Identifying the end user requirements for a novel, field-based environmental DNA (eDNA) forensic collection method

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Declarations of interest: none

Acknowledgements: The authors would like to thank the Liverpool John Moores VC Scholarship scheme and the Chartered Society of Forensic Science for providing funding to support this research.

Keywords: Environmental DNA (eDNA), forensic method development, wildlife crime investigation, freshwater pearl mussel

Abstract

Environmental DNA (eDNA) refers to the DNA derived from species and found in environmental samples such as water, air and soil. eDNA has been used in biodiversity and conservation studies but not routinely implemented in forensic investigations. Currently physical surveys are implemented at freshwater pearl mussel, *Margaritifera margaritifera,* crime scenes which are costly, invasive, reliant on practitioner experience and susceptible to false negative results. The aim of this study was to identify key end-user requirements for a novel eDNA sampling method collected through stakeholder discussion and questionnaire. Stakeholder discussions identified the following key criteria for method development: the end-user group, ease of collection, risk to end-user, speed of collection, cost of collection, and data accuracy. These criteria were used to develop three possible eDNA collection approaches: basic (2L water bottles for bankside collection), intermediate (Whirl-Pak® bags for bankside collection), and advanced (onsite Sterivex™ filtration equipment to use on samples collected by entering the watercourse). To compare the three possible methods in respect to their ease and risk, a questionnaire was disseminated to government officers, police officers, postgraduate students and university staff with expertise in either wildlife crime and/or eDNA approaches (N=63). The time taken to collect samples together with the associated costs of equipment and analysis time were also calculated. Finally, impact of collection method on data accuracy was assessed through the development of a qPCR assay to measure the sensitivity, specificity and concentrations across two different DNA fragment sizes from hatchery reared populations of *M.margaritifera*. End-user questionnaire data reveals a preference for the basic and intermediate methods in respect to their ease of use and identified risks. Furthermore, these sampling strategies were quicker and cheaper to use although the advanced method could offer logistical benefits in terms of longer preservation times and reduced laboratory processing time. In respect to impact on data accuracy, the advanced method also showed poor detection rates of eDNA, a surprising result given the popularity of the method in conservation studies. Together, these data show the benefits of a co-developed and usable method for police and conservation first responders to use at wildlife crime investigations concerning *M.margaritifera* habitat destruction. Further research to develop a robust qPCR assay and assess the usability of the basic and intermediate collection methods on wild populations is recommended to determine the overall effectiveness of the described methods.

1. Introduction

Environmental DNA (eDNA) generally refers to the DNA found in soil, water or air which can be isolated from environmental samples. Intracellular, intraorganellar, dissolved or particle-adsorbed DNA in these samples [1] can derive from skin, mucous, saliva, secretions, sex cells, waste products, blood, plant materials, rotting bodies and entire microorganisms present within an ecosystem [2–4]. Freshwater eDNA methods have been used widely for effective monitoring of invasive species such as European carp, *Cyprinus carpio* [5,6] ,and crayfish, *Pacifastacus leniusculus, Faxonius limosus, Faxonius immunis, Procambarus clarkii* [7,8], and endangered species such as the eastern hellbender, *Cryptobranchus alleganiensis* [9,10], great crested newt, Triturus cristatus [11,12] and freshwater mussel, *Margaritifera, margaritifera* [13–16]. While there is growing research exploring the potential for eDNA methods in forensic applications [17,18] these procedures are still under development and have not been utilised in wildlife crime investigations despite evidence that they could be readily adapted into forensic frameworks [19].

Freshwater pearl mussels *(Margaritifera margaritifera)*, native to rivers across Europe and parts of North America [20]), are listed as critically endangered on the IUCN Red List [21]. *M. margaritifera* is legally protected under several international frameworks, such as Annexes II and V of the EU Habitats Directive 92/43/EEC [22], as well as under national legislations- for example, in the UK via the Wildlife and Countryside Act [23] where it is a criminal offence to disturb, injure or kill the species. Commonly cited threats include habitat destruction, water pollution and pearl fishing [24,25], though the full scale of these are unknown due to inconsistent data and the under-reporting of crimes [26].

Currently, suspected cases of illegal activity are investigated across Europe through labour-intensive physical surveys involving wading upstream and performing spot-checks of riverbed transects positioned obliquely across the flow with a glass-bottomed viewing bucket [27,28]. While this approach offers physical proof if individuals are observed, it is costly, requires specialist training and is difficult to scale. As such, there is a growing need for non-invasive, cost-effective, and scalable forensic tools. eDNA analysis offers a promising alternative. By serving as a presumptive test, eDNA could be used to prioritise physical inspections and better allocate enforcement resources [19].

Forensic tools may be developed through academic research or via stakeholder groups such as the PAW Forensic Working Group [29], but co-development with end-user consultation is strongly recommended to ensure practical adoption. This study develops and evaluates methods for collecting forensic eDNA samples in suspected wildlife crime cases affecting *M.margaritifera* populations. Potential users include nature agencies, national park authorities and the police, all of whom have varying expertise, constraints, and requirements. By assessing cost and time efficiency, ease of use, perceived safety, and detection accuracy, this study aims to identify the most suitable approaches and inform the development of field-appropriate eDNA methodologies. While this study focuses on the UK context, where *M.*

margaritifera is one of six national wildlife crime priorities under the National Police Chief's Council [30], the tools and evaluation framework are designed to have broader application. As eDNA technologies advance, integrating such standardised criteria into forensic workflows could enhance their role as a reliable, non-invasive tool for wildlife crime detection and enforcement.

2. Methods

2.1 Method development

An initial literature review to determine the features of traditional physical survey methods and novel eDNA methods was conducted in Google Scholar using the search term 'freshwater pearl mussel eDNA vs traditional approaches'. The search was then expanded to include identified areas using terms such as 'false positives and negatives eDNA studies' and 'environmental effects eDNA'. This search was used to support stakeholder discussions (Table 1) leading to the collaborative identification of six key end-user specifications (end user group, safety, speed, cost, ease of use and data quality) for forensic eDNA sampling (Table 2). Three methods for water collection were developed to accommodate three end-user groups:

- Basic (designed for end users with no materials available for impromptu sampling events). Sampling from the bank with shop bought 2L water bottles emptied and washed out with water from the sample location. Two litre bottles were used as they are most common in UK supermarkets.
- Intermediate (designed for end users with materials available but unable to enter the water [31]). Sampling from the bank with 36oz (1.023L) sterile Whirl-Pak® bags (Nasco).
- Advanced (designed for end users with materials available who are able to enter the water [31]). Sampling from within the watercourse using Whirl-Pak® bags (Nasco) and onsite filtration of 1L of water with Sterivex™ (Merck Millipore). Filters preserved with 100% ethanol.

Each method was assessed according to the key requirements as detailed in Table 2.

