Research Article

First assessment of eDNA-based detection approach to monitor the presence of Eurasian otter in Southern Italy

Pushpinder Singh Jamwal[1], Antonia Bruno[2,], Andrea Galimberti[2],
Davide Magnani[2], Hannah Krupa[1], Maurizio Casiraghi[2], Anna Loy[1]

[1]*Department of Biosciences and Territory, University of Molise, Pesche, Italy*
[2]*Biotechnology and Biosciences Department, University of MIlano-Bicocca, Milan, Italy*

Abstract

The assessment of the occurrence of rare and endangered species in freshwater environments is crucial for ecological studies and conservation issues, but it can be time-consuming and challenging in harsh environments. Detecting DNA traces from the environment (environmental DNA, eDNA) can provide innovative and reliable solutions for the monitoring and conservation of rare and elusive species, such as the Eurasian otter *Lutra lutra*. We tested an experimental workflow based on target qPCR assay to detect *L. lutra* eDNA from water samples as a speditive monitoring tool at large scale to be coupled with fine-scale traditional field surveys. This is the first application of an eDNA-based approach to monitor the presence of *L. lutra* in Italy. We compared the eDNA-based results with traditional survey observations and confirmed the reliability of this innovative approach for the large-scale monitoring of such aquatic elusive species.

Introduction

Monitoring the presence of rare and/or endangered species is essential for ecology and conservation purposes, but often can be conducted only adopting non-invasive methods, or actively searching for indirect signs (e.g. roadkills, footprints, scats, spraints, hairs). In the past, efforts have been made to explore mammal species diversity by using various techniques, such as transect sampling (Thomas et al., 2010), camera trapping (Jamwal et al., 2016), and sign surveys (Sadlier et al., 2004). Camera trapping has largely been used to study ecology worldwide (Rovero et al., 2014). This approach has an advantage over other types of surveillance since cameras can operate in the field for more extended periods and do not require the same level of attention as human observers (Meek et al., 2014). However, the issue of theft and the ongoing costs of using camera traps are significant drawbacks. Sign surveys using trails and transects are not only effective for wildlife monitoring, but they are also relatively inexpensive in terms of dedicated facilities and analytics requirements and straightforward to conduct (Jathanna et al., 2003). Nonetheless, sign survey is often incapable of identifying congeneric species or to quantify their relative abundance (Harrington et al., 2010).

On the other hand, scat survey is a widely used strategy in mammal monitoring studies to assess distribution through space and time of a certain species (Lee et al., 2019; Prat-Mairet et al., 2017), such as the Eurasian otter (*Lutra lutra*) (Reuther et al., 2000). However, when dealing with elusive species and field surveys, conducting a capillary and long-term monitoring can be challenging: reaching sampling sites difficult to access and following signs of the target species in areas with extreme topography and ruggedness require manpower, investment, and is time-consuming. Especially when sampling campaigns are over a wide spatial scale and extended in time these issues appear to be determinant (Lerone et al., 2015). Within the last decade, we faced a revolution in species detection, due to the implementation of molecular techniques

able to detect DNA traces from non-invasive samples as well as environmental matrices (e.g. water) (Taberlet et al., 2018). Environmental DNA (eDNA) is released by organisms into the environment from faeces, mucus, skin cells, or extracellular DNA (Taberlet et al., 2012). Indeed, the possibility to gain relevant information from non-invasive sampling through an eDNA-based approach facilitates biomonitoring efforts, allowing species presence and abundance estimation and biodiversity assessments (Bruno et al., 2017).

eDNA detection is becoming an invaluable ally in wildlife conservation studies (Valsecchi et al., 2021; Cristescu and Hebert, 2018). This is the case of many top predator carnivores (Harper et al., 2019; Lyet et al., 2021; Wilcox et al., 2018), including the Eurasian otter.

