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Review

The forensic potential of environmental DNA (eDNA) in freshwater wildlife crime investigations: From research to application

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ABSTRACT

Environmental DNA (eDNA) is widely used in biodiversity, conservation, and ecological studies but despite its successes, similar approaches have not yet been regularly applied to assist in wildlife crime investigations. The purpose of this paper is to review current eDNA methods and assess their potential forensic application in freshwater environments considering collection, transport and persistence, analysis, and interpretation, while identifying additional research required to present eDNA evidence in court. An extensive review of the literature suggests that commonly used collection methods can be easily adapted for forensic frameworks providing they address the appropriate investigative questions and take into consideration the uniqueness of the target species, its habitat, and the requirements of the end user. The use of eDNA methods to inform conservationists, monitor biodiversity and impacts of climate change, and detect invasive species and pathogens shows confidence within the scientific community, making the acceptance of these methods by the criminal justice system highly possible. To contextualise the potential application of eDNA on forensic investigations, two test cases are explored involving i) species detection and ii) species localisation. Recommendations for future work within the forensic eDNA discipline include development of suitable standardised collection methods, considered collection strategies, forensically validated assays and publication of procedures and empirical research studies to support implementation within the legal system.

1. 1. Background

Wildlife forensic science is the application of a range of scientific disciplines to legal cases involving non-human biological evidence [1]. Wildlife forensic cases often involve the illegal trade in protected plant and animal species, the poaching of trophy and game animals, and wildlife mortality events caused by habitat destruction, i.e oil spills and/or building works. Such acts are perpetrated by both opportunistic individuals and large scale organised criminal gangs. Wildlife crimes can occur across international borders requiring expertise from multiple investigating agencies and have been compared to the illegal arms trade, drug smuggling and people trafficking [2,3]. Indeed, the illegal trade in wildlife has an estimated annual black-market cost of ~$23 billion [4]. Currently 80 % of wildlife forensic cases require some form of species identification [5]. Typically performed using DNA sequencing approaches, these forensic data will support or refute the prosecution or defence testimony as to the species identity of a seized item or the presence of a protected species DNA in a sample. The application of DNA sequencing in forensic casework has required general scientific acceptance and extensive forensic validation [6] prior to use in casework. As novel approaches are developed for species detection and identification in ecological contexts, their ability to be applied in a forensic context becomes a point of interest and discussion in the forensic community.

Environmental DNA (eDNA) represents a new approach for species detection and refers to the genetic material extracted from environmental samples [7] allowing species detection without their direct observation in samples from water [8,9], air [10,11] and sediments including soil [12,13]. This ubiquitous genetic material consists of

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intracellular, intraorganellar, dissolved or particle-adsorbed DNA [14] and can originate from skin, mucous, saliva, secretions, gametes, waste products, blood, plant materials, rotting bodies and entire microorganisms [7,15,16]. Its application to quantify biodiversity in terms of number of species, abundance, biomass, and communities, and detect physically elusive species mitigates some of the pitfalls associated with traditional survey work [17,18,19]. Furthermore, the increased accessibility and sensitivity of massively parallel sequencing (MPS) and rapid DNA profiling from complex mixtures and low biomass samples has led to the assertion that eDNA may become a tool in forensic science to link genetic signature to habitats by determining provenance of unknown samples [20,21].

Common approaches used in eDNA studies include multi-species (e.g., metabarcoding) and single-species analyses such as real-time quantitative (q) PCR. eDNA metabarcoding is often used in macro-organismal survey work [22], biodiversity monitoring, paleology, botany, habitat management and invasive species detection [23] and has the potential to determine provenance of forensic soil samples [20], dust samples [24], pollen samples [25] and diatom assemblages [26]. PCR approaches are often used for the detection of native and non-native species for conservation management [27,28,29] and have been introduced as a supplementary monitoring tool for protected species such as the great crested newt, *Triturus cristatus* [30] and the freshwater pearl mussel, *Margaritifera margaritifera* [31]. Such PCR approaches ask similar questions to current wildlife forensic tests with species identification a common forensic tool to improve compliance and enforcement [32] and identify trafficked animal derivatives [33,34]. Broadly speaking, eDNA approaches have been suggested to address forensic identification issues, provide evidence for illegal wildlife trade (IWT) [35] and yield evidence conforming to legal standards for admissibility [36,37] yet inconsistencies in collection methods [38,39], storage techniques [40,41], and analysis and interpretation [42,43] are all documented. For results to be admissible in a criminal court, methods and procedures must be transferred to wildlife forensic frameworks, standardised, validated and quality assured with appropriate data analysis and interpretation [44]. It should be noted that *de novo* eDNA evidence has been found admissible in cases of environmental management for the Asian carp (*Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis*) and the designation of the black warrior waterdog as an endangered species [37].

When considering the literature base, the use of the term 'forensics' within eDNA studies has increased in recent years (Fig. 1), possibly due to the increase in eDNA studies in general or usage of 'forensics' as an explanatory term for the methodology [45]. There is also an increase in publications which mention 'wildlife' in conjunction with 'eDNA' and 'forensics', although interest is lesser possibly due to the prioritisation of human forensic applications [46,47] compared to wildlife applications [48]. Regardless, the literature base indicates that forensic applications are being considered by the scientific community. Despite the interest, eDNA approaches have not yet been successfully adopted as a routine forensic tool.

