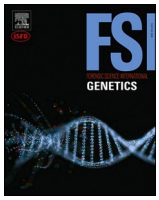
Forensic Science International: Genetics 87 (2027) 103598

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen



Forensic environmental DNA (eDNA) validation for protected species: An integrated framework for wildlife crime investigation

Matthew Lewis [a,b,*], Catherine Moir [a], Kirstie Scott [b,c], Stefano Mariani [b,c], Bernd Hänfling [d], Nick Dawnay [a,b]

[a] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, United Kingdom
[b] Forensic Research Institute, Liverpool John Moores University, Byrom Street, Liverpool, United Kingdom
[c] School of Biological and Environmental Sciences, Liverpool John Moores University, Byrom Street, Liverpool, United Kingdom
[d] Institute for Biodiversity and Freshwater Conservation, University of the Highlands and Islands, Inverness, United Kingdom

ARTICLE INFO

Keywords:
Environmental DNA
Forensic validation
Freshwater pearl mussel
Multiplex PCR
Wildlife crime

ABSTRACT

Environmental DNA (eDNA) analysis enables non-invasive detection of aquatic species, but no established framework integrates eDNA best practices with forensic validation standards. The critically endangered freshwater pearl mussel (*Margaritifera margaritifera*) exemplifies the need for forensically validated eDNA assays as traditional survey methods are invasive and time-consuming. This research provides a framework that bridges both ecological monitoring standards and forensic legal admissibility requirements in wildlife crime investigations. Here we describe the validation of a multiplex quantitative PCR (qPCR) assay targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene, using the highest forensic standards and eDNA best practice. *In silico* and *in vitro* testing confirmed species specificity, with alignment against reference sequences showing 100% identity. The multiplex assay LOD and LOQ were calculated at 2.5 and 3.14 copies/μL respectively, determined by the performance of the short fragment. The assay demonstrated robust repeatability (R^2, 0.986–1.000), precision (coefficient of variation, 6–28%), and accuracy (recovery, 62–137%), meeting forensic thresholds. Field validation detected a previously undetected Welsh population through intelligence-led sampling, while blind proficiency testing in the Lake District achieved 95.8–100% detection rates across juvenile release sites and successfully detected distribution patterns later confirmed by stakeholders. We establish the first forensic eDNA validation of a protected species, providing a transferable methodology for conservation, regulatory enforcement, and wildlife crime investigations, ready for deployment in an operational environment. We believe that this integrated approach can be transferred to similar forensic and conservation scenarios across a broad range of species and geographical contexts.

1. Introduction

Freshwater pearl mussels, *Margaritifera margaritifera*, are bivalve molluscs that live in clean, fast-flowing freshwater streams and rivers. The protection of *M. margaritifera* is critical, both to conserve the species themselves and to maintain the freshwater river environments on which they and other species depend [1]. The species is listed as endangered on the International Union for Conservation of Nature (IUCN)'s Red List [2], and is protected in the European Union [3] and the United Kingdom under *The Wildlife and Countryside Act 1981* [4], where they have been a priority for the National Wildlife Crime Unit since 2018 [5]. Despite this legal protection, populations face persistent threats from river engineering, pollution, poaching, and other deliberate illegal activities [6], necessitating tools capable of supporting both wildlife crime investigations and conservation management.

To protect *M. margaritifera* and prosecute any crimes relating to their disturbance, their presence at sites of disturbance must be confirmed. Traditional sampling methods to detect *M. margaritifera* include manually surveying the riverbed using a bathyscope, snorkelling, or SCUBA diving [7]. Traditional surveys also require a protected species licence, limiting the pool of practitioners available to support investigations, whereas eDNA sampling can be conducted by non-specialists following standard protocols. However, these methods are dependent on practitioner experience, may cause disturbance, and are challenging in turbid

* Corresponding author at: School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, United Kingdom
E-mail address: lewismattty@live.co.uk (M. Lewis).

https://doi.org/10.1016/j.fsigen.2026.103598
Received 11 May 2026; Received in revised form 3 July 2026; Accepted 28 July 2026
Available online 29 July 2026

waters [8,9]. Furthermore, *M. margaritifera* burrow into benthic substrates and, while adults tend to remain partially visible, juvenile populations are fully buried, rendering them visually undetectable [7]. Low-density and cryptic populations may be missed, while surveyors also face safety risks in fast-flowing or deep water. These limitations emphasise a need for non-invasive, sensitive detection methods that can inform both conservation decision-making and evidential casework.

Environmental DNA (eDNA) analysis offers a non-invasive, specific, sensitive approach for detecting aquatic species through genetic material shed into the environment [10–12]. Quantitative PCR (qPCR) methods provide accessible, sensitive, cost-effective tools for species-specific detection [13], with extensive applications in aquatic conservation monitoring [14–17]. eDNA validation guidelines [18,19] outline rigorous method development but focus primarily on ecological monitoring rather than legal admissibility. Forensic validation guidelines, such as those provided by the Scientific Working Group on DNA Analysis Methods (SWGDAM) in North America [20] and the European Network of Forensic Science Institutes (ENFSI) [21], provide frameworks for court-admissible evidence but lack protocols specific to eDNA analysis. This creates a validation gap: no established framework bridges both eDNA and the forensic standards required for wildlife crime prosecution. While forensically validated DNA extraction kits [22] and capture method optimisation for human eDNA [23] support pre-analytical aspects of forensic eDNA work, species-specific qPCR assays require development and forensic validation to support potential admissibility in criminal proceedings.

Multiplex qPCR assays targeting different eDNA fragment lengths provide complementary information for forensic applications. Short fragments are hypothesised to maximise detection sensitivity through extended environmental persistence, while long fragments may offer improved spatial resolution for source localisation due to faster degradation [24–28]. This study therefore aimed to validate a multiplex qPCR assay for *M. margaritifera*, incorporating three amplification targets: a long fragment (180 bp), a short fragment (83 bp), and an internal positive control (IPC) for inhibition detection. The approach integrated forensic standards [20] with qPCR and eDNA best practices [18,19,29,30]. Objectives were to 1) assess specificity, sensitivity, repeatability, standard consistency across tissue sources, robustness, and inhibition tolerance; 2) evaluate operational capability through directed field testing and blind proficiency testing; and 3) establish a transferable framework for forensic eDNA validation of protected species to support wildlife crime investigation and conservation management.

2. Methods

2.1. Assay development

2.1.1. qPCR assay development

An optimised qPCR assay was developed with each reaction containing 1 × TaqPath™ ProAmp™ Multiplex Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), oligonucleotides as described in Table 1, and 4 μL of DNA (or nuclease-free water as a no-template control (NTC)) in a 20 μL reaction. The primers amplified 83 bp (short) and 180 bp (long) fragments of the *M. margaritifera* mitochondrial COI region [31], and the IPC template. Primers and probes were designed using National Centre for Biotechnology Information (NCBI) tools [32] or taken from existing literature [33,34].

Amplification was performed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with an initial activation step of 95 °C for 10 min, followed by 50 cycles of 90 °C denaturation for 15 s and annealing/extension at 60 °C for 60 s. The data were then processed in Q-Rex software, with the quantification cycle (Cq), which reflects the position of the amplification curve relative to the cycle number [35], determined using thresholds set at 0.01 for the short fragment, 0.008 for the long fragment, and 0.035 for the IPC to maintain consistency between different runs.

Table 1
Oligonucleotides used in this study to amplify the *M. margaritifera* short fragment (83 bp), long fragment (180 bp) and internal positive control (IPC). Concentrations indicate the final concentration (μM) of each oligonucleotide in the 20 μL reaction (IPC template in nM).

Name	Type	Sequence	Concentration
SF	Primer	5′-TTGTTGATTCGTGCTGAGTTAGG–3′	0.1 μM
SR	Primer	5′-GCATGAGCCGTAACAATAACATTG–3′	0.1 μM
LF	Primer	5′-TGGTGGAGAGTGGTGTTGGT–3′	0.4 μM
LR	Primer	5′-CCACTCCCGGAGAACGTATG–3′	0.4 μM
SP	Probe	[JOE] 5′-CCTGGTTCTTTGCTGGGTGATGATC–3′ [BHQ1]	0.5 μM
LP	Probe	[FAM] 5′-TTTCTTTGCACCTTGCCGGTG–3′ [BHQ1]	0.5 μM
IPC-F	Primer	5′-CGCGAGATACACTGCCAGAA–3′	0.5 μM
IPC-R	Primer	5′-GACCACAGCCAGATTAAATTTACCA–3′	0.5 μM
IPC-P	Probe	[ROX] 5′-TCCGGTGATTACGAGTCCGTATCG–3′ [BHQ2]	0.5 μM
IPC	Template	5′-AACGCGAGATACACTGCCAGAAAGCCAATCCGCGTGATTACGAGTCCGTATCGCCTGAGCCAGTGTGGTAAATTTAATCTGGCTGTGGTCAA–3′	0.002 nM

All oligonucleotides were supplied by Eurofins Genomics (Ebersberg, Germany).

2.1.2. Generation of qPCR DNA standards

DNA was extracted from three approximately 25 mg tissue samples of *M. margaritifera* sourced from a licensed hatchery using a QIAgen Blood & Tissue Kit (QIAgen, Hilden, Germany). These extracts were used to amplify a 427 bp fragment of the *M. margaritifera* COI gene containing both the short and long target fragments of the multiplex assay. Each reaction consisted of 1 × MyFi reaction mix (Meridian Bioscience, Cincinnati, OH, USA), 20 mM SF primer, 20 mM LR primer, 2 μL DNA, and nuclease-free H_2O up to 20 μL per reaction, alongside a negative control. PCR conditions were optimised to 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. PCR products were run on a TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA) and quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific). Amplified product was normalised to 10,000 copies/μL prior to use as the amplicon standard in qPCR. Tissue-derived DNA standards (normalised to 10 ng/μL, providing concentrations in pg/μL) were used for the earlier Welsh case study, which preceded development of the amplicon standard. Throughout, eDNA concentrations reported in copies/μL were quantified against the amplicon standard, while those reported in pg/μL were quantified against the tissue-derived DNA standard.

2.2. Assay validation

2.2.1. Analytical performance

Repeatability was evaluated using seven independent standard curves from separate qPCR runs. Inter-operator repeatability was assessed by comparing standard curves generated by two independent operators using identical assay protocols and standard concentrations (10^2–10^5 copies/μL). Tissue standard consistency was assessed by generating a separate amplicon standard from each of three tissue extracts (I6, I8, I10) and comparing their standard curves within a single run. Robustness was tested by varying primer concentrations by ±10% and ±20% from optimised levels. All standard curves used four concentrations (10^2 to 10^5 copies/μL) in duplicate with NTCs. Internal positive control (IPC) performance was monitored with $\Delta Cq \leq 2.0$ indicating acceptable performance.

