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Design and Validation of a Single-Species Environmental DNA qPCR Assay for the Detection of the Invasive Chinese Mitten Crab *Eriocheir sinensis*

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ABSTRACT

Biological invasions are a leading driver of biodiversity loss and generate significant economic costs, either through direct impact on native ecosystems or through repairs and remediation. Reducing the impact of invasive species is a key aspect of environmental management targets, necessitating early detection and comprehensive distribution data for effective management. Environmental DNA (eDNA) has been demonstrated to enable sensitive monitoring, able to infer the presence of a target organism without physical observations and is particularly advantageous in aquatic environments where invasive species detection is challenging. The Chinese mitten crab (*Eriocheir sinensis*) is amongst the world's top 100 invasive species and is considered amongst the most damaging invasive species globally, causing significant detriment to riverbanks, fishing practices, and native populations, for example, through generalist predation and as a carrier of crayfish plague. In the UK, its distribution remains poorly understood, with current management relying on reporting of ad hoc sightings. This study developed and validated a species-specific qPCR assay for detecting *E. sinensis* eDNA against a standardized scale. Primer design utilized genome skimming of *E. sinensis* and related species collected in the UK, with the final assay achieving a detection limit of 15.6 copies/μL. Field tests in the UK detected target species eDNA at three sites with historical sightings, despite no recent visual records. Overall, the assay shows potential as a tool to support environmental monitoring and offer insights into the distribution, population dynamics, and invasion pathways, to support informed management of *E. sinensis*.

1 | Introduction

Invasive species are amongst the most important direct drivers of biodiversity decline worldwide (IPBES 2019; Jaureguiberry et al. 2022), and generate significant economic costs, either through direct impact on ecosystem services, or through remediation requirements. Aquatic invasions cost the global economy US$345 billion per year; however, this figure is likely to be underreported due to large gaps in taxonomic, geographic, and temporal recording of aquatic invasives (Cuthbert

et al. 2021). Damage that accrues to aquaculture, water management systems, as well as coastal and riverine facilities has a significant economic consequence, costing €12 billion each year to repair damages and control invasive species populations in Europe alone (European Commission 2008). Consequently, there is both a need and increasing pressure to prevent their introduction and spread. Early detection of recently introduced or establishing populations can help facilitate rapid responses to reduce ecological and economic impacts, as well as providing valuable information to support the identification of introduction pathways, and their subsequent management (Dougherty et al. 2016; Woodell et al. 2021). As the return on investment associated with management actions decreases dramatically throughout the course of the invasion process, emphasis is placed on prevention and rapid response (Leung et al. 2005). This is reflected in the European Union Marine Strategy Framework Directive (MSFD), which outlines methods to facilitate reducing the impact of invasive species through coordinated monitoring programmes (European Commission 2008), and in the UK's Environmental Improvement Plan 2023, aiming to reduce the number of invasive species establishments by at least 50% in 2030 compared to 2000 (DEFRA 2023). Prerequisites to achieve such targets include the collection of complete and accurate presence and absence data for the assessment of risk, and the introduction of early detection systems for new invasive species.

The Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853 is listed as one of the top 100 invasive species worldwide (Lowe et al. 2000) and cited in The Handbook of Alien Species in Europe (DAISIE 2009); it is considered to be amongst the most harmful invasive species in the world in terms of ecological, economic, and societal impacts (Lowe et al. 2000; Rudnick et al. 2005). Native to Southern China and Korea, it has now been reported from Northeast Europe, Russia, North America, and Canada (Veilleux and de LaFontaine 2007) and is estimated to have cost the global economy US$ 62.9 million since 1960 (Kouba et al. 2022). In the United Kingdom, the first substantial population was detected in the Thames Estuary in the late 1970s (Eno et al. 1997) and investigations into the genetic divergence between populations have indicated transport via ballast water to be the main introductory route (Herborg et al. 2006; Dittel and Epifanio 2009; Zhang et al. 2019). Annual downstream mass migrations, from freshwater to higher salinity water spawning grounds, impact heavily on fishing activities and cause wider ecological impact through damage to riverbanks and competition as a generalist predator (Veilleux and de LaFontaine 2007; Dittel and Epifanio 2009; Morritt et al. 2013). Further harm is likely to be caused to native species via the spillover effect of introduced pathogens (Foster et al. 2021). For example, as a carrier of the crayfish plague pathogen, *Aphanomyces astaci* Schikora, 1906, which has decimated populations of the endangered white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) throughout their European range (Füreder et al. 2010; Schrimpf et al. 2014). The known distribution of *E. sinensis* in the UK currently includes southern coastlines and major established populations in riverine systems including the Rivers Thames, Severn, Dee, Medway, Ouse, and Nene Washes (NBN Trust 2024a). Efforts to record their spread have traditionally included the use of fyke nets, electrofishing (May and Brown 2001; Schmidt et al. 2009), and accidental capture by anglers on rod and line, but the current known distribution is based on reporting of individual sightings (www.mittencrabs.org.uk). Capture methods are time-consuming, hard to scale, and lead to bycatch of non-target species, for example, the endangered European eel, *Anguilla anguilla* (Linneaus, 1758) (Clark et al. 2017). Further highlighting the importance of developing monitoring tools and elucidating introduction pathways, a cryptic species of *E. sinensis* has recently been discovered in the River Dee, Wales, and the full extent of their distribution is unclear (Palero et al. 2022).

