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Environmental DNA as a tool for monitoring species of EU interest in Italian freshwaters. Management and conservation implications.

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Chapter 1 – Introduction to the research project

Freshwater conservation

The loss of biodiversity has reached an unbelievable alarming pace nowadays, raising rapidly over recent decades. This crisis is taking a toll on ecosystems worldwide, as well as on the natural capital that sustains human life on this planet. Nevertheless, the anthropogenic factors driving these harmful outcomes have not impacted all environments equally. Among them, freshwater ecosystems stand out as perhaps the most endangered globally (Ahmed et al., 2022). Freshwaters indeed face several threats, including water extraction, flow alteration, water pollution, over-fishing, introduction of invasive alien species (IAS), and finally climate changes (Costa et al., 2021; Dextrase & Mandrak, 2006; Francis, 2012; Jaureguiberry et al., 2024). These are directly translated into habitat loss, habitat degradation, and habitat fragmentation for multiple species linked to water for any of their life cycle. As a consequence, numerous freshwater species are significantly declining on a worldwide scale (Sanders et al., 2024). Although freshwater ecosystems are characterized by unique biodiversity and provide essential ecosystem services (Vary et al 2022), they are globally poorly protected and suffer a continuous decline in conservation measures (Moilanen et al., 2008).

Native aquatic fauna: cryptic, endangered, and sensitive species

Freshwater habitats occupy less than the 1% of the Earth but host almost 10% of the known biodiversity and act as hotspots of biological diversity (Strayer and Dudgeon, 2010). Lentic and lotic ecosystems support up to 5700 dragonflies, 17.800 fishes, and 1.600 crustaceans, 250 turtles, 700 birds, and 70 freshwater mammals (Fricke et al., 2019). Moreover, freshwater environments exhibit high levels of endemism (Abell et al., 2008). Unaltered aquatic communities are fragile and often affected by some complications that can hinder their management and conservation. Moreover, native species related to water environments can be cryptic and difficult to detect and monitor. This is especially concerning since many native freshwater species are in danger of extinction and sensitive to environmental changes (Sanders et al 2024; Sayer et al. 2025). Specifically, amphibians and freshwater fish are the most threatened group of vertebrates of the world (Saunders et al., 2002). Compared to terrestrial and marine environments, freshwater vertebrates are currently experiencing the largest biodiversity decline (84% decline since 1970) (WWF, 2020), and it is often believed this is just the tip of the iceberg. Indeed, monitoring efforts primarily focus on vertebrates, and the conservation status of neglected species and lower taxonomic groups is often unknown (Šmejkal et al., 2025). To reverse this decline, accurate and effective action plans are urgently needed (Tickner et al., 2020).

Freshwater species that are rare often have a narrow ecological niche and may also play as bioindicator species (Cucherousset et al., 2008). These species are sensitive to changes in environmental conditions, such as differences in chemical concentrations as exhibited by freshwater mussels (Wang et al., 2025). Therefore, sensitive species can be used to detect changes in natural surroundings as well as to indicate negative impacts which can affect the entire freshwater biodiversity (Parmar et al., 2018). In this way, an early warning of pollutants can be turned into rapid conservation actions to favour all biological components of aquatic ecosystem, and nominating these species as sentinels of the freshwater community for all purposes. Water quality are periodically assessed by several countries though monitoring population density of a bunch of animal bioindicators (Lopez-Lopez and Sedeno-Diaz,2015). A specific group of bioindicators is represented by aquatic invertebrates, typically bottom-dwelling species such as molluscs and arthropods like crustaceans and insects during their aquatic stages (Khatri and Tyagi, 2015). Fish also can be sensitive to different changes in water condition, and are particularly affected by anthropogenic stressors, like the introduction of allochthonous species (Šmejkal et al., 2025).

Cryptic species are elusive wildlife hardly to be observed through traditional techniques due to several factors linked to their biology, such as nocturnal behaviours, harsh habitat requirements, camouflaging, and low population densities (Martin et al., 2022). For example, fully-aquatic species residing on single locations within the core of the river may impede their visual detection (Sternhagen et al., 2024). Furthermore, for their elusiveness, scientists also need well-trained specialists to scrutiny the reliability of their observations (Ebner et al., 2011). These traits limit both the understanding of ecological requirements and the monitoring of population trends (Doak et al., 2015). These limited conditions often reflect a poor knowledge of the conservation status of species that are both cryptic and rare (Hill et al., 2007; Ebner et al., 2011; Cerqueira et al., 2013). Thus, most of these species are often reported as endangered species (Zhi-Gang and En-Yuan, 2003). This is particularly true for species dependent on freshwater habitats that are at risk of extinction (Zhang et al., 2020).

Invasive alien species

Invasive Alien Species (IAS) are among the most impacting threats to biodiversity globally, but are particularly harmful for freshwater ecosystems (Dueñas et al., 2021). Biological invasions alter ecosystem composition and structure, leading to the impoverishment of local native communities, in which endemic species are particularly vulnerable (Ricciardi et al., 2017). Native species are directly threatened due to the negative interactions of competition, predation, and disease transmission (Porco et al., 2024). IAS can also adversely affect human health, leading to severe pathologies, allergies, and burns (Lombardo et al., 2022; Pilaczyk et al., 2022; Souty-Grosset et al., 2016; Longshaw, 2011). This degradation results in reduced ecosystem functions, and a decrease on resilience and resistance (IPBES, 2023). Consequently, it follows a deterioration of ecosystem services and nature's contributions to human well-being, for a global economic loss annually estimated to $423 billion (IPBES, 2023). Among all continents, Europe is the most invaded by freshwater alien species, mainly colonized by fish, crustaceans, and molluscs (IPBES, 2023). Effective strategies to mitigate this threat to the native biodiversity include establishing a coordinated system for the early detection of new invasions and implementing rapid eradication and control measures (Sepulveda et al., 2023). More than 19% of threatened species in Europe are directly endangered by IAS competition, predation, or disease transmission (EU, 2020). Established IAS need rapidly effective management actions informed by spatial planning methodologies.

Italian freshwaters biodiversity

Italian ecosystems support the highest animal biodiversity in Europe (ISPRA, 2009; German Federal Agency for Nature Conservation, 1997; CBD, 2025). As to freshwater ecosystems, the country hosts at least 100,000 species (Cazzolla Gatti, 2016), and the highest number of endemism and diversity on fish in Europe (Fliervoet et al., 2013). Due to its unique biogeographic position at the intersection of north-central and south-central species ranges, species diversity is particularly high in Central Italy (Iannella et al., 2017; Mattoccia et al., 2011; Solano et al., 2018). This region is home to several endemic and endangered freshwater species (Balletto et al., 2010), such as the Italian crayfish (*Austropotamobius italicus*), the Apennine yellow-bellied toad (*Bombina v. pachypus*), the Italian crested newt (*Triturus carnifex*), and the Tuscan stream chub (*Squalius lucumonis*) (Brusconi et al., 2008; Canestrelli et al., 2013; Rondinini et al., 2022; Tancioni et al., 2013). Central Italy also represents a critical potential expansion area for the endangered Eurasian otter (*Lutra lutra*) (Giovacchini et al., 2018). Despite this natural capital, Italian freshwaters suffer the same conditions observed across Europe, with large human alterations leading to a diffuse habitats degradation (Haubrock et al., 2021). The first European Red List of Habitats in Italy highlighted the high level of threat that freshwaters are suffering at the national scale (Gigante et al., 2018). Furthermore, Italian freshwater ecosystems are significantly experiencing additional pressures from the introduction of IAS, including fish, crustaceans, amphibians, and molluscs (IPBES, 2023). In fact, the number of non-indigenous fish species in Italy has now surpassed that of native fish (Lorenzoni et al., 2019;

Nocita et al., 2017). The increasing trend of invasive freshwater species recorded in Italian rivers and lakes makes Italy one of the most impacted country in Europe (Gherardi et al., 2008; Magliozzi et al., 2020).

