



# Comparing traditional vs. environmental DNA monitoring protocols. The case of the Eurasian otter (*Lutra lutra*) re-expansion in Central Italy

Simone Giovacchini<sup>1,2</sup> · Enrico Mirone<sup>1</sup> · Pushpinder Singh Jamwal<sup>1,2</sup> · Pamela Monaco<sup>1</sup> · Fausto Ramazzotti<sup>3</sup> · Emiliano Pioltelli<sup>3</sup> · Andrea Galimberti<sup>3</sup> · Antonia Bruno<sup>3</sup> · Francesco Belluardo<sup>1</sup> · Antonio Canu<sup>2,4</sup> · Marco Galaverni<sup>4</sup> · Mirko Di Febbraro<sup>1</sup> · Anna Loy<sup>1,2,5</sup>

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## Abstract

Once widespread in Italy, in the last century the Eurasian otter (*Lutra lutra*) went to the brink of extinction in this country, only surviving in a few southern river basins. Being included in the Habitats Directive 92/43/EEC, it is mandatory for State Members to monitor its conservation status over time, making cost effective and accurate monitoring protocols a relevant issue. We compared the efficiency of environmental DNA (eDNA) and traditional field survey techniques in detecting the presence of Eurasian otter at the border of the current expansion range of the Eurasian otter in Latium, Central Italy. Water sampling for eDNA detection through species-specific assay and systematic field surveys were run in six river basins. Results from the two approaches were compared at the scale of sampling site, 5 km and 10 km radius upstream. Both techniques agreed in revealing the expansion of Eurasian otter in southern Latium and in confirming otter absence in the northern part of the study area. eDNA allowed to detect otters at two more river catchments compared to field surveys, one of which is located northward in a tributary of the river Tiber, and showed a higher detection rate (0.41), both at sampling site (0.06,  $p=0.01$ ), 5 km (0.23) or 10 km radius (0.35) scale. These results suggest that eDNA is a more sensitive method in revealing Eurasian otter presence, providing a useful reference for future standardized monitoring of the species at the country level, especially in expansion areas.

**Keywords** Otter · Survey · eDNA · Protocol · Expansion · Recolonization · eDNAjoint

## Introduction

Once spread all over Europe, the Eurasian otter (*Lutra lutra*) suffered a severe decline in the last century (Loy et al. 2022). In Italy, otter's decline was among the most pronounced. Once distributed throughout the whole peninsula, it survived only in a few river basins in southern

Italy, forming a genetically distinct and isolated population from the other European populations (Cassola 1986; Mucci et al. 2010). The implementation of legal protection of both the species and its habitats, coupled with banning of harmful pollutants, fostered the recovery of otter populations in many European countries including Italy (Roos et al. 2012). This partial recovery led to downgrade of the species' conservation status to Vulnerable (Rondinini et al. 2022). However, the recovery in Italy has progressed more slowly compared to other European countries (Panzacchi et al. 2011), and the species remains confined to South-Central Italy (Giovacchini et al. 2021).

Otter distribution is typically surveyed through a standardized field survey protocol (Reuther et al. 2000), based on the recording of presence signs along riverbanks. This method is simple but highly time-consuming, requiring field skills, and appropriate conditions for implementing sampling transects (Jeffress et al. 2011). Some limitations, due to environmental factors and local conditions, have long been debated for their

✉ Simone Giovacchini  
s.giovacchini@studenti.unimol.it

<sup>1</sup> University of Molise, Environmetrix Lab, Contrada Fonte Lappone, Pesche 89090, IS, Italy  
<sup>2</sup> IUCN SSC Otter Specialist Group, Gland, Switzerland  
<sup>3</sup> University of Milano-Bicocca, piazza della Scienza 2, Milan 20126, Italy  
<sup>4</sup> WWF Italia, via Po 25/c, Rome 00100, Italy  
<sup>5</sup> CNR-IRET, Montelibretti 00015, RM, Italy

potential to impair survey results, leading to false-absence data, especially where otter densities are very low (Jefferies 1986; Kruuk and Conroy 1987; Mason and Macdonald 1987).