Laboratory validation standard curves used four concentrations (10^2 to 10^5 copies/μL) with NTCs. These standard curves were used to characterise repeatability, tissue standard consistency, and robustness. The limit of detection (LOD) and limit of quantification (LOQ) were determined using dose-response modelling following Klymus et al. [19]. Eleven DNA standards ranging from 0.0016 to 5000 copies/μL were tested in quadruplicate with four NTCs. LOD was calculated at 95% detection probability (automatic model selection) and LOQ at CV < 35% (exponential decay model) using R (v4.5.1).

2.2.2. Inhibition

Inhibition tolerance was assessed using three dissolved organic carbon (DOC) environmental inhibitors common in British freshwater systems: humic acid, fulvic acid, and tannic acid [36]. Inhibitor concentration selection was based on environmental monitoring data from Natural Resources Wales (n = 2490 measurements; 2020–2025). Test concentrations ranged from environmentally realistic levels (5 mg/L) to extreme stress conditions (500 mg/L) for each inhibitor, to define operational thresholds. Working stocks of 5, 50, 100, and 500 mg/L were prepared in nuclease-free water from 1000 mg/L master stocks. Reactions (20 μL) contained 16 μL mastermix, 2 μL template DNA (10,000 copies total input), and 2 μL inhibitor working stock (n = 3 per concentration per inhibitor). Positive template controls (PTCs) contained template DNA without inhibitor. Internal positive control (IPC) performance was monitored in all reactions. Inhibition was defined as $\Delta Cq > 3$ relative to PTC [29].

2.2.3. Specificity

The specificity of the primers and probes used in the study was analysed by downloading voucher sequences from the NCBI database [37] of *M. margaritifera* and other co-occurring or genetically similar species. The sequences were aligned *in silico* using MUSCLE [38] in MEGA12 [39]. BLAST searches [37,40] were also conducted against bacterial genomes to predict cross-reactivity. Potential primer binding was characterised as less than 10% of mismatches overall and 100% homology of the last four 3′ bases [41]. Probe binding was assessed by the overall homology as binding (100% homology), unlikely binding (≥90%), or no binding (<90%). EcoPCR [42] analysis was performed using a COI database that included both the broad COI/mitochondrial reference records and *M. margaritifera* target records. Mismatch thresholds from 0 to 5 were used to assess target recovery and off-target behaviour for *in silico* PCR against the database. Candidate ecoPCR amplicons were then screened for exact matches to the corresponding internal hydrolysis probe.

Tissue samples of quagga mussel, *Dreissena bugensis*, and zebra mussel, *Dreissena polymorpha,* were obtained from the University of Hull for *in vitro* testing, being the only co-occurring UK freshwater bivalve species for which tissue samples were obtainable. DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen), quantified on a Qubit 4 Fluorometer (Thermo Fisher Scientific), and 1 ng/μL of each extract undergoing qPCR triplicate to check for non-target species amplification. This concentration represents a conservative upper boundary for eDNA in freshwater systems where concentrations are inherently low.

2.3. Case-type samples

2.3.1. Sample collection

Two field validation case studies were conducted: a Welsh river system sampled in September 2024, and a Lake District river system sampled in August 2025. Sample collection timing was determined by field access and logistical constraints rather than a targeted seasonal design. Exact sampling locations are withheld due to the legal protection status of *M. margaritifera* and the risk of disclosing sensitive population locations. The Welsh system supports a recruiting *M. margaritifera* population of ~150 adults, confirmed by traditional surveying methods. Six sampling sites were positioned along a 7.8-km transect encompassing locations upstream and downstream of the known population to capture spatial eDNA dynamics. The multiplex assay was applied to validate detection of this population identified through prior physical surveillance. The Lake District system supports ~300 adults and has been subject to juvenile releases. West Cumbria Rivers Trust provided eight sampling sites along an 8.65-km transect as a blind validation test to assess the multiplex assay's field detection capabilities without prior knowledge by the authors of mussel distribution patterns. At both locations, bankside samples were collected in triplicate at each site using two-litre water bottles [24]. Bottles were rinsed three times with river water by inversion before collection, then submerged facing upstream until filled. Samples were stored in opaque bags to minimise UV exposure and transported for laboratory filtration within 24 h [43].

2.3.2. Filtration and extraction

The water samples were all filtered in the laboratory. The water was filtered using a Millipore EZ-Fit® Base3 manifold base and EZ-Stream1® vacuum pump (Millipore, Merck KGaA, Darmstadt, Germany) through Nalgene™ Single Use Analytical Filter Funnels (Thermo Fisher Scientific) with a volume of 250 mL and a 0.45 μm cellulose nitrate membrane. The filters from each replicate site were then removed from the filter cups and stored at −20°C in 50-mL universal tubes before DNA extraction. Filtration controls were run alongside by filtering 1 L of bottled water of the same brand purchased from the same shop at the same time as the bottles used to collect the water samples.

Filtration of each 1 L replicate sample required two 0.45 μm cellulose nitrate membrane filters. Both filters from each replicate were cut and placed into a single 2 mL extraction tube, and extracted using the swab protocol from the DNA Investigator Kit (Qiagen), following the

manufacturer's protocol for a cotton swab, and eluted in 30 μL Buffer ATE. Samples were stored at −20°C until qPCR analysis (2.1.1).

2.4. Statistical analyses

Standard curve data were analysed using linear regression to determine R^2 values, slopes, and intercepts. One-way analysis of variance (ANOVA) was performed to determine if there was a significant difference between standard curves across different runs (repeatability), biological replicates (reproducibility), and primer concentration variations (robustness). Effect sizes were calculated using eta squared (η^2), with thresholds of 0.01 (small), 0.06 (medium), and 0.14 (large) [44]. Statistical significance was defined as $p < 0.05$. Precision was assessed using coefficient of variation (CV), calculated as (standard deviation / mean) × 100. Accuracy was evaluated through recovery percentages, calculated as (observed concentration / expected concentration) × 100. Tissue standard consistency was also assessed by Bland-Altman analysis [45].

Inhibition was quantified using ΔCq values relative to positive template controls (PTCs), with $\Delta Cq > 3$ defined as the inhibition threshold [29]. Internal positive control (IPC) performance was monitored throughout, with $\Delta Cq \leq 2.0$ considered acceptable.

For case study data, eDNA concentrations were $\log_{10}$-transformed after adding a small constant (0.001), a standard approach for accommodating zero values in right-skewed concentration data prior to statistical analysis. This value was selected to be small relative to the overall range of observed concentrations. For the Lake District case study, the standard curve was extended to include a 10 copies/μL point (10–10^5 copies/μL), informed by LOD/LOQ sensitivity characterisation. Following eDNA best practices [46], all detections were retained in datasets regardless of concentration, with non-detections reported as true negatives, values below LOQ flagged for reduced precision, and values below LOD interpreted as positive detections with increased uncertainty. Statistical analyses were performed in Python (v3.11; scipy v1.11, numpy v1.24, pandas v2.0) with visualisation using matplotlib (v3.7) and seaborn (v0.12).

3. Results

3.1. Repeatability

The seven standard curves, generated from serial dilutions of the amplicon standard, demonstrated consistent performance. Internal positive control (IPC) ΔCq values ranged from −1.08 to 0.94 (overall mean ± SD: 33.48 ± 0.49 Cq, n = 56), all within the $\Delta Cq \leq 2.0$ threshold.

Linear regression analysis (Fig. 1) showed R^2 values of 0.986–1.000 (mean ± SD: 0.997 ± 0.005) for the long fragment (180 bp) and 0.989–1.000 (mean ± SD: 0.998 ± 0.004) for the short fragment (83 bp). Slopes ranged from −3.358 to −3.753 (mean ± SD: −3.605 ± 0.131) for the long fragment and −3.325 to −3.696 (mean ± SD: −3.542 ± 0.134) for the short fragment. ANOVA detected no significant differences in intercepts or slopes between runs for either fragment (all $p > 0.05$, $\eta^2 = 0.09$–0.17). A precision and accuracy assessment showed coefficient of variation (CV) values ranging from 6.2 to 26.0% for the short fragment and 5.5–28.1% for the long fragment, with quantification accuracy percentages between 62–137% (Table 2).

Table 2
Precision and accuracy metrics for the multiplex assay. CV represents coefficient of variation.

Run	SHORT FRAGMENT		LONG FRAGMENT	
	Quantification accuracy (% range)	CV (%)	Quantification accuracy (% range)	CV (%)
1	92.7–115.3	7.8	86.0–113.9	8.3
2	87.6–115.3	9.3	92.0–109.4	5.5
3	62.0–137.7	26.0	58.2–136.0	28.1
4	84.8–114.7	11.1	76.3–117.5	12.5
5	86.5–109.8	8.7	87.5–129.1	14.2
6	78.3–115.0	12.1	70.8–126.9	18.6
7	93.2–109.4	6.2	83.2–121.9	13.5

Standard curve R^2 values were comparable between operators (Operator 1: short fragment 0.990–0.999, long fragment 0.988–0.999; Operator 2: short fragment 0.990–0.995, long fragment 0.989–0.994), demonstrating inter-operator repeatability of assay performance.

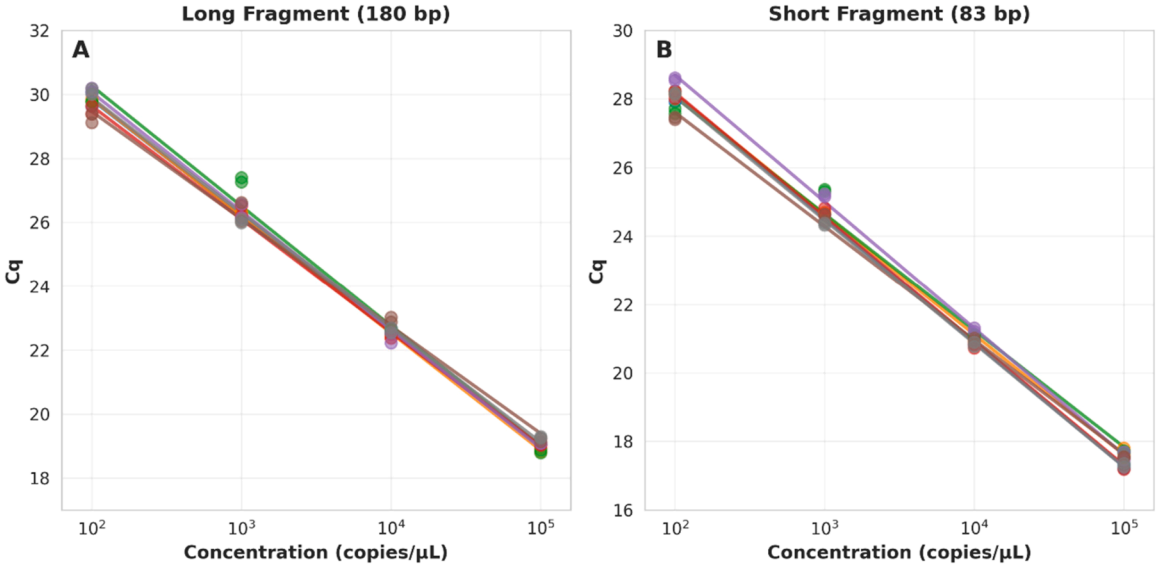


Fig. 1. Repeatability of the *M. margaritifera* multiplex qPCR assay across different runs. Standard curves for (A) long fragment (180 bp) and (B) short fragment (83 bp) from seven independent technical replicates. Each coloured line represents one complete standard curve run across four concentrations (10^2, 10^3, 10^4 and 10^5 copies/μL).