Irrespective of its application eDNA analysis, due to its rapid emergence, has received warnings that its conclusions need to be toned down, and that eDNA analyses need to be interpreted with caution as there are often many factors, including unknowns about the ecology of eDNA, environmental influence, animal biology, and sensitivity of eDNA techniques [49]. It has been recognised that even best practices in the laboratory and field sites cannot 'exclude the occurrence of false positive and false negative results' but that these effects can be mitigated by using process-based or occupancy-based models which will be further assisted by increased data sets [50], perhaps coupled with traditional diagnostic measures of test accuracy [51]. Confidence in eDNA methods has been met with uncertainty by some end users with the presence of false positives and this scepticism should be met with evidence from the method to avoid lost opportunities [52]. Darling et al. [53] highlighted the need for communication in distinguishing 'false-positive eDNA detection at the sample level and false-positive inference of taxa presence at the site level', error definitions and the limitations of traditional sampling methods. Inferences from eDNA analysis and interpretation should be discussed with end users as part of method development protocol. In its current guise it is more likely that eDNA analysis would be able to contextually support other forms of evidence in court and be used as an intelligence tool in investigations.

Wildlife forensic methods support investigations by providing data that supports or refutes a specific legislative act or code, such as the Wildlife and Countryside Act 1981(England and Wales) [54] and The Conservation of Habitats and Species Regulations 2017 (Europe) [55]. Consequently, forensic assays tend to be developed with specific criminal investigations in mind and investigations may require different data to support different hypotheses. Questions such as *'is the species eDNA present at the crime scene?'* are relatively easy to answer while questions such as *'where is the species relative to the crime scene?'* become more complex. To explore the forensic potential of eDNA in wildlife crime investigations, and progress experimental research through to meaningful application we need to consider key components of the wildlife forensic framework such as collection from the crime scene, laboratory analysis, and courtroom measures. Within the scope of this paper this will be confined to single species work in freshwater environments.

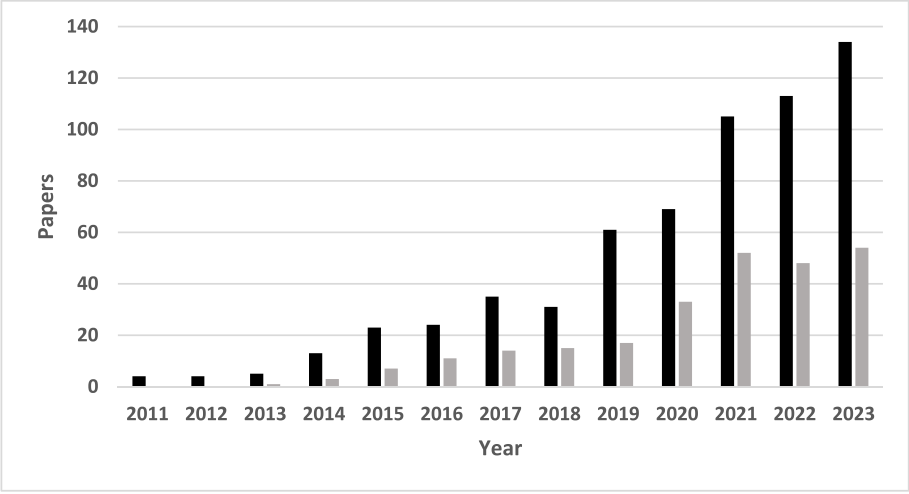

Fig. 1. The search results from SCOPUS from 2011 to 2023 within all fields for 'eDNA AND forensics' shown in black and 'eDNA AND forensics AND wildlife' shown in grey (4th April 2024).

Literature searches were performed in Google Scholar using terms such as 'eDNA collection', 'eDNA methodology', 'eDNA analysis', 'wildlife forensic evidence admissibility' and narrowed down to approximately 400 articles. These research papers combined with official government and scientific working group reports formed the 184 references included in this review. Together these resources represent many of the most highly cited papers from the last 10 years (papers performing within the top 1 % based on the number of citations received when compared to other papers published in the same field in the same year); niche papers relevant to the transfer and adoption of eDNA in forensics; and older papers when deemed contextually relevant. Although case studies pertain to European species the articles reviewed include international research representative of the globally expanding eDNA body of literature.

2. Freshwater crime scene considerations.

2.1. Collection method

Collection of a water sample for analysis is the first step in eDNA recovery. Within wildlife forensics, sample collection may be carried out by first responders without a crime investigation background potentially compromising an investigation through lack of training and awareness [56]. Each stakeholder may have varying experience of field-based collection methods and specific forensic requirements such as anti-contamination controls, sample storage and chain of custody requirements. End users may have limited time and/or resources (Fig. 2D) or may not be able to work in certain habitats due to licensing requirements or safety concerns. Methods and procedures from other disciplines need to be carefully mapped and measured against forensic best practice to ensure successful knowledge transfer. A range of collection vessels and equipment is available for eDNA collection (Fig. 2).

Collection methods can be developed for water to be filtered post collection (in the laboratory) (Fig. 2C) or directly filtered onsite through a filter (Fig. 2B and 2F). The re-use of sample collection equipment is not permitted in forensic casework, which instead employ single-use items such as swabs, forceps, scalpels, and disposable lab coats. Equipment used in eDNA studies may also be single-use such as Whirl-Pak® (Fig. 2A and 2E) and similar "fold and twist" (FAT) bags for collecting biological samples [58] and the use of enclosed filter capsules such as Sterivex™ (Merck Millipore) (Fig. 2F) for onsite filtering. However, equipment such as chest-waders and life-vests do not exist in a disposable form and in these instances, decontamination is required before re-use. Soaking in a sodium hypochlorite solution (e.g., commercial bleach) has been proven effective to decontaminate commonly used laboratory used items [59,60] and 10 % bleach solution is routinely used to sterilise sample containers and filtration equipment *in situ* in eDNA studies [61–63]. However, the effectiveness on eDNA fieldwork items for forensic use has not been ascertained and is likely dependent on exposure to diluted bleach with thorough rinsing required to remove the cleaning product after use [64,65]. Strategies to prevent contamination should consider the proximity of materials to the sample and prioritise accordingly [66]. Decontamination procedures of all equipment would have to be subjected to strict controls, appropriately validated and negative control samples implemented to ascertain sample integrity [42].

