers used were:

- mICOIintF 5’-GGWACWGGWTGAACWGTWTAYCCYCC-3’ and
- jgHCO2198 5’-TAIACYTCIGGRTGICCRAARAAYCA-3’

PCR amplicons were purified as per DNA purification above and sent for Sanger sequencing (Source Bioscience). Returned sequence data was submitted to BLAST for species identification.

H. 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; 0.8 µmol/L reverse primer; 0.5 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 and each plate included 8 positive controls (4 replicates each at $1x10^{-3}$ and $1x10^{-4}$ ng/µl *C. chinensis* DNA) and 4 negative controls (ultrapure water in place of DNA) on a Bio-Rad CFX Connect real-time PCR machine as follows: an initial incubation for 5 minutes at 56.3ºC 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 56.4ºC for 1 minute.

Appendix 3. *C. chinensis* sequence information

Potential Primer/Probe Combinations

Table 9. Potential primer/probe combinations for the species-specific amplification of *C. chinensis*.

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	GTGATTGTAACT GCTCACGCA	GTGATTGTAAC TGCTCACGCA	GTGATTGTAACTG CTCACGCA	214	Yes, multiple species
2	TGTGATTGTAAC TGCTCACGC	TGTGATTGTAA CTGCTCACGC	TGTGATTGTAACT GCTCACGC	202	Yes, multiple species
3	GGGTGTTGGGA CTGGTTGAA	GGGTGTTGGG ACTGGTTGAA	GGGTGTTGGGAC TGGTTGAA	109	Yes, multiple species
4	TGATTGTAACTG CTCACGCA	TGATTGTAACT GCTCACGCA	TGATTGTAACTGC TCACGCA	201	Only *C. chinensis* and *C. cathayensis*
5	CTGCTGTTGAAG GGGGTGTT	CTGCTGTTGAA GGGGGTGTT	CTGCTGTTGAAG GGGGTGTT	119	Yes, multiple species
6	TGTAACTGCTCA CGCATTTGT	TGTAACTGCTC ACGCATTTGT	TGTAACTGCTCAC GCATTTGT	201	Only *C. chinensis* and *C. cathayensis*
7	TTCAGCTGCTGT TGAAGGGG	TTCAGCTGCTG TTGAAGGGG	TTCAGCTGCTGTT GAAGGGG	130	Yes, multiple species
8	GTGATTGTAACT GCTCACGCAT	GTGATTGTAAC TGCTCACGCAT	GTGATTGTAACTG CTCACGCAT	204	Only *C. chinensis* and *C. cathayensis*
9	ATGTGATTGTAA CTGCTCACGC	ATGTGATTGTA ACTGCTCACGC	ATGTGATTGTAAC TGCTCACGC	209	*C. chinensis* and *C. cathayensis*
10	GTGATTGTAACT GCTCACGC	GTGATTGTAAC TGCTCACGC	GTGATTGTAACTG CTCACGC	215	Yes, multiple species

Cross-species amplification refers to *in silico* analysis of primers/probes using primerBLAST software.

Potential Primer/Probe Positions on C. chinensis Sequence Alignment

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LC437753.1      GTATTAGGAGACGATCAGTTATATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437751.1      TTATTAGGAGACGATCAGTTATATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437761.1      TTATTAGGAGACGATCAGTTATATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
MK053826.1      TTATTAGGAGACGATCAGTTATATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437802.1      TTATTAGGAGACGATCAGTTATATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60 [9]
LC028537.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60 [8]
LC437807.1      TTATTAGGAGATGATCAGTTGTACAATGTGATTGTAACTGCTCATGCATTTGTTATAATT 60 [6]
LC437771.1      TTATTAGGAGATGATCAGTTGTACAATGTGATTGTAACTGCTCACGCATTTGTTATAATT 60 [4]
LC028539.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437757.1      TTATTAGGAGATGATCAGCTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437758.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437760.1      TTATTAGGAGATGATCAATTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028538.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028536.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028540.1      TTATTAGGAGATGATCAGTTGTACAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC437800.1      TTATTAGGAGATGATCAGTTGTATAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028543.1      TTATTAGGAGATGATCAGTTGTACAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028548.1      TTATTAGGAGATGATCAGTTGTACAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
LC028546.1      TTATTAGGAGATGATCAGTTGTACAATGTAATTGTAACTGCTCATGCATTTGTTATAATT 60
                 ********** *****  * ** ***** ************** ***************

LC437753.1      TTCTTTATGGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
LC437751.1      TTCTTTATGGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
LC437761.1      TTCTTTATAGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
MK053826.1      TTCTTTATAGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
LC437802.1      TTCTTTATAGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
LC028537.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTAATTCCTTTAATA 120
LC437807.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTAATTCCTCTAATA 120
LC437771.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTAATTCCTTTAATA 120
LC028539.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGATTTGGAAATTGATTAATTCCTTTAATA 120
LC437757.1      TTTTTTTTAGTTATGCCTATAATAATTGGGGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC437758.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC437760.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC028538.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTAATTCCTTTAATA 120
LC028536.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTAATTCCTTTAATA 120
LC028540.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC437800.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC028543.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC028548.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
LC028546.1      TTTTTTTTAGTTATGCCTATAATAATTGGTGGGTTTGGAAATTGATTGATTCCTTTAATA 120
                ** *** * ******************** ** ************** ****** *****

LC437753.1      TTAGGTGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTCTGATTATTACCC 180
LC437751.1      TTAGGTGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTCTGATTATTACCC 180
LC437761.1      TTAGGTGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTCTGATTATTACCC 180
MK053826.1      TTAGGTGCTCCTGATATAGCTTTTCCTCGTTTAAATAACATAAGTTTTTGATTATTACCC 180
LC437802.1      TTAGGTGCTCCTGATATAGCTTTTCCTCGTTTAAATAACATAAGTTTCTGATTATTACCC 180
LC028537.1      TTAGGAGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180
LC437807.1      TTAGGAGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180
LC437771.1      TTAGGAGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180 [P]
LC028539.1      TTAGGAGCTCCAGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180
LC437757.1      TTGGGAGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180
LC437758.1      TTGGGAGCTCCTGATATAGCTTTTCCTCGTTTAAATAATATAAGTTTTTGATTATTACCT 180
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Figure 10. *C. chinensis* sequence alignment showing the positions (some bases differ from primer sequence) of the four potential species-specific primer/probe combinations. Primer pair 4 shown in yellow; primer pair 6 in blue; primer pair 8 in red; and primer pair 9 in grey. The probe [P] was identical for all four primer pairs and is shown in pink. Only the relevant part of the alignment is shown. Stars denote that all bases are the same, where there is no star there is variation between the sequences shown in the alignment.

Appendix 4. COASTER

This was the first use of the COASTER tool developed during NECR359 (Harper et al. 2021) and as such there are some explanations required from its use during this study.

PCR efficiency and R^2 values

As the qPCR being developed was to be used to show presence/absence, after measuring the sensitivity of the assay (LOD, LOQ) we used positive controls on the PCR plates rather than standards. However, once using the COASTER tool it became apparent that for the tool to assess the data, standards rather than positive controls were required on every plate of data to be entered. We therefore changed the positive controls to standards and this meant that the PCR efficiencies and R^2 values for each plate were not always within acceptable limits. We were unable to enter the data from the three sensitivity plates performed which showed that the assay developed was within acceptable limits for both the PCR efficiency and R^2 value. We are therefore confident that the assay developed here was both optimal and thoroughly optimised.

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