3.2. Tissue standard consistency

Three biological replicates (independent extractions from three individual mussels) were used to generate standard curves. IPC ΔCq values ranged from −0.73 to 0.83, within the ΔCq ≤ 2.0 threshold. Linear regression analysis showed R^2 values of 0.992–0.998 (mean ± SD: 0.996 ± 0.003) for the long fragment (180 bp) and 0.986–0.999 (mean ± SD: 0.995 ± 0.008) for the short fragment (83 bp). Slopes ranged from −3.779 to −3.965 (mean ± SD: −3.897 ± 0.103) for the long fragment and −3.597 to −3.853 (mean ± SD: −3.693 ± 0.139) for the short fragment. ANOVA detected no significant differences in intercepts or slopes between biological replicates for either fragment (all $p > 0.05$, $\eta^2 = 0.02–0.07$). Bland-Altman analysis confirmed interchangeability of amplicon standards from three tissue sources, with maximum deviations < 0.5 Cq (Fig. 2).

3.3. Robustness

Primer concentration variations of ±10% and ±20% demonstrated consistent assay performance. IPC ΔCq values ranged from −1.03 to 2.12 across all conditions. Linear regression analysis showed R^2 values of 0.990–0.999 (mean ± SD: 0.996 ± 0.004) for the long fragment (180 bp) and 0.995–1.000 (mean ± SD: 0.997 ± 0.002) for the short fragment (83 bp). Slopes ranged from −3.377 to −3.836 (mean ± SD: −3.551 ± 0.212) for the long fragment and −3.388 to −3.720 (mean ± SD: −3.495 ± 0.157) for the short fragment. ANOVA detected no significant differences in intercepts or slopes between deviated conditions for either fragment (all $p > 0.05$, $\eta^2 = 0.14–0.22$), with maximum Cq deviations < 2.0 cycles across all conditions (Fig. 3).

3.4. Limits of detection and quantification (LOD and LOQ)

Curve-fitting modelling yielded LOD values of 1.25 copies/μL (5.0 copies/reaction) for the long fragment and 2.5 copies/μL (10.0 copies/reaction) for the short fragment at 95% detection probability (Fig. 4). LOQ values (CV < 35%, exponential decay model) were 2.0 copies/μL (8.0 copies/reaction) for the long fragment and 3.14 copies/μL (12.56 copies/reaction) for the short fragment. The multiplex assay LOD and LOQ are 2.5 and 3.14 copies/μL (10.0 and 12.6 copies/reaction), determined by the performance of the short fragment. Internal positive control (IPC) Cq values ranged from 29.48 to 30.22 (overall mean ± SD: 29.94 ± 0.16 Cq, n = 44), all within the ΔCq ≤ 2.0 threshold.

3.5. Inhibition

Humic acid (HA), fulvic acid (FA) and tannic acid (TA) were tested at concentrations of 5, 50, 100, and 500 mg/L (Fig. 5). Inhibition was defined as ΔCq > 3 [29]. Positive template control (PTC) mean ± SD Cq values were 24.06 ± 0.16 (long fragment), 22.82 ± 0.26 (short fragment), and 30.90 ± 0.18 (IPC). At 5–100 mg/L, HA showed ΔCq values ranging from −0.22 to 1.01 (long fragment) and −0.30 to −0.07 (short fragment), with 100% DNA detection rate across all replicates. At 500 mg/L, HA caused complete long fragment failure while the short fragment amplified with ΔCq = 9.84 ± 1.91. FA showed ΔCq values ranging from −0.16 to 0.86 (long fragment) and −0.31 to −0.11 (short fragment) at 5–100 mg/L, with complete failure of IPC and both fragments at 500 mg/L. TA showed ΔCq values ranging from −0.13 to 0.25 (long fragment) and −0.21–0.08 (short fragment) across all concentrations (5–500 mg/L) with 100% detection rate (long fragment: ΔCq = 0.03 ± 0.33; short fragment: ΔCq = −0.09 ± 0.34). Internal positive control (IPC) ΔCq values ranged from −0.36 to 0.19 at concentrations ≤ 100 mg/L. At 500 mg/L, IPC successfully detected inhibition (HA: ΔCq = 7.59 ± 1.98; FA: no amplification; TA: ΔCq = 0.19 ± 0.10).

3.6. Specificity

Primer and probe sequences were tested *in silico* against sequences from the NCBI reference database [37]. Five *M. margaritifera* voucher sequences were included to check for intraspecies genetic variation. Off-target species tested included seven other UK freshwater mussel species, food contamination sources, salmonid hosts, freshwater crustaceans and terrestrial contaminants (Supplementary Material 1). Sequence alignments in MEGA12 [39] using the MUSCLE algorithm [38] showed that there were key sequence differences between the off-target species and *M. margaritifera*. No off-target species exhibited complete binding sites for the three oligonucleotides (forward primer, probe, reverse primer) in either the short or long fragment assay components. The multiplex assay is predicted to be 100% specific to *M. margaritifera* (Supplementary Material 1). BLAST searches [37,40] against bacterial genomes showed no predicted cross-reactivity. *Dreissena bugensis* and *Dreissena polymorpha* did not show any amplification *in vitro*.

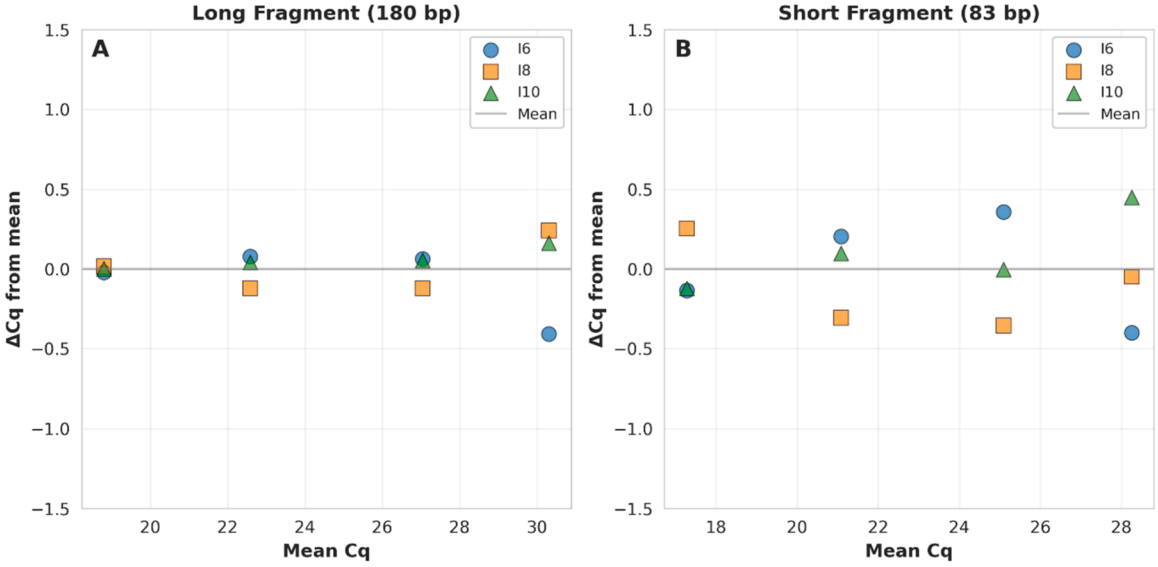


Fig. 2. Tissue standard consistency assessed by Bland-Altman analysis [45]. Deviations from mean Cq values are shown for three biological replicates (I6, I8, I10) across four concentrations (10^2, 10^3, 10^4 and 10^5 copies/μL) for (A) long fragment (180 bp) and (B) short fragment (83 bp). Grey line indicates the mean Cq at each concentration.

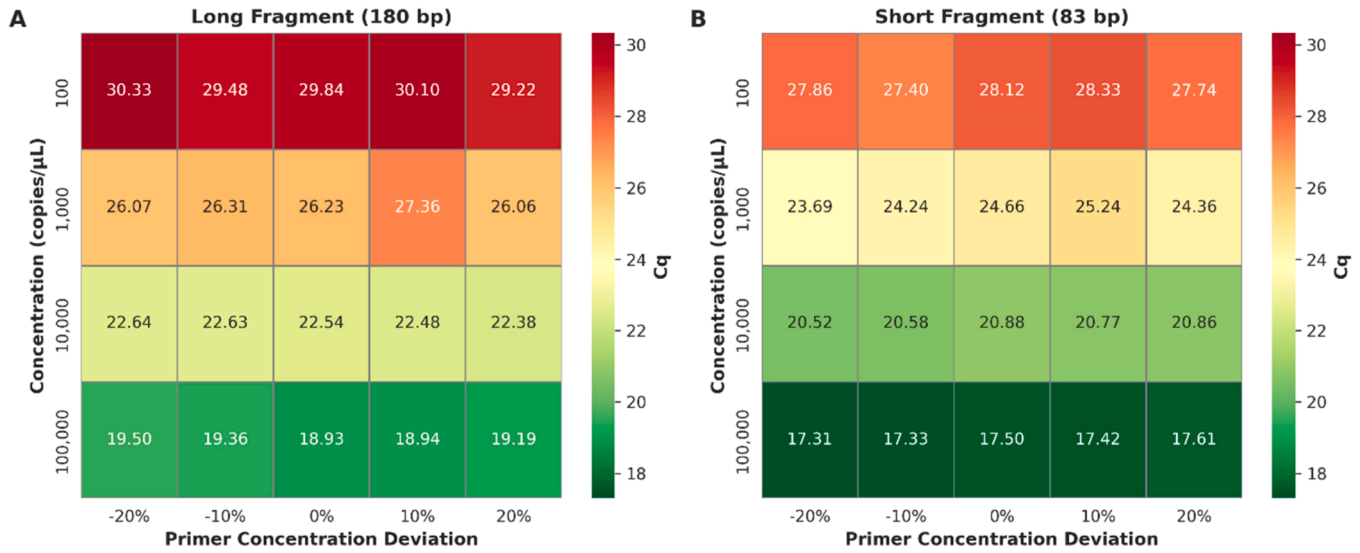


Fig. 3. Robustness of multiplex qPCR assay. Mean Cq values for (A) long fragment (180 bp) and (B) short fragment (83 bp) across ±10% and ±20% primer concentration variations at four DNA standard concentrations. The optimised primer concentration (0% deviation) Cq values are included for visual reference, derived from two independent runs. Colour gradient indicates Cq value.

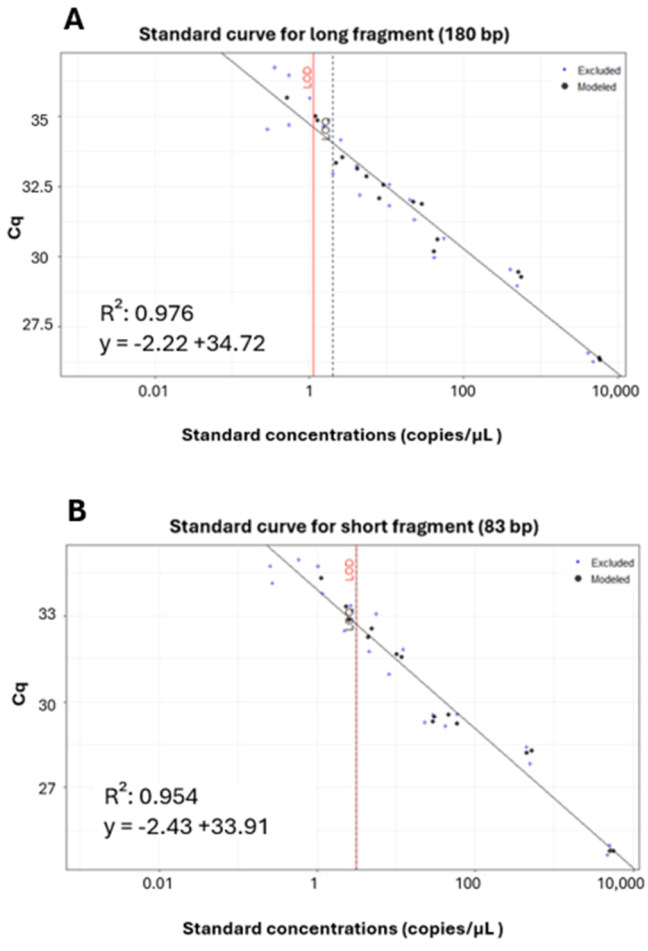


Fig. 4. Standard curves for qPCR validation of long (A) and short (B) fragment assays targeting *M. margaritifera* eDNA. Black diamonds represent data points included in models; blue crosses indicate replicates excluded. Vertical lines indicate the limits of detection (LOD) and quantification (LOQ) for each assay.

EcoPCR [42] analysis confirmed consistent recovery of *M. margaritifera* by both the short and long assays across mismatch thresholds from 0 to 5 (Supplementary Material 2). At exact primer matching, both assays recover only *M. margaritifera*. For the short assay, non-target primer-pair hits appeared only once two or more mismatches per primer were permitted and increased as threshold was relaxed; however, none of the non-target amplicons contained the exact short-probe sequence, indicating that probe specificity was retained even when relaxed primer binding was allowed. For the long assay, no non-target hits occurred between 0 and 4 mismatches per primer, with a small number appearing only at the most permissive setting (5 mismatches per primer). Of these, one carried an exact long-probe match, representing a theoretical low-likelihood off-target under highly relaxed *in silico* conditions rather than a likely assay-compatible target. Overall, EcoPCR supported assay specificity: the target was recovered strongly, while off-target matches emerged only as primer binding was made increasingly permissive, with full primer-probe compatibility absent or extremely limited.

3.7. Case study 1: Welsh river system

Excluding Site 1 (upstream of the habitat), the long fragment amplified in 17 of 60 samples (28.3% detection rate) (Fig. 6). The short fragment amplified in 31 samples (51.7% overall detection rate). The multiplex assay confirmed a second localised increase in concentration at Site 4 at 3.5 km (long fragment, -2.56 ± 0.17 $\log_{10}$(pg/µL); short fragment, -2.06 ± 0.16 $\log_{10}$(pg/µL)) indicative of a localised eDNA source. This deviates from the expected pattern of steady concentration decrease downstream from the known population (Site 2: long, -2.69 ± 0.15; short, -1.85 ± 0.14 $\log_{10}$(pg/µL)). PTC baseline IPC Cq values were 29.87 ± 0.35 (mean ± SD, $n = 16$) compared to field IPC Cq values of 30.13 ± 0.48 ($n = 72$). All ΔCq values were < 3 [29], indicating no detectable inhibitory effects.

3.8. Case study 2: Lake District river system

Sampling was conducted at eight sites along an 8.65-km transect of a Lake District river system (Fig. 7). The long fragment exhibited consistent detection across all sites (144/144 amplifications). The short fragment showed reduced detection rates at upstream sites, with non-detects recorded at Site 2 (2/12 samples) and Sites 3–5 (1/12 samples each), giving an overall detection rate of 95.8% (138/144 samples positive).

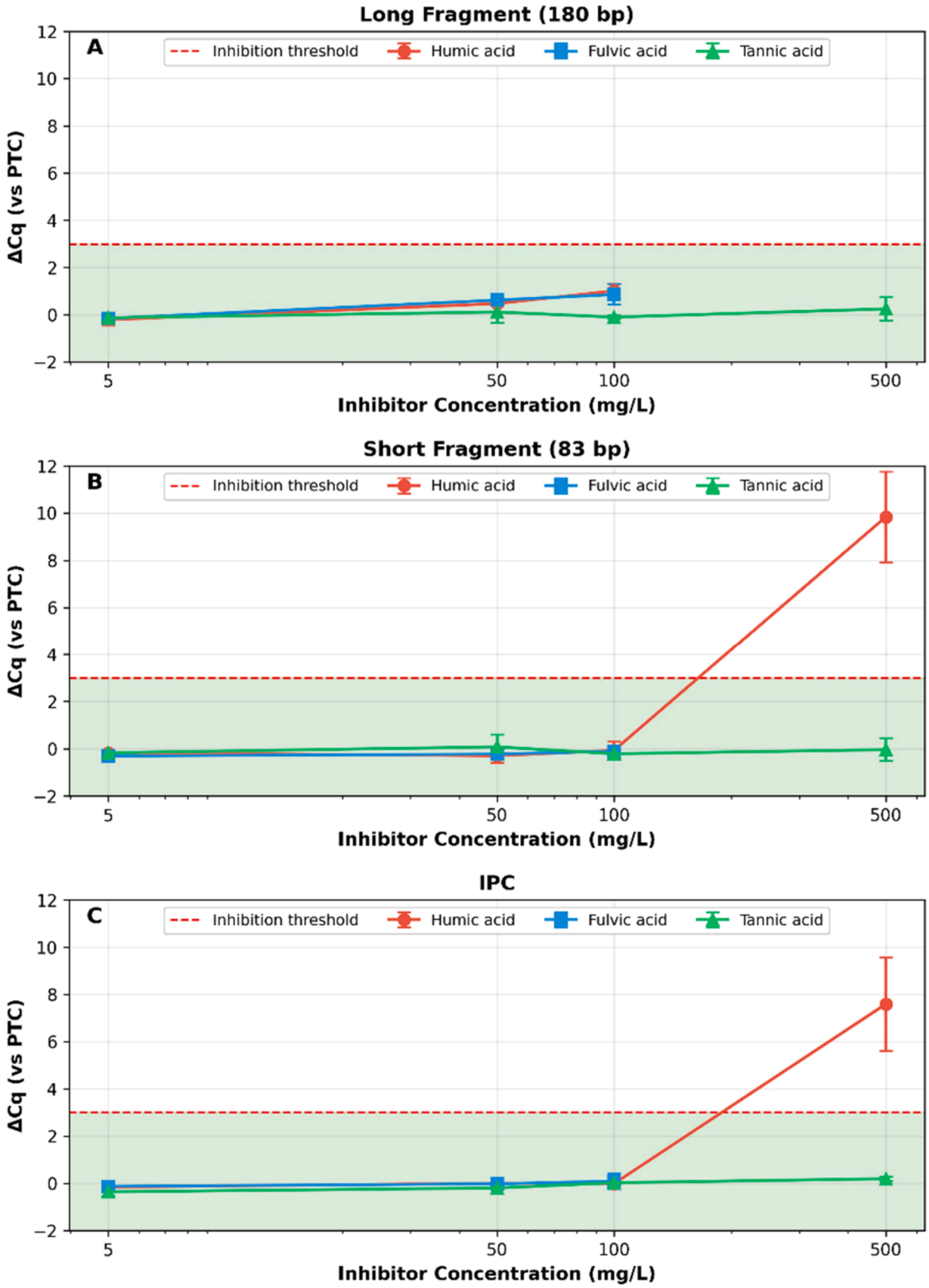


Fig. 5. Inhibition tolerance of the *M. margaritifera* multiplex assay. ΔCq values (mean ± SD) for (A) long fragment (180 bp), (B) short fragment (83 bp), and (C) internal positive control (IPC) in the presence of humic acid (red), fulvic acid (blue), and tannic acid (green) at concentrations of 5–500 mg/L. ΔCq values represent the shift in Cq relative to the positive template control (PTC). The red dashed line indicates the defined inhibition threshold (ΔCq = +3); green shading represents the acceptable range (ΔCq −3 to +3). Concentrations are displayed on a logarithmic scale.