Table 1. *The features of eDNA and traditional approaches when surveying Margaritifera margaritifera habitats to monitor species presence in a forensic capacity.*

Sampling consideration	eDNA sampling	Traditional approach
Evidence of FWPM presence	Presence is inferred after detection of mussel DNA [14].	Physical presence can be confirmed visually [27].
False positives	Possible due to contamination, vertical transport, historical DNA persistence or insufficient specificity of assays [32].	Unlikely unless dead shells are misreported as live mussels.
False negatives	Possible in the field due to inappropriate sampling method, habitat heterogeneity, low DNA persistence due to rapid degradation, seasonal effects and hydrological events. Possible in lab due to inhibition, inadequate extraction or storage of samples, and low sensitivity and specificity of assays [32].	High risk [13]. It may be impractical to physically survey every single square metre of a habitat dependent on size. Mussels may be burrowed into the substrate or undetected due to crypsis [33].
Sensitivity and detection limits	Highly sensitive. Small mussel populations (N=20) have been detected 8km downstream [34]. Accuracy dependent on robust collection methods and procedures [19].	Efficiency may vary dependent on practitioner experience and poor visibility in turbid waters [13].
Spatial and temporal resolution	Can cover large spatial scales. Determination of exact mussel distribution wihin the habitat not possible. Sampling can be performed across sites simultaneously.	Dependent on strategy a good spatial resolution could be achieved but would be time consuming or labour intensive.
Effects of environmental conditions	Turbid waters can decrease the ease of filtration and increase levels of inhibition [35]. eDNA degradation in aquatic habitats may be increased by higher temperature, neutral to acidic pH and moderately high UV-B levels [36]. Increasing stream size can decrease detection rate [7].	Poor visibility if low water clarity. High water levels and adverse weather conditions could cause a health and safety risk.
Data interpretation	eDNA datasets must be interpreted accounting for spatiotemporal variation with a knowledge of DNA dynamics within the water body [37]. Interpretation may require use of occupancy or process-based modelling [32].	Observations easily logged and interpreted.
Practitioner experience	First responders could compromise an investigation due to lack of training and awareness [38]. Training in forensic collection methods would be required.	Practitioners' knowledge from many years of experience invaluable to identify hotspot areas of high concern. Novices would need training by an experienced sampler.
Effect on target species	Mussels unlikely to be affected if water samples are collected from the bank. Samples taken from the water body would require due diligence and could require prerequisite permissions or licensing.	Mussels could be accidentally disturbed or dislodged by sampling activity. If mussels are handled, this could cause indirect mortality due to short term behavioural costs [33]

Table 2. *The 6 key factors in eDNA forensic method development, their associated constraints and the mitigations required for operationalisation. Perceived ease of use and safety of the collection methods were assessed by circulation of an anonymous questionnaire.*

Factor	Considerations	Constraints	Mitigations
Users	End users- Nature Scot and Police Scotland.	Police unlikely to have access to eDNA equipment/unable to enter the water. Nature Scot may or may not be able to enter the water depending on licensing requirements/time.	Development of basic, intermediate, and advanced eDNA collection strategies for different groups.
Safety	Entering the water. River conditions and terrain. Variable weather conditions.	May need to enter the water to collect samples. River conditions (flow, depth, temperature) may make it unsafe to enter the water. Environmental conditions (wind, rain etc) may increase risk and limit time in the field. Different equipment required for potential risks.	Risks associated with different collection strategies must form basis for method development. User groups need consultation on different collection strategies. Training (third party or internal) required around risks and risk management when working in and near rivers. Risk assessments required for different collection methods can be shared with each end-user group.
Speed	eDNA unstable. Adverse field conditions likely to limit time at crime scene. Additional time required for CSI duties.	Sample filtration/preservation on side of bank takes a long time. Sample collection in adverse weather and river conditions more limited than in fair conditions. Weather will not prevent sample collection but will impact strategy chosen. CSI paperwork for QC purposes is essential but time consuming.	Basic strategy not time consuming (<60 minutes) Maximum 3 hours on site for all methods. Develop evidence management strategy that ensures documenting scene and labelling evidence in ≤ 1 hour.
Cost	Different collection strategies will vary in cost. Government budgets for forensic and consultation work.	Can't control cost of required consumables and kits. Volume of work is may not to be high enough to reduce costs through bulk purchasing. Sample preservation on side of bank more costly.	Develop collection methods that provide end-users with a choice of cost options. Total cost of sampling must be less than an independent ecologist (£330 including VAT).
Ease	Methods need to have minimal steps, be easily adopted and be reproducible. Equipment needs to be easily sourced and transported.	Police have limited eDNA collection experience. Nature Scot Officers have limited CSI experience. Sample preservation on side of bank complicated.	Training in each eDNA collection strategies needs to be provided to each user group. Videos of each method to be generated for stakeholder discussion. Three different strategies developed with end-users picking most appropriate for their staff.
Data Quality	Data should be specific, sensitive, reproducible, and repeatable. Impact of different collection methods on 'data quality' needs assessing.	Increased eDNA sensitivity may lead to false positives. Data from different population sizes need assessing. Data from different seasons need assessing.	Validated qPCR assay needs developing before sample collection. Include short and long DNA fragments in qPCR. Assess specificity/sensitivity to determine accuracy under a range of conditions.

2.2 Data collection

2.2.1 Ease of use and perceived safety

Perceived ease of use and safety of the three collection methods were assessed by distribution of an online questionnaire created in Google Forms. Participants gave their job role and current level of field activity. They watched videos of each collection technique followed by completion of Likert scale questions to assess ease of use, ease of implementation, training requirements, and perceived safety; followed by the opportunity to leave additional comments. Finally, they ranked different scenarios in order of safety concern, different skills in order of priority at any potential training event, and were invited to name any barriers to operationalisation (Supplemental Material 1). The survey was distributed at the 2023 UK Annual Wildlife Crime Conference [39] and via email to staff and postgraduate students in (redacted) and (redacted). Respondents represented four key groups with fieldwork, applied conservation and crime response backgrounds: 23 academic researchers with training in relevant subject areas (forensic bioscience, conservation and ecology), 12 wildlife crime police officers from various UK forces, 18 government officers (Natural Resources Wales and NatureScot) and 5 non-government officers (affiliated conservation bodies). The survey was anonymous, voluntary and no personal information was collected. The survey was granted ethical approval (redacted) from the University Research Ethics Committee (UREC). Including other job roles, a total of 63 individuals completed the survey. Response data was exported to Microsoft Excel for Microsoft 365 (Version 2509) for analysis.

2.2.2 Time

Researchers performed field sampling at six field sites in the Lake District, England and North Wales. Timings were performed at field sites only as they best reflected conditions of a crime scene (N=24 for each method). The mean time taken to collect each sample from entering the water to securing the sample was recorded. Data was checked for normality and variances with post hoc Mann-Whitney U tests with Bonferroni correction chosen to identify significant differences between methods ($p=0.017$).

2.2.3 Cost

The cost of sample collection materials was calculated based on the average costs of three suppliers, whilst lab reagent costs were taken directly from the manufacturer's websites. These estimates include consumables needed for sampling, filtration, kits for eDNA extraction, PCR master mix, and staff costs (including overheads). They do not include travel to field sites or costs for assay optimisation. Processes assumed a well-equipped eDNA laboratory (Supplemental data 2). Data was checked for normality and variances with one-way ANOVA and post hoc Tukey honestly significant difference (HSD) tests chosen to identify significant differences between methods ($p=0.05$). All statistics were calculated in SPSS 29 (IBM, 2023).

2.2.4 Impact of collection method on data quality

The impact of collection method on data quality was explored through the development of two single-plex qPCR assays, one amplifying an 83bp fragment of COI and the other an 821bp fragment of COI. qPCRs were carried out on the Rotor Gene Q (Qiagen, Hilden, Germany) using a universal forward primer (5'-TTGTTGATTCGTGCTGAGTTAGG-3') and separate short reverse (5'-GCATGAGCC GTAACAATAACATTG-3') and long reverse (5'-GCCGCAGTAAAATAAGCCCG-3') primers. The short amplicon primers were selected based on their application in previous research [40] and sensitivity for detecting low-concentration samples. The long amplicon reverse primer was selected after wet lab screening of different primer pairs across variable amplicon sizes and its potential utility as a relative indicator of specificity (or proximity to source population) in field applications. Amplified product was detected using a TaqMan® minor groove binding (MGB) FAM probe (5'-CCTGGTTCTTTGCTGGGTGATGATC-3') (Thermofisher). Primer sequences were aligned in MegaX [40] to check for specificity to *M.margaritifera*, other genetically similar freshwater species and species whose DNA is commonly encountered within PCR testing of freshwater samples. qPCR was carried out in 20µl final volumes consisting of 10µl 1X TaqMan Path Pro Master Mix, 4.5µl ddH_2O and 0.5µM of forward primer, 0.5uM reverse primer with 0.5uM probe, allowing 4µl DNA input. Thermal cycling parameters consisted of 95ºC enzyme activation (10 minutes) followed by 50 cycles of 90°C denaturation (15 seconds) and annealing/extension at 60°C (60 seconds). RotorGene analysis settings were as follows: Filter data: Light data smoothing. Remove non-amplified curves with fluorescence change <5%/ reaction efficiency <-100%. Crop cycles: before 1 and after 50. Normalization: Check dynamic tube: use noise slope correction, ignore first 5 cycles, adjust take-off points- if take off point<12 use take-off cycle 20. Tissue standards were used as positive controls. No-template controls (NTCs) were included in each qPCR run, along with negative extraction controls (generated by filtering the bottled water) for each sampling visit and collection method were also included. This allowed quality control over contamination and assay consistency.

Following primer selection and qPCR optimisation and characterization, the assays were applied to compare collection method effectiveness. Sensitivity of the qPCR assay was first assessed through a dilution series of DNA using donated mussel tissue while the impact of the three different collection methods on the qPCR accuracy was assessed using water samples recovered from two hatcheries in Wales (operated by Natural Resources Wales) and the Lake District (operated by the Fresh Water Biological Association) under permission from the regulatory bodies. From each hatchery, three tanks (one tank with ~50 adults, one with ~15 adults, and one juvenile tank with ~200 juveniles) were chosen to act as positive control sites and mimic populations of different biomass with additional water collected from six natural negative control sites (N=4 for each method at each tank/control site). Negative control sites were situated in the Afon Llugwy, Llyn Padarn and Afon Glaslyn in Wales, and Langdale Beck, Yewdale Beck and Esthwaite Water in the Lake District, all locations where surveys

have shown no population of *M.margaritifera* through the catchment. Field and hatchery sampling was conducted in July 2023. All sampling methods were conducted on the same day for each hatchery visit and for each of group of three negative control sites. Hatchery tanks were maintained with controlled temperature conditions (8ºC Wales, 10ºC Lake District), regulated flow rates (~27Lmin-1 for adults; 10Lmin^{-1} for juveniles) and sand/gravel based substrates, minimising any seasonal influence on DNA persistence. Field sampling during the UK summer corresponded with peak eDNA concentration and detectability in *Margaritifera margaritifera* associated with increased biological activity and water temperature [41]. Samples were transported to the lab in cool boxes and filtered with a randomised study design. Filtration of water samples from basic and intermediate methods was performed using the EZ Fit Vacuum Manifold (Merck Millipore) and 250 ml Thermofisher disposable cups (Thermofisher) within 24 hours of sampling [42]. 1L of sample water was filtered and filters frozen. DNA Investigator Kit [43] swab protocol was used for extraction with an elution volume of 30 µL. The swab protocol was chosen as filters from both bottle and bag samples occupied similar volumes within extraction tubes. This approach ensured consistency across these sample types. Sterivex™ filters were extracted with the DNeasy® PowerWater® Sterivex™ extraction kit with an elution volume of 50µL as per protocol [44]. These extraction kits were selected to align with their respective filtration formats, following manufacturer recommendations and to preserve the integrity of each workflow. This integrated approach reflects real-world application where end-users implement complete collection-to-extraction systems. The impact of each collection method on the sensitivity (proportion of true positives) and specificity of the qPCR assay (proportion of true negatives) was calculated based on established methods [45]. DNA concentrations were compared between tanks to determine any relationship to population biomass. Data were assessed for normality using Shapiro-Wilk tests and homogeneity of variance using Levene's test prior to statistical analysis. When data met parametric assumptions (normal distribution, equal variances), independent samples t-tests or one-way ANOVA with Tukey's HSD post-hoc tests were used for group comparisons. When normality assumptions were met but variances were unequal, Welch's t-test or Welch's ANOVA with Games-Howell post-hoc tests were applied to account for heterogeneity of variance. When data violated normality assumptions, Mann-Whitney U tests were used for two-group comparisons. All statistical tests were conducted at $\alpha=0.05$ significance level using SPSS 29 (IBM, 2023). Effect sizes were calculated and reported for all statistical comparisons to assess the magnitude of observed differences. For Mann-Whitney U tests, effect size r was calculated as $r = z/\sqrt{N}$ [46]. For one-way ANOVA and Welch's ANOVA, eta-squared (η^2) was calculated to quantify the proportion of variance explained. Effect size interpretations followed Cohen's [47] guidelines, and were reported alongside p-values to provide a complete assessment of both statistical significance and practical importance of findings [48].

3. Results and discussion

3.1 Ease of use and training needs

The results of the end user survey indicate that the methods shown in the demonstrative videos were perceived as progressively harder to perform from basic to advanced (Figure 1). Among respondents, 87% felt that the basic method was very easy or somewhat easy in comparison to 81% for intermediate and 46% for advanced. Figure 2 shows that most respondents believe that they could confidently perform the basic (84%) and intermediate (76%) methods with minimal training and that the advanced method would require more training to carry out with 41% needing minimal training. Additionally, 100% of respondents believed that they would need half a day basic method training or less compared to 95% and 79% for the intermediate and advanced. These trends are not surprising. The advanced method is multi-faceted with elements of filtration, preservation, and manipulation of filters. It also requires more health and safety considerations due to sampling from within the water body which requires PPE and risk assessment before entering. The intermediate method may require training on correct sealing of the FAT bag. There was concern that "*if the wire ends aren't carefully tucked bags get punctured*", "*user friendliness*" (of the bags) posed a "*potential for leak and unwanted contamination*" and that the bags should be "*folded*" to seal rather than whirled around the wire-supported mouth band. Comments support findings that manufacturers presume sealing of FAT bags is intuitive to all, the process is prone to error and bags are occasionally reported leaking or improperly sealed after transport [49].

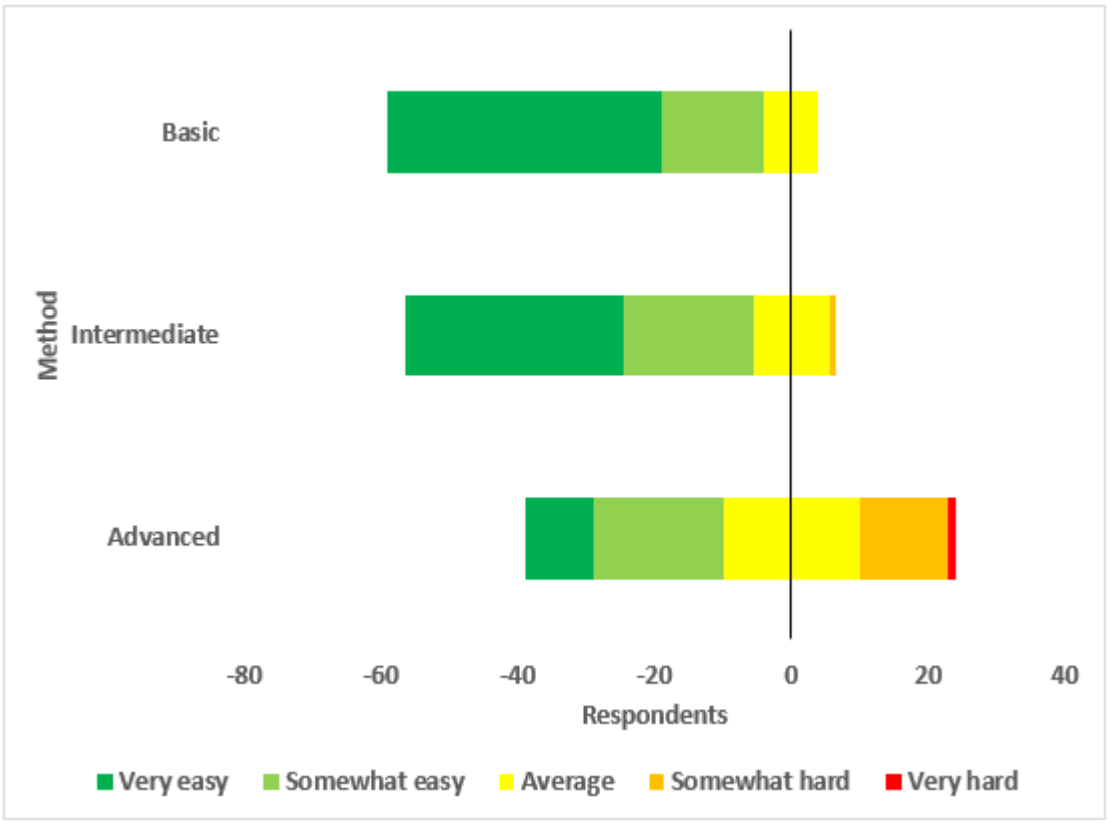

Figure 1. *The 'ease of use' indicated by questionnaire respondents (N=63) from the questions: "If you were performing field work how easy would it be for you to carry out the basic/intermediate/advanced method?"*

Respondents thought that large amounts of equipment would be difficult to transport by foot over long distances. *M.margaritifera* habitats are often located in remote and protected special areas of conservation (SACs) [20] with no direct access via the roads. This supports the use of onsite filtration using the less bulky Sterivex™ in remote areas and reinforces collaborative working practices to ease equipment management. Within wildlife forensics, sample collection may be carried out by first responders without a crime investigation background which can compromise evidential integrity through insufficient training [38] and understanding of forensic protocols. Forensic collection approaches must be performed following standard operating procedures (SOPs) with objective and clear instructions [50] to minimise human bias and error [51] and prepare practitioners for potential legal scrutiny. The survey's indication that many users believe minimal training suffices, even for complex tasks, may point to a wider imbalance between perceived and actual understanding of forensic requirements [52]. Tailored training and stakeholder-specific knowledge sharing could help address this disconnect. Furthermore, the wider forensic sampling process—from anti-contamination measures and chain of custody documentation to secure transport and storage [35,53,54]—must be contextualised within the entire forensic pipeline [19] and communicated to end users. Together, these results suggest that successful adoption of eDNA methods for forensic purposes will depend not only on the physical ease of sampling, but also on robust procedural understanding and training that bridges the gap between field utility and evidential integrity.

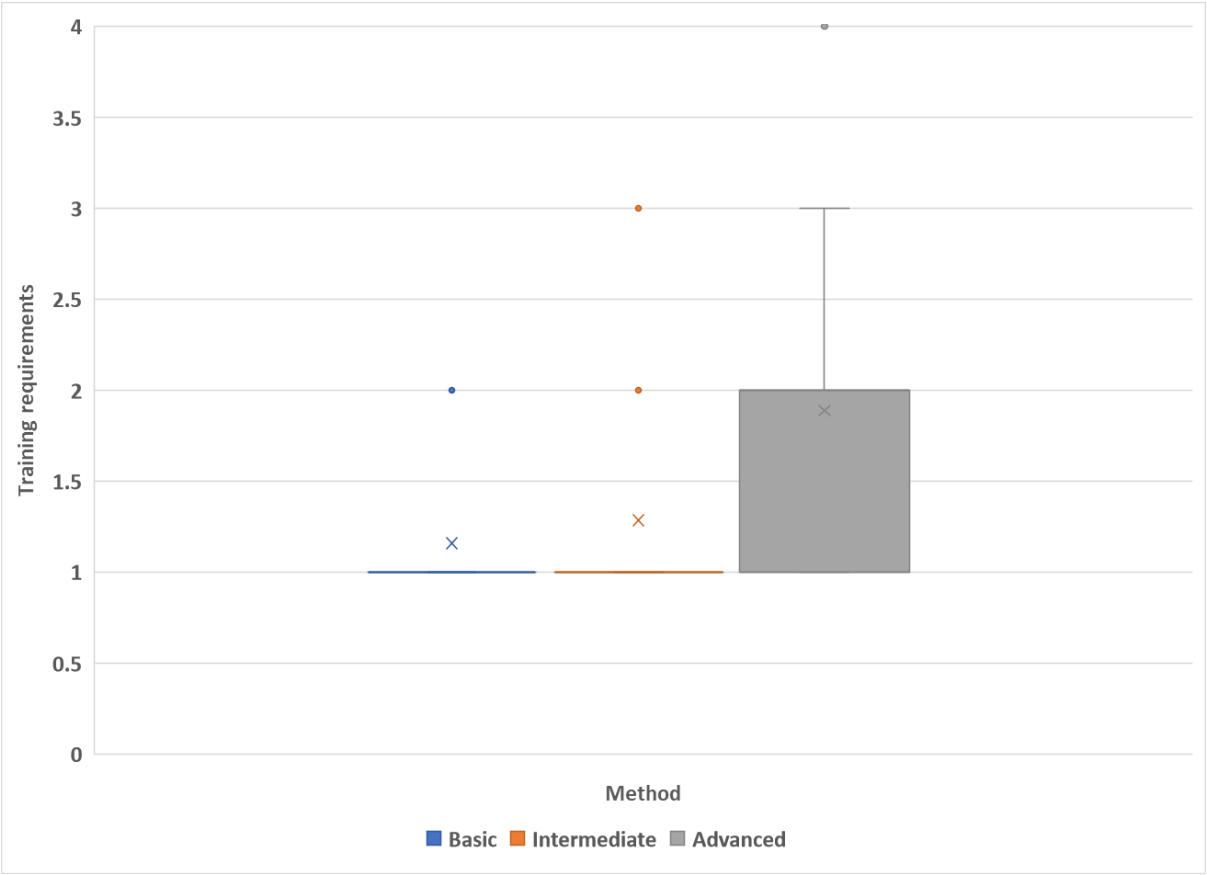

Figure 2 *The training requirements indicated by questionnaire respondents (N=63) from the questions: "How much training do you think you'd need to confidently carry out the basic/intermediate/advanced method?". 1=Minimal (≤1hour), 2= Intermediate (half a day), Advanced (1 day) and Extensive (2-day workshop and field training).Xs show the mean response value and dots show outliers (beyond 1.5 times the interquartile range from the third quartile).*

3.2 Perceived safety

Figure 3 shows that most respondents would feel safe or very safe performing the basic (95.3%), intermediate (89.1%) and advanced (78.1%) methods, with a decrease in perceived safety with increased complexity. Whilst the basic and intermediate methods are performed from the bankside, the advanced method, which requires entering the water with personal protective equipment (PPE), presents additional risks. Respondents highlighted concerns such as lone working, fast river currents, fluctuating water levels, and low temperatures, which could make water entry unsafe or impractical. However, midstream sampling may be beneficial when surveying in wide rivers, when mussel populations are suspected in central channel areas, or when time allows for more comprehensive spatial coverage. This may improve collection when eDNA from central channel sources start as a narrow plume before it laterally disperses to bankside [55].Where midstream access is utilised but direct water entry poses safety risks, extended-reach collection methods could provide safer alternatives [56], though these will increase costs. Simpler adaptations such as collection bottles or bags attached to poles deployed from the bankside offer more accessible alternatives, though such approaches would require an assessment

to ensure comparable sample quality. It has been shown that source organisms often shed eDNA in spatially constrained plumes before a 'breakout phase' where lateral mixing and fragmentation cause a more homogenous mixture with higher detection probability and consequently higher recorded concentrations [57]. It has also been hypothesised that, when considering a midstream source, detections will be highest midstream and lowest bankside at habitat level before homogenising up to about 1000m, where bankside detections may be favourable due to deposition [55]. These processes would have varying effects dependent on speed of river currents and discharge rate, [34,58] and spatial target distribution [41,59]. Entering the water to collect midstream samples could be preferable to increase detection probabilities in certain contexts yet these benefits must be weighed against significant safety considerations.

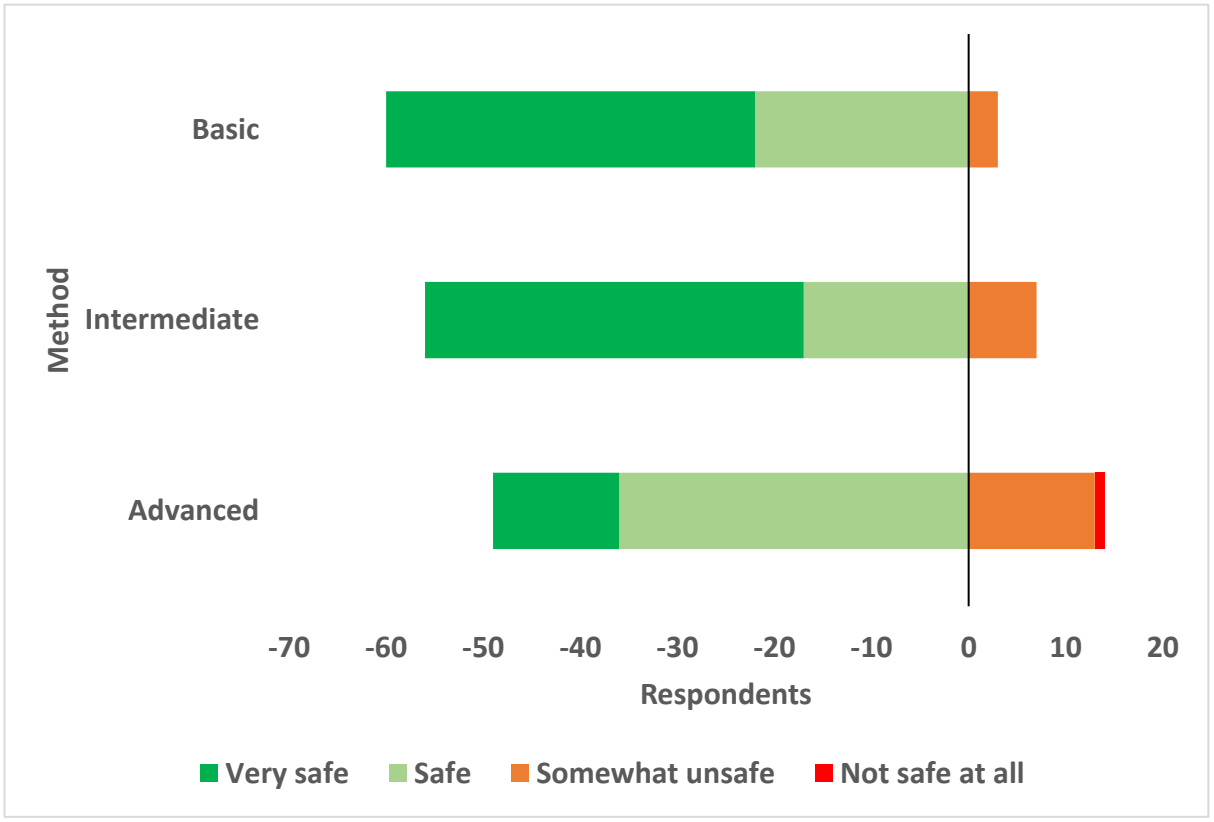

Figure 3. *The perceived safety of each method indicated by questionnaire respondents (N=63) from the questions: "How safe would you feel carrying out the basic/intermediate/advanced method?"*

3.3 Time efficiency

Mann-Whitney U tests indicated that the intermediate method took significantly longer than the basic method (z=-2.795, p=0.005, r=-0.40, medium effect) and that the advanced method took significantly longer than the basic (z=-5.939, p<0.001, r=-0.86, large effect) and intermediate methods (z=-5.939, p<0.001, r=-0.86, large effect) (Figure 4).The basic ($\bar{x}$: 1.16±0.2 mins) and intermediate ($\bar{x}$: 1.52±0.89 mins) methods both had average times below 2 minutes. The intermediate method took longer due to the sealing of the Whirl-Pak® bag despite the 2L bottles for the basic method needing to be washed out

with river water before use. The advanced method ($\bar{x}$: 14.06±3.38 mins) included filtration of water through the Sterivex™ filter, addition of ethanol and sealing.

The basic method serves to prove that forensic collection of eDNA can be performed quickly without the need for specialist equipment or complicated methodology. Species-specific studies simply require that the collection vessel is free from target species contamination and be transported safely for analysis. [60]. The 'fold-and-twist' (FAT) Whirl-Pak® bags used for intermediate collection are sterile, lightweight, easily transportable, and cheap. However, the times recorded in this study may not be repeated by untrained operatives. Respondents to our survey reported that they had found Whirl-Pak® bags "*difficult*", "*not as sturdy as the plastic mineral bottles*" and "*could definitely see people spilling the contents whilst trying to close the bag*". It was experienced in this study that it was difficult to achieve the volume needed from a flowing water body whilst leaving space for sealing. Sharp debris was often removed from samples before sealing and alignment of the mouth bands occasionally required resealing of the sample. FAT bags remain a viable option for eDNA collection but user experience and feedback from the community suggest that prerequisite training would increase reproducibility in terms of time and effectiveness.

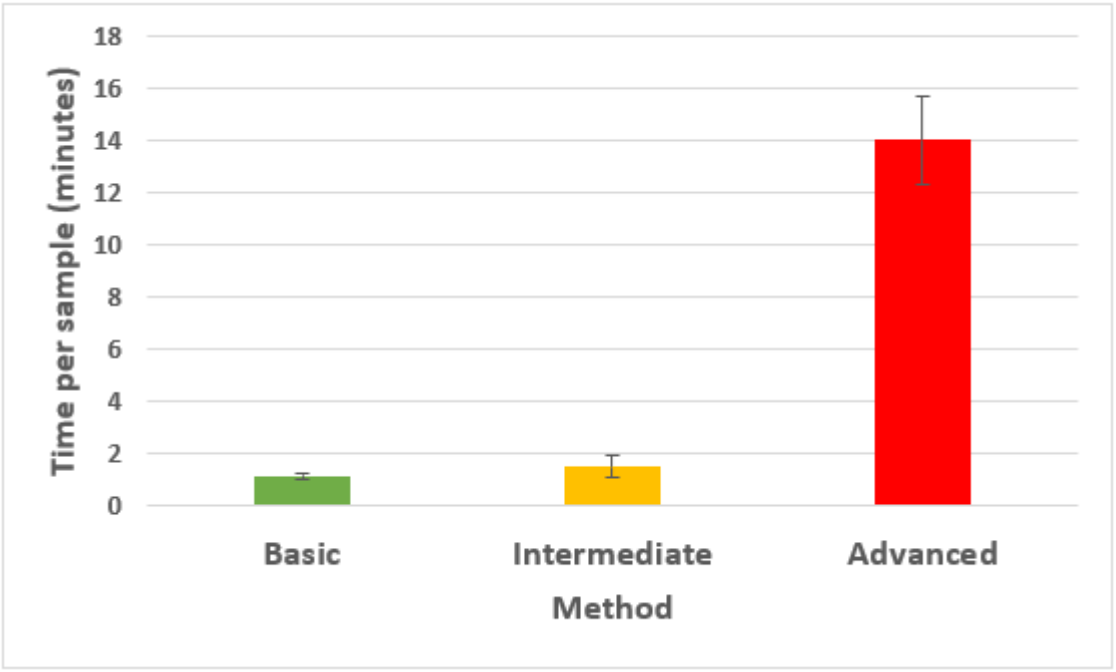

Figure 4. *The mean time in minutes for sample collection using each of the three methods. Timings for each method (N=24) were taken from the negative control sites which better reflected the conditions of a crime scene than the hatchery tanks. Bars show standard deviation.*

The filtration of samples onsite in the advanced method saves time in the laboratory and is considered a logistical advantage as long as filters are preserved accordingly [61] with ethanol or Longmire's buffer recommended [62]. Other direct filtration methods may be used in the field which may be similarly

time consuming [63] and total sampling time when considering number of replicates may involve a few hours at the crime scene. In general, filtration rate and efficiency are dependent on the filter material [64], pore size [65,66], volume filtered [67], water composition and turbidity [35]. Provided filtration materials have been selected accordingly, the associated time considerations may simply be a question of whether the end user has the time to allocate to field filtration, and the facilities to stock and transport filtration and preservative materials.

3.4 Cost efficiency

The mean costs of the basic, intermediate and advanced methods were £65.14, £95.31 and £122.81 respectively. One-way ANOVAs were performed to compare the effect of method upon cost. Collection method had a very large effect on total cost ($F(2,6)=11.717$, $p=0.008$, $\eta^2=0.796$), explaining approximately 80% of the variance in sampling costs. Tukey's HSD test found that the mean cost of the advanced method was significantly higher than the basic method ($p=0.007$, Cohen's $d = 3.67$, very large effect) at the 95% confidence level. There were no statistically significant differences between the basic and intermediate methods ($p=0.126$), and intermediate and advanced methods ($p=0.102$). The cost of all methods fell below that of an independent ecologist visit (£330 inc. VAT) (pers. comm). eDNA methods can be more cost-efficient than traditional approaches when primer/probe development and sample processing costs are low though totality of costs may favour traditional techniques [68]. The field-based collection methods in this study would necessitate instruction in collection technique, water safety and anti-contamination procedures with further training on field filtration and preservation protocols for the advanced method. While these represent initial costs compared to recurring consumable expenses, these requirements constitute a practical barrier to implementation, particularly for resource-limited agencies. An important factor to consider for end users sampling in a forensic capacity is the frequency at which surveying may be necessary. Whereas eDNA sampling has been found to be cheaper in 15 of the 19 conservation studies which directly compare the approaches (N=194) [69], it could be argued that conservation and monitoring studies are relatively prescribed as opposed to forensic investigations which only occur when a crime has been suspected. Low frequency forensic sampling could result in lower cost efficiency due to staff training costs, the purchase of safety equipment, forensic validation of qPCR assays [70], and the processing of less samples simultaneously. However, high frequency projected sampling could justify the implementation of an eDNA workflow, provided the method delivered similar or better results than traditional approaches (Table 1). Another key consideration could be the viability of the future transfer of eDNA methods to sample other species of interest within an organisation.

The basic (£195.04, N=4) and intermediate (£225.31, N=6) methods varied in cost mainly due to the differing amount of similar equipment (Figure 5). The advanced method (£252.81, N=4) was the most expensive due to the high unit cost of the Sterivex™ filters and associated extraction kit. Sterivex™ filters are often used to reduce contamination and allow for onsite sampling [71] which relieves the

pressure of having to safely transport water samples to a laboratory in a timely fashion to avoid excessive DNA degradation [72]. This gives the end user flexibility to sample in remote areas whilst preserving sample integrity before downstream processing. The forensic end user would have to determine whether the additional equipment cost was required based upon the most likely crime scene locations and the potential impact of being unable to process an unfiltered sample within an acceptable timeframe such as 24 hours [42]. The 2L collection bottles used in this study provided practical advantages in terms of availability and filtering only 1L of the collected volume is recommended for routine applications. Furthermore, the additional 1L provides contingency against spillages or leaks during transport and filtration while avoiding the increased processing time, additional filter cups (each processing a maximum of 1L within an acceptable timeframe), and greater consumable costs associated with filtering larger volumes. Together these results demonstrate that 1L provides sufficient sensitivity for detecting *M. margaritifera.*