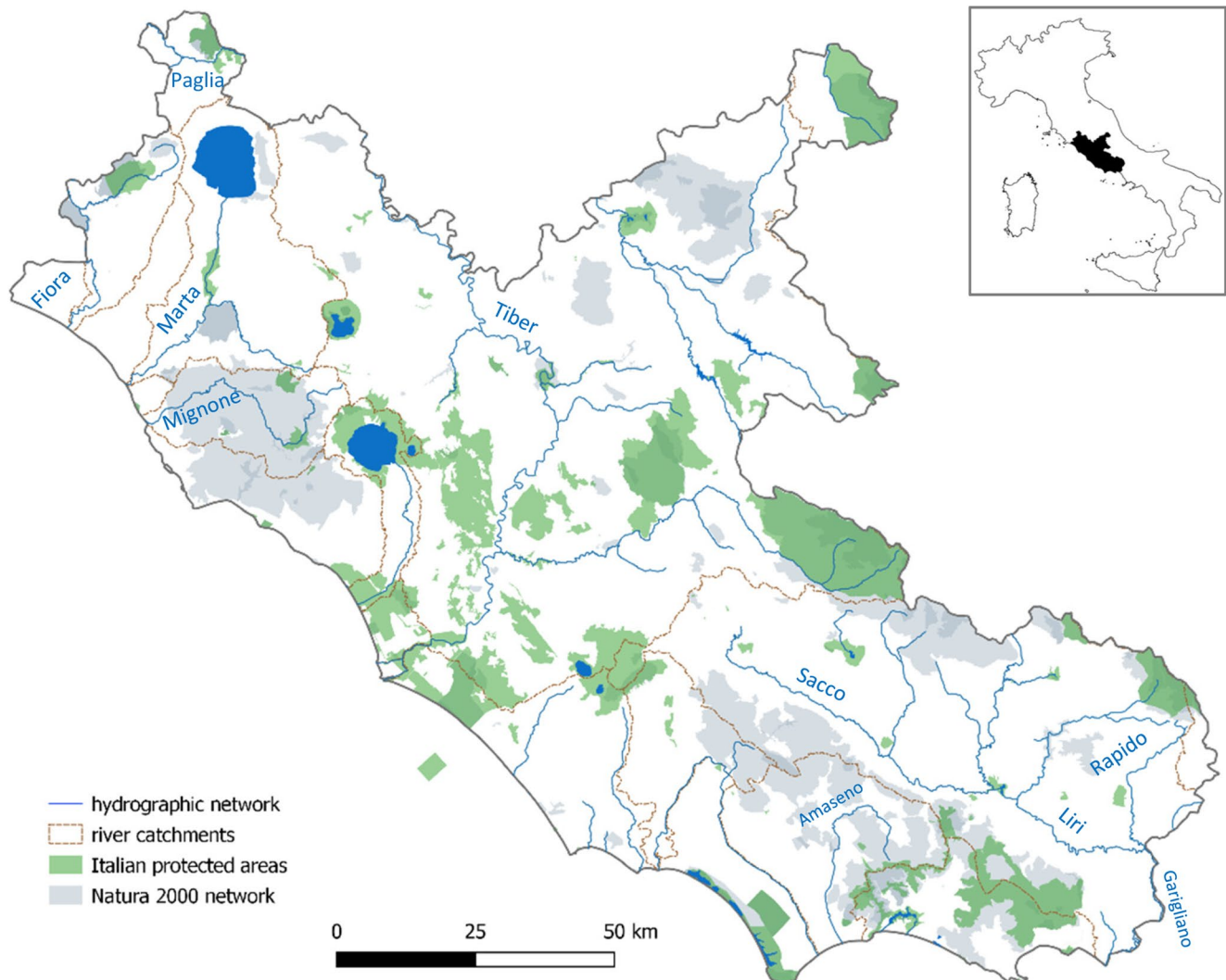
Environmental DNA (eDNA) has emerged as a cost-effective alternative to complement traditional species survey (Keck et al. 2022), and is particularly effective in detecting rare and elusive species (Johnson et al. 2024). Quantitative PCR (qPCR) assays offer a higher sensitivity and species-specific detection technique, outperforming broader approaches like eDNA metabarcoding (Harper et al. 2018). A qPCR-based approach is particularly advantageous for rapidly detecting endangered species outside their known distribution range, where low population densities occur. Despite this method has successfully been used to detect Eurasian otters (Ballini et al. 2024; Jamwal et al. 2021, 2023), these studies did not assess the relative efficiency of the eDNA vs. traditional field survey.