European legislation and monitoring obligations

Since 1992 the European Union adopted several regulations to implement the international Convention on Biological Diversity to halt the loss of biodiversity at the global scale.

Among these, the Habitats Directive 92/43/EEC (HD hereafter) marked a milestone in European policies for biodiversity conservation. The primary objective of the directive is to preserve natural habitats and wild fauna and flora in a favourable conservation status by implementing active measures or restoration efforts. Specifically, HD focuses on safeguarding a range of habitats and wildlife species that are endangered, rare, or endemic. These, referred to as species and habitats of Community Interest, are listed in Annexes I (habitats), II, IV, and V (animal and plant species) of the directive. Specifically, all forms of killing, capture, and disturbance of wild individuals are strictly prohibited for species listed in Annexes II and IV. HD obliges member states to report every six years on the distribution of species of Community Interest through the establishment of a surveillance monitoring (HD, Art. 17). The directive also mandates the creation of a network of European protected areas, the Special Areas of Conservation, for species listed in Annex II and habitats listed in Annex I. These areas integrate other protected sites designed under the Bird Directive 2009/147/EC to form the world largest network of protected areas, known as the Natura 2000 Network (N2KN hereafter). Currently, the network includes 27,193 Natura 2000 sites and covers 18.6% of land and 9% of marine European territory (EEA, 2022).

Recognizing protected areas as one of the most powerful tool for effective conservation (Rodrigues & Cazalis, 2020), the European Union declared the willingness to expand protected areas coverage up to the 30% of the European land territory until 2030, a target in line with the COP16 Kunming-Montreal Global Biodiversity Framework. The Natura 2000 Network is the backbone of the protected areas network in the Europe and it is complemented by additional areas designated at national level. Together, they reached 26.1% of the European terrestrial coverage by the end of 2022. It is advisable to increase coherently this network to ensure a well-connected ecological network of protected areas to guarantee species movements and habitats preservation within countries and across borders. The commitment of 30% is transferred also to Member States. Italy is covered almost by the 19% of Natura 2000 Network, mostly overlapped with national areas whose contribution increases Italian terrestrial surface being protected up to the 21.5%. This will need to significantly intensify Italian efforts to reach the target.

The Water Framework Directive 2000/60/EC (WFD hereafter) provides a different perspective on conservation of freshwater biodiversity (Weigelhofer et al., 2020). Instead of merely addressing specific actions toward individual species, conservation objectives are addressed to the entire water bodies. WFD focuses on promoting the sustainable use of water, reducing pollution, and enhancing the protection of aquatic environments to ensure adequate water quality to support local biodiversity. Specifically, all European surface and underground water bodies were expected to achieve a good chemical and ecological status by 2015. On 2022, the European Commission published a proposal for amendment of the WFD to align the directives with the 2020 European Green Deal by updating lists of pollutants, updating quality standards, improving assessment of combination effects and ensuring that the legal framework of the directives can swiftly align with scientific findings.

In accomplishment with WFD provisions, the ecological status of rivers and lakes is evaluated using various parameters, many of which are based on the conditions of biological indicators like aquatic flora, benthic invertebrates, and fish communities (Hering et al., 2018). Benthic invertebrate communities are typically analysed using multimetric indexes based on sensitive species, while fish communities are assessed based on the composition and structure of native species and IAS (Hering et al., 2018). Every six years each

Member State should report on the ecological status of national freshwater bodies, including the benthic invertebrates and fish components metrics (WFD, Art. 18). WFD also mandates the establishment of protected areas known as '*protection areas for the conservation of habitats and species directly depending on water*' (WFD, Art. 4, Art. 6, and Annex VII), where to implement several conservation measures for wetlands restoration and negotiated environmental agreements (WFD, Annex VI).

Freshwater ecosystems are also acknowledged as crucial for biodiversity conservation, still needing attention and greater efforts. Thus, Europe supports freshwater restoration, especially directed on large rivers and floodplain, and increases the natural functionality of rivers and lake to clean European water quality and improve the Water Framework Directive. The aim is to enhance flood protection and nursery habitats for native fish, control water regulation and remove chemical pollution at large scale, with a sustainable development toward restoration, tourism, and recreation economic sectors. To achieve these goals, rivers barriers will be removed or adjusted for fish movements, and floodplains will be restored along at least 25,000 km to have free-flowing river courses, depending on the human necessities of hydropower generation, flood management, water supply, agriculture and navigability.

The Regulation on IAS 1143/2014 was adopted by the European Union to prevent new introductions and manage the spreading of invasive alien species in the Union territory (Genovesi et al., 2015). In line with the guidelines of organizations such as the IUCN and IPBES, this Regulation emphasizes the establishment of a robust surveillance system (Pagad et al., 2015). The core of the Regulation is the list of IAS of Union concern (Union List). The species included on this list are subject to restrictions and measures set out in the Regulation. These include restrictions on keeping, importing, selling, breeding, growing and releasing into the environment. Member States are required to implement a monitoring framework to swiftly detect the presence of any of the 88 IAS of Union concern (amended version 2022/1203) within their territories and promptly initiate eradication efforts during the early stages of invasion.

The surveillance system must update every six years the distribution of IAS across the European territory and assess the effectiveness of rapid eradication measures (Art. 24). Simultaneously, management measures must be applied to IAS that are already widely spread. To address this, a spatial prioritization approach is essential, focusing on prevention in areas where species are absent, eradication where IAS have recently emerged, and mitigation in heavily colonized areas (EC, 2014). Italian freshwaters host 40 over the total 88 IAS of Union concern (Colangelo et al. 2017), making monitoring protocols and management plans of these species a priority to accomplish the Regulation provisions.

The European Biodiversity Strategy for 2030 boosts the three regulations illustrated above, committing several ambitious goals in relation to protected areas, freshwater conservation, and IAS management. Specifically, the Strategy includes a pledge to manage established IAS in order to reduce by 50% the number of native Red List species directly threatened by IAS by 2030 (EC, 2020). EU regulations and the European Biodiversity Strategy for 2030 urge to adopt rapid and sensitive monitoring protocols of both rare and endangered species, as well for early warning of IAS (Leese et al., 2018; Pawlowski et al., 2021). To achieve good conservation status of species and habitats protected by HD it is also important to provide a spatial planning framework to establish new protected areas that could fill gaps and improve the connectivity among protected areas, thus contributing to the extension of European protected areas by 2030.

Species monitoring

Monitoring freshwater species is central to all European regulations dealing with biodiversity conservation, and to achieve objectives of the European Biodiversity Strategy for 2030. Monitoring through periodic systematic surveys is essential for understanding environmental systems, and to fulfil three key purposes: detecting deviations from desired conditions, assessing the success of management and conservation

initiatives, and tracking the effects of disturbances over time (Primack and Boitani, 2013). Understanding the initial state of environments and follow the trends over time and space is crucial to orient actions and plan recovery programs to modify the status itself. Also, the early detection of rare species is relevant for rapid adoption of protection measures to support new individuals colonizing new territories (Duarte et al., 2023). This underlines the importance of accurate and sensitive monitoring programs and protocols to describe wildlife population densities, species distribution, and habitat conditions. However, monitoring multiple species across different taxa and habitats is labour-intensive, costly, and requires taxon-specific expertise, often hindering the implementation of targeted monitoring programs, particularly for rare and endangered species and IAS at the initial stages of colonisations. This is specifically true for freshwater species, where traditional monitoring techniques involve specific protocols tailored to each taxon habitat, behaviour, and ecological requirements (Stoch and Genovesi, 2016). Moreover, these traditional survey techniques often fail to detect populations at low densities, and demand intensive sampling efforts, often causing stress or even damage to the animals being handled (Bo et al., 2011). Accordingly, financial resources for periodical multi-specific monitoring programs are highly demanding in terms of both costs and time (Barnes & Turner, 2016).