2.2. Standardisation

Collection approach should be standardised. The quality assurance practices within the field of forensic science necessitate the need for standard operating procedures (SOPs) to minimise bias and error [67]. Any deviations from the published SOP are subject to cross-examination later in court that may undermine the expert witness testimony. Many eDNA methods reported in a 2018 *meta*-analysis were not considered reproducible due to inaccurate and/or incomplete reporting and the use of subjective protocols, with only 5 % able to be replicated [38]. Although an emerging discipline at that time, such a low level of reproducibility was certainly poor in academic research but would be negligent in forensic casework [68]. Standardised forensic methods should be safe, cost-effective, simple, have longevity yet be adaptable, be based on accessible data [38] and could integrate existing user-friendly eDNA tools [69]. Given the myriad of different approaches, tools, and procedures used for sample collection it is possible that some of these have a greater impact on data quality than others, with authors choosing to remove those that are perceived to have a minimal effect. For example, a review of 160 studies suggests better reporting of

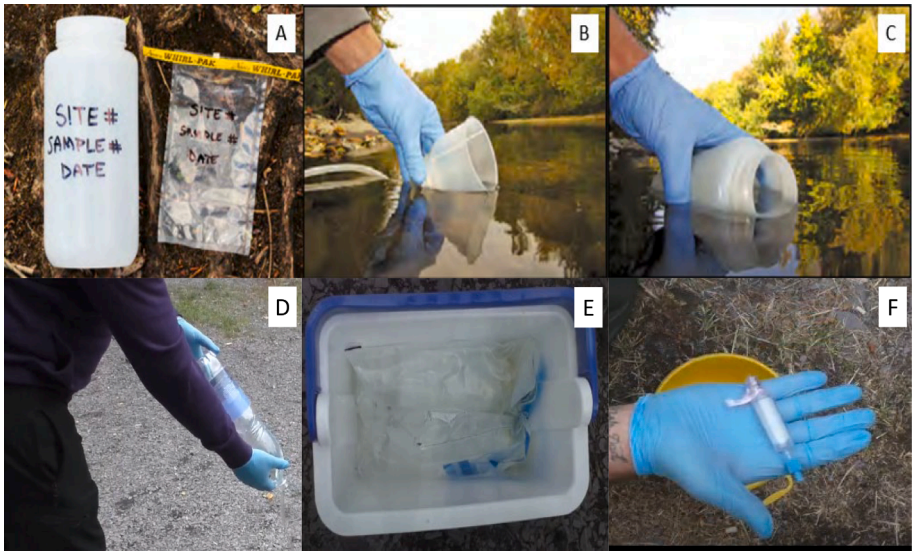

Fig. 2. 2A- A sterile sample collection bottle (e.g. Nalgene™) and sterile sample bag (Whirl-Pak®) used to collect samples. Samples can be taken away from the scene to be stored appropriately, preservative added or filtered at the scene with pumps. 2B- A funnel can be used to draw water directly from the sampling point usually through a filter paper assembly. 2C– Collection can be made from the sampling point to be poured through a filter assembly post collection. Images taken from [57]. 2D- A store bought water bottle may be the only collection vessel available at a crime scene. 2E- Whirl-Pak® bags or other collection vessels may be placed in crime scene bags and transported via a cool box. 2F- Sterivex™ filters can be preserved at the scene and transported at ambient temperature.

categories such as sample volume, filter pore size and filter preservation method while poorer reporting in other more nuanced categories, such as level of UV exposure (reported in 4.4 % of studies), time of day (4.4 %) and precipitation (3.7 %) [70]. These factors may be of particular importance to the forensic community who may attend a wildlife crime scene at any time. Other studies in the literature base exist to provide such context and often give details of suitable, readily available, cost-effective equipment to use, experimental designs, examples of negative and positive controls, contamination prevention procedures and adaptable documentation [42,71–74]. The forensic practitioner can use these approved documents which may also be relevant to eDNA methods as a basis to further refine techniques before validation following best practice guidelines from the Scientific Working Group on DNA Analysis Methods [75] and the European Network of Forensic Science Institutes [76]. It should be noted that there may not be an optimised test developed which can be used for every possible scenario due to differences in species behaviour, habitat conditions and the forensic question posited. However, standardisation of a variety of species-specific methods for different purposes may be achievable if coupled with reflective SOPs.

2.3. Sampling strategy

Another fundamental aspect of eDNA collection is the sampling strategy. Critical decisions concerning sample collection from an ecological perspective [38] remain the same in forensic applications yet subtly differ in flexibility. The temporal nature of eDNA persistence [77,14] and its low abundance [78], particularly in lotic systems [79] make it challenging to ensure that sampling at a crime scene is representative. Statistical sampling strategies for river systems have been developed for eDNA biomonitoring work [80] with guidance based on species' spatial distribution and availability of sampling sites. Carraro et al. [80] highlighted the need for sampling design to be informed by 'preliminary knowledge on the expected distribution of the taxon, and on whether or not estimates of eDNA decay rates in the environment are available' suggesting that forensic sampling strategies also be designed and tailored for their target species' behavioural and distribution patterns. Mächler et al. [81] recommend that volumes of 1L with at least 14 μL of extracted eDNA per sample could reduce detection uncertainty with macroinvertebrates in rivers. However, taxonomic composition in biodiversity studies has been found to vary considerably between aliquots of equal volumes [82] suggesting that the level of eDNA recovered is not sufficient to prevent stochastic effects such as signal dropout or preferential amplification of markers. These observations are common in forensic applications of trace human DNA and guidance on how to appropriately set stochastic thresholds exist [83]. Due to the diversity and complexity of behaviour, optimal sampling strategies considering water volumes need to be established for each species before being validated, and then disseminated to first responders.