We combined both eDNA-based analysis and field survey to detect Eurasian otter at the northern boundary of its current range along the Tyrrhenian side in south-central Italy to (i) update the position of the expansion front, and (ii) compare the efficiency of eDNA versus field survey in recent colonized areas.

## Methods

### Study area

The study area encompasses seven river basins located in Central Italy, Latium Region, including river Tiber (405 km in length), river Liri (158 km), and five smaller coastal rivers (Amaseno, Mignone, Marta, and Fiora) (Fig. 1). Sampling sites were selected based upon the proximity to the current



**Fig. 1** The study area overlaps the administrative boundaries of Latium region and encompasses six different river catchments

species range, the high habitat suitability for the species and the inclusion in previous surveys.

### Habitat suitability model

Otter habitat suitability was assessed using an expert based modelling approach (Loy et al. 2009). The model integrates results from multiple inferential models developed at different spatial scales on the Eurasian otter in Italy and Europe (Cianfrani et al. 2011, 2013; Carone et al. 2012, 2014; Weinberger et al. 2019). Accordingly, we reclassified the following environmental variables into three suitability categories for otters (Suppl. Mat. S1): river networks ranging from first- to fourth-order, a 10 m-scale DEM (Tarquini et al. 2023), and a 10 m-scale riverine vegetation (De Fioravante et al. 2021). Results are available in Suppl. Mat. S2.

### Environmental DNA sampling and laboratory procedures

A full description of lab procedures is available in Giovacchini et al. (n.d.). Three litres of water were collected at 17 sampling sites, performing two separate replicates (one in July 2021 and one in June 2022). Samples were filtered through an 8  $\mu\text{m}$  and then a 0.22  $\mu\text{m}$  filter of cellulose mixed esters (Merck-Millipore, Burlington, USA). eDNA was extracted using Power Soil Pro extraction kits (Qiagen, Venlo, Netherlands) and amplified using real-time qPCR (StepOnePlus, Applied Biosystem, Waltham, USA) with Eurasian otter species-specific primers (Park et al. 2011). To circumvent false negatives due to PCR inhibitors, each sample was run in two dilutions (1:1, and 1:10) and in three technical replicates, including a negative technical, and environmental positive and negative controls (Klymus et al. 2020). Samples were considered positive when

amplification was observed in at least two technical replicates (Bustin et al. 2009).