Environmental DNA

Genomic techniques based on the detection of environmental DNA (eDNA) are a highly effective non-invasive method for investigating the presence of animal or plant species over large areas and in short periods of time. They can be applied to several environmental matrices, like water, air, soil, snow, ice, biofilms (Taberlet et al., 2018). eDNA also revealed potentials retrieving fragments of DNA natural samplers like for example spider webs filtering air, sponges filtering thousands of water litres per day as well as freshwater mussels, and leeches ingesting blood of vertebrate community (Gregoric et al., 2022; Mariani et al., 2019; Schnell et al., 2015; Weber et al., 2023). The collection of the DNA shred into the environment is particularly accurate and sensitive (Beng & Corlett, 2020; Mynhardt et al., 2024).

The potentiality of eDNA techniques lies in the fact that they don't require a direct contact with organisms, i.e. they are highly non-invasive techniques, and, unlike other non-invasive techniques they don't imply time consuming efforts for the detection of indirect presence signs (Jamwal et al., 2023). This is particularly true for rare species that live at low population densities, exhibiting elusive behaviours, or are cryptic and difficult to locate, i.e., due to their small size. Thus, eDNA could be particularly powerful to unveil relict populations, discover initial stages of colonisations of IAS, and preventing wildlife disturbance to sensitive populations as it is completely harmfulness (Barnes & Turner, 2016). Additionally, eDNA sampling allows for the simultaneous detection of multiple species, even if they belong to very different taxa, thereby providing insight on extended biotic communities within a specific area (Pont et al., 2018).

Molecules of DNA spread into the environment are usually rare, implying the adoption of accurate and reliable methods for the species detection (Johnson et al., 2024). In this context, the quantitative PCR (qPCR) approach offers high sensitivity and species-specific resolution, outperforming other common eDNA alternatives like metabarcoding (Harper et al., 2018; McColl-Gausden et al., 2023). The use of PCR (Polymerase Chain Reaction) makes this method very easy and fast to be implemented both in the field and in the lab (Taberlet et al., 2018). Thus, eDNA species-specific monitoring protocols can represent a powerful tool to accomplish EU regulations monitoring provisions, as witnessed by their adoption by the United States Geological Survey (Pilliod et al., 2019).

Accordingly, eDNA could represent an effective complementary tool to fulfill the monitoring obligations under the Habitats Directive and for the early detection of IAS of Union Concern, as to the 1143/2014 EU Regulation, as well as to accomplish biological assessments about water quality under the Water Framework Directive, such as those on benthic invertebrates and fish fauna (Leese et al., 2018; Pawlowski et al., 2021; Thomsen et al. 2012). Simultaneous multispecific eDNA surveys results could also guide the extension of protected areas by 2030, especially those addressed to the protection of freshwater habitats

and species. The usefulness of eDNA to support the Water Framework Directive and the European strategy to make European river in a free-flowing condition has been highlighted also by recent studies showed potentials in evaluations the propensity of local communities toward dam removal (Fernandez et al., 2022).

A multispecific eDNA approach could also inform spatial planning on IAS management through the detection of direct and indirect threats to native endangered species, i.e. prioritizing efforts for eradication where IAS occur with native species.

One of the primary challenges in expanding and solidifying a species-specific approach for the simultaneous monitoring of multiple species through eDNA lies in the need of well-validated qPCR assays. Despite the growing need, many species still lack these critical assays (Thalinger et al., 2021). Furthermore, for a ready-to-use tool for routine monitoring at local scale, a proper validation at the local scale is essential to account for various detection biases due to biogeographic and genetic variation and mismatches with similar syntopic species, leading to false negatives (Goldberg et al., 2016; Klymus et al., 2020; Langlois et al., 2021). These factors highlight the importance of investing time and resources in the design and validation phases to ensure both robustness and efficiency (Nicholson et al., 2020; Sepulveda et al., 2020; Thalinger et al., 2021).

Despite some qPCR eDNA protocols have been recently developed to quickly inform on species presence (Thomas et al., 2020), this technique is still rarely used to simultaneously detect different species (King et al., 2022; Minegishi et al., 2023; Tsuji et al., 2018; Wozney & Wilson, 2017), and it has been even more rarely applied to wide taxonomic inventories (Leblanc et al., 2020; Seymour et al., 2018; Thomsen et al., 2012).

Furthermore, despite qPCR based eDNA has been successfully applied to detect several freshwater species, studies focusing on single species often did not evaluate the relative efficiency of the eDNA vs traditional field surveys (Ballini et al., 2024; Jamwal et al., 2023).

Also, the scientific community has been recently focusing on bridging the knowledge gaps regarding the ecological parameters that affect the eDNA, whose understanding is crucial for increasing the accuracy of the results. These types of information are vital to guide a planning process of an eDNA project, and guide context-specific standard procedures to the sampling design, e.g. time and space selection.

Setting up an environmental DNA study, may entail the arise of several strategic, logistical, scientific, technological, and economic issues. To put qPCR eDNA at the service of European regulations, guidelines and best-practices need to be improved in different ecoregions. Currently, Italy lacks eDNA guidelines for its use to accomplish monitoring obligations under EU regulations, except those proposed for the transnational Alpine context, although relying on metabarcoding and led by a Slovenian team (Domaizon et al., 2021).

Results from qPCR eDNA could also be combined with species distribution modelling to further increase the optimization of monitoring protocol, to implement spatial planning based on species presence/absence, and to implement connectivity and species distribution modelling to forecast future conservation needs

Aims of the project

My PhD research project has been addresses to explore the potential applications of qPCR eDNA techniques to contribute monitoring obligations by European regulations for freshwater species of Community Interest occurring in Central Italy.

The specific aims were as follows:

Test a qPCR eDNA protocol for multi-species detection in freshwaters of Central Italy (chapter 2)

- to simultaneously detect native endangered species and IAS of Community Interest,
- to design new qPCR assays for freshwater species,
- to orient sampling timing and location,
- to identify small- and large-scale environmental factors affecting eDNA detection,
- to compare costs of a multi-taxa survey between qPCR eDNA and traditional monitoring techniques.

Explore the conservation planning implications of a qPCR eDNA multi-specific assessment results (chapter 3)

- to identify potential areas for new protected areas and IAS management under three EU regulations
- to update the distribution of endangered species listed in Habitats Directive,
- to early warn new IAS of Union Concern identified in the study area,
- to update the distribution of IAS species listed in the Regulation on IAS 1143/2014,
- to integrate routine monitoring under the Water Framework Directive.

Compare the efficiency of qPCR eDNA *versus* traditional monitoring techniques (chapter 4)

- on the case study of *Lutra lutra* along the Tyrrhenian side of central Italy

Develop guidelines for eDNA monitoring in Central Italy (chapter 5)

- to provide information for planning a qPCR eDNA project on freshwater species.

Each aim is the topic of a specific chapter that is already in the form of a paper submitted or to be submitted.

Literature

Abell, R., Thieme, M. L., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., Coad, B., Mandrak, N., Balderas, S. C., Bussing, W., Stiassny, M. L. J., Skelton, P., Allen, G. R., Unmack, P., Naseka, A., Ng, R., Sindorf, N., Robertson, J., Armijo, E., … Petry, P. (2008). Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. BioScience, 58(5), 403–414. https://doi.org/10.1641/B580507

Ahmed, S. F., Kumar, P. S., Kabir, M., Zuhara, F. T., Mehjabin, A., Tasannum, N., ... & Mofijur, M. (2022). Threats, challenges and sustainable conservation strategies for freshwater biodiversity. Environmental Research, 214, 113808.