Lutra lutra population showed a decline in the last century, due to habitat fragmentation and destruction across its range, especially in Western and Central Europe (Pigneur et al., 2019; Marcelli et al., 2012; Mucci et al., 2010; Koelewijn et al., 2010; Janssens et al., 2008; Ferrando et al., 2004; Dallas et al., 2002). Law enforcement and banding of harmful chemical compounds used in agriculture allowed the species to recover in many European countries, and in 2007 *L. lutra* moved from Vulnerable (VU) to Near Threatened (NT) category in the IUCN Red List. However, the status of *L. lutra* is still perilous in Italy, and the species is listed as Endangered (EN) in the national red list (Rondinini et al., 2013). Remnant viable populations only occurred in southern Italy (Loy et al., 2004). The return of otters in the river Sangro in 2007 (De Castro and Loy, 2007)(marked the onset of a still ongoing recovery in south-central regions (Giovacchini et al., 2018; Loy et al., 2015). In this context, traditional field monitoring of otters can be highly demanding in terms of time and costs. eDNA analysis could be a valuable alternative, especially to assess the species occurrence in peripheral areas of the species range (Sales et al., 2020). In the present study, we tested for the very first time in Italy an eDNA-based experimental workflow to detect *L. lutra* DNA from water samples by a target qPCR assay. As a speditive monitoring tool for aquatic elusive species, *L. lutra* eDNA-based assay can be proficiently coupled with traditional field surveys to
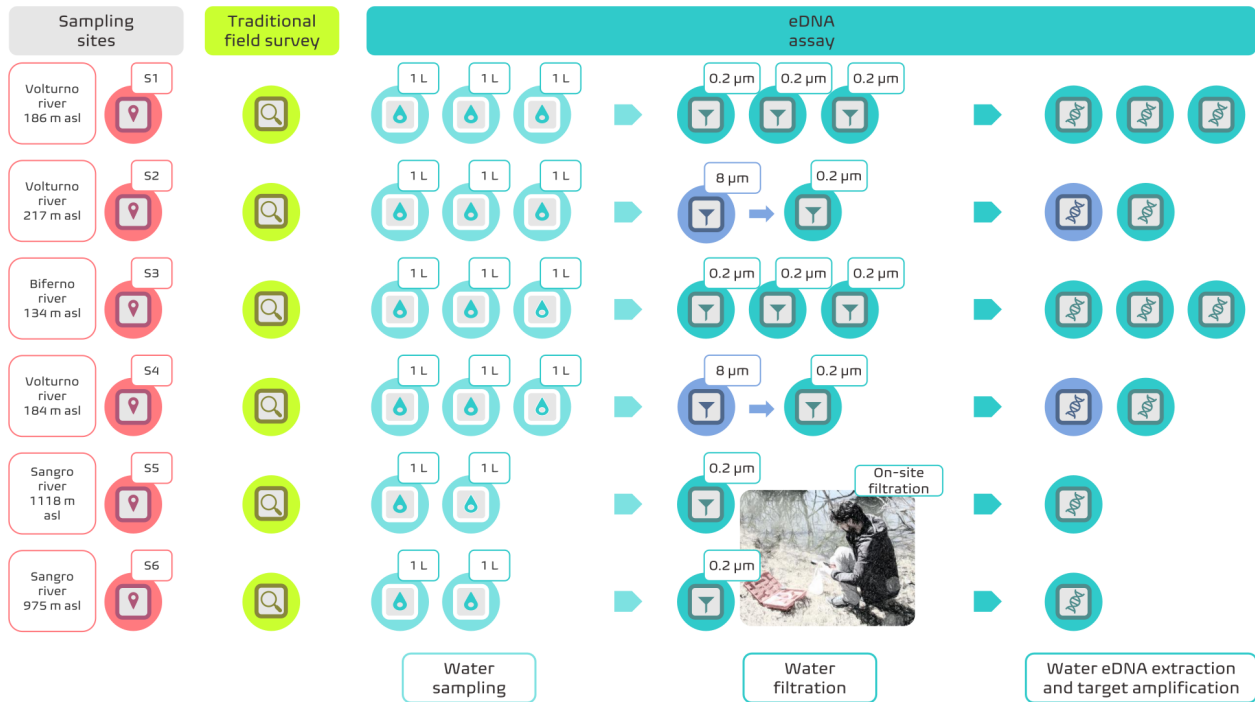
Email address: antonia.brunbo@unimib.it (Antonia Bruno)