The number and location of sampling sites should also be considered carefully. It has been shown that eDNA detections can be a function of the volume of "reverse flow" (defined as infrequent reversals in flow direction caused by dry spells [84]), in large water bodies (up to 5000 m downstream) low lateral mixing can render single species eDNA detection only achievable in direct line of the source [85], and that preferential sampling sites should be selected based on taxon's spatial distribution due to non-uniform patterns of eDNA concentration caused by hydrological factors [80]. More recently, Altermatt et al. [86] also suggest that eDNA sampling should be scaled to the size of the watercourse to provide reliable estimates. These recommendations, despite mainly considering biodiversity studies, indicate that hydrology can affect eDNA dispersion and that targeted sampling could result in an increased likelihood of positive detections for any given target species. Although at a crime scene the hydrological nature of the watercourse may be relatively unknown, it might be prudent to sample from multiple points across a large watercourse to account for hydrological effects. However, sampling a large transect presents difficulties to responders; banks may be inaccessible, robot or boat sampling would be required to sample from deep areas, and it would take considerably more time and expertise. Responders would have to receive guidance in SOPs on appropriate sampling strategy for different sizes of watercourse. Furthermore, investigating agencies employing eDNA methods would have to assess whether whole transect sampling is viable in terms of cost, staff training, logistics and level of accuracy required from the method itself.

2.4. Equipment

eDNA can be found in a range of particle sizes [87] dependent on its biological source, origin and degradation. Filter size selection has been shown to affect the captured quantity of eDNA and subsequent sensitivity of downstream applications [88]; for instance, small pore size filters can get clogged easily in turbid waters by suspended material which may introduce high levels of PCR inhibition [89]. Inhibition in freshwater can cause non-amplification of high eDNA copy numbers [90] although careful PCR reagent selection can reduce the impact of plant derivative inhibitors such as humic, fulvic and tannic acids [91]. Different filter materials have also been shown to yield varying levels of eDNA due to different DNA binding affinities [92] with glass fibre [93], cellulose based [92,94] and polyethersulfone [95] filters all shown to be advantageous in different studies. Crimes may alter the existing habitat. For example, a low turbidity freshwater habitat subject to illegal river works such as dredging may result in increased turbidity levels. Forensic practitioners should be aware of the advantages and disadvantages of different filter sizes (Table 1) and validate a range of methods for use in casework. Data to support the decision making and selection of filter types is lacking in the forensic literature. It has even been suggested that optimal methods cannot exist for eDNA collection due to the variation introduced by the many possible target species and environmental factors [96], however it remains necessary to validate a standardised method for forensic application.

2.5. Transport to laboratory

Microcosm experiments have shown that eDNA degradation in aquatic habitats may increase with temperature, UV-B levels [100] and decreasing pH. Increased degradation has also been shown with increased amplicon lengths in a *meta*-analysis (N = 28) [101] and microbial activity [102]. It is important from a collection point of view that the eDNA concentration within the sample at the time of collection is preserved optimally as analysis will then give an accurate result reflective of the sample site. Although direct filtration, filtration onsite following collection, and laboratory filtration the following day have been shown to not significantly affect the quantity of eDNA recovered [103], filtration of collected samples on site may be beneficial if transport is lengthy or problematic [104]. Refrigeration of water samples may be recommended if samples are to be filtered within one day to avoid a freeze/thaw cycle which has been shown to adversely affect DNA integrity [105,94]. Freezing is also an option for prolonged storage though refrigeration or freezing only slows degradation of eDNA rather than preventing it [106,94]. Nevertheless, there may be a lack of a cold chain available to first responders due to a lack of mobile cooling equipment and materials such as dry ice. Furthermore, the sampling site may be in a remote location away from a facility with cooling units or there may be too large a volume of samples to fit in a particular unit. Improvised storage may introduce the risk of contamination of external sample surfaces and subsequent cross contamination in the lab [107]. Methods of filter preservation exist with Longmire's solution used to inhibit enzyme activity and inhibit bacterial growth [108,109], ethanol used to dehydrate bacterial cells and denature proteins [94], silica beads used to desiccate the sample [110], ATL buffer which acts in similar way to Longmire's [111] and self-preserving filters which desiccate the sample on capture [112]. Different approaches are available dependent

Table 1
The advantages and disadvantages of the range of filter sizes commonly used in eDNA on site filtration.

Filter size (μm)	Advantages	Disadvantages
	Allow larger volumes of water (and total eDNA capture) to be processed [97] due to reduced clogging.	Increased water volumes increases the potential for inhibition [97]
≥1.2 (large pore)	Cell-bound eDNA sufficiently captured whilst water particulates allowed to pass through [40].	Short eDNA fragments may not be captured increasing the ratio of long:short fragments for analysis [98].
	Can be used in turbid waters with reduced chance of clogging.	Large volumes of water take longer to collect/process.
		Large volumes of water may be difficult to transport/store. May involve bulky specialised equipment e.g. large volume samplers with dolphin bucket [97].
~0.45–0.7 (medium pore)	0.6 μm filter may be considered an optimal balance between total yield and quantification efficacy' [99].	Slower filtration rates compared to large pore filters.
	Less potential inhibition introduced with smaller filter volumes.	Increased risk of clogging in turbid waters compared to large pore filters.
	More short eDNA fragments captured than large pore method.	Large volumes of water take longer to collect/process.
	Smaller volumes than large pore method may take a shorter time to collect/process.	Large volumes of water may be difficult to transport/store.
	Smaller volumes than large pore method may be easier to transport/store.	
0.2 (small pore)	High capture efficiency may increase sensitivity [99].	Clog easily in turbid waters.
	Lowest levels of inhibition.	Slow filtration rates.
	Small volumes of water easier to collect/store.	May involve bulky specialised equipment e.g. filtration pumps [40].

on circumstances with a combination of preservation method and temperature control optimal (Table 2). Forensic practitioners may favour preservation methods that are readily available, such as ethanol or silica beads, over methods which may involve prior preparation and large volumes (Longmire's). All these considerations need to be measured against the capabilities of the forensic laboratory conducting the work, many of which will have a backlog of casework and may not be able to generate results for many weeks and will simply freeze the sample once it has been delivered.