Balletto, E., Bonelli, S., Borghesio, L., Casale, A., Brandmayr, P., C Vigna Taglianti, A. (2010). Hotspots of biodiversity and conservation priorities: A methodological approach. Italian Journal of Zoology, 77(1), 2–13. https://doi.org/10.1080/11250000902758923

Ballini, L., Ottonello, D., Repetto, V., Natali, C., Chini, G., Tolve, L., Ciofi, C., Fratini, S., & Iannucci, A. (2024). Early detection of rare and elusive endangered species using environmental DNA: a case study for the Eurasian otter and the white-clawed crayfish in northwestern Italy. *Conservation Genetics*, *25*(4), 999–1005. https://doi.org/10.1007/s10592-024-01619-5

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Bo, T., Cammarata, M., Candiotto, A., Fenoglio, S., López-Rodríguez, M. J., Tierno de Figueroa, J. M., C Malacarne, G. (2011). Feeding habits of Padogobius bonelli (Bonaparte, 1846) (Osteichthyes, Gobiidae): The importance of fish dimensions and hydrological conditions. Italian Journal of Zoology, 78(3), 390–397. https://doi.org/10.1080/11250001003777168

Brusconi, S., Bertocchi, S., Renai, B., Scalici, M., Souty-Grosset, C., & Gherardi, F. (2008). Conserving indigenous crayfish: Stock assessment and habitat requirements in the threatened Austropotamobius italicus. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *18*(7), 1227–1239. https://doi.org/10.1002/aqc.935

Canestrelli, D., Zampiglia, M., & Nascetti, G. (2013). Widespread Occurrence of Batrachochytrium dendrobatidis in Contemporary and Historical Samples of the Endangered Bombina pachypus along the Italian Peninsula. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0063349

Cazzolla Gatti, R. (2016). Freshwater biodiversity: A review of local and global threats. International Journal of Environmental Studies, 73(6), 887–904. https://doi.org/10.1080/00207233.2016.1204133

CBD (2025). https://www.cbd.int/countries/profile?country=it#:~:text=Status%20and%20trends%20of%20biodiversity,a%20high%20rate%20of%20endemism [accessed on 20/01/2025]

Cerqueira, M. C., Cohn-Haft, M., Vargas, C. F., Nader, C. E., Andretti, C. B., Costa, T. V. V, Sberze, M., Hines, J. E., & Ferraz, G. (2013). Rare or elusive? A test of expert knowledge about rarity of Amazon forest birds. *Diversity and Distributions*, *19*(7), 710–721. https://doi.org/https://doi.org/10.1111/ddi.12033

Colangelo, P., Fontaneto, D., Marchetto, A., Ludovisi, A., Basset, A., Bartolozzi, L., ... & Boggero, A. (2017). Alien species in Italian freshwater ecosystems: a macroecological assessment of invasion drivers. *Aquatic Invasions*, *12*(3), 299-309.

Costa, M. J., Duarte, G., Segurado, P., & Branco, P. (2021). Major threats to European freshwater fish species. *Science of The Total Environment*, *797*, 149105. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.149105

Cucherousset, J., Santoul, F., Figuerola, J., & Céréghino, R. (2008). How do biodiversity patterns of river animals emerge from the distributions of common and rare species? *Biological Conservation*, *141*(12), 2984–2992. https://doi.org/10.1016/j.biocon.2008.09.004

Dextrase, A. J., & Mandrak, N. E. (2006). Impacts of alien invasive species on freshwater fauna at risk in Canada. *Biological Invasions*, *8*(1), 13–24. https://doi.org/10.1007/s10530-005-0232-2

Doak, D. F., Himes Boor, G. K., Bakker, V. J., Morris, W. F., Louthan, A., Morrison, S. A., Stanley, A., &

Crowder, L. B. (2015). Recommendations for Improving Recovery Criteria under the US Endangered Species Act. *BioScience*, *65*(2), 189–199. https://doi.org/10.1093/biosci/biu215

Domaizon, I., Riccioni, G., Pindo, M., Vasselon, V., Kurmayer, R., Boscaini, A., ... & Salmaso, N. (2021). Technical guidelines for eDNA monitoring in Alpine waters for stakeholders and end-users. Interreg Alpine Space Eco-AlpsWater.

Duarte, S., Simões, L., & Costa, F. O. (2023). Current status and topical issues on the use of eDNA-based targeted detection of rare animal species. *Science of The Total Environment*, *904*, 166675. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.166675

Dueñas, M.-A., Hemming, D. J., Roberts, A., & Diaz-Soltero, H. (2021). The threat of invasive species to IUCN-listed critically endangered species: A systematic review. *Global Ecology and Conservation*, *26*, e01476. https://doi.org/https://doi.org/10.1016/j.gecco.2021.e01476

Ebner, B. C., Kroll, B., Godfrey, P., Thuesen, P. A., Vallance, T., Pusey, B., Allen, G. R., Rayner, T. S., & Perna, C. N. (2011). Is the elusive Gymnothorax polyuranodon really a freshwater moray? *Journal of Fish Biology*, *79*(1), 70–79. https://doi.org/https://doi.org/10.1111/j.1095-8649.2011.02987.x

EC (2014). Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. Official Journal of European Union, L 317, 04.11.2014.

EC (2020). Communication from the commission to the European parliament, the Council, the European economic and social committee and the Committee of the regions. EU Biodiversity Strategy for 2030 - Bringing nature back into our lives. Brussels, 20.5.2020 COM(2020) 380 final

EEA (2022). Natura 2000 barometer. European Environment Agency. https://www.eea.europa.eu/data-and-maps/dashboards/natura-2000-barometer [accessed November 22, 2022].

EU (1992). Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Official Journal of European Communities, L 206, 22.7.1992, p. 7-50.

Fernandez, S., Arboleya, E., Dopico, E., & Garcia-Vazquez, E. (2022). Dams in South Europe: socio-environmental approach and eDNA-metabarcoding to study dam acceptance and ecosystem health. *Wetlands Ecology and Management*, *30*(2), 341–355. https://doi.org/10.1007/s11273-022-09864-6

Fliervoet, J. M., Van den Born, R. J. G., Smits, A. J. M., & Knippenberg, L. (2013). Combining safety and nature: A multi-stakeholder perspective on integrated floodplain management. *Journal of Environmental Management*, *128*, 1033–1042. https://doi.org/https://doi.org/10.1016/j.jenvman.2013.06.023

Francis, R. A. (2012). A Handbook of Global Freshwater Invasive Species. In *A Handbook of Global Freshwater Invasive Species*. https://doi.org/10.4324/9780203127230

Fricke, A. T., Nittrouer, C. A., Ogston, A. S., Nowacki, D. J., Asp, N. E., C Souza Filho, P. W. M. (2019). Morphology and dynamics of the intertidal floodplain along the Amazon tidal river. Earth Surface Processes and Landforms, 44(1), 204–218. https://doi.org/10.1002/esp.4545

German Federal Agency for Nature Conservation, 1997. European Biodiversity. In: Biodiversity and Tourism. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-60689-2_8

Gherardi, F., Bertolino, S., Bodon, M., Casellato, S., Cianfanelli, S., Ferraguti, M., Lori, E., Mura, G., Nocita, A., Riccardi, N., Rossetti, G., Rota, E., Scalera, R., Zerunian, S., & Tricarico, E. (2008). Animal xenodiversity in Italian inland waters: Distribution, modes of arrival, and pathways. *Biological Invasions*, *10*(4), 435–454. https://doi.org/10.1007/s10530-007-9142-9

Gigante, D., Acosta, A. T. R., Agrillo, E., Armiraglio, S., Assini, S., Attorre, F., Bagella, S., Buffa, G., Casella, L., Giancola, C., Giusso del Galdo, G. P., Marcenò, C., Pezzi, G., Prisco, I., Venanzoni, R., C Viciani, D. (2018). Habitat conservation in Italy: The state of the art in the light of the first European Red List of Terrestrial and Freshwater Habitats. Rendiconti Lincei. Scienze Fisiche e Naturali, 2S(2), 251–265. https://doi.org/10.1007/s12210-018-0688-5

Giovacchini, S., Marrese, M., & Loy, A. (2018). Good news from the south: filling the gap between two otter populations in Italy. *IUCN Otter Specialist Group Bulletin*, *35*(4), 212-221.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler-McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, *7*(11), 1299–1307. https://doi.org/10.1111/2041-210X.12595

Gregorič, M., Kutnjak, D., Bačnik, K., Gostinčar, C., Pecman, A., Ravnikar, M., & Kuntner, M. (2022). Spider webs as eDNA samplers: Biodiversity assessment across the tree of life. *Molecular ecology resources*, *22*(7), 2534-2545.