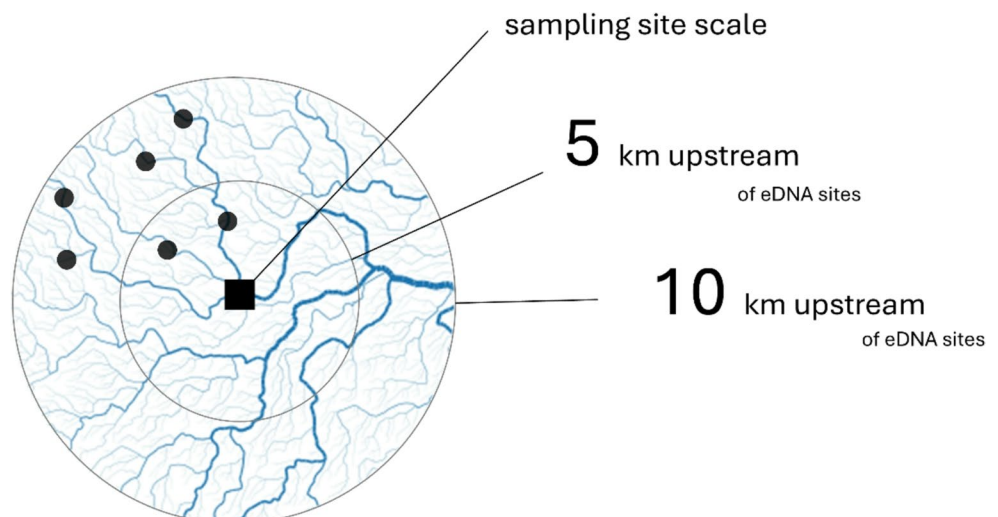
### Traditional field survey

A systematic survey based on traditional field methods was run in July 2022 at 192 sites, following the standardized protocol recommended by Reuther et al. (2000). Up to four sites were selected on each  $10 \times 10$  km cell grid (ETRS89 LAEA 5210 projection). Otter signs (spraints, anal jellies, and footprints) were searched for along 600 m of riverbank by walking or using a kayak to navigate and inspect the river (Giovacchini et al. 2019). Where transects were unfeasible, we inspected up to six bridges per grid cell as an accurate complementary method (Schenekar et al. 2022; Weinberger et al. 2022).

### Data analysis

Results from eDNA and field surveys were imported into a GIS environment (QGIS 3.28, <http://qgis.org>). Detection rates were compared at the sampling sites, as well as within 5 km and 10 km distance radii to account for potential downstream transport of eDNA (Burian et al. 2021). At broad scales, results from field survey were pooled, considering the pooled area positive if at least one of the sites was found positive (Fig. 2). Detection rates (DR) for eDNA and field surveys were calculated as the number of positive sites divided by the sites tested. Differences in detection rates between eDNA and field surveys at all spatial scales were assessed using Fisher's exact tests (Freeman and Campbell 2007), implemented in the R (R Core Team 2013). All analyses were repeated after excluding negative sites from both eDNA and traditional surveys (i.e. sites potentially representing true absences).

**Fig. 2** Environmental DNA (black square) techniques are compared at sampling site scale, at 5 km, and at 10 km scale on upstream watersheds with traditional field monitoring (black dots)



Finally, we analysed the mean probability of a false positive eDNA detection ( $p_{10}$ ), the median value of otter density under eDNA detections become unreliable ( $\mu$ ), and the number of survey units necessary to detect species presence for eDNA and the cumulative traditional samplings (within 10 km upstream) through the calculation of expected catch rate for each site running a Bayesian model with the *eDNA-joint* R package (Keller and Kelly 2025).

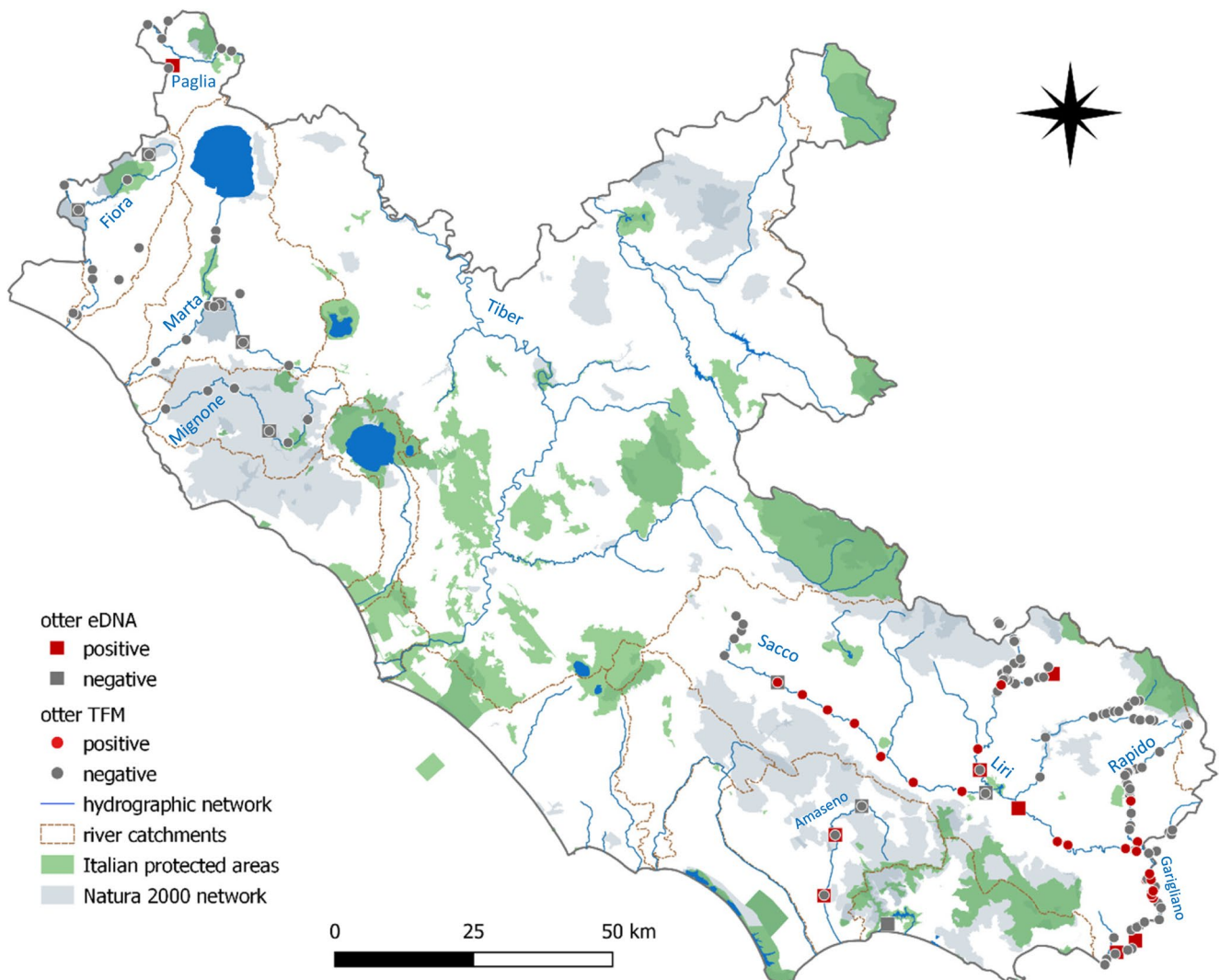
## Results

eDNA detected otters at 8 out of 17 sampling sites. Seven positive sites were located in two southern catchments (Liri and Amaseno River, while one positive site in a tributary of the Tiber River in the northernmost portion of the study area (Paglia River) (Fig. 3). Field surveys confirmed otter

presence along Liri river at 23 sites. Conversely, no otter signs were found in the northern rivers.

At sampling site scale, DR was higher for eDNA (DR=0.41) compared to traditional survey (DR=0.059). Despite the DR of field surveys increased when results were pooled at 5 km scale and 10 km distances, it remained lower than the eDNA DR at all scales (Table 1). When sites representing potential true absences were excluded, the eDNA DR remained higher than that of the field surveys. Differences in DR between the two methods decreased with increasing distance from the eDNA sampling point (Table 1). Fisher's exact tests revealed a statistically significant difference between the DR of eDNA and field surveys only at the sampling site scale (Table 2).

