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Non-invasive monitoring of freshwater species in Central and Southern Italian basins. From standard survey to environmental DNA

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Introduction

Freshwater Ecosystems: conservation status and threats

Freshwater ecosystems, comprising rivers, lakes, wetlands, and riparian zones, serve as vital components of the Earth's biosphere. However, significant planetary changes driven by human activities, has profoundly altered these ecosystems (Dudgeon et al., 2006). In fact, freshwater ecosystems have been massively affected due to their sensitivity to environmental changes. Human activities, particularly water resource development and land cover modifications, have led to widespread alterations in freshwater ecosystems (Jansson et al., 2007). The construction of numerous dams globally, coupled with extensive land transformation, has disrupted the natural dynamics of riverine systems (Graf, 1999; Nilsson & Renöfält, 2008). These alterations have profoundly impacted the magnitude, timing, and duration of river flows, essential for sustaining the dynamic nature of freshwater ecosystems. Moreover, habitat destruction, fragmentation, eutrophication, chemical pollution, invasive species, overexploitation, and climate change further exacerbate the threats faced by freshwater biodiversity (Sanchez-Bayo & Wyckhuys, 2019). The cumulative impacts of these stressors have accelerated biodiversity loss in freshwater ecosystems, damaging the natural ecosystems and services they provide (McRae et al., 2017).

Despite covering less than 1% of the Earth's surface, freshwater ecosystems play a critical role in supporting biodiversity and providing essential ecosystem services. These habitats host a remarkable biodersity, including approximately one-third of vertebrate species and 10% of all species (Strayer & Dudgeon, 2010). For instance, they are home to around 70 species of freshwater mammals, 5700 dragonflies, 250 turtles, 700 birds, 17.800 fishes, and 1.600 crabs (Balian et al., 2008; Fricke et al., 2019). Moreover, freshwater environments exhibit high levels of endemism, with many species confined to specific ecoregions (Abell et al., 2008). In addition to supporting biodiversity, freshwater ecosystems provide indispensable services crucial for human well-being. These services include fisheries, water provisioning for drinking and agriculture, transportation, and cultural benefits (Green, 2003; Balian et al., 2008; Dodds et al., 2013). Despite their significance, the management of freshwater ecosystems has often prioritized a narrow range of services for economic gain, leading to habitat degradation and loss. Consequently, the current rate of wetland loss exceeds that of forest loss, and populations of freshwater vertebrate species are declining at alarming rates (Gardner & Finlayson, 2018; Grooten & Almond 2018).

Freshwater ecosystems face an array of threats driven by human activities, posing significant risks to their integrity and biodiversity. The ongoing Anthropocene era has intensified these threats, making necessary urgent conservation efforts (Ehlers & Krafft, 2006). These threats stem from various anthropogenic stressors acting within these habitats (Abell et al., 2008; McRae et al., 2017). The vulnerability of freshwater environments is exacerbated by their role as recipients of pollutants and wastes from terrestrial landscapes, coupled with their limited capacity to mitigate these impacts (Gerbersdorf et al., 2011). Climate change intensifies the detrimental effects, intensifying issues like eutrophication (Strayer & Dudgeon, 2010). Moreover, the inability of many freshwater species to adapt or disperse efficiently increase the risk of local extinctions (Alahuhta et al., 2019). Invasive species further increase these challenges, disrupting ecosystems and outcompeting native species (Luque et al., 2014; Pelicice et al., 2014; Ricciardi et al., 2017). Despite increased understanding, the complexity and urgency of freshwater environmental challenges continue to escalate (Jackson et al., 2016;

Jeppesen et al., 2015; Zarfl et al., 2015). These findings underscore the critical need for concerted global efforts to address the crisis facing freshwater ecosystems, as current research and conservation endeavors remain insufficient (Mazor et al., 2018; Tydecks et al., 2019).

Italian Freshwaters

Italy's freshwater ecosystems, comprising rivers, lakes, and wetlands, are vital components of the country's ecology (Cantonati et al., 2020; Forchetti et al., 2010). They have historically played a pivotal role in sustaining biodiversity and facilitating human activities, but today, they face multifaceted challenges arising from human intervention and environmental shifts (Arora et al., 2018; Svenning et al., 2024). Despite constituting a small fraction of Italy's land surface, freshwater ecosystems wield profound ecological significance, sustaining a rich variety of biodiversity and furnishing indispensable ecosystem services (Carosi et al., 2023; Cifoni et al., 2022; Rui Rivaes et al., 2021). These habitats host a multitude of aquatic species, including numerous endemic taxa of evolutionary importance (Bolpagni et al., 2018; Facca et al., 2020; Gigante et al., 2018). The endemic biodiversity housed within Italian freshwater environments underscores their ecological richness, supporting at least 100,000 species (Cazzolla Gatti, 2016). However, the ecological integrity of Italian freshwater ecosystems is increasingly imperilled by anthropogenic activities, necessitating urgent conservation interventions to safeguard their ecological functions and ensure their resilience in the face of mounting environmental pressures (Carpenter et al., 2011).

Italian freshwater ecosystems confront a myriad of threats, driven predominantly by human activities and environmental perturbations. Beyond the realm of invasive species, which have posed a persistent threat, with the introduction of non-native species such as crayfish and fish altering the delicate ecological balance, Italy's freshwater ecosystems are now grappling with escalating pollution levels and habitat degradation (Boggero et al., 2014; Colangelo et al., 2017; Haubrock et al., 2021). One example above all is the emergence of crayfish species like *Procambarus clarkii* and *Faxonius limosus* in Italian waters. These invasive species triggered ecological disruptions, leading to habitat destruction and intense interspecies competition (Aquiloni et al., 2010; Chiesa et al., 2006). These invasions not only endanger indigenous species but also homogenize Italy's freshwater biota, eradicating regional biodiversity distinctions (Giangrande et al., 2020). Pollution, including chemical contaminants and nutrient runoff, poses a significant threat, directly impacting aquatic organisms and altering ecosystem dynamics (Birk et al., 2020; Strayer & Dudgeon, 2010). Despite increased understanding, the complexity and urgency of freshwater environmental challenges continue to escalate (Jackson et al., 2016; Jeppesen et al., 2015; Zarfl et al., 2015). These findings underscore the critical need for concerted global efforts to address the crisis facing freshwater ecosystems, as current research and conservation endeavors remain insufficient (Tydecks et al., 2019). Pollution emanating from urban and agricultural sources poses a pervasive menace, contaminating water bodies and disrupting aquatic ecosystems (Morin-Crini et al., 2022; Pastorino et al., 2023). Moreover, habitat degradation resulting from dam construction, river channelization, and the modification of riparian zones further exacerbates ecological disturbances, impinging on the viability of native species populations (Sabater et al., 2018). The insidious proliferation of non-native species compounds these challenges, altering ecological dynamics and outcompeting indigenous fauna. Climate change amplifies these threats, inducing shifts in precipitation patterns, elevating temperatures, and exacerbating the frequency and intensity of extreme weather events (Ludovisi et al., 2013; Portoghese et al., 2013). These environmental transformations disrupt the delicate balance of freshwater ecosystems, precipitating declines in native

species abundance and compromising the delivery of crucial ecosystem services (Carpenter et al., 2011). To avert irreversible ecological degradation, concerted efforts are imperative to mitigate pollution, restore degraded habitats, and implement adaptive management strategies that foster the resilience of Italian freshwater ecosystems amidst mounting environmental pressures.

Invasive alien species

Invasive alien species (IAS) refer to animals, plants and even pathogens introduced accidentally or intentionally into natural environments where they are not native, resulting in significant negative consequences for the ecosystem (Keller et al., 2011). They pose a major threat to native biodiversity in Europe and are among the top five causes of biodiversity loss (Suietnov & Tulina, 2021). Moreover, IAS can adversely affect human health, leading to severe allergies and burns. The EU is committed to preventing, minimizing, and mitigating the adverse impacts of these species on native biodiversity and ecosystem services. Regulatory measures aim to also limit social and economic damage (Caffrey et al., 2014). The EU Biodiversity Strategy for 2030 includes a pledge to manage established invasive alien species and reduce the number of Red List species they threaten by 50% by 2030. Currently, 88 invasive alien species are strictly regulated in the EU, including 47 animal species and 41 plant species of Union concern. The Invasive Alien Species Regulation (Regulation (EU) 1143/2014) outlines measures to be implemented across the EU regarding invasive alien species. Central to the Regulation is the Union List, which identifies invasive alien species subject to restrictions and measures. These measures include limitations on keeping, importing, selling, breeding, growing, and releasing into the environment (Genovesi, 2005; Roy et al., 2018). Member States are obligated to take action to prevent unintentional introductions of invasive species, implement measures for early detection and rapid eradication, and manage species that are already widespread within their territories (Roy et al., 2023).

Monitoring techniques for Freshwater biodiversity

Monitoring plays a critical role in studying environmental systems, serving three primary purposes: providing insights into deviations from the desired state, evaluating the effectiveness of management and conservation efforts, and identifying the impacts of disturbances. For the data gathered in a monitoring program to be truly valuable, careful attention must be given to designing a scheme that aligns with its intended purposes. This involves striking a suitable balance between the effort invested in sampling (often the most resource-intensive aspect in terms of time and cost) and the nature of the information acquired. It's important to recognize that while more information may be gathered through monitoring, it doesn't always directly lead to conservation benefits (McDonald-Madden et al., 2010). The Habitats Directive marks a milestone in European biodiversity conservation policies. Its primary objective is to protect biodiversity by preserving natural habitats and the diverse wildlife within the European territory of the Member States. To achieve this goal, the Directive establishes measures aimed at ensuring the preservation or restoration, to a favorable conservation status, of the habitats and species of Community interest listed in its annexes. One key measure involves establishing a cohesive European ecological network of protected areas, accomplished through the designation of Special Areas of Conservation (SACs) for habitats listed in Annex I and species listed in Annex II. These SACs, along with Special Protection Areas (SPAs) designated under Article 4 of the Birds Directive, collectively form the Natura 2000 Network, as outlined in Article 3. Among other measures, monitoring and drawing up national reports are particularly relevant. In order to preserve biodiversity and ecosystems, it is not sufficient to assess the conservation status based solely on current conditions; it is necessary to

forecast their future evolution through the identification of factors that may influence them, whether threats, pressures, or actions with positive or negative effects.

Traditional monitoring techniques

Standard monitoring techniques obtained from the ISPRA manual for monitoring species in the Habitats Directive are given (Stoch, F., & Genovesi, P. - 2016).

Standard monitoring techniques for various animal species involve specific protocols tailored to each species' habitat, behaviour, and ecological requirements. However, several challenges and limitations are associated with these methods. For instance, for molluscs the collection of material and sieving process cannot be standardized due to the wide variety of habitats occupied, resulting challenging and time consuming to access sampling points and often requiring numerous transects with obstacles and submerged animals that are difficult to observe (Larsen, 2016). Expert recognition is also required for accurate species identification. The same problems arise in the monitoring of *Austropotamobius pallipes* and Odonata species (*Lindenia tetraphylla, Oxygastra curtisii* and *Coenagrion castellani*), and for amphibian species such as *Bombina pachypus*, *Triturus carnifex* and *Lissotriton italicus* (Chiesa et al., 2006; Maletzky et al., 2007; Watts et al., 2007). The monitoring of most fish species, especially where it is required to take biometric data or tissue samples of the animals, is done by electrofishing, a technique that, although subject to very careful regulation, is nonetheless invasive and causes stress and disturbance to the animals (Bo et al., 2011; Marconato et al., 1989). Finally, for many species, the equipment needed for regular monitoring can be very specific and sometimes expensive, and in terms of multi-specific or multi-taxa monitoring, this can be inconvenient. For IAS, the rapidity of detection of the species in a territory is by far the most important aspect to contain their expansion (Early et al., 2016; Shackleton et al., 2019). While the aspect of disturbing individuals is certainly less important since management actions, where possible, are mainly aimed at eradicating the invasive species from the territory. These challenges highlight the need for innovative and alternative monitoring methods tailored to each species' specific requirements and behaviours to improve detection and conservation efforts.

Environmental DNA – eDNA

Environmental DNA (eDNA) analysis emerges as a revolutionary technique for large-scale biodiversity monitoring, encompassing the extraction and simultaneous analysis of billions of DNA sequences from various environmental samples like water, soil, and air (Deiner et al., 2016; Ficetola et al., 2019, 2021; Pawlowski et al., 2020; Xia et al., 2021). Its versatility has been demonstrated across different ecosystems, particularly in characterizing freshwater communities from lakes, rivers, and wetlands (Clusa & García-Vázquez, 2018; Edwards et al., 2018; Jerde et al., 2011; Thomsen et al., 2012). Notably, eDNA stands out for its sensitivity and non-invasiveness, enabling the detection of cryptic, rare, and elusive species where traditional survey methods often fall short (Hobbs et al., 2019). A significant advantage of eDNA lies in its ability to detect multiple species from a single environmental sample, facilitating multispecies surveys without the need for numerous specialized surveyors (Pawlowski et al., 2020). This capability is particularly beneficial for species that are challenging to detect using conventional methods, including both endangered and invasive species (Leese et al., 2021). With the rising importance of monitoring programs for rare and invasive species, eDNA offers a solution to fulfil reporting obligations under various directives such as the Habitat Directive and the Regulation on Invasive Alien Species (Pawlowski et al., 2021). Moreover, standardized methods proposed for eDNA analysis align with monitoring commitments mandated by directives like the Water Framework Directive

200/60/EC, ensuring consistency and reliability in monitoring efforts (Leese et al., 2021). Traditionally, species detection through conventional methods can be time-consuming and resource-intensive, posing challenges for monitoring rare species and invasive alien species (IAS). In contrast, eDNA techniques, including metabarcoding multispecies assessment and high-throughput sequencing, offer rapid and efficient species detection (Pont et al., 2023; Ushio et al., 2017). Single species approaches based on quantitative polymerase chain reaction (qPCR) have proven to be particularly sensitive, making them valuable for detecting rare and invasive species during the early stages of colonization (Harper et al., 2020; Xia et al., 2018, 2021). Recent advancements have also led to the development of protocols for rapid confirmation of invasive species presence in the field (Thomas et al., 2018, 2020). The fundamental principle behind eDNA lies in retrieving DNA from environmental samples, holding immense potential for addressing challenges associated with biodiversity monitoring (Kelly et al., 2014; Mary Kelly-Quinn et al., 2020; Staley et al., 2018). Species continuously release DNA into their surroundings through various means such as excreted cells, tissue, and dead individuals, forming the basis for eDNA analysis (Jamwal et al., 2021; Valsecchi et al., 2022; White et al., 2020). Once released, DNA persists in the environment, albeit with varying preservation times, ranging from weeks to thousands of years, depending on environmental conditions (Dejean et al., 2012; Willerslev et al., 2003). The advent of next-generation sequencing (NGS) technologies has revolutionized biodiversity surveys, enabling comprehensive analysis of entire communities across taxonomic groups from a single standardized sample (Taberlet et al., 2012, 2018). As interest in eDNA assays for species detection grows, the importance of assay reliability and performance becomes paramount (Goldberg et al., 2016; MacDonald & Sarre, 2017). Quantitative PCR (qPCR) assays, widely used for sequence-specific detection and quantification of nucleic acids, have become routine in eDNA research (Goldberg et al., 2016; Lacoursière-Roussel & Deiner, 2021). However, assay performance can vary significantly due to differences in design, optimization, and testing, emphasizing the need for standardized protocols and transparent reporting of assay performance measures (Jerde et al., 2011). eDNA analysis holds immense promise for revolutionizing biodiversity monitoring by offering rapid, sensitive, and non-invasive species detection across various ecosystems. With ongoing advancements in technology and methodology, eDNA stands poised to become a cornerstone in conservation and management efforts aimed at preserving and restoring biodiversity.

eDNA strengths

The environmental DNA (eDNA) monitoring technique presents numerous advantages and strengths, making it a valuable tool for biodiversity assessment and conservation efforts. Firstly, eDNA sampling can be conducted in a highly standardized manner across different habitats, offering consistency in data collection compared to traditional methods, which often rely on personnel expertise and may yield variable results (Deiner et al., 2016; Takahara et al., 2015). Moreover, eDNA sampling is entirely non-invasive, posing no harm to the studied species or their habitats, unlike traditional survey methods that may disturb organisms and ecosystems (Biggs et al., 2015; Dejean et al., 2012). One of the notable strengths of eDNA is its sensitivity, particularly in detecting species with cryptic lifestyles or during juvenile life stages that are challenging to identify using traditional methods alone (Biggs et al., 2015; Dejean et al., 2012). This sensitivity makes eDNA methods superior in certain scenarios, enhancing the detection of species of conservation importance. Cost- effectiveness is another significant advantage of eDNA monitoring, although its superiority over traditional methods depends on the target species (Thomsen et al., 2012). Nevertheless, several studies have reported shorter handling times and lower costs associated with eDNA compared to traditional techniques (Jerde et al., 2011; Sigsgaard et al.,

2015). With the continuous decline in sequencing costs, eDNA is expected to become increasingly cost-efficient, especially when employing a metabarcoding approach. Furthermore, eDNA sampling offers independence from weather conditions, extending the monitoring window for certain species that may be difficult to survey using traditional methods during specific seasons or weather conditions (Rees, Bishop, et al., 2014; Rees, Maddison, et al., 2014). This flexibility enhances the reliability and effectiveness of biodiversity monitoring efforts, particularly for species like amphibians and fish. In the context of aquatic organisms, including freshwater mussels, eDNA sampling has emerged as a powerful survey tool, capable of improving the efficiency and accuracy of sampling compared to traditional methods (Goldberg et al., 2016). It enables the collection, extraction, and amplification of genetic material shed by organisms into the water column, facilitating the detection of multiple taxa across various aquatic systems (Thomsen & Willerslev 2015). Despite these advantages, it's important to acknowledge potential challenges associated with eDNA sampling, such as the presence of co-extracted compounds that can inhibit PCR amplification, leading to false negatives (Barnes et al., 2014; Jane et al., 2015). However, advancements in understanding and mitigating these challenges continue to enhance the reliability and effectiveness of eDNA monitoring techniques.

eDNA disadvantages

Environmental DNA (eDNA) monitoring, while holding great promise, presents several significant challenges that need careful consideration. Chief among these is the risk of contamination, which can lead to false positive results at various stages, from field sampling to laboratory analysis (Taberlet et al., 2018). Cross-contamination between samples during fieldwork and the spread of DNA within the lab environment, especially with the widespread use of PCR and high-throughput sequencing technologies, are particularly concerning (Champlot et al., 2010; Ficetola et al., 2021; Pedersen et al., 2015). To mitigate this risk, strict protocols involving decontamination procedures and physical lab separation are crucial, along with the inclusion of extraction blanks, PCR blanks, and field blanks for monitoring contamination levels. Another challenge is inhibition caused by humic acids or humic substances present in environmental samples, which can strongly interfere with PCR reactions, especially in soil and sediment-contaminated water samples (McKee, Calhoun, et al., 2015; McKee, Spear, et al., 2015; Sigsgaard et al., 2015). Inhibition can result in false negative results, leading to underestimation of species occurrence and potentially impacting conservation efforts. Errors in DNA sequences, whether occurring before sampling, during PCR, or during sequencing, also pose a significant challenge (Cooper et al., 2021; Pedersen et al., 2015). PCR-generated errors and sequencing errors can lead to biased results and overestimation of microbial diversity, as well as obscuring biodiversity signals in animals (Goldberg et al., 2016; Hadziavdic et al., 2014; Thomsen et al., 2012). Careful filtering of raw sequence data is essential to minimize false positives and ensure the reliability of taxon lists. Moreover, the reliance on reference DNA-sequence databases for the identification of DNA sequences derived from environmental samples introduces additional challenges (Creer et al., 2016; Honka et al., 2023; Lear et al., 2018; Pereira et al., 2008). While global initiatives aim to address the geographical and taxonomic gaps in these databases, there remains a significant knowledge gap at lower taxonomic levels, hindering the effectiveness of eDNA monitoring, especially in cases where all species have not yet been sequenced (Beng & Corlett, 2020; Fonseca et al., 2023; Ruppert et al., 2019). Furthermore, the sub-optimal nature of COI sequences in existing databases for eDNA metabarcoding suggests a need for future databases to focus on complete mitochondrial or nuclear genomes to enhance the applicability of eDNA monitoring (Deagle et al., 2018). In summary, while eDNA monitoring holds immense potential for biodiversity assessment, addressing challenges related to contamination, inhibition, errors, and

database limitations is crucial for ensuring the reliability and effectiveness of this technique in conservation and management efforts.

PhD project objectives

The main objective of this PhD was to: I) investigate and contribute to the development of new techniques for monitoring species of conservation interest; II) Evaluate the strengths of standard monitoring techniques and the potential of new non-invasive monitoring techniques to improve the current state of the art; III) to assess the weaknesses of these techniques, especially in the case of innovative techniques, so as to propose new directions of study that fill current knowledge gaps as soon as possible. Evaluating the strengths and weaknesses between the two could thus open scenarios that would see the contextualized application of one of the two, or if complementarily useful, the coordinated application of both. The target were mainly animal species of freshwater ecosystems, listed in the annexes II, IV and V of the Habitats Directive 92/43/cee as threatened and in need of rapid monitoring, to be reported by Member States every 6 years, and with them, invasive alien species that may pose a threat to the survival of the former, directly impacting them or the habitats in which they survive.

Target species

The target species studied during the PhD have varied over the three-year period. Since the PhD project was part of a PSR project (Programma di Sviluppo Rurale) funded by the Lazio Region, the initial target list was provided by the Region, whose main interest was focused on developing a protocol to rapidly monitoring rare and elusive freshwater species using eDNA. Although they were all species whose ecology is closely linked to freshwater environments, some were found to be unsuitable.

During the preparatory stages of the protocol, the research team collaborated with experts in the region to assess which species could be worked on. The technique involves the preparation of species-specific primers, exploiting DNA sequences deposited in genetic databases. A preliminary limitation is that for some species, few genetic sequences are deposited in the databases, while other species (especially invertebrates) can be genetically close and thus, they are difficult to distinguish genetically, or may incur hybridisation phenomena in areas of co-presence. For these technical reasons, some species have been removed from the target list. After the preliminary part of the work, aimed at deciding which species were most suitable to work on using environmental DNA monitoring, the final target list consisted of 22 species belonging to 10 taxa (Tab.1).

Below is the list of species that have been studied during the three-year PhD programme and the species that were discarded from the project (Tab. 1-2).

Table 1 – *Target species of the PSR project with indication of the conservation status (Annexes of the Habitat Directive 42/93/CE – II, IV, V), if they are invasive alien species (IAS) or trasfaunated (T), and their conservation status according to IUCN global red list.*

Species	IAS; Annexes	IUCN red list global status
Bivalvia		
Unio elongatulus	V	NT
Dreissena polymorpha	IAS	LC
Gastropoda		
Vertigo spp.	II	//

Malacostraca (Decapoda)		
Austropotamobius pallipes	II, V	EN
Faxonius limosus	IAS	LC
Procambarus clarkii	IAS	LC
Insecta (Odonata)		
Coenagrion castellani	II	VU
Lindenia tetraphylla	II, IV	LC
Oxygastra curtisii	II, IV	LC
Actinopterygii		
Alosa fallax	II, V	LC
Padogobius bonelli	T	LC
Pseudorasbora parva	IAS	LC
Squalius lucumonis	NA	EN
Chytridiomicetes		
Batrachochytrium dendrobatidis	IAS	//
Amphibia (Anura)		
Bombina pachypus	II, IV	EN (*Bombina variegata*)
Lithobates catesbeianus	IAS	LC
Amphibia (Urodela)		
Triturus carnifex	II, IV	VU
Lissotriton italicus	II	LC
Reptilia (Testudines)		
Emys orbicularis	II	NT
Trachemys scripta	IAS	LC
Mammalia (Carnivora)		
Mustela putorius	V	LC
Lutra lutra	II, IV	NT

Table 2 – *Species discarded from the PSR project due to technical issues with indication of the conservation status (Annexes of the Habitat Directive 42/93/CE – II, IV, V), if they are invasive alien species (IAS) or trasfaunated (T).*

Species	IAS(UI); Att. 42/93/CE
Petromyzon marinus	II, IV
Hirudo verbana	V
Eriocheir sinensis	IAS (UI)
Pacifastacus leniusculus	IAS (UI)
Procambarus fallax f. virginalis	IAS (UI)
Barbus barbus	T
Barbus plebejus	T

Protected species

Bivalvia

Unio elongatulus (Pfeiffer, 1825): **Biology**: Can be found in waters with moderate flow, such as rivers and channels with sandy bottoms. Its reproductive strategy involves a parasitic larva attaching to the

external surface of host fish until metamorphosis occurs (Araujo et al., 2015). **Distribution**: Present in Italy and coastal areas of Slovenia and Croatia. The species, according to recent studies, may be allopatric with Unio mancus and distributed along the Adriatic side of the peninsula (Marrone et al., 2019). **Monitoring protocol**: Currently, no standardized monitoring techniques have been proposed for *U. elongatulus*, so guidelines suggest to apply the same methods used for other Unionoidea species, hence, it is suggested to utilize a monitoring approach derived from that proposed by (Young et al., 2001), which involves conducting visual censuses in 50x1m transects and 1x1m squares positioned in habitats characterized by suitable conditions (e.g., clean sand protected by pebbles or rocks). Transects should be traversed against the current, either on foot with a clear-bottomed bucket or by swimming with a mask, while counting all encountered bivalves. Along a 50m transect, a series of five 1m2 squares at 10m intervals should be thoroughly examined by counting bivalves, including those potentially concealed under rocks and pebbles or submerged, and measuring their maximum length. Any remains of deceased bivalves (shells) should be counted separately. It is advisable to conduct three transects per site, each with a series of squares, under normal circumstances.

Gastropoda

Genus Vertigo: **Biology**: *Vertigo* is a genus of terrestrial pulmonate gastropods, represented by small (1-2 mm long) species. Their favored microhabitats include areas adjacent to or within permanent wetlands like swamps and marshes, or along lake shores characterized by calcareous substrates and moisture-loving vegetation. V. angustior is also observed near backdune wetlands and salt marshes. It's classified as an annual species with a lifespan of approximately 18 months. While the breeding season primarily occurs in autumn, juvenile specimens can be encountered throughout the year (Stoch & Genovesi, 2016). **Distribution**: In Italy, two species (V. geyeri and V. genesii) are found exclusively in the Alps, while V. moulinsiana and V. angustior are regularly distributed throughout the peninsula. **Monitoring protocol**: Two protocols exist for sampling and monitoring *Vertigo* species. One method, outlined in (Horsák & Hájek, 2003), entails sampling a surface layer of soil, encompassing litter and herbaceous vegetation, at a rate of approximately 12l of material per 16m2 of uniform area. The gathered material undergoes washing through a sieve to eliminate soil particles. Any plant residues are manually removed, preceded by rinsing to recover any attached individuals. A second approach involves manual collection of individuals within plots or along transects (Moorkens & Killeen, 2011).

Malacostraca (Decapoda)

Austropotamobius pallipes (Lereboullet, 1858): **Biology**: *A. pallipes* is an adaptable crustacean that lives in various aquatic habitats, preferring clean, cool water at altitudes between 350 m and 1500 m. It is mainly active between 15°C and 18°C and feeds on both organic material and small animals. It has crepuscular and nocturnal habits, resting during the day in riparian zones. It breeds in autumn, with females carrying the eggs until the following spring. (Stoch & Genovesi, 2016). **Distribution**: This species is distributed across the western European continent, encompassing the United Kingdom and Ireland. In Italy, it can be found throughout the peninsula and in Sardinia but is notably absent from Sicily. **Monitoring protocol**: For both lake and river ecosystems protocols are documented in the literature (Trizzino et al., 2013). The optimal time for sampling is typically July-August when juveniles become independent and there's minimal risk of disturbing egg-laying females on the water body bed. In lake environments, three sampling methods (outlined by Reynolds et al., 2010) are commonly employed, each executed manually. The first method involves observing and collecting shrimp with

snorkeling gear. The second, suitable for vegetation-rich, silty, and shallow seabeds, entails using a macroinvertebrate net for collection. The third method involves gathering active crayfish from the banks at night. In river environments, however, monitoring entails walking upstream from valley to valley. However, this method requires ongoing surveillance to prevent illegal harvesting, as it exposes the population to such risks.

Insecta (Odonata)

Coenagrion castellani (Galimberti & Assandri 2020): **Biology**: It predominantly inhabits small flowing water bodies, such as springs and streams abundant in aquatic vegetation and well-oxygenated. Larvae typically settle near the roots of aquatic plants, particularly in areas where mud accumulates. Adults tend to remain close to their breeding sites, exhibiting limited movement away from them (Watts et al., 2004). **Distribution**: This species has a broad distribution across Central Western Europe and Spain. It's documented in northwestern and central southern Italy, excluding Abruzzo, Molise, and Sardinia, found from sea level up to altitudes of 850 meters. Recent research by Dijkstra et al. (2023) has confirmed that the Italian populations belong to the species *C. castellani*. **Monitoring protocol**: Monitoring for this species can be conducted in two manners (Hassall & Thompson, 2012). In both approaches, the initial step involves selecting the study area, determined by confirming the species' presence or ensuring the environmental conditions are suitable. The operator delineates a 100m linear transect along the stream bank, where riparian vegetation is not overly dense, and proceeds to walk slowly along it. If the counting method is chosen, the operator records all sighted individuals. Alternatively, if the CMR (Capture-Mark-Recapture) method is preferred, the operator traverses the transect equipped with a rigid circle net, aiming to capture all observed individuals, which are then temporarily housed in a container. Upon reaching the end of the transect, each captured individual is marked with a small spot on the thorax and an alphanumeric code on the left forewing, using an indelible marker with a fine but not excessively fine tip, before being promptly released. The operator records the marked individuals; recaptures may be conducted by reading the alphanumeric codes applied on the wings with binoculars or by physically recapturing individuals if there are doubts about the initial identification.

Lindenia tetraphylla (Vander Linden, 1825): **Biology**: The flying season goes from May to August. It inhabits both natural and man-made lakes of small to medium size, as well as streams in lowland areas. **Distribution**: Italy marks the western boundary of this species' range, which stretches to central Asia and is also documented in Egypt and Libya. In Italy, its presence is recorded in only a handful of locations in Tuscany, Campania, Sardinia, and Latium (Crescia et al., 2020). **Monitoring protocol**: Currently, due to limited ecological information available, there is no dedicated monitoring protocol for this species (Trizzini et al., 2013). In the absence of specific methodologies, periodic (annual) monitoring of known populations is advised. Inspections should occur during periods of adult activity (May- August). These inspections involve conducting a 500m transect along lake shores and known presence sites, tallying the observed individuals. Given the males' home range, this count can also provide an estimate of potential breeding pairs.

Oxygastra curtisii (Dale, 1834): **Biology**: It flies from May to the beginning of August. It frequents the calmest stretches of rivers and small streams, with banks rich in vegetation. It is reported at lowland altitudes and rarely above 500 m above sea level. **Distribution**: This species is widespread in Western Europe and Morocco, while in Italy it is reported in the north-west in the centre (except for Abruzzo and Molise). **Monitoring protocol**: According to the LIFE Manage Project of the Lombardy Region,

monitoring *O.curtisi*i can be conducted using two methods: the adult sighting and the exuviae collection, either simultaneously or separately. When combining both methods, each session can be divided into two phases: the first phase involves practicing the exuviae collection method, followed by the adult sighting method in the second phase. Regardless of the method used, fishing boots or a diving suit can be helpful for inspecting the habitat comfortably, along with a stopwatch for timing. For exuviae collection, the operator identifies a suitable stretch of bank and walks along it slowly for 30 minutes, collecting exuviae and storing them in plastic containers. If collection is interrupted due to obstacles such as steep banks or fallen trees, the stopwatch is paused, the obstacle is overcome, and the search resumes with the stopwatch restarted. Transects of 300 meters in length are selected for adult sighting, located within the area designated for exuviae collection. During each sampling session, the operator walks the entire transect at a very slow pace, counting the sighted *O. curtisii* adults.

Actinopterygii

Alosa fallax (Lacépède, 1803): **Biology**: An anadromous migratory species with gregarious habits, it carries out its trophic phase in the high seas and makes reproductive migrations to spawn in inland waters. Spawning and fertilisation take place collectively in the central hours of the night, with water temperatures above 15 °C. It can swim up rivers for very long distances. **Distribution**: *Alosa fallax* is widespread in European basins on the Mediterranean and Atlantic sides. It is present in most Italian coastal watercourses and in the large rivers Po, Piave, etc. In Sardinia it is present in Lake Omodeo and the middle Flumendosa. **Monitoring protocol**: Electrofishing is used to monitor Alosa populations during their spawning migration, both in wadeable areas and those requiring a boat. This method involves non-lethal sampling, with specimens released at capture sites. The sampling area's length along the river stretch is proportional to the width of the riverbed. Each specimen is counted and measured for total length and weight, and sometimes mini-invasive sampling is conducted for molecular analysis. It's important to note that Alosa species, like cuttlefish and agoni, are highly sensitive to handling, with survival outside water limited to seconds.

Squalius lucumonis (Bianco, 1983): **Biology**: Adapted to swift, clear watercourses with a diverse substrate of rock, sand, and gravel, as well as vegetation-filled tunnels. It avoids lakes, reservoirs, and stagnant water, thriving in the Mediterranean's fluctuating temperatures and flows. During dry seasons, it persists in perennial pools. It exhibits less social behavior, congregating solely for breeding purposes. **Distribution**: *S. lucumonis* is native to the Tuscan-Lazio region. Initially, its habitat encompassed the river basins along the Tyrrhenian coast. Presently, the species exhibits a widespread yet fragmented distribution across the river basins of Tuscany, Umbria, and Lazio. **Monitoring protocol**: Monitoring *S. lucumonis* populations can be accomplished using electric fishing techniques, which employ an electrostunner. The sampling protocol entails capturing specimens (progressing from downstream to upstream) within a section of the watercourse proportional to the width of the wetted riverbed. Captured specimens are then kept alive to minimize handling stress by administering light sedation during recognition, counting, biometric parameter detection, scale sampling, and photographic phases. Subsequently, the specimens are placed in tanks or small pens submerged in the water for an 'awakening' phase before being released back into the same capture sites following light disinfection.