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S., & Hänfling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (Triturus cristatus). *Ecology and Evolution*, *8*(12), 6330–6341. https://doi.org/https://doi.org/10.1002/ece3.4013

Haubrock, P. J., Cuthbert, R. N., Tricarico, E., Diagne, C., Courchamp, F., C Gozlan, R. E. (2021). The recorded economic costs of alien invasive species in Italy. NeoBiota, c7, 247–266. https://doi.org/10.3897/neobiota.67.57747

Hering, D., Borja, A., Jones, J. I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., & Kelly, M. (2018). Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Research*, *138*, 192–205. https://doi.org/https://doi.org/10.1016/j.watres.2018.03.003

Hill, S. L. L., Sayer, C. D., Hammond, P. M., Rimmer, V. K., Davidson, T. A., Hoare, D. J., Burgess, A., & Okamura, B. (2007). Are rare species rare or just overlooked? Assessing the distribution of the freshwater bryozoan, Lophopus crystallinus. *Biological Conservation*, *135*(2), 223–234. https://doi.org/10.1016/j.biocon.2006.10.023

Iannella, M., Cerasoli, F., C Biondi, M. (2017). Unraveling climate influences on the distribution of the parapatric newts Lissotriton vulgaris meridionalis and L. italicus. Frontiers in Zoology, 14(1), 55. https://doi.org/10.1186/s12983-017-0239-4

IPBES. (2023). *Summary for Policymakers of the Thematic Assessment Report on Invasive Alien Species and their Control of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services* (H. E. Roy, A. Pauchard, P. Stoett, T. Renard Truong, S. Bacher, B. S. Galil, P. E. Hulme, T. Ikeda, K. V Sankaran, M. A. McGeoch, L. A. Meyerson, M. A. Nuñez, A. Ordonez, S. J. Rahlao, E. Schwindt, H. Seebens, A. W. Sheppard, & V. Vandvik (eds.)). IPBES Secretariat. https://doi.org/10.5281/zenodo.7430692

ISPRA (2009). Key topics. Italian Environmental Data Yearbook. Chapter 2 – Biodiversity and Natural, Agricultural and Forest Areas. Italian Institute for Environmental Protection and Research.

Jamwal, P. S., Bruno, A., Galimberti, A., Magnani, D., Casiraghi, M., & Loy, A. (2023). Environmental DNA revealed high accuracy in detection of the Eurasian otter in Himalaya. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *33*(11), 1309–1320. https://doi.org/https://doi.org/10.1002/aqc.4010

Jaureguiberry, P., Titeux, N., Wiemers, M., Bowler, D. E., Coscieme, L., Golden, A. S., Guerra, C. A., Jacob, U., Takahashi, Y., Settele, J., Díaz, S., Molnár, Z., & Purvis, A. (2024). The direct drivers of recent global anthropogenic biodiversity loss. *Science Advances*, *8*(45), eabm9982. https://doi.org/10.1126/sciadv.abm9982

Johnson, M., Tetzlaff, S., Katz, A., & Sperry, J. (2024). Comparison of qPCR and metabarcoding for environmental DNA surveillance of a freshwater parasite. *Ecology and Evolution*, *14*(5), e11382. https://doi.org/https://doi.org/10.1002/ece3.11382

Khatri, N., & Tyagi, S. (2015). Influences of natural and anthropogenic factors on surface and groundwater quality in rural and urban areas. Frontiers in life science, 8(1), 23-39.

King, A. C., Krieg, R., Weston, A., & Zenker, A. K. (2022). Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. *Journal of Environmental Management*, *302*, 113929. https://doi.org/https://doi.org/10.1016/j.jenvman.2021.113929

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282. https://doi.org/https://doi.org/10.1002/edn3.29

Langlois, V. S., Allison, M. J., Bergman, L. C., To, T. A., & Helbing, C. C. (2021). The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. *Environmental DNA*, *3*(3), 519–527. https://doi.org/https://doi.org/10.1002/edn3.164

Leblanc, F., Belliveau, V., Watson, E., Coomber, C., Simard, N., Di Bacco, C., Bernier, R., & Gagné, N. (2020).

Environmental DNA (eDNA) detection of marine aquatic invasive species (AIS) in Eastern Canada using a targeted species-specific qPCR approach. *Management of Biological Invasions*, *11*(2), 201–217. https://doi.org/10.3391/mbi.2020.11.2.03

Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., Ekrem, T., Čiampor, F., Čiamporová-Zaťovičová, Z., Costa, F. O., Duarte, S., Elbrecht, V., Fontaneto, D., Franc, A., Geiger, M. F., Hering, D., Kahlert, M., Kalamujić Stroil, B., Kelly, M., … Weigand, A. M. (2018). Chapter Two - Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action. In D. A. Bohan, A. J. Dumbrell, G. Woodward, & M. B. T.-A. in E. R. Jackson (Eds.), *Next Generation Biomonitoring: Part 1* (Vol. 58, pp. 63–99). Academic Press. https://doi.org/https://doi.org/10.1016/bs.aecr.2018.01.001

Lombardo, A., Brocherel, G., Donnini, C., Fichi, G., Mariacher, A., Diaconu, E. L., ... & De Liberato, C. (2022). First report of the zoonotic nematode Baylisascaris procyonis in non-native raccoons (Procyon lotor) from Italy. *Parasites & Vectors*, *15*(1), 24.

Longshaw, M. (2011). Diseases of crayfish: a review. *Journal of invertebrate pathology*, *106*(1), 54-70.

López-López, E., & Sedeño-Díaz, J. E. (2015). Biological indicators of water quality: The role of fish and macroinvertebrates as indicators of water quality. Environmental indicators, 643-661.

Lorenzoni, M., Borghesan, F., Carosi, A., Ciuffardi, L., De Curtis, O., Del Mastro, G. B., Di Tizio, L., Franzoi, P., Maio, G., Mojetta, A., Nonnis Marzano, F., Pizzul, E., Rossi, G., Scalici, M., & Zanetti, M. (2019). Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane the Check-List of the Italian Freshwater Fish Fauna. *Italian Journal of Freshwater Ichthyology*, *5*(1), 239–254.

MacDonald, A. J., C Sarre, S. D. (2017). A framework for developing and validating taxon-specific primers for specimen identification from environmental DNA. Molecular Ecology Resources, 17(4), 708–720. https://doi.org/10.1111/1755-0998.12618

Magliozzi, C., Tsiamis, K., Vigiak, O., Deriu, I., Gervasini, E., & Cardoso, A. C. (2020). Assessing invasive alien species in European catchments: Distribution and impacts. *Science of the Total Environment*, *732*, 138677. https://doi.org/10.1016/j.scitotenv.2020.138677

Mariani, S., Baillie, C., Colosimo, G., & Riesgo, A. (2019). Sponges as natural environmental DNA samplers. *Current Biology*, *29*(11), R401-R402.