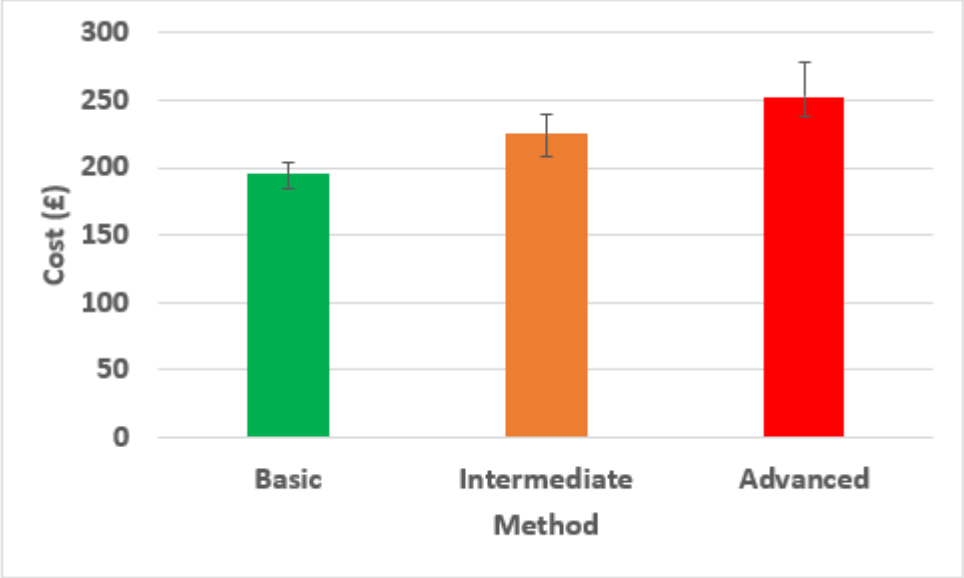

Figure 5. *The total cost of sample collection in £ Sterling (inc. VAT) using each method including sampling, filtration, kits for eDNA extraction, PCR master mix, and staff costs (including overheads). They do not include travel to field sites or costs for assay optimisation. Processes assumed a well-equipped eDNA laboratory. Bars show the minimum and maximum costs from the item prices obtained.*

3.5 Accuracy

The qPCR assays were optimised and characterised prior to application (see Section 2.2.4) and demonstrated robust and reproducible amplification of standard curves (data not shown). These data therefore suggest that the majority of variation observed in reported accuracy metrics is the result of the prior processing steps (collection-filtration-extraction).

3.5.1 Sensitivity

Table 3 shows the overall lack of sensitivity of the Sterivex™ method compared to samples filtered in the laboratory. The Sterivex Powerwater Extraction Kit™ was selected for use due to its easy-to-follow protocol [44], that allows extraction directly from the Sterivex™ capsule. Yields may be improved by aseptic filtration of the filter before processing [73], backflushing with lysis buffer [71], decreasing elution volume [74] or using an alternative method such as Mu-DNA [75]. Low detection could also have been caused by PCR inhibition [76] from plant derivatives [77]. An internal positive control (IPC) within a multiplex assay could detect this inhibition [78].

Table 3. *The sensitivity of the two primer pairs amplifying 83bp and 821bp fragments. Sensitivity values were calculated from the presence/absence of qPCR amplification from Welsh and Lake District hatchery tank samples [45]. Laboratory filtered samples (basic and intermediate methods) were collected using 2L water bottles (N=4) and Whirl-Pak® bags (N=4) from each tank at each location before transport, lab filtration and extraction. Sterivex filtered samples (advanced method) were filtered at the hatchery and extracted in the lab (N=4 for each tank at each location).*

		Wales		Lakes		Overall	
	Fragment size amplified (BP)	83	821	83	821	83	821
Laboratory filtered Sensitivity (%)	Large tank	100.00	87.50	100.00	100.00	**100.00**	**93.75**
	Small tank	100.00	75.00	100.00	62.50	**100.00**	**68.75**
	Juvenile tank	0.00	0.00	100.00	37.50	**50.00**	**18.75**
Sterivex filtered Sensitivity (%)	Large tank	50.00	0.00	75.00	0.00	**62.50**	**0.00**
	Small tank	25.00	0.00	50.00	0.00	**37.50**	**0.00**
	Juvenile tank	0.00	0.00	25.00	0.00	**12.50**	**0.00**

Laboratory filtered samples (Table 3) yielded higher detection rates, with the smaller fragment marker (83.33% across all samples, N=48) exhibiting greater sensitivity than the longer markers (72.50%, N=48). Longer fragments have been shown to be more heterogenous in their spatial distribution [79]. Sensitivity of the longer marker also decreased from the large tanks to the small tanks with less individuals. This suggests lower eDNA concentrations of long fragments in these tanks which has been shown to cause increased stochasticity [80] due to heterogeneity.

Long fragment sensitivity values in the adult tanks (N=32, 81.25%) proves that this marker can be used for detection. Longer fragments exhibit a higher decay rate than smaller fragments, due to larger surface area, lower adsorption rates [81] and higher susceptibility to environmental factors [82]. Presence of the more persistent [79] shorter fragments and absence of longer fragments in a lotic system could indicate further distance from a source population provided there are no confounding factors such as additional mussel beds and sediment resuspension [83]. This could be useful as a conservation detection

tool. Furthermore, presence of shorter fragments and longer fragments could provide forensic intelligence at a destroyed habitat crime scene when mussels are no longer physically observable, whereby positive detections suggest species presence and proximity.

Sensitivity in the juvenile tanks was variable with 0% detection in the Welsh tank across both markers, and 100% and 37.5% in the Lake District tank for 83bp and 821bp markers. Post encystation to a host fish, juveniles generally burrow to avoid being predated until they reach around 2-3 cm after 4-5 yo and establish on the sediment surface [84]. The older juveniles in the Lake District hatchery (estimated 3-6 yo as opposed to 2-3 yo) were noticeable above the surface meaning that their shed eDNA would have entered the water column as opposed to the younger, burrowed Welsh juveniles whose eDNA would likely remain adsorbed to the sediment substrate. eDNA release rate is lower in juvenile bluegill sunfish, *Lepomis macrochirus* than adults [85]. Smaller size mussels (zebra mussel*, Dreissena polymorpha*) have also displayed lower clearance rates of suspended particles [86]. This low detection rate for some juveniles could result in false negative data and certainly be a limitation of the methodology when used for conservation monitoring or site survey work where traditional methods [24] may be preferable. However, potential crime scene habitats would usually involve established adult populations identified by extensive river survey work [87].

3.5.2 Specificity

100% specificity was recorded for all markers across all negative control sites which suggests assay suitability for these field sites. Reduced specificity may occur due to amplification of a non-target species which can result in false positives or false negatives due to cross amplification, template competition [88] and contamination. All species aligned in *in silico* alignment displayed low similarity except for the sympatric duck mussel, *Anodonta anatine* (Supplemental data 3). *A.anatine* amplification seems unlikely because Taqman MGB probes are very sensitive to base pair mismatches [89]. However, in vitro studies testing assays against non-target species and mixtures should be performed as part of effective development [90]. Technical contamination can arise from inappropriate procedures [32]. In this study, negative control samples were used which showed no amplification. However, negative control samples, especially from the field are not always used or documented [91] and are crucial in eDNA studies to rule out sources of contamination or adjust workflows to include extra anti-contamination measures.

3.5.3 Biomass

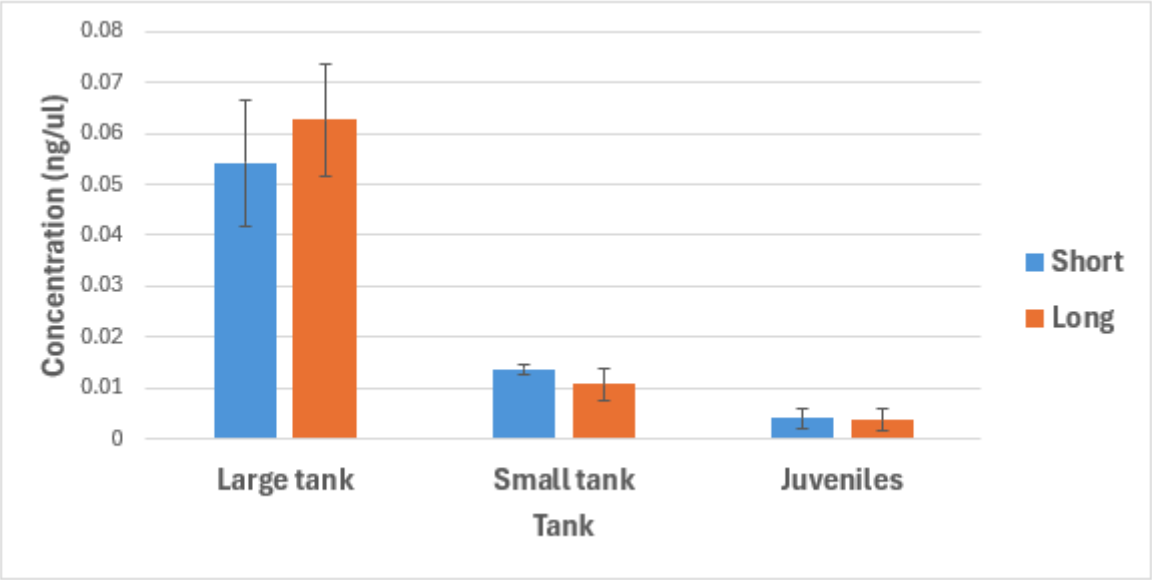

Figure 6. *The eDNA concentrations of the small (83bp) and large (821bp) fragments in the large, small and juvenile tanks (N=16 in each), containing ~50, ~15 and ~200 individuals respectively.*

Independent samples t tests showed that there was no significant difference in the eDNA concentrations between the short (83bp) and long (821bp) fragment sizes in the large ($t(30)=0.499$, $p=0.622$) and juvenile ($t(30)=0.133$, $p=0.895$) tanks. Welch's t test (unequal variances) also showed no significant difference between small tank fragment sizes ($t(18.742)=1.052$, $p=0.306$) at $\alpha=0.05$. Welch's ANOVA revealed a large effect of tank type on eDNA concentration (Welch $F(2,55.45)=26.574$, $p<0.001$, $\eta^2=0.429$), with tank identity explaining approximately 43% of the variance in eDNA concentration. The Games-Howell post-hoc test revealed that all pairwise comparisons were significant: large vs small ($p<0.001$, Cohen's $d = 1.36$, large effect), large vs juvenile ($p<0.001$, $d = 1.60$, large effect), and small vs juvenile ($p<0.001$, $d = 1.05$, large effect) (Figure 6). Sterivex™ concentrations were not included in this study due to low concentration yields.

The approximate 1:1 ratios of fragments in the tanks indicates that the eDNA detected was fresh or minimally degraded. eDNA degradation can be affected by temperature, pH, UV-B levels [36] and microbial effects [92]. These effects could be slowed in an indoors tank with low UV-B levels, controlled temperature and pH, and a filtration system to limit microbial activity. Furthermore, eDNA particles in such proximity to the species would have experienced limited water abrasion which can cause fragmentation [81]. This relationship is also observed in samples of human DNA where ratios of ~ 1:1 (short/long) fragments occur in non-degraded samples [93].

The decrease in eDNA concentration from the large adult tank to the small tank supports findings that eDNA concentration is correlated to organism biomass [10,94,95]. The juvenile tank was found to

contain the lowest concentration. The reduced biomass within the tank coupled with burrowing behaviour could have contributed to the low yield. In practice, these data suggest that a ratio of close to 1:1 in wild sampling could indicate minimally degraded eDNA and close proximity to a source population.

3.5.4 Practical application and method selection

In comparison to the controlled hatchery environment, forensic field samples may be collected under varying environmental conditions that affect eDNA persistence and detection reliability. Our sampling data demonstrated consistent detection, suggesting that one-off sampling events can provide reliable presence/absence determination under stable conditions. However, eDNA persistence and degradation are influenced by multiple abiotic and biotic factors including temperature, pH, ultraviolet radiation [36], hydrology [96], particulate dynamics [97], and microbial activity [98]. Seasonal variation in biological activity can also affect eDNA concentrations [41], with highest detectability typically occurring during warmer months when metabolic rates increase. Field validation across different seasonal conditions, water chemistries, and flow regimes would strengthen confidence in one-off sampling reliability for forensic applications. From a technical perspective, the development of a multiplex assay that allows for short and long fragment detection together within an internal control would be a logical step towards application [99]. Furthermore, the use of PCR technical replicates [100] would provide additional confidence in detection results, particularly in forensic contexts requiring high evidentiary standards.

The choice of collection method should align with operational constraints. Based on our findings, we recommend the basic bottle collection method for routine forensic applications due to its cost efficiency (Section 3.4), ease of use (Section 3.1), and safety advantages of bankside sampling (Section 3.2). However, Whirl-Pak® bags offer a practical alternative when water bottles are unavailable or procurement time is constrained, providing comparable safety and detection performance to bottle collection. Users adopting bag collection must ensure proper sealing to prevent leakage and careful storage/transport to maintain bag integrity. The advanced Sterivex^{TM} method may be justified in specific circumstances where trained personnel with appropriate site access permissions cannot filter samples within an acceptable timeframe [42]. However, agencies considering field filtration should note that our data did not demonstrate superior detection rates with this method compared to laboratory filtration approaches, and workflow optimisation would be necessary to ensure comparable performance. End-users can therefore adapt their strategy based on bottle availability, processing timeline constraints, and staff training levels, while recognising that the basic method provides the most practical and cost-effective approach for the majority of forensic eDNA collection scenarios.

4. Conclusion

eDNA offers a valid approach to supporting wildlife crime investigations involving habitat destruction incidents. This study recommends bankside sampling with 2L containers followed by laboratory filtration based on time, cost efficiency, ease of use and safety to the end user. Additionally, primers amplifying short and longer fragments should be used as these have been shown to both infer species presence and could indicate DNA quality in proximity. These primers should be coupled with an internal positive control (IPC) to detect inhibition to the sample. Further research is recommended to develop a multiplex assay and collect data to inform the effectiveness of different fragment size amplification in water courses with wild *M.margaritifera* populations. Collaborative approaches in method development are helpful to establish clear requirements and then assess experimental designs to help identify perceived areas of difficulty and safety concerns, and inform appropriately designed training plans, improvements to methodology and effective SOPs. This collaborative approach can facilitate timely implementation of developed approaches into wildlife crime casework.

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