Amphibia (Urodela)

Lissotriton italicus (Peracca, 1898): **Biology**: It is a frequent visitor to still water bodies of anthropogenic origin, such as cattle troughs and fountains. Among natural environments it colonises puddles and ponds, even those subject to complete desiccation in the summer season. **Distribution**: *Lissotriton italicus* is an Italian endemism, widespread from central southern Italy to Calabria, which represent the southern limit of the areal. It lives from sea level up to 1855 m altitude, with higher densities in the 700 to 1000 m range. **Monitoring protocol**: The monitoring technique for this species involves counting. Counts can be conducted at a suitable number of sampling sites within each biogeographical region. Sampling stations will be designated in as many 10x10km cells where this newt species is documented, with allocation across regions based on the species' distribution. Within individual SCIs/SACs (Sites of Community importance/ Special Areas of Conservation), it is necessary to verify breeding occurrences at all breeding sites if fewer than 5 are identified, at a minimum of 6 breeding sites if between 5 and 9 are known, and at least half plus one if there are 10 or more breeding habitats.

Triturus carnifex (Laurenti, 1768): **Biology**: Adults breeds in aquatic environments (still, permanent and temporary waters) but during the post-reproductive period, they live in a wide variety of terrestrial habitats, from deciduous forests to xeric environments to modified environments. Some individuals may remain in water throughout the year. **Distribution:** *T. carnifex* is present in continental and peninsular Italy, but it is absent in western Liguria, Trentino-Alto Adige and Puglia. On the western Alos it is almost absent, with the exception of very few populations in the main valleys. **Monitoring protocol**: Standardized repeated counts will be conducted nationally at an appropriate number of sample sites per biogeographical region, selected within as many 10x10km cells where the species is documented. Within individual SCIs/SACs, it is essential to confirm breeding occurrences at all breeding sites if fewer than 5 are identified. If there are 5 to 9 known sites, verification is required at a minimum of 6 sites. For 10 or more known sites, validation is needed at half plus one. National range assessment will utilize models based on detecting the number of locations within the 10x10km national grid. Each survey year will consider the number of reports per cell and the total number of cells with reports. The total reports of all amphibian species in these cells will serve as a measure of sampling effort. Species frequency will then be analysed using hierarchical models.

Bombina pachypus (Bonaparte, 1838): **Biology**: It is found in hilly and mountainous environments. It frequents waters of modest size, such as temporary pools, dead or stagnant loops of rivers and streams, sunny and shallow in forests and open areas. Larval development occurs in puddles. It is also present in modified habitats including areas of non-intensive agriculture, pastures, irrigation canals. **Distribution**: The Apennine yellow-bellied toad is an endemic species of peninsular Italy, where it occurs in the Apennines. It is not present on the islands. It prefers altitudes between 300 and 700 m in central-northern Italy and 600-1000 in central-southern Italy. It can also exceed 1900m **Monitoring protocol**: For *B. pachypus*, a species with insufficient reports, confirmation of presence in the 10x10 km cells will be utilized. Monitoring will primarily involve estimating population trends through repeated counts at sample locations. Within individual SCIs/SACs, separate from national monitoring sites, it is necessary to confirm breeding occurrences for all known breeding sites if they total 10 or fewer, for 10 sites if there are 11-19, and for 50% of known sites if there are 20 or more. Each year, the number of reports per cell and the total number of cells with reports will be considered. The total reports of all amphibian species in these cells will serve as an indicator of sampling effort.

Reptilia (Testudines)

Emys orbicularis (Linnaeus, 1758): **Biology**: It is mainly found in two types of wetland habitats: ponds, puddles, swamps, or artificial canals and small uncultivated areas between rice fields. In northern Italy it is found almost exclusively in the plains, while in central and southern Italy it can also be found in the hills and mountains. It is a long-lived species and sexual maturity occurs at around 7-11 years. **Distribution**: It occupies all the Italian peninsula (except Alps) and the major islands, from sea level up to over 1500 m above sea level. **Monitoring protocol**: At sites with water body access, population estimates will use the (CMR) method, which requires ministerial authorization. Alternatively, in sites with challenging water body access but good bank visibility, repeated counts can occur in known areas or along transects. Sampling stations will be established within appropriate 10×10km cells where the species is recorded. For monitoring within individual SCIs/SACs, population studies via capture-mark-recapture or repeated counts are suggested. All selected sites will be documented and transects, or observation/capture points mapped for standardized repetitions over time.

Mammalia (Carnivora)

Mustela putorius (Linnaeus, 1758): **Biology**: *M. putorius* prefers damp environments like banks of rivers, but it can live in very different environments, from wetlands to forested mountain areas and agricultural areas, to man-made environments, where it sometimes uses human dwellings as daytime refuges. However, it needs a dense vegetation cover to hunt and for daytime rest. **Distribution**: In Italy, *M. putorius* is present, discontinuously, throughout the peninsula, from sea level up to about 1300 m in the Alps, and absent in the islands. **Monitoring protocol**: Photo trapping offers a promising approach for monitoring large-area polecat populations. However, its effectiveness may be limited due to factors such as the polecat's typically low densities and their preference for riparian habitats and dense vegetation cover (Fusillo & Marcelli, 2014). To enhance photo-trapping success, sampling sites should be selected from a map of the study area's hydrographic network, ensuring adequate representation of riparian habitats for sufficient photographic surveys. Field characteristics, particularly the proximity of shrub cover, should also be considered during site selection. The use of decoys can significantly increase polecat detectability in photographs. Photo traps should be positioned no more than 30cm above the ground to capture animals at an optimal distance.

Lutra lutra (Linnaeus, 1758): **Biology**: Closely linked to the aquatic environment, it lives mainly near rivers, streams and mountain lakes up to 1500 m above sea level. It uses coastal areas sporadically. The frequented environments must be characterized by a good availability of trophic resources (especially fish, but also crustaceans and amphibians) and by abundant riparian vegetation that provides diurnal shelter. It also uses other animals dens as nocturnal shelter. **Distribution**: In Italy the population is confined to the central-southern area, although it is expanding. It has recently reappeared in Trentino and Friuli thanks to the expansion of the Austrian and Slovenian populations. **Monitoring protocol**: Otter populations across Europe are systematically monitored using the standard methodology recommended by the IUCN Otter Specialist Group (Reuther et al., 2000). This approach involves searching for signs of otter presence, such as tracks and droppings, along 600m of shoreline at four randomly selected sites within each 10x10km national grid cell. At each site, both positive and negative results are recorded, along with various environmental parameters useful for site characterization. Monitoring activities require experienced operators capable of accurately identifying otter signs, especially droppings (spraint). Typically, monitoring of riverbanks and emergent features in the riverbed is conducted on foot, although in certain situations, it may be carried out using canoes or other boats.

Invasive species

Bivalvia

Dreissena polymorpha (Pallas, 1771): **Biology**: It lives in lakes, artificial reservoirs, large rivers and brackish waters. Requires substrates rocks or macrophytes on which to attach. **Distribution**: Originally from the Black Sea, Caspian Sea, and Aral Sea, the zebra mussel is believed to have entered Europe via river navigation channels towards the late 19th century. Subsequently, it was intentionally introduced into various European nations to filter water and address issues of eutrophication. **Monitoring protocol**: Early detection of zebra mussels, especially in isolated settings from natural ecosystems, can enhance the success and cost-effectiveness of removal operations. Consequently, there is an urgent need to develop methods for early invasive species detection (Thomsen & Willerslev, 2015). Traditional capture-based methods for visual inspection and identification of specimens have limitations, particularly in correctly identifying closely related species (Xiong et al., 2016). Additionally, conventional sampling protocols often have low detection probabilities unless population densities are already high (Harvey et al., 2009). Environmental DNA (eDNA) analysis offers a promising alternative for species identification, with greater sensitivity compared to traditional methods (Dejean et al., 2012; Jerde et al., 2011; Laramie et al., 2015; Takahara et al., 2015). These molecular techniques provide more accurate species distribution data and can differentiate between various invasive mussel species (Carvalho et al., 2021). Consequently, eDNA methods have the potential to significantly enhance early detection and monitoring capabilities (Darling & Mahon, 2011).

Malacostraca (Decapoda)

Procambarus clarkii (Girard, 1852): **Biology**: Suitable for living in a wide variety of aquatic habitats. It's an omnivorous species diet includes eggs of fish, amphibians, and aquatic insects, but also algae and plants aquatic. **Distribution**: The Louisiana crayfish is native to the southern United States. Due to its remarkable adaptability and highly valued meat for dining purposes, it has been extensively introduced in numerous countries, becoming the most widespread freshwater crayfish globally. In Italy, it was introduced in Tuscany and is now present mostly along all the peninsula, with highest concentrations in central Italy (Lazio, Marche, Tuscany, and Umbria). It's also found in Sardinia and Sicily. **Monitoring protocol**: *Procambarus clarkii* presence leads to significant destruction of macrophytes and heavy predation on insects and mollusks, resulting in reduced resources for native species and substantial ecosystem alterations. Additionally, it can transmit lethal diseases such as crayfish plague and chytridiomycosis to European native crayfish and amphibians, respectively. Loureiro et al., (2018) proposes a standard monitoring approach through traps deployment underwater, baited with dry fish-flavored cat food (Bueno et al., 2007), and checked the following morning for crayfish presence. Traps were distributed along the pond edges and in the middle, with baits replaced daily and used baits properly discarded outside the study area.

Faxonius limosus (Rafinesque, 1817): **Biology**: The species thrives in calm, shallow, and muddy waters, displaying resilience to high levels of both organic and inorganic pollution, as well as to elevated temperatures and low oxygen levels. It can reproduce successfully in environments with salinity levels of up to 7%. Its diet consists primarily of a variety of organisms, including both plant material and debris. **Distribution**: Native to the eastern United States. In Italy the species was introduced accidentally through batches of fish coming from from Poland. It is reported in northern and central Italy and in Sardinia. **Monitoring protocol**: This species of crayfish can destabilize riverbanks, and modify other

habitats, due to its burrowing behavior, aggressively interact and compete for shelters, impact on the fish and amphibian communities because they can feed on eggs (Nystrom, 2002). Active diurnal searches by turning stones and checking holes and trapping can prove the presence of the species. The occasional findings of dead crayfish on the banks can be also taken into consideration (Puky et al., 2005; Puky & Schád, 2006). Besides crayfish-oriented surveys, valuable observations or collections are to be made. Captures by hand, netting, diving and dredging and also by electro fishing during ichthyological surveys are possibilities (Nesemann et al.,1995(Puky & Schád, 2006).

Actinopterygii

Pseudorasbora parva (Temminck & Schlegel, 1846): **Biology**: It lives in shallow waters with little current, sandy bottoms or rich in vegetation. It feeds on insect larvae, small fish and fish eggs. **Distribution**: Species native to Asia and introduced into many European countries for sport fishing. **Monitoring protocol**: The species has been classified as highly impactive by the UK Technical Advisory Group on the WFD (Water Framework Directive) (Panov et al., 2009) due to its rapid reproductive rate, and competition for resources with native fish (Britton et al., 2017). In addition to direct competition, *P. parva* is also a vector for several fish pathogens (Robinson et al., 2019). Electrofishing, fish-trapping and throw- netting seem to be the most convenient sampling methods to detect small invasive fish like *P. parva* in ponds for fishery research, nature conservation and pond management (Kajgrová et al., 2023).

Padogobius bonelli (Bonaparte 1846): **Biology**: Species widespread in lakes, rivers, streams, with rocky or mixed sand and gravel substrates, and with clear and well-oxygenated waters. The typical habitat consists of the stretch middle of the rivers, in the stretches with shallow waters and moderate currents. It is active mainly in the twilight and night hours for feeding. **Distribution**: *P. bonelli* is a subendemic species found mainly in the Po Valley region, where it is distributed widely across the Po Basin, as well as in Veneto and Friuli-Venezia Giulia. In central Italy, certain populations have been introduced through transfaunation, likely originating from accidentally introduced individuals along with Cyprinids of commercial interest approximately 20 years ago. **Monitoring protocol**: *P. bonelli* invaded new ecosystems thanks due to its aggressive behaviour, its broad environmental tolerance (Zerunian, 2002), and a high reproductive energy allocation (Pompei et al., 2016). One significant negative impact attributed its invasions is competition with native fishes, particularly for species with similar ecological requirements (Grabowska et al., 2016). Sampling for the species can be conducted via electrofishing using the removal method during the low flow period (June–September).

Chytridiomicetes

Batrachochytrium dendrobatidis (Longcore, Pessier & D.K. Nichols 1999): **Biology and distribution**: Mycete with cosmopolitan distribution transmitted by a susceptible flagellated zoospore to drying and heat. **Monitoring protocol**: *B. dendrobatidis* is currently considered to be the only pathogen consistently identified as a key factor in loss of amphibian biodiversity and abundance (Baláž et al., 2014; Bosch et al., 2006; Lips et al., 2006; Vredenburg et al., 2010). Identifying species that are susceptible to both infection and mortality attributable to chytridiomycosis (Bielby et al., 2009; Bosch & Martínez-Solano, 2006; Havlíková et al., 2015; Sztatecsny & Glaser, 2011) is of the highest importance for the preservation of amphibians.

Amphibia (Urodela)

Lithobates catesbeianus (Shaw, 1802): **Biology**: This species thrives in diverse freshwater habitats, favouring permanent aquatic environments abundant in vegetation and deep water. During humid conditions, it can venture over 1 km away from water sources. Reproduction occurs in bodies of water that remain unfrozen for at least two consecutive years. Females lay up to 20,000 eggs per mating, depositing them either freely on the water's surface or attaching them to vegetation. Tadpole growth is influenced by water temperature and nutrient availability. **Distribution**: The bullfrog was introduced to Europe from North America. The largest European population is present in the Po Valley, the result of repeated introductions, it was introduced in the central-south at the end of 1900. **Monitoring protocol**: The American bullfrog adults are noted as important predators and competitors for food resources (Laufer et al., 2021), while tadpoles also act as predators and disrupt habitats through bioturbation (Gobel et al., 2018; Ruibal & Laufer, 2012). *L. catesbeianus* has been identified as a vector and reservoir of global amphibian diseases, particularly Ranavirus and Chytridiomycosis (Garner et al., 2006; Ribeiro et al., 2019; Schloegel et al., 2012). Large-sized bullfrog adults are suggested to behave as important predators of amphibians, while juveniles can also be strong competitors (Bissattini et al., 2018, 2019; Veronez et al., 2016). Sampling can be conducted using two methods: fishing for tadpoles with hand nets and trawls and detecting post-metamorphic individuals through sightings and/or listening for adult nocturnal vocalizations during the breeding season. Net sampling involves sweeps covering approximately 7–10m per body of water, while adult samplings consist of specialized surveyors conducting slow-paced walks around the perimeter of water bodies on consecutive nights. This method is particularly effective for highly conspicuous species like the bullfrog (Chang et al., 2022; Laufer et al., 2018).

Reptilia (Testudines)

Trachemys scripta (Thunberg and Schoepff, 1792): **Biology**: It inhabits lakes, ponds, and sluggish, muddy rivers rich in aquatic vegetation. During summer, when water pools dry up, they either burrow holes in the mud or seek refuge in wooded areas or tall grass. **Distribution**: Originally from the Mississippi basin and introduced to Italy in the 1970s, they are currently naturalized populations present in many regions. **Monitoring protocol**: *Trachemys scripta* poses a significant threat to aquatic ecosystems by preying on various animal species and consuming aquatic vegetation (Prévot-Julliard et al., 2007). It exhibits higher aggression in direct competition for food compared to native species, leading to ecological disruptions (Lindeman, 1999). Additionally, its release into the environment can transmit pathogens to other species, further endangering ecosystem health (Demkowska-Kutrzepa et al., 2018; Martínez-Silvestre et al., 2015). Finally, competition over basking places may occur and can have a strong impact just after wintering when water temperature is low and the need for heat high. Underwater pursuits between Trachemys males and Emys females can be observed. This behaviour suggests a kind of interference competition (Cadi & Joly, 2004). The Visual Encounter Survey (VES) is the simplest technique for confirming species presence, involving direct observation and counting of individuals along transects or in known areas. This method is particularly useful in locations with limited access to water bodies but good visibility of the banks. Following the protocol outlined for *E. orbicularis* and *E. trinacris* by (Lo Valvo et al., 2016), which is also applicable for *T. scripta*, sampling stations should be identified within appropriate 10×10km cells where the species presence is confirmed.

Structure of the thesis

This Ph.D. thesis was developed working on two main monitoring techniques, explored in three chapters, as presented in the following workflow.

Environmental DNA (Chapter I): Environmental DNA (eDNA) emerges as an effective method for species detection, especially for species challenging to detect using traditional methods (Pawlowski et al., 2020). Standardized methods, such as metabarcoding and high-throughput sequencing, enable the detection of multiple species from environmental samples, overcoming the limitations of traditional monitoring approaches Ficetola et al., 2019; Pont et al., 2023). In Italy, characterized by high biodiversity, both native endangered species and invasive alien species (IAS) are present, posing significant conservation challenges (Roy et al., 2023). The Central Italy region, particularly the Latium region, represents a heterogeneous environmental context with high landscape diversity and a rich biodiversity of wildlife and plants (Balletto et al., 2010). Endemic endangered species, such as the Italian crayfish *Austropotamobius italicus* and the Apennine yellow-bellied toad *Bombina pachypus,* are found in this region (Canestrelli et al., 2006, 2013; Secchi et al., 2022) Additionally, Central Italy serves as a hybrid zone for various species of insects and amphibians, further contributing to its biodiversity (Iannella et al., 2017). However, the region is also susceptible to biological invasions, with high incidences of IAS in freshwater ecosystems (Roy et al., 2023). The presence of invasive species, including fish, crustaceans, and molluscs, poses a significant threat to native biodiversity (Gherardi et al., 2008; Lorenzoni et al., 2018). In this study, we aimed to design accurate and sensitive eDNA assays for 22 native endangered and invasive alien freshwater species found in Central Italy's freshwater bodies. The study area, encompassing the Latium region, provides a representative environmental context for assessing the distribution of these species. The selected target species include 14 endangered species listed in the Habitat Directive 92/43/EC and 8 invasive species representing a direct threat to native biodiversity. The development and validation of species- specific eDNA assays in this study area are essential for robust molecular analyses and effective conservation management (Nicholson et al., 2020; Sepulveda et al., 2020). **Environmental DNA (Chapter II)**: In the second chapter we focused on *Bombina pachypus*, a species endemic to the Italian Apennines, who faces severe decline and fragmentation of its habitat, with populations often consisting of only a few individuals (Caputo et al., 2004; Romano et al., 2012; Spilinga et al., 2010). Despite its status as an Endangered species according to the IUCN Red List and protection under various conservation directives, such as the Bern Convention and the European Union Habitats Directive, *B. pachypus* continues to experience population declines due to threats like habitat drying, water pollution, and stochastic factors affecting small populations (Sindaco et al., 2006; Griffiths, 1997). The Environmental DNA (eDNA) analysis presents a promising solution for monitoring *B. pachypus* and its habitat. This non-invasive technique, capable of detecting DNA sequences from environmental samples like water, enables the detection of cryptic, rare, and elusive species where traditional survey techniques often fail (Hobbs et al., 2019). Furthermore, eDNA analysis allows for the simultaneous detection of multiple species from the same sample, streamlining surveys without requiring many specialized surveyors (Thomsen & Willerslev, 2015). While eDNA has been successfully applied to detect various amphibian species in European freshwaters, including *Rana temporaria* and *Triturus cristatus*, the genus *Bombina* has not yet been investigated using this technique, nor has the co-occurrence of the pathogenic fungus *Batracochytridium dendrobatidis* (*B. dendrobatidis*) been explored. The aim of our project is threefold: a) to develop specific primers for eDNA detection of *B. pachypus*, b) to detect *B. pachypus* and *B. dendrobatidis* in 20 freshwater bodies of Central Italy from eDNA water samples, and c) to evaluate

small- and large-scale factors that could affect the detection of *B. pachypus* through eDNA. By leveraging the efficiency and sensitivity of eDNA analysis, we aim to provide valuable insights into the distribution and conservation status of *B. pachypus* and its habitat. **Survey standard (Chapter III):** In the third chapter we report our work on Eurasian otter trophic niche to report the relevance of survey standard monitoring and conservation strategies for a (formerly) endangered top predator of riverine ecosystems. In fact, in the past century, the Eurasian otter (*Lutra lutra*) in Europe faced severe declines due to human persecution, habitat degradation, and pollution (Loy et al., 2022). However, conservation efforts, including law enforcement and pollutant bans, have led to the species' recovery in most European countries, including Italy, where it has recently been downgraded from Endangered to Vulnerable on the national red list (Leoncini et al., 2023). Despite this progress, the otter's range remains limited to the south-central portion of the peninsula, with a population highly isolated from other European populations (Giovacchini et al., 2018; Mirone et al., in press, p. *Hystrix*). The recovery of the Eurasian otter in Italy has been hindered by threats such as habitat degradation, pollution, and human persecution (Loy et al., 2022). Additionally, the genetic uniqueness of the Italian otter population underscores the need for specific conservation efforts (Mucci et al., 2010; Randi et al., 2003). Standard survey techniques are essential for monitoring the Eurasian otter population in Italy to assess population trends, habitat use, and the species' ecological requirements (Freitas et al., 2007). Understanding the otter's trophic niche is crucial for effective conservation and management strategies (Loy et al., 2023). Otters are opportunistic predators, primarily feeding on fish but also consuming amphibians, crustaceans, small mammals, reptiles, birds, and invertebrates (Kruuk, 2006; Roos et al., 2015; Panzacchi et al., 2011). However, variations in diet composition along river gradients and among neighbouring rivers have not been thoroughly analysed. To address this gap, our project aims to analyse the fine-scale spatial pattern of the trophic niche of otters along three neighbouring rivers in Southern Italy by examining prey remains in otter scats (spraints). We hypothesize that prey remains in otter scats will change along each river gradient and reflect the local composition of prey. Additionally, we expect that prey composition in scats will vary more along the river gradient than among the same sectors of different rivers (Huet, 1949; Marconato et al., 2002; Kruuk, 2006; Loy et al., s.d. 2009). This information will provide valuable insights into the resource exploitation pattern by otters and aid in the conservation of endangered native prey species (IUCN, 2023).

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CHAPTER 1

Multiple detection of endangered and alien invasive species in freshwater bodies of Central Italy using environmental DNA

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Abstract

Environmental DNA (eDNA) is a novel and effective technique involving the collection of environmental samples containing living cells of the target species that is transforming biodiversity monitoring worldwide, especially of freshwater and marine communities. The use of eDNA single species approach based on qPCR is rarely used for the simultaneous detection of different species. However, eDNA single species approach has proven to be a sensible tool, making it particularly useful for detecting rare and elusive species, such as endangered species or invasive species during the initial stages of colonization. Italy hosts the highest faunal biodiversity in Europe, with several freshwater species both endemic and endangered. We aimed at i) design and test a protocol to simultaneously detect through eDNA a set of both endangered and invasive alien species occurring in freshwater bodies of Central Italy; ii) designing and validate new species-specific eDNA primers and probes for those species lacking this information; iii) identify small- and large-scale environmental factors affecting species and eDNA detection. We selected 22 species belonging to Fungi, Mollusca, Arthropoda, and Chordata. Specifically, 14 out of 22 were endangered species listed in the Habitat Directive 92/43/EEC, and eight alien invasive species representing a direct threat to the first ones. New primers and probes derived from mitochondrial DNA successfully passed the three validation steps for 12 species, and for the remaining species we used already published eDNA assays (validating them accordingly). By carrying out the work to develop species-specific primers, a considerable amount of information was gathered on which taxa are best suited to be monitored using this technique. Environmental DNA allowed to detect 18 over the 22 target taxa, and Detection Rate were calculated to evaluate which taxa responded better. We also performed PCA analysis to better understand which environmental parameters can influence detection of the species in the sampled sites and which parameters influence eDNA detection. Our study demonstrates the efficacy of eDNA techniques in simultaneously detecting multiple species, offering potential for large-scale monitoring of freshwater wildlife. These methods can aid in invasive species eradication efforts and improve the effectiveness of protected areas. Future research should focus on refining methods for species with low detectability and exploring seasonal variability and sampling designs. Additionally, species distribution modelling holds promise for optimizing sampling strategies and identifying potential occurrences.

Introduction

Freshwater ecosystems are threatened globally due to various anthropogenic stressors (Sanders et al., 2024). Consequently, many freshwater species are experiencing substantial declines worldwide, mainly due to habitat loss and degradation, pollution, and introduction of invasive alien species (IAS) (Costa et al., 2021; Jaureguiberry et al., 2024; Dextrase & Mandrak, 2006; Francis, 2012). A sound knowledge and monitoring of the conservation status of endangered species and the rapid detection of invasive species are crucial for the proper management of freshwater ecosystems (Meira et al., 2019; Simberloff et al., 2013). Specifically, monitoring is mandatory for endangered species included in Annexes II, IV, and V of the European Union Council Directive 92/43/EEC also known as Habitat Directive, and for alien invasive species of Union concern included in the EU Regulation 1143/2014 (Pawlowski et al., 2021). Freshwater bodies of Central Italy host 23 species included in Annexes of the Habitat Directive, and 39 IAS (Ercole et al., 2021). Standardized methods have been proposed to meet monitoring commitments required by EU Directives, including also the Water Framework Directive 2000/60/EEC (Leese et al., 2018, Stoch & Genovesi, 2016). However, detecting multiple species through traditional methods is time-consuming and resource-intensive, often hindering monitoring programs of most of them, especially in the case of rare and elusive species.

Environmental DNA (eDNA) is a novel and effective technique involving the collection of environmental samples containing living cells of the target species that is transforming biodiversity monitoring worldwide, especially of freshwater and marine communities (Taberlet et al., 2018). Usually, environmental DNA is processed through metabarcoding multispecies assessment (Ficetola et al., 2008) or high-throughput sequencing for studying a whole community (Pont et al., 2023; Ushio et al., 2018). The use of eDNA single species approach based on qPCR is rarely used for the simultaneous detection of different species. However, eDNA single species approach has proven to be more sensitive than metabarcoding, making it particularly useful for detecting rare and elusive species, such as endangered species or invasive species during the initial stages of colonization (Harper et al., 2018; Thomas et al., 2020; Xia et al., 2021). But scientific experiences involving several species are still few and limited to single taxa. For example, qPCR techniques have been adopted to assess fish communities (Pont et al., 2023; Wozney & Wilson, 2017), to infer fish biomass of commercial fish (Knudsen et al., 2019), to study predator-prey relationships between a fish and its prey (Minegishi et al., 2023), to assess marine invasive tunicates and bryozoans responsible of biofouling (Leblanc et al., 2020), or other studies involving two or three species (Seymour et al., 2018; Tsuji et al., 2018).

One of the main constrain in the expansion of single species approach through eDNA monitoring is the need of well-validated assays. As eDNA is still a novelty in biodiversity monitoring, many species lack properly designed species-specific primers. Furthermore, efficient assays must undergo to a proper and long process of validation in the study area (Goldberg et al., 2016). For this reason, many qPCR assays are currently not reliable for monitoring applications (Nicholson et al., 2020; Sepulveda et al., 2020; Thalinger et al., 2021).

Italy hosts the highest faunal biodiversity in Europe (ISPRA, 2009), with several freshwater species both endemic and endangered (Balletto et al., 2010). Among these, the Italian crayfish *Austropotamobius italicus*, the Apennine yellow-bellied toad *Bombina pachypus*, and the Tuscan stream chub *Squalius lucumonis* (Brusconi et al., 2008; Canestrelli et al., 2014; (Rondinini et al., 2022; Tancioni et al., 2013). Species diversity is particularly high in Central Italy due to its biogeographic location at the boundary of

north-central and south-central species ranges (Iannella et al., 2017; Mattoccia et al., 2011; Solano et al., 2018). On the other hand, Italy hosts among the highest number of IAS in its freshwater ecosystems, including fish, crustaceans, and molluscs (IPBES, 2023), and the number of non-indigenous fish species has surpassed that of native fish in Italian rivers and lakes (Lorenzoni et al., 2019; Nocita et al., 2017; Gherardi et al., 2008; Magliozzi et al., 2020). Considering EU reporting obligations under Art. 17 of Habitat Directive and the commitment to manage established invasive alien species and decrease the number of Red List species they threaten by 50% by 2030 (https://environment.ec.europa.eu/topics/nature-and-biodiversity/invasive-alien-species_en), it is urgent to adopt rapid and sensitive monitoring protocols to optimize monitoring of both rare and endangered species and for early warning for IAS in Italian freshwaters (Leese et al., 2021; Pawlowski et al., 2021).

Accordingly, we aimed at i) design and test a protocol to simultaneously detect through eDNA a set of both endangered and invasive alien species they threaten occurring in freshwater bodies of Central Italy; ii) designing and validate new species-specific eDNA primers and probes for those species lacking this information; iii) identify small and large scale environmental factors affecting species and eDNA detection.

Material and Methods

Study area. We sampled 53 sites in eight main river catchments in the Latium region in west central Italy, including Tiber, the main river basin of Central Italy (river length 405 km), Liri (158 km), and six small catchments flowing close to the coast, i.e. Fiora, Marta, Mignone, Arrone, Astura, Amaseno (Fig. 1, Suppl. Mat. S1). The area is characterized by a high environmental heterogeneity, ranging from the seacoast and lowlands (19.9%) to hills of volcanic plateaus and piedmont zones (54%) and mountains (26.1%). Large volcanic lakes are located in the central and northern part of the region, whereas natural coastal lakes are found in the southern part. Artificial lakes are located along the rivers Velino, Turano, and Liri. The Italian capital city, Rome, is located at the center of the region, and likely acts as a source of several invasive alien species (Spear et al., 2013)

Target species. We selected 22 species of interest living in freshwater bodies of Central Italy, belonging to Fungi, Mollusca, Arthropoda, and Chordata (Tab. 1). Specifically, 14 out of 22 were endangered species listed in the Habitat Directive 92/43/EEC for which is mandatory to report on distribution and conservation status every six years (*Unio elongatulus, Vertigo spp, Coenagrion castellani, Oxygastra curtisii, Lindenia tetraphylla, Austropotamobius italicus, Alosa fallax, Squalius lucumonis, Bombina pachypus, Triturus carnifex, Lissotriton italicus, Emys orbicularis, Mustela putorius, Lutra lutra*), and eight alien invasive species representing a direct threat to the first ones (*Dreissena polymorpha, Faxonius limosus, Procambarus clarkii, Pseudorasbora parva, Padogobius bonelli, Lithobates catesbeianus, Batrachochytrium dendrobatidis, Trachemys scripta*). Six over the eight alien species are of Union Concern under the Regulation 1143/2014, and three are included in the Black List of the IUCN 100 of the World's Worst Invasive Alien Species (Lowe et al., 2000) (Tab.1). Although not listed in any regulation, the fungus *B. dendrobatis* was included as it represents a main threat to amphibians worldwide (Olson et al., 2013), and its detection through qPCR eDNA was not yet tested in Italy. The fish *Padogobius bonelli*, is naturally endemic to northern Italy, has been introduced to several rivers in central Italy outside of its native range in northern Italy, and it is threatening the native P. nigricans (Bianco & Ketmaier, 2001; Pompei et al., 2016)

Table 1. *Details of target species. HD: Habitat Directive 43/92/ECC; UC IAS: Invasive Alien Species of Union Concern (s. EU regulation 1143/2014; 100 WWS: 100 of the World's Worst Invasive Alien Species (Lowe et al 2000). Species for which new primers and probes were designed in this study are reported with **

Phylum	Clade	Species	Synonym	Species ID	Legislation	IUCN national Red List/100 WWS
Fungi	Chytridiomycota	*Batrachochytrium dendrobatidis*	-	BD	-	IUCN 100 WW IAS
Mollusca	Bivalva	*Unio elongatulus **	*U. elongatulus*	UE	HD, Ann. V	EN
Mollusca	Gasteropoda	*Vertigo spp. **	*V. angustior V. moulinsiana*	VE	HD, Ann. II	NT-VU
Mollusca	Bivalva	*Dreissena polymorpha*	-	DP	-	IUCN 100 WW IAS
Arthropoda	Insecta	*Coenagrion castellani **	*C. mercuriale*	CM	HD, Ann. II	NT
Arthropoda	Insecta	*Oxygastra curtisii **	-	OC	HD, Ann. II, IV	NT
Arthropoda	Insecta	*Lindenia tetraphylla **	-	LT	HD, Ann. II, IV	VU
Arthropoda	Crustacea	*Austropotamobius italicus*	*A. pallipes*	AI	HD, Ann. II, IV	EN
Arthropoda	Crustacea	*Faxonius limosus*	*Orconectes limosus*	FL	UC IAS (2016)	-
Arthropoda	Crustaceans	*Procambarus clarkii*	-	PC	UC IAS (2016)	-
Chordata	Osteichthyes	*Alosa fallax*	-	AF	HD, Ann. II, IV	EN
Chordata	Osteichthyes	*Squalius lucumonis **	-	SL	HD, Ann. II	CR
Chordata	Osteichthyes	*Pseudorasbora parva*	-	PP	UC IAS (2016)	-
Chordata	Osteichthyes	*Padogobius bonelli **	*P. martensii*	PM	introduced outside its native range	-
Chordata	Amphibia	*Bombina pachypus **	-	BP	HD, Ann. II, IV	EN
Chordata	Amphibia	*Triturus carnifex **	-	TC	HD, Ann. II, IV	NT
Chordata	Amphibia	*Lissotriton italicus **	-	LI	HD, Ann. IV	LC
Chordata	Amphibia	*Lithobates catesbeianus*	-	LC	UC IAS (2016)	IUCN 100 WW IAS
Chordata	Chelona	*Emys orbicularis **	-	EO	HD, Ann. II	EN
Chordata	Chelona	*Trachemys scripta*	-	TS	UC IAS (2016)	IUCN 100 WW IAS
Chordata	Mammalia	*Mustela putorius **	-	MP	HD, Ann. V	LC
Chordata	MammaliaI	*Lutra lutra*	-	LL	HD, Ann. II, IV	VU

Assay design. We first searched in literature for already published primers and probes used in single species approach and qPCR. Published primers were found for 10 species, i.e. the pathogen *Batrachochytrium dendrobatidis*, the mollusc *Dreissena polymorpha*, the crayfish *Austrapotamobius pallipes, Faxonius limosus* and *Procambarus clarkii*, the fish *Alosa fallax* and *Pseudorasbora parva*, the amphibian *Lithobates catesbeianus*, the terrapin *Trachemys scripta*, and the semiaquatic mammal *Lutra lutra* (Suppl. Mat. S2). We designed new species-specific primers and probes for the remaining 12 species, i.e. the molluscs *Unio elongatulus* and *Vertigo spp*., the damselflies *Coenagrion castellani, Lindenia tetraphylla,* and *Oxygastra curtisii*, the fish *Padogobius bonelli* and *Squalius lucumonis*, the amphibians *Bombina pachypus*, *Triturus carnifex*, and *Lissotriton italicus*, the pond terrapin *Emys orbicularis*, and the riparian mammal *Mustela putorius*. Probes were designed following the protocol by Thalinger et al. (2021). All sequences available as genetic marker (i.e. COI, CytB, 16S; REF THOMSEN ET AL 2012) of the target and closely related species were searched in BOLD Systems and GenBank NCBI (https://www.ncbi.nlm.nih.gov/) and compared using BioEdit (Hall, 1999). Target and non-target sequences were merged in the same file and aligned using MAFFT (Multiple alignment program for amino acid or nucleotide sequences (Katoh et al., 2009). Using BioEdit we manually selected and designed primers with the following molecular characteristics: i) around 60% GC content, ii) length between 18-24 bp, iii) melting temperature between 58-68 °C and within 2 °C of each other. Probes were selected with a length between 20-25 bp and a melting temperature of 4-6 °C higher than the primers. Total length of the amplicon was about 150 bp (Jo, 2023). All primers and probes were evaluated for possible effects of intraspecific genetic variability of Italian populations and for detection of false

positive due to co-amplification of phylogenetically close non-target species (e.g. Galimberti et al., 2021).