Martin, M. E., Delheimer, M. S., Moriarty, K. M., Early, D. A., Hamm, K. A., Pauli, J. N., Mcdonald, T. L., & Manley, P. N. (2022). Conservation of rare and cryptic species: Challenges of uncertainty and opportunities for progress. *Conservation Science and Practice*, *4*(11), e12809. https://doi.org/https://doi.org/10.1111/csp2.12809

McColl-Gausden, E. F., Weeks, A. R., Coleman, R., Song, S., & Tingley, R. (2023). Using hierarchical models to compare the sensitivity of metabarcoding and qPCR for eDNA detection. *Ecological Informatics*, *75*, 102072. https://doi.org/https://doi.org/10.1016/j.ecoinf.2023.102072

Minegishi, Y., Wong, M. K.-S., Nakao, M., Nishibe, Y., Tachibana, A., Kim, Y.-J., & Hyodo, S. (2023). Species-specific patterns in spatio-temporal dynamics of juvenile chum salmon and their zooplankton prey in Otsuchi Bay, Japan, revealed by simultaneous eDNA quantification of diverse taxa from the same water samples. *Fisheries Oceanography*, *32*(3), 311–326. https://doi.org/https://doi.org/10.1111/fog.12631

Moilanen, A., Leathwick, J., & Elith, J. (2008). A method for spatial freshwater conservation prioritization. *Freshwater Biology*, *53*(3), 577–592. https://doi.org/https://doi.org/10.1111/j.1365-2427.2007.01906.x

Mynhardt, S., Matthew, E., le Roux, J. P., Little, I., Bloomer, P., & Theron, C. (2024). Environmental DNA from soil reveals the presence of a "lost" Afrotherian species. *Biodiversity and Conservation*, *33*(1), 31–50. https://doi.org/10.1007/s10531-023-02728-2

Nicholson, A., McIsaac, D., MacDonald, C., Gec, P., Mason, B. E., Rein, W., Wrobel, J., de Boer, M., Milián-García, Y., & Hanner, R. H. (2020). An analysis of metadata reporting in freshwater environmental DNA research calls for the development of best practice guidelines. *Environmental DNA*, *2*(3), 343–349. https://doi.org/https://doi.org/10.1002/edn3.81

Nocita, A., Tricarico, E., & Bertolino, S. (2017). Fine-scale analysis of heavily invaded Italian freshwater fish assemblages. *Integrative Zoology*, *12*(6), 500–511. https://doi.org/https://doi.org/10.1111/1749-4877.12267

Pagad, S., Genovesi, P., Carnevali, L., Scalera, R., & Clout, M. (2015). IUCN SSC Invasive Species Specialist Group: invasive alien species information management supporting practitioners, policy makers and

decision takers. *Management of Biological Invasions*, *6(2)*, 127–135. https://doi.org/10.3391/mbi.2015.6.2.03

Parmar, T. K., Rawtani, D., & Agrawal, Y. K. (2016). Bioindicators: the natural indicator of environmental pollution. Frontiers in life science, 9(2), 110-118.

Pawlowski, J., Bonin, A., Boyer, F., Cordier, T., & Taberlet, P. (2021). *Environmental DNA for biomonitoring*. *June*, 2931–2936. https://doi.org/10.1111/mec.16023

Pilarczyk, B. M., Tomza-Marciniak, A. K., Pilarczyk, R., Rząd, I., Bąkowska, M. J., Udała, J. M., ... & Havryliak, V. (2022). Infection of raccoon dogs (Nyctereutes procyonoides) from northern Poland with gastrointestinal parasites as a potential threat to human health. *Journal of Clinical Medicine*, *11*(5), 1277.

Pilliod, D. S., Laramie, M. B., MacCoy, D., & Maclean, S. (2019). Integration of eDNA-based biological monitoring within the US Geological Survey's National Streamgage Network. *JAWRA Journal of the American Water Resources Association*, *55*(6), 1505-1518.

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., & Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, *8*(1), 10361. https://doi.org/10.1038/s41598-018-28424-8

Porco, D., Purnomo, C. A., Glesener, L., Proess, R., Lippert, S., Jans, K., Colling, G., Schneider, S., Stassen, R., & Frantz, A. C. (2024). eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. *BMC Ecology and Evolution*, *24*(1), 1–16. https://doi.org/10.1186/s12862-023-02189-9

Primack, R. B., & Boitani, L. (2013). *Biologia della conservazione*. Zanichelli editore. ISO 690

Reyjol, Y., Hugueny, B., Pont, D., Bianco, P. G., Beier, U., Caiola, N., Casals, F., Cowx, I., Economou, A., Ferreira, T., Haidvogl, G., Noble, R., De Sostoa, A., Vigneron, T., & Virbickas, T. (2007). Patterns in species richness and endemism of European freshwater fish. *Global Ecology and Biogeography*, *16*(1), 65–75. https://doi.org/https://doi.org/10.1111/j.1466-8238.2006.00264.x

Ricciardi, A., Blackburn, T. M., Carlton, J. T., Dick, J. T. A., Hulme, P. E., Iacarella, J. C., Jeschke, J. M., Liebhold, A. M., Lockwood, J. L., MacIsaac, H. J., Pyšek, P., Richardson, D. M., Ruiz, G. M., Simberloff, D., Sutherland, W. J., Wardle, D. A., C Aldridge, D. C. (2017). Invasion Science: A Horizon Scan of Emerging Challenges and Opportunities. Trends in Ecology & Evolution, 32(6), 464–474. https://doi.org/10.1016/j.tree.2017.03.007

Rodrigues, A. S. L., & Cazalis, V. (2020). The multifaceted challenge of evaluating protected area effectiveness. *Nature Communications*, *11*(1), 5147. https://doi.org/10.1038/s41467-020-18989-2

Rondinini, C., Battistoni, A., & Teofili, C. (2022). Lista rossa 2022. *Lista Rossa IUCN Dei Vertebrati Italiani 2022*, *Comitato I*.

Sanders, E., Wassens, S., Michael, D. R., Nimmo, D. G., & Turner, J. M. (2024). Extinction risk of the world's freshwater mammals. *Conservation Biology*, *38*(1), e14168. https://doi.org/https://doi.org/10.1111/cobi.14168

Saunders, D. L., Meeuwig, J. J., & Vincent, A. C. (2002). Freshwater protected areas: strategies for conservation. Conservation biology, 16(1), 30-41.

Schnell, I. B., Sollmann, R., Calvignac-Spencer, S., Siddall, M. E., Yu, D. W., Wilting, A., & Gilbert, M. T. P. (2015). iDNA from terrestrial haematophagous leeches as a wildlife surveying and monitoring tool–prospects, pitfalls and avenues to be developed. *Frontiers in zoology*, *12*, 1-14.

Sepulveda, A. J., Hutchins, P. R., Forstchen, M., Mckeefry, M. N., & Swigris, A. M. (2020). The Elephant in the Lab (and Field): Contamination in Aquatic Environmental DNA Studies . In *Frontiers in Ecology and Evolution* (Vol. 8). https://www.frontiersin.org/articles/10.3389/fevo.2020.609973

Sepulveda, A. J., Dumoulin, C. E., Blanchette, D. L., McPhedran, J., Holme, C., Whalen, N., ... & Smith, D. R. (2023). When are environmental DNA early detections of invasive species actionable?. Journal of Environmental Management, 343, 118216.

Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., Colbourne, J. K., Wilgar, G., Carvalho, G. R., de Bruyn, M., Edwards, F., Emmett, B. A., Bik, H. M., & Creer, S. (2018). Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. *Communications*

Biology, *1*(1), 4. https://doi.org/10.1038/s42003-017-0005-3
Šmejkal, M., Kalous, L., Auwerx, J., Gorule, P. A., Jarić, I., Dočkal, O., Fedorčák, J., Muška, M., Thomas, K., Takács, P., Ferincz, Á., Choleva, L., Lamatsch, D. K., Wanzenböck, J., & Van Wichelen, J. (2025). Wetland fish in peril: A synergy between habitat loss and biological invasions drives the extinction of neglected native fauna. *Biological Conservation*, *302*, 110948. https://doi.org/https://doi.org/10.1016/j.biocon.2024.110948
Souty-Grosset, C., Anastácio, P. M., Aquiloni, L., Banha, F., Choquer, J., Chucholl, C., & Tricarico, E. (2016). The red swamp crayfish Procambarus clarkii in Europe: impacts on aquatic ecosystems and human well-being. *Limnologica*, *58*, 78-93.
Sternhagen, E. C., Davis, M. A., Larson, E. R., Pearce, S. E., Ecrement, S. M., Katz, A. D., & Sperry, J. H. (2024). Comparing cost, effort, and performance of environmental DNA sampling and trapping for detecting an elusive freshwater turtle. *Environmental DNA*, *6*(2), e525. https://doi.org/https://doi.org/10.1002/edn3.525
Stoch, F., & Genovesi, P. (2016). Manuali per il monitoraggio di specifiee habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. *ISPRA, Serie Manuali e linee guida*, *141*(2016), 1-364.
Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. Journal of the North American Benthological Society, 29(1), 344-358.
Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For biodiversity research and monitoring*. Oxford University Press.
Tancioni, L., Russo, T., Cataudella, S., Milana, V., Hett, A. K., Corsi, E., & Rossi, A. R. (2013). Testing Species Delimitations in Four Italian Sympatric Leuciscine Fishes in the Tiber River: A Combined Morphological and Molecular Approach. *PLoS ONE*, *8*(4), 1–10. https://doi.org/10.1371/journal.pone.0060392
Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., Traugott, M., Goldberg, C. S., & Bruce, K. (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA*, *3*(4), 823–836. https://doi.org/10.1002/edn3.189
Thomas, A. C., Tank, S., Nguyen, P. L., Ponce, J., Sinnesael, M., & Goldberg, C. S. (2020). A system for rapid eDNA detection of aquatic invasive species. *Environmental DNA*, *2*(3), 261–270. https://doi.org/https://doi.org/10.1002/edn3.25
Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/https://doi.org/10.1111/j.1365-294X.2011.05418.x
Tickner D., Opperman J.J., Abell R., Acreman M., Arthington A.H., Bunn S.E., et al. (2020). Bending the curve of global freshwater biodiversity loss: an emergency recovery plan. BioScience, 70: 330–342.
Tsuji, S., Iguchi, Y., Shibata, N., Teramura, I., Kitagawa, T., & Yamanaka, H. (2018). Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese medaka species. *Scientific Reports*, *8*(1), 9138. https://doi.org/10.1038/s41598-018-27434-w
Vári, Á., Podschun, S. A., Erős, T., Hein, T., Pataki, B., Iojă, I. C., ... & Báldi, A. (2022). Freshwater systems and ecosystem services: Challenges and chances for cross-fertilization of disciplines. *Ambio*, *51*(1), 135-151.
Wang, N., Ivey, C. D., Cleveland, D., Kunz, J. L., Schapansky, R., Lane, T., & Barnhart, M. C. (2025). Evaluation of the sensitivity of a federally endangered freshwater mussel (Venustaconcha trabalis) to selected chemicals. Environmental Toxicology and Chemistry, vgae092.
Weber, S., Junk, I., Brink, L., Wörner, M., Künzel, S., Veith, M., ... & Krehenwinkel, H. (2023). Molecular diet analysis in mussels and other metazoan filter feeders and an assessment of their utility as natural eDNA samplers. *Molecular Ecology Resources*, *23*(2), 471-485.
Weigelhofer, G., Feldbacher, E., Trauner, D., Pölz, E., Hein, T., & Funk, A. (2020). Integrating Conflicting Goals of the EC Water Framework Directive and the EC Habitats Directives Into Floodplain Restoration Schemes. *Frontiers in Environmental Science*, *8*(November), 1–14. https://doi.org/10.3389/fenvs.2020.538139
Wozney, K. M., & Wilson, C. C. (2017). Quantitative PCR multiplexes for simultaneous multispecies detection of Asian carp eDNA. *Journal of Great Lakes Research*, *43*(4), 771–776. https://doi.org/https://doi.org/10.1016/j.jglr.2017.05.001
WWF. (2020). Living Planet Report 2020 — Bending the curve of biodiversity loss. Edited by R.E.A. Almond,

M. Grooten, and T. Petersen. WWF, Gland, Switzerland.
Zhang, H., Jarić, I., Roberts, D. L., He, Y., Du, H., Wu, J., Wang, C., & Wei, Q. (2020). Extinction of one of the world's largest freshwater fishes: Lessons for conserving the endangered Yangtze fauna. *Science of The Total Environment*, *710*, 136242. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.136242
Zhi-Gang, J. I. A. N. G., & En-Yuan, F. A. N. (2003). Exploring the endangered species criteria: rethinking the IUCN Red List Criteria. Biodiversity Science, 11(5), 383.

Chapter 2 - Using species-specific environmental DNA for multi-taxa detection of endangered and invasive alien species in Italian freshwater ecosystems. Implications for monitoring obligations under EU regulations.

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Abstract

Environmental DNA (eDNA) using quantitative PCR (qPCR) is a sensitive tool to detect rare and elusive species, such as endangered or invasive alien species (IAS) during the initial stages of colonization. In this study, we aimed at i) designing and testing an eDNA protocol for the simultaneous detection of 22 freshwater species of conservation concern, ii) identifying environmental factors that influence species detection, iii) assessing the efficiency and cost-effectiveness of eDNA-based multi-species detection versus taxon-specific traditional survey methods. Target species belonged to Fungi, Mollusca, Arthropoda and Chordata, including 14 endangered species listed in the Habitats Directive 92/43/EEC and eight IAS listed under the EU Regulation 1143/2014. New eDNA assays were ex-novo designed for 13 species. The protocol was tested in two temporal replicates at 53 sampling sites located on standing and running water of Central Italy. PCA and biplots were used to explore the relationships between species detection and 25 fine-and large-scale environmental variables measured at each sampling site. Cost for eDNA included assay design, validation and purchase, laboratory equipment and consumables, personnel and cost for sampling, filtration, extraction, and amplification stages. Costs for traditional surveys were based on taxon and habitat specific sampling techniques according to published guidelines. We detected 18 out of the 22 target species at 45 sites. Highest detection rates were observed for fish (0.59 ± 0.2), amphibians (0.43 ± 0.3), molluscs (0.30 ± 0.12) and mammals (0.27 ± 0.29). Species were mostly detected during spring. PCA highlighted a relationship between a subset of environmental factors for four species. Compared to traditional techniques, a qPCR-based eDNA approach demonstrated as a faster (<46% of time), accurate (+49 new occurrences), and affordable (<88% of costs) approach for multiple species detection, offering

great potential for large-scale monitoring of freshwater wildlife, especially for routine monitoring under EU regulations.

1. Introduction

Freshwater ecosystems are threatened globally and many freshwater species are experiencing substantial declines worldwide (Sanders et al., 2024), mainly due to habitat loss and degradation, pollution and introduction of invasive alien species (IAS) (Costa et al., 2021; Dextrase and Mandrak, 2006; Francis, 2012; Jaureguiberry et al., 2024). A solid understanding and monitoring of the conservation status of endangered species, along with rapid detection of threatening invasive alien species (IAS), are essential for implementing effective management strategies (Meira et al., 2019; Simberloff et al., 2013). Accordingly, periodic monitoring and reporting every six years are mandatory for European native freshwater species included in Annexes II, IV, and V of the European Union Council Directive 92/43/EEC, known as Habitats Directive (hereafter referred to as HD). Additionally, EU Regulation 1143/2014 requires member states to monitor freshwater bodies for the early detection of IAS of Union Concern (Pawlowski et al., 2021). Central Italy's freshwater bodies host at least 23 endangered native animal species listed in the HD, as well as approximately 40 IAS, half of which are classified as of Union Concern under EU regulation 1143/2014 (Ercole et al., 2021; Magliozzi et al., 2020; Monaco, 2014). Although standardized methods have been proposed to fulfill the monitoring obligations established by EU (Leese et al., 2018, Stoch and Genovesi, 2016), traditional methods for detecting multiple species can be time-consuming and resource-intensive, often hindering monitoring programs, particularly in case of rare and elusive species.

Environmental DNA (eDNA)-based approaches are transforming biodiversity monitoring worldwide, especially for freshwater and marine communities (Taberlet et al., 2018). Relying on the collection and analysis of the DNA released by organisms in the environment, eDNA-based approaches proved to be sensitive and versatile tools. Indeed, eDNA metabarcoding usually refers to the method of targeting and sequencing many DNA fragments belonging to different taxa that are contained in a mixed biological sample (Pont et al., 2023; Ushio et al., 2018). An alternative approach involving species-specific PCR-based assays allows the target detection of taxa (Ficetola et al., 2008). Quantitative PCR (qPCR) techniques have been used to assess fish communities (Pont et al., 2023; Wozney and Wilson, 2017), infer fish biomass of commercial fish (Knudsen et al., 2019), study predator-prey relationships between a fish and its prey (Minegishi et al., 2023), assess marine invasive tunicates and bryozoans responsible of biofouling (Leblanc et al., 2020), or in studies involving two or three species belonging to different taxa (Seymour et al., 2018; Tsuji et al., 2018). As a matter of fact, a qPCR eDNA-based approach has proven to be more sensitive than eDNA metabarcoding, making it particularly useful for detecting rare and elusive species, such as highly endangered species or IAS at their initial colonization phase (Duarte et al., 2023; Harper et al., 2018; Thomas et al., 2020; Xia et al., 2021). However, the qPCR species-specific approach has been rarely used to detect multiple species at the same time (Leblanc et al., 2020; Thomsen et al., 2012b), even though it could prove a valid approach when high sensitivity and specificity is required to monitor multiple IAS and elusive species with high conservation value from the same geographic area (Duarte et al., 2023; Hii et al., 2024; Rund et al., 2024).

One of the primary challenges in expanding and solidifying the species-specific approach for eDNA monitoring lies in the need of well-validated eDNA species-specific qPCR assays. Despite the growing need, many species still lack these critical assays, leading to the proposal of a validation scale to highlight reliable and ready-to-use tests (Thalinger et al., 2021). Furthermore,a proper validation in the study areas is essential to account for various detection biases. These biases can result from genetic variation of local populations and co-occurrence of phylogenetically closely related species as well as the potential low DNA detectability of environmental samples (Goldberg et al., 2016; Klymus et al., 2020; Langlois et al., 2021). These factors highlight the importance of investing time and resources in the validation phases to ensure it is both robust and efficient (Nicholson et al., 2020; Sepulveda et al., 2020; Thalinger et al., 2021).

Italy hosts the highest faunal biodiversity in Europe (ISPRA, 2009; German Federal Agency for Nature Conservation, 1997; https://www.cbd.int/countries/profile?country=it#:~:text=Status%20and%20trends%20of%20biodiversity,a%20high%20rate%20of%20endemism). 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). This area is also characterized by several endemic and endangered freshwater species (Balletto et al., 2010), including the Italian crayfish *Austropotamobius italicus*, the Apennine yellow-bellied toad *Bombina v. pachypus*, the Italian crested newt *Triturus carnifex,* and the Tuscan stream chub *Squalius lucumonis* (Brusconi et al., 2008; Canestrelli et al., 2014; Rondinini et al., 2022; Tancioni et al., 2013). By counterpart, Italian freshwater ecosystems host among the highest number of IAS in Europe, including fish, crustaceans, amphibians and molluscs (IPBES, 2023), and the number of non-indigenous fish species has surpassed that of native fish (Gherardi et al., 2008; Lorenzoni et al., 2019; Magliozzi et al., 2020; Nocita et al., 2017). Given the EU reporting obligations under HD, and the commitment to manage IAS while reducing by 50% the number of Red List species they threaten 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. These protocols are essential to optimize monitoring of both rare and endangered species, as well as providing an early warning system for IAS (Leese et al., 2018; Pawlowski et al., 2021).

Thus, we designed and tested a panel of protocols to detect through eDNA coupled with qPCR assay a set of 22 species occurring in freshwater bodies of Central Italy, including 14 endangered species listed in HD annexes, six IAS of Union Concern as in the Regulation 1143/2014, one pathogen they threaten, and one translocated fish. We specifically aimed at i) design new species-specific assays for 13 species lacking available eDNA primers and probes; ii) assessing sampling timing and location we selected for multi species detection in Central Italy; iii) identify small- and large-scale environmental factors affecting either species or eDNA detection; iv) compare costs of eDNA multispecific detection with respect to traditional survey techniques.

2. Material and Methods

2.1 Target species

We selected 22 target species occurring in freshwater bodies of Central Italy, representing a diverse taxonomic coverage that includes: Fungi, Mollusca, Arthropoda and Chordata. We selected 14 species that are protected according to the HD 92/43/EEC (Annexes II, IV, V) or endangered according to the IUCN Italian Red List (Rondinini et al., 2022) that are cryptic, difficult to detect with traditional techniques, and therefore with scarce occurrences in the study area. We also included eight species posing a direct threat to the former, that are either IAS of Union Concern under the EU regulation 1143/2014, or are listed among the IUCN Blacklist of the World's Worst Invasive Alien Species (Lowe et al 2000), or represented translocated species or harmful pathogens (Tab 1). We discarded all the species fitting these criteria but hardly to be correctly detected as primers designed with short genetic sequences may undergo possible hybridizations with congeneric species living in the same range. Details on conservation and legal status of target species are reported in Tab.1.

Table 1. Details of target species. HD: Habitats Directive 92/43/EEC, Annexes II, IV, and V; UC: Invasive Alien Species of Union Concern (s. EU regulation 1143/2014; 100 WWIAS: IUCN 100 of the World's Worst Invasive Alien Species (Lowe et al 2000). The asterisk indicates the species for which new primers and probes were designed in this study.

Phylum	Clade	Species	Synonym	Species ID	Regulation	IUCN national Red

						List/100 WW IAS
Fungi	Chytridiomycota	*Batrachochytrium dendrobatidis*	-	BD	-	100 WWIAS
Mollusca	Bivalva	*Unio elongatulus* *	*U. mancus*	UE	HD, Ann. V	EN
Mollusca	Gasteropoda	*Vertigo spp.* *	*V. angustior V. moulinsiana*	VE	HD, Ann. II	NT-VU
Mollusca	Bivalva	*Dreissena polymorpha*	-	DP	-	100 WWIAS
Arthropoda	Crustacea	*Austropotamobius italicus*	*A. pallipes*	AI	HD, Ann. II, IV	EN
Arthropoda	Crustacea	*Faxonius limosus* *	*Orconectes limosus*	FL	UC (2016)	-
Arthropoda	Crustaceans	*Procambarus clarkii*	-	PC	UC (2016)	-
Arthropoda	Insecta	*Coenagrion castellani* *	-	CC	HD, Ann. II	NT
Arthropoda	Insecta	*Oxygastra curtisii* *	-	OC	HD, Ann. II, IV	NT
Arthropoda	Insecta	*Lindenia tetraphylla* *	-	LT	HD, Ann. II, IV	VU
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 (2016)	-
Chordata	Osteichthyes	*Padogobius bonelli* *	*P. martensii*	PB	introduced out native range	-
Chordata	Amphibia	*Bombina v. 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 (2016)	100 WWIAS
Chordata	Chelona	*Emys orbicularis* *	-	EO	HD, Ann. II	EN
Chordata	Chelona	*Trachemys scripta*	-	TS	UC (2016)	100 WWIAS
Chordata	Mammalia	*Mustela putorius* *	-	MP	HD, Ann. V	LC
Chordata	Mammalia	*Lutra lutra*	-	LL	HD, Ann. II, IV	VU

2.2 Study area

We sampled 53 sites across eight river catchments in the Latium region of central western Italy. The study area is characterized by high environmental heterogeneity, ranging from the coastal and lowