eDNA detection of *Cipangopaludina chinensis* in the UK, 2022

Pevensey Levels, Sussex and Southampton Common, Hampshire

August 2024

Natural England Commissioned Report NECR554

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Citation

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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 and at two lakes at Southampton Common, Hampshire in 2022. This project delivers important baseline data on the applications of DNA technologies, specifically the development of species-specific eDNA assays to survey and monitor the spread of non-native molluscs.

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Executive summary

Based on eDNA and manual survey data carried out in 2021 and 2022 at the Pevensey Levels, Sussex, Natural England has erected four coffer dams in the main colonised ditch and side ditches to prevent further spread of the invasive snail *Cipangopaludina chinensis*. Natural England wished to undertake further in-field validation of the qPCR assay developed by ADAS in 2021 (NECR410) to determine the likely presence and spread of *C. chinensis,* an invasive non-native species of snail that has recently been found in ditches within the Pevensey Levels, Sussex. This site is within an internationally important area of coastal grazing marshes which is also a Special Area of Conservation and supports numerous SSSIs. 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 on the Pevensey Levels, a habitat for many rare invertebrate species including rare freshwater Mollusca such as the Little Whirlpool Ram's-horn Snail *Anisus vorticulus.* Water samples taken by Natural England and ADAS staff in August 2022 confirmed the continued presence of *C. chinensis* within the ditch system albeit in fewer sites than in 2021. Notably *C. chinensis* DNA was not found in or around the coffer dam erected between sites 9 and 11 but was found within side ditch H and at site 4 to J (where it has not previously been found).

C. chinensis has also recently been found at Southampton Common SSSI, Hampshire. The site contains two large artificial lakes that support nationally important breeding assemblages of amphibians. In January 2022, reports were received that the shells of *C. chinensis* had been found, likely when the boating lake was drained. A manual survey in August 2022 confirmed the presence of *C. chinensis* and eDNA analysis found that 6 out of the 7 sites sampled were positive for *C. chinensis* DNA.

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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. RSK ADAS is an environmental consultancy which exists to provide ideas, specialist knowledge and solutions to secure our food and enhance the environment.

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. Due to the success of environmental DNA (eDNA) approaches to survey for great crested newts and freshwater fish species there has been an increased interest in the use of these techniques for the detection of invasive non-native species. 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). In 2021, Natural England commissioned a project with RSK ADAS to develop an eDNA assay for the detection of *Cipangopaludina chinensis* (Gray, 1834), Chinese mystery snail. This invasive species is a problem in many parts of the world (Global Invasive Species Database, 2021) and was first found in the UK within ditches at the Pevensey Levels, Sussex in September 2018.

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. 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 the 2021 eDNA study.

Pevensey Levels, East Sussex

Survey work carried out in July 2019 found the presence of a recruiting population of the snail in an approximately 400m stretch of the ditch. Further surveys carried out at this ditch system in February 2021 found a number of smaller juveniles which suggested that a

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

breeding population was present (Willing 2021a). Confirmation of the breeding population was made during eDNA surveys in August 2021 when specimens were collected and returned to the laboratory. During tissue removal for DNA extraction one of the specimens was found to contain juvenile snails (Figure 1) - this species is known to give live birth during June to October potentially having more than 160 young in their lifetime (Jokinen, 1992).

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 juveniles (© Helen Rees, ADAS).

In August 2021, additional manual survey work carried out following eDNA sample collection, showed that *C. chinensis* had colonised an additional 138m of ditch (downstream) since it was surveyed in February 2021 (Willing 2021b). The August 2021 eDNA survey confirmed the presence of *C. chinensis* in the areas of known *C. chinensis* colonisation (including the additional 138m of ditch) but also identified 5 other areas where *C. chinensis* eDNA was present despite not being found during manual survey (Rees et al. 2022) (Figure 2). Following these combined findings, four coffer dams were installed to prevent further spread of *C. chinensis* within the ditch system (Figure 2). In April 2022, further manual searches found no live *C. chinensis* in ditch sectors lying beyond (outside) any of the coffer dams or in any of the other side channels surveyed (side ditches B, C, D, G, H and J) (Willing 2022).

Southampton Common SSSI

Southampton Common is a 365-acre area of open space featuring woodland, rough grassland, ponds, wetlands, lakes and parkland and was designated a Site of Special Scientific Interest (SSSI) in 1988. The site contains two large artificial lakes that support nationally important breeding assemblages of amphibians. In January 2022, reports were received that the shells of *C. chinensis* had been found, likely when the boating lake was drained. A manual survey of the two lakes on Southampton Common SSSI and at a third lake (Cemetery Lake) took place on the 24th and 25th August 2022 entailing visual inspection and netting (Figure 3). Evidence of *C. chinensis* was found during this survey with multiple adult and juvenile shells and adult specimens being found in both the Boating Lake (sites 1-4) and Ornamental Lake (sites 5-8) (Figure 4).

Aims and Objectives

The overall aim of the 2022 eDNA surveys at the Pevensey Levels was to confirm the continued presence of *C. chinensis* in the main colonised section of the ditch and to survey around the sites of the coffer dams to determine their effectiveness. A further aim was to compare the sterivex filters used in 2021 with two other types of enclosed filter in terms of their ease of use, the volume of water that could be filtered and the results of the *C. chinensis* eDNA assay performed.

The aim for the 2022 eDNA surveys at Southampton Common SSSI was to confirm the presence of *C. chinensis* found during the manual survey by eDNA analysis.

This report details the methodology employed in these studies, the results obtained and, discussion of the survey results. 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.

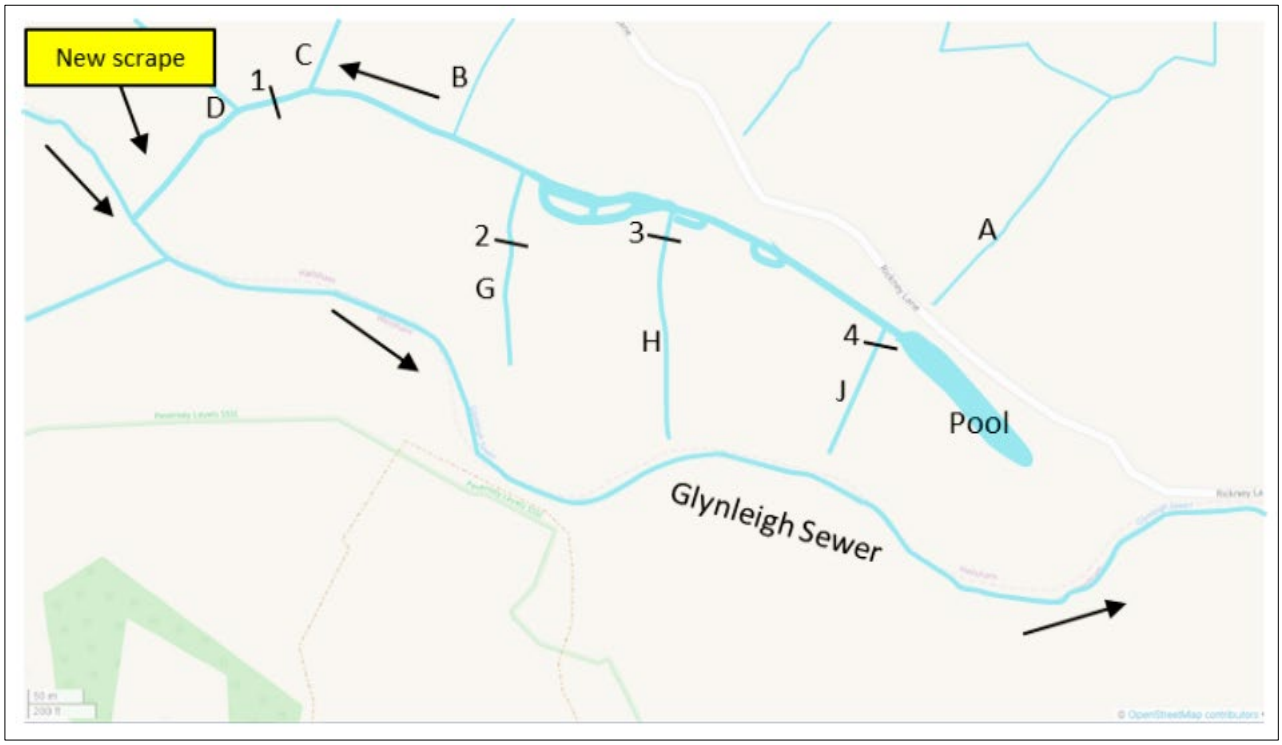

Figure 2. Map of the ditch system at Pevensey Levels showing the locations of the four coffer dams (1-4). Black arrows show direction of flow of main ditch. Side ditches are labelled A-J. OpenStreetMap © (data available under the Open Database License).

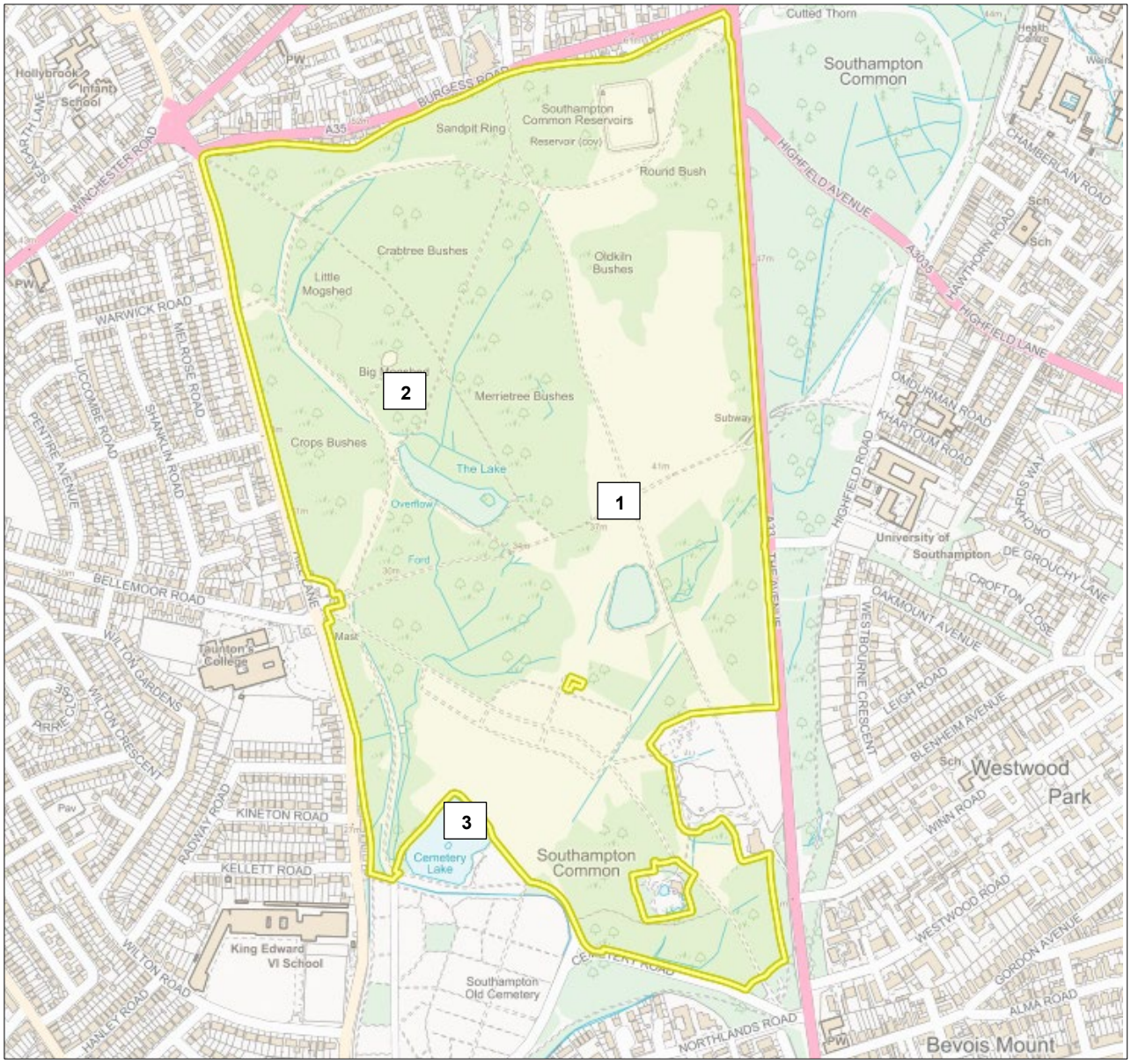

Figure 3. Location of the three lakes on Southampton Common SSSI

Yellow lines indicate SSSI site boundary. 1. Boating Lake, 2. Ornamental Lake, 3. Cemetery Lake. Contains, or is derived from, information supplied by Ordnance Survey. © Crown copyright and database rights 2022.

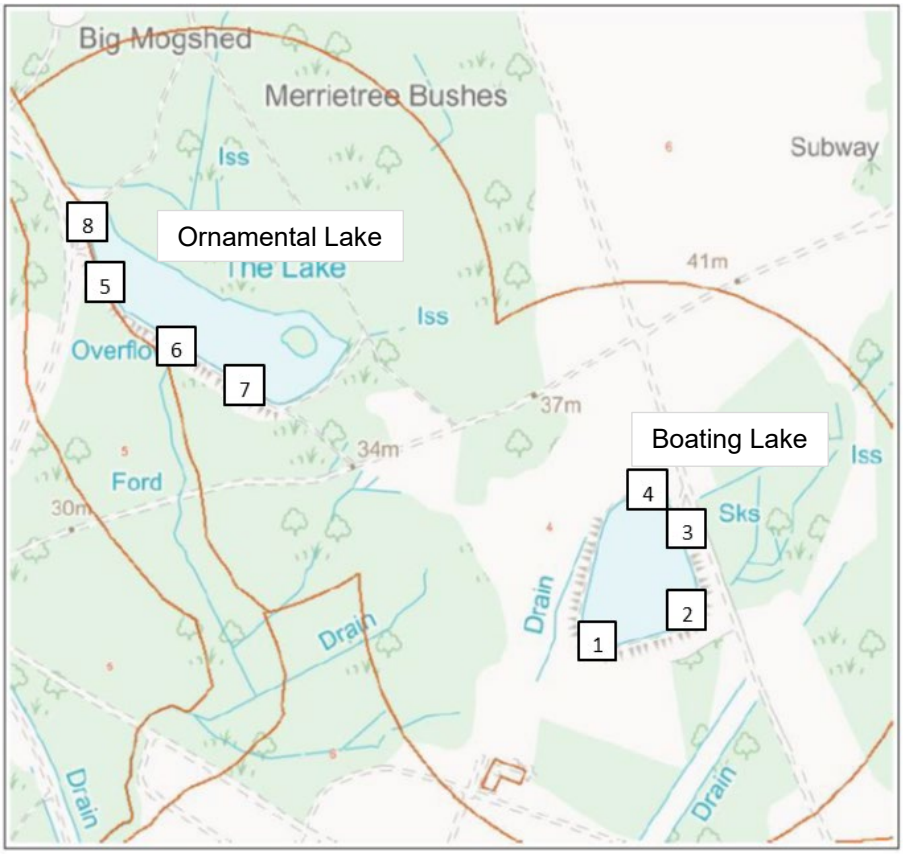

Figure 4. Sample locations on boating and ornamental lakes, Southampton Common SSSI (taken from Measures, 2022). Orange lines indicate SSSI management units. Contains, or is derived from, information supplied by Ordnance Survey. © Crown copyright and database rights 2022.

Materials and Methods

For the full materials and methods please see NECR410 (Rees et al, 2022), a summary of any alterations to this is provided below.

Sample Collection – Pevensey Levels

Eighteen ditch water samples were collected by Natural England and RSK ADAS staff at Pevensey Levels on the 3rd August 2022 (Appendix 1). Two control field blanks (same volume of distilled water) were also filtered by ADAS staff next to sampling site 8.

For all samples two litres of water was collected to allow for three types of filters to be tested for their ease of use and filterable volume of water. As in the 2021 eDNA survey a 0.22µm Sterivex polyethersulfone (PES) filter (Merck Millipore, Darmstadt, Germany) was used alongside two further filter types (Figure 5):

- a 0.48µm PES filter (GVS, Stanford, North America) in combination with a 3.1µm glass fibre pre-filter (GVS, Stanford, North America) (recommended where water to

be sampled is turbid - used to remove larger particles of sediment and plant material before capturing eDNA on the main filter); and

- a 0.8µm Sylphium PES filter (Sylphium, Groningen, The Netherlands).

Figure 5. Filter types. Images of each different filter type used: A) 0.22µm Sterivex PES filter; B) 0.48µm PES filter in combination with 3.1µm glass fibre pre-filter; C) 0.8µm Sylphium PES filter (© Helen Rees, ADAS).

Sample Collection – Southampton Common SSSI

Seven water samples were collected by Natural England staff at Southampton Common SSSI on the 24th August 2022 (Appendix 2). One control field blank (distilled water) was also filtered by Natural England staff on site 7. All samples were collected using 0.22µm Sterivex PES filters.

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.

DNA extraction

DNA was extracted from ditch samples using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (Appendix 2), with the following exceptions:

1. For sterivex filters - 760µL of ATL buffer/proteinase K (10:1 ratio) was added to each filter.
2. For the PES/GF filter combination – 580µL ATL buffer/proteinase K (10:1) was added to each filter.
3. For the Sylphium filters – 2mL ATL buffer/proteinase K (10:1) was added to each filter.

The ends of the filters were sealed and briefly agitated by vortexing. Proteinase K Digestion was carried out overnight at 37°C.

All DNA extracts were quantified using a Qubit 3.0 Fluorometer (Invitrogen) following the manufacturer's instructions then stored at -20 ºC prior to PCR set up.

eDNA assay

All 60 extracted samples (18 Pevensey Levels samples with the three different filter types plus the two field blanks with the three different filter types) were diluted to a DNA concentration of 1ng/µL then applied to the *C. chinensis* eDNA assay developed in NECR410 (Rees et al. 2022). PCRs were set up in a total volume of 25µL consisting of: 12.5µL TaqMan Environmental Mastermix 2.0 (containing AmpliTaq GOLD DNA polymerase), 1µL of forward primer (final concentration of 0.2 µmol/L), 1 µL of reverse primer (final concentration of 0.8 µmol/L), 1µL probe (final concentration of 0.5 µmol/L), and 6.5µL ddH_2O (see Table 1 for primer/probe sequences). 3µL of DNA was added to each reaction and 12 replicates were performed per DNA sample. All controls were performed in quadruplicate with a dilution series of $1x10^{-1}$ to $1x10^{-4}$ ng/µL *C. chinensis* DNA being used as positive controls and ddH_2O in place of DNA for the negative control. PCRs were run 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.