Assay validation. Candidate sequences underwent three validation steps: i) in gene bank (in situ); ii) on tissues samples (in vitro), and iii) on two environmental samples from ascertained presence and absence of the target species (in situ) (e.g. Langlois et al., 2021). For validation in situ, molecular parameters of primers and probes were evaluated using Oligo Analysis (http://www.operon.com/tools/oligo-analysis-tool.aspx), also checking for hairpin or self-annealing could impede correct amplifications (Kibbe, 2007). The final primer couple was compared with all sequences available in NCBI using Primer BLAST (Altschul et al., 1997) to identify sequences of non-target species most likely to be amplified showing. We excluded any overlap with non-target species in the study area for less than 3 bp mismatches (Klymus et al., 2020). Candidate primers were obtained from Eurofins Genomics srl (Hamburg, Germany) and validated in vitro on tissue samples of target specie kindly provided by various species experts. Genomic DNA (gDNA) was extracted from tissue samples using DNeasy Blood and Tissue kits (Qiagen). PCR reactions were set up using gDNA extracts as templates using only the external primers. To select the optimal annealing temperature of primer (Thalinger et al., 2021), a gradient end-point PCR run were conducted in 20-µL total volume reactions containing 4 µL of 10x Buffer solution (5x WonderTaq reaction Buffer EuroClone®), 1 µL of each primer (10 µM), 0.25 µL Taq polymerase (WonderTaq EuroClone®) and 2 µL of DNA template, under the following conditions: an initial step at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 60 seconds, with annealing temperature ranging from 50 to 60 °C for 45 seconds, elongation at 72 °C for 60 seconds, and a final extension at 72°C for 7 minutes. Agarose gel electrophoresis (2%) was used to verify the presence of a sequence corresponding in size to the expected target fragment (Wilcox et al., 2015). Evaluation of eDNA assay accuracy was based on amplification efficiency and limit of detection (LOD) (Bustin et al., 2009; Klymus et al., 2020). The correct sequence of the designed amplicons was verified through electrophoresis and Sanger sequencing, using BOLD Systems IDS (Ratnasingham & Hebert, 2007) and NCBI Primer BLAST (Altschul et al., 1997). For in situ validation, we collected and filtered water samples from two locations of ascertained presence of the target species and from two locations of ascertained absence. A total of 35 presence sites were selected in both enclosures and wild conditions, following expert recommendations, personal sightings, or recent literature data (Suppl. Mat. S3). Absence sites were identified outside the species range and in unsuitable habitats (Suppl. Mat. S4). We used tap water only in one case. Water sampling was carried out during the best sampling season from each species, as recommended for traditional monitoring by Stoch & Genovesi (2016). The combination of external primers and probes were used to set up qPCR assays with TaqMan technology (Bylemans et al., 2016), including in each run i) PCR positive controls, using the gDNA extracts of target species; ii) environmental positive control: eDNA extracts deriving from water samples collected in two sites of ascertained presence; iii) environmental negative control: eDNA extracts from water samples from two sites of ascertained absence of the species; PCR negative control: no template samples to verify the absence of contamination with DNA of target or untargeted species during the reaction set-up steps (Karlsson et al., 2022) Klymus et al., 2020; Thalinger et al., 2021).

Sampling sites. Most sampling sites were positioned in protected areas (national or regional), in Natura 2000 sites (n = 28), and in the regional ecological network designed to connect the regional protected areas (Scalisi et al., 2011) (n = 20). Sampling sites were located in different water bodies according to habitat requirements of the target species (Fig. 1; Tab. 2; Suppl. Mat. 1). To perform cost effective

analyses, we selected subsets of target species to be searched at each sampling site. Selection was based on ecological requirements, species range, most recent presence data, and potential expansions from neighboring areas (Petrassi et al., 2017; Stoch and Genovesi, 2016; Suppl. mat. S3, S5). To account for the species phenology and sampling errors we run two sampling replicates in 2021 and 2022. The first session was run in summer (July-August) at 52 sites. Twelve sites were found in drought conditions and were replaced by new sites in the second year. In 2022 sampling sessions were run in spring (May-June, 35 sites) and autumn (October, 17 sites) (Fig. 1).

Table 2. *Number (N) and relative percentage (%) of sampling sites in each habitat type, and suitability for target species (ID, as in Tab. 1).*

Habitat type	N	%	Suitable habitat for target species (ID)
Lowland Rivers	5	9.6	UE, VE, OC, LT, AF, TC, LI, EO, LL, PC, PP, LC, TS
Rivers	16	30.7	UE, VE, CM, OC, AI, AF, SL, BP, EO, MP, LL, DP, FL, 17, PP, PM, LC, TS
Creeks	13	23.0	UE, VE, CM, AI, SL, BP, MP, FL, PC, PM, BD
Channels	3	5.7	EO, MP, PC, PP, PM, TS
Ponds	5	9.6	CM, BP, TC, LI, EO, MP, TS, BD
Lakes	10	19.2	UE, VE, CM, OC, LT, TC, LI, EO, MP, DP, FL, PC, TS

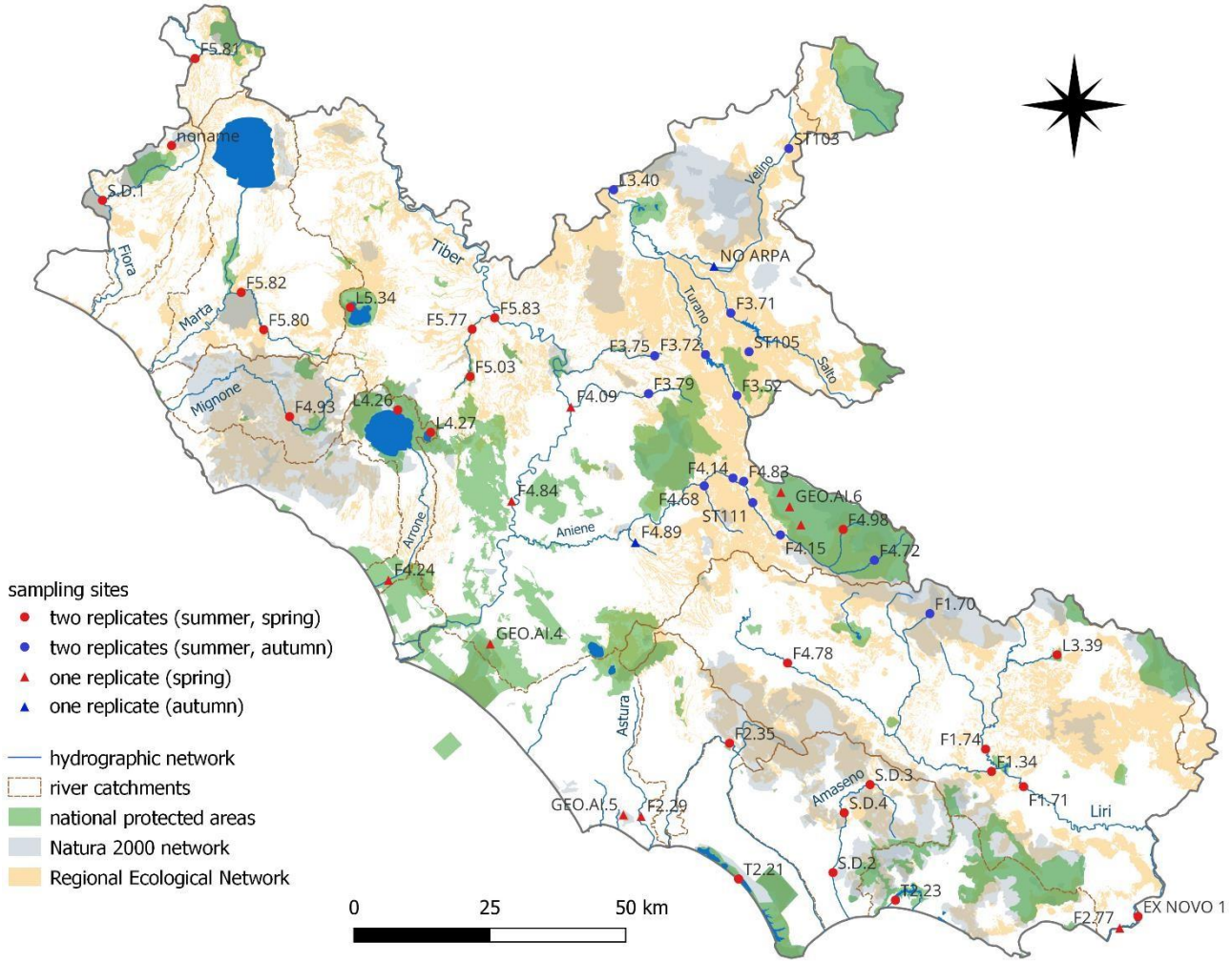

Figure 1. *Location of 52 sampling sites. Each site was sampled once (triangles) or twice (dots). Sites with two replicates were sampled during summer and in either autumn (blue) or spring (red).*

Water sampling. At each site we collected 3 liters of water using reusable bottles (first replicate) or single-use sterile bottles (second replicate). After each sampling, bottles were first washed three times with tap water, then with 10% bleach and distilled water solution, and finally three times with environmental water at the next sampling site. We sampled water by handling the bottle with a single-use glove and pooling it up to 20 cm under the surface. For running waters, we sampled different microhabitats transecting the channel section wherever possible, based on walkability of the river, otherwise we sampled just on shore. For standing waters, we walked along 300 m along the shore starting from the sampling site and collecting 200 ml of water every 20 m. Water was filtered within 24 hours using an electric vacuum pump (KNF Laboport, N86 KN.18, 6l/min, final vacuum: 100 mbar). Water was prefiltered through 8 µm mixed cellulose ester (MCE) filters (Millipore, diameter 47 mm), and then filtered through 0.22 µm MCE filters (Millipore, diameter 47 mm. For extra-turbid waters we changed prefilters and filters as soon as they clogged up to reach at least 1 l (n=4; 4,3%). Filters were immediately stored at -21°C. For each site replicate we recorded the time of filtering, the number of filtered liters, and the filtering protocol.

Environmental variables. Nine fine scale environmental variables were measured at each site during the 2022 sampling session, including air temperature, water temperature, river depth, river width, pH, nitrites and nitrates, water turbidity and water speed (Suppl. mat. S6 and S7). These variables have been measured just once during the sampling and are representative of the environmental condition at the moment of the DNA sampling. For each site we also collected 16 environmental parameters derived from digital maps and converted to uniform raster format with a resolution of 250 m. These included six bioclimatic variables extracted from the CHELSA database (https://chelsa-climate.org/), i.e. annual temperature (BIO2), maximum temperature of the warmest month (BIO5), average temperature of the warmest quarter (BIO10), precipitation during the wettest quarter (BIO16), and precipitation during the coldest quarter (BIO19); the Enhanced Vegetation Index (EVI) sourced from the MODIS database (https://lpdaac.usgs.gov/products/mod13q1v006/), topographical information (altitude, aspect, and slope) obtained from the Digital Elevation Model (DEM) provided by the CGIAR-CSI database (http://www.cgiarcsi.org/2010/03/108/uot;http:/srtm.csi.cgiar.org), and Euclidean distance from agricultural areas, artificial surfaces, forests, semi-natural regions, inland marshes, water bodies, and water courses, derived from CORINE Land Cover dataset from the Copernicus database (https://www.copernicus.eu/it).

eDNA processing. eDNA was extracted from filters after an average 53±37 days using DNeasy PowerSoil Pro kit (Qiagen). Prefilters and filters belonging to the same sample were extracted individually from membranes and then joint onto the same spin column. eDNA extracts were finally diluted with 75 µL bidistilled Miili-Q sterile water and preserved at -21 °C. Pre and post amplification phases were carried out in separate rooms to avoid contaminations, and each step was conducted in a laminar flow cabinet. Real-time quantitative PCR (qPCR) with TaqMan technology (StepOnePlus - Applied Biosystem) were run on eDNA extracts. To reduce inhibitions and avoid false negatives, each eDNA sample was analyzed in six technical replicates, three for each dilution (1:1, and 1:10), together with positive (PTC) and negative technical controls (NTC), respectively. PTC consisted of 2 µL of gDNA extracted from tissue samples, while NTC included ultrapure water. qPCR conditions included 10' initial denaturation at 95 °C and 40 cycles of denaturation at 95 °C for 15 s and annealing-elongation at 58 °C for 1 min. Amplification reaction consisted of 10 µL reaction mix with 5 µL of buffer, 0.25 µL of each primer (10 µM), 0.15 µL of dual-labelled Taqman probe (10 2 µM), 2 µL of extracted DNA. To ensure that the absence of target

species eDNA in water samples was not due 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 untargeted DNA metabarcoding characterization of fishes (REF Miya et al., 2015). Samples were considered as positive when a sigmoidal signal was observed in at least two qPCR technical replicates for each run and above the LOD (Ficetola et al., 2015). Cycle threshold (Ct) values were converted into counts, as number of DNA copies (Bruno et al., 2017). We considered positive detections also when counts were under the LOQ but equal or more than five copies per reaction (Bustin et al., 2009).

Data analyses. The detection rate (DR) of each species/taxon was computed as the number of occurrences divided by the number of sites tested for that species. A principal component analysis (PCA) was run to explore the relationship between environmental factors and the presence or absence of target species. PCA were run separately for fine and broad scale environmental variables for species with more than five eDNA occurrences. Chi-Squared and Fisher's exact tests are performed to assess statistically significative differences among replicates, seasons, and habitat types. A principal component analysis (PCA) was run to explore fine and large-scale factors affecting either presence or absence of target species. PCA were run separately for fine and broad scale environmental variables for species with more than five eDNA occurrences.

Results

eDNA assays. Tab. 3 lists the details of new primers and probes derived from mtDNA that successfully passed the three validation steps for 12 species. For three species primers and probes were derived from COI, for four species from CytB, and for two species from 16S. Length of primers and probes varied from 18 to 24 bp. Tab.4 shows the accuracy parameters (LOD, Amplification efficacy, DNA count) of all newly developed assays that successfully passed the in vitro validation. LOD values ranged between 4,00*10-7 and 2,90*10-3 ng. Efficiency of amplification on tissue sample (in vitro) ranged from 82.2% to 191.9%. Finally, all couples of primers with probe showed no amplification in the field negative controls (environmental samples of ascertained absence of the species). In situ validation successfully amplified the two environmental samples of ascertained presence of the species for 18 over 22 species (Suppl. mat., Tab. S4, L. Caprotti, com. pers.). Odonates failed the in situ validation step with environmental positive controls (*C. castellani*, *O. curtisii*) or were validated just partially (*L. tetraphylla*: one out of two presence sites). The polecat was partially validated as well.

Table 3. *List of qPCR-eDNA primers and probes used to detect target species during the project design for the first time.*

TARGET SPECIES	MARKER	PRIMER FW (5'-3') PRIMER REV (5'-3') PROBE (5'-3')	PRIMER/PROBE LENGTH (BP)	MELTING T (°C)	AMPLICON LENGTH (BP)	ANNEALING T (°C)
Unio elongatulus	COI (mtDNA)	GGTTATTAGGTGATGATCAATTG	23	57.5	257	54 °C
		CGGAGGATATACTGTTCAACC	21	60		
		[FAM]AGTGATGCCAATAATAATTGGCGG[MGBEQ]	24	61.5		
Vertigo spp. (genere)	COI (mtDNA)	CAAGAATAGTTGAAGGCGGTGC	22	62.6	94	60 °C
		GCCAAATCAACGGATGCTCC	20	62.4		
		[FAM]GAACAGGGTGGACTGTTTATCCC[MGBEQ]	23	64.5		
Coenagrion castellani	NA	CTCATGCAGGAGGTTCAG	18	59.9	NA	NA
		ACCTGCTGGATCAAAGAATGAT	22	58.9		
		[FAM]ACCAGTATTAGCTGGTGCCATC[MGBEQ]	22	62.6		
		CCTCCACTAGCAGGTGCTA	19	57.8		

Lindenia tetraphylla	NA	GGCCCCTAGAATTGAGGATACT	22	62.6	NA	NA
		[FAM]GCACACGCTGGAGGGTCG[MGBEQ]	18	66.7		
Oxygastra curtisii	NA	TTAGGACAACCAGGCTCTT	19	58	NA	NA
		TACTAGTCAGTTCCCAAACC	20	58.3		
		[FAM]AACAGCACATGCATTTGTCATG[MGBEQ]	22	58.9		
Padogobius bonelli	COI (mtDNA)	CCTTTTGGCCTCCTCTGG	18	62.1	129	60 °C
		CCCGCCAGGTGTAAGG	16	61.8		
		[FAM]GGCACAGGATGAACCGTGTACC[MGBEQ]	22	66.4		
Squalius lucumonis	CytB (mtDNA)	GCATTGTGAAACTTCGGTTCC	21	60.6	153	58 °C
		GGTGACGGATGAAAACGC	18	59.9		
		[FAM]CCTGGCAATGCACTACACTTCCG[MGBEQ]	23	66.3		
Bombina pachypus	16s (mtDNA)	CCTAATTCACCCCTTAGGG	19	60.5	166	59 °C
		CTGATTGGATCAGTGTGTTAGACG	24	62.8		
		[FAM]CCCTCCGAGATGAAAGGGAC[MGBEQ]	20	64.5		
Triturus carnifex	CytB (mtDNA)	GGCGTCATCTTACTATTCTTG	21	58	80	57 °C
		GAAGGTTGGTAATAACTGTGG	21	58		
		[FAM]GCTTTCGTTGGGTATGTCCTGCCA[MGBEQ]	24	66		
Lissotriton italicus	16s (mtDNA)	TTATTACCTACCTACGGGC	19	58	95	57 °C
		TCTATCGCGGAGGATGGCTT	18	62.4		
		[FAM]AAATTTTCGGTTGGGGCGACCGC[MGBEQ]	23	66		
Emys orbicularis	CytB (mtDNA)	CCAATGGTGCCTCCCTATT	19	60.1	114	56 °C
		AGAGTAAAAGGGTGGTTCCTG	21	60.6		
		[FAM]CATCTATCTCCACATCGGACGG[MGBEQ]	22	64.5		
Mustela putorius	CytB (mtDNA)	CGGATTCTCAGTAGATAAAGC	21	58	130	57 °C
		GGAGGGGTTATTAGATCCTG	20	60		
		[FAM]CCTAGCAGCAGTCCACCTAC[MGBEQ]	20	64.5		

Table 4. *Results obtained for the parameters used to assess accuracy for each species assay.*

TARGET SPECIES	concentration tissue DNA	CT	LOD	Amplification efficacy	DNA counts (copies)
Unio elongatulus	53,5	34,0301	$5{,}35*10^{-4}$	95,180	$6{,}48*10^{11}$
Vertigo spp. (genere)	98,5	34,552	$9{,}85*10^{-7}$	191,908	$2{,}74*10^{12}$
Coenagrion castellani	1,38	NA	NA	NA	NA
Lindenia tetraphylla	45,7	33,445	NA	NA	NA
Oxygastra curtisii	50,5	35,795	NA	NA	NA
Padogobius bonelli	215	32,928	$2{,}15*10^{-6}$	150,250	$2{,}48*10^{15}$
Squalius lucumonis	150	33,8449	$1{,}50*10^{-6}$	137,573	$1{,}46*10^{13}$
Bombina pachypus	290	35,2565	$2{,}90*10^{-3}$	89,573	$1{,}82*10^{9}$
Triturus carnifex	87	28,766	$8{,}70*10^{-5}$	150,122	$2{,}82*10^{24}$
Lissotriton italicus	83,5	42,9613	$8{,}35*10^{-6}$	82,247	$2{,}13*10^{-6}$
Emys orbicularis	73	38,154	$7{,}30*10^{-5}$	101,594	$5{,}07*10^{3}$
Mustela putorius	40	39,92	$4{,}00*10^{-7}$	99,345	1,44

Species detection. Environmental DNA allowed to detect 18 over the 22 target taxa, at 45 over 53 sampling sites, for a total 101 occurrences (Fig. 2). Only native species were found at 24 over 53 (45%)

sites, mainly concentrated in mountains and at the headwaters. Only IAS were found at 5 sites (9%), mainly located along primary rivers and artificial lakes. Native and invasive species were found in co-occurrence at 16 sites (30%). The number of species detected at each site varied from 0 (8 sites) to 4 (8 sites). Highest number of native species (3-4) was found at 10 sites, mainly located in the hilly and mountainous areas, whereas highest numbers of IAS (2-3) were retrieved at six sites, primarily located in Southern Latium and the area between the river Tiber and the mountainous reservoirs. The Eurasian otter *L. lutra* was detected for the first time in the study area after 50 years from its extinction in the region (Battisti et al. 2011). Also, the fungal pathogen *B. dendrobatidis* was detected for the first time through eDNA inItaly, co-occurring with the endangered amphibian *B. pachypus* (Mirone et al., submitted).

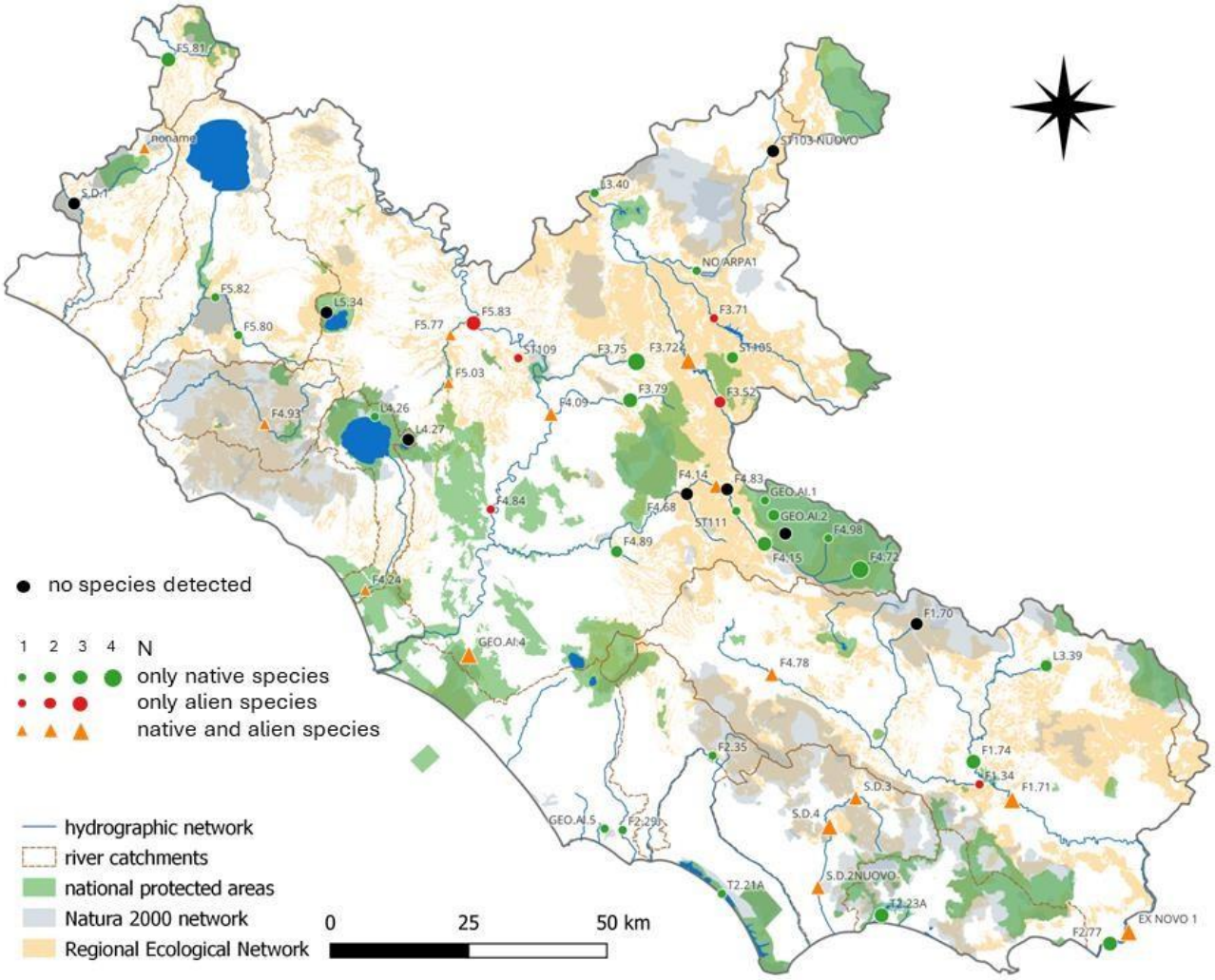

Figure 2. *Environmental DNA reuslts revealed sites with no species detections, and sites with native species only, with alien species only, and with the co-occurrence of both native and alien species.*

Highest detection rates (DR) for both sampling replicates were obtained for fish (0.59 ± 0.2), amphibians (0.43 ± 0.3), molluscs (0.30 ± 0.12), and mammals (0.27 ± 0.29); the least for terrapins (0.02, tested over 31 sites), and dragonflies (no detection for any of the three species) (Tab. 5). DR for 10x10 km grid cells

confirmed fish (0.83), molluscs (0.72), and amphibians (0.61) as the taxa with the highest values (Tab.5). For individual sampling replicates, positive occurrences increased in the second (spring and autumn) vs the first (summer) replicate with 44 vs 73 total occurrences respectively, even if not significant (X=1.52, p=0.22). This result, that was likely related to the highest probability of finding live cells in the water, i.e. accounting for phenological stages of species, was confirmed for all taxa except molluscs (Tab. 5). Most occurrences were detected in spring (n=51), followed by summer (n=44) and autumn (n=21), even if this difference is not significant (X=1.83, p=0.39). A higher DR in spring was found for molluscs, reptiles, and mammals, but it was statistically significant only for amphibians (p=0.01). DR of fish and crustaceans were comparable for both spring and summer for the firsts (p=0.67). Crustaceans also showed a comparable DR across all the seasons (p=0.93). The unique occurrence of the pathogenic fungus was recorded in autumn.

Table 5. *eDNA single species approach detection rates (DR) on sampling sites, on a 10x10 km grid of presence cells, and distinguished by sampling replicate, explained by mean standard ± deviation and summarized by taxa*.

	DR	**DR on 10x10 km presence grid**	**Replicates**		**Seasons**		
Taxon			**1st**	**2nd**	**spring**	**summer**	**autumn**
Fungi	0.05	-	0	0.05	0	0	0.07
Mollusca	0.30 ± 0.12	0.72 ± 0.47	0.27 ± 0.16	0.23 ± 0.19	0.45 ± 0.48	0.27 ± 0.16	0.14 ± 0.14
Insecta	0	0	0	0	0	0	0
Crustacea	0.16 ± 0.09	0.49 ± 0.44	0.09 ± 0.11	0.09 ± 0.02	0.06 ± 0.1	0.09 ± 0.11	0.09 ± 0.09
Osteychtes	0.59 ± 0.20	0.83 ± 0.19	0.39 ± 0.18	0.42 ± 0.07	0.39 ± 0.07	0.39± 0.18	0.29 ± 0.34
Amphibia	0.43 ± 0.30	0.61 ± 0.25	0.19 ± 0.10	0.36 ± 0.29	0.31 ± 0.26	0.19 ± 0.26	0.16 ± 0.32
Chelona	0.02 ± 0.02	0.02 ± 0.02	0	0.02 ± 0.03	0.02 ± 0.03	0	0
Mammalia	0.27 ± 0.29	0.06	0.10 ± 0.14	0.21 ± 0.20	0.21 ± 0.21	0.10 ± 0.14	0.04 ± 0.05

Highest detected native species (DR>0.6) included *T. carnifex*, *A. fallax*, and *B. pachypus*. Among IAS P. bonelli was the most detected taxon (0.76), followed by *D. polymorpha* (0.44) and *P.* parva (0.35). In contrast, some species were detected at very low unexpected rate, i.e. the European pond turtle E. orbicularis (0.04), the alien crayfish *P. clarkii* (0.09), and the European polecat *M. putorius* (0.06). Moreover, four species were not detected in eDNA samples. As expected, these included the Odonates *L. tetraphylla* (0/23), *O. curtisii* (0/21), and *C. castellani* (0/26), but also the pond slider *T. scripta*, although this latter was directly observed during field sampling (S. Giovacchini, pers. obs.).

Seasonal detection varied according to species. Highest DR during summer was found for *U. elongatulus* (0.26 vs 0.07 in spring/autumn), *A. fallax* (0.57 vs 0.45), *L. catesbeianus* (0.14 vs 0). For eight species DR was maximum during spring: *D. polymorpha* (1), *Vertigo spp*. (0.24), *P. clarkii* (0.18), *P. parva* (0.32 vs 0 in summe), *T. carnifex* (0.63 vs 0.33 ini summer), L. italicus (0.27 vs 0.12 in summer), *E. orbicularis* (0.04) and *L. lutra* (0.35 vs 0.2 in summer). The highest DR during autumn was found for *S. lucumonis* (0.67 vs 0.33 in summer and spring) *B. pachypus* (0.64 vs 0.33 in spring), *B. dendrobatidis* (0.07) and *M. putorius* (0.07*). A. italicus* showed a higher DR both in summer and in autumn (0.2 vs 0.12). Finally, some species did not displayed any specific seasonality in DR, as for *F. limosus*, and *M. putorius*). Comparing habitat types, channels had the highest DR in spring and summer, and creeks during autumn (Tab. 6). Figure 3. Average detection rates obtained for each taxon in the three sampling seasons.

In contrast, DR on habitat types (i.e., total occurrences over the total number of times that a specific habitat was tested) did not show any appreciable difference among sampling habitats, ranging from 0.20 for lakes to 0.30 for lowland rivers (X=2.7, p=0.73) (Tab. 6).

Table 6. *Detection rate (DR) by habitat based on single species eDNA detections.*

	Lowland river	River	Lake	Creek	Channel	Pool
Detections	15	31	15	24	8	8
Tested	50	147	75	109	28	31
DR	0.3	0.21	0.20	0.22	0.29	0.26

Table 7. *Detection rate (DR) by species based on positive over tested sites, and on positive sites over a 10x10 km grid cell of target species presence.*

Species	**N. positive/tested sites**	**eDNA survey DR**	**10x10 km grid DR**
Batrachochytrium dendrobatidis	1/21	0.05	0
Unio elongatulus	7/28	0.25	1
Vertigo spp.	7/32	0.22	0.18
Dreissena polymorpha	4/9	0.44	1
Oxygastra curtisii	0/21	0	0
Coenagrion castellani	0/26	0	0
Lindenia tetraphylla	0/23	0	0
Austropotamobius pallipes	7/26	0.27	0.26
Faxonius limosus	2/15	0.13	0.50
Procambarus clarkii	3/34	0.09	0.17
Alosa fallax	15/20	0.75	1
Squalius lucumonis	5/10	0.50	0.6
Pseudorasbora parva	7/20	0.35	0.75
Padogobius bonelli	10/13	0.77	1
Bombina pachypus	12/20	0.60	0.67
Lithobates catesbeianus	1/10	0.10	-
Triturus carnifex	6/8	0.75	0.83
Lissotriton italicus	3/12	0.25	0.33
Trachemys scripta	0/21	0	0
Emys orbicularis	1/26	0.04	0.05
Mustela putorius	2/31	0.06	0.06
Lutra lutra	8/17	0.47	-

PCA performed on fine scale variables recorded at sampling sites allowed to distinguish between positive and negative sites for one native (*T. carnifex*) and two alien (*D. polymorpha* and *P. parva*) species (Fig. 4a). For *T. carnifex* negative sites were mainly clustered on the upper part of the second PC axis

(22% of cumulative variance), characterized by high water turbidity, low PH and low river width. For D. polimorpha negatives sites were distinct along the first PC axis (47.8% of variance explained), and mainly characterized by low ambient and water temperature, and to a lesser extent by low depth and width of the water course, low levels of nitrates and low pH. For *P. parva* positive sites were clustered in the negative portion of PC2 (23.3% of explained variance) and characterized by high pH and low river width. Also, PCAs performed for large scale variables showed a distinction of positive and negative sites only for *S. lucumonis* (Fig. 4b), with positive sites concentrated in the upper part of the plot along the PC2 (28,3% of cumulative variance) and characterized by low extent of agricultural areas and high level EVI, precipitation during the wettest (BIO16) and the coldest (BIO19) quarter, and at a lower extent by high aspect.