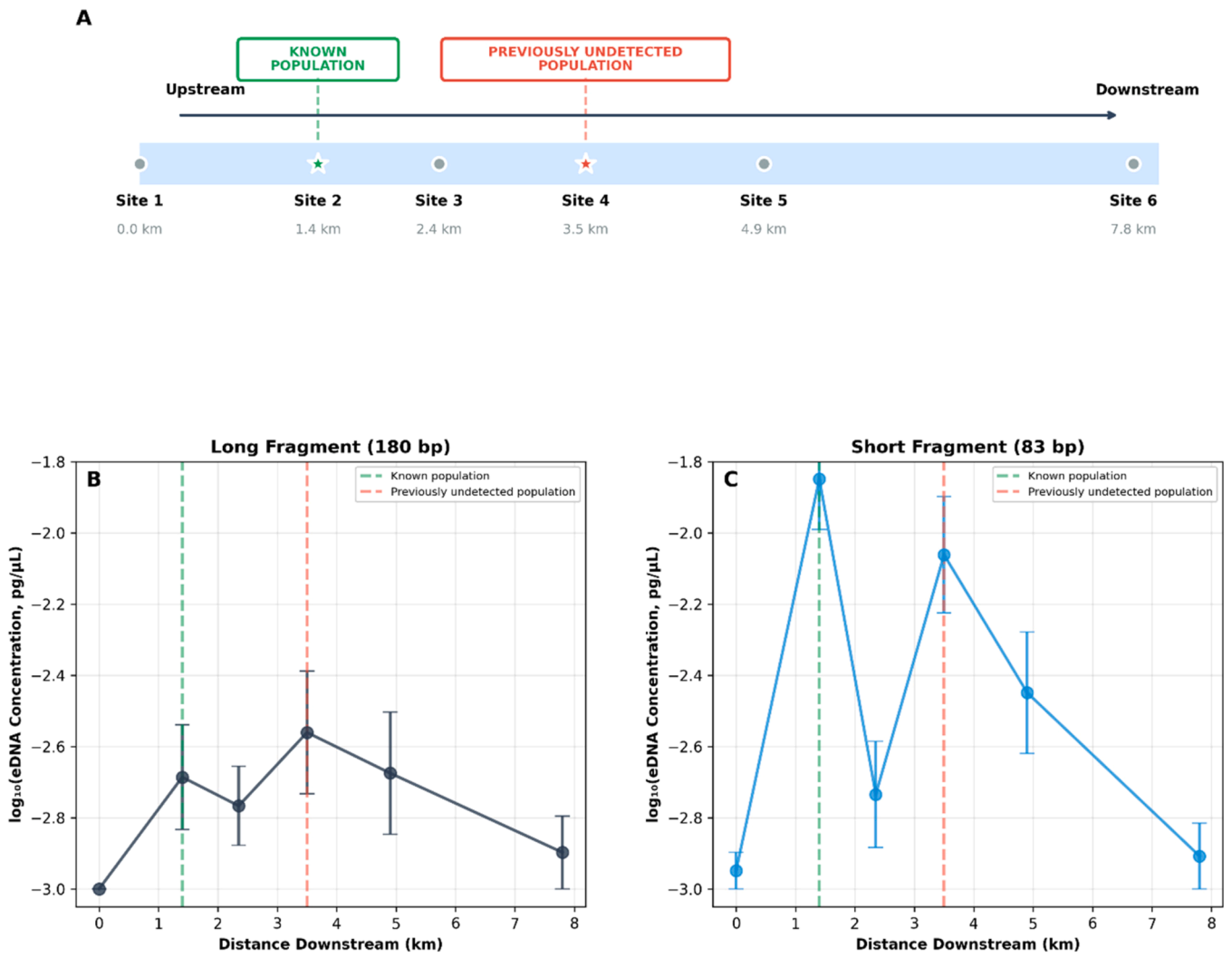


Fig. 6. Field validation case study: Detection of previously undetected *M. margaritifera* population in Wales. (A) River schematic showing six sampling sites along the main channel from upstream. Green star indicates the known population detection at Site 2; red star indicates the population detected at Site 4 following eDNA-based intelligence. (B) Long fragment (180 bp) and (C) short fragment (83 bp) log eDNA concentrations along the transect show an unexpected secondary increase in concentration at Site 4 (red dotted line) after the known population (green dotted line). Natural Resources Wales later surveyed this location and confirmed previously undetected mussels. Error bars represent standard error (n = 12 replicates per site).

Site 3 contained a sample with comparatively high concentration values (short, 99.81 copies/µL and long, 103.28 copies/µL, compared with site means of short, 14.91 copies/µL and long, 12.57 copies/µL, excluding this outlier). This data point, although reflective of eDNA stochasticity resulting from capture of large aggregate particles [47], was removed from further analysis to provide a fairer reflection of typical site concentrations. Comparatively elevated concentrations were observed at Sites 7–8 in both fragments (Site 7: long = 1.54 ± 0.06, short = 1.49 ± 0.05 $\log_{10}$(copies/µL); Site 8: long = 1.72 ± 0.03, short = 1.64 ± 0.02 $\log_{10}$(copies/µL)). PTC baseline IPC Cq values were 30.55 ± 0.93 (mean ± SD, n = 48) compared to field IPC Cq values of 30.27 ± 0.85 (n = 96). All ΔCq values were < 3 [29], indicating no detectable inhibitory effects.

Results indicated significant *M. margaritifera* populations upstream of Site 7 (sharp increase in long fragment concentrations). Long fragment concentrations increased steadily from Site 1–6. In contrast, short fragments displayed detection at Site 1, a decrease at Site 2 (suggesting degradation from upstream sources), followed by an unexpected increase at Site 3. This Site 3 increase in short fragments, combined with large standard errors at Sites 2 and 3, may reflect either stochastic variation in eDNA capture or an unidentified local population upstream of Site 3. Based on the spatial eDNA patterns, it was inferred that there may be further dispersed individuals between Site 3 and Site 8 which caused eDNA concentrations to steadily increase rather than decline with degradation effects. Following completion of eDNA analysis, West Cumbria Rivers Trust revealed that there were juvenile releases (2021 and 2023 [48]) around Site 4 (789 juveniles contributing to steady increase in concentrations) and around Sites 7 and 8 (two release sites of 1789 and 964 totalling 2753 juveniles, resulting in the larger increase) with interspersed adults throughout the system. The unexpected short fragment increase at Site 3 will be investigated through physical surveys to determine if additional populations exist in this region.

4. Discussion

4.1. Forensic validation of the assay

Environmental DNA science is mature for monitoring and policy implementation [18,49] while suggested forensic working group protocols [20,21] offer recommendations to establish reliable analytical approaches in legal contexts. Wildlife crime investigations require both the monitoring of protected species and the detection of illegal activities

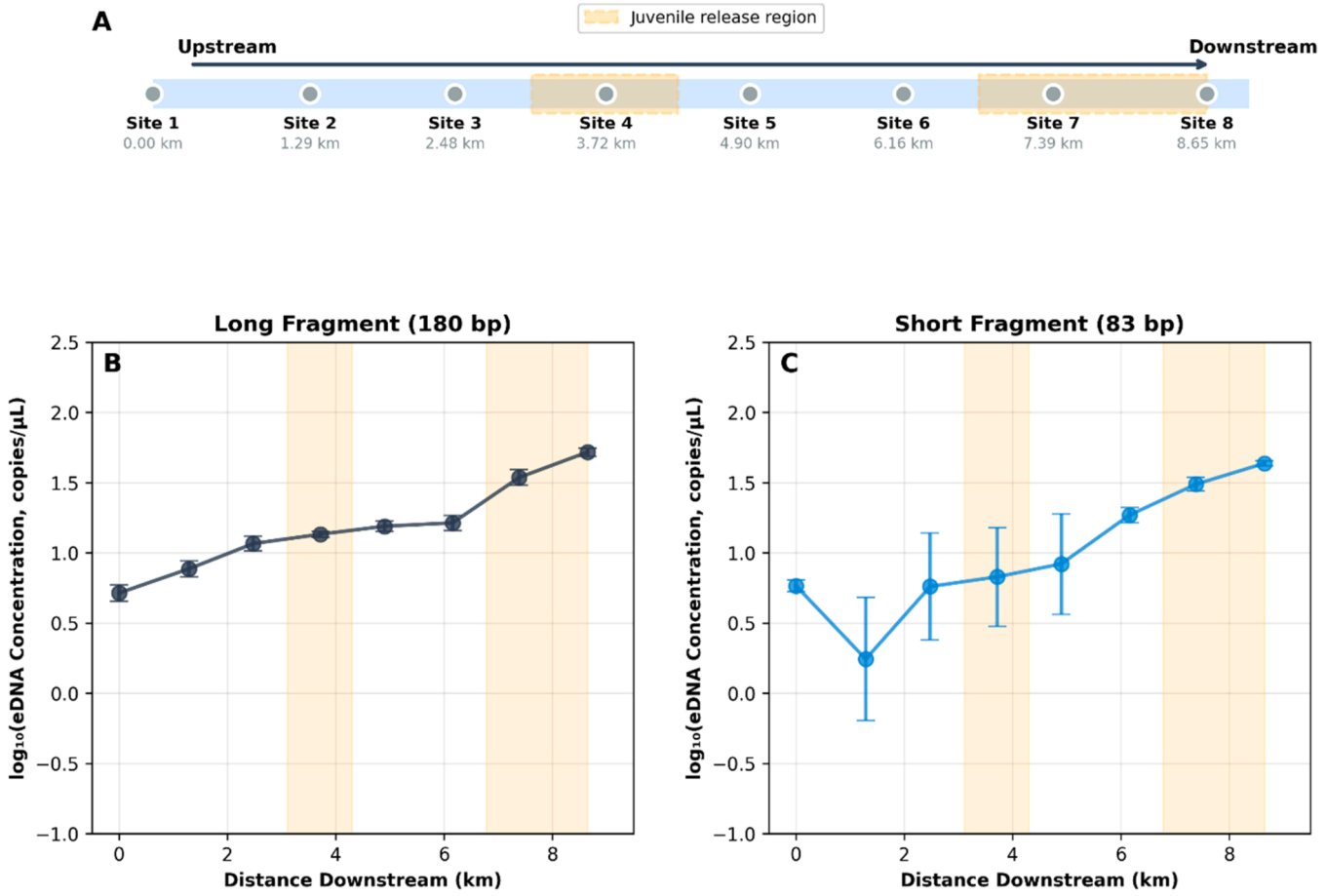


Fig. 7. Blind field validation: Detection of juvenile mussel release sites in the Lake District. (A) River schematic showing eight sampling sites along an 8.65-km transect from upstream to downstream. Two release regions (shaded areas) indicate general zones where juvenile mussels were released (disclosed post-analysis) through conservation programmes [48]. (B) Long fragment (180 bp) and (C) short fragment (83 bp) show elevated concentrations in both release regions. Error bars represent standard error (n = 12 replicates at all sites apart from Site 3 where n = 11 after outlier removal).

threatening them; frameworks integrating eDNA methodology with forensic validation are therefore essential. No previous research, technical guidance, or casework protocols have addressed this integration. This study demonstrates the first explicit forensic validation of an eDNA assay for a protected species, combining eDNA and qPCR best practices [18,30] with forensic validation requirements [20]. This cross-disciplinary framework addresses critical gaps: forensic guidelines lack eDNA-specific protocols for environmental samples, while eDNA guidelines were not designed for legal admissibility standards required to support wildlife crime prosecutions. Formal method-specific validation guidance for eDNA-based forensic approaches, developed by forensic working groups analogous to existing addenda for newer methods, e.g. interpretation of single nucleotide polymorphism (SNP) analysis [50], would support broader standardised implementation. The resulting validation framework enables *M. margaritifera* detection for both investigative intelligence and potential evidential use in prosecutions, and is transferable to other protected aquatic species.