Table 1. *C. chinensis* primer and probe set 8 details (Rees et al. 2023).

Oligo Name	Sequence (5' - 3')
CMS 8F	GTGATTGTAACTGCTCACGCAT
CMS 8R	CCCAACACCCCCTTCAACAG
CMS.probe	FAM-GGAGCTCCTGATATAGCTTTTCCTCGT-BHQ1

All samples were also tested for the presence of inhibitors (e.g. humic and phytic acids) 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.

Any samples that were 1 out of 12 were retested twice more (i.e. 24 further replicates) to confirm the result.

COASTER analysis

COASTER: Confidence Assessment Tool for eDNA qPCR Results (Harper et al. 2021) was used to verify the results of the qPCR assay and to understand the confidence in the qPCR assay. Default settings were used except that: the number of plates which was set to 12; 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.

Results

Pevensey Levels

All Pevensey Levels ditch water samples were subjected to the optimised assay, with 4 water samples from the Pevensey Levels being positive for *C. chinensis* DNA when using Sterivex filters, 7 water samples being positive when using the PES/GF combination or 5 being positive when using the Sylphium filters (Figure 6). Upon retesting of samples that were 1 out of 12 this reduced the number of samples being positive for *C. chinensis* to 3 water samples when using Sterivex filters, 6 water samples when using the PES/GF combination, or 3 water samples being positive when using the Sylphium filters.

All remaining samples were negative for *C. chinensis* DNA, therefore other than any ditches where *C. chinensis* was found by manual survey, *C. chinensis* is likely to be absent from these ditches (Table 2). It was also noted that fewer live *C. chinensis* were

observed during the eDNA surveys in 2022 than were observed in the eDNA surveys in 2021, although lots of empty shells were seen in 2022.

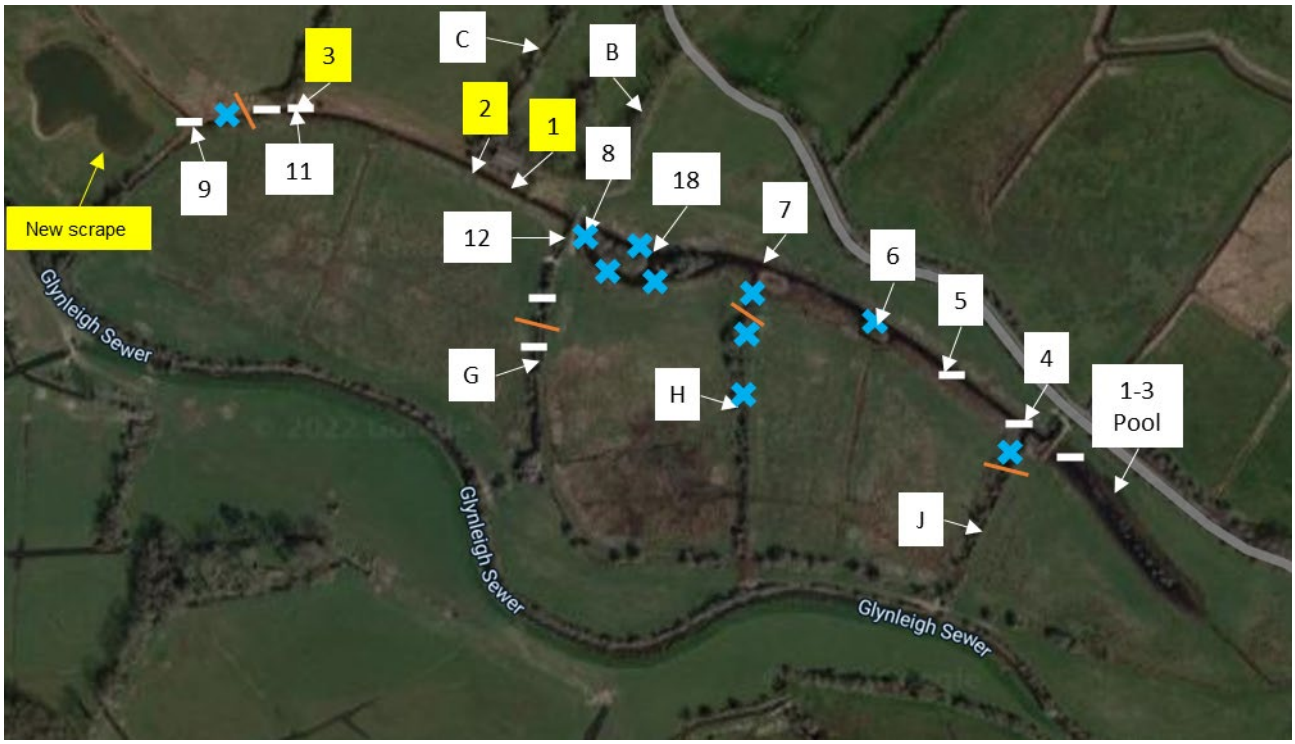

Figure 6. Satellite image of ditch system at Pevensey Levels showing sites with or without *C. chinensis* as per qPCR analysis August 2022 (© Google 2022). Numbers and letters indicate sampling previous sampling sites: 1, 2, and 3 (in yellow) were sampled by M. Willing and indicate the further spread of *C. chinensis* in February 2021. Sites 7, 7 to coffer, 8, coffer to 9, 18 lower, 18 upper, 8 to 18 lower, and side ditches coffer to H, H and 4 to J were all found to be positive (blue cross) for *C. chinensis* DNA by initial eDNA assay in August 2022, and sites 1-3 pool, 4, 5, 9, 11, coffer to 12, and side ditch G were found to be negative (white dash) for C. chinensis DNA in August 2022. Orange lines indicate the sites of the four coffer dams installed in 2021/2022 (numbered 1 to 4 from left to right). (results from all three filter types have been pooled to create this figure).

COASTER – Pevensey Levels

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 when the results from all three filter types were taken into account. Those that were tentative were either 1/12 positive amplifications as the COASTER default settings require 2/12 positive replicates to call a sample positive or were below the limit of detection. All qPCR negatives were recorded as negative or inconclusive by COASTER when the results from all three filter types were taken into account. The inconclusive results were due to inhibition found during the inhibition control test carried out on all the samples. COASTER assumes that a sample is inconclusive if the inhibition control qPCR results are not within certain limits as you do not know if the sample is negative because of PCR inhibition preventing amplification or if the sample was truly negative. It was notable

that where Sterivex filter qPCR results were negative for *C. chinensis* the majority were also found to contain inhibitors which was not the case with the corresponding PES/GF or Sylphium filter qPCR results. This suggests that the 0.22µm filter retained a component that later caused inhibition of qPCRs which was not retained by the larger pore sized filters.

Southampton Common SSSI

Six of the seven water samples collected at Southampton Common SSSI were positive for *C. chinensis* DNA. The filterable volume of water at sites 3 and 4 was less than that at sites 1 and 2 due to the presence of blue-green algae in the Boating Lake. The presence of the blue-green algae did not cause PCR inhibition but it did make it much more difficult to push the water through the filters and is why only 80 mLs water were filtered at these sites. The presence of the blue-green is also likely to be the reason why a higher concentration of DNA was extracted from these two samples.

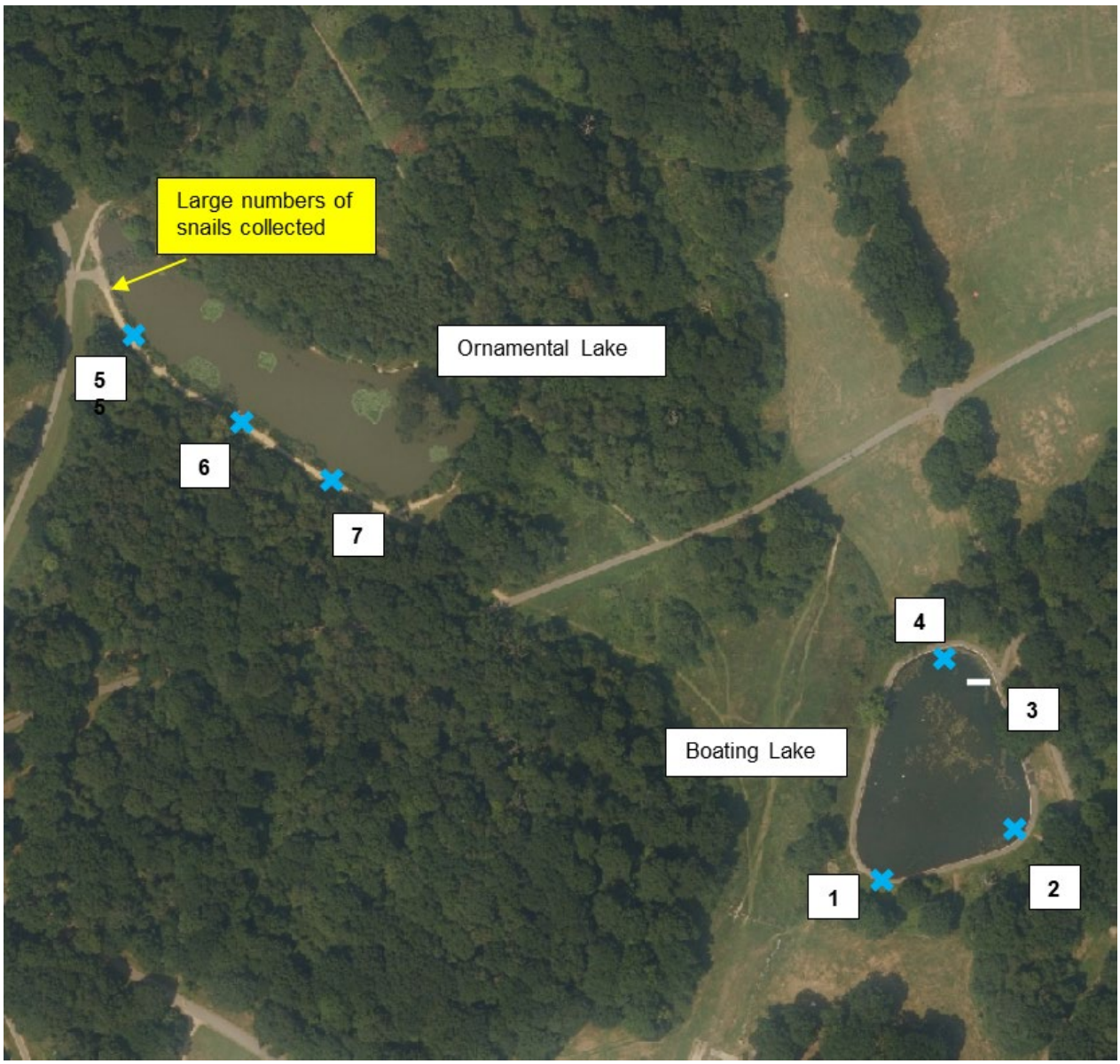

Figure 7. Satellite image of the lakes at Southampton Common SSSI showing sites with or without *C. chinensis* as per qPCR analysis August 2022 (© Google 2022). Numbers indicate sampling sites. Sites found to be positive (blue cross) for *C. chinensis* DNA by eDNA assay and sites found to be negative (white dash) for C. chinensis DNA in August 2022 are marked.

COASTER – Southampton Common

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. All qPCR negatives were recorded as negative or by COASTER.

Table 2. Pevensey Levels ditch water sample information.

Sampling site	Volume filtered (mL)			DNA (ng/µL)			*C. chinensis* species-specific qPCR result (out of 12)			COASTER result 2022			COASTER result 2021 (equivalent site in brackets)	2022 Manual survey result
	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm		
	EASTERN SECTION DITCH													
1-3 pool	160	285	1000	30.10	40.90	0.125	0	0	0*	I	N	N	N	Not found
4	250	400	800	8.54	7.04	6.16	0	0	0*	I	N	N	N	Not found
5	350	300	1000	14.80	14.50	<0	0	0	0*	I	N	N	P	Not found
6	350	300	1000	8.49	16.30	10.20	0	**4**	0*	I	P	N	P	Present
8	300	250	1000	2.40	3.08	14.40	**5**	**9**	0*	P	P	N	N	Present
8 to 18 lower side	400	300	1000	8.91	2.67	<0	0	1, **2**, 0#	0*	I	T	N	P	Present
18 lower side	300	300	1000	9.64	12.40	5.00	0	**11**	**8***	I	P	P	P	Present
18 upper side	400	400	1000	17.80	15.70	2.79	0	0	**4***	I	N	T	T	Present
	WESTERN SECTION DITCH													
9	150	200	1000	11.60	5.19	0.925	0	0	0*	I	N	N	T	Not found
Coffer to 9	300	400	1000	32.10	55.00	47.30	0	1, 0, 0#	1, 0, 0#	I	T	T	T	Not found
11 opposite ditch C	325	300	1000	38.50	35.00	30.60	0	0	0*	N	N	N	T	Not found
Coffer to 11	250	350	1000	14.10	32.90	43.40	0	0	0*	N	N	N	T (site 11)	Not found

Sampling site	Volume filtered (mL)			DNA (ng/µL)			*C. chinensis* species-specific qPCR result (out of 12)			COASTER result 2022			COASTER result 2021 (equivalent site in brackets)	2022 Manual survey result
	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm	0.22µm	0.48µm	0.8µm		
	SIDE DITCHES													
Coffer to G	200	200	1000	27.10	28.30	18.50	0	0	0	I	N	N	N (G upper)	Not found
Coffer to 12	200	200	1000	20.10	27.00	4.55	0	0	0*	N	N	N	P (site 12)	Not found
H	150	200	900	12.10	0.848	1.64	**4**	1, 1, 1#	0*	P	T	P	P (H upper)	Not found
Coffer to H	300	250	1000	54.00	9.40	194.80	0	0	1, 0, 0#	I	N	T	P (H lower)	Not found
Coffer to 7	200	200	1000	32.40	8.54	17.40	1, 0, **3**#	**12**	**11***	T	P	P	P (H lower)	Not found
4 to J	200	200	1000	20.90	7.92	57.00	1, 0, 0#	0	0*	N	T	I	N	Not found
	FIELD BLANKS													
FB1	250	250	1000	<0	<0	<0	0	0	0	N	N	N	N/A	N/A
FB2	250	250	1000	<0	<0	<0	0	0	0	N	N	N	N/A	N/A

Note that >0 = Out of range (too low to measure). * Denotes broken/cracked luer locks on the ends of the filter. # Denotes samples that were initially found to be 1 out of 12 which were retested twice more (i.e. a total of 24 further replicates) to confirm the results. Please note that these samples went through a second freeze-thaw to allow repeat analysis.

P = positive (≥2 out of 12 according to COASTER); I = inconclusive (not detected but inhibition was evident); T = tentative (below the limit of detection or <2 out of 12); N = negative.

Table 3. Southampton Commons SSSI water sample information (0.22µm Sterivex filters).

Sampling site	Volume filtered 0.22µm (mL)	DNA (ng/µL) 0.22µm	*C. chinensis* species-specific qPCR result (out of 12)	COASTER result	2022 manual survey result
BOATING LAKE					
1	200	10.90	**11**	P	Present (shells)
2	200	7.02	**2**	T	Present (shells)
3	80*	11.60	0	N	Present (shells)
4	80*	36.10	**3**	T	Present (shells)
ORNAMENTAL LAKE					
5	100#	2.15	**12**	P	Present (live)
6	100#	2.21	**2**	T	Present (live)
7	100#	3.34	**11**	P	Present (live)
FIELD BLANK					
7	470	<0	0	N	N/A

Note that >0 = Out of range (too low to measure). * Due to the presence of blue-green algae clogging the filters only 80 mLs water was filtered. # Filtering was difficult at these sites due to the presence of a fine sediment in the water samples.

P = positive (≥2 out of 12 according to COASTER); I = inconclusive (not detected but inhibition was evident); T = tentative (below the limit of detection or <2 out of 12); N = negative.

Discussion

Pevensey Levels

Fewer samples were found to be positive for *C. chinensis* DNA during the 2022 surveys than in the 2021 surveys. Although the addition of the four coffer dams could partly explain this for the side ditches (except H which was still positive for *C. chinensis* DNA), there was a period of very hot weather prior to the 2022 sampling which caused water levels to drop considerably compared with 2021 levels and in addition the water quality was poor (see photos in Appendix 3). There were significant areas of exposed mud at all sampled sites and lots of *C. chinensis* shells were found in these. It is known that *C. chinensis* favour marginal areas during the summer months (favouring the deeper more central areas outside of this time). This suggests that the number of *C. chinensis* could have been reduced compared with previous years which could also help to explain why sites which were positive for *C. chinensis* were now negative for *C. chinensis* DNA. It is also the case that eDNA will have been more likely to degrade at a faster rate due to the hot weather

and the high UV levels associated with this hot weather (Dejean *et al.* 2011; Treguier *et al.* 2014; Barnes *et al.* 2014; Pilliod *et al.* 2014). This could also have played a part in the reduced detection levels at the site in 2022. 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).

The fact that site 'Coffer to H' was found to be positive for *C. chinensis* DNA at low qPCR score (1 out of 12) with the Sylphium filter (no evidence of damage to the filter) suggested that the coffer dam was not placed far enough along side ditch H - the coffer dams were put into place before the eDNA results were completed which could explain the potential positive result in 2022. The sample was, however, found to be negative for *C. chinensis* upon retesting. This was also the case for sites 4 to J and coffer to 9 (both initially found to be positive at 1 out of 12). It should be noted that these samples went through an additional round of freeze-thawing which can lead to DNA degradation. This could in part account for the sample now being negative although it should also be noted that three other samples were still positive for *C. chinensis* DNA when retested despite also going through an additional round of freeze-thawing. In contrast, both sites 9 and 11 which were found to be positive for *C. chinensis* DNA in 2021 were no longer positive in 2022 which goes some way to confirming their absence at these sites. All these sites will require further eDNA and/or manual surveys to confirm and give more confidence in these results.

A new shallow water scrape has been created in a field lying on the far western side at the outflow end of the infested ditch (Figure 2). Without access permission the scrape was not surveyed. With no apparent ditch connection with the infested ditch colonisation by *C. chinensis* seems unlikely, but if the snail was accidentally (or intentionally) introduced, the shallow water conditions are likely to suite it.

Filter Type

Three different filter types were trialled (different pore sizes) to compare their ease of use and the total overall filterable volume. The Sylphium filters (0.8µm) were the easiest to use in field as for most samples it was easy to filter 1000mL of water through them. With these filters however, the luer locks at each end of the filter were prone to cracking and/or breaking off completely during sample collection or transit and storage (Figure 8). We found that a number of these filters had leaked their preservative ethanol into the storage bags and these had to be wiped with 5% Hypochlorite solution prior to the DNA extraction and during this process sealing the broken ends as best as possible with parafilm/tape. It was difficult to quantify how much of this preservative was lost due to leakage in these filters, we do know however that the ethanol preservative can wash eDNA containing material off the surface of the filter during storage and transit of the samples (evidenced here by a change in colour of the preservative solution) which is why we extract DNA from both the filter and the preservation solution (Bruce et al. 2021). This would mean that any preservative lost due to leakage would have resulted in a decrease in the amount of eDNA recovered from these samples this is evident in Sylphium filter sample Coffer to H (which

did not suffer from damaged or broken luer locks) and resulted in the highest concentration of DNA recovered from this type of filter. We also note that the pressure required to push the DNA extraction buffers and ethanol out of the Sylphium filters meant that a few of the filters started to leak around the edges of the filter unit. These observations could be an explanation for the negative eDNA qPCR results achieved with these filters compared with the Sterivex and PES/GF combination filters for the samples: 5, 7, 8, 8-18 lower side, and H and the lower qPCR score achieved for sample 18 lower side despite the successful filtering much larger volumes of water. An exception to this was sample 18 upper side which achieved a higher qPCR score. We recommend that these filters are not used in future surveys. These issues were not seen with either the Sterivex or PES/GF filter combinations. We note in this study the larger number of inconclusive results that were seen with the 0.2 µm Sterivex filters, with these filters it is possible that they could have retained higher amounts of inhibitory components due to their smaller pore size.

Figure 8. Image of the Sylphium filters upon commencing sample processing (© K. Bishop). Many of the Sylphium filters had damaged luer lock adaptors at one or both ends. See Figure 5 for example of undamaged Sylphium filter.

Substances that can cause inhibition of PCR reactions and cause false negative results are known as PCR inhibitors, these can include humic and fulminic acids from dead biomass which are likely to occur in environmental samples and can inhibit PCR even at low concentrations (Schrader et al. 2012; Ijzerman et al 1997). The Environmental mastermix 2.0 (ThermoFisher) that was used here is specifically designed for use in the presence of high levels of inhibitors that are likely in this kind of samples so their affect should be minimised. Inhibition can be further overcome by use of a PCR inhibitor removal kit or by dilution of the DNA extract, although this latter method will also reduce the concentration of target DNA and therefore will affect detection probability. We recommend that in the future (and where samples show sign of sample inhibition) that DNA extracts are treated with a PCR inhibitor removal kit prior to PCR analysis for the target DNA.

Accepting that the variation in the amounts of DNA extracted from the 0.8 µm filters (compared to the other two filter types) was likely due to leakage of the preservation

solution in a number of instances and some loss of sample, the variation in total DNA extracted between different sites is relatively consistent. Comparing the two smaller filter sizes (0.22 µm and 0.45 µm pore sizes), both the amounts of water volume filtered and the amounts of DNA recovered are similar to our other studies. Using both filter systems the recovery of all the extraction buffer (added to the filter device) is prone to sample losses and can account for some variability between extracted samples. Two samples (H and Coffer to H) differed more than we would have expected with the Sterivex filter (particularly sample H), consistent with poor recovery of the eDNA from the surface of the filter.

Small pore size filters (0.20 to 1.5 µm) have been shown to yield the most eDNA but are prone to clogging when sampling turbid water or where there are algal blooms (Eichmiller et al, 2016; Turner et al, 2014; Liang et al, 2013). In such conditions, either larger pore-size filter or pre-filtration can be used as was the case with the PES/GF filter combination. A larger pore size filter allows an increased volume of water to be filtered but there is a trade-off in that the smallest particles containing eDNA may pass through the filter. Pre-filtration can increase single species detection probability (Robson et al, 2016) and give more consistent results for community compositions. However, the use of pre-filters can lower the DNA yield and the number of detected taxa (Majaneva et al 2018). In the current study DNA was extracted from both the pre-filter and the main filter to minimise any DNA loses (Bruce et al. 2021). Similar volumes of water were filterable through the Sterivex and PES/GF filters and although this was a smaller volume of water than was filtered through the Sylphium filters more effort was required to push the water through these smaller filter types. We would recommend that surveys use the 0.45 µm PES/GF filter combination in future studies as they allow a good volume of water to be filtered; were robust and did not retain the PCR inhibitors that the Sterivex filters seem to have been prone to.

Southampton Common SSSI

Manual surveys confirmed the presence of *C. chinensis* at the ornamental lake but only empty shells and no live specimens were found at the boating lake within Southampton Common SSSI. The presence of shells of all ages at the boating lake confirms a former recruiting population but does not confirm the continued presence of *C. chinensis.* Positive amplification of *C. chinensis* DNA was found in 6 out of the 7 water samples collected from these sites. Where *C. chinensis* DNA was not found at site 3 of the boating lake empty *C. chinensis* shells were found so it possible that when the boating lake was drained the snails at this site were killed off. Alternatively the presence of blue-green algae could have affected the DNA extraction by affecting the efficiency of the DNA extraction (blocking the filter column used). Two of the other sampling sites at the boating lake were PCR positive but had low qPCR scores/were below the LOD (2 or 3 out of 12) and were classed as tentative positives by COASTER analysis. As for the Pevensey Levels samples the eDNA present will be subject to degradation. eDNA will have been more likely to degrade at a faster rate due to the hot weather and the high UV levels associated with this hot weather (Dejean *et al.* 2011; Treguier *et al.* 2014; Barnes *et al.* 2014; Pilliod *et al.* 2014). The boating lake has steep vertical sides with no margin shelf or marginal vegetation although

there are large areas of aquatic weeds in the lake. This means that there will not be many shaded areas where DNA degradation could be slower due to less UV penetration.

The draining of the boating lake may have been detrimental to the *C. chinensis* population. However, site 1 at the boating lake had a very high qPCR score (11 out of 12) suggesting high amounts of *C. chinensis* DNA could have been present in the water sample. Further survey of the boating lake will be required to investigate if the snails survive drainage of the lake which takes place in December and remains empty until February or March. It will also be useful to understand where the drainage occurs and if any part of the lake is kept full to preserve any aquatic species present.

Two of the three samples collected from the ornamental lake (site 5 and 7) had high qPCR scores (11 or 12 out of 12) and the third (site 6) had a lower qPCR score (2 out of 12) and was classed as a tentative positive by COASTER analysis. This was despite the same volume of water being filtered, similar amounts of DNA being extracted, and all three sites having known presence of similar numbers of adult *C. chinensis* found in the shallow waters. These results could be due to the collection of water near to localised populations such that the water collection was not carried out close enough to these populations meaning that the amount of *C. chinensis* DNA in the water was lower. It is also possible that poor water quality contributed to these results with the presence of fine sediment affecting some samples more than others.

Conclusions

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* detected DNA at the Southampton Common SSSI where no animals were found. This could therefore enhance management decisions when it comes to planning lake management at Southampton Common SSSI. Coffer dams were introduced at three sites at the Pevensey Levels prior to the completion of the eDNA study in 2021 and the results of the eDNA analysis in 2022 suggest that two of these may not be beyond the current range of *C. chinensis*.

Recommendations

- Manual survey should be conducted at Pevensey Levels sample sites coffer to 9, 4 to J and side ditch 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.
- The new shallow scrape at Pevensey Levels was found to be suitable for colonisation by *C. chinensis* (Willing 2022), therefore this site should be surveyed by both manual and eDNA survey.

- It is recommended that in order to maximise confidence in PCR results it would be advisable to carry out replicate sampling at each sampling site with one type of filter.
- Further survey of other water bodies (ponds and streams/ditches) at Southampton Common SSSI could be carried out using eDNA surveys to assess the snail's presence across the SSSI and inform management decisions for the eradication of *C. chinensis* at this SSSI site. It will also be important to investigate the potential connectivity via the stream/ditches/drains on the site.

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Appendix 1

Table A1. Pevensey Levels ditch water samples

Sample ID	Location (what3words)	Sampler	Collection date/time	Volume filtered (ml) 0.22µm/0.48µm/0.8µm	Turbidity of sample	Site conditions
EASTERN SECTION DITCH						
1-3 pool	removers. elects. repaying	DC/RA	15:15	160/285/1000	Low-medium*	Breezy, sunny, warm
4	shocks. composts. dares	BM/HR	15:00	250/400/800	Low*	Breezy, sunny, warm
5	smiled. reference. estimate	GM/EW	15:15	350/300/1000	Low	Breezy, sunny, warm
6	removers. duties. exposes	BM/HR	12:30	350/300/1000	Low	Breezy, cloudy, warm
8	campfires. brittle. hairpin	HS/LD	14:15	300/250/1000	Low	Breezy, cloudy, warm
8 to 18 lower side	materials. scrap. consults	GW/EW	14:00	400/300/1000	Low	Breezy, cloudy, warm
18 lower side	caramel. restore. conspired	BM/HR	14:15	300/300/1000	Low	Breezy, cloudy, warm
18 upper side	sprint. consented. noble	DC/RA	14:15	400/400/1000	Low	Breezy, cloudy, warm
WESTERN SECTION DITCH						
9	moment. leafing. toads	GM/EW	11:00	150/200/1000	Low	Windy, overcast
Coffer to 9	landscape. chilling. inspects	DC/RA	11:00	300/400/1000	Low	Windy, overcast
11 opposite ditch C	loved. activity. breaches	BM/HR	11:00	325/300/1000	Low	Windy, overcast
Coffer to 11	roadblock. grapes. scrapped	HS/LD	11:00	250/350/1000	Low	Windy, overcast

Sample ID	Location (what3words)	Sampler	Collection date/time	Volume filtered (ml) 0.22µm/0.48µm/0.8µm	Turbidity of sample	Site conditions
SIDE DITCHES						
Coffer to G	consented. rear. strong	GM/HR	16:00	200/200/1000	Low*	Breezy, sunny, warm
Coffer to 12	error. crackling. unhappily	HS/EW	16:00	200/200/1000	Low	Breezy, sunny, warm
H	pouch. listen. gasp	GM/EW	12:30	150/200/900	Medium	Breezy, cloudy, warm
Coffer to H	capers. dragonfly. postcard	HS/LD	12:30	300/250/1000	Medium	Breezy, cloudy, warm
Coffer to 7	alike. blazing. occupations	DC/RA	12:30	400/400/1000	Low	Breezy, cloudy, warm
4 to J	boarding. interests. annotated	HS/LD	15:15	110/200/500	High*	Breezy, sunny, warm
FIELD BLANKS						
FB1	Next to site 8	HR	13:00	250/250/1000	N/A	Breezy, cloudy, warm
FB2	Next to site 8	BM	13:00	250/250/1000	N/A	Breezy, cloudy, warm

*indicates that there was a low water level at the sampling site.

Appendix 2

Table A2. Southampton Common SSSI water samples

Sample ID	Location (grid reference)	Sampler	Collection date	Volume filtered (ml) 0.22µm	Turbidity of sample	Site conditions
BOATING LAKE						
1	SU 41619 14345	GM	24/08/22	200	Medium	Cloudy
2	SU 41679 14361	GM	24/08/22	200	Medium	Cloudy
3	SU 41668 14431	GM	24/08/22	80	Blue-green algae bloom	Sunny, some cloud
4	SU 41657 14443	GM	24/08/22	80	Blue-green algae bloom	Sunny, some cloud
ORNAMENTAL LAKE						
5	SU 41294 14586 SU 41301 14575	GM	24/08/22	100	High (turbid/find sediment)	Sunny, some cloud
6	SU 41353 14540 SU 41340 14547	GM	24/08/22	100	High (turbid/find sediment)	Sunny, some cloud
7	SU 41380 14517 SU 41373 14527	GM	24/08/22	100	High (turbid/find sediment)	Sunny, some cloud
FIELD BLANK						
8	Next to site 7	GM	24/08/22	470	N/A	Sunny, some cloud

Appendix 3.

Table A3. Pevensey Levels ditch site photographs, August 2022 © Gavin Measures (NE)

Photograph	Site/description
	Sites 1-3 (pool)
	Site 4 and ditch J junction
	Site 12 (looking to site 17) Low water levels and cattle poached margins

Photograph	Site/description
	Site 8-18 upper side Low water levels exposing margins
	Site 7 looking to ditch H with installed coffer dam
	Site 9-11 with installed coffer dam

Table A4. Southampton Common SSSI site photographs, August 2022 © Gavin Measures (NE)

Photograph	Site/description
	General view over the boating lake
	Boating lake sites 3, 4 Blue-green algae blooms
	Snail shells collected at boating lake (adult and juveniles)

Photograph	Site/description
	General view of the ornamental lake to the island in the background. Low water levels exposing lake bed
	Ornamental lake - fishing points with shallow waters and emergent vegetation
	Cemetery Lake

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