The mean probability  $p_{10}$  to have a false positive eDNA detection resulted to be  $0.038 \pm 0.032$ . The median value  $\mu$  of otter density under eDNA detections become unreliable



**Fig. 3** Otter occurrences detected using environmental DNA (eDNA) and traditional field monitoring (TFM) techniques within the study area, Latium region

**Table 1** Detection rates (DR) based on occurrences divided tested sites and computed on all sites, and on sites resulted negatively to both eDNA and traditional field monitoring (TFM) surveys

	eDNA	TFM (sampling sites)	TFM (5 km)	TFM (10 km)
DR	0.471	0.059	0.235	0.353
DR without true absences	0.800	0.100	0.400	0.600

**Table 2** Number of survey units necessary to detect species presence for the cumulative traditional samplings within 10 km upstream eDNA sites, and eDNA

	TFM (sampling sites)	TFM (5 km)	TFM (10 km)
eDNA (all sites)	0.016 *	0.281	0.728
eDNA (without true absences)	0.005*	0.169	0.628

is equal to 0.193. The number of survey units necessary to detect species presence is greater for traditional methods than eDNA (Table 3).

## Discussion

Our results revealed an expansion of the otter range along the Tyrrhenian side of Central Italy. New occurrences in southern river catchments of Amaseno and Liri-Garigliano rivers confirm the ongoing recovery within the historic otter range (Giovacchini et al. 2018; Marcelli et al. 2023). This evidence claims the need to protect the Liri river in accordance with the prescriptions of the Habitats Directive 42/93/EC. Conservation measures targeting an apex predators such as the otters are likely to confer broader benefit to the freshwater biodiversity of river ecosystems (Macdonald et al. 2018). In addition, community-based initiatives should be implemented to prevent the emerging of human-otter conflicts, promoting coexistence and supporting long-term conservation goals (Sales-Luís et al. 2009).

The species appears to have recolonized the area within a few years, and its current widespread distribution is expected to promote further northward expansions. However, the low marking frequencies and the advanced state of deterioration of spraints suggest low revisitation rates of marking sites. This pattern could indicate an initial phase of territory occupation, with unstable home ranges over time, and suggest that the carrying capacity of Liri-Garigliano river catchment may not yet have been reached (Chapman and Byron 2018). This hypothesis is supported by the observation that field surveys failed to detect otters along many rivers in the southern coast of the region, where qPCR eDNA analysis unveiled otter presence. Wandering individuals exhibiting

**Table 3** Number of survey units necessary to detect species presence for the cumulative traditional samplings within 10 km upstream eDNA sites, and eDNA

catch rate	traditional survey	eDNA
0.1	24	54
0.5	5	12
1	3	6

dispersal behaviours may not have marked their presence, as the sprinting behaviour is known to serve for communication purposes to conspecifics within well-established home ranges (Kruuk 2006).

qPCR eDNA analysis is recognized to be capable of identifying even a few individuals engaged in long-distance dispersal movements (Stewart and Taylor 2020). The sensitivity of qPCR eDNA is further demonstrated in the Tiber river catchment, where otter occurrence was detected along the tributary river Paglia, located far from the current known range boundaries. Notably, one individual otter was also recently camera trapped in the Tiber basin near Rome (Cervoni et al. 2024), and a direct observation was retrieved in an adjacent river catchment (Fiora River) (<http://therio.unimol.it:8080/lontra/>). The higher sensitivity of eDNA was also highlighted by positive detections at the river mouths, where field techniques failed to identify otter signs. Conversely, results from eDNA perfectly matched those of the field survey conducted along the northern river basins of the region, confirming the absence of otter where a remnant non-viable population was declared extinct in 2000 (Reggiani et al. 2001). Conversely, we think eDNA has underestimated otter presence along Sacco for ecological reasons, instead of being related to technical issues on the eDNA analysis process. Otter signs retrieved by traditional monitoring were very old and dry. This causes two possible effects: a non-presence of otters in that specific water body at the time (and much earlier) of eDNA collection, and, more important, a complete degradation until the loss of DNA available for the detection of the species.

qPCR-based eDNA is more sensitive in revealing otter presence, particularly at the sampling site scale, suggesting that standard surveys can be affected by false absences when otters occur at low densities (Janssens et al. 2006). This is proved also by the values of  $p_{10}$  and  $\mu$  that resulted very low. At the local scale, the availability of accessible substrates for otter markings vary according to riverbank morphology and hydrological conditions (Hong et al. 2022), highlighting the critical importance of site selection. The underestimation of traditional monitoring is particularly evident in case of embanked channels, like river Amaseno, where the absence of emerging marking sites leads to false negatives. However, we acknowledge that rivers can act as conveyor belts, transporting molecules shed by organisms many kilometres upstream (Deiner et al. 2016). Consequently, the

probability to detect a species via eDNA may remain high even in areas where the species is not physically present at the sampling site, potentially leading to mismatch presence/absence results obtained through field techniques (Eiler et al. 2018). This phenomenon is particularly evident at river mouth, where river sections are wider, emerged substrates are excessively numerous to investigate (Elmeros and Busenius 2002).

Only one site showed otter detection by field surveys where eDNA failed. Inhibition mechanisms related to chemical properties and microbial communities, as well as DNA degradation, can reduce eDNA amplification (Barnes and Turner 2016). Nevertheless, qPCR eDNA is well recognized as a cost-effective alternative method for biological monitoring, offering substantial saving in both time and financial resources (Johnson et al. 2024). An estimation of time and cost for otter monitoring at our 17 sampling sites indicated that qPCR based analysis requires 14 days and a total cost of € 1,800, whereas the traditional survey requires 6 days and € 2,116 (Giovacchini et al. submitted; Suppl. Mat. 3). Moreover, in our experience, along Liri river, seven sites sampled using eDNA outperformed 141 sites surveyed with traditional techniques, considering the aim to rapidly inform the new presence of a protected and totemic animal. This is translated directly into substantial time savings for wildlife assessments, as water sampling required only two days, compared to 35 days needed for conducting field transects in search for otter tracks. Thus, even if traditional techniques seem to have a higher catching rate, they are characterized by the trade-off of a huge sampling effort.

Molecular approaches have once again demonstrated the high sensitivity in detecting species presence at low concentrations, highlighting its potential to guide biodiversity monitoring in freshwater ecosystems (Porco et al. 2022). The use of eDNA techniques has proven crucial for monitoring expansion stages of highly mobile mammals (Valsecchi et al. 2022). Nonetheless, we recommend the combination of both techniques to better inform the real distribution of this species and balance the bias of false absences offered by both methods. An integrated approach that combines both eDNA and traditional methods can provide more accurate spatial data to better inform conservation planning (Tolrà et al. 2024). In this context eDNA can serve as a rapid tool for large-scale monitoring of areas where the expansion of endangered species is anticipated. Once a species has been detected, traditional survey techniques can be deployed to collect more reliable and detailed on the spatial occupation, giving the opportunity to also monitor habitat degradation, to support targeted conservation actions (Duarte et al. 2023).

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10344-026-02079-7>.

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**Author contributions** S.G. carried out field surveys, wrote the main manuscript, and prepared the figures. S.G., E.M., P.S.J., and F.B. undertook eDNA sampling. P.M., F.R., and E.P. performed lab procedures. A.G., A.B. supervised lab and statistical analysis. M.D.F. implemented species distribution modeling. S.G., A.C., M.G., M.D.F., and A.L. planned project design and financial covers. All authors reviewed and accepted the manuscript.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Competing interests** The authors declare no competing interests.

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