2.6. Chain of custody

The maintenance of chain of custody, the continuity of possession of evidence, its correct documentation from crime scene to courtroom and its importance should be considered an ethical and professional necessity at a crime scene [116]. In all methodologies, but particularly in the case of novel forensic techniques, the chain of custody may identify areas to improve in terms of timescale of transfer of evidence, risk of contamination, storage conditions and even documentation practices themselves. However, when implementing new techniques into a framework, the end user may not always be aware of the information to include on forms, the different evidence bags/vessels appropriate and the necessity of unique identifiers such as position within a location matrix [56]. It is the responsibility of developers of forensic methodology to educate practitioners in these procedures, consider educational needs and produce documents such as end user specifications to identify existing expertise and areas for development.

In summary, there is a variety of different factors that need to be considered at scene that can affect the amount of eDNA recovered and their impact should be measured and understood to help develop forensic collection methods. However, these factors are similar to those already facing wildlife crime scene investigators who are required to choose the most appropriate method of sample recovery (i.e., swab or mini-tape or cutting) to capture as much relevant DNA from the site as possible. To apply eDNA collection methods in a forensic context it should be validated and standardised.

3. Analytical considerations

3.1. Analytical method

eDNA as a wildlife forensic tool is likely to focus on questions pertaining to species identification. The COI mitochondrial region is often targeted in both eDNA studies and wildlife forensic casework when authenticated reference data exists [6]. mtDNA is a popular marker as the relative abundance compared to nuclear DNA and its slower decay rate [77] makes it detectable in the environment longer than nuclear DNA.

Quantitative PCR (qPCR) is cited as the most suitable method because of its high sensitivity [117–120], specificity [121], no requirement for a database [122] and therefore rapid testing time [123] and better resistance to false negatives because of no preferential amplification of more abundant species [117]. Greater detection has also been cited for qPCR compared to MPS [118,93,124]. More complex protocols such as multiplex PCR [125,126,] would be transferrable for eDNA forensic validation whilst digital PCR [127,123,128] and perhaps CRISPR [129,130] may be the forensic tools of the future. Digital droplet PCR (ddPCR) shows promise due to its intolerance to inhibitory substances such as humic acid inhibition which may be a significant hurdle in the analysis of samples [131,127,132]. ddPCR is highly sensitive [128], cost-effective and gives absolute quantification [127]. CRISPR-Cas technology has been proven in concept to enhance differential detection of closely related species, may be adapted to detect any species from an eDNA sample and could be used for onsite monitoring [129]. RPA-CRIPSR-Cas assays have been adapted for use on portable fluorometers and lateral flow tests to further simplify the process [133] though further research may be needed before its widespread use [134]. MPS whilst an invaluable tool for biodiversity and conservation survey work isn't suitable for forensic analysis with a species-specific focus. The area for contention regarding qPCR is whether the assumption of presence based solely on the presence/absence of amplification is adequate for use in court. Harper et al. [118] suggest that Sanger sequencing could be utilised to verify a true positive, especially in the case of rare species. Despite the high sensitivity and specificity of qPCR, and even if the assay has been fully validated using comprehensive frameworks [69], sequencing would increase confidence and could act as a confirmatory test for qPCR amplification.

The type of analytical method used in forensic casework and the subsequent data generated are typically simple, specific and require few assumptions or caveats to the interpretation of data. Such a conservative approach may be at odds with research which tends to seek to explore and expand on methods to demonstrate novelty and advancements. As such, traditional quantitative PCR (qPCR) [136] methods with limited variation in laboratory and bioinformatic workflows could be more

Table 2
The impact upon sample eDNA detection rate from a range of different preservation methods and the drawbacks of each method.

Method of preservation	Sample preserved	Duration	Sample treatment	Result	Cons of method	Reference
Temperature control						
Refrigeration	250 ml water samples	3–5 days	Refrigeration at 4 °C	Refrigeration at 4 °C for short term storage (i.e. 3–5 days) may yield higher copy number than freezing due to absence of a freeze–thaw cycle.	Significant decrease in DNA copy number after 24 h of raw sample regardless of storage method.	[94]
Freezing	2L water sample from aquaria	28 days	Frozen at −20 °C	Significant effect of time upon copy number for samples kept at room temperature (20 °C) and refrigerated (4 °C) but not for frozen samples.	Availability of freezing facilities within 24 h from sampling difficult from some field sites.	[94]
Room temperature (RT)	Various filters	2 weeks	Stored at room temperature	Sterivex filters highest detection rate when DNA extracted from preservation buffer (95 % perch/96 % pike). All filters > 50 % detection.	Open filters (CN, GF, PCTE) show significant degradation as opposed to enclosed capsule filters (SX).	[95]
Chemical additives						
Longmire's solution (LS)	10 ml water samples	Up to 56 days	5 ml of high concentration Longmire's added.	Perfect detection above limit of detection after 56 days. DNA concentration significantly lower after this period.	Longmire's must be used in a 3:1 ratio. Only small sample sizes may be preserved. Decrease in DNA yield.	[108]
Ethanol or LS	Sterivex (0.22 μm)	2 weeks	Ethanol or LS added to Sterivex capsule at RT	Addition of ethanol/LS immediately after SX filtration provides the lowest Cq-values, and is significantly better than freeze storage or extraction within 5 h.	Preservative needed at sampling site and anti-contamination measures stringently implemented.	[95]
Ethanol and sodium acetate	15 ml water samples	3 days	1.5 mL of 3 M sodium acetate and 33 mL of absolute ethanol added.	DNA yield remained high after 3 days (>50 %) but did not maintain original quantity of DNA. After this period target DNA in each sample decreased.	Small sample volumes required. May provide more unstable preservation environment than temperature control.	[113]
Ethanol	Glass fibre filter	6 days	15 ml of ethanol added after filtration	Number of eDNA copies did not significantly decrease after 6 days at ambient temperature.	Not ideal for long term storage at RT as degradation can occur. Handling and storage considerations.	[114]
Benzalkonium chloride (BAC)	1L water samples	8 h	0.01 % BAC added (v/v).	Water stored in ice provided better results than those preserved in BAC (239 and 142 vs. 194 and 71 MOTUs)'	Cationic surfactants such as BAC less effective than temperature control for recovery and detection.	[115]
Silica gel beads	Mixed cellulose ester filter (0.45 μm)	1 month	15–30 ml of 2–4 mm rechargeable silica gel beads added to protected filter	Brief storage of 1 month preserved low abundance eDNA regardless of storage temperature. Only freezing prevented noticeable decrease in detectability at 5 and 12 months.	Recommended to be used with freezing for long term sample preservation.	[110]
Combinations						
Ethanol/silica gel beads at −20 °C	Mixed cellulose ester filter (0.45 μm)	15 months	Frozen at −20 °C with each preservation method	Relatively abundant DNA stable as indicated by consistent C_t values.	Freezer storage space required.	[110]

* Sterivex, SX (0.22 μm), cellulose nitrate, CN (0.45 μm), glass fibre, GF (0.6 μm), polycarbonate track-etched, PCTE (0.2 μm).

'forensically transferable' compared to eDNA metabarcoding.

3.2. Addressing the investigative questions

Forensic questions such as 'is the species eDNA present at the crime scene?' and 'where is the species relative to the crime scene?' may be asked in cases of habitat destruction, water pollution, poaching and the introduction of nonindigenous species [137] where species protected by legislation [54,55] are suspected to have been killed, injured, or disturbed. The question of 'how many of a species are present at the crime scene?' cannot be answered using eDNA. eDNA has been used to infer species abundance/biomass of a range of fish species [39], amphibians [103] and molluscs [138] under certain conditions but generally eDNA concentrations have been found to have a stronger correlation with abundance in maintained lab experiments with controlled abiotic variables than in the natural environment [139]. Similarly, eDNA concentrations taken from, for example, tanks in transit, are not only affected by the density of species, but by variation in decay and shedding rates. eDNA decay rates might be affected by various factors such as temperature and pH [93,140] whilst shedding rates may differ between species or life stages and might be affected by seasonal variation in behaviour [62].

Case study 1: Is the species eDNA present at the crime scene? Amphibians are among nature's most threatened classes due to habitat loss and changes to their environment [141]. The greater crested newt (GCN), *Triturus cristatus*, is an emblematic European example, as its habitats, which require both ponds and humid lowland forests, are increasingly threatened by anthropogenic activity, such as building developments. Given their protected status, GCN are often translocated to mitigate anthropogenic disturbance [142]. Developers proposing a change in land-use that may affect this species are obliged by law to carry out survey work as the species is protected by both British [54] and European law [55]. eDNA analysis has been shown to detect species presence previously undetected by traditional macro surveys suggesting improved detection rates [143]. eDNA has been shown to persist in freshwater bodies for 2–4 weeks dependent on original species density and degradation rates influenced by temperature, pH, UV light levels and microbe activity [144,145]. As such, these assays need to ensure they are species-specific and extremely sensitive.

The proven persistence of eDNA suggests that a positive reading within an enclosed lentic system could indicate either presence of a species or very recent historical presence. From an investigative

perspective, this could suggest a subsequent, traditional, physical search to confirm species presence or absence within the habitat. The data generated in such applications is binary (present/absent) or a derivative of such binary data (detected in 4/6 samples analysed) and therefore easily understood. Such an application in a legal context is already performed with forensic laboratories in the UK offering detection services for this species [146]. The development of detection tools for new species simply requires optimisation, validation, and transfer to a forensic laboratory. As such this represents the easiest and likely most common eDNA approach in wildlife forensic casework.

Case study 2 – Where is the species relative to the crime scene? Freshwater pearl mussels *(Margaritifera margaritifera)*, native to European rivers, are listed on the IUCN red list as critically endangered [147]. Crimes associated with habitat destruction, water pollution and pearl harvesting are most frequently cited [148]. eDNA has significant forensic potential in cases of habitat destruction and therefore forensic questions will require the answering of '*where are species present in a specific area?*'to assess impact upon protected species. In such a situation, the forensic analyst may need to analyse samples across a series of zones either side of the crime scene and thereby indicate the presence of the species in these areas (Fig. 3) or the likelihood of species' absence based on extensive sampling. However, the interpretation of the data may be confounded by the distance that eDNA can be transported in a lotic system [149,106,150–152,135], flow/discharge rates [131] and degradation [49]. In such instances interpreting data under a variety of different scenarios needs considering (Table 3).

Issues remain if eDNA is detected across all three zones (Table 3, scenario 4), an issue than can only be solved with accurate quantification of eDNA through qPCR. If there is more eDNA in Zone B than both Zone A and C it could infer that this is where most of the species are localised. Further spatial accuracy could be achieved by building on the hypothesis of Hänfling et al. [153] and Harper et al. [118] that larger eDNA fragment lengths occur close to species location because longer fragments indicate less degradation and subsequent closer proximity to species location [152]. Such an approach would require the development and validation of a species-specific multiplex qPCR assay that gave accurate concentration estimates across a variety of different eDNA fragment sizes. Sampling from a limited number of sites downstream could be coupled with the use of predictive models proven to be effective in inverse modelling for species such as *Margaritifera margaritifera* and consider hydrology and geomorphology [80]. It should be noted that Fig. 3 shows a narrow watercourse in a simplistic scenario. A much wider watercourse would be impacted by the hydrological factors discussed in 3.4 and require a considered sampling strategy to increase confidence in determination of eDNA presence for investigative use.

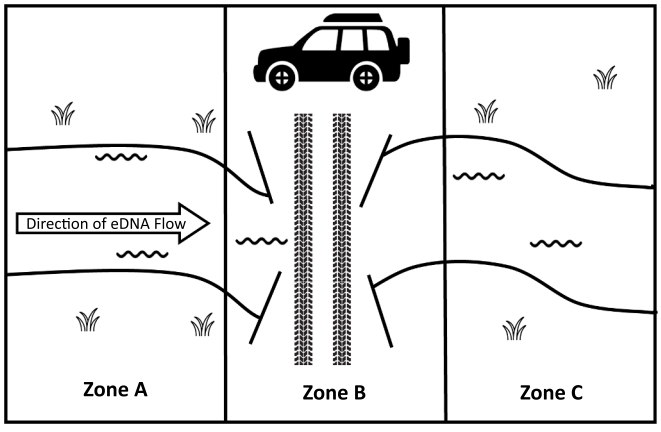

Fig. 3. sampling zones to determine the locality of an eDNA signature to assess whether illegal activity (disturbance/damage of freshwater pearl mussels) has taken place in zone B (crime scene). Zone A relates to upstream sampling and zone C downstream sampling.

3.3. Validation

eDNA methodology validation is necessary for any results to be recognised as biological truth with statistical backing recognised by others in official statements such as white papers and within the legal system [154]. eDNA as a method has been suggested to meet Daubert standards when validated protocols are used [36], yet widespread acceptance by ecological end-users has been slowed due to the need for decision-support trees [36] and eDNA application components that need addressing. For instance, the creation of properly validated, accessible eDNA assays [69] would ensure that in-silico predictions of specificity lead to in-vitro performance [155].

eDNA analysis for use within the legal system can build on recognised practices, guidelines, and recommendations in forensic DNA analysis such as from the Scientific Working Group on DNA Analysis Methods (SWGDAM) [75] and the Forensic Science Regulator (FSR) [156]. It has been suggested that eDNA practitioners could learn from such standards [154] and species assays which are developed using forensic precision would be of cross-disciplinary benefit to practitioners and increase confidence of end users.

3.4. Cost

eDNA sampling has been found to be cheaper and more cost-effective than traditional approaches [157] though traditional techniques may be favoured if primer/probe development and sample processing costs are high [158] and the method used sparingly. 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 [159], and the processing of less samples simultaneously. High frequency projected sampling could justify the implementation of an eDNA workflow, provided the method delivered similar or better results than traditional approaches.

Another key consideration could be the availability of wildlife forensic testing in a laboratory with quality assurance (QA) and accreditation such as ISO17025. A well-equipped operating lab such as a dedicated wildlife forensics lab, commercial DNA lab or university labs would save analytical equipment costs though may vary in evidential quality, level of quality assurance, availability of experts and cost of analysis due to differential overheads and running costs [160]. Some wildlife forensic labs might not have the time or expertise to develop the specific assays and methodologies required for eDNA analysis. The development process could rely on academic research and funding from end user groups which could be a potential barrier to near term implementation.

3.5. Occupancy modelling and imperfect detection

eDNA analysis can be used to confirm the presence of species DNA at the sampling point but cannot be used to absolutely prove absence. Occupancy modelling [161] helps to account for imperfect detection in aquatic environments, which can arise when false negatives occur during sampling or the PCR process [162], by considering normal species distribution ranges and dispersal. Occupancy modelling places extra demands on the sampler [163] so it is not suggested that it should be considered at a crime scene from a collection point of view or that biostatisticians compile data relating to every potential crime scene. Due to time constraints, expertise, and logistical considerations this is most likely not possible. However, data generated from these types of study [164,165] combined with location data of sampling, historical sampling record, and potentially re-examination of the crime scene could lead to probability estimates being recounted in court.

eDNA detection may also be affected by the presence of dead individuals within the water body. eDNA from dead goldfish, *Carassius auratus* has been detected in microcosm experiments at the bottom of the water column [166] and silver carp, *Hypophthalmichthys molitrix* carcass

Table 3
Various scenarios and the meaning of such results based on DNA detection alone. Result indicates inference of species presence in zone B.

Scenario	Zone A	Zone B	Zone C	Result
1	DNA Absent	DNA Present	DNA Absent	✓
2a	DNA Absent	DNA Absent	DNA Absent	✗
2b	DNA Absent	DNA Absent	DNA Present	✗
2c	DNA Present	DNA Absent	DNA Absent	✗
2d	DNA Present	DNA Absent	DNA Present	✗
3	DNA Absent	DNA Present	DNA Present	✓
4	DNA Present	DNA Present	DNA Present	?

Scenarios 1 and 3 are the easiest to support mussel presence- upstream absence suggests local eDNA as opposed to transient eDNA in the target zone B. 2a and 2b support mussel absence at the target zone- no upstream presence lends confidence to the zone B reading. 2c and 2d may be unlikely occurrences unless zone A detection levels were at low concentrations indicating dilute transient eDNA. This could not be confirmed with a binary detection method. Issues with scenario 4 are detailed below.

eDNA detected for up to 28 days in chamber experiments [167] whilst a more recent study using red swamp crayfish, *Procambarus clarkia* suggests that in cases of rare species, such as endangered species or new invasive species, carcasses may not produce detectable eDNA [168]. Any analytical method employed must consider the potential contribution of species eDNA from different vectors including dead individuals. This is an area in need of further research which needs addressing. However, procurement and availability of dead rare or endangered species in significant quantities may make this research difficult to conduct.