Figure 1 – Experimental workflow. For each sampling site, samples collected, filtered and processed for eDNA extraction and target amplification are reported. The volume of water collected for each sample is indicated. The membrane filter pore size used is reported and highlighted with different colours (0.2 μm pore size in cyan and 8 μm pore size in blue).

facilitate rapid, large-scale biomonitoring, representing a natural progression in bioassessment and ecosystem surveillance.

Methods

Study area and sampling sites

The study area is located at the northern boundary of the Eurasian otter range in Italy. Six sampling sites (S1–S6) were selected along the rivers Sangro, Volturno and Biferno, including areas (S1–S4) where otters have been monitored and detected by sign surveys (spraints and tracks) since 2000 (Loy et al., 2015; De Castro et al., 2013; Loy et al., 2004), and an area (S5–S6) upstream of a steep dam located along the river Sangro in the National Park of Abruzzo, Lazio and Molise (PNALM). Here, otter signs were never recorded (Marcelli, 2006; De Castro, 2007; Imperi, 2013; Lerone , 2013), with the exception of S6, where spraints were first found in September 2018 (Caldarella, *pers. comm.*; Lerone, *pers. comm.*; Giovacchini, *pers. comm.*; see Tab. 1 for detailed information).

During our survey, at each sampling site, indirect signs of otter presence (spraints and footprints) were actively searched and recorded along 600 m of riverbank. According to the standard protocol for otter survey in Europe, this is the searching distance that allows to decide whether a site is positive or negative for otter occurrence (Reuther et al., 2000).

Field surveys for four sampling sites (S1, S2, S3 and S4) were carried out in June 2018, and for two sites (S5 and S6) in February 2019.

eDNA survey and field survey were carried out at the same time, in order to ensure the most up-to-date information on otter presence.

For eDNA analyses, three litres of water for sites S1–S4 and two liters of water in the case of sites S5–S6 were aseptically collected using sterile 1 L bottles. Samples from S1–S4 sites were transported (within 24 h, kept at 4 °C) in a laboratory equipped for eDNA analyses for water filtration; orthogonal water filtration was performed under a laminar flow cabinet and vacuum was generated by a vacuum pump (Vacuubrand™) connected to a filtering apparatus. Samples from S5–S6 sites were tested for on-site filtration by a portable hand vacuum pump connected to a polypropylene flask (1 L, Nalgene®) using sterile disposable filter units (Sartorius). Due to the heterogeneity of water turbidity among samples, we developed an experimental scheme designed as follows. Briefly, where water was evaluated as transparent at

naked eye, it was filtered directly on a nitrocellulose membrane filter with a pore size = 0.2 μm (sites S1, S3, S5, S6). Moreover, in the case of S1 and S3 sites, each 1 L bottle of the three liters of water collected was filtered in parallel, obtaining three replicas, whereas in the case of on-site filtration (sites S5 and S6) the entire volume of water (2 L) was directly filtered on the disposable filter unit, to limit sample handling. In the case of high turbid waters (sites S2 and S4), the water of each bottle was mixed thoroughly and preliminary filtered (for a total of 3 L) with 8 μm-based nitrocellulose membranes; then, the water recovered was further filtered by 0.2 μm membrane filters. In this case, no filtration replicates were accounted for, but to increase eDNA concentration the entire volume (3 L) was directly filtered on the filter.

All tubes and containers were sterilised with sodium hypochlorite between samples.

DNA extraction

All the instrumentations used, if not disposable, were sterilized with sodium hypochlorite or autoclaved prior to each experiment. Pre and post amplification phases were carried out in separate laboratory rooms with dedicated equipment and every step was conducted in the laminar flow cabinet, in order to avoid any possible contamination with exogenous eDNA.

eDNA was extracted from each filter using the DNeasy® PowerWater® Kit (Qiagen), following the Manufacturer's protocol with minor modifications; briefly, eDNA was eluted in 75 μL of warmed (40 °C) elution buffer, to increase the final eDNA concentration. If not processed immediately, the filter samples were stored at −80 °C (Fig. 1).

Positive controls for eDNA assay were obtained from two *L. lutra* tissue samples stored in ethanol at the University of Molise. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) following the Manufacturer's protocol.

qPCR assay set up

First, we tested with end-point PCR the otter positive controls obtained from the reference *L. lutra* tissue samples. To do that, we used otter specific primers reported by Park et al. (2011), LutCyt-F and LutCyt-R, which were designed to amplify a 227 bp long region of the mitochondrial cytochrome b of *L. lutra* (positions 32–259). Amplifica-

Table 1 – Sampling sites details and references of past surveys.

Site name	River name	Altitude (m asl)	Sampling date	References for past field surveys	Otter signs searched in past field surveys
S1	Volturno	186	June 2018	Loy et al. (2004); De Castro et al. (2013); Loy et al. (2015)	Spraint and tracks
S2	Volturno	217	June 2018	Loy et al. (2004); De Castro et al. (2013); Loy et al. (2015)	Spraint and tracks
S3	Biferno	134	June 2018	Loy et al. (2004); De Castro et al. (2013); Loy et al. (2015)	Spraint and tracks
S4	Volturno	184	June 2018	Loy et al. (2004); De Castro et al. (2013); Loy et al. (2015)	Spraint and tracks
S5	Sangro (PNALM)	1118	February 2019	Marcelli (2006); Imperi (2013); Lerone (2013) – all negative –	Spraint and tracks
S6	Sangro (PNALM)	975	February 2019	Marcelli (2006); De Castro (2007); Imperi (2013); Lerone (2013) – always negative – First spraint found in September 2018 (Caldarella, *pers. comm.*; Lerone, *pers. comm.*; Giovacchini, *pers. comm.*).	Spraint and tracks

tion conditions consisted in an initial denaturation for 5 min at 94 °C, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min (Park et al., 2011). The reaction mix was composed as follows: 0.25 μL of Wonder Taq Thermostable DNA polymerase© (EuroClone S.p.A.), 4 μL of Wonder Taq Reaction Buffer (EuroClone S.p.A.), 0.2 μL each primer [100 μmol], 2 μL of DNA sample and Milli-Q water to reach the volume of 20 μL. Amplicons were purified from agarose using EuroGOLD Gel Extraction Kit® (EuroClone S.p.A.) following Manufacturer's protocol.

Cyt b amplicons obtained by end-point PCR were bidirectionally Sanger-sequenced at Eurofins Genomics (www.eurofinsgenomics.eu). A GenBank-NCBI BLAST analysis of the obtained consensus sequences confirmed that the two reference tissue samples corresponded to the expected Cyt b fragment of *L. lutra* (i.e. maximum identity >99%; query coverage 100%).

Quantitative Real Time PCR (qPCR) assays were performed with an AB 7500 (Applied Biosystem) instrument. To set up the qPCR assay, first a standard curve was generated. Briefly, we amplified the DNA of positive controls using *L. lutra* specific primer pairs (LutCyt-F, LutCyt-R). qPCR conditions included 10' initial denaturation at 95 °C and 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation at 55 °C for 1 min. Amplification reaction consisted of 5.0 μL SsoFast EvaGreen Supermix with Low ROX (Bio-Rad), 0.1 μL each [10 μmol] primer solution, 2 μL of extracted DNA. Ten-fold serial dilutions of positive control were tested in triplicate and the assay was repeated in triplicate as well, in order to calculate qPCR amplification efficiency and limit of detection (LoD). The LoD is defined as the lowest concentration (and as a consequence the highest Ct) at which 95% of the positive samples are detected (Bustin et al., 2009).

All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems).

otter eDNA qPCR assay

Environmental water samples, together with positive controls and negative controls (no template), were amplified in the same run in triplicate. We further included, in the same assay, otter negative controls, constituted by eDNA extracts of water samples (AQU01–05) from a sampling site characterized by the ascertained absence of otter eDNA and the likely occurrence of heterogeneous DNA and inhibitors. These samples were collected from the river Lambro. They were processed previously, according to the protocol tested for the other environmental samples, i.e., 1 L of water was filtered on a membrane filter (0.2 μm pore-sized) and eDNA was extracted using the DNeasy® PowerWater® Kit (Qiagen). For all the filtered water samples (both those from S1–S6 sites and AQ01–05), 5 μL of eDNA template was used in the reaction to take into account low target eDNA concentration. To ensure that no otter detection in environmental samples was due to the absence of *L.*

lutra eDNA and not to a failure of eDNA extraction, the same samples were also tested with qPCR using the MiFish primer pairs, a set of universal 12SrRNA oligonucleotides for the untarget DNA metabarcoding characterization of fishes (Miya et al., 2015). The amplification reaction conditions were the same tested for LutCyt assay. Amplification profile included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation for 1 min at 58 °C. All the amplification data were collected and analyzed with the SDS 7500 Real-Time PCR System Software (Applied Biosystems).

Ct (Threshold Cycles) values were converted into counts (DNA copies) using the formula

$$\text{Counts} = E^{(40-Ct)}$$

where E is the efficiency of amplification (Bruno et al., 2017).

To avoid any possible contamination with exogenous DNA, pre and post amplification phases were carried out in separate rooms, and every step was conducted in a laminar flow cabinet.

Results and Discussion

Amplification efficiency of qPCR assay, estimated by means of serial dilutions of positive controls, approached 100%. The limit of detection (LoD) approximated at Ct=35.4. According to the calculated LoD, the tested environmental samples were considered negative (no detection) if Ct was greater than 35.4 in at least 2/3 of amplification replicates. If at least one sampling replicate resulted in positive detection, the site was considered otter-positive for eDNA assay. Water eDNA of *L. lutra* measured by qPCR ranged from 0 to 178 DNA counts/μl. Negative controls (no otter DNA: AQU01–05 and no DNA) provided no amplification signal. Considering otter-positive environmental samples (n=4), we reported an average of 22 DNA counts/μl (s.d.: 29 DNA counts/μl), indicating low DNA concentration of the target species. However, this value matches with other studies on rainbow trout eDNA detection from water samples (Wilcox et al., 2018). MiFish qPCR assay, used for the untarget DNA metabarcoding characterization of fishes (Miya et al., 2015), confirmed the successful DNA extraction for each tested sample, with the exception of positive and no template controls, as expected.

Figure 2 shows the results of eDNA assay for each sampling site, whereas otter presence estimation through both visual observation and eDNA assay is reported in Fig. 3. eDNA samples collected at S1 and S3 sites (positive for otter visual records) produced positive results in 1/3 of sampling replicates. Previous studies (Kasai et al., 2020; van Bochove et al., 2020; Bylemans et al., 12018; Tsuji et al., 2017) suggest that low target DNA concentration together with DNA degradation can have a role in false negative results and recommend replicates to
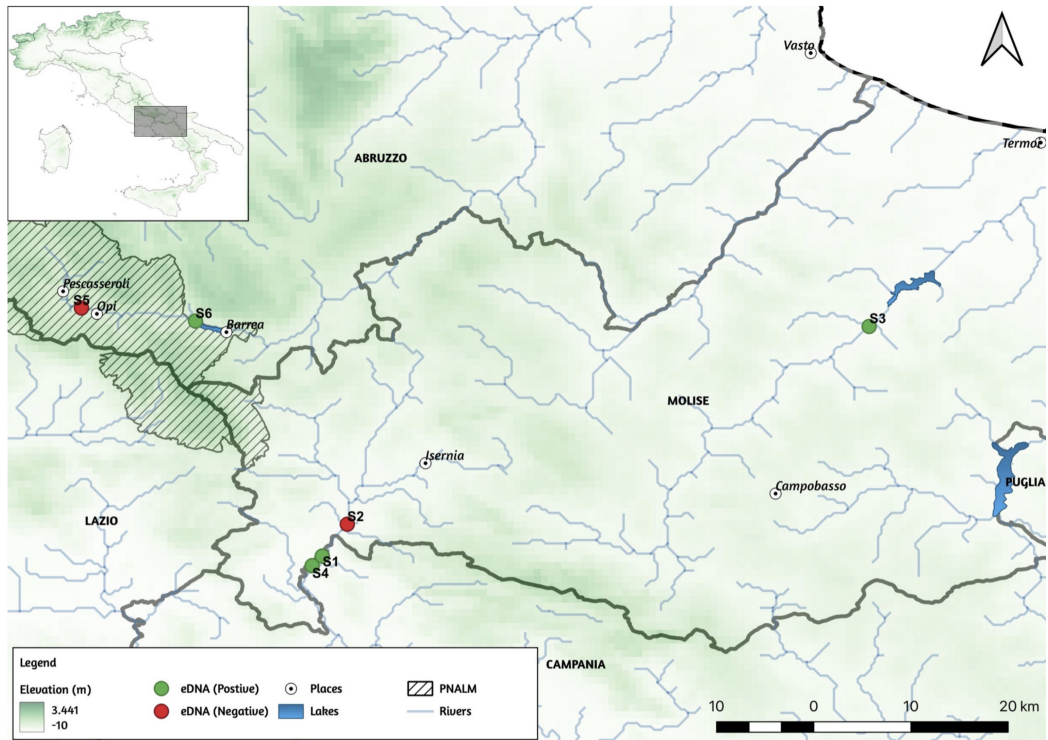
Figure 2 – Map of eDNA results for each sampling site.

detect eDNA traces of target species. Samples collected at site S2 (otter records only in the past) resulted negative for eDNA otter detection. For site S4 (positive for otter signs), eDNA amplification occurred for the 0.22 μm fraction. The 8 μm pore size filter, used to remove water turbidity, showed no amplification of target DNA. In the case of S5 (on-site filtration), we obtained no detection with both visual observation and eDNA assay. Conversely, site S6 (on-site filtration) (otter presence reported only by previous studies) resulted positive for eDNA assay.

In this study, considering sampling sites, and taking into account the eDNA and traditional survey contextually carried out, we obtained 5 out of 6 sampling sites concordant in results. S6 only was not congruent in the results obtained, with positive otter eDNA signal, but no otter signs recorded at the time of our survey. If we include the information gained from past field surveys, S2 represents an "otter-positive" site for data collected in past surveys, but negative in otter eDNA detection; conversely, S6 reported otter records from the past (but not for the present survey) and an otter eDNA signal in our assay. Consider-

Figure 3 – Otter detection from field survey and eDNA assay at each sampling site (S1–S6), compared to negative controls (AQU01–05). The membrane filter pore size used is reported and highlighted with different colours (0.2 μm pore size in cyan and 8 μm pore size in blue). *otter detection in previous field surveys only.

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ing this last evidence regarding S6, eDNA could reveal otter presence when no visual signs are reported, increasing the positive location for otter presence from 50% (3 out of 6 sites) to 67% (4 out of 6 sites).

On the whole, our data revealed that an eDNA approach based on the amplification of a specific genetic marker from water samples could be a reliable tool to monitor the occurrence of the elusive Eurasian otter in a non-invasive way. We tested different filtration strategies (i.e., preliminary filtration to remove water turbidity vs. direct filtration) and different experimental scenarios (i.e., laboratory vs. on-site filtration), in order to account different fieldwork requirements and conditions. Pre-filtration with a wide-mesh filter can be useful to avoid filter clogging in case of high water turbidity, enabling the recovery of eDNA in the downstream fine-mesh filter. Further, on-site filtration could be useful in harsh environments, to avoid issues related to transport and storage of bulky water samples. Despite the low number of samples, we obtained a good congruence (5 out of 6 sampling sites) of results, suggesting the protocol we adopted could be used in areas where field survey is unfeasible or unaffordable and in large scale monitoring. However, the accuracy of site detection still needs to be tested thoroughly, taking into consideration specific environmental variables such as water temperature, water flow, water chemical composition, and all the other factors (including minimum water volume and sampling replicates) that could affect DNA detection (Furlan et al., 2016; Ficetola et al., 2015). One step forward the use of eDNA as a well validated approach for monitoring can be achieved by modelling eDNA detection considering effects of intrinsic sample characteristics, environmental conditions in the field and sample treatment in the molecular laboratory (Song et al., 2017; Harrington et al., 2010; Jo and Minamoto, 2021). Increasing efforts in this direction can help in correctly defining a threshold accounting for positive or negative sites for a stable otter presence.

In this study, several measures were taken in the field and in the lab to circumvent false positive and false negative results. To obtain a reliable test of the method for a target species we strongly recommend to 1) select sampling localities where the target taxon is surely absent (as a negative control) or present (integrating data from well validated traditional methods and recording indirect signs of the presence of the target species); 2) evaluate and record environmental variables, such as water turbidity, which could affect experimental procedures; 3) in case of turbid water, a pre-filtration step with a large mesh filter can be useful to avoid filter clogging and it could improve detectability of eDNA reducing particulate matters and inhibitors; 4) use disposable instruments and carefully clean non disposable ones among samples. Considering that a key factor that strongly influences the reliability of eDNA results is the laboratory set-up, we also recommend to 5) employ a pre-amplification room specific for low quantity DNA samples, and a post amplification room, with a unidirectional workflow through the different rooms of the laboratory; 6) use dedicated equipment for each phase; 7) include quality controls for each experimental step (i.e. DNA extraction positive and negative controls, as well as DNA amplification positive and negative controls); 8) include technical (repeated measures of the same sample) and biological (repeated measures of biologically distinct samples that capture random biological variation) replicates (Blainey et al, 2014) to avoid false negative results.

Conclusions

Under a reliable experimental design and laboratory conditions / procedures, eDNA investigations offer several advantages compared to traditional field surveys of rare and elusive species. Among these, we can mention the null disturbance to the ecosystem or to the target species, fast analytical time, possibility of being applied in harsh field conditions, and the possible scalability to multiple species detection from one environmental sample, that is especially useful for required monitoring obligations under European regulation. Considering the pros and cons of both field survey and eDNA assays, we believe that to make sound wildlife management decisions (e.g.: evaluation of the effectiveness of conservation measures or regulating plans) it is critical to adopt an integrated approach, exploiting the advantages of sensitivity of molecular assays and the robustness of traditional well-validated methods. In addition, comparing the distribution of the target species through both target eDNA assays and field surveys will broaden the understanding of limits and caveat of these monitoring strategies. Finally, environmental water samples collected for target species detection can be proficiently exploited to unlock the huge amount of information hidden in the nucleic acids extracted: a DNA metabarcoding approach, relying on the high-throughput analyses of DNA sequences, can be usefully applied for species occupancy modelling, population genetics, biodiversity estimation, and food web and trophic niche exploration (Compson et al., 2020), deepening our knowledge about the state of an ecosystem on the whole. 🖋

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