Detection of target species through extraction of environmental DNA (eDNA) from samples such as soil, water, or air has become a powerful tool in biological monitoring (Blackman et al. 2024). Industry, researchers, and governments are increasingly incorporating eDNA into their monitoring toolkits because of the degree of sensitivity, cost-effectiveness, scalability, ability to measure "hard-to-se" and cryptic species, and ease of deployment by non-experts (Thomsen and Willerslev 2015). Many studies have now shown that molecular surveys can produce highly correlated data on species distributions compared to morphology-based surveys, and DNA-based data frequently informs management decisions (Hering et al. 2018; Ruppert et al. 2019; Yu and Matechou 2021), including management of invasive species, for example, signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), mosquito vectors (Schneider et al. 2016), Burmese python, *Python molurus bivittatus* (Kuhl, 1820) (Hunter et al. 2015; Harper et al. 2018), and green shore crab, *Carcinus maenas* (Linnaeus, 1758) (Roux et al. 2020).

Technologies related to eDNA have advanced rapidly, and consequently there is a considerable diversity in approaches to field sampling, laboratory validation, assay development, DNA extraction, and data interpretation across published studies. This variability poses challenges for end-users aiming to implement these assays confidently in monitoring activities. Recognizing the need for consistency and validation in methods based on eDNA, there is a push for standardization within the scientific community and amongst end-users engaged in on-the-ground surveys and decision-making processes. Thalinger et al. (2021) proposed a standardized validation scale to enhance the reliability and reproducibility of species-specific qPCR eDNA assays, transparently documenting the validation steps taken during the study to instill greater confidence amongst end-users when applying the assay for statutory monitoring purposes (Stein et al. 2023). In designing a robust qPCR assay, it is important to determine the assay specificity based on known sequence diversity, by including sequences from: (a) closely related and co-occurring species; (b) closely related but geographically distinct species; and (c) distantly related but co-occurring species which could co-amplify and produce false positive results (Thalinger et al. 2021).

qPCR is preferable to standard end-point PCR because the use of a probe increases the specificity, however it is also more expensive than traditional end-point PCR requiring only primers, or non-probe-based qPCR for example, with SYBR Green. A qPCR method has been previously developed for *E. sinensis*, using a High-Resolution Melt (HRM) peak analysis of a multiplex qPCR (Robinson et al. 2019). The possibility, however, of

amplifying homologous regions within closely related taxa increases the chance of non-specific detection with an HRM approach (Winder et al. 2011).

In the present study, we describe the development and subsequent validation of a qPCR assay for the detection of *E. sinensis* eDNA using complete mitochondrial genomes generated from *E. sinensis* specimens collected in the Thames at Erith, Kent, as well as UK-caught specimens of co-occurring and closely related crab species: (i) brush-clawed shore crab (*Hemigrapsus takanoi*, Asakura & Watanabe, 2005); (ii) Asian shore crab (*H. sanguineus*, De Haan, 1835); and (iii) green shore crab (*C. maenas*, Linnaeus, 1758). In-field testing was conducted to confirm the potential of the assay's use on filtered water and sediment from freshwater, brackish, and marine sites, and performance of the assay was validated against known distributions of *E. sinensis* from visual observations. This study provides the wider community with a species-specific assay to be used as a tool to assemble a fuller picture of *E. sinensis* distribution, detect further introductions and population expansions, and aid eradication and mitigation efforts.

2 | Materials and Methods

2.1 | Specimen Collection

Crab specimens were collected between 2018 and 2023, either by hand on the shoreline (turning over rocks) or trawling (Table 1). Five *E. sinensis* specimens were collected on the Thames Southbank, Surrey, at Hammersmith and used to extract DNA sequences for *E. sinensis* (July 28, 2022). Trawling was permitted by the Environment Agency and the Port of London Authority at Erith, Kent. Individual crabs were euthanised by placing them on ice prior to dissection for DNA extraction, conforming to UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039). Where fresh specimens could not be collected due to their rarity, DNA was extracted from specimens stored in ethanol from the collections of the Natural History Museum, London, as was the case for the *Eriocheir* cryptic species, *H. takanoi* and *H. sanguineus*. The *H. sanguineus* specimen used for mitogenome sequencing represents the only individual of this species collected in UK waters.

2.2 | DNA Extraction, Sequencing, and Assembly

DNA was extracted from crab pereiopod tissue using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol. To sequence the mitochondrial genomes of *E. sinensis*, *C. maenas*, *H. takanoi*, and *H. sanguineus*, extracted genomic DNA was quantified from each sample using the Qubit II fluorometer (Invitrogen) and 1 ng was used to prepare each sequencing library using the sparQ DNA Frag & Library Prep Kit (Quantabio) prior to sequencing on either an Illumina iSeq or MiSeq sequencing platform (illumina). Mitogenome assembly was conducted following the protocol as described in Briscoe et al. (2016) for genomic DNA (gDNA). Briefly, the sequence library was trimmed using default settings in Geneious Prime 2019.1.3 (Biomatters); the mitogenome was then assembled using available reference sequences (GenBank accession numbers: *E. sinensis* NC_006992; *H. sanguineus* KX456205; *H. takanoi* MW446895.1; *C. maenas* AY919125.1). Mismatches and gaps in alignment were subsequently iteratively re-mapped until circularization could be achieved. Gene boundaries were annotated with MITOS (Bernt et al. 2013) and open reading frames were visualized in Geneious Prime 2019.1.3 (Biomatters) which were compared against published mitochondrial gene alignments for verification.

2.3 | Assay Design

Thirty potential *E. sinensis* primer sets were generated using Primer 3 (Untergasser et al. 2012), based on the complete mitochondrial genomes generated in this study, using default parameters with the following modifications: primer length of 18–30 bp; product size 70–200 bp; primer Tm 55°C–65°C, with no more than ±2°C difference between the forward and reverse primers. Primers with secondary structures (e.g., hairpins) and primers producing amplicons that cross gene boundaries were excluded to avoid reducing qPCR efficiency (Dieffenbach et al. 1993). The primer pairs were checked for

TABLE 1 | Details of specimens collected for Chinese mitten crab qPCR primer design and analytical validation.

Family	Species	Life stage	Location	Date collected	NHM registration
Carcinidae	*Carcinus maenas*	Adult*	Ribble Estuary, Ainsdale	03/07/2019	NHM UK 2024.147
Carcinidae	*Carcinus maenas*	Adult	Greenhithe, Kent	07/06/2023	NHMUK 2024.109–120
Varunidae	*Eriocheir* sp. cryptic species*	Adult*	Dee, Estuary	25/09/2014	NHMUK 2024.104
Varunidae	*Eriocheir sinensis*	Adult	Erith, Kent	04/12/2018	See McGoran et al. (2020)
Varunidae	*Eriocheir sinensis*	Subadult	Hammersmith, London	28/07/2022	NHMUK 2024.105–108
Varunidae	*Hemigrapsus sanguineus*	Adult*	Osmington Mills, East of Weymouth, Dorset	21/08/2020	NHMUK 2024.121
Varunidae	*Hemigrapsus takanoi*	Adult*	Thanet Coastline, Kent	15/09/2016	NHMUK 2017.21–22

Note: Specimens marked with * were sourced from the collections at the Natural History Museum.

TABLE 2 | Details of CMC_2 qPCR primers and probe for Chinese mitten crab designed in this study.

Name	Description	Sequence	Product size (bp)	Location-Gene
CMC_2F	Forward primer	5′-GAAGTATCAGGGTTCCGGCT-3′	163	COX2
CMC_2R	Reverse primer	5′-TTGATTGATTTAGCCGCCCG		
CMC_2_P	Probe	5′-/6-FAM/TCCATTCCTGGACAGTCCCATCCT/BHQ1/-3′		

cross amplification against online reference libraries with Primer Blast (National Center for Biotechnology Information (NCBI) 2024). All primers generated, meeting the specified parameters, were located in cytochrome c oxidase subunits 1 (COX1) or 2 (COX2). Sequences were aligned with the mitochondrial genomes generated in this study of closely related or abundant co-occurring crabs to investigate mismatches in the primer regions including *E. japonica*, *E. hepuensis*, *H. sanguinensis*, *H. takanoi*, *C. maenas* (Geneious "MultipleAlign" tool; Clustal Omega). All primers designed had at least 2 mismatches per primer binding site and variability in the probe region to increase the specificity of the assay (Table S1). Based on the laboratory validation steps described in this study, primer set CMC_2 (Table 2) was taken forward as it performed well in terms of efficiency, LOD and LOQ, and was highly specific to *E. sinensis*. Additionally, the slightly longer amplicon length can be preferable if confirmatory Sanger sequencing of amplicons is required. All other primer sets were discounted as they did not meet the standards for specificity to varying degrees. The final assay, including forward and reverse primers and the hydrolysis probe, was also tested against the eDNAssay machine learning tool (Kronenberger et al. 2022) as an additional validation step, predicting a negligible risk of non-target amplification in the other crab species (Table S2). Hydrolysis probes were generated using Geneious Prime Software 2022.2.1 (Biomatters Ltd.) to be specific to *E. sinensis*.

2.4 | Laboratory Specificity and Efficiency Testing

Genomic DNA from *E. sinensis*, *C. maenas*, *H. takanoi*, *H. sanguineus*, and *Eriocheir* cryptic sp. was normalized to 1 ng/μL for qPCR specificity testing. Molecular grade water was used for no template controls (NTCs). Quantitative PCR cycling conditions involved an initial denaturing step of 95°C for 10 min, then 40 cycles of 95°C for 30 s and 60°C for 1 min. Each reaction comprised 1× TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 0.4 μM CMC_2_F and CMC_2_R primers, 0.1 μM CMC_2-Probe, 2 ng template DNA, and molecular grade water up to a final volume of 15 μL. The experiment was carried out on a StepOnePlusTM Real-Time PCR instrument (ThermoFisher Scientific). The qPCR was analyzed in triplicate. A synthetic DNA sequence (gBlock Gene Fragment, IDT) was developed in-house for use as a positive control, consisting of the mitten crab specific primer and probe sequences and random nucleotides not matching any known sequence (full sequence in Supporting Information S1). Optimum annealing temperature was ascertained by gradient PCR. Efficiency testing was carried out with serial dilutions from $\times 10^7$ to $\times 10^0$ ng/μL of *E. sinensis* synthetic DNA, each in triplicate with triplicate NTCs.

The limit of detection (LOD) and limit of quantification (LOQ) were ascertained based on Forootan et al. (2017), by running 20 replicates of a 12-point, 1 in 2 dilution series of the synthetic sequence, starting at 1000 copies/μL. Only Ct values below 40 were accepted as a positive amplification. The LOD was measured as the concentration that produces at least 95% positive replicates and was calculated by plotting the fraction of positive replicates (where $n = 20$) against the concentration of each dilution. An approximation of LOD was made by fitting a line at $y = 95$, with data points above this deemed detectable. In this case, concentrations with fewer than 19/20 detections were deemed below the LOD. The LOQ was calculated based on the mean and standard deviation of Ct values for replicates at each concentration. The coefficient of variation (CV) was calculated using CV = 100 × standard deviation/mean, whereby negative amplification replicates were set to zero. Plotting percentage CV against concentration allowed for estimation of LOQ based on a threshold line at $y = 35$. Data points below this were deemed quantifiable. This threshold can vary depending on sample complexity, but a CV ≤ 35% has previously been suggested by TATAA Biocenter (Forootan et al. 2017).

2.5 | Pilot Field Testing: Filtered Water and Sediment Samples

Environmental samples used in this study were collected from riverine, estuarine, and marine locations (Figure 1; Table 3). Sampling was carried out at high tide during the late summer and autumn (end of July to end of September). Sampling periods and locations were selected to coincide with the annual spawning and migration of juvenile *E. sinensis* upstream from marine sites, meaning eDNA was expected to be detected from larvae, juveniles (Clark 2011; Morritt et al. 2013; Kamanli et al. 2018) and likely increased adult shedding during spawning as witnessed in other Brachyuran species (Crane et al. 2021). Sites were chosen based on records of confirmed sightings of the target species (Table S3 for details of records). The Thames Southbank, Chelsea, London, was chosen as a positive control site for environmental samples as it has a well-established population of the target species that is monitored routinely. Two historic sightings of *E. sinensis* individuals were confirmed by the NBN Trust (2024a) from Gosport (2010) and Southampton (2018). These isolated records would suggest that this invasive was not established in the Solent, Hampshire area. Consequently, sampling in this waterway was considered a test control site.

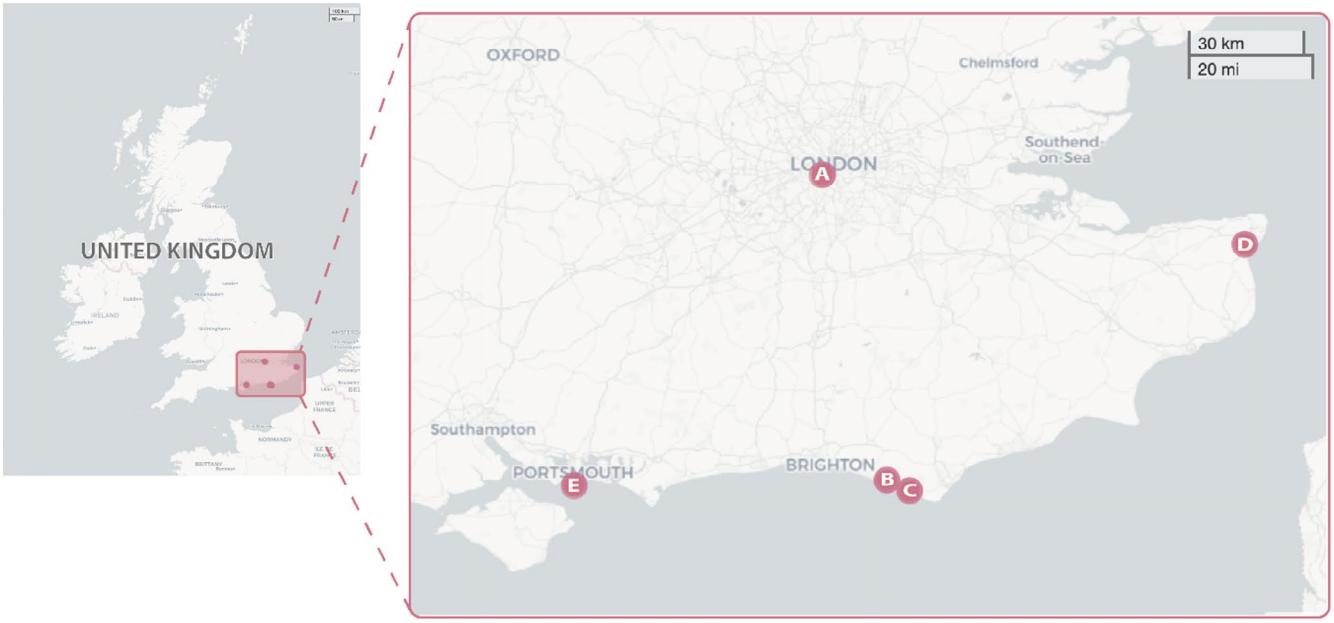

FIGURE 1 | Map of eDNA sampling sites in England, United Kingdom, as follows: (A) Thames Southbank, Chelsea, London; (B) Mill Creek, Newhaven; (C) Cuckmere Haven, East Sussex; (D) Pegwell Bay, Kent; (E) Solent, Hampshire. Map projection WGS84.

2.6 | eDNA Sampling and Extraction

Water samples were collected in the intertidal zones of the shore at low tide. A maximum of 6 water samples, dependent on access, were taken at each sampling location. At each site, 0.26–2 L of water was filtered per sample depending on turbidity. Samples were collected using NatureMetrics eDNA Aquatic Kits (consisting of sterile 5 L plastic collection bags and 60 mL syringes, filtered with 0.8 μm Polyethersulfone filters with Longmire's preservative solution), transported on ice, and stored at −20°C prior to extraction. Sediment samples were collected where access to the foreshore was possible (Thames Southbank, Mill Creek, Cuckmere Haven and Pegwell Bay). Approximately 20 g of sediment was collected with a sterilized spatula and stored in ethanol at −20°C prior to extraction. For both sample types, freeze–thaw cycles were kept to a minimum, and all samples were extracted within 1 week of collection. Measures were taken to avoid contamination when collecting samples, including the use of sterile gloves and sampling from downstream so as not to contaminate or disturb the sediment. No target species were handled while sampling for eDNA, and during sampling at Thames Southbank, Chelsea, London, roles were split between personnel for eDNA and specimen collection, with specimens collected after eDNA sampling to minimize contamination and disturbance. No in-field negative controls were used, limiting the ability to detect contamination introduced during sampling.

DNA was extracted from the 0.8 μm PES filters using a DNeasy Blood and Tissue Kit (Qiagen) following a modified version of the Spens et al. (2017) SXcapsule method for disc filters in buffer, with proteinase K added directly to the filter housing to minimize the risk of contamination arising from handling of the filter. A negative extraction control, consisting of molecular grade water, was processed with each batch of samples to monitor for exogenous DNA contamination. Sediment samples were extracted using a DNeasy Powersoil kit (Qiagen) according to the manufacturers' protocol. An extraction blank containing no sample was processed with each batch of samples to assess potential contamination in the extraction process. Purified DNA extracts from both water and sediment samples were quantified using the Qubit dsDNA Broad Range and High Sensitivity Assay Kit, respectively, on a Qubit 3.0 fluorometer (Thermo Scientific).

2.7 | eDNA qPCR Analysis and Inhibition Testing

The *E. sinensis* qPCR reaction for eDNA samples was conducted in a final reaction volume of 15 μL, comprising 1× TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 0.4 μM CMC_2_F and CMC_2_R, 0.1 μM CMC_2_P, 2 μL eDNA sample, 0.2× IPCC Master Mix (Eurogentec), 0.5× IPCC DNA (Eurogentec) and molecular grade water. Cycling conditions consisted of an initial denaturation of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. qPCR runs were carried out on a LightCycler480 Instrument (Roche). To ensure consistent performance of the assay across qPCR cyclers, a synthetic standard curve was run in triplicate on all plates to measure assay efficiency; all qPCR runs performed within the expected R^2 and efficiency thresholds. Each eDNA sample was analyzed across six replicates. Successful amplification, below a Ct of 40, in one of the six replicates deemed a sample as positive for *E. sinensis*. Following Klymus et al. (2020) all positive amplifications were deemed to be suspected qualitative detections, even if below the reported LOD. In addition, each plate had NTCs and a six-point 1:10 serial dilution of synthetic DNA ranging from 1×10^6 to 1×10^1 copies/μL run in triplicate. This serial dilution was used to generate a standard curve for calculation of assay efficiency and quantification of target DNA in a sample. Extraction blanks were processed under the same conditions to control for contamination during DNA extraction. The Eurogentec IPCC (Cy5-QXL670 Probe) was used to test for the presence of PCR inhibitors in our samples, based on the "Ct shift" method whereby a mean shift of greater than two Ct values compared to the NTCs (no eDNA sample) was considered a sign of inhibition.

TABLE 3 | Locations and details of sampling, corresponding to Figure 1 map labels.

Figure 1 map label	Sample site	Site Description	Type of environment	Latitude (DD)	Longitude (DD)	Total water samples	Total sediment samples	Date of sampling
A	Thames Southbank, Chelsea, London	Mixed sand, mud and stony substrate with retaining walls. Part of the tidal Thames with brackish water environment	Brackish	51.485175	−0.152438	6	0	26/09/2022
B	Mill Creek, Newhaven	Industrial area with surrounding countryside. Deep mud with wide banks at low tide	Brackish	50.783333	0.068618	3	5	09/08/2022
C	Cuckmere Haven, East Sussex	Streams with low flow sheltered areas leading to large stony/ shingle beach	Brackish to Marine	50.759000	0.148300	6	6	09/08/2022
D	Pegwell Bay, Kent	Edge of salt marsh into sandy beach. Fine silt/mud with vegetation patches	Brackish to Marine	51.323456	1.366256	6	6	10/08/2022
E	Solent, Hampshire	Beach, docks and river locations. (1) Hamble River (2) Hill Head Beach (3) Camber Dock Port (4) Langstone Village	(1) Brackish (2) Marine (3) Brackish (4) Brackish	(1) 50.878454 (2) 50.817259 (3) 50.792428 (4) 50.80482	(1) −1.299253 (2) −1.246874 (3) −1.107039 (4) −1.006059	4	0	19/07/2022 to 21/07/2022

3 | Results

3.1 | Specificity, Assay Efficiency and LOD/LOQ

The assay developed in this study (CMC_2) (Table 2) amplified *E. sinensis* from tissue DNA extractions (Average Ct 17.6) with no non-target amplifications for *C. maenas*, *H. takanoi*, *H. sanguineus*, or *Eriocheir cryptic sp*ecies. The primers had a high assay efficiency (97.48% amplification efficiency; R^2 0.9986) (Figure 2). The addition of the exogenous control IPCC had no significant effect on assay efficiency. The LOD and LOQ were calculated at 15.6 copies/μL, respectively (Figures S1 and S2; Table S4).

3.2 | Field Testing

Field testing detected *E. sinensis* eDNA in filtered water and sediment samples at all sites of expected establishing populations (Mill Creek, Cuckmere Haven and Pegwell Bay), despite no individuals of the target species being observed during sampling (Figure 3, Table 4). There were strong detections of *E. sinensis* eDNA at the Thames Southbank (all six filtered water samples had 6/6 positive qPCR replicates), where many mitten crabs were collected and positively identified. eDNA of *E. sinensis* was detected in fewer sediment samples compared to water samples from the same locations (Table 4). There were no positive detections in the Solent, where *E. sinensis* is not yet believed to be established (NBN Trust 2024a). All extraction blanks and NTCs showed no amplification. During sampling, a total of 89 juvenile mitten crabs were observed during the 45-min sampling window at the Thames Southbank positive control site, and none were observed at other sites. However, *C. maenas* were frequently observed at all marine locations.

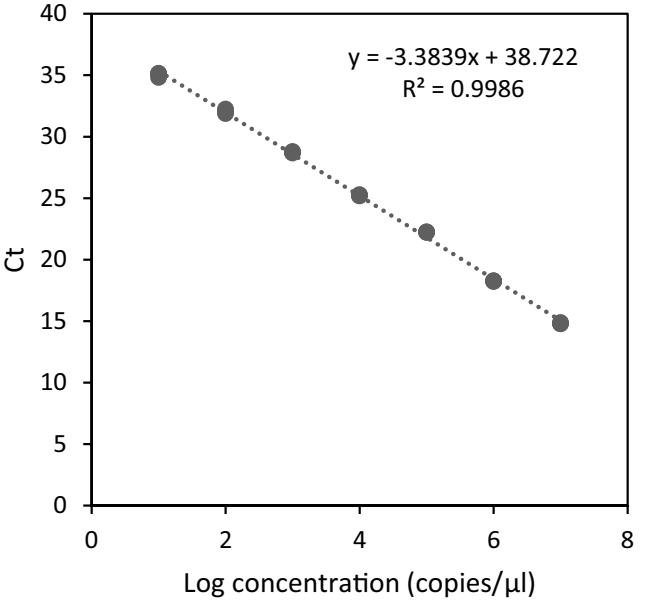

FIGURE 2 | Assay efficiency for *E. sinensis* species-specific qPCR assay based on serial dilutions of *E. sinensis* synthetic DNA from $10^7 \times 10^0$ ng/μL. Trendline equation and R^2 value displayed on chart.

4 | Discussion

4.1 | Summary of Results

In the present study, we have developed a new qPCR assay for detecting *E. sinensis* eDNA using mitogenomes from UK-caught specimens of target and non-target species and conducted initial field testing on water and sediment samples from five sites in the UK. Validation was conducted according to the steps recommended by Thalinger et al. (2021) on which it would receive a level 4 validation score. Additionally, we generated mitochondrial genomes from local specimens for populations in their invasive range to improve the reference database for UK specimens and enhance the robustness of primer design. The *E. sinensis* assay's LOD and LOQ were determined to be 15.6 copies/μL, which is within the range reported for other qPCR assays designed for high sensitivity species-specific detection (Matejusova et al. 2021; Moyer et al. 2023). For those sites where sediment sampling was possible, there were fewer detections of *E. sinensis* DNA compared to filtered water from the same site.

4.2 | Validation of the Assay Using UK Specimens

Included in the species-specific qPCR assay design for *E. sinensis* were non-target species of brachyuran crabs, maximizing sequence diversity in primer design and using the tissue DNA extractions for specificity testing. These included: *H. takanoi*, *H. sanguineus*, a newly discovered *Eriocheir* cryptic species, and *C. maenas*. The invasions of brush-clawed and Asian shore crabs were first discovered in the UK in England in 2014 (Seeley et al. 2015; Wood et al. 2015). Both *Hemigrapsus* species have been recorded throughout the rest of the UK and Europe, though *H. sanguineus* is not yet considered an established invasive species in Great Britain (NBN Trust 2024b). Since 2014, there have been seven reported UK sightings of *H. takanoi*, reported near the Thames Estuary and Kent (NBN Trust 2024c), and six reported sightings of *H. sanguineus*, and only one official caught specimen (NBN Trust 2024b). The presence of a second cryptic *Eriocheir* species was confirmed in 2022 by morphological and molecular data and is recorded to have been introduced independently from *E. sinensis* through Dutch dredging activities in the early 2000s, resulting in a population inhabiting the Dee Estuary, Wales (Palero et al. 2022). The green shore crab (*C. maenas*) is the most dominant crab species co-occurring with UK *E. sinensis* populations (NBN Trust 2024d) and it was thus included in the specificity analysis despite being a more distant relative to the target organism. Observation during field sampling confirmed the dominance of *C. maenas* in coastal sites. Sequencing of the full mitochondrial genomes of *E. sinensis*, *H. takanoi*, *H. sanguineus*, and *C. maenas* allowed for a more robust primer design considering the lack of suitable genomic data available in online repositories and adds to UK reference databases for these species. Incorporating the recently identified *Eriocheir* cryptic species into the specificity testing confirmed that the assay does not amplify this species.

4.3 | Using the qPCR Assay in Invasive Species Management

Given the promising results of this testing, the assay could be used to further elucidate the distribution of mitten crabs

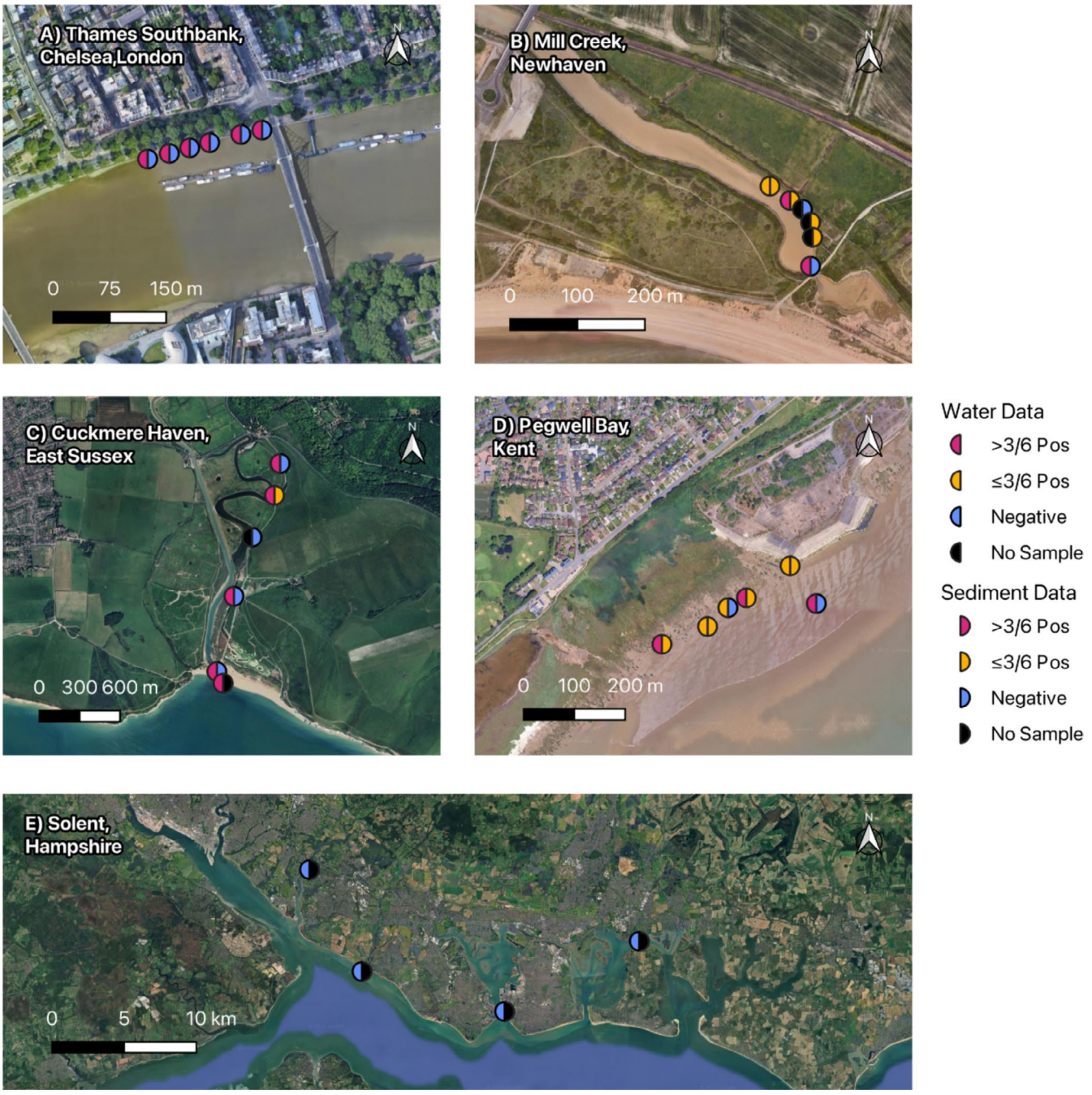

FIGURE 3 | qPCR results from filtered water and sediment samples from field sites in England, showing field replicates for detection of Chinese mitten crab eDNA using the *E. sinensis* species-specific qPCR assay designed in this study.

elsewhere in the UK. To advance the assay's readiness for deployment, further in-field testing is recommended, particularly in sites with known population sizes, and including negative field blanks to further understand the rate of false positives and negatives. With additional modeling of detection probabilities, this assay would be useful for early warning signals of an expanding range, or testing introductory pathways (e.g., by testing ballast water) (Darling and Frederick 2018). Once fully operational, application of the assay could aid conservation management of the endangered native white-clawed crayfish (*A. pallipes*) by identifying rivers less likely at risk of pathogen transfer, and therefore more suitable for reintroduction programmes. This could be in addition to current monitoring for reintroduction schemes, which do not currently consider the risk of *E. sinensis* presence as carriers of the crayfish plague pathogen (*A. astaci*) (Chucholl et al. 2021; Casabella-Herrero et al. 2023).

To increase the adoption of eDNA surveys for biodiversity monitoring outside of research, end-users call for methods to be standardized and show a high level of validation (Darling et al. 2017). The need for standardized assay validation will become critical as eDNA approaches develop towards use as fundamental management tools for invasive species, bioindicators, pathogens, or species at risk of extinction (Makiola et al. 2020; Cordier et al. 2021). The assay validation performed in this study meets the minimum criteria for "Level" validation on the scale

TABLE 4 | Quantitative PCR results of field testing with *E. sinensis* qPCR species-specific assay.

Sample location	Sample type	Field replicate	Water volume filtered (ml)	qPCR replicate concentration (copies/μL)						No. detections (/6)
				1	2	3	4	5	6	
Thames Southbank, Chelsea, London (positive control site)	Filtered water	1	1000	**23.87**	**17.30**	9.68	2.20	**17.92**	5.93	6
	Filtered water	2	1000	**25.60**	10.90	13.73	6.06	8.53	**23.05**	6
	Filtered water	3	550	7.02	**20.75**	12.02	12.11	**16.71**	10.38	6
	Filtered water	4	260	**43.56**	**18.42**	**22.89**	7.17	7.12	4.27	6
	Filtered water	5	350	**29.03**	**22.89**	**24.54**	**23.37**	**24.20**	11.86	6
	Filtered water	6	300	**18.04**	9.09	11.06	6.10	**21.79**	7.12	6
	Sediment	1								0
	Sediment	2								0
	Sediment	3								0
	Sediment	4								0
	Sediment	5								0
	Sediment	6								0
Mill Creek, Newhaven	Filtered water	1	1000			2.39				1
	Filtered water	2	1000	**333.55**	**328.92**	**219.22**	**262.95**	**205.84**	**186.64**	6
	Filtered water	3	1000	**18.55**	**27.45**	**21.34**	6.63	14.22	5.93	6
	Sediment	1						12.65		1
	Sediment	2		10.31	**17.42**			5.94		3
	Sediment	3								0
	Sediment	4		**40.82**						1
	Sediment	5		12.82						1
	Sediment	6								0
Cuckmere Haven, East Sussex	Filtered water	1	1000	6.59	13.35		2.21	5.85		4
	Filtered water	2	1000	9.41	7.58	3.51	4.12	3.14		5
	Filtered water	3	800	**89.54**	**113.58**	**101.55**	**126.14**	**112.00**	**61.80**	6
	Filtered water	4	1000	**22.10**	**19.76**	**18.55**	**28.23**	12.63	**24.03**	6
	Filtered water	5	1000	10.60	2.23	3.93	11.53	2.53		5
	Sediment	1								0

(Continues)

TABLE 4 | (Continued)