4. Courtroom considerations.

4.1. Intelligence or Evidence?

Data generated in forensic laboratories can be used for different purposes. Often presumptive tests are performed that provide 'activity level' information that add context to the case but fail to reach evidential standards due to limited accuracy [169]. Such tests are routinely used and support decision making during the investigation. Such intelligence tests tend to be cheap, quick, easy to use and are not required to be as accurate as tests used to generate evidential data [170]. eDNA methods have potential to generate both intelligence data and evidential data. The issues surrounding the reliability of the technique and its admissibility to court as evidence are complex and have led to some fields of forensic analysis such as blood pattern analysis labelled as 'subjective rather than scientific' by the Committee on Identifying the Needs of the Forensic Sciences Community [67]. However, DNA evidence has been more scrutinised than most due to its unique ability for identification based on a few cells [171]. As such it becomes necessary to determine accurate interpretation models for eDNA prior to use as evidence in casework.

eDNA data is often subject to scrutiny due to sources of error resulting in false positive and false negative results [52]. However, robust detection assay design which incorporates wet lab testing against confounding taxa (to increase specificity) and in silico sequence alignments of well-designed primers and probes followed by thorough validation practices both within the lab and the field [116] (3.3), alongside 'methodologically sound field sampling' [38] and recommended lab practice [172] would reduce error rates. Occupancy modelling [173] has advanced to a level whereby rates of false positive and negative results can be assessed more effectively with increasing eDNA data sets combined with traditional and citizen-science approaches [50]. Statistical analysis combined with process-based models incorporating eDNA decay curves [50] can be used to increase the reliability of eDNA interpretation. Misunderstandings surrounding the terminology used to communicate error could also be clearly addressed to increase confidence in the application and inferences of eDNA methodology [53].

4.2. Admissibility

The context of an admissibility challenge is subtly different to the reliability of evidence when viewed from an analytical perspective as described above. Admissibility of forensic evidence involves the judge who acts as a gatekeeper for evidence and the expert witness who presents it. A study of judges [174] found that 'their error rate estimates were more supported by research than many estimates by laypersons, who often assume forensic methods are nearly infallible' but they reported 'having very different backgrounds in relevant scientific concepts and having forensic science education needs'. Judges reported needs in accessing better material concerning reliability of forensic science methods (N = 164). This also supports the need to produce eDNA white papers, standards, and reports at a government level. Furthermore, Murrie et al. [175] showed that from 181 forensic analysts- 'Most analysts could not specify where error rates for their discipline were documented or published. Their estimates of error in their fields were widely divergent – with some estimates unrealistically low.' This study indicates a need for establishing error rates where possible, collating and centralising for availability to judges and scientists. It should also be recognised that in the USA the expert should be prepared to show that the evidence meets admissibility standards set out by Daubert [176] and Frye [177]- demonstrating that the science is generally accepted within the scientific community, has published studies to show reliability and validity, has recognised error rates and has appropriate administrative controls. Similar suggestions exist across most jurisdictions, for example, from cases in the UK [178] and Crown Prosecution service guidance for new or novel techniques [179]. Daubert admissibility standards require methodology which is published and peer reviewed. Peer review is recognised as the 'premier approach to ensure the validity of methods and conclusions, to detect errors and fraud, and to improve the quality of learned papers' [180]. The style of peer review is important and the review itself may not be sufficient indicator of reliability until empirical evidence supports it [181]. As such, it becomes important that scientists working at the interface between eDNA and forensics begin publishing their data in forensic and legal focused journals rather than ecological or environmental based journals as the reviewers and audience will have the required expertise to critically evaluate the work appropriately. It should be noted that where indigenous peoples have rights and governance over specific areas, they should be brought in as partners so that

work can be done ethically according to the FAIR and CARE guidelines [182].

5. Conclusion

Environmental DNA (eDNA) has the potential to be implemented into the framework of wildlife forensics from crime scene to courtroom. Forensic investigators can extract genetic material from environmental samples, such as freshwater, to identify species DNA. The technique offers numerous advantages over traditional forensic methods, such as non-invasiveness and greater sensitivity. Various eDNA collection methods have been developed for conservational and ecological use. Their implementation into a forensic methodology is dependent on suitable adaptation to answer forensic questions. The impacts of challenges associated with eDNA analysis, such as sample contamination, degradation, and interpretation can be dissipated by further research, development of appropriate methodology for the target species tailored for use by the end user, and cross disciplinary collaboration. eDNA analysis, and indeed DNA analysis in general, has made considerable advances in technology and cost efficiency of newly developed methods. This analytical power must be used appropriately, dependent on the forensic question posed and the data required as evidence, for further research to be undertaken or directly for the courtroom.

The key recommendations going forward for implementation of eDNA methods into wildlife crime investigations from existing research are as follows:

1. Establish standardised eDNA sampling methods using single use materials and ensure end users are trained in crime scene collection procedures.
2. Develop filtration and preservation methods to recover optimal eDNA concentrations within samples to ensure analysis reflects the habitat and target species behaviour.
3. Work with investigating authorities to develop and validate DNA tests which address the relevant forensic question.
4. Collaborate with the criminal justice system to develop eDNA statistical reporting methods appropriate for courtroom presentation.

Together these observations and discussions should be used to create a policy framework that describes where, when and how eDNA methods can be used in criminal casework.

CRediT authorship contribution statement

Matthew Lewis: Conceptualization, Methodology, Writing – original draft. **Katie Lainé:** Writing – original draft. **Louise Dawnay:** Visualization. **David Lamont:** . **Kirstie Scott:** Supervision. **Stefano Mariani:** Supervision. **Bernd Hänfling:** Supervision. **Nick Dawnay:** Conceptualization, Writing – review & editing.

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.

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