4.2. Assay performance

The multiplex assay demonstrated species specificity to *M. margaritifera*. Although no variation was detected with voucher species [51], this does not preclude the possibility that isolated *M. margaritifera* populations could begin to differ genetically from each other [52] and mutations could arise in key primer or probe binding regions that affect the performance of the assay. Studies to genetically characterise local mussels and confirm assay efficiency could be carried out if false negatives are suspected, whether in forensic casework or broader conservation monitoring. *In silico* analysis of all off-target species predicted no false positives. The nature of this approach (testing UK co-occurring species plus potential contaminants [53]) provides confidence that *M. margaritifera* will be correctly identified in field deployment scenarios. Complementary ecoPCR [42] analysis across a broad COI reference database further supported specificity, recovering *M. margaritifera* across all mismatch thresholds while non-target primer–probe compatibility remained absent or negligible even under highly permissive *in silico* conditions. *In vitro* testing was limited to *Dreissena* spp. due to tissue availability constraints. While this represents a limitation relative to the comprehensive guidelines of Thalinger *et al.* [18], the negative results support *in silico* predictions. For forensic casework, this multi-evidence approach (*in silico* + targeted *in vitro* testing) meets SWGDAM [20] specificity requirements, though expanded *in vitro* testing could further define empirical assay limits.

The multiplex assay met forensic precision and accuracy standards [20] across the three parameters of repeatability, tissue standard consistency, and robustness. Repeatability assessments showed acceptable linearity and no significant differences in slopes or intercepts between qPCR runs, with effect sizes indicating minimal technical variation. Reporting effect sizes alongside p-values [44] represents enhanced statistical rigour relative to standard eDNA practices, aligning with forensic requirements for comprehensive quantification [54]. Precision and accuracy met accepted thresholds [20] for forensic quantitative assays, establishing reliable performance for investigative intelligence. Amplicon standards generated from three independent tissue sources

(biological replicates) validated the amplicon standard generation method, demonstrating consistent performance independent of tissue mitochondrial copy number variation [55]. Robustness testing confirmed operational reliability (±20% primer concentration variation). This tolerance to pipetting error and reagent batch variation is essential for routine casework deployment where perfect conditions cannot be guaranteed, especially when sample collections involve different, often non-specialist stakeholders in the field. The work in this study characterises unintentional variation rather than intended operational changes. Developmental validation experiments were conducted by two independent operators using identical protocols, with comparable standard curve R^2 values across operators, indicating that inter-operator variability did not meaningfully impact assay performance. This supports the suitability of the assay for deployment across multiple analysts within a single laboratory setting.

The assay demonstrated analytical sensitivity with LOD values of 2.5 copies/μL (10 copies/reaction, short fragment) and LOQ of 3.14 copies/μL (12.6 copies/reaction) at 95% detection probability and CV < 35%, respectively. These thresholds were determined using modelling following Klymus et al. [19], which provides probabilistic confidence intervals superior to simple binary presence/absence determination. The multiplex assay sensitivity (defined by short fragment performance) falls within the range of published eDNA assays validated using comparable methodologies [19,56] demonstrating appropriate analytical performance for trace eDNA detection. For eDNA applications, LOD and LOQ serve as assay characterisation metrics rather than absolute rejection thresholds. Following Sansom [46], detections below LOQ represent concentration estimates with reduced precision, while detections below LOD indicate presence with increased uncertainty.

The assay exhibited robust performance under the inhibitory stress caused by environmentally relevant concentrations of humic, fulvic, and tannic acids. At concentrations ≤ 100 mg/L (exceeding typical UK freshwater DOC levels) both assay fragments maintained consistent performance (ΔCq < 3 [29]) for all three inhibitors tested. Tannic acid showed complete tolerance even at 500 mg/L, suggesting TaqPath™ ProAmp™ Master Mix-specific resistance to this inhibitor. These inhibitors represent different chemical classes with distinct inhibitory mechanisms [29], enabling assessment of assay resilience across diverse environmental contaminants typical of UK freshwater systems. The internal positive control successfully detected inhibition at 500 mg/L for humic and fulvic acids (ΔCq > 3 or complete failure), supporting its intended function as a quality assurance mechanism [18]. This performance supports the assay's operational suitability for forensic casework, including samples from mussel inhabited peat-dominated catchments [57] where dissolved organic carbon concentrations are naturally elevated [58].

4.3. Case studies

As recommended within validation guidelines [18,20], representative case-type samples were incorporated within this research to test assay effectiveness in forensically pertinent scenarios. The Welsh study confirmed *M. margaritifera* presence at the known population (Site 2) with declining eDNA concentrations at Site 3, before detecting a secondary, localised increase in concentration at Site 4. The long fragment concentration increase acted as a primary indicator of more localised eDNA [24,59] while the short marker corroborated the findings. Considering eDNA downstream transport [60] and lateral transport to the bankside [61], further investigation upstream of Site 4 was suggested. A physical survey discovered populations previously undetected using traditional survey methods, demonstrating the assay's capacity to direct conservation efforts toward populations beyond known distributions. In this exercise, the IPC indicated no inhibitory substances within the river which reflects the water quality of this habitat, with mussels preferring oligotrophic waters free of fine sediment [62].

Blind proficiency testing is recommended forensic practice for eliminating confirmation bias [63]. In the Lake District study, samples were provided by West Cumbria Rivers Trust with no prior disclosure of mussel distribution. Only after eDNA analysis was complete were juvenile release locations (two sites totalling 2753 juveniles [48]) and adult distribution revealed, providing unbiased assessment of detection capability. Data showed a marked increase in long fragment concentrations from Site 7 onwards which corresponded to the largest juvenile population, demonstrating its utility as a proximity marker due to more rapid degradation compared with the persistent short fragment [24]. Increasing concentrations in the downstream direction indicated the dispersed juveniles and adults throughout the system. Detection successes of 100% and 95.8% for the long and short markers showed operational reliability while IPC results again pointed towards suitable habitat conditions. Concentration ranges spanned from below LOQ to > 100 copies/μL, validating the assay's characterised sensitivity across field-relevant conditions. The eDNA patterns provide actionable intelligence for West Cumbria Rivers Trust, highlighting upstream areas (particularly Site 3) warranting physical survey to confirm the presence of previously undetected populations. Blind test results showed operational readiness for deployment to inform conservation management.

4.4. Broader implications

Forensic guidelines for court admissibility standards [20], supplemented by recommendations from the eDNA community [18,19,29,64] were implemented in this validation study. While the immediate applications are conservation-focused, establishing validation precedent is essential for future forensic intelligence uses as wildlife crime applications continue to develop. The methodological framework is transferable to other protected species where eDNA assays exist but lack forensic validation [65,66], for which molecular evidence may support regulatory enforcement or wildlife crime investigation. Whilst eDNA evidence has been found admissible in environmental management cases [67], its application within criminal proceedings is currently limited due to the lack of testing and validation to determine admissibility. Further research should consider forensic validation across additional species, environments, and the development of approaches to determine eDNA provenance and persistence in forensic contexts.

The validated assay supports multiple operational applications. For post-incident investigation, eDNA provides forensic intelligence to direct targeted searches where physical evidence has been destroyed following habitat disturbance [24]. For pre-incident prevention, developers requiring ecological surveys can use eDNA screening before planning applications, reducing disturbance to protected populations [68]. The assay's sensitivity supports discovery of undetected populations in suitable habitats, as demonstrated by the Welsh case study where eDNA detection led to Natural Resources Wales confirming previously undocumented individuals. Finally, regulatory compliance monitoring benefits from non-invasive assessment of translocation success, as validated by the Lake District juvenile release detection.

4.5. Future directions

Formal reproducibility testing (where aliquots of the same samples are analysed using identical assay protocols, reagents and controls) in different laboratories [69] would be recommended before broader forensic implementation. Precision and accuracy of quantitative results could then be evaluated between different operators and detection instruments [20]. This validation progression aligns with standard forensic practice, where initial internal validation precedes external validation as assays are adopted by additional facilities. The use of certified synthetic DNA standards of known copy number would provide an independent reference material for quantification accuracy assessment, complementing the amplicon standard approach employed here. Extension of the laboratory validation standard curve range (10^2–10^5 copies/μL) to encompass field-relevant concentrations across validation

parameters (repeatability, tissue standard consistency, and robustness) would make the formal validation more comprehensive and internally consistent with field application.

In vitro specificity testing was limited to *Dreissena* spp., the only co-occurring UK freshwater bivalves for which tissues were available. Tissue availability constraints are widely recognised in eDNA validation, particularly for rare, endemic, or protected species [70]. More extensive testing would be recommended as freshwater mussel tissue becomes available from other institutions and biobanks. For laboratories processing both human forensic DNA and wildlife eDNA samples, *in vitro* testing against human DNA [53] would verify no cross-reactivity, a quality assurance consideration for multi-discipline forensic facilities. Inhibition testing was focused on environmental factors and DOC compounds. While this is standard in the field [29], testing with biological inhibitors (such as blood) or synthetic compounds (such as cyanine dye [71]) could further define operational boundaries. Advanced *in situ* validation [18] data collection is planned to characterise biotic and abiotic factors, including seasonal dynamics (e.g., life stage, flow rate, river depth, temperature [60]) and spatial sampling strategies (bankside vs. midstream sampling [61]), to optimise field protocols. While the validation framework is transferable across protected aquatic species, spatial interpretation of eDNA detections requires species-specific considerations [72,73]. Sessile species like *M. margaritifera* enable straightforward detection-to-presence inference. For mobile species (fish, amphibians, and crustaceans), transport modelling [73] and higher-tier validation approaches [18] may be required to account for species mobility alongside eDNA dispersal from source populations.

4.6. Conclusion

This study establishes the first forensic validation framework integrating environmental DNA best practices with legal admissibility standards, addressing a critical gap in wildlife crime investigations. Demonstrated through *M. margaritifera* as a model species, this framework is transferable to other protected aquatic species requiring eDNA evidence for regulatory enforcement. This framework provides a methodological precedent for expanding forensic eDNA applications beyond conservation monitoring into evidential casework. The validation approach demonstrated here strengthens the molecular toolkit available for biodiversity conservation and wildlife crime enforcement, facilitating development of defensible assays for threatened species to complement traditional survey methods.

CRediT authorship contribution statement

Matthew Philip Lewis: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Catherine Moir**: Writing – review & editing, Investigation, Formal analysis. **Kirstie Scott**: Writing – review & editing, Supervision. **Stefano Mariani**: Writing – review & editing, Supervision. **Bernd Hänfling**: Writing – review & editing, Supervision. **Nick Dawnay**: Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the Liverpool John Moores VC Scholarship scheme for providing PhD student funding. This work has been funded in part by The Chartered Society of Forensic Sciences Research Grant 2023. The authors thank Dr Peter Shum for his contribution to the ecoPCR bioinformatics analysis, and Mountain Equipment for providing discounted fieldwork equipment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2026.103598.

References

[1] J. Geist, Strategies for the conservation of endangered freshwater pearl mussels (Margaritifera margaritifera L.): a synthesis of conservation genetics and ecology, 2010 644, Hydrobiologia 644 (1) (2010) 69–88, https://doi.org/10.1007/S10750-010-0190-2.
[2] Margaritifera margaritifera: Moorkens, E., IUCN Red List of Threatened Species (2022). https://doi.org/10.2305/IUCN.UK.2024-1.RLTS.T12799A212995510.EN.
[3] The Habitats Directive - Environment - European Commission, (n.d.). https://environment.ec.europa.eu/topics/nature-and-biodiversity/habitats-directive_en (accessed October 16, 2025).
[4] Wildlife and Countryside Act 1981, c.69, UK.
[5] uk | National Wildlife Crime Unit - NWCU, (n.d.). https://www.nwcu.police.uk/ (accessed October 16, 2025).
[6] P.J. Cosgrove, M.R. Young, The status of the freshwater pearl mussel M. margaritifera (L.) in Scotland, Aquat. Conserv. 25 (2000) 2093–2112.
[7] P.J. Boon, S.L. Cooksley, J. Geist, I.J. Killeen, E.A. Moorkens, I. Sime, Developing a standard approach for monitoring freshwater pearl mussel (Margaritifera margaritifera) populations in European rivers, Aquat. Conserv. 29 (2019) 1365–1379, https://doi.org/10.1002/aqc.3016.
[8] B.C. Stoeckle, R. Kuehn, J. Geist, Environmental DNA as a monitoring tool for the endangered freshwater pearl mussel (Margaritifera margaritifera L.): a substitute for classical monitoring approaches? Aquat. Conserv. 26 (2016) 1120–1129, https://doi.org/10.1002/aqc.2611.
[9] C.D. Wilson, G. Arnott, N. Reid, D. Roberts, The pitfall with PIT tags: marking freshwater bivalves for translocation induces short-term behavioural costs, Anim. Behav. 81 (2011) 341–346, https://doi.org/10.1016/j.anbehav.2010.10.003.
[10] L. Gasparini, S. Crookes, R.S. Prosser, R. Hanner, Detection of freshwater mussels (Unionidae) using environmental DNA in riverine systems, Environ. DNA 2 (2020) 321–329, https://doi.org/10.1002/EDN3.71.
[11] C.S. Goldberg, C.R. Turner, K. Deiner, K.E. Klymus, P.F. Thomsen, M.A. Murphy, S. F. Spear, A. McKee, S.J. Oyler-McCance, R.S. Cornman, M.B. Laramie, A.R. Mahon, R.F. Lance, D.S. Pilliod, K.M. Strickler, L.P. Waits, A.K. Fremier, T. Takahara, J. E. Herder, P. Taberlet, Critical considerations for the application of environmental DNA methods to detect aquatic species, Methods Ecol. Evol. 7 (2016) 1299–1307, https://doi.org/10.1111/2041-210X.12595.
[12] H.C. Rees, B.C. Maddison, D.J. Middleditch, J.R.M. Patmore, K.C. Gough, The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology, J. Appl. Ecol. 51 (2014) 1450–1459, https://doi.org/10.1111/1365-2664.12306.
[13] V.S. Langlois, M.J. Allison, L.C. Bergman, T.A. To, C.C. Helbing, The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories, Environ. DNA 3 (2021) 519–527, https://doi.org/10.1002/EDN3.164.
[14] Q. Mauvisseau, E. Kalogianni, B. Zimmerman, M. Bulling, R. Brys, M. Sweet, eDNA-based monitoring: Advancement in management and conservation of critically endangered killifish species, Environ. DNA 2 (2020) 601–613, https://doi.org/10.1002/EDN3.92.
[15] E.P. Preece, M. Bryan, S.M. Mapes, C. Wademan, R. Dorazio, Monitoring for freshwater mussel presence in rivers using environmental DNA, Environ. DNA 3 (2021) 591–604, https://doi.org/10.1002/edn3.156.
[16] E.R. Waits, L.M. Smith, K.A. Patnode, J.L. Clayton, M.A. McGregor, A.J. Bergdale, Development and validation of environmental DNA assays for the detection of endangered and threatened freshwater mussels, 2025 17, Conserv. Genet. Resour. 17 (4) (2025) 237–244, https://doi.org/10.1007/S12686-025-01398-2.
[17] S. Fernandez, M.M. Sandin, P.G. Beaulieu, L. Clusa, J.L. Martinez, A. Ardura, E. García-Vázquez, Environmental DNA for freshwater fish monitoring: Insights for conservation within a protected area, PeerJ 2018 (2018) e4486, https://doi.org/10.7717/PEERJ.4486/SUPP-2.
[18] B. Thalinger, K. Deiner, L.R. Harper, H.C. Rees, R.C. Blackman, D. Sint, M. Traugott, C.S. Goldberg, K. Bruce, A validation scale to determine the readiness of environmental DNA assays for routine species monitoring, Environ. DNA 3 (2021) 823–836, https://doi.org/10.1002/EDN3.189.
[19] K.E. Klymus, C.M. Merkes, M.J. Allison, C.S. Goldberg, C.C. Helbing, M.E. Hunter, C.A. Jackson, R.F. Lance, A.M. Mangan, E.M. Monroe, A.J. Piaggio, J.P. Stokdyk, C. C. Wilson, C.A. Richter, Reporting the limits of detection and quantification for environmental DNA assays, Environ. DNA 2 (2020) 271–282, https://doi.org/10.1002/EDN3.29.
[20] SWGDAM Validation Guidelines for DNA Analysis Methods, (2016). https://www.swgdam.org/_files/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf (accessed November 18, 2025).
[21] ENFSI, Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process Aim2010. https://enfsi.eu/wp-content/uploads/2016/09/minimum_validation_guidelines_in_dna_profiling_-_v2010_0.pdf (accessed November 18, 2025).

[22] QIAamp DNA Investigator Handbook, Qiagen, Hilden, Germany, 2020.
[23] M.A. Dass, C.D.H. Sherman, R.A.H. van Oorschot, K. Tuohey, D. Hartman, G. Carter, A. Durdle, Assessing eDNA capture method from aquatic environment to optimise recovery of human mt-eDNA, Forensic Sci. Int. 361 (2024) 112085, https://doi.org/10.1016/j.forsciint.2024.112085.
[24] M. Lewis, K. Lainé, K. Scott, B. Hänfling, N. Dawnay, Identifying the end user requirements for a novel, field-based environmental DNA (eDNA) forensic collection method, Sci. & Justice (2025) 101371, https://doi.org/10.1016/J.SCIJUS.2025.101371.
[25] B. Hänfling, L. Lawson Handley, D.S. Read, C. Hahn, J. Li, P. Nichols, R. C. Blackman, A. Oliver, I.J. Winfield, Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods, Mol. Ecol. 25 (2016) 3101–3119, https://doi.org/10.1111/mec.13660.
[26] K.M. Zarzyczny, J.C. Robidart, P.B. Fenberg, A. Forryan, M.E. Hellberg, S. T. Williams, D.A. Paz-García, A.C. Naveira Garabato, M. Rius, Combining Environmental DNA Data With Oceanography, Life History and Ecology for Detecting Climate-Induced Range Shifts, Divers. Distrib. 31 (2025) e70064, https://doi.org/10.1111/DDI.70064.
[27] T. Jo, K. Takao, T. Minamoto, Linking the state of environmental DNA to its application for biomonitoring and stock assessment: Targeting mitochondrial/nuclear genes, and different DNA fragment lengths and particle sizes, Environ. DNA 4 (2022) 271–283, https://doi.org/10.1002/EDN3.253.
[28] T. Jo, H. Murakami, R. Masuda, M.K. Sakata, S. Yamamoto, T. Minamoto, Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA, Mol. Ecol. Resour. 17 (2017) e25–e33, https://doi.org/10.1111/1755-0998.12685.
[29] K. Uchii, H. Doi, T. Okahashi, I. Katano, H. Yamanaka, M.K. Sakata, T. Minamoto, Comparison of inhibition resistance among PCR reagents for detection and quantification of environmental DNA, Environ. DNA 1 (2019) 359–367, https://doi.org/10.1002/edn3.37.
[30] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clin. Chem. 55 (2009) 611–622, https://doi.org/10.1373/CLINCHEM.2008.112797.
[31] N. Dawnay, R. Ogden, R. McEwing, G.R. Carvalho, R.S. Thorpe, Validation of the barcoding gene COI for use in forensic genetic species identification, Forensic Sci. Int. 173 (2007) 1–6, https://doi.org/10.1016/J.FORSCIINT.2006.09.013.
[32] National Center for Biotechnology Information, (2025). https://www.ncbi.nlm.nih.gov/ (accessed November 28, 2025).
[33] J.E.L. Carlsson, D. Egan, P.C. Collins, E.D. Farrell, F. Igoe, J. Carlsson, A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera L.*), Aquat. Conserv. 27 (2017) 1341–1344, https://doi.org/10.1002/aqc.2788.
[34] N. Dawnay, P. Riley, L. Dawnay, R. Ogden, S. McColl, Development of a qPCR assay for the quantification of canine autosomal DNA recovered from livestock attacks, Sci. & Justice 64 (2024) 688–697, https://doi.org/10.1016/J.SCIJUS.2024.10.003.
[35] A. Ruiz-Villalba, J.M. Ruijter, M.J.B. van den Hoff, Use and misuse of Cq in qPCR data analysis and reporting, Life 11 (2021) 496, https://doi.org/10.3390/LIFE11060496.
[36] B.E. Watt, R.L. Malcolm, M.H.B. Hayes, N.W.E. Clark, J.K. Chipman, Chemistry and potential mutagenicity of humic substances in waters from different watersheds in Britain and Ireland, Water Res. 30 (1996) 1502–1516, https://doi.org/10.1016/0043-1354(95)00319-3.
[37] M. Johnson, I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, T.L. Madden, NCBI BLAST: a better web interface, Nucleic Acids Res. 36 (2008) W5–W9, https://doi.org/10.1093/NAR/GKN201.
[38] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (2004) 1792–1797, https://doi.org/10.1093/NAR/GKH340.
[39] S. Kumar, G. Stecher, M. Suleski, M. Sanderford, S. Sharma, K. Tamura, MEGA12: molecular evolutionary genetic analysis version 12 for adaptive and green computing, Mol. Biol. Evol. 41 (2024) 1–9, https://doi.org/10.1093/MOLBEV/MSAE263.
[40] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.
[41] Primers - Geneious Prime User Manual, (n.d.). https://manual.geneious.com/en/latest/Primers.html (accessed March 11, 2026).
[42] G.F. Ficetola, E. Coissac, S. Zundel, T. Riaz, W. Shehzad, J. Bessière, P. Taberlet, F. Pompanon, An In silico approach for the evaluation of DNA barcodes, 2010 11, BMC Genom. 11 (1) (2010) 434, https://doi.org/10.1186/1471-2164-11-434.
[43] R. Hinlo, D. Gleeson, M. Lintermans, E. Furlan, Methods to maximise recovery of environmental DNA from water samples, PLOS One 12 (2017) e0179251, https://doi.org/10.1371/JOURNAL.PONE.0179251.
[44] J. Cohen, Statistical Power Analysis for the Behavioral Sciences, Routledge, 2013, https://doi.org/10.4324/9780203771587.
[45] J. Martin Bland, D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 327 (1986) 307–310, https://doi.org/10.1016/S0140-6736(86)90837-8.
[46] B.J. Sansom, D.V. Ruiz-Ramos, N.L. Thompson, M.O. Roberts, Z.A. Taylor, K. Ortiz, J.W. Jones, C.A. Richter, K.E. Klymus, Detection and transport of environmental DNA from two federally endangered mussels, PLoS. One 19 (2024) e0304323, https://doi.org/10.1371/JOURNAL.PONE.0304323.
[47] M.C. Yates, M. Gaudet-Boulay, E. Garcia Machado, G. Côté, A. Gilbert, L. Bernatchez, How much is enough? Examining the sampling effort necessary to estimate mean eDNA concentrations in lentic systems, Environ. DNA 5 (2023) 1527–1540, https://doi.org/10.1002/EDN3.461.
[48] L. Lavictoire, C. West, Population Reinforcement of the Endangered Freshwater Pearl Mussel (Margaritifera margaritifera): Lessons Learned, 2024, Vol. 16, Page 187, Diversity 16 (2024) 187, https://doi.org/10.3390/D16030187.
[49] K.N. Lee, R.P. Kelly, E. Demir-Hilton, E. Laschever, E.A. Allan, Adoption of environmental DNA in public agency practice, Environ. DNA 6 (2024) e470, https://doi.org/10.1002/EDN3.470.
[50] SCIENTIFIC WORKING GROUP ON DNA ANALYSIS METHODS 1 SWGDAM Interpretation Guidelines for Single Nucleotide Polymorphism (SNP) Analysis by Forensic DNA Testing Laboratories Short Title: SNP Interpretation Guidelines2024.
[51] E.W. Sayers, J. Beck, E.E. Bolton, J.R. Brister, J. Chan, D.C. Comeau, M. Feldgarden, A.M. Fine, K. Funk, J. Hoffman, S. Kannan, C. Kelly, W. Klimke, S. Kim, S. Lathrop, Z. Lu, A. Malheiro, T.L. Posey, Y. Wang, J. Ye, E. Zellers, V. A. Schneider, K.D. Pruitt, Database resources of the National Center for Biotechnology Information in 2025, Nucleic Acids Res. 53 (2025) D21–D29, https://doi.org/10.1093/nar/gkae979.
[52] J. Geist, R. Kuehn, Genetic diversity and differentiation of central European freshwater pearl mussel (Margaritifera margaritifera L.) populations: Implications for conservation and management, Mol. Ecol. 14 (2005) 425–439, https://doi.org/10.1111/J.1365-294X.2004.02420.X.
[53] L.M.I. Webster, T.L. Prigge, G.J. Frankham, A guide for the validation of DNA based species identification in forensic casework, Forensic Sci. Int. Anim. Environ. 5 (2024) 100080, https://doi.org/10.1016/J.FSIAE.2023.100080.
[54] H.S. Stern, Statistical issues in forensic science, Annu. Rev. Stat. Appl. 4 (2017) 225–244, https://doi.org/10.1146/ANNUREV-STATISTICS-041715-033554.
[55] J. Naue, C. Xavier, S. Hörer, W. Parson, S. Lutz-Bonengel, Assessment of mitochondrial DNA copy number variation relative to nuclear DNA quantity between different tissues, Mitochondrion 74 (2024) 101823, https://doi.org/10.1016/J.MITO.2023.11.006.
[56] M.H. Fu'Adil Amin, J.H. Lee, A.R. Kim, J.K. Kim, C. Il Lee, H.W. Kim, Development of a quantitative PCR assay for four salmon species inhabiting the yangyangnamdae river using environmental DNA, 2021, Vol. 10, Page 899, Biology 10 (2021) 899, https://doi.org/10.3390/BIOLOGY10090899.
[57] R. Flynn, C. McVeigh, F. Mackin, F.R. Wilson, Sources of stream base flow in blanket peat covered catchments, J. Hydrol. (Amst.). 603 (2021) 126965, https://doi.org/10.1016/J.JHYDROL.2021.126965.
[58] F. Worrall, R. Harriman, C.D. Evans, C.D. Watts, J. Adamson, C. Neal, E. Tipping, T. Burt, I. Grieve, D. Monteith, P.S. Naden, T. Nisbet, B. Reynolds, P. Stevens, Trends in dissolved organic carbon in UK Rivers and Lakes, 2004 70, Biogeochemistry 70 (3) (2004) 369–402, https://doi.org/10.1007/S10533-004-8131-7.
[59] T. Jo, H. Yamanaka, Fine-tuning the performance of abundance estimation based on environmental DNA (eDNA) focusing on eDNA particle size and marker length, Ecol. Evol. 12 (2022) 9234, https://doi.org/10.1002/ece3.9234.
[60] S. Wacker, F. Fossøy, B.M. Larsen, H. Brandsegg, R. Sivertsgård, S. Karlsson, Downstream transport and seasonal variation in freshwater pearl mussel (Margaritifera margaritifera) eDNA concentration, Environ. DNA 1 (2019) 64–73, https://doi.org/10.1002/edn3.10.
[61] Z.T. Wood, A. Lacoursière-Roussel, F. LeBlanc, M. Trudel, M.T. Kinnison, C. Garry McBrine, S.A. Pavey, N. Gagné, Spatial heterogeneity of eDNA transport improves stream assessment of threatened salmon presence, abundance, and location, Front. Ecol. Evol. 9 (2021), https://doi.org/10.3389/fevo.2021.650717.
[62] J. Geist, K. Auerswald, Physicochemical stream bed characteristics and recruitment of the freshwater pearl mussel (Margaritifera margaritifera), Freshw. Biol. 52 (2007) 2299–2316, https://doi.org/10.1111/J.1365-2427.2007.01812.X.
[63] C. Hundl, M. Neuman, A. Rairden, P. Rearden, P. Stout, Implementation of a blind quality control program in a forensic laboratory, J. Forensic Sci. 65 (2020) 815–822, https://doi.org/10.1111/1556-4029.14259.
[64] K.E. Klymus, D.V. Ruiz Ramos, N.L. Thompson, C.A. Richter, Development and testing of species-specific quantitative PCR assays for environmental DNA applications, J. Vis. Exp. (2020), https://doi.org/10.3791/61825.
[65] Z.H. Olson, J.T. Briggler, R.N. Williams, An eDNA approach to detect eastern hellbenders (Cryptobranchus a. alleganiensis) using samples of water, Wildl. Res. 39 (2012) 629, https://doi.org/10.1071/WR12114.
[66] J. Biggs, N. Ewald, A. Valentini, C. Gaboriaud, T. Dejean, R.A. Griffiths, J. Foster, J. W. Wilkinson, A. Arnell, P. Brotherton, P. Williams, F. Dunn, Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus), Biol. Conserv. 183 (2015) 19–28, https://doi.org/10.1016/j.biocon.2014.11.029.
[67] M. Lewis, K. Lainé, L. Dawnay, D. Lamont, K. Scott, S. Mariani, B. Hänfling, N. Dawnay, The forensic potential of environmental DNA (eDNA) in freshwater wildlife crime investigations: from research to application, Sci. & Justice 64 (2024) 443–454, https://doi.org/10.1016/j.scijus.2024.06.003.
[68] A. Sahu, M. Singh, A. Amin, M.M. Malik, S.N. Qadri, A. Abubakr, S.S. Teja, S. A. Dar, I. Ahmad, A systematic review on environmental DNA (eDNA) Science: an eco-friendly survey method for conservation and restoration of fragile ecosystems, Ecol. Indic. 173 (2025) 113441, https://doi.org/10.1016/J.ECOLIND.2025.113441.
[69] De Brauwer, M; Chariton, A; Clarke, LJ; Cooper, MK; Di Battista, J; Furlan, E; Giblot-Ducray, D; Gleeson, D; Harford, A; Herbert, S; MacDonald, AJ; Miller, A; Montgomery, K; Mooney, T; Noble, LM; Rourke, M; Sherman, CDH; Stat, M; Suter, L; West, KM; White, N; Villacorta-Rath, C; Zaiko, A; Trujillo-Gonzalez, A, Environmental DNA test validation guidelines, 2023.

[70] J.A. Kronenberger, T.M. Wilcox, M.K. Young, D.H. Mason, T.W. Franklin, M. K. Schwartz, Large-scale validation of 46 invasive species assays using an enhanced in silico framework, Environ. DNA 6 (2024) e548, https://doi.org/10.1002/EDN3.548.
[71] M. Sidstedt, P. Rådström, J. Hedman, PCR inhibition in qPCR, dPCR and MPS—mechanisms and solutions, 2020 412, Anal. Bioanal. Chem. 412 (9) (2020) 2009–2023, https://doi.org/10.1007/S00216-020-02490-2.
[72] L. Carraro, E. Mächler, R. Wüthrich, F. Altermatt, Environmental DNA allows upscaling spatial patterns of biodiversity in freshwater ecosystems, 2020 11, Nat. Commun. 11 (1) (2020) 3585, https://doi.org/10.1038/s41467-020-17337-8.
[73] A. Burian, Q. Mauvisseau, M. Bulling, S. Domisch, S. Qian, M. Sweet, Improving the reliability of eDNA data interpretation, Mol. Ecol. Resour. 21 (2021) 1422–1433, https://doi.org/10.1111/1755-0998.13367.