Sample location	Sample type	Field replicate	Water volume filtered (ml)	qPCR replicate concentration (copies/μL)						No. detections (/6)
				1	2	3	4	5	6	
	Sediment	2				13.82				1
	Sediment	3								0
	Sediment	4								0
	Sediment	5								0
Pegwell Bay, Kent	Filtered water	1	1000	5.53			1.91		0.51	3
	Filtered water	2	1000	**24.20**	15.25	10.09	13.73	**24.03**	8.30	6
	Filtered water	3	1000	2.08			7.96	5.97	2.26	4
	Filtered water	4	1000	2.14				2.15		2
	Filtered water	5	1000	3.64	3.25		6.23	1.78	4.39	5
	Filtered water	6	1000	**69.60**	**56.43**	**96.02**	**70.58**	**81.75**	**53.73**	6
	Sediment	1		14.20						1
	Sediment	2		**24.16**						1
	Sediment	3								0
	Sediment	4				7.09				1
	Sediment	5				7.75				1
	Sediment	6								0
Solent, Hampshire	Filtered water	1	2000							0
	Filtered water	2	800							0
	Filtered water	3	2000							0
	Filtered water	4	2000							0

Note: Detections above the calculated LOD (15.6 copies/μL) are highlighted in bold.

proposed by Thalinger et al. (2021) ("Substantial"). To obtain a full Level 5 validation score, there would need to be some statistical modeling of detection probability and further understanding of the ecological and physical factors influencing eDNA in the environment.

4.4 | Interpreting qPCR Detections Into Wider Ecological Contexts

Understanding how qPCR detections translate into actual species presence requires consideration of the ecological and physical factors that influence eDNA dynamics in the environment. It has been suggested that for some invertebrates including true crabs and crayfish, eDNA detection is hindered by low DNA shedding rates, but these rates vary depending on the degree to which individuals are most active and stressed, and the stage of their reproductive and migratory cycles (Forsström and Vasemägi 2016; Dunn et al. 2017; Stewart 2019). Additionally, as *E. sinensis* are catadromous, the eDNA sampling will occur across a gradient of freshwater, brackish, and marine habitats, which poses complications in modeling the detection probabilities across such varying systems. To date, there is more research into freshwater eDNA, and less is understood about the variation in eDNA signal in the marine environment (Shogren et al. 2017; Snyder et al. 2023). Additionally, eDNA decay and persistence rates can vary between species and ecosystems, and these dynamics have not been investigated for Chinese mitten crab, leaving gaps in our understanding that make interpreting eDNA results for this species somewhat challenging.

eDNA records are beginning to be included alongside conventional biodiversity records in repositories such as the Atlas of Living Australia (Belbin et al. 2021), and the Global Biodiversity Information Facility (GBIF) (GBIF Secretariat 2024). Transparency in how eDNA observations are derived will be key to their acceptance by database users. Although care should be taken to compare results between surveys, some degree of association may be possible for other studies using the same validation scale (e.g., Moyer et al. 2023; Nolan et al. 2023; Chevrinais and Parent 2023; Simpson et al. 2023). While eDNA provides a sensitive means of detecting species presence, physical evidence may still be required to support eradication efforts or regulatory decisions, as eDNA data alone is not yet accepted in some management or legal frameworks for invasive species (Royal Society 2025).

4.5 | Sediment Versus Filtered Water

Variability in physical environmental factors can lead to the DNA signal of the same species being variously present or absent from different sampling matrices (e.g., sediment and water) in the same location, leading to contradictory conclusions (Nevers et al. 2020; Aalismail et al. 2021; Lanzén et al. 2021). eDNA of macrofauna present in sediments will likely come from DNA traces originating from tissue fragments, secretions, free cells, organelles, or extracellular DNA molecules (Pawlowski et al. 2022). These traces may be too fragmentary for detection in the few grams of sediment that are used in a standard sediment DNA extraction. Despite this, some previous studies have shown that sediment samples can result in higher detection rates than water sampling for certain species (Hartikainen et al. 2016; Shogren et al. 2017), including with other brachyuran crabs (Forsström and Vasemägi 2016). These trends are not widely researched, and the opposite was observed from the *E. sinensis* results of the present study.

The lower detectability observed in sediment samples, and the improved practicality makes water sampling preferable for *E. sinensis* monitoring. Limited access to foreshores made collection of all six water and sediment replicates at certain locations challenging. Sediment sampling was also more difficult where the substrate was coarse, sandy, or stony, as was the case at Cuckmere Haven (Stoeckle et al. 2017). Use of on-site filtering to obtain larger water samples overcomes the portability issues often associated with water sampling (Darling et al. 2017; Hunter et al. 2018). Furthermore, the filters can be stored in the preservation buffer at room temperature until they can be frozen in the laboratory. These practical qualities make water sampling preferable to sediment sample collection for the implementation of this eDNA tool.

5 | Conclusion

This present study has demonstrated that, in addition to effectively detecting *E. sinensis* eDNA, a standardized method can be successfully applied for the development and validation of qPCR assays in environmental samples. The use of reference genomes from local populations enhances the reliability of the assay for UK management contexts and improves UK reference databases for these species. With further in-field validation and detection probability modeling, continued testing across UK catchments could help to build a clearer picture of Chinese mitten crab distribution and population establishment. This technique may also support management efforts aimed at mitigating their detrimental and costly impacts, including the introduction of pathogens and the degradation of native habitats, ecosystems, and infrastructure.

Author Contributions

A.G.B. conceptualized the idea and acquired funding for the project. L.S.J.C., A.G.B., and M.A.W. designed the qPCR primers and detailed methodology. L.S.J.C., M.A.W., D.M., and P.F.C. conducted field sampling. P.F.C., P.S., and D.M. advised on field sampling locations. L.S.J.C., M.A.W., and A.G.B. conducted the laboratory work and analyzed the data. P.F.C. provided taxonomic expertise for specimens stored and newly collected. L.S.J.C. wrote the first draft of the manuscript. A.G.B., M.A.W., D.B., P.S., and P.F.C. edited and provided feedback. All authors gave final approval for publication.

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Ethics Statement

Euthanising of crabs conformed to UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

On acceptance of the paper, the raw mitogenome sequencing data will be uploaded to the sequence read archive (SRA).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370166-sup-0001-Supinfo.docx.