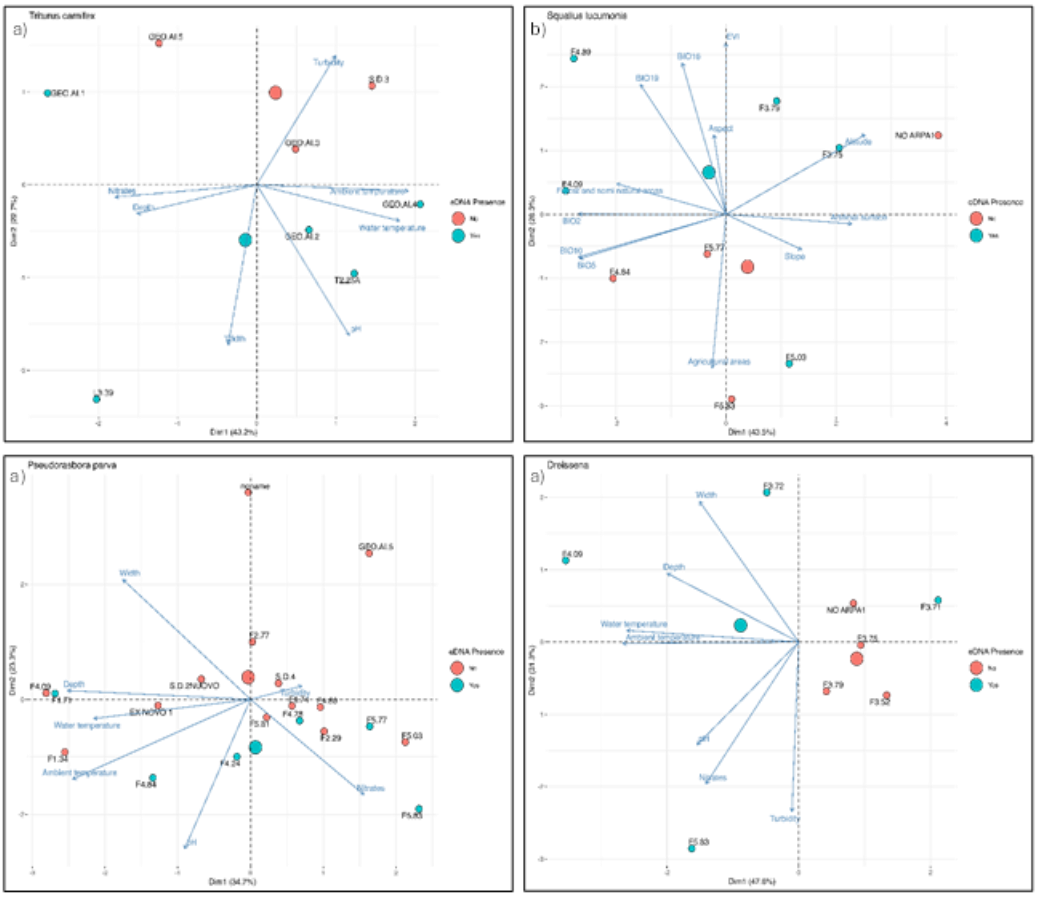

Figure 4. *Results of PCA run on a) fine scale and on b) large scale variables, recorded at sampling sites, showing the distribution of positive and negative sites for two native (T. carnifex [a] and S. lucumonis [b]) and two alien (D. polymorpha [a] and P. parva [a]) species. Biplot superimposed to PCA axes indicate the direction and relative importance of the original variables.*

Discussion

The main objective of this work was to develop a protocol to monitor simultaneously and on a large scale, endangered freshwater species and invasive species that pose a threat to them. By carrying out the work to develop species-specific primers, a considerable amount of information was gathered on which taxa are best suited to be monitored using this technique.

The new assays developed for molluscs, fish, amphibians, and mammals were accurate and did not reveal any constrains during primer design, and validation phases. This is partly due to the fact that,

unlike invertebrates, the genetic target regions for these taxa are more conserved at species level, ensuring primer selectivity. Most of these primers developed reached level 4 out of 5 in the classification proposed by Thalinger et al. (2021). In fact, most primers were characterized by high percentage of LOD, extensive field testing with positive results, and can be considered operational. Our multispecific approach allowed the simultaneous detection of several species for 18 out of 22 target species belonging to a wide range of taxonomic groups, confirming the efficiency of multiple taxa detection reported by Thomsen et al. (2012b), although these authors analyzed only six species. In our case, higher detection rates were observed for amphibians, fish, molluscs, and mammals, confirming both the reliability of eDNA assays and the effectiveness of site selection for multiple species detection. In contrast, for damselflies, crayfish and terrapins our eDNA essays either failed to detect the species or detection rates were below their expected occurrence in the study area.

Effectiveness in eDNA detection for organisms with high metabolic rates like fish and amphibians (Collins et al., 2018; Eichmiller et al., 2016; Goldberg et al., 2016) is likely related to a major shedding of skin cells and mucus released in the water during all their life stages. As an example, a high accuracy in eDNA detection of the crested newt *Triturus cristatus* was also reported by Harper et al. (2018), and eDNA technique is largely applied to monitor amphibian worldwide (Osathanunkul and Minamoto, 2021; Pilliod et al., 2014; Reyne et al., 2021; Rodgers et al., 2023). PCAs run on the sampled sites of *Triturus carnifex* did not show any particularly remarkable patterns. However, this result is not surprising as the species tends to be generalist (Mori et al., 2017). Also for fish eDNA is known to represent a particularly promising survey method (Pont et al., 2023; Wozney and Wilson, 2017; Knudsen et al., 2019; Minegishi et al., 2023; Tsuji et al., 2018), at the point that several initiatives are exploring the praticabibility of inferring other information from eDNA sampling such as biomass and population size (Lacoursière-Roussel et al., 2016; Rourke et al., 2022). A confirmation came also from our results, where the native *S. lucumonis*, *A. fallax*, the translocated *P. bonelli* and to a lesser extent the IAS *P. parva*, were among the most detected taxa in our study. The numerous occurrences of *S. lucumonis* have made it also possible to interpret in greater detail the parameters that could influence its eDNA detection. The most favourable environments seem to be characterised by low ambient and water temperatures as well as low turbidity levels and slow-flowing waters. Furthermore, most of the sites where the species was not detected are characterised by high rainfalls, presence of cultivated areas and low riparian vegetation cover. This is in agreement with the ecology of the species, which prefers streams and lateral watercourses with lower water flow, characterised by high vegetation cover and clear, highly oxygenated waters in which they reproduce by laying their eggs on the bottom (Giannetto et al., 2013; Rossi et al., 2021; Tancioni et al., 2013). Instead, the presence of the IAS *P. parva* is negatively affected by high rainfall, high riparian vegetation cover and high water turbidity. High rainfall likely swell the watercourses whereas the species prefers calm, shallow waters, sandy/muddy bottoms, and high vegetation is more tricky, as it is considered a characteristic element of its habitat (Sunardi et al., 2005). However, it should be considered that these two parameters could also be correlated with the detection of environmental DNA, i.e. DNA more dispersed along the fast running than slow moving watercourses. A variation of water sampling technique or direct extraction from the muddy/sandy sediment could provide useful information in this regard. Also, our results allowed to witness the expanding range of the translocated fish *P. bonelli* (Sarrocco et al., 2012) that is threatening the native *P. nigricans* (Pompei et al., 2018, 2016a, 2016b, 2014). *P. bonelli* is known to prefer midstream areas with slow waters and in central Italy it is accidentally released both along the river Tiber and in those areas closed by dams used for recreational fishing, where it proliferates in the shallow, sandy waters along riverbanks.

Molluscs are other freshwater organisms known to be easily detected by qPCR-based assays in eDNA water samples. Unionids bivalves are fully living in the water and thus they have been effectively retrieved by other eDNA studies (Deiner et al., 2015; Prié et al., 2021; B. C. Schmidt et al., 2021). Specifically, our assay designed for *U. elongatulus* was positively validated on *U. mancus* samples, overlooking their taxonomical splitting recently proposed (Marrone et al., 2019; Prie et al., 2012). This assay was designed with just one mismatch in the forward primer and two in the reverse primer and make us unaware if our results revealed new translocations of the *U. elongatulus* in the Tyrrhenian environments, or acknowledged new populations of *U. mancus* in the study area (G. Manganelli, pers. comm.). Instead, eDNA has been more rarely applied to terrestrial pulmonated Gastropods, as they often are riparian or linked to marshes and turbid waters hard to filter for eDNA purposes (Rees et al., 2023). We had some difficulties to obtain a specie-specific assay for *Vertigo* species. Here again, new eDNA locations need confirmation by local traditional survey to ascertain either the presence of the protected *V. angustior* and *V. moulinsiana* (Ghezzi, 2021; Manganelli et al., 2020) or the alien species belonging to the same genus (*V. pygmaea* and *V. pusilla*; M. Bodon, pers. comm.). Finally, we confirmed qPCR as an accurate technique to detect the IAS zebra mussel *D. polymorpha* (Monaco, 2014; Peñarrubia et al., 2016; Gingera et al., 2017), confirming a recent finding in the Turano lake (Grano et al., 2020), and detecting three new occurrences in the Tiber river catchment, likely derived from the source population of the Turano lake (Grano, 2022).

As to mammals, we obtained a different, and somehow unexpected, response for the European polecat and the Eurasian otter. As the first species is widespread distributed it was likely under detected (Russo et al., 2020), whereas the second was found at multiple sites, although still considered extinct in the region (Battisti et al., 2011; Giovacchini et al., 2021). We suggest this different detection rates to be related to the time these animals spend in the water. The Eurasian otter is in fact more strictly linked to freshwater habitats, as it exclusively catches its prey in the water, and spends most of its active time swimming in search of prey (Kruuk, 2006). In contrast, the polecat is known to live preferentially close to water course, but it is not an obligate aquatic predator, thus likely spending less time in the water. In this context, the probability of releasing DNA in the water is much higher for otters than for polecats. Environmental DNA survey for the Eurasian otter also revealed to be cost effective in harsh environments were traditional techniques require strenous field efforts Specifically, our eDNA survey was very efficient in discovering the Eurasian otter in the study area, where it was considered extinct since 1975. New findings were found at the boundary of its known current range (Liri river and Pontine marshes), and, more unexpectedly, and at one isolated site in the river Paglia at the northern boundary with Tuscany.

Finally, the pathogen fungus *B. dendobatidis* was successfully retrieved by environmental samples using a qPCR-based eDNA technique, confirming this technique as a cost effective survey tool for this concerning threat to amphibians world wide (Kamoroff, 2017; Kirshtein et al., 2007; Lastra González et al., 2021; Porco et al., 2024).

Also, our outcomes stressed the validity of a multiple single species qPCR-based eDNA technique by highlighting areas characterized by highest diversity of endangered native species, and areas where only alien, or both native and alien species occurred. This relevant adding value of a multispecific approach contributed to identify highly pristine biotopes and to prioritize areas where eradication and mitigation measures of alien species should be undertaken, i.e. areas where they directly threaten endangered native species (Caffrey et al., 2014). As an example, the pathogen *B. dendrobatidis* was successfully retrieved at the same site where also the sensitive and highly endangered endemic Apennine yellow bellied toad *B. pachypus* was found (Aniene river, Roviano, RM). that allow to detect both the species and its threatening taxa, and confirmed the accuracy of this technique for the early detection of the

pathogen reported by other authors also in our study area (Canestrelli et al., 2013; Porco et al., 2024; Tessa et al., 2013).

Limiting factors of our approach were represented by the low applicability of multiple species eDNA detection to same taxa which likely release low number of cells in the water, although they either spend much of their time in the water like the two species of pond terrapins, or have fully aquatic life stages like the three species of damselflies. However, we should remark that other freshwater macroinvertebrates were successfully detected through qPCR-based eDNA (Deiner et al., 2015; Mächler et al., 2014; Seymour et al., 2018) showing the sensitivity of this technique also to fulfill the objective claimed by the Water Framework Directive 2000/60/EC (Leese et al., 2021).

Some species, on the other hand, were either not detected or had such low detection rates that the environmental DNA technique cannot be considered efficient for large-scale eDNA surveys. Two invasive species (*P. clarkii* and *T. scripta*), specifically, went undetected with eDNA although spread in the study area (Gherardi et al., 2009; Monaco, 2014; Scalici et al., 2009). Some individuals were spotted at sampling sites during our water collection, and same trouble arose also on positive control sites, suggesting a low production of target DNA in the environment by these species. A similar low detection rate was obtained also for the *Emys orbicularis*, masking its real distribution (Capizzi and Bellavita, 2014; Ferri et al., 2019; Vignoli et al., 2015). One hypothesis concerns the possibility that the exoskeleton of crayfish and the highly keratinised skin of terrapins severely limit exchanges of live cells with the environment and that, at least at low densities of individuals, the amount of DNA released into the environment is too low to be detected. A first confirmation was provided by Adams et al. (2019) who detected eDNA of *Trachemys scripta* only at higher density values (13 kg/ha). It is thus possible that a dilution effect hindered our probability of detecting very few eDNA molecules produced by these reptiles in large rivers characterized by high water volumes. Alternatively, a more sensitive assay would be beneficial to enhance detection rates and control false absences (Tréguier et al., 2014). A similar case is represented by the European polecat *M. putorius* whose detection rate was 0.18 with just two occurrences out of 31 sites investigated. The species is known to be broadly distributed in study area (Russo et al., 2020). Primer and probes in this case were validated positively just on one of the two water samples where the species was certainly present. Few studies are conducted on polecats using eDNA. Broadhurst et al. (2021) had similarly low values on polecat detection probability (i.e. p=0.01) adopting a metabarcoding approach on mammalian communities in Great Britain. Over 24 species, the polecat resulted to be the less detected, found only on 2 over 27 sampling sites along the river network. The low detection rate could then be related to the low amount of target DNA in the water compared to the high detection rate of *L. lutra* and could be linked to the ecology of the polecat. In fact, polecats are less closely related to the aquatic environment than the other semiaquatic mammals, like the Eurasian otter (Kruuk, 2006).

Conclusion

The utilization of eDNA techniques has demonstrated success in detecting multiple species simultaneously, offering significant potential for future large-scale monitoring of freshwater wildlife. Additionally, eDNA surveys can serve as a driving force in implementing eradication plans for invasive species. However, refinement of these methods is necessary, particularly for species with low detectability, especially those that release minimal DNA. Adjustments in sampling procedures may be needed to accurately capture the distribution of armored invertebrates and reptiles.

Future research directions could involve assessing eDNA performance across various sampling periods to determine its seasonal variability and explore whether sampling designs can capitalize on

phenological stages. Moreover, this new method holds promise for evaluating the effectiveness of protected areas. Finally, we highlight the potential for species distribution modeling to enhance the identification of potential occurrence sites and optimize sampling strategies.

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Supplementary materials

Suppl. Mat. S1. *Length of rivers and surface of lakes sampled*.

Waterbody	Length [km]	Surface [sq-km]	Number of sampling sites
Fiora	80	-	1
Marta	54	-	2
Mignone	62	-	1

Arrone	37	-	1
Tevere	405	-	6
- Aniene	99	-	8
- Velino	90	-	2
- Salto	55	-	2
- Turano	90	-	1
Astura	18	-	1
Amaseno	40	-	3
Liri - Garigliano	158	-	7
Ventina	-	0.12	1
Mezzano	-	0.45	1
Vico	-	12.93	1
Bracciano	-	57.5	1
Martignano	-	2.44	1
Turano	-	5.6	1
Posta Fibreno	-	0.29	1
Ninfa	-	0.02	1
Caprolace	-	2.3	1
Fondi	-	3.8	1

Suppl. Mat. S2. *List of published qPCR-eDNA primers and probes used for eDNA detection.*

TARGET SPECIES	MARKER	PRIMER FW (5'-3') PRIMER REV (5'-3') PROBE (5'-3')	PRIMER/PROBE LENGTH (BP)	MELTING T (°C)	AMPLICON LENGTH (BP)	ANNEALING T (°C)	REFERENCES
Dreissena polymorpha	COI (mtDNA)	TGGGCACGGGTTTTAGTGTT	20		105	56	Amberg et al. 2019
		CAAGCCCATGAGTGGTGACA	20				
		[FAM]GCACCTGGAAGCGTCCTTGGTG[MGBEQ]	22				
Austropotamobius pallipes	16s (mtDNA)	CCCAGGAAATTTAAGCCTTTTCA	23	59.2	138	64	Manfrin et al. 2022a
		TATTATGACCGTGCTAAGGTAGCA	24	61.1			
		[FAM]CAACCATTCATACCAGCCTTC[MGBEQ]	21	60.6			
Faxonius limosus	NA	[Manfrin et al. in press]	NA	NA	NA	NA	Manfrin et al. in press
		[Manfrin et al. in press]	NA	NA			
		[Manfrin et al. in press]	NA	NA			
Procambarus clarkii	COI (mtDNA)	GGAGTTGGAACAGGATGGACT	21	62.5	73	56	Mauvisseau et al. 2018
		AATCTACAGATGCTCCCGCA	20	60.4			
		[FAM]CCTCCTTTAGCTTCTGCTATTGCTC[MGBEQ]	25	64.6			
Alosa fallax	COI (mtDNA)	GCGGCTTTGGGAATTGACTAG	21	62.6	114	60	Antognazza et al. 2019
		CAAGGAGGAGGAGGAATGAG	20	62.4			
		[FAM]GGCACCAGACATGGCATTCCCACG[MGBEQ]	24	69.7			
Pseudorasbora parva	COI (mtDNA)	CCCTGACATAGCATCCCC	18	62.2	79	64	Manfrin et al. 2022b
		AGAGGCTAAGAGTAGGAGGA	20	60.4			
		[FAM]AAGGGGGAAGTAGTCAGAAGCT[MGBEQ]	22	62.7			
Batrachochytrium dendrobatidis	ITS1	CCTTGATATAATACAGTGTGCCAT	24	59.4	121	60	Canessa et al. 2017
		AGCCAAGAGATCCGTTGTCAAA	22	60.8			
		[FAM]CGAGTCGAACAAAAT[MGBEQ]	15	47.9			

Lithobates catesbeianus	CytB (mtDNA)	TGCCAACGGAGCATCATTC	19	60.2	168	54	Sanz et al. 2023
		GATATTTGGCCCCATGGT	18	57.6			
		[FAM]ATTTCCACATCGGCCGAGGCCTC[MGBEQ]	23	68.1			
Trachemys scripta	CytB (mtDNA)	CCTCCAACATCTCTGCTTGA	20	60.4	153	60	Kakuda et al. 2019
		ATTGTACGTCTCGGGTGATG	20	60.4			
		[FAM]CGGAATTTTCTTGGCTATAC[MGBEQ]	20	56.3			
Lutra lutra	CytB (mtDNA)	CCACAATCCTCAACAACTCGC	21	62.6	240	58	Park et al. 2011
		CTCCGTTTGGGTGTATGTATCG	22	62.7			
		[FAM]CACTACACATCAGACACAACCACAGC[MGBEQ]	26	66.2			

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Suppl. Mat. S3. List of papers and grey literature used to assess habitat requirements and distribution of the target species in the study area.

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Suppl. Mat. S4. *Positive control (PCS) and negative control (NCS) sites sampled for in situ validation of probes. Target species are refereed as ID (see Tab. 1).*

PCS for target species	NCS for target species	Coordinates (WGS 84 EPSG: 4326)	River basin	Water body	Locality	Sampling date dd/mm/yy	Habitat type	Season
BP	AF, SL, DP, PP, PM, TS	41.50211, 14.34536	Biferno	-	Castelpizzuto	13/5/2022	Water dwell	Spring
BP, TC, LI	-	41.5479, 14.30183	Volturno	-	Castelpetroso	18/5/2022	Water dwell	Spring
-	BP, TC, LI, MP, LL, BD	41.93355, 12.42077	-	-	Roma	5/11/2022	Tap water	Autumn
SL	AI, BP, TC, LI, MP, LL, BD	41.85086, 12.63037	-	-	Tor Vergata	7/7/2022	Ex situ aquarius with non-target species	Summer

SL	-	41.92267, 12.76691	Tevere	Fosso S. Vittorino	Villa Adriana	27/6/2022	Creek	Summer
UE	-	43. MP526, 11.47604	Ombrone	Arbia	Buonconvento	06/7/2022	River	Summer
UE	-	43.2952, 11.40263	Ombrone	Arbia	Taverne d'Arbia	06/7/2022	River	Summer
TC, LI	-	41.76682, 14.22953	-	-	Vastogirardi	3/5/2022	Water dwell	Spring
TS	-	45.54639, 9.20565	-	Parco Nord	Cinisello Balsamo	16/5/2023	Pond	Spring
TS	-	45.58754, 9.24424	-	Parco Villoresi	Monza	04/5/2023	Channel	Spring
MP	-	41.60717, 14.2653	-	-	Pesche	26/1/2023	Tap water	Winter
MP	-	41.55717, 15.87866	-	Lago Salso	Manfredonia	2/3/2023	Lake	Spring
PP	-	41.66002, 12.99007	-	-	Giuliano di Roma	16/3/2023	Pond	Spring
PM	-	43.48912, 12.62057	Metauro	Bevano	Pontedazzo	4/11/2022	River	Autumn
OC	-	41.56068, 14.84775	Biferno	Biferno	Pietracatella	16/5/2022	River	Spring
OC	-	42.2088, 12.41533	Tevere	Treia	Mazzano	20/5/2022	River	Spring
-	OC	NA	NA	NA	NA	NA	NA	NA
-	OC	NA	NA	NA	NA	NA	NA	NA
LT	-	41.62459, 14.9268	Biferno	Occhito	S. Elia	16/5/2022	Lake	Spring
LT	-	41.78403, 14.97748	-	-	Montorio	16/5/2022	Pond	Spring
AF	-	42.40986, 11.6352	Fiora	Fiora	Vulci	23/5/2022	River	Spring
AF	-	41.98671, 12.49803	Tevere	Tevere	Castel Giubileo	29/5/2022	River	Spring
CM	-	41.45116, 13.1284	Amaseno	Ufente	Sezze	2/6/2022	Channel	Summer
CM	-	41.5441, 12.98213	-	-	Sermoneta	2/6/2022	Pond	Summer
-	AF, SL, DP, PP, PM, TS	41.7817, 13.41128	Liri	Capo d'acqua	Trisulti	2/6/2022	Creek	Summer
-	UE, VE, CM, LT, FL, PC, LC, EO	41.7817, 13.41128	Liri	Capo d'acqua	Trisulti	2/6/2022	Creek	Summer
-	UE, VE, CM, LT, FL, PC, LC, EO	41.7817, 13.41128	Liri	Capo d'acqua	Trisulti	24/6/2022	Creek	Summer
LL	-	41.74257, 14.30367	Trigno	Trigno	Vastogirardi	1/3/2023	River	Spring
LL	-	42.00244, 14.7202	Trigno	Trigno	San Salvo	1/3/2023	River	Spring
PC	-	42.60938, 11.76684	Mezzano	Mezzano	Valentano	23/5/2022	Lake	Spring
PC	-	42.60938, 11.76684	Mezzano	Mezzano	Valentano	14/8/2021	Lake	Spring
PM	-	41.50396, 13.26024	Amaseno	Amaseno	Prossedi	5/6/2022	River	Summer
PP	-	41.46887, 12.75324	Astura	Astura	Latina	29/5/2022	Channel	Spring
DP, FL	-	42.22825, 12.94169	Tevere	Turano	Rocca Sinibalda	17/10/2022	Lake	Autumn
DP, FL	-	42.22825, 12.94169	Tevere	Turano	Rocca Sinibalda	9/8/2021	Lake	Summer

AI	-	41.84755, 13.78181	Sangro	Sangro	Pescasseroli	12/10/2022	Creek	Autumn
AI	-	41.84755, 13.78181	Sangro	Sangro	Pescasseroli	27/10/2022	Creek	Autumn
VE	-	45.16123, 9.95539	-	-	Cavatigozzi	26/10/2022		Autumn
VE	-	45.16117, 9.95556	-	-	Cavatigozzi	26/10/2022		Autumn
EO, TS	-	45.21712, 8.155 MP	-	Fontanetto lago	Fontanetto Po	7/5/2022	Pond	Spring
LC	-	45.21742, 8.15508	-	Fontanetto tank 1	Livorno Ferraris	7/5/2022	NA	Spring
LC	-	NA	NA	NA	NA	NA	NA	NA
TS	-	45.21846, 8.15570	-	Fontanetto tank2	Fontanetto Po	7/5/2022	NA	Spring
EO	-	45.21796, 8.15592	-	Fontanetto v1	Fontanetto Po	7/5/2022	NA	Spring
EO	-	45.21796, 8.15592	-	Fontanetto v3	Fontanetto Po	7/5/2022	NA	Spring
EO	-	45.21796, 8.15592	-	Fontanetto vLab	Livorno Ferraris	7/5/2022	NA	Spring
AI	-	45.767695, 8.658320	NA	Lentate Verbano 1	Sesto Calende	1/8/2021	NA	Summer
AI	-	45.767695, 8.658320	NA	Lentate Verbano 1	Sesto Calende	1/8/2021	NA	Summer
AI	-	45.767695, 8.658317	NA	Lentate Verbano 2	Sesto Calende	1/8/2021	NA	Summer
AI	-	45.767695, 8.658317	NA	Lentate Verbano 2	Sesto Calende	1/8/2021	NA	Summer
AI	-	NA	NA	Montevecchia 1	Montevecchia	27/9/2021	NA	Autumn
-	AI	NA	NA	Moriggia 3	NA	1/8/2021	NA	Summer
-	AI	NA	NA	Moriggia 3	NA	1/8/2021	NA	Summer
-	AI	NA	NA	Moriggia 4	NA	1/8/2021	NA	Summer
-	AI	NA	NA	Moriggia 4	NA	1/8/2021	NA	Summer
PC	-	41.47093, 14.09331	-	-	Capriati a Volturno	17/06/2021	Channel	Summer
TC, EO	-	41.47093, 14.09331	-	-	Capriati a Volturno	17/06/2021	Pond	Summer
TC, EO	-	41.47093, 14.09331	-	-	Capriati a Volturno	17/06/2021	Pond	Summer
BD	-	NA	NA	NA	NA	NA	NA	NA
BD	-	NA	NA	NA	NA	NA	NA	NA

Suppl. Mat. S5. *Presence (P) and absence (A) of species at each site, irrespective of replica. Coloured cells indicate the tested species at each site*

		alien species								**native species**														
	Year	*Dreissena polymorpha*	*Faxonius limosus*	*Procambarus clarkii*	*Batrachochytrium dendrobatids*	*Lithobates catesbeianus*	*Trachemys scripta*	*Pseudorasbora parva*	*Padogobius bonelli*	*Unio elongatulus*	*Vertigo spp.*	*Coenagrion castellani*	*Lindenia tetraphylla*	*Oxygastra curtisii*	*Austropotamobius pallipes*	*Alosa fallax*	*Squalius lucumonis*	*Bombina pachypus*	*Triturus carnifex*	*Lissotriton italicus*	*Emys orbicularis*	*Lutra lutra*	*Mustela putorius*
EX NOVO 1	2021 -22			A			A	P		A	P		A	A		P				A	A	P	

F1.34	2021-22							A	P	A	A		A	A		A					A	A	
F1.70	2021-22			A	A										A			A		A			A
F1.71	2021-22			A				P	P	A	A	A	A	A		P					A	P	
F1.74	2021-22							A	A	A	P		A	A		P					A	P	
F2.29	2022							A				A	A	A		P					A		
F2.35	2022						A					A								P	A		A
F2.77	2022			A			A	A		A	A		A	A		P				P	A	P	
F3.52	2021-22	A	P	A	A				P	A	A				A			A					
F3.71	2021-22	P	A	A	A						A				A			A					A
F3.72	2021-22	P	P	A	A				A	A					P			P					A
F3.75	2021-22	A	A	A	A					P	P				A		P	P					A
F3.79	2021-22	A	A	A	A					P	A				A		P	P					A
F4.09	2022	P		A		A	A	A			P	A					P						
F4.14	2021-22		A	A	P					A	A				P			P					A
F4.15	2021-22		A	A	A					P	A				P			P					A
F4.24	2022			A		A	A	P			A	A		A		P					A		
F4.68	2021-22			A						A	A	A			A						A		A
F4.72	2021-22		A	A	A					P	A				P			P					P
F4.78	2022						A	P	P	A	A		A	A		P				A	A	A	
F4.83	2021-22		A	A							A				A								A
F4.84	2022				A	A	A	P		A		A		A	A	A	A	A			A		
F4.89	2022						A	A		P	A	A		A		A	P						
F4.93	2021-22			A		A	A		P			A	A	A	A	P					A	A	A
F4.98	2021-22		A	A	A					A	A				A			P					A
F5.03	2021-22					A	A	A	P			A	A		A		P						A
F5.77	2021-22					A	A	P	A			A	A		P		A						A
F5.80	2021-22			A						A	P	A	A	A		A					A	A	
F5.81	2021-22							A		P	A	A			P						A	P	
F5.82	2021-22			A			A				A	A	A	A		P					A	A	
F5.83	2021-22					A	A	P	P			A	A		A		A						P
GEO.AI.1	2022				A							A						A	P				
GEO.AI.2	2022				A													P	P				
GEO.AI.3	2022				A													A	A				
GEO.AI.4	2022			P	A		A					A	A	A				P	P	P	A		A
GEO.AI.5	2022				A		A	A				A	A	A				A	A	A	P		
L3.39	2021-22			A						A	A				A				P	A		P	A
L3.40	2021-22		A	A			A			A	P										A		
L4.26	2021-22			A		A						A	A	A		P							A

L4.27	2021-22			A		A							A	A									A
L5.34	2021-22		A										A		A						A		A
NO ARPA1	2022		A	A	A					A	A				A		A	P					A
noname	2022			P			A	A			P	A		A	A						A	A	A
S.D.1	2022						A				A				A	A					A	A	A
S.D.2NUOVO	2022			A				A	P	A		A	A			P				A	A	P	A
S.D.3	2021-22			A			A		P	A	A	A	A	A		P			P	A	A	A	A
S.D.4	2021-22			P				A	P	A		A	A			P				A	A	P	A
ST103 NUOVO	2021-22			A	A						A				A			A					A
ST105	2021-22		A	A	A										P			P					A
ST109	2021					P		A			A	A		A			A						A
ST111	2021-22		A	A	A					A	A				A			P					A
T2.21A	2021-22			A			A			A		A	A			P					A		A
T2.23A	2021-22						A			P	A	A	A	A		P			P	A	A	A	A

Suppl. Mat. S6. *Environmental variables measured on site during the second replicate only.*

environmental variable	**unit**	**type**	**method**
air temperature	°C	continuous	a Suunto portable watch after 30 minutes of acclimatization
water temperature	°C	continuous	a Deeper Pro portable sonar
river depth	meters	continuous	a Deeper Pro portable sonar, average value of a straight river stretch with no curves
river width	meters	continuous	by remote sensing, using Google Earth Pro
pH	range between 6.4 and 8.4	categorical (6.4, 6.8, 7.2, 7.6, 8.0, 8.4)	test strips
nitrites	range between 0 and 50 mg/l	categorical (0, 1, 5, 10, 25, 50)	test strips
nitrates	range between 0 and 500 mg/l	categorical (0, 10, 25, 50, 100, 250, 500)	test strips
water turbidity	low, medium, high	categorical	by visual checks
water speed	m/s	continuous	a pin ball attached with a 10 m wire floating in the main reachable current stream and measuring the time of the path in [s/m] three times, taking the average value and using the inverse formula to calculate water speed in [m/s]

Suppl. Mat S7. *Environmental parameters measured on site in 2022.*

Site ID	Spring/Autumn sampling	water temperature	environment temperature	pH	nitrites	nitrates	turbidity	water speed	depth
EX NOVO 1	S	24	35	8	0	10	L	0,00	5
F1.34	S	31	36	8.4	0	10	H	0,05	6

F1.70	A	12	12	7.6	0	0	L	0,34	0.3
F1.71	S	30	36	8	0	10	H	0,01	7
F1.74	S	25	29	8	0	10	L	0,24	0.7
F2.29	S	29	27	8	0	25	M	0,00	1.8
F2.35	S	23	29	7.6	0	10	L	0,00	1.3
F2.77	S	22	27	8	0	10	L	0,00	5
F3.52	A	14	14	7.2	0	0	L	0,18	0.5
F3.71	A	10	13	6.8	0	0	L	1,05	0.7
F3.72	A	22	25	6.8	0	0	L	-	0.5
F3.75	A	15	18	7.6	0	0	L	0,64	0.2
F3.79	A	17	19	8	0	0	L	0,16	0.3
F4.09	S	27	37	8	0	10	H	0,09	7.6
F4.14	A	14	23	7.6	0	0	L	0,91	0.9
F4.15	A	11	19	7.6	0	0	L	0,79	0.5
F4.24	S	29	34	8	0	10	H	0,82	0.5
F4.68	A	18	19	7.6	0	0	L	0,00	0.7
F4.72	A	11	16	7.6	0	0	L	0,78	0.3
F4.78	S	26	30	8	1	10	M	0,22	0.8
F4.83	A	14	20	8	0	10	L	1,03	0.3
F4.84	S	31	39	8	0	10	M	0,49	0.5
F4.89	S	22	29	8	0	0	L	0,55	0.3
F4.93	S	29	37	8	0	10	M	0,91	0.5
F4.98	A	12	20	7.6	0	0	L	1,06	0.5
F5.03	S	20	28	8	0	25	L	0,67	0.5
F5.77	S	20	31	7,6	0	25	L	0,57	0.4
F5.80	S	24	39	7.6	0	25	M	0,09	0.6
F5.81	S	26	33	7.6	0	10	M	0,00	0.5
F5.82	S	26	36	8.4	0	25	M	0,57	0.5
F5.83	S	22	30	8	0	50	L	1,09	0.5
GEO.AI.1	S	21	22	6.4	0	10	H	-	1
GEO.AI.2	S	30	27	8	1	10	H	-	0.5
GEO.AI.3	S	26	24	7.6	0	0	H	-	0.6
GEO.AI.4	S	29	36	7.6	0	0	H	-	0.5
GEO.AI.5	S	25	25	6.4	0	10	M	-	0.9
L3.39	S	22	24	8	0	10	L	-	1
L3.40	A	NA	19	7.2	0	0	M	-	0.5
L4.26	S	29	36	8	0	0	L	-	0.7
L4.27	S	25	26	7.6	1	10	M	-	-
L5.34	S	28	36	7.6	0	0	L	-	0.7
NO ARPA1	A	13	15	7.6	0	0	L	0,40	2.1
noname	S	25	28	6.4	0	0	L	-	0.8
S.D.1	S	25	29	7.6	0	25	M	0,31	0.8
S.D.2NUOVO	S	29	33	7.6	0	0	M	0,00	3
S.D.3	S	25	34	7.6	0	0	L	0,40	0.7

S.D.4	S	27	33	7.2	0	10	M	0,00	1.2
ST103 NUOVO	A	12	15	7.6	0	0	L	0,51	0.2
ST105	A	13	12	6.8	0	0	L	0,10	0.1
ST111	A	15	18	7.6	0	0	L	0,64	0.5
T2.21A	S	30	32	7.6	0	0	L	-	0.8
T2.23A	S	31	32	7.6	0	0	L	-	1

Suppl. Mat S8– *Details and source of data for environmental variables collected in the field and from digital map layers*.

Variable	**From field (F) or maps (M)**	**Source**	**Measure used**
Air temperature	F	Suunto portable watch after 30 minutes of acclimatization	°C
Water temperature	F	Deeper Pro portable sonar	°C
Maximum riverbed depth	F	Deeper Pro portable sonar, measured along straight river stretches with no curves.	Average value (m)
PH	F	Test strips, average values	Average value (range 6,4 - 8,4)
Water speed	F	Pin ball attached to a 10 m wire floating in the main reachable current stream. Three replicates	Average time to flow 10 m (s/m)
River width	M	Google Earth Pro maps	m
Mean annual temperature (BIO2)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Maximum temperature of the warmest month (BIO5)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Average temperature of the warmest quarter (BIO10)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Precipitation during the wettest quarter (BIO16)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Precipitation during the coldest quarter (BIO19)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Vegetation Index (EVI)	M	MODIS (https://lpdaac.usgs.gov/products/mod13q1v006/)	Rasterized at 250 m
Altitude	M	Digital Elevation Model (DEM) from CGIAR-CSI (http://www.cgiarcsi.org/2010/03/108/uot;http:/ srtm.csi.cgiar.org)	Rasterized at 250 m
Aspect	M	Digital Elevation Model (DEM) from CGIAR-CSI (http://www.cgiarcsi.org/2010/03/108/uot;http:/ srtm.csi.cgiar.org)	Rasterized at 250 m
Slope	M	Digital Elevation Model (DEM) from CGIAR-CSI (http://www.cgiarcsi.org/2010/03/108/uot;http:/ srtm.csi.cgiar.org)	Rasterized at 250 m
Distance from agricultural areas (2)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from artificial surfaces (1)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from forests and semi-natural areas (3)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance

Distancer from marshes (4.1.1.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from water bodies (5.1.2.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from water courses (5.1.1.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from lakes	M	Hydrosheds https://www.hydrosheds.org/	Rasterized at 250 m –Euclidean distance

CHAPTER 2

Amphibia-Reptilia

New eDNA-based insights for the detection of endemic and endangered Apennine yellow bellied toad (*Bombina pachypus*) and its plague *Batrachochytrium dendrobatidis*

--Manuscript Draft--

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Abstract:	Amphibian populations are experiencing a global significant decline with habitat loss, climate change, pathogen outbreaks, and alien species rank first. The Anuran family Bombinatoridae includes three species occurring in Europe including the Apennine yellow-bellied toad (*Bombina pachypus* (Bonaparte, 1838)), endemic to the Italian Apennines. This species is declining and is listed as Endangered (EN) in the Italian red list due to different threats, among which, infection by *Batrachochytrium dendrobatidis* has not been assessed yet. This study aimed at detecting the occurrence of both *B. pachypus* and *B. dendrobatidis* in twenty freshwater bodies of Central Italy through an environmental DNA (eDNA)-based approach. Here, a new Taqman-based (qPCR) assay has been developed for eDNA-based detection of *B. pachypus*. Moreover, in situ eDNA-based screening for *B. dendrobatidis* was carried out. Two sampling replicates were run in 2021-2022 in Latium (central Italy). For 14 sites sampled along rivers, we collected seven fine scale and 18 large scale environmental variables that could affect both species occurrence and eDNA detection of *B. pachypus*. This species was detected in 33 water samples from 13 out of 20 sampling sites, whereas *B. dendrobatidis* was only found at one sample site, although co-occurring with B. pachypus. Environmental DNA of *B. pachypus* was detected at all the rivers surveyed but the Liri river. Nine out of 12 positive sites were new occurrences for the species, most of which (n = 8) located outside Natura 2000 sites. Positive sites included 57% of running waters sites (n= 8), and 50% of standing water sites (n = 3). These latters included a pool in a lowland protected area where *B. pachypus* was considered extinct since 1997. Outcomes confirmed eDNA as a speditive and cost-effective method for large scale surveys of both the Apennine yellow bellied toad and its potential threat *B. dendrobatidis*.
Keywords:	Chytridiomycosis; Environmental DNA; RealTime qPCR; TaqMan
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New eDNA-based insights for the detection of endemic and endangered Apennine yellow bellied toad (*Bombina pachypus*) and its plague *Batrachochytrium dendrobatidis*

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Abstract

Amphibian populations are experiencing a significant decline at global scale. Habitat loss, climate change, viral and fungal diseases, pollution, and introduction of alien species are the primarily drivers. The Anuran basal family Bombinatoridae includes three species occurring in Europe, comprising the Apennine yellow-bellied toad (*Bombina pachypus* (Bonaparte, 1838)), endemic to the Italian Apennines. This species is experiencing a severe decline and is listed as Endangered (EN) in the Italian red list. Decline is due to multiple threats. Despite the impact on *B. pachypus* has not been ascertained yet, *Batrachochytrium dendrobatidis* is threatening amphibian populations. Environmental DNA (eDNA)-based assays are highly sensitive and non-invasive, providing effective results for large-scale monitoring of freshwater biodiversity, especially for rare and elusive species. This study aimed at detecting the occurrence of both B. pachypus and *B. dendrobatidis* in twenty freshwater bodies of Central Italy through an eDNA approach. Here, a new Taqman-based (qPCR) assay has been developed for eDNA-based detection of B. pachypus. Moreover, in situ validation of the available probes for *B. dendrobatidis* was carried out. Two sampling replicates were run in summer 2021 and spring-autumn 2022 in Latium (central Italy). For 14 sites sampled along rivers, we collected seven fine scale and 18 large scale environmental variables that could affect both species occurrence and eDNA detection of *B. pachypus*. *Bombina Pachypus* was detected in 33 water samples from 13 over 20 sampling sites, whereas *B.* dendrobatidis was only found at one sample site, although co-occurring with *B. pachypus*. Environmental DNA of *B. pachypus* was detected at all the rivers surveyed but the Liri river. Nine over 12 positive sites were new occurrences for the species, most of which (n = 8) located outside Natura 2000 sites.

Positive sites included 57% of running waters sites (n= 8), and 50% of standing water sites (n = 3). These latters included a pool in a lowland protected area where *B. pachypus* was considered extinct since 1997. Outcomes confirmed eDNA as a speditive and cost-effective method for large scale surveys of both the Apennine yellow bellied toad and its potential threat *B. dendrobatidis*.

Keywords: Chytridiomycosis; Environmental DNA; RealTime qPCR; TaqMan

Introduction

Worldwide amphibian populations are experiencing significant decline and are on the brink of extinction. While disease and habitat loss are the primary drivers (Rachowicz et al., 2005), these deteriorations may also be influenced by intricate and often not entirely elucidated factors. These include climate change (Stuart et al., 2004; Corn, 2005), ultraviolet radiation (Kiesecker et al., 2001), pollution (Mirabile et al., 2009) in conjunction with predation and competition from introduced species (Beebee & Griffiths, 2005). The Anuran family Bombinatoridae includes three species occurring in Europe, i.e., *Bombina bombina* (Linnaeus 1761), distributed in eastern Europe, *Bombina variegata* (Linnaues 1758), occurring in Central and South-East Europe, and the Apennine yellow-bellied toad *Bombina pachypus*, endemic to the Italian Apennines. The Apennine yellow bellied toad, considered as a subspecies of *B. variegata* by some authors (Fijarczyk et al., 2011; Pabijan et al., 2013), is mainly distributed in hilly and mid-mountainous environments from 300 to 700m a.s.l. in the central-northern Apennines, and from 600 to 1000 m a.s.l. in the central-southern Apennines, with some populations reaching 1900 m a.s.l. on the Pollino massif (Guarino et al., 2007; Sindaco et al., 2006). It lives in a wide range of modestly sized water bodies, such as temporary pools, dead or stagnant loops of rivers and streams, sunny and shallow in forests and open areas (Guarino et al., 2007; Lanza et al., 2007; Scheele et al., 2014). Preferred habitats also include drinking troughs, fountains, irrigation tanks, irrigation channels, and other small artificial water pools found in human-modified habitats including non-intensive agriculture and pastures. This species breeds in temporary pools and other shallow and unshaded freshwater environments (Lanza 1983), spending the daylight hours in the water and feeding nocturnal activity is recorded on terrestrial invertebrates around the ponds (Guarino et al., 2007; (de

Meis et al., 2022). Larval development occurs in puddles (Mirabile et al., 2009). The species is experiencing a severe decline throughout its range, which is fragmented, and includes populations often consisting of no more than a few individuals (Doria & Salvidio 1994; Barbieri et al., 2004). As an example, Barbieri et al. (2004) revealed that from 1996 to 2004 the species disappeared from more than half of known locations. In the following years significant declines have also been recorded in the many areas of Central and Southern Italy (Bologna et al., 2000, 2007; Fiacchini, 2007; Guarino et al., 2007; Piazzini et al., 2005; Stagni et al., 2004). The Apennine yellow-bellied has not been yet recognized as a separate species from *B. variegata* by the IUCN Red List program, whereas it is listed as Endangered (EN) in the national IUCN Red List (Rondinini et al., 2022), and it is included in Appendix II of the Bern Convention and in Annexes II and IV of the European Union Habitats Directive (92/43/EEC). According to Article 17 of the Habitats Directive, the latest assessment confirmed an unfavourable conservation status and a declining trend for this species (Stoch and Grignetti, 2021). Among main threats to the species are the drying out of wetlands, particularly of still and stagnant waters, water pollution, and the effects of stochastic factors acting on small populations, which are a characteristic feature of this species (Bologna and La Posta 2004, Sindaco et al., 2006). The chytridiomycosis caused by the mycete *B. dendrobatidis* is an emerging infectious disease threatening amphibian populations and several authors have suggested that it is a primary driver of the worldwide amphibian declines (Berger et al., 1998; Daszak et al., 1999, 2003; Pessier et al., 1999; Skerratt et al., 2007). Although direct effects on *B. pachypus* have not been investigated yet, it has been suggested it could be responsible for its recent decline (Bologna e La Posta 2004, Sindaco et al., 2006). Environmental DNA (eDNA)-based analysis is one of the most promising techniques for large scale monitoring of biodiversity (Deiner et al., 2016; Ficetola et al., 2019; Pawlowski et al., 2020; Xia et al., 2018, 2021). The eDNA metabarcoding approach has been successfully applied to characterize freshwater communities from lakes, rivers, and wetlands environmental samples (Clusa & García-Vázquez, 2018; Edwards et al., 2018; Jerde et al., 2011; Thomsen et al., 2012; Thomsen & Willerslev, 2015). It is a highly rapid and non-invasive technique, able to detect cryptic, rare and elusive species where traditional survey techniques often failed (Hobbs et al., 2019). Moreover, it allows the detection of multiple species from the same environmental sample, opening the way to multispecies surveys without employing many specialized surveyors. Multispecific survey can be carried out with eDNA single species approach, consisting in developing species-specific primers and probe whose sensitivity has been recognized to be higher than other eDNA approaches (Harper et al., 2018; Xia et al., 2021). The eDNA has specifically been successfully applied to detect many amphibians occurring in European freshwaters, including European brown frog (*Rana temporaria* Linneaus, 1758) (Zanovello et al., 2023), American bullfrog (*Lithobates catesbeianus*) (Ficetola et al., 2008), European yellow bellied toad (*Bombina variegata* L., 1758) (Knudsen et al., 2023), great crested newt (*Triturus cristatus*) (Biggs et al., 2015; Harper et al., 2018), and the smooth newt (*Lissotriton vulgaris*) (Strandt et al., 2022). However, at date the genus *Bombina* and *B. dendrobatidis* have not yet been investigated together through eDNA techniques. To fill this gap, we aimed at: a) developing specific primers for eDNA detection of *B. pachypus*; b) detecting *B. pachypus* and *B. dendrobatidis* in twenty freshwater bodies of Central Italy from eDNA water samples and c) evaluating fine- and large-scale factors that could affect the eDNA detection of *B. pachypus* in running waters.

Material and methods

Primers development and validation

Primers for *B. pachypus* were developed accounting for intraspecific genetic variability of *B. pachypus* in Italy (Canestrelli et al., 2006), "false positives" that could occur by detecting congeneric species (e.g. *B. variegata*), and co-amplification of non-target species (Jamwal et al., 2021; Langlois et al., 2021). International molecular databases (BOLD Systems, GenBank NCBI) were then explored to identify the most common molecular markers (e.g. COI, CytB, NaDH, 16S; Thomsen et al., 2012) that could account for both intra- and interspecific genetic variability, phylogenetically proximate, and potentially syntopic taxa (Klymus et al., 2020). Each candidate marker DNA sequence in the reference databases was aligned using the MAFFT online tool (Katoh et al., 2009). Candidate marker DNA sequences were also evaluated for parameters required to enhance eDNA detection accuracy and specificity, including the presence of missing data or polymorphic sites, mismatch between taxonomic ranking and molecular assignment, and sequence length (Wilcox et al., 2013). High intra-specific conserved regions and high frequency of mismatches among congenerics guided the final selection (Klymus et al., 2020; Wilcox et al., 2013). Finally, the Forward and Reverse primers were identified within the suitable nucleotide regions using BioEdit (Hall, 1999). Potential primers were then subject to a three steps validation, i.e. *in silico*, *in vitro*, and *in situ* (Thalinger et al., 2021). For *in silico* validation, the following chemical-physical parameters affecting eDNA amplification were taken into account: annealing temperatures of the outer primers, ranging between 55 and 62 °C, a higher melting temperature for the probes of around 4 °C above that of the primers, about 50% of the nitrogenous bases G (Guanine) and C (Cytosine) in the docking regions, length of the primers (about 18-22 bp) and probe (about 25 bp), as well as the length of the amplicon (about 150 bp). This last parameter is relevant to avoid fragmentation that often affect eDNA (Jo, 2023; Langlois et al., 2021). The final alignment was created by considering 16s nucleotide sequencing belonging to all species of Anura occurring in Central Italy and to congeneric European species, for a total of 199 sequences (Supplementary text S1 - Supplementary table S1). Primers and probes that passed the first validation step were obtained from Eurofins Genomics and tested *in vitro* on *B. pachypus* tissue samples stored in the collection of University of Roma Tre. DNA (gDNA) was extracted from tissues using the commercial DNA Blood and Tissue Kit (Qiagen) following the manufacturer's instructions (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit). PCR reactions were set up using gDNA extracts as templates using only the external primers. To select the optimal annealing temperature of primer (Thalinger et al., 2021), a gradient end-point PCR run were conducted in 20-µL total volume reactions containing 4 µL of 10x Buffer solution (5x WonderTaq reaction Buffer EuroClone®), 1 µL of each primer (10 µM), 0.25 µL Taq polymerase (WonderTaq EuroClone®) and 2 µL of DNA template, under the following conditions: an initial step at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 60 seconds, with annealing temperature ranging from 50 to 60 °C for 45 seconds, elongation at 72 °C for 60 seconds, and a final extension at 72°C for 7 minutes. Agarose gel electrophoresis (2%) was used to verify the presence of a sequence corresponding in size to the expected target fragment (Wilcox et al., 2015). The sequence was then subjected to Sanger sequencing to verify the correct assignment of the reference sample to the target genetic region and species of interest by means of NCBI's BLAST tool (Altschul, 1997; Ye et al., 2012). In the final *in situ* validation step, the combination of external primers and probes were used to set up qPCR assays with TaqMan technology (Bylemans et al., 2016), including in each run i) PCR positive control: the gDNA extracts of *B. pachypus*; ii) environmental positive control: eDNA extracts deriving from water samples collected in two sites of ascertained presence of *B. pachypus* (Castelpizzuto (IS) and Castelpetroso (IS); iii) environmental negative control: eDNA extracts from water samples from two sites of ascertained absence of the species (tap water and water from a controlled *ex situ* sample (aquarius) not hosting the target species); PCR negative control: no template samples to verify the absence of

contamination with DNA of target or untargeted species during the reaction set-up steps (Karlsson et al., 2022; Klymus et al., 2020; Thalinger et al., 2021) (Supplementary table S2). Presence sites were sampled either in summer or fall, corresponding to maximum concentration of the species in water bodies (Di Cerbo et al. 2016). For *B. dendrobatidis,* primers and a probe developed from Canessa et al., (2017) were used following the protocols by (Kamoroff & Goldberg, 2017) and a positive sample stored at ZooPlantLab at University of Milano-Bicocca. To evaluate primers and probe quality, the amplification efficiency (E) and limit of detection (LOD) were calculated, according to (Bustin et al., 2009) and (Klymus et al., 2020). To generate the standard curve, tenfold serial dilutions of quantified positive tissue controls were used, at the starting concentration of 290 ng/µl in both cases.

eDNA sampling

B. pachypus and *B. dendrobatidis* were searched at 20 sampling sites, both lentic (n = 14) and lotic (n = 6) within the distribution range of the species in West-Central Italy (Sindaco et al., 2006). Specifically, five sites were located in isolated pools in the lowland plains (n=2) and mountain uplands (n=3) of Latium region, and along the rivers Aniene, Tiber, Velino, Turano, Salto, and Liri-Garigliano (Supplementary figure S1).
Sampling sessions were run in July and August 2021, and in May and October 2022. For 13 sites, two sampling replicates in 2021 and 2022 were run, for a total 33 samples collected in the two years (Supplementary table S3). At each site, 3 L of water were collected by immersing a sterilized glass or plastic bottle to a depth of 20 cm from the water surface, using disposable gloves. For lentic waters, 200 ml of water were collected every 20 m along 300 m of shoreline. In case of high turbid water near the shore, samples were collected by walking in the water using boots and quickly (1 second) dipping the bottle at a distance of 1.5 m (Di Cerbo et al., 2016). Water samples were filtered either directly in the field or in laboratory. Field filtering was run by connecting a car inverter to a vacuum pump (KNF Laboport, N86 KN.18, 6l/min) coupled to a 5-litre jar and a 3-station filtration ramp (Fig. 1).

Figure 1. *Filtering operations run in the field. An inverter was connected to the car battery to provide power for the three-rump pump (Photo A. Loy)*

A sterilized metal mesh strainer was used to pre-filter out coarse materials and prevent clogging of the filter (e.g., algae, leaves, etc.). Three 0.22 µm pore size cellulose mixed ester filters (Millipore, 47 mm diameter, individually packed by the manufacturer) were placed on the sterilized ramps. Each filter was used to filter 1 L of water. Tweezers, filtration ramp and strainer were sterilized with alcohol and flame to be reused at the next site. The three filters were placed in a sealed and labelled petri dish and placed in a portable refrigerator to be transferred within 12 hours in the laboratory and stored at -21 °C. When field filtering was not possible, water samples were transferred to the laboratory, stored overnight in a fridge, and filtered the next day under a laminar flow hood using sterile instruments. Under conditions of high-water turbidity, multiple filters were used for the same sample. In this case, the extraction process was conducted separately for each filter and the extracted DNA pooled in one spin column (Schmidt et al., 2021). Prior moving to another sampling site, bottles were washed with domestic tap water for three times, then with distilled water with 1:10 concentration of chlorine, and rinsed three times with environmental water in the proximity of the next sampling site.

eDNA extraction and amplification

The eDNA was extracted from the filters using DNeasy PowerSoil Pro kit (Qiagen®) following the manufacturer's instructions (available at https://www.qiagen.com/us/products/discovery-andtranslational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powersoil-pro-kit). Multiple serial dilutions of each eDNA extract (from 10^{-1} to 10^{-3}) were used with three replicates

in order to reduce the presence of false negatives caused by the possible presence of inhibitors and assess the concentration threshold of detection (Jamwal et al., 2021; Klymus et al., 2020; Langlois et al., 2021). The qPCR analysis were run on a RealTime PCR, StepOnePlus (Applied Biosystem™). Each run included an environmental water sample from ascertained location of *B. pachypus and B. dendrobatidis,* and negative (no template) controls, all run in triplicate. The amplification reaction conditions were the same set for both assays. 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. The qPCR analysis was performed in 10 µL reaction mix containing 5 µL of buffer, 0.25 µL of each primer (10 µM), 0.15 µL of dual-labelled (5'-FAM, 3'- MGBEQ) Taqman Probe (10 µM) and 2 µL of eDNA template. To ensure that the absence of *B. pachypus* eDNA in water samples was not due 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 untargeted DNA metabarcoding characterization of fishes (Miya et al., 2015).

eDNA extracts of water samples from a site of ascertained absence of *B. pachypus* (Rome) and *B. dendrobatidis* (Rome), and the likely occurrence of heterogeneous DNA and inhibitors was added as environmental negative control sample. Ct (Threshold Cycles) values were converted into counts (DNA copies) using the formula Counts = E^(40-Ct), where E is the efficiency of amplification (ref). We considered a negative sample (no Apennine yellow bellied toad or fungus detection) for DNA counts/microL < 5. We considered a reliable result when at least ⅔ replicas were in concordance. To avoid any possible contamination with exogenous DNA, the pre and post amplification phases were carried out in separate rooms, and all steps were conducted in a laminar flow cabinet.

Environmental variables at running water sites.

Six environmental variables that could influence species and/or eDNA resilience were recorded in the field in 2022 at each sampling sites on running waters. These included water and ambient temperature, average pH, nitrates concentration, water speed, and water depth, and river width (Supplementary table S4). We also recorded 16 large scale environmental parameters from digital maps that could potentially affect the species occurrence. Variables included Enhanced Vegetation Index (EVI), altitude, aspect, slope, Euclidean distance from eight land use variables (inland marshes, water bodies, water courses, lakes, agricultural areas, artificial surfaces, forests and semi-natural areas), and five bioclimatic variables, i.e. annual temperature (BIO2), maximum temperature of the warmest month (BIO5), average temperature of the warmest quarter (BIO10), precipitation during the wettest quarter (BIO16), and precipitation during the coldest quarter (BIO19). Vector layers were converted to raster format with a resolution of 250 m. (Supplementary table S4).

Principal Component Analyses (PCA) were run separately for variables measured *in situ* and those obtained from maps to explore the relationship presence or absence of *B. pachypus* eDNA.

Results

eDNA assay development and validation

The 16S sequences of primers and probes for *B. pachypus* that successfully passed the three validation steps are reported in Tab. 1. In detail, primer specificity was evaluated *in silico* aligning 199 sequences of *Bombina pachypus* and 7 other species belonging to *Bombina* genus, downloaded on 8 April 2022 (Supplementary text S1 - Supplementary table S1). We searched for areas of the sequence that were highly conserved intra-specifically but highly diversified from the sequences of aligned congeneric species. They amplify a DNA fragment of 166 bp, located in the position 165-330. Efficiency of amplification on tissue sample (*in vitro*) was 89.5%. LOD estimated was 2.90E-03 ng. The couple of

primers with probe showed successfully amplified the two environmental samples of ascertained presence of the species and showed no amplification in the field negative controls (environmental samples of ascertained absence of the species).

Table 1. *Sequences, length of the amplicon, molecular marker, and annealing temperature for the forward and the reverse primers and the probes for B. pachypus that successfully passed the three validation steps*.

Molecular marker	PRIMER FW (5'-3')	PRIMER REV (5'-3')	PROBE (5'-3')	Length (bp)	Annealing T°
16s (mtDNA)	CCTAATTCACCCCTTAGGG	CTGATTGGATCAGTGTGTT	[FAM]CCCTCCGAGATGAAAGGGAC[MGBEQ]	166	59 °C

eDNA survey outcomes

Environmental DNA extracted from filters from the 33 water samples allowed to detect *B. pachypus* in 13 samples, collected at 12 over 20 sites (60%) (Fig. 2, Supplementary table S3).

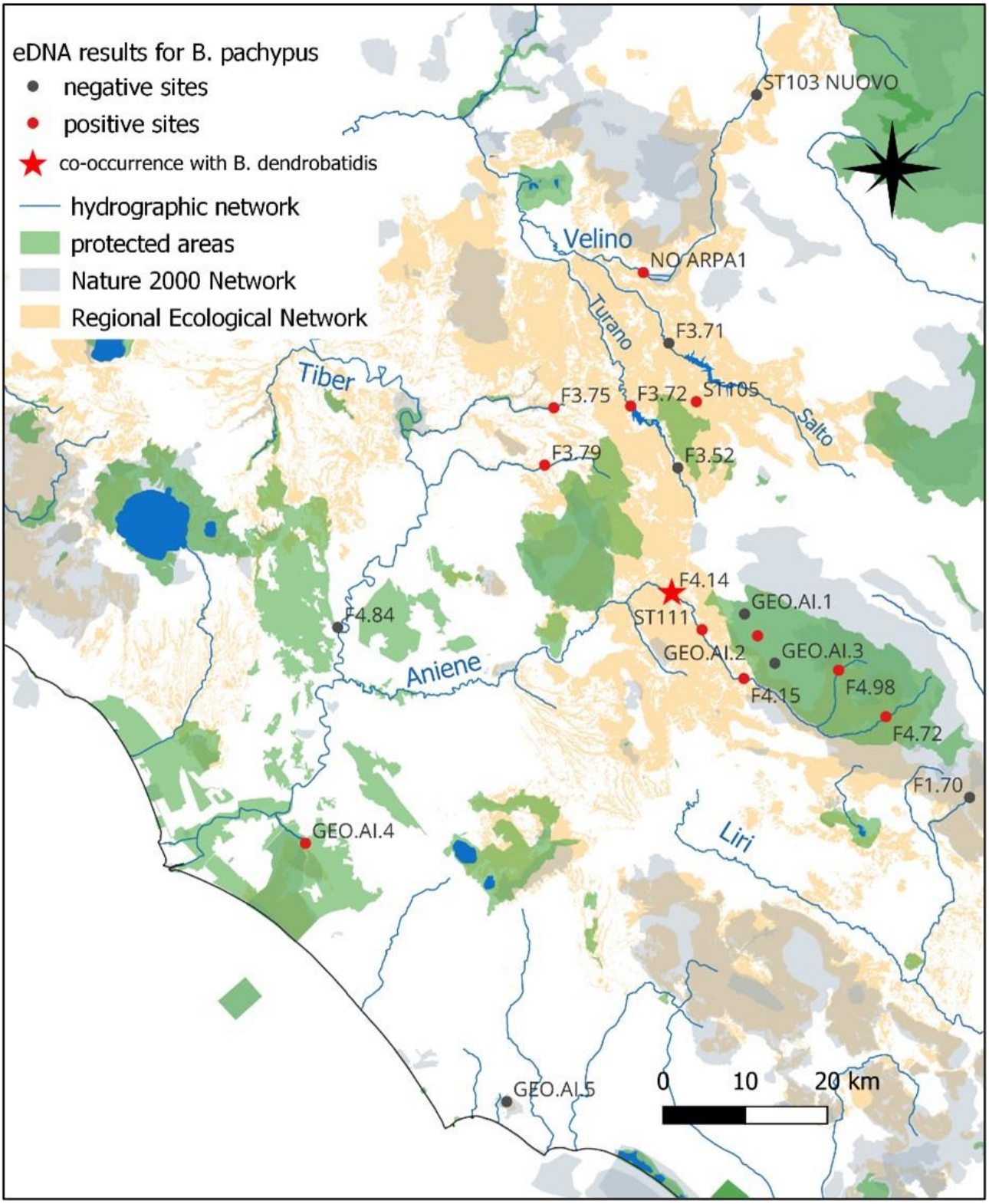

Figure 2. *Environmental DNA detection of Bombina pachypus (red circles) and Batrachochytrium dendrobatidis (red star, co-occurring with B. pachypus). Black circles indicate negative sites for both species.*

B. pachypus was detected both freely in water and bound to suspended sediments. At least one positive site was found in each river surveyed but the Liri river, where the only tested site was negative (Fig. 2). Nine over twelve positive sites were new occurrences for the species, most of which (n = 8) located outside Natura 2000 Network sites. Positive sites included 57% out of 14 running waters sites (n= 8), and 50% over six standing water sites (n = 3). The latter included a pool in a lowland protected area (GEO

AI4, Piscina Malpasso in Castelporziano State Reserve, Fig. 3) where *B. pachypus* was considered extinct since 1997 (Bologna et al. 2007, D. Cecca e D. Capizzi, com. pers.). The remaining 11 positive sites were in both areas of known occurrence of the species (n=5; Marini et al., 2021) where the presence needed confirmation (n=6; Sindaco et al., 2006; Lanza et al., 2007; Marini et al., 2021), although they might represent new point locations. Positive sites were concentrated in the Tiber River basin (n = 9), where detection rate was very high (9/12= 75% positive sites).

Figure 3. *The sampling site GEO AI4, Piscina Malpasso in Castelporziano State Reserve where B. pachypus was detected through eDNA after having been declared extinct in the last century (Photo S. Giovacchini).*

The pathogen *B. dendrobatidis* was only detected at one site along the Aniene river (site F4.14 in Fig. 2), where it co-occurred with Apennine yellow bellied toad. Most detections of *B. pachypus* (n = 8, 66%) were from samples collected in autumn, followed by spring and summer (n= 2 each); only one site was found positive both in summer and autumn (F4.15 on the Aniene river). All sites found positive in autumn were from running waters (rivers Velino, Aniene, Turano, Salto, and Tiber), those found positive in spring from standing waters (GEO.AI.2 and GEO.AI.4) (Supplementary figure S1). Fig. 4 shows the scatter plot of the first two principal component scores from variables collected in the field (Fig. 4, top) and derived from maps (Fig. 4, bottom) for the 14 sites of lotic waters, accounting for 61.9% (PC1 = 32.6; PC2 = 29.3) and 57.4% (PC1 = 38.4; PC2 = 28.4) of cumulative variance, respectively. Scatter plots showed a clear clustering of negative with respect to positive sites, the latter showing a higher environmental variability (Fig. 4, Supplementary table S6).

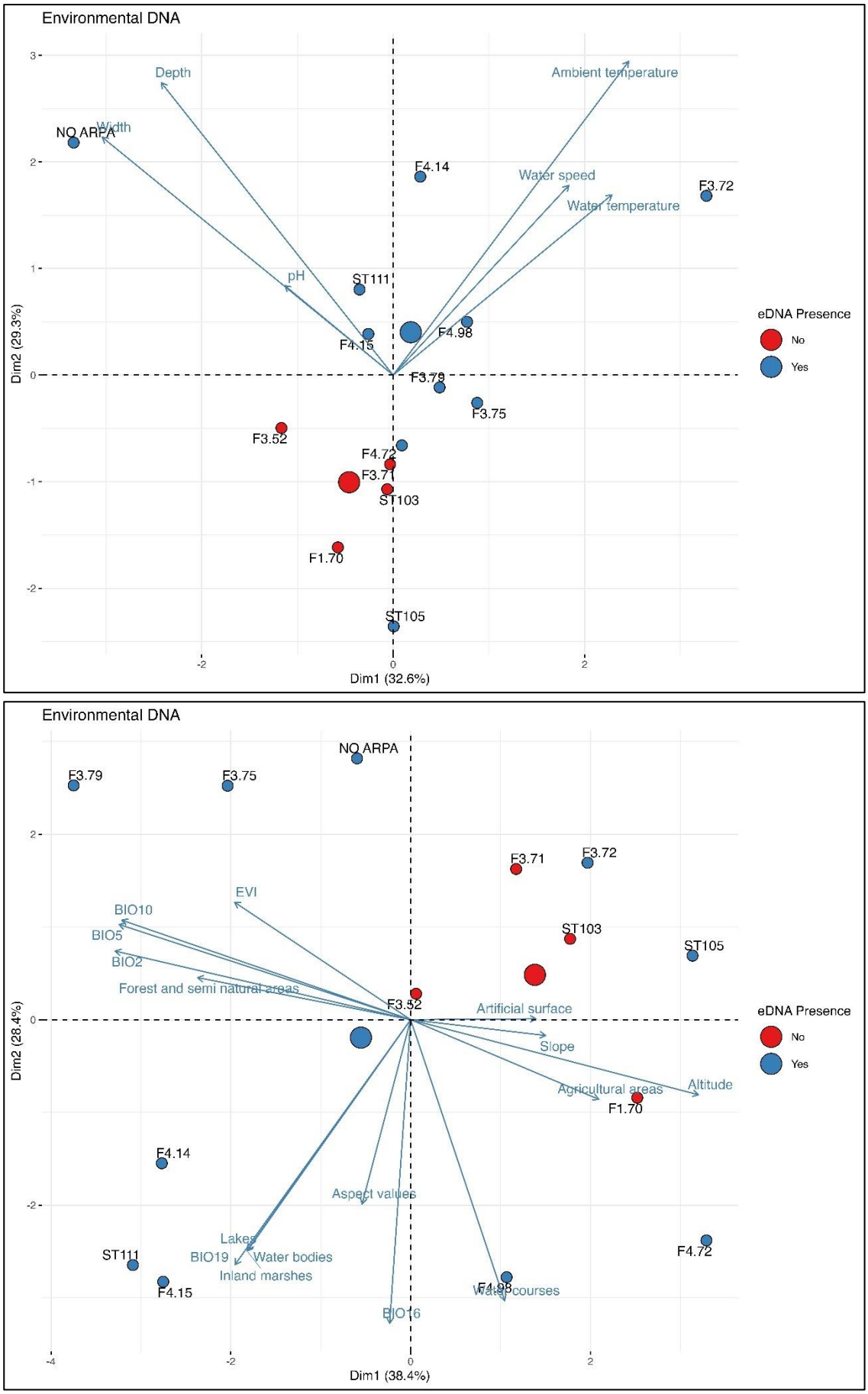

Figure 4. *Scatter plot of the first two PC scores derived from seven environmental variables collected in the field (top) and 16 variables derived from digital maps (bottom) for 14 sampling sites in lotic waters. Small circles*

indicate the presence (blue) and absence (red) of B. pachypus; large circles indicate the corresponding average score.

Biplots of variable vectors superimposed to scatter plots revealed that negative and positive sites mainly differed by ambient and water temperature ($mean_N$ = 13.5 ± 1,29 and 12.0 ± 1.63 in negative sites *vs* $mean_P$ = 18.5 ± 3.75 and 14.30 ± 3.30 in positive sites respectively), water depths ($mean_N$ = 0.43 m ±0.22 vs $mean_P$ = 0.59 m ± 0.57) river width ($mean_N$ = 5.75 ± 6.18 m *vs* $mean_P$ = 8.0 m ± 8.0), temperature during the warmest months (BIO 05 $mean_N$ =25.9°C ± 1.27 vs $mean_P$ = 27.21 °C ± 2.06) and warmest season (BIO 10, $mean_N$ = 19.5°C ± 0.9 vs $mean_P$ = 20.53 °C ±1.45), and by variation in diurnal temperature along the year (BIO 02 $mean_N$ = 8.63°C ±0.2 vs $mean_P$ = 9.18 °C ± 0.56) (Fig. 4, top, Supplementary table S6-S7).

Discussion

The 16S eDNA probe developed for the Apennine yellow bellied toad (*B. pachypus*) allowed to successfully detect the species at 60% (n = 12) of sites, confirming eDNA as a valuable technique for large scale surveys of this rare and endemic toad. Detection capabilities were also highlighted by a presence record in a pool where the species was considered extinct since 1997 (Bologna et al., 2007). However, the species was reported in neighbouring Nature Regional Reserve of Decima Malafede (Bologna et al., 2000) which is bordering with the State Reserve and connected through 1,019 meters of Fosso dell'Ortaccio stream. Also, as eDNA detection. might be due to old DNA remained bound to the sediment mobilized from the bottom by convective water motions (Barnes & Turner, 2016), further field surveys are needed to confirm the presence of the species in the pool. The high detection rate suggests *B. pachypus* is in a good conservation status in the study area, evidence also supported by the low detection rate of the pathogen fungus *B. dendrobatis*. The fungus was in fact only detected at one site (F4.14 in Fig. 2). However, since it co-occurred with *B. pachypus* at a site located along the river Aniene where the species was also detected at other four sites upstream (ST111, F4.15, F4.98, and F4.72 in Fig. 2), this finding claims for a rapid disinfection to prevent the further spreading of the fungus along the river (Thumsová et al., 2024). Detection rate for *B. pachypus* was higher for lotic compared to lentic waters, suggesting standing waters be more exposed to pollution and habitat degradation (Fiacchini, 2007; Mirabile et al., 2009), but also characterized by lower pH levels (mean PH = 7.00 *vs* 7.46 for standing and running waters respectively) and high water temperatures (mean = 26.2 °C vs 14.8 °C for standing and running waters respectively) that could favour eDNA degradation (Klymus et al., 2020). Occurrence of *B. pachypus* in standing waters was only detected in summer, whereas in running waters it was mainly detected during the autumn sampling. High detection in rate in standing waters during summer is easily explained by the reproductive biology of the species, as *B. pachypus* is known to reproduce mainly in temporary water bodies from April to September (Barbieri et al., 2004; Guarino et al., 1998; Mirabile et al., 2009), and that highest probability of finding adults in the water occurs from May to July (Di Cerbo et al., 2016). Instead, the high, and higher, detection rate in running waters during autumn was only partially explained by the biology of the species. PCA evidenced positive and negative sites differed mainly by local and climate temperature conditions, i.e. positive sites showed higher mean ambient temperature (18.5 °C) and mean water temperature (14.30 °C), as well as higher mean annual temperature (9.18 °C), higher maximum temperature during the warmest months (27.21 °C), and higher mean temperature during the warmest season (20.53 °C). These evidence are in agreement with the thermal needs highlighted by a suitability model for *B. pachypus* provided by Bernabò et al. (2022), i.e. high temperature during summers and high annual temperature range, that were related to

post-metamorphic survival and reproduction thermal requirements (Cayuela et al., 2016). However, in contrast with these authors, our analyses did precipitations to only influenced a limited number of positive sites along PC2, and a concentration of positive sites at low altitudes (mean = 496 m a. s. l.) compared to the suitable range of 1000 -1250 m a.s.l. reported by (Bernabò et al., 2022). However, Bologna et al. (2000) already reported the occurrence, although rare, of *B. pachypus* at low altitudes in the Latium region. Future field survey will help to either confirm the species occurrence at low altitudes, or assess if it could result from eDNA flowing from upstream tributaries (Deiner et al., 2016). Finally, the higher detection reported characterized by higher water depths (0.59 m) and river width (8.0 m) at sampling points is more difficult to explain in terms of either eDNA dilution or probability of occurrence of any life stage of the species and will deserve further investigations.

Concluding remarks

Primers specifically designed for *B. pachypus* were able to accurately depict the distribution and conservation status of the species in the study area. We confirmed the strict thermal needs of the species highlighted by Bernabò et al (2022), thus confirming the risk posed by the global warming, and the need of a continuous monitoring of the populations and their habitat quality. In this context, the eDNA-based assay developed proved to be a valuable and effective tool for monitoring *B. pachypus* and *B. dendrobatis* at a large scale. Future field surveys will likely help to identify factors affecting eDNA resilience and diluition due to water flowing and identify the degree of accuracy for fine scale monitoring of both species using eDNA approach.

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Supplementary material

Suppl. Text S1. *GenBank accession numbers of the Bombina pachypus 16S sequences used to evaluate species specificity of the assay. The sequences were downloaded on 08 04 2022. Each sequence was trimmed to match only the region targeted by the assay. In bold are reported the local sequences.*

EU531353 **AY500227 AY500226 AY500228 AY500225**

Suppl. Table S1. *List of GenBank from 16S congeneric species used to check species specificity of the assay.*

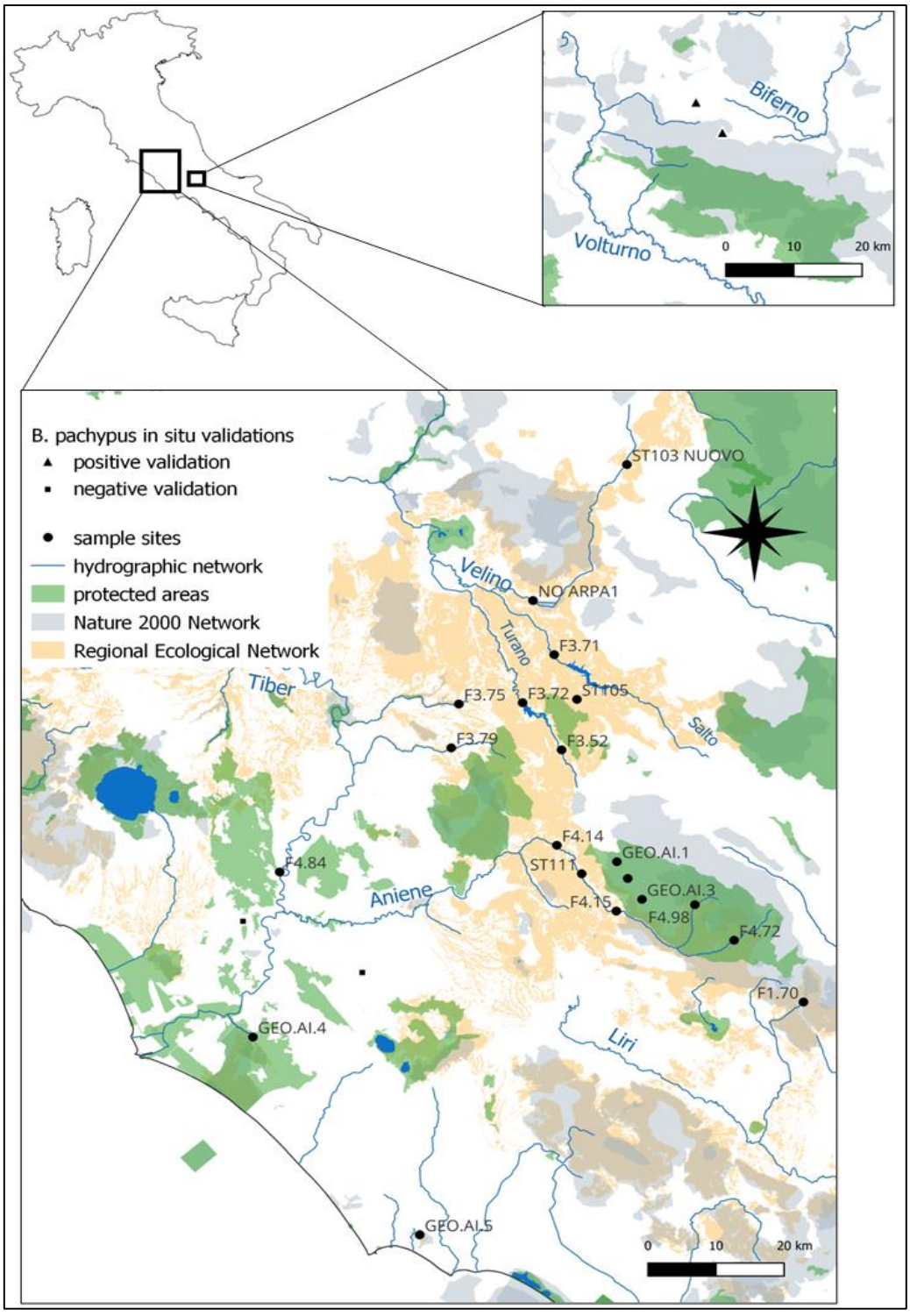
Bombina variegata	Bombina bombina	Bombina orientalis	Bombina maxima	Bombina microdela-digitora	Bombina lichuanensis	Bombina fortinuptialis
AY333694 AJ440764 KY496592 KY496591 KY496590 AJ440803 KY496589 KY496588 EU531355 EU531354 AY333691 AY333699 AY333692	MH105110 AJ440762 AJ440801 AY333701 AY333695	JQ815303-KR869605-KR869607- KR869591-KR869577-KR869590 KR869587-KR869606-KR869608- KR869627-KR869625-KR869614 KR869626-KR869624-KR869623- KR869595-KR869528-KR869621 KR869520-KR869615-LC640571- EU531351-EU531343-EU531337- DQ925775-DQ925774-AY333698- JQ815301JQ815299-KR869545 KR869620-KR869594-KR869547- KR869529-KR869618-KR869523 KR869596-KR869514-KR869521- EU531332-AY364368-EU531347 KR869617-KR869603-KR869538- JQ815300-KR869598-KR869589 KR869602-KR869539-KR869540- KR869543-KR869599-KR869604 KR869586-KR869513-KR869613- KR869611-KR869609-KR869612 KR869610-KR869517-JQ815302- KR869593-KR869584-KR869544 KR869533-KR869532-KR869542- KR869600-KR869585-KR869534 KR869583-KR869581-KR869576- KR869569-KR869559-KR869554 KR869567-KR869566-KR869564- KR869562-KR869560-KR869549 KR869578-KR869563-KR869574- KR869582-KR869579-KR869580 KR869573-KR869572-KR869568- KR869565-KR869550-KR869557 KR869551-KR869558-KR869556- KR869553-KR869541-KR869616 KR869597-AY333696-KR869537- KR869588-X86227-KR869524 KR869622-KR869536-KR869516- KR869519-KR869526-KR869518 KR869515-KR869575-KR869571- KR869570-KR869619-AJ440802 KR869527-X86295-AY364346- KR869535-KR869525-KR869592	EU531349 EU531348 EU531346 EU531335 DQ925770 DQ925765 DQ925776 DQ925764 AY333697 EU531344 EU531345 EU531342 EU531336 DQ925777 DQ925780 EU531339 DQ925768 DQ925769 DQ925773 AJ440761 AJ555151 AJ440800	DQ925778 DQ925761 DQ925760 EU531338 DQ925771 EU531352 DQ925772 EU531350	EU531341 EU531334 DQ925766 DQ925767	EU531333 DQ925779 DQ925759

		KR869561-AJ440763-KR869546-KR869548-KR869531-KR869555 KR869522-KR869552-X86261-				

Suppl. table S2. *Positive control (PCS) and negative control (NCS) sites sampled for in situ validation of probes for B. pachypus and B. dendrobatidis.*

Target	**Coordinates**	**River basin**	**Water body**	**Locality**	**Sampling date dd/mm/yy**
PCS *B. pachypus*	41.50211, 14.34536	Biferno	Water dwell	Castelpizzuto	13/5/2022
PCS *B. pachypus*	1.5479, 14.30183	Volturno	Water dwell	Castelpetroso	18/5/2022
NCS *B. pachypus*	41.93355, 12.42077	-	Tap water	Roma	5/11/2022
NCS *B. pachypus*	41.85086, 12.63037	-	Ex situ aquarius with non-target species	Roma	7/7/2022
PCS *B. dendrobatidis*	NA	NA	NA	NA	NA
PCS *B. dendrobatidis*	NA	NA	NA	NA	NA
NCS *B. dendrobatidis*	41.93355, 12.42077	-	Tap water	Roma	5/11/2022
NCS *B. dendrobatidis*	41.85086, 12.63037	-	Ex situ aquarius with non-target species	Roma	7/7/2022

Suppl. Figure S1. *Study area showing the location of water sampled for in-situ validation (index map) and location of sampling sites for eDNA. Ecological corridors belong to the Regional Ecological Network.*


B. pachypus in situ validations
positive validation
negative validation
sample sites
hydrographic network
protected areas
Nature 2000 Network
Regional Ecological Network
Biferno
Volturno
0
10
20 km
Velino
Turano
Tiber
Aniene
Salto
Liri
ST103 NUOVO
NO ARPA1
F3.71
F3.75
F3.72
ST105
F3.79
F3.52
F4.14
GEO.AI.1
ST111
GEO.AI.3
F4.15
F4.98
F4.72
F4.84
F1.70
GEO.AI.4
GEO.AI.5

Suppl. Table S3. *Details of sampling sites and results for B. pachypus detection. PA (Protected areas): N2K = Natura 2000 Network (SITE ID); SNR = State Nature Reserve; REN = Regional Ecological Network. Result: P= positive, N = negative. R = Running waters; S = Standing waters.*

Site_ID	Coordinates (WGS 84 EPSG: 4326)	Sampling Locality	Water body	R/S	River basin	PA	Sampling date mm/dd/yy	Result
F1.70	41.70224, 13.68757	Collepardo	Cosa Creek	R	Liri - Garigliano	N2K(IT6050008)-REN	08/11/2021 10/24/2022	N
F3.52	42.15759, 13.00641	Pozzaglia Sabina	Turano River	R	Tiber	REN	08/09/2021 10/22/2022	P
F3.71	42.29449, 13.0023	Concerviano	Salto River	R	Tiber	REN	08/09/2021 10/17/2022	N
F3.72	42.22825, 12.94169	Rocca Sinibalda	Turano Lake	S	Tiber	REN	08/14/2021 10/17/2022	P
F3.75	42.23023, 12.82833	Monteleone Sabino	Farfa Creek	R	Tiber	REN	07/23/2021 10/14/2022	P
F3.79	42.16732, 12.81097	Scandriglia	Fosso Corese Creek	R	Tiber	REN	07/23/2021 10/14/2022	P
F4.14	42.0218, 12.99009	Roviano	Aniene River	R	Tiber	REN	06/26/2022 10/22/2022	P
F4.15	41.92516, 13.08988	Subiaco	Aniene River	R	Tiber	REN	08/11/2021 10/24/2022	P
F4.72	41.87501, 13.295	Filettino	Aniene River	R	Tiber	N2K (IT605000) – REN	08/11/2021 10/24/2022	P
F4.98	41.92828, 13.22893	Vallepietra	Simbrivio Creek	R	Tiber	N2K (IT605000) - REN	08/11/2021 10/24/2022	P
F4.84	41.99987, 2.49631	Roma	Fosso della Mola Creek	R	Tiber	-	06/27/2022	N
NO ARPA1	42.61081, 11.7699	Cittaducale	Velino River	S	Tiber	N2K (IT601001)	10/19/2022	P
GEO.AI.1	41.99474, 13.0947	Cervara di Roma	Pool	S	Tiber	SNR (PNR Monti Simbruini) - N2K (IT605000)	05/25/2022	N
GEO.AI.2	41.97015, 13.11252	Campaegli	Pool	S	Tiber	SNR (PNR Monti Simbruini) - N2K (IT605000)	05/25/2022	P

GEO.AI.3	41.93927, 13.13568	Subiaco	Pool	S	Tiber	SNR (PNR Monti Simbruini) - N2K (IT605000)	05/25/2022	N
GEO.AI.4	41.76482, 12.43576	Roma	Piscina Malpasso Pool	S	Tiber	SNR (RNS Tenuta di Castelporziano) - N2K (IT603008) – REN	05/27/2022	P
GEO.AI.5	41.47254, 12.7144	Nettuno	Piscina di Vallone Cupo Pool	S	Tiber	N2K (IT603004) – REN	05/29/2022	N
ST103 NUOVO	42.56249, 13.14963	Cittareale	Velino River	R	Tiber	-	20/10/2022	N
ST105	42.22971, 13.03883	Marcetelli	Fosso della Foresta Creek	R	Tiber	REN	08/09/2021 10/20/2022	P
ST111	41.97978, 13.03125	Agosta	Aniene River	R	Tiber	REN	08/11/2021 10/22/2022	P

Suppl. Table S4. *Details and source of data for environmental variables collected in the field and from digital map layers*.

Variable	From field (F) or maps (M)	Source	Measure used
Air temperature	F	Suunto portable watch after 30 minutes of acclimatization	°C
Water temperature	F	Deeper Pro portable sonar	°C
Maximum riverbed depth	F	Deeper Pro portable sonar, measured along straight river stretches with no curves.	Average value (m)
PH	F	Test strips, average values	Average value (range 6,4 - 8,4)
Water speed	F	Pin ball attached to a 10 m wire floating in the main reachable current stream. Three replicates	Average time to flow 10 m (s/m)
River width	M	Google Earth Pro maps	m
Mean annual temperature (BIO2)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Maximum temperature of the warmest month (BIO5)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Average temperature of the warmest quarter (BIO10)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Precipitation during the wettest quarter (BIO16)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Precipitation during the coldest quarter (BIO19)	M	CHELSA (https://chelsa-climate.org/)	Rasterized at 250 m
Vegetation Index (EVI)	M	MODIS (https://lpdaac.usgs.gov/products/mod13q1v006/)	Rasterized at 250 m
Altitude	M	Digital Elevation Model (DEM) from CGIAR-CSI (http://www.cgiarcsi.org/2010/03/108/uot;http:/srtm.csi.cgiar.org)	Rasterized at 250 m
Aspect	M	Digital Elevation Model (DEM) from CGIAR-CSI	Rasterized at 250

		(http://www.cgiarcsi.org/2010/03/108/uot;http:/ srtm.csi.cgiar.org)	m
Slope	M	Digital Elevation Model (DEM) from CGIAR-CSI (http://www.cgiarcsi.org/2010/03/108/uot;http:/ srtm.csi.cgiar.org)	Rasterized at 250 m
Distance from agricultural areas (2)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from artificial surfaces (1)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from forests and semi-natural areas (3)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distancer from marshes (4.1.1.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from water bodies (5.1.2.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from water courses (5.1.1.)	M	CORINE Land Cover from Copernicus (https://www.copernicus.eu/it).	Rasterized at 250 m –Euclidean distance
Distance from lakes	M	Hydrosheds https://www.hydrosheds.org/	Rasterized at 250 m –Euclidean distance

Suppl. Table S5. *Sequences, length of the amplicon, molecular marker, and annealing temperature for the forward and the reverse primers and the probes for primers and probe developed by Canessa et al. (2017) for Batrachochytrium dendrobatidis.*

Molecular marker	PRIMER FW (5'-3')	PRIMER REV (5'-3')	PROBE (5'-3')	Length (bp)	Annealing T°
ITS1	CCTTGATATAATACAGTGTGCCAT	AGCCAAGAGATCCGTTGTCAAA	[FAM]CGAGTCGAACAAAAT[MGBEQ]	121	60 °C

Suppl. Table S6. *Eigenvector coefficients of the first six PCs derived from nine environmental variables recorded in situ (above), and for the first seven PCs derived from 16 environmental variables derived from digital maps (below).*

Field variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
Water Temperature	**0,410312**	0,32028	0,448583	-0,51096	0,142171	-0,49653
Ambient Temperature	**0,441681**	**0,557143**	0,138422	0,145192	-0,2211	0,636696
pH	-0,203	0,158848	0,581276	0,715845	0,180442	-0,22513
Water Speed	0,329567	0,337025	-0,63039	0,384541	-0,02174	-0,48173
Depth	**-0,43484**	**0,519331**	-0,20966	-0,18329	0,660862	0,164082
Width	**-0,54558**	**0,42248**	0,019442	-0,15472	-0,67907	-0,19598

Large scale variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7
BIO 02	**-0,37928**	0,099348	0,127323	-0,11397	-0,0697	0,045233	-0,0698
BIO 05	**-0,37436**	0,137875	0,041733	-0,17585	0,017788	0,074676	-0,0898
BIO 10	**-0,37082**	0,14411	0,01405	-0,19498	0,037191	0,07094	-0,09864
BIO 16	-0,0269	**-0,43993**	0,083001	0,096794	0,18648	0,199099	-0,18735
BIO 19	-0,22551	**-0,35486**	-0,0524	-0,02302	0,215042	0,237192	-0,23109
Agricultural areas	0,241344	-0,11535	-0,32833	-0,24646	-0,21054	-0,37822	-0,6687
Artificial surface	0,160909	0,001257	0,636006	0,254883	0,194923	0,108039	-0,18536
Forest semi natural areas	-0,27349	0,060956	-0,06574	0,482342	-0,0748	-0,50357	0,000607
Inland marshes	-0,20984	**-0,33521**	0,151557	-0,23406	-0,00479	-0,34716	0,274348
Water bodies	-0,20984	**-0,33521**	0,151557	-0,23406	-0,00479	-0,34716	0,274348
Water courses	0,120728	**-0,40698**	0,136632	0,085536	-0,20416	-0,09507	-0,02792
Dem	**0,369585**	-0,10882	-0,00158	0,214707	0,006473	-0,12984	0,286327
Slope	0,17338	-0,02302	-0,20748	-0,25282	0,811646	-0,16204	0,064532
Aspect values	-0,06228	-0,26704	-0,52822	0,106723	-0,16139	0,384403	0,31971
Lakes	-0,20914	**-0,33302**	-0,05327	0,336041	0,072853	0,058546	-0,25922
EVI	-0,22600	0,170036	-0,25705	0,445639	0,304327	-0,19685	-0,02103

Suppl. Table S7. *Environmental variables recorded in the field (F) and from digital maps (M) at the 20 sampling sites*.

site ID	F1.70	F3.52	F3.71	ST103 NUOVO	F3.72	F3.75	F3.79	F4.14	F4.15	F4.72	F4.98	NO ARPA 1	ST105	ST111	F4.84	GEO.AI.1	GEO.AI.3	GEO.AI.6	GEO.AI.4	GEO.AI.5
R/S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S
B. pachypus	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
PH	7,60	7,20	6,80	7,60	6,80	7,60	8,00	7,60	7,60	7,60	7,60	7,60	6,80	7,60	8,00	6,40	7,60	8,00	7,60	6,40
Water speed (m/s)	0,345	0,182	1,053	0,510	0,640	0,637	0,161	0,909	0,787	0,775	1,064	0,402	0,097	0,641	0,488	-	-	-	-	-
Water depth (m)	0,3	0,5	0,7	0,2	0,5	0,2	0,3	0,9	0,5	0,3	0,5	2,1	0,1	0,5	0,5	1	0,6	0,5	0,5	0,9
river width (m)	2	15	3	3	-	2	2	11	10	3	3	25	1	15	14	-	-	-	-	-
T amb °C	12	14	13	15	25	18	19	23	19	16	20	15	12	18	39	22	24	27	36	25
T water °C	12	14	10	12	22	15	17	14	11	11	12	13	13	15	31	21	26	30	29	25
EVI	2453,2	2097,3	1705,8	2473,1	1632,3	2567,5	3202,7	2617,9	2189,1	1853,8	1848,8	2787,7	2299,9	2265,5	2101,36	2544,48	3886,25	1164,74	2683,27	27,99
Altitude	750,0	550,2	469,2	779,3	582,9	302,2	272,5	346,0	376,3	767,7	763,0	425,5	792,5	334,2	20,57	1290,14	19,76	1337,33	24,82	1413,65
Slope	12,4	1,8	2,0	2,5	13,6	3,3	2,7	11,4	4,7	7,3	4,0	5,1	11,2	0,8	0,82	9,81	0,86	5,01	0,28	1,69
Aspect	295,4	234,7	48,5	93,3	81,5	267,7	239,8	215,5	224,4	322,5	325,0	41,8	79,3	201,3	111,77	236,72	35,81	223,72	160,06	98,12
BIO 02	7,7	9,2	9,0	8,6	8,7	10,1	10,0	10,0	9,8	7,7	8,3	9,6	7,8	9,9	10,5	6,7	9,4	6,6	9,1	64
BIO 05	24,5	26,9	27,0	25,1	26,6	29,3	29,5	28,7	28,2	23,9	24,9	27,8	24,6	28,5	31	22	29,4	21,6	30	21,1
BIO 10	18,7	20,2	20,4	18,8	20,1	22,0	22,2	21,6	21,1	18,2	18,8	20,9	18,8	21,4	23,48	16,5	23,03	16,27	23,6	15,72
BIO 16	310,0	268,3	259,3	268,0	245,3	234,0	236,0	301,8	323,0	293,0	300,0	250,0	258,0	319,0	230,64	254	296	260	356	257
BIO 19	200,0	168,0	157,7	145,0	148,0	166,0	174,0	220,8	232,5	180,7	185,0	155,0	144,0	234,0	190,05	147	249	153	291	150
Agricultural areas	353,6	250,0	500,0	1,0	500,0	1,0	0,0	1,0	250,0	2517,8	1,0	1,0	957,1	1,0	250	2207,11	1	1560,66	707,11	2500
Artificial surface	14914,2	8985,3	16263,5	27278,2	14849,2	10621,3	3103,6	11253,1	11210,2	9389,1	9285,5	9742,6	12810,7	14553,3	2621,32	11621,32	2207,11	11113,96	4474,87	14992,64
Forest & semi nat. areas	1,0	1,0	1,0	500,0	1,0	250,0	957,1	250,0	603,6	1,0	250,0	353,6	1,0	250,0	3371,32	250	500	1	1	250
Inland marshes	26063,7	46655,6	30991,4	34253,1	36773,9	32942,4	39424,6	61344,9	59673,4	40205,8	48234,0	23152,5	39587,6	65020,8	26834,52	62633,51	42380,46	56398,23	1457,11	59994,43
Water bodies	26063,7	46655,6	30991,4	34253,1	36773,9	32942,4	39424,6	61344,9	59673,4	40205,8	48234,0	23152,5	39587,6	65020,8	26834,52	62633,51	42380,46	56398,23	1457,11	59994,43
Rivers	32824,1	31000,0	32942,4	35364,0	25750,0	16500,0	14957,1	33788,6	46637,8	46966,3	54848,0	26541,6	33707,1	39256,1	603,55	43677,67	4578,43	49912,95	46123,11	46316,76
Lakes	13362,7	3250,0	2850,4	10982,9	250,0	9500,0	12083,1	18006,9	22803,5	13704,5	18884,5	11371,6	2850,4	23099,0	13950,81	22718,38	12260,2	22137,36	20615,53	25842,07

CHAPTER 3

I mark where I eat: a fine scale spatial pattern of the Eurasian otter diet along three rivers in southern Italy

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Abstract

The Eurasian otter *Lutra lutra* suffered a sharp decline in the last century caused by multiple pressures, including human persecution, habitat loss, and water pollution. In Italy, the species is currently listed as Vulnerable in the Italian Red List. For top predators a sound knowledge of the feeding ecology is essential to adopt effective conservation strategies, especially at local scale. We analysed the trophic niche of the Eurasian otter in three rivers located in the core area of the Italian otter range and compared niche width and prey composition among river sectors. Prey remains were examined in 415 spraints collected from July to October 2019 at 17 frequent marking sites along seven river sectors (one upstream, three medium course, and three downstream). Differences in the probability of occurrence of five prey categories (fish, crustaceans, amphibians, reptiles, and insects) were compared among rivers and river sectors through Generalized Linear Mixed Models. Pianka index (O_{jk}) was used to evaluate the trophic niche overlap among rivers sector pairs. Results showed that fish was the main prey in the whole study area, followed by crustaceans, amphibians, insects, and reptiles. However, prey remains in spraints reflected prey composition at very local scale, with fish prevailing in all downstream sectors, while amphibians, crustaceans, and insects mainly represented in the middle sectors. The endemic crayfish *Austropotamobius italicus* was almost the unique prey in the single upstream investigated. Accordingly, trophic niche differed more among river sectors within the same river (O_{jk} = 0.21-0.28) than among same sectors (i.e., downstream and middle course) of different rivers (O_{jk} > 0.6). This spatial pattern is discussed both in terms of behavioural ecology and digestive physiology of the Eurasian otter.

Introduction

In the past century, several stressors, such as human persecution, habitat degradation, and pollution, led to a severe decline of the Eurasian otter *Lutra lutra* in Europe (Loy et al. 2022). Following law enforcement and the banning of harmful pollutants (Roos et al., 2012; Stocholm Convention, 2001), the species is now recovering in most European countries (Loy et al., 2022), including Italy, where it has been recently downgraded from Endangered to Vulnerable in the national red list (Loy et al., in press;

Rondinini et al., 2022). Once widespread in the whole peninsula, in the ’90 of the last century the species went extinct in northern and central Italy, and a viable population only survived in southern regions, completely isolated from other European populations (Panzacchi et al., 2011). The ongoing recovering allowed a recent return to central (Loy et al., 2023; Marcelli et al., 2023; Giovacchini et al., 2018, 2023) and northeastern Italy (Stokel et al., 2022; Giovacchini et al., 2021; Lapini et al., 2020; Malthieux, 2020; Marcolin et al., 2020; Pavanello et al., 2015; Righetti, 2011). However, the core otter range is still limited to the south-central portion of the peninsula, still isolated from other European populations (Giovacchini et al., 2021). Moreover, the genetic uniqueness of this population suggests it represents an Evolutionary Significant Unit (Mucci et al., 2010; Randi et al., 2003) in need of specific conservation efforts. Successful conservation efforts of ESU should account for their local adaptations and ecological requirements. For threatened top predators, it is crucial to thoroughly understand feeding ecology and identify both key feeding resources and prey resources that might expose populations to conflicts with humans or other endangered species (Arrizabalaga-Escudero et al., 2015; Freitas et al., 2007). Otters are opportunistic predators that feed on aquatic and semi-aquatic prey (Roos et al., 2015; Panzacchi et al., 2011; Kruuk, 2006). When available, otters eat almost exclusively fish (Remonti et al., 2007; Fusillo, 2006; Prigioni et al., 1991a,1991b; Ruiz-Olmo et al., 1989). However, the diet also includes amphibians, crustaceans and, to a lesser extent, small mammals, reptiles, birds, and other invertebrates (Remonti et al., 2007; Fusillo, 2006; Polednik et al., 2004; Clavero et al., 2003; Ruiz-Olmo et al., 2002; Prigioni et al., 1991a, b).

Although the otter diet has been studied in many European countries (Bedmar et al., 2022; Boyi et al., 2022; Dettori et al., 2022; Sittenthaler et al., 2021; Harper et al., 2020) including Italy (Loy et al., 2023; Buglione et al., 2020; Smiroldo et al., 2019b), no authors have yet analysed if and how diet composition varies along the river gradient or among neighbouring rivers. This information is valuable to both infer prey resource exploitation pattern by otters, and to manage prey communities at both local and river basin scales (Arrizabalaga-Escudero et al., 2015). To fill this gap, we analysed the fine-scale spatial pattern of the trophic niche of otters along three neighbouring rivers in the core area of the Italian otter range in Southern Italy.

We specifically examined prey remains in otter scats (spraints) to first characterize otter diet in the study area, and then to answer three main questions that could contribute to understanding the spatial pattern of resource exploitation by otters. As community composition (e.g., fish, macroinvertebrates, etc.) is known to vary along the river gradient (Marconato et al., 2002; Huet, 1949) and otters are opportunistic predators (Kruuk, 2006) do prey remain composition change along the river gradient according to the local composition of prey (RQ1)? Does prey composition in spraints vary among different rivers (RQ2)? Does feeding niche overlap across same sectors of different rivers (RQ3)?

Materials and methods

Study area

We investigated the rivers Bussento, Mingardo, and Lambro flowing in the Cilento, Vallo di Diano and Alburni National Park, within the core otter range in southern Italy in the region of Campania (Fig. 1). The Bussento river is 37 km long, with a catchment area of 352 km^2 and runs across a heterogeneous landscape, including a 5 km sinkhole. The Mingardo river is 38 km long with catchment area of about 230 km^2, while the Lambro river as a total length of about 24 km and a catchment area of approximately 77 km^2. The mean annual rainfall is about 998 mm (www.scia.isprambiente.it).

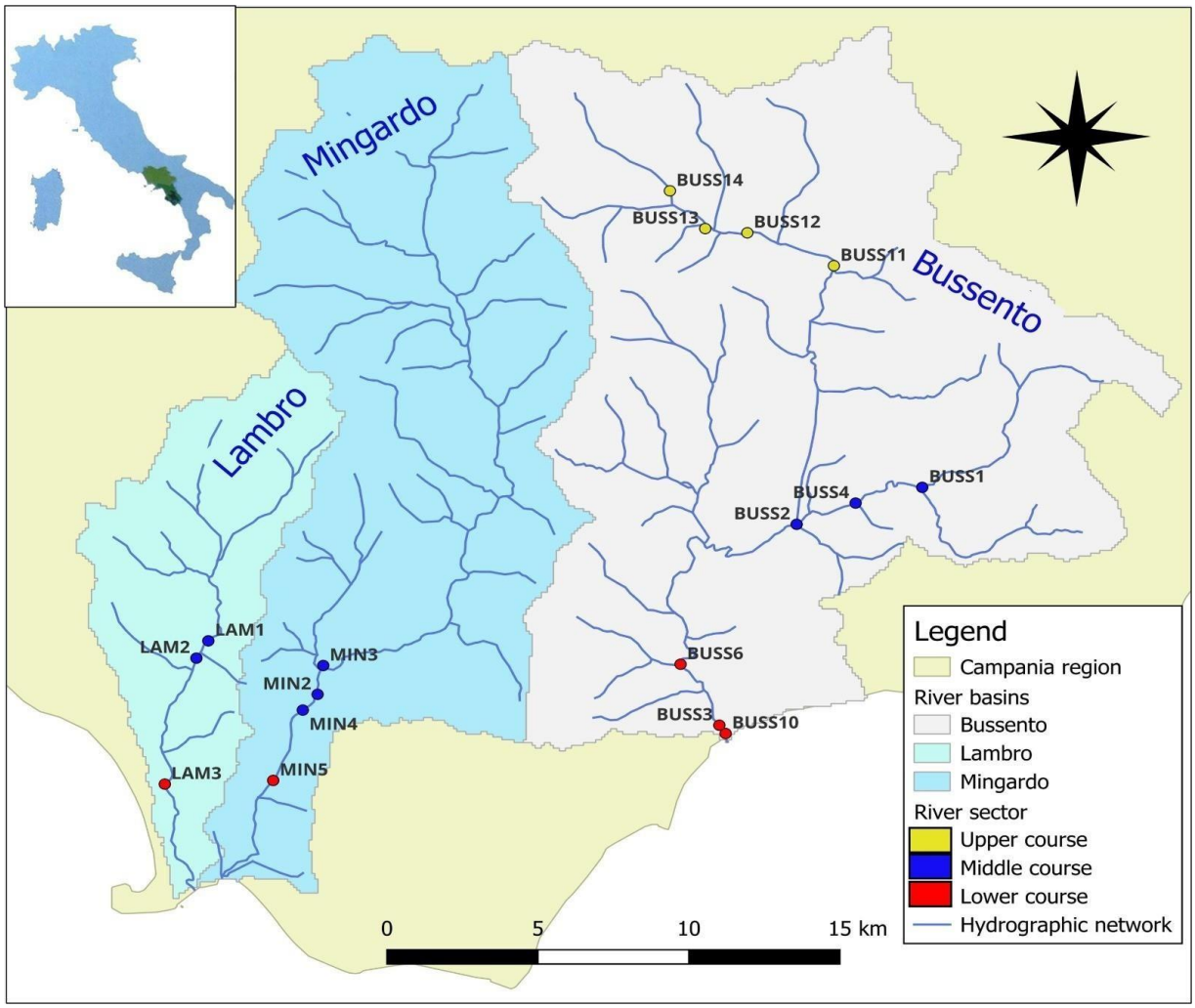

Figure 1. *Sampling sites along the rivers Mingardo, Bussento and Lambro in Campania, Italy. Colors of the sites indicate the different rivers sectors sampled.*

Spraint collection and prey identification

Otter spraints were collected at 17 sampling sites along the main courses of Bussento (n=10, river length: 30 km from the river mouth up to 435 m a.s.l.), Mingardo (n=4, river length: 9 km from the river mouth up to 60 m a.s.l.), and Lambro (n = 3, river length: 11 km from the river mouth up to 85 m a.s.l.) (Suppl. Mat. 1). Sampling sites were selected among frequently marking river stretches. Sampling sites were assigned to the lower (<5 km from the river mouth), middle (5-25 km), and upper (>25 km) course of each river. Accordingly, sampling sites were then assigned to two river sectors (lower and middle course) for Lambro and Mingardo rivers, and three for Bussento river (Fig. 1). Details on specific distances among sampling sites and river sectors are reported in Supplementary material 1.

Spraints were collected during five sampling sessions ran in July, September, and October 2019, for a total of 40 sampling days. Spraints were collected by walking along both riversides for 600 m of river stretch (Jamwal et al., 2021; Balestrieri et al., 2011; Reuther et al., 2000). Each spraint was preserved in a paper bag and then soaked in water containing a cleaning capsule of potassium monopersulphate for at least 6 h (Jenkins et al., 1979). Remains were then washed using a 0.5 mm mesh sieve under running water to clean and separate hard parts from the matrix. Hard parts were examined using a binocular microscope and assigned to the lowest taxonomic level, using identification keys for fish (Gagliardi et al., 2006; Conroy et al., 2005; Dondolin, 1999; Webb, 1976), amphibians (Smiroldo et al., 2019c; Di Palma and Massa, 1981), reptiles (Di Palma and Massa, 1981), birds (Brom et al., 1986; Day, 1966), and

mammals (Chaline et al., 1974; Day, 1966). Fish were identified based on hurophore complexes, pharyngeal teeth, opercular and preopercular bones, scales, dentary, maxillary, caudal and thoracic vertebrae, and branchial arches and otholits. Identification of amphibians was based on femur, tibia-fibula, scapula, ilion, premaxillary and maxillary bones. Exoskeleton remains were used to identify crustaceans (crayfishes and crabs). Exoskeleton, legs, head, and wings remains were used to detect the presence of insects. Hairs, teeth, feathers, and claws revealed the presence of mammals and birds.

Data analysis

Prey remains were assigned to eight prey categories: fish, amphibians, crustaceans, reptiles, insects, molluscs, birds, and mammals. For each prey category we recorded the presence (1) or absence (0) in each spraint (Suppl. Mat. 2) (Prigioni et al., 2009; Remonti et al., 2007). Presence/absence data were first converted into Percentage Frequency of Occurrence (PFO), i.e. the total number of spraints containing a specific prey item by the total number of spraints × 100 (Smiroldo et al., 2019a; Conroy et al., 2005; Webb, 1976). To evaluate the minimum sample size needed to represent the whole prey category assemblage, we computed accumulation curves for the whole study area and for each river sector. All curves showed that the minimum sample size was reached in every river stretch but the upper sector of Bussento (Suppl. Mat. 4).

Probability of Occurrence (PO) of each prey category was obtained by fitting four General Linear Mixed Models (GLMMs; McCullagh et al., 1989) to the presence/absence matrix. A first model (M1) was calibrated to assess PO of prey categories in the whole sample, setting prey presence/absence in each spraint as the response variable (see Suppl. Mat. 3. for an example of the input matrix), and the prey category as the explanatory variable. The same analysis was applied to other three models, to test the effect on PO of i) prey category, river sector irrespective of river (lower or medium), and their interaction (M2), ii) prey category, river sectors within Bussento river (upper, middle, and lower), and their interactions (M3), prey category, river, and their interaction (M4). Prey categories with PO < 0.1 were not analysed. To account for spatial autocorrelation in model residuals, we set the sampling site as a random effect nested within sector and river (M1), within river (M2 and M3) or within sector (M4). Model goodness-of-fit was assessed through conditional R^2 (Nakagawa et al., 2013), while predictive performance was quantified through a five-fold cross-validation approach, calculating the area under the receiver operating characteristic curve (AUC; Hanley and McNeil, 1982). As a post–hoc test, we performed a pairwise comparison between marginal means (i.e., the difference in fitted means between each river and sector) through ANOVA with F-tests and p-values quantified via the Kenward-Roger's method for denominator degrees-of-freedom and F-statistic. The statistical significance was assessed through Kenward–Roger p-values (Gomez et al., 2005). All the analyses were carried out using "lme4", "lmerTest" and "performance" R packages (Lüdecke et al., 2021; Kuznetsova, 2017; Bates, 2015).

The feeding niche overlap among river sector pairs within each river and among lower and medium sectors of the three rivers was evaluated through the Pianka index (Baghli et al., 2002) on PFO values, as follows:

$$O_{jk} = \frac{\sum_i^n \left(p_{ij} \times p_{ik}\right)}{\sqrt{\sum_i^n p_{ij}^2 \times \sum_i^n p_{ik}^2}}$$

where p_{ij} and p_{ik} indicate PFO in the j sector and *k* river respectively. Pianka's index ranges from 0 (no overlap) to 1 (total overlap). A Spearman's correlation test was also performed to test the hypothesis of independence between the overlap niche (Pianka index) and the distance between the river sectors. The

result ρ can range between -1 to +1. Extreme values indicate maximum correlation, whereas the correlation is null close to 0.

Results

A total of 415 spraints were collected along 17 sampling sites: 188 (45.3%) in Bussento, 123 (29.7%) in Mingardo, and 105 (25%) in Lambro rivers (Suppl. Mat. 1). Results from model M1 comparing PO of each prey category in the whole sample pooled for the three rivers (Fig. 2) produced a fair predictive performance (AUC = 0.75), even though with a relatively low goodness-of-fit (cond. R^2 = 0.24). As expected, fish showed the highest average probability (PO = 0.67), followed by crustaceans (PO = 0.39), amphibians (PO = 0.25), insects (PO = 0.23), and reptiles (PO = 0.11). All pairwise comparisons were significant at p< 0.001, with the only exception of the difference between amphibians and insects (Suppl. Mat. 5). The remaining categories, i.e., mollusks, mammals and birds were the least exploited resources (PO < 0.1).

According to PFO, fish were the main prey in the otter diet in five out of seven river sectors. Crustaceans resulted to be almost the unique food item in the upper Bussento sector. Amphibians prevailed as primary trophic source in the middle course of two out of three rivers (Bussento and Lambro). Finally, insects were the first food category in one river sector only (middle course of Lambro) (Tab. 1).

Table 1. *Percentage of frequency of occurrence (PFO) of prey categories in the whole study area, in each river, in pooled lower and middle sectors, and in each individual sector.*

	Fish	amphibians	crustaceans	reptiles	insects	molluscs	birds	mammals
Bussento upper course	2	2	97.9	2	2	0	0	0
Bussento middle course	72.2	72.2	27.7	5.6	44.4	5.6	0	0
Bussento lower course	72	15.5	21.3	10.7	13.1	5.7	4.1	1.6
Mingardo middle course	100	15.9	39.3	12.7	14.9	0	0	2.1
Mingardo lower course	88	25	45.8	20.8	0	4.2	0	0
Lambro middle course	50	50	30.6	0	52.2	1.1	0	0
Lambro lower course	69	18.8	37.5	6.3	25	0	12.5	0
Middle course	67	36	34.5	6.5	33.8	1	0	1
Lower course	73.5	17.2	23.3	12.3	12.3	4.9	3.1	1.2
Bussento river	88.7	23.4	22.7	9.9	17	5.7	3.5	1.4
Mingardo river	83.0	17.8	40.6	14.4	11.8	0.9	0	1.8
Lambro river	52.9	45.1	26.9	1.9	48.7	1	0	0
Overall sample	62.7	25.1	38.1	8.2	22.2	2.4	1.2	1

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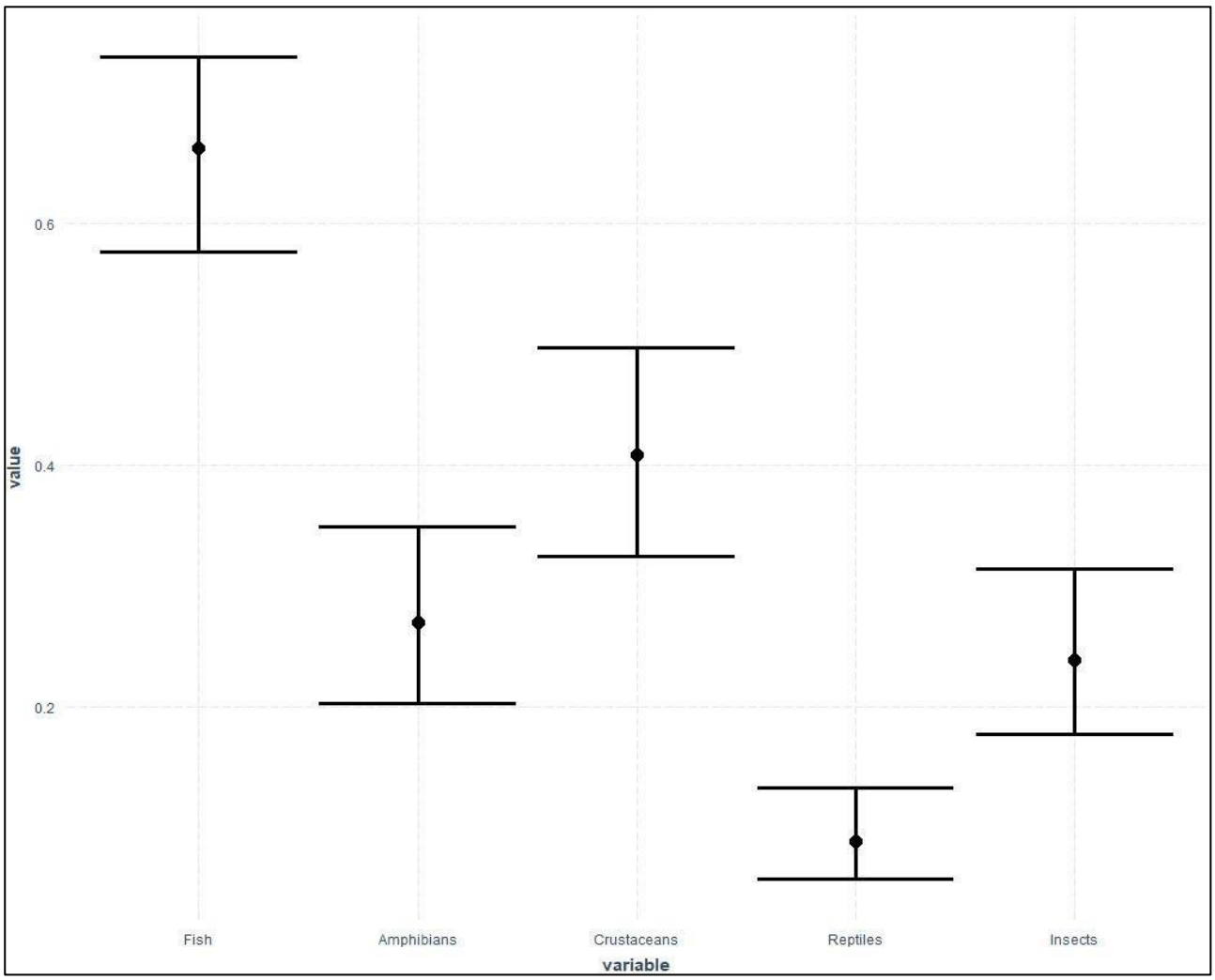

Figure 2. *Box plots showing average probability of occurrence (PO) and standard deviation of each category of prey in the total sample, as modelled by M1. Molluscs, mammals, and birds are not shown (average PO < 0.1).*

A comparison of otter prey composition between the middle and lower course of the rivers (model M2) confirmed fish as the main prey category in both sectors (Fig. 3). However, the proportion of fish was significantly higher in the lower (PO always > 0.6) than in the middle (PO always < 0.6) sector. Conversely, the other prey categories were more represented in the middle than in the lower course (Suppl. Mat. 6). The model achieved a good predictive accuracy (AUC = 0.77) and a goodness-of-fit equal to 0.29 (cond. R^2).

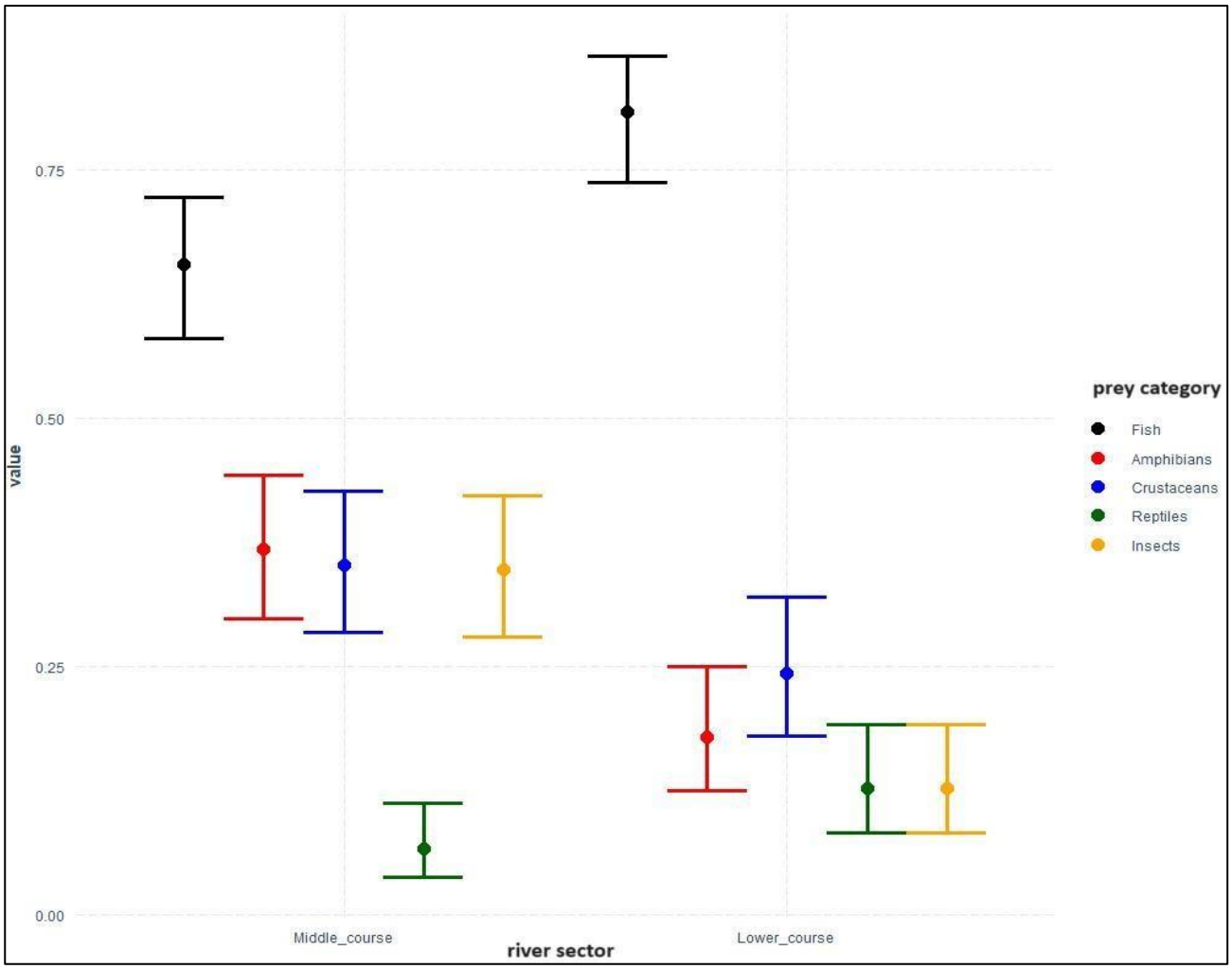

Figure 3. *Box plots of PO of prey categories in middle and lower course sectors of the three rivers, as modelled by M2. Molluscs, mammals, and birds are not shown (PO<0.1).*

Model M3 exploring changes in prey composition along the river gradient of Bussento achieved a good predictive accuracy (AUC = 0.808) and a goodness-of-fit slightly higher than M1 and M2 (cond. R^2 = 0.39). Prey composition differed significantly in the upper, middle, and lower sectors of the river (Suppl. Mat. 7). Specifically, in the upper course, prey remains were almost only represented by crustaceans, mainly belonging to the endemic crayfish *Austropotamobius italicus* (PO close to 1). It should be underlined that according to accumulation curves (Suppl. Mat. 3) this latter result could be biased by an inadequate sample size to represent other prey categories. However, as PFO of crayfish was 97.9%, the influence of alternative prey could be considered negligible (Tab. 1). Moving from the upper to the lower course, prey composition shifted from crayfish to mainly fish (Fig. 4). Specifically, fish was the almost exclusive prey item the lower course (PO > 0.75), while the middle course was mainly characterized by fish and amphibians (PO close to 0.75), followed by crustaceans and insects (PO close to 0.5), and reptiles (PO close to 0.10). (Suppl. Mat. 7).

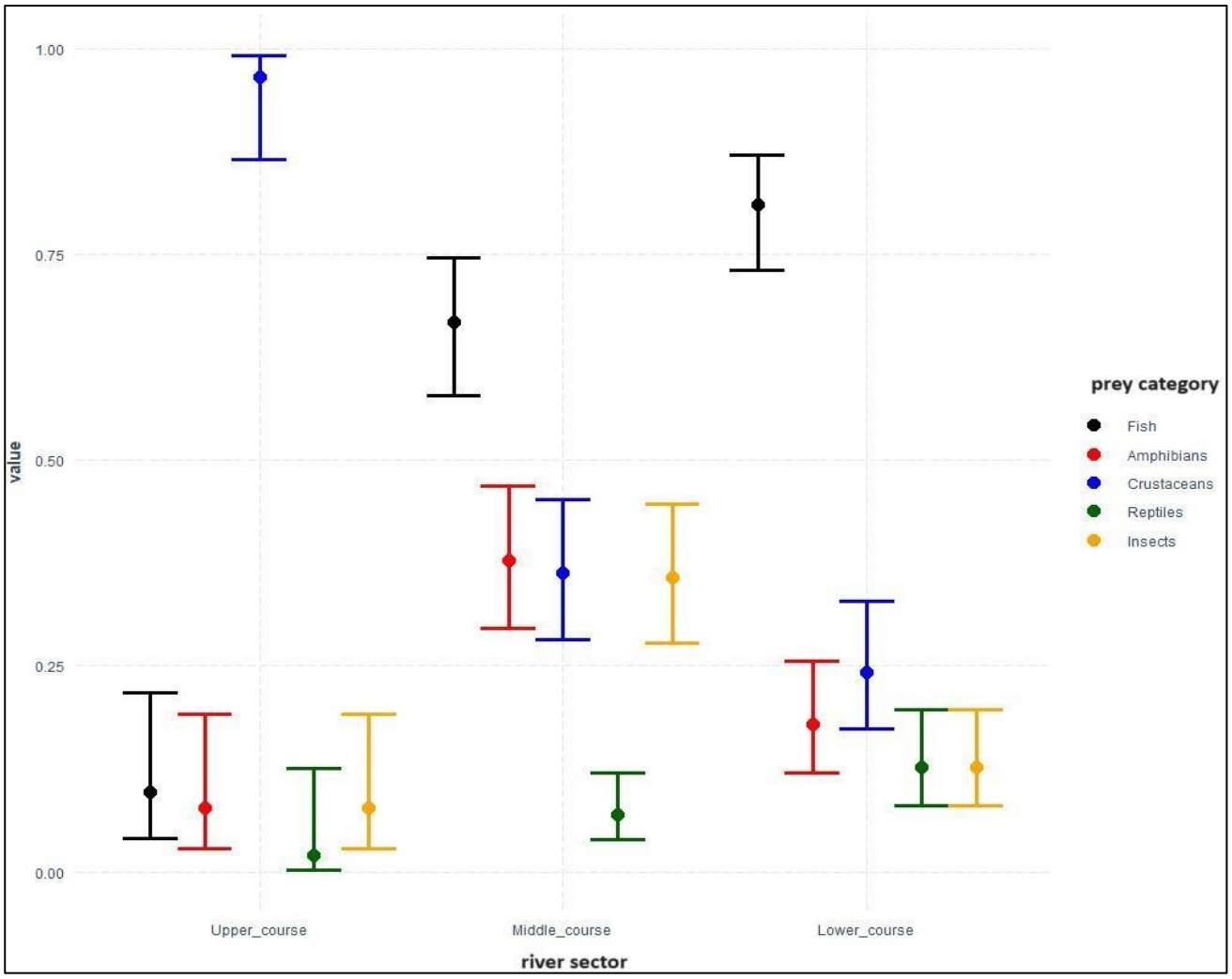

Figure 4. *Box plots of PO of prey categories along different sectors of the Bussento river, as modelled by M3. Molluscs, mammals, and birds are not shown (PO<0.1).*

Finally, when comparing prey categories among rivers (model M4), the model obtained a fair predictive accuracy (AUC = 0.78) and a similar fitting value (cond. R^2 = 0.35) compared to M3. Fish were again the most represented prey category in each river, although with some difference in their prevalence (Fig. 5). Specifically, in the Bussento river fish were significantly higher than all the other prey categories (PO > 0.75) (Suppl Mat. 6). In the Mingardo river fish (PO > 0.75) and crustaceans (PO > 0.35) were significantly higher than other categories (Suppl. Mat. 6). Finally, in Lambro river fish frequency was not significantly higher than amphibians, crustaceans or insects, and only reptiles were significantly lower than all other categories (PO > 0.05) (Suppl. Mat. 8).

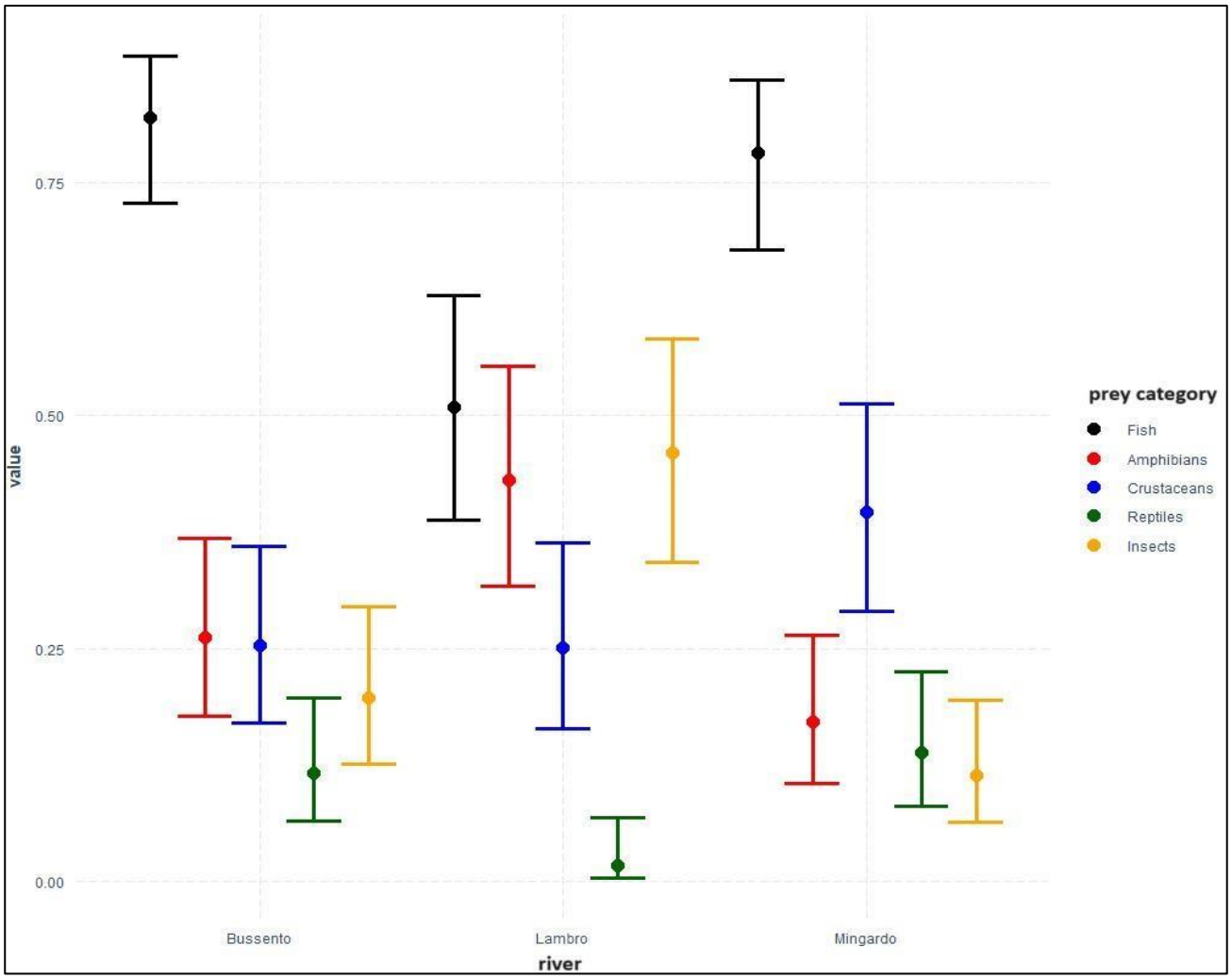

Figure 5. *Box plots showing mean and standard deviation of Probability of Occurrence (PO) of prey categories in the three rivers, obtained by pooling the middle and lower course, as modelled by M4. Molluscs, mammals, and birds are not shown (PO < 0.1).*

The mean niche overlap index between sector pairs of the same rivers (mean O_{jk} = 0.43, SD = 0.25) was lower than the niche overlap between sectors of different rivers (mean O_{jk} = 0.71, SD = 0.34). The least overlap was found among the sectors of Bussento river (all values lower than 0.3), whereas Mingardo and Lambro rivers showed a high degree of overlap between the middle and the lower sectors of the river, ranging from 0.68 to 0.72 (Tab. 2). When we explored the overlap index between the same sectors of different rivers, all values were above 0.5, with the highest overlap shown between the middle course of Bussento and Lambro rivers (Tab. 3). Instead, Spearman correlation between overlap indices and distance among sectors was low (ρ = - 0.314), suggesting that niche similarity was not related to distance between sectors.

Table 2. *Pianka niche overlap index Ojk and linear distances between sector pairs of the same river. Distances are measured following the hydrographic network.*

Sector pairs	O_{jk}	Distance (km)
Bussento upper course* middle course	0.28	10.5
Bussento upper course* lower course	0.21	17.5
Bussento middle course* lower course	0.25	7.1
Mingardo middle course* lower course	0.68	3
Lambro middle course* lower course	0.72	6.3

Table 3. *Pianka overlap index Ojk and linear distances between the same sectors (middle or lower course) of different rivers. Distance between sampling sites of different river basins are linear distances.*

Sector pairs	O_{jk}	Distance (km)
Bussento middle course*Mingardo middle course	0.61	15.7
Bussento middle course*Lambro middle course	0.93	19.5
Mingardo middle course*Lambro middle course	0.55	4.2
Bussento lower course*Mingardo lower course	0.62	15
Bussento lower course*Lambro lower course	0.66	18.6
Mingardo lower course*Lambro lower course	0.78	4.1

Discussion

Our study allowed us to detect up to eight categories of prey in the diet of the Eurasian otter in the study area, confirming a broad trophic niche of otters in Mediterranean areas compared to other European biomes (De Sanctis, 2020; Krawczyk et al., 2016; Lanszki et al., 2016; Clavero et al., 2003). As expected, fish was the main prey category in our study area. In fact, it is widely recognized that otters prefer fish and that their demographic parameters are directly influenced by fish abundance (Kruuk, 2006; Ruiz-Olmo et al., 2001). Prey preference tests conducted in captivity always showed fish as the favorite food item (Erlinge, 1968). However, a broader feeding niche observed in Mediterranean otters has been related to fluctuations in the water regime of rivers and water extraction, which are reflected in fluctuations in fish stocks, making alternative prey relevant food resources in specific seasons or areas (Rytwinski et al., 2023; Gil-Sánchez and Antorán-Pilar, 2020; Remonti et al., 2009; Ruiz-Olmo et al., 2001). More specifically, in Mediterranean areas fish are less consumed, with crayfish and reptiles as main alternative prey (De Sanctis, 2020; Marcolin et al., 2020; Lanszki et al., 2016; Remonti et al., 2007; Fusillo, 2006; Prigioni et al., 1991a, 1991b; Ruiz-Olmo et al., 1989). Our study partially confirms this evidence, as crustaceans were the main alternative prey, but amphibians replaced reptiles as the second main alternative item. Interestingly, both fish and crustaceans' exploitation were significantly higher than other prey categories, whereas reptiles were significantly less likely to occur in the diet. A prevalence of amphibians over reptiles was also observed in other southern Italian rivers by Remonti et al. (2009).

Surprisingly, insects were another locally important food consumed by otters in our study area, especially in the Lambro river, where they represented the second most frequent prey, and the main prey in the middle sector of the river. Insects as alternative prey for otters were observed by Anton and Delibes (1987) in Donana (Spain) and are known to be actively preyed by otters (Taylor et al., 2010). According to Meaking et al. (1976) and Drive et al. (1981), insects provide a high caloric intake and are particularly easy to catch and ingest (Hansen et al., 2003; Reid et al., 1994). Thus, predation upon insects is in accordance with the optimal foraging theory (Pyke and Starr, 2021). However, as amphibians and insects were represented almost equally in all river sections, the latter could represent the undigested prey of amphibians rather than being directly preyed on by otters, as suggested by other authors (Remonti et al., 2007; Hansen et al., 2003; Larsen et al., 1984; Toweill et al., 1974).

Focusing on otter diet along the river gradient, our results showed that fish were more abundant in the otter diet in lower sections of all rivers, whereas amphibians and insects were more represented in the

medium sections, and crustaceans were the exclusive prey in the upstream section. A similar shift in otter diet along the river has been observed also by Macarthur (2022). Also, electrofishing campaigns conducted along the same rivers showed a high fish density in the lower sectors, whereas in the upper Bussento, native crayfish was the unique freshwater species (Guida et al., 2019; Bianco et al., 2011; Marconato et al., 2002). Moreover, a survey on amphibian conducted in the same rivers revealed an altitudinal gradient of species linked to running waters (i.e. *Rana italica*, *Pelophylax kl. hispanicus*, *Bufo bufo*, and *Hyla intermedia*), with increasing abundances from the mouth of the rivers upward (Romano, 2014). As otters are opportunistic feeders, the prevalence of fish in their diet observed in lower river sections reflects their higher availability in this part of the river, as already suggested by Dettori et al. (2022). In fact, fish composition and abundance are known to vary along the river gradient (Sutela et al., 2020; Huet, 1949). Specifically, fish distribution along rivers responds to the River Continuum Concept that depicts a downstream gradient of organisms following the accumulation of nutrients downward (Welcomme, 1985; Vannote et al., 1980) and leads to a general increase in fish diversity and biomass in the lower stretches of the rivers (Muneepeerakul et al., 2008; Matthews, 1998; Zamora Hernandez et al., 1996). Otter capability to include amphibian prey according to their increase in abundance with the altitude is witnessed by our data in middle river sectors (Remonti et al. 2009). Moreover, amphibians are known to typically follow a seasonal trend in otter diet, being more exploited during summer, a season that corresponds to our sampling period (Bauer-Haaz et al. 2014). Finally, in the upper stream section of the river Bussento, fish and amphibians disappeared from the otter diet, as otters fed exclusively on the endemic endangered river crayfish *Austropotamobius italicus*. Based on local surveys conducted in the study area, it has been found that crayfish are only found in this particular section of the river. Additionally, they are the only prey present in this part of the headwater. This region is physically separated from the downstream river sections by a 5 km stretch that runs into a sinkhole. This physical separation is believed to prevent fish recolonization (Bianco et al., 2011; Marconato et al., 2002). Preference tests on food items showed crayfish is the least preferred prey by otters (Elringe, 1968), evidence also confirmed by Melero et al (2008) in Spain. This supports the idea of otter feeding opportunistic behaviour as no other prey were available in the upper stretch of Bussento river. Nevertheless, besides being just a secondary prey where fish biomass is scarce (Remonti et al., 2007; Polednik et al., 2004), crayfish seem to be selected by otters when massively present, as they are very easy to catch (Beja, 1996). Otters direct their predation to slower prey (Erlinge, 1968) and switch their diet from fish to crayfish when or where the latter are abundant (Route and Peterson, 1988; Dettori et al., 2021). Abundance of slow-moving prey may have led to a local preference for crayfish over amphibians in the upper Bussento river. To respond to our question RQ1, this evidence suggests that prey remains in otter spraints do change along the river gradient and reflect the local abundance and composition of prey. This hypothesis is also supported by Remonti et al. (2009), who suggested fish reduction at high elevations as the main factor in shaping otter diet at the local scale.

When comparing different rivers, we revealed that fish communities were more exploited in the lower and medium sectors of the Bussento river compared to Mingardo and Lambro rivers. Bussento, Mingardo and Lambro rivers are adjoining river catchments with descending order flow rates. As fish communities are affected by flow rates (Rytwinski et al., 2023; Baran et al., 1995), different river bodies having different flow rates may lead to different carrying capacities for fish. Accordingly, larger rivers like the Bussento river can support higher fish productivity. Moreover, the Bussento river has a more stationary regime due to the hydropower dam located upstream that regulates water release, prevent river drought, and buffers the flow rate during the summer period guaranteeing appropriate habitats for

strictly aquatic species like fish (Bovolin et al., 2017). Therefore, higher selection of secondary prey in the Mingardo and Lambro rivers could be related to their lower river flow, which favors availability of alternative prey, as also suggested by Amhaouch et al. (2020). Consumption of amphibians and crustaceans as alternative feeding resources can also represent a seasonal shift in prey abundance, as our study has been carried out during the dry summer season, when high temperatures and water drought might limit fish abundance and favour alternative prey like crustaceans and amphibians (Prenda et al., 2001). This evidence supports the hypothesis that the otter trophic niche changes among rivers (question RQ2) but also that a similar pattern along every river gradient can also be observed (question RQ3). These outcomes were confirmed by Pianka indices of niche overlap, which evidenced that prey composition differed more along a river gradient than among different rivers. Otter prey remains thus reflect prey community at the very local scale.

Possible explanations of this spatial pattern arise from movement ecology and digestive physiology of otters. Even if occasional long-distance movements are possible, core areas within home ranges are restricted to few kilometres along the river (Lerone et al., 2022; Quaglietta et al., 2019; Quaglietta et al., 2013; Durbin, 1996; Kruuk, 1995). Usually, the core area of an individual otter is around 2-5 km (Quaglietta et al., 2019; Kruuk, 2006; Mason and Macdonald, 1986; Green et al., 1984), corresponding to the average length of river sectors analysed in this study. Moreover, in Mediterranean habitats otters shrink their activities around few remaining feeding sites during the dry season, as well as during freezing winters in northern Europe (Saavedra, 2002; Erlinge, 1967). Accordingly, we hypothesize that river sectors correspond to areas that are exploited intensively before moving to other unexploited portions of the home range (Mitchell and Powell, 2012; Spencer, 1992, 2012), and may corresponds to the daily core feeding areas of 3.5 km of river explored by individuals in a single night reported by Quaglietta (2011) in Portugal. During this hunting activities otters move quiet slowly along the river, with a net displacement of a couple of hundred meters every hour (Quaglietta, 2011).

Additionally, otters have high metabolic rates due to their elongated body shape associated with a remarkable heat dispersion during immersions (McNab, 2008; Iversen, 1972). This, together with the short colon trait, leads to a fast transit time of food during the digestion process (Sà et al., 2014). Time digestion can also vary with activity levels, food caloric contents, and digestive physiology (McGrosky et al., 2016; Carss et al., 1998; Markussen, 1993). Carss et al. (1998) reported an average time of otter digestion of about an hour during active patrolling and hunting.

Accounting for slow movements and short digestion time, the strict relation between prey composition in otter spraints and local feeding resources accurately depict otter diet at fine spatial scale. Effective conservation measures on freshwater top predators should be addressed to also protect prey ecological needs rather than only focusing on the single target species. It is the case of the rare and endemic crayfish *Austrapotamobius pallipes* (Chiesa et al., 2010; Brusconi et al., 2008), that in our study area was the exclusive prey in the upstream sector of the Bussent river. These strategies are common for carnivores as they typically are elected as umbrella species, whose protection implies the protection of the whole trophic network (Roberge and Angelstam, 2004). In this sense, it should be paid particular attention to conservation priority issues related to endangered species that are linked by prey-predator relationships, especially if they are jeopardized with few little remnant populations (Fedriani et al., 2017). A proper understanding of how feeding requirements differ in different areas of a river can

guide conservation efforts and river management strategies aimed at safeguarding specific components of the river community, particularly those that are restricted to limited areas. In general, our findings confirmed the opportunistic feeding behaviour of the otter in southern Italy and highlighted the diversity of prey offered by the different parts of rivers as a key factor for otter ecological plasticity in the highly Italian human modified landscapes (Loy et al, 2022).

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Supplementary material

Suppl. Mat. 1. *Number of spraints collected each sample sites and relative distance from the river mouth.*

River	Sector	Site	Distance from the mouth	N. spraints per site	N. spraints per sector	N. spraints per river
Bussento	Upper Course	BUSS14	29.7 km	10	48	188
		BUSS13	28.5 km	11		
		BUSS12	27.8 km	11		
		BUSS11	27 km	16		
	Middle Course	BUSS1	17.7 km	5	18	
		BUSS4	15.5 km	5		
		BUSS2	11 km	8		
	Lower Course	BUSS6	3.6 km	61	122	
		BUSS3	600 m	37		
		BUSS10	200 m	24		
Mingardo	Middle Course	MIN3	8.7 km	4	94	123
		MIN2	7.84 km	58		
		MIN4	7.25 km	32		
	Lower Course	MIN5	5 km	24	24	
Lambro	Middle Course	LAM1	10.6 km	42	89	105
		LAM2	9.55 km	47		
	Lower Course	LAM3	2.7 km	16	16	

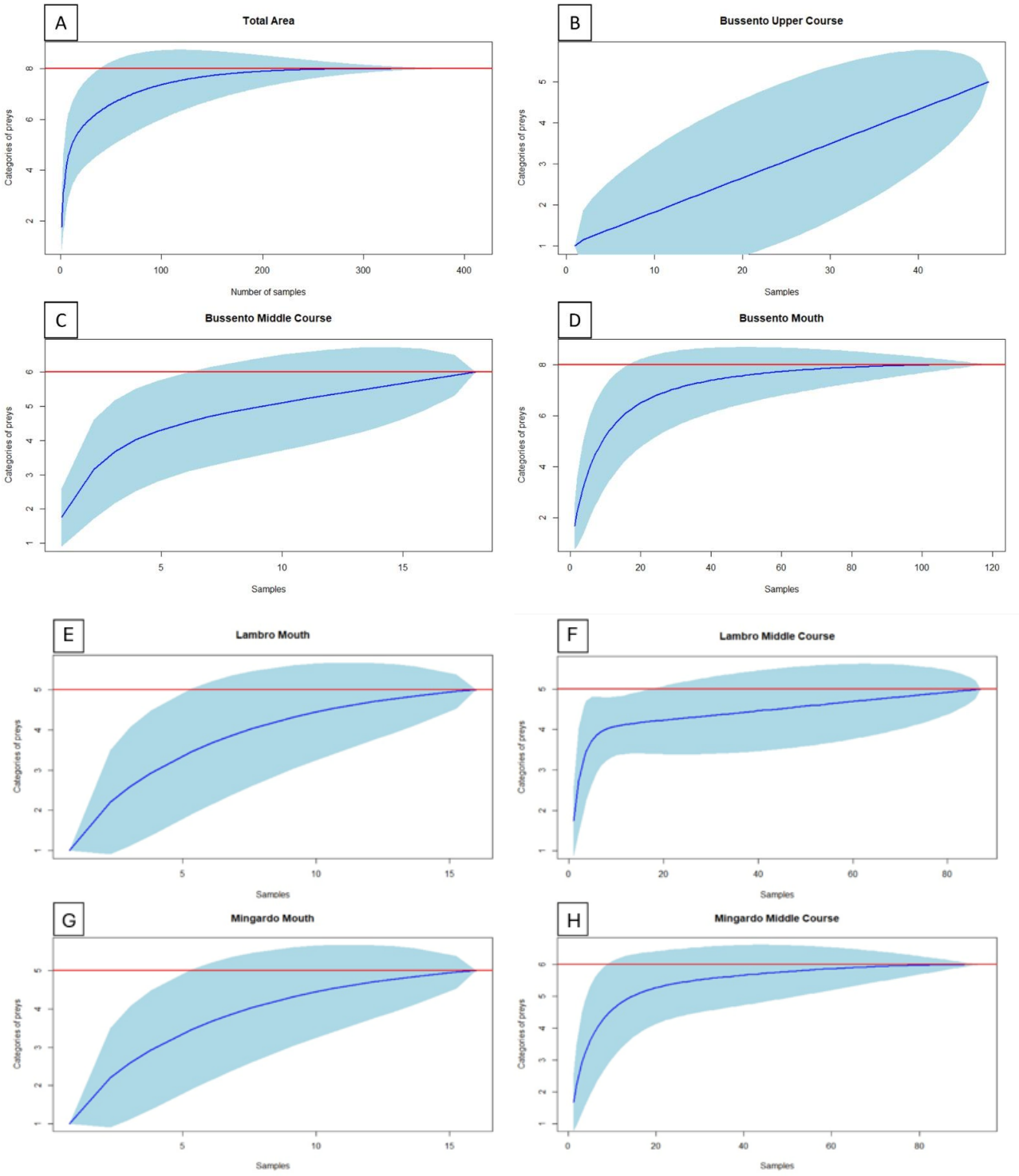
Suppl. Mat. 2. *Occurrences of prey categories in the study area, sorted also by river, river sector, and individual river sector.*

	fish	amphibians	crustaceans	reptiles	insects	birds	mammals	molluscs	samples analysed
Overall sample	260	104	158	34	92	5	4	10	415
Bussento	13	13	5	1	8	0	0	1	18
Mingardo	94	15	37	12	14	0	2	0	94
Lambro	44	44	27	0	46	0	0	1	88
Middle course	88	19	26	13	16	5	2	7	122
Lower course	21	6	11	5	0	0	0	1	24
Bussento upper course	11	3	6	1	4	2	0	0	16
Bussento middle course	1	1	47	1	1	0	0	0	48
Bussento lower course	134	72	69	13	68	0	2	2	200
Mingardo middle course	119	28	38	20	20	5	2	8	162
Mingardo lower course	108	33	78	15	25	5	2	8	188
Lambro middle course	98	21	48	17	14	0	2	1	118
Lambro lower course	55	47	28	2	50	0	0	1	104

Suppl. Mat. 3. *Matrix with the first four observations of each category provided as example of the source file to perform Model 1 (M1) of the analysis. In the last column are reported presence (1) or absence (0) for each category in each sample analysed.*

ID	SITE	River	Sector	Variable	Absence/presence
BUSS1_10	BUSS1	Bussento	Middle_course	Amphibians	1
BUSS1_13	BUSS1	Bussento	Middle_course	Amphibians	1
BUSS1_21	BUSS1	Bussento	Middle_course	Amphibians	0
BUSS1_24	BUSS1	Bussento	Middle_course	Amphibians	1
					[…]
BUSS1_10	BUSS1	Bussento	Middle_course	Crustaceans	1
BUSS1_13	BUSS1	Bussento	Middle_course	Crustaceans	1
BUSS1_21	BUSS1	Bussento	Middle_course	Crustaceans	0
BUSS1_24	BUSS1	Bussento	Middle_course	Crustaceans	0
					[…]
BUSS1_10	BUSS1	Bussento	Middle_course	Fish	1
BUSS1_13	BUSS1	Bussento	Middle_course	Fish	0
BUSS1_21	BUSS1	Bussento	Middle_course	Fish	1
BUSS1_24	BUSS1	Bussento	Middle_course	Fish	0
					[…]
BUSS1_10	BUSS1	Bussento	Middle_course	Reptiles	0
BUSS1_13	BUSS1	Bussento	Middle_course	Reptiles	0
BUSS1_21	BUSS1	Bussento	Middle_course	Reptiles	0
BUSS1_24	BUSS1	Bussento	Middle_course	Reptiles	1
					[…]
BUSS1_10	BUSS1	Bussento	Middle_course	Insects	0
BUSS1_13	BUSS1	Bussento	Middle_course	Insects	1
BUSS1_21	BUSS1	Bussento	Middle_course	Insects	1
BUSS1_24	BUSS1	Bussento	Middle_course	Insects	0
					[…]

Suppl. Mat. 4. *Accumulation curves for the whole study area (A), and for each individual river sector (B-H).*



Suppl. Mat. 5. *Significant pairwise comparisons for the whole sample of 415 spraints (model M1).*

contrast	estimate	SE	df	z.ratio	p.value	stars
Fish - Amphibians	1,67	0,16	inf	10,76	4,5E-14	***
Fish - Crustaceans	1,04	0,15	inf	7,13	9,8E-12	***
Fish - Reptiles	3,01	0,21	inf	14,45	0,0E+00	***
Fish - Insects	1,83	0,16	inf	11,54	0,0E+00	***

Amphibians - Crustaceans	-0,63	0,15	inf	-4,07	4,6E-04	***
Amphibians - Reptiles	1,34	0,21	inf	6,29	3,1E-09	***
Crustaceans - Reptiles	1,97	0,21	inf	9,49	4,2E-14	***
Crustaceans - Insects	0,79	0,16	inf	5,01	5,3E-06	***
Reptiles - Insects	-1,18	0,22	inf	-5,46	4,9E-07	***

Suppl. Mat. 6. *Significant pairwise comparisons of prey categories in middle and lower course of the three rivers (model M2)*.

contrast	estimate	SE	df	z.ratio	p.value	stars
Fish Upper_course - Crustaceans Upper_course	-5,55	0,87	inf	-6,39	1,8E-08	***
Fish Upper_course - Fish Middle_course	-2,92	0,52	inf	-5,66	1,6E-06	***
Fish Upper_course - Fish Lower_course	-3,68	0,53	inf	-6,88	6,4E-10	***
Amphibians Upper_course - Crustaceans Upper_course	-5,80	0,90	inf	-6,46	1,1E-08	***
Amphibians Upper_course - Fish Middle_course	-3,18	0,56	inf	-5,63	1,8E-06	***
Amphibians Upper_course - Amphibians Middle_course	-1,99	0,56	inf	-3,53	3,2E-02	*
Amphibians Upper_course - Crustaceans Middle_course	-1,92	0,56	inf	-3,41	4,7E-02	*
Amphibians Upper_course - Fish Lower_course	-3,93	0,58	inf	-6,77	1,3E-09	***
Crustaceans Upper_course - Reptiles Upper_course	7,24	1,25	inf	5,82	6,2E-07	***
Crustaceans Upper_course - Insects Upper_course	5,80	0,90	inf	6,46	1,1E-08	***
Crustaceans Upper_course - Fish Middle_course	2,63	0,75	inf	3,48	3,7E-02	*
Crustaceans Upper_course - Amphibians Middle_course	3,82	0,76	inf	5,06	4,4E-05	***
Crustaceans Upper_course - Crustaceans Middle_course	3,88	0,76	inf	5,14	2,8E-05	***
Crustaceans Upper_course - Reptiles Middle_course	5,92	0,80	inf	7,45	9,9E-12	***
Crustaceans Upper_course - Insects Middle_course	3,91	0,76	inf	5,17	2,4E-05	***
Crustaceans Upper_course - Amphibians Lower_course	4,85	0,78	inf	6,21	5,7E-08	***
Crustaceans Upper_course - Crustaceans Lower_course	4,46	0,78	inf	5,75	9,1E-07	***
Crustaceans Upper_course - Reptiles Lower_course	5,25	0,79	inf	6,64	3,3E-09	***
Crustaceans Upper_course - Insects Lower_course	5,25	0,79	inf	6,64	3,3E-09	***
Reptiles Upper_course - Fish Middle_course	-4,62	1,03	inf	-4,48	7,1E-04	***
Reptiles Upper_course - Fish Lower_course	-5,37	1,04	inf	-5,17	2,4E-05	***
Insects Upper_course - Fish Middle_course	-3,18	0,56	inf	-5,63	1,8E-06	***
Insects Upper_course - Amphibians Middle_course	-1,99	0,56	inf	-3,53	3,2E-02	*
Insects Upper_course - Crustaceans Middle_course	-1,92	0,56	inf	-3,41	4,7E-02	*
Insects Upper_course - Fish Lower_course	-3,93	0,58	inf	-6,77	1,3E-09	***
Fish Middle_course - Amphibians Middle_course	1,19	0,21	inf	5,65	1,7E-06	***
Fish Middle_course - Crustaceans Middle_course	1,26	0,21	inf	5,94	3,0E-07	***
Fish Middle_course - Reptiles Middle_course	3,30	0,32	inf	10,16	1,4E-13	***
Fish Middle_course - Insects Middle_course	1,28	0,21	inf	6,04	1,7E-07	***
Fish Middle_course - Amphibians Lower_course	2,22	0,31	inf	7,21	5,7E-11	***
Fish Middle_course - Crustaceans Lower_course	1,83	0,29	inf	6,26	4,1E-08	***
Fish Middle_course - Reptiles Lower_course	2,62	0,33	inf	7,94	4,2E-13	***
Fish Middle_course - Insects Lower_course	2,62	0,33	inf	7,94	4,2E-13	***
Amphibians Middle_course - Reptiles Middle_course	2,11	0,32	inf	6,51	7,8E-09	***
Amphibians Middle_course - Fish Lower_course	-1,94	0,30	inf	-6,56	5,7E-09	***
Amphibians Middle_course - Reptiles Lower_course	1,43	0,33	inf	4,38	1,1E-03	**
Amphibians Middle_course - Insects Lower_course	1,43	0,33	inf	4,38	1,1E-03	**
Crustaceans Middle_course - Reptiles Middle_course	2,04	0,32	inf	6,29	3,3E-08	***
Crustaceans Middle_course - Fish Lower_course	-2,01	0,30	inf	-6,77	1,4E-09	***

Crustaceans Middle_course - Reptiles Lower_course	1,36	0,33	inf	4,17	2,8E-03	**
Crustaceans Middle_course - Insects Lower_course	1,36	0,33	inf	4,17	2,9E-03	**
Reptiles Middle_course - Insects Middle_course	-2,02	0,32	inf	-6,22	5,2E-08	***
Reptiles Middle_course - Fish Lower_course	-4,05	0,38	inf	-10,56	1,4E-13	***
Reptiles Middle_course - Crustaceans Lower_course	-1,46	0,38	inf	-3,88	9,1E-03	**
Insects Middle_course - Fish Lower_course	-2,03	0,30	inf	-6,84	8,3E-10	***
Insects Middle_course - Reptiles Lower_course	1,34	0,33	inf	4,10	3,8E-03	**
Insects Middle_course - Insects Lower_course	1,34	0,33	inf	4,10	3,8E-03	**
Fish Lower_course - Amphibians Lower_course	2,97	0,29	inf	10,16	1,4E-13	***
Fish Lower_course - Crustaceans Lower_course	2,59	0,28	inf	9,32	1,5E-13	***
Fish Lower_course - Reptiles Lower_course	3,37	0,32	inf	10,69	1,4E-13	***
Fish Lower_course - Insects Lower_course	3,37	0,32	inf	10,69	1,5E-13	***

Suppl. Mat. 7. *Pairwise comparisons between categories from different sectors of the Bussento river (model M3).*

contrast	estimate	SE	df	z.ratio	p.value	stars
Fish Middle_course - Amphibians Middle_course	1,18	0,21	inf	5,64	7,6E-07	***
Fish Middle_course - Crustaceans Middle_course	1,25	0,21	inf	5,93	1,4E-07	***
Fish Middle_course - Reptiles Middle_course	3,28	0,32	inf	10,14	9,9E-14	***
Fish Middle_course - Insects Middle_course	1,27	0,21	inf	6,02	7,8E-08	***
Fish Middle_course - Amphibians Lower_course	2,16	0,27	inf	8,03	1,8E-13	***
Fish Middle_course - Crustaceans Lower_course	1,77	0,25	inf	7,03	9,4E-11	***
Fish Middle_course - Reptiles Lower_course	2,56	0,29	inf	8,71	8,3E-14	***
Fish Middle_course - Insects Lower_course	2,56	0,29	inf	8,71	8,2E-14	***
Amphibians Middle_course - Reptiles Middle_course	2,10	0,32	inf	6,50	3,7E-09	***
Amphibians Middle_course - Fish Lower_course	-1,98	0,26	inf	-7,55	2,1E-12	***
Amphibians Middle_course - Amphibians Lower_course	0,98	0,27	inf	3,65	9,7E-03	**
Amphibians Middle_course - Reptiles Lower_course	1,38	0,29	inf	4,70	1,1E-04	***
Amphibians Middle_course - Insects Lower_course	1,38	0,29	inf	4,70	1,1E-04	***
Crustaceans Middle_course - Reptiles Middle_course	2,03	0,32	inf	6,28	1,5E-08	***
Crustaceans Middle_course - Fish Lower_course	-2,05	0,26	inf	-7,78	4,0E-13	***
Crustaceans Middle_course - Amphibians Lower_course	0,91	0,27	inf	3,40	2,4E-02	*
Crustaceans Middle_course - Reptiles Lower_course	1,31	0,29	inf	4,47	3,4E-04	***
Crustaceans Middle_course - Insects Lower_course	1,31	0,29	inf	4,47	3,4E-04	***
Reptiles Middle_course - Insects Middle_course	-2,01	0,32	inf	-6,20	2,5E-08	***
Reptiles Middle_course - Fish Lower_course	-4,08	0,36	inf	-11,35	0,0E+00	***
Reptiles Middle_course - Crustaceans Lower_course	-1,51	0,35	inf	-4,29	7,5E-04	***
Insects Middle_course - Fish Lower_course	-2,07	0,26	inf	-7,85	3,3E-13	***
Insects Middle_course - Amphibians Lower_course	0,89	0,27	inf	3,31	3,1E-02	*
Insects Middle_course - Reptiles Lower_course	1,29	0,29	inf	4,39	4,8E-04	***
Insects Middle_course - Insects Lower_course	1,29	0,29	inf	4,39	4,8E-04	***
Fish Lower_course - Amphibians Lower_course	2,96	0,29	inf	10,15	9,5E-14	***
Fish Lower_course - Crustaceans Lower_course	2,57	0,28	inf	9,32	1,0E-13	***
Fish Lower_course - Reptiles Lower_course	3,36	0,31	inf	10,68	9,9E-14	***
Fish Lower_course - Insects Lower_course	3,36	0,31	inf	10,68	9,8E-14	***

Suppl. Mat. 8. *Significant pairwise comparisons of prey categories in the three rivers, obtained by comparing the middle and lower course only (model M4). Pairs marked with [a] refer to the same prey category resulted significantly different in PO between different rivers.*

contrast	estimate	SE	df	z.ratio	p.value	stars
Fish Bussento - Amphibians Bussento	2,55	0,30	inf	8,59	1,3E-13	***
Fish Bussento - Crustaceans Bussento	2,59	0,30	inf	8,69	1,3E-13	***
Fish Bussento - Reptiles Bussento	3,55	0,36	inf	9,94	1,3E-13	***
Fish Bussento - Insects Bussento	2,92	0,31	inf	9,32	1,5E-13	***
Fish Bussento - Fish Lambro [a]	1,48	0,35	inf	4,20	2,5E-03	**
Fish Bussento - Amphibians Lambro	1,79	0,35	inf	5,07	4,0E-05	***
Fish Bussento - Crustaceans Lambro	2,61	0,37	inf	7,06	1,7E-10	***
Fish Bussento - Reptiles Lambro	5,55	0,77	inf	7,17	7,7E-11	***
Fish Bussento - Insects Lambro	1,67	0,35	inf	4,75	2,0E-04	***
Fish Bussento - Amphibians Mingardo	3,09	0,37	inf	8,46	1,5E-13	***
Fish Bussento - Crustaceans Mingardo	1,94	0,33	inf	5,83	5,8E-07	***
Fish Bussento - Reptiles Mingardo	3,35	0,38	inf	8,80	1,1E-13	***
Fish Bussento - Insects Mingardo	3,57	0,40	inf	9,01	9,8E-14	***
Amphibians Bussento - Reptiles Lambro	3,00	0,77	inf	3,91	8,0E-03	**
Amphibians Bussento - Fish Mingardo	-2,31	0,34	inf	-6,78	1,3E-09	***
Crustaceans Bussento - Reptiles Lambro	2,96	0,77	inf	3,85	1,0E-02	**
Crustaceans Bussento - Fish Mingardo	-2,36	0,34	inf	-6,88	6,4E-10	***
Reptiles Bussento - Fish Lambro	-2,07	0,39	inf	-5,32	1,0E-05	***
Reptiles Bussento - Amphibians Lambro	-1,75	0,39	inf	-4,51	6,4E-04	***
Reptiles Bussento - Insects Lambro	-1,87	0,39	inf	-4,82	1,5E-04	***
Reptiles Bussento - Fish Mingardo	-3,31	0,39	inf	-8,42	1,5E-13	***
Reptiles Bussento - Crustaceans Mingardo	-1,61	0,37	inf	-4,31	1,5E-03	**
Insects Bussento - Fish Lambro	-1,44	0,35	inf	-4,11	3,6E-03	**
Insects Bussento - Reptiles Lambro	2,63	0,77	inf	3,41	4,7E-02	*
Insects Bussento - Insects Lambro [a]	-1,24	0,35	inf	-3,55	3,0E-02	*
Insects Bussento - Fish Mingardo	-2,68	0,35	inf	-7,55	4,5E-12	***
Fish Lambro - Crustaceans Lambro	1,13	0,30	inf	3,79	1,3E-02	*
Fish Lambro - Reptiles Lambro	4,07	0,74	inf	5,49	4,1E-06	***
Fish Lambro - Fish Mingardo [a]	-1,24	0,32	inf	-3,82	1,1E-02	*
Fish Lambro - Amphibians Mingardo	1,61	0,33	inf	4,83	1,3E-04	***
Fish Lambro - Reptiles Mingardo	1,87	0,35	inf	5,34	9,6E-06	***
Fish Lambro - Insects Mingardo	2,09	0,37	inf	5,70	1,2E-06	***
Amphibians Lambro - Reptiles Lambro	3,76	0,74	inf	5,07	4,0E-05	***
Amphibians Lambro - Fish Mingardo	-1,55	0,33	inf	-4,78	1,8E-04	***
Amphibians Lambro - Amphibians Mingardo [a]	1,30	0,33	inf	3,89	8,7E-03	**
Amphibians Lambro - Reptiles Mingardo	1,55	0,35	inf	4,44	8,8E-04	***
Amphibians Lambro - Insects Mingardo	1,78	0,37	inf	4,84	1,3E-04	***
Crustaceans Lambro - Reptiles Lambro	2,94	0,75	inf	3,94	7,2E-03	**
Crustaceans Lambro - Fish Mingardo	-2,37	0,34	inf	-6,94	4,1E-10	***
Reptiles Lambro - Insects Lambro	-3,88	0,74	inf	-5,23	1,7E-05	***
Reptiles Lambro - Fish Mingardo	-5,31	0,76	inf	-6,99	2,9E-10	***
Reptiles Lambro - Crustaceans Mingardo	-3,61	0,75	inf	-4,82	1,4E-04	***
Insects Lambro - Fish Mingardo	-1,44	0,32	inf	-4,42	9,4E-04	***
Insects Lambro - Amphibians Mingardo	1,42	0,33	inf	4,25	2,0E-03	**
Insects Lambro - Reptiles Mingardo	1,67	0,35	inf	4,78	1,8E-04	***
Insects Lambro - Insects Mingardo [a]	1,90	0,37	inf	5,17	2,4E-05	***
Fish Mingardo - Amphibians Mingardo	2,86	0,33	inf	8,64	1,3E-13	***
Fish Mingardo - Crustaceans Mingardo	1,70	0,29	inf	5,78	7,7E-07	***

Fish Mingardo - Reptiles Mingardo	3,11	0,35	inf	8,97	1,0E-13	***
Fish Mingardo - Insects Mingardo	3,33	0,36	inf	9,16	1,8E-13	***
Amphibians Mingardo - Crustaceans Mingardo	-1,16	0,31	inf	-3,79	1,3E-02	*
Crustaceans Mingardo - Reptiles Mingardo	1,41	0,32	inf	4,37	1,2E-03	**
Crustaceans Mingardo - Insects Mingardo	1,63	0,34	inf	4,79	1,7E-04	***

Conclusions

The overall aim of this thesis was to contribute to the state of the art on freshwater's biodiversity monitoring.

First, the new species-specific primers that have been designed for the eDNA detection of 12 species of conservation interest living in freshwater habitats of Central Italy provided a new tool for monitoring these species and assess their conservation status. Primers were developed so that, provided the three steps validation made according to most advanced protocols (Goldberg et al., 2016; Klymus et al., 2020), could be used in other geographical areas of occurrence of the species.

The monitoring, using primers developed ex novo, and primers available from literature, but properly validated for the study area, allowed to obtain new information on the species of interest. These include the detection in unknown areas of presence, co-occurrence between species at risk and alien invasive species, and ascertaining range expansion and first occurrence of species in the study area (e.g. *Lutra lutra*).

More specifically, results showed that this technique was effective in detecting molluscs, fish, amphibians, and mammals. The species belonging to these taxa show an ecology closely linked to the aquatic environment and a biology that likely favours the release of DNA into the environment. In support of this hypothesis is the differences in detection rate (DR) between *Lutra lutra* and *Mustela putorius* (0.46 and 0.06 respectively) that are likely linked to the different time they spend in water, i.e the Eurasian otter feeds exclusively on aquatic prey, whereas the European polecat on both acquatic and terrestrial prey (Rondinini et al., 2006). The technique showed limitations in detecting terrapins, damselflies, and crayfish. For crayfish DR ranged between 0.09 and 0.27. The thick exoskeleton of this group may be the most influential factor in the release of DNA in sufficient quantities to be efficiently detected. The same hypothesis can be made for the two species of terrapins, *Emys orbicularis* and *Trachemys scripta*, whose highly sclerified skin could be responsible for the low DNA release and consequent low DR (0.04 and 0 respectively), even in areas where the species were directly observed during water sampling (Giovacchini com pers.). Studying and resolving the limitations of this technique may open interesting scenarios for refining eDNA monitoring of these taxa.

Also, the analysis of environmental parameters provided relevant information to implement the knowledge about factors influencing to the presence of each of the species and detectability of the eDNA. This, in terms of future research opportunities, becomes of paramount importance in order to further refine a method with enormous potential (Ficetola et al., 2008; Taberlet et al., 2018).

Finally, the results of this work will contribute to further management plans for protected areas, and in synergy with traditional monitoring techniques, improve the monitoring network, improving both the management of invasive species of EU importance and the conservation of species under Habitat Directive 43/92/EEC.

The potential application of this technique was specifically demonstrated by the results obtained from eDNA monitoring of *Bombina pachypus*, one of the species for which an ex-novo eDNA assay was developed, and *B. dendrobatidis*, which represent a major risk factor for these species (Bologna e La Posta 2004), and which detection through eDNA was tested for the first time in this study. *B. pachypus*, endemic to the central-Italian territory, is the perfect representative of a taxon, the amphibians, whose global status is strongly endangered by multiple impacts of anthropogenic origin (Beebee & Griffiths,

2005; Mirabile et al., 2009) and it is of fundamental importance to investigate all the possibilities offered by new monitoring techniques, such as the application of environmental DNA (Taberlet et al., 2018; Thomas et al., 2018; Xia et al., 2021). In fact, the technique has proved highly effective in practical terms and in terms of results for the study of animals closely linked to the aquatic environment, such as the the Appenine yellow-bellied toad, whose physiology greatly favours DNA release within the environment (Knudsen et al., 2023). Here too, the work offered confirmation, having demonstrated the applicability of the method, and detected an area of co- presence between *B. pachypus* and *B. dendrobatidis*.

Lastly, the work carried out to assess the trophic ecology of the Eurasian otter *Lutra lutra* in three rivers in southern Italy offered a new design for the analyses of data derived from traditional monitoring techniques. Through the collection of droppings (spraint) and the analysis of undigested prey remains, the work has in fact provided detailed and accurate information, not only on the trophic habits of the species but also on spatial feeding behaviour of this endangered species. Indeed, our results showed that this generalist and opportunist species (Kruuk, 2006; Roos et al., 2015) very localised diversity of prey in the different river sectors. The analysis of prey selection along the course of the Bussento river agrees with what has been reported on the distribution of fish along the river course (Blanco-Garrido et al., 2008; BIanco et al., 2002; Bianco et al., 2011). This work confirmed the role of the otter as an umbrella species, which, in addition to being of considerable conservation interest, also enables the assessment, and consequently the development, of monitoring and conservation plans for the species with which it shares its ecosystem.

In conclusion, this work highlighted the potential of the new non-invasive monitoring techniques, which allow for simultaneous, large-scale analysis in a reduced time and cost and with minimal disturbance to biodiversity. But it also emphasised the still essential contribution of traditional monitoring techniques, thus highlighting the importance of the complementary application of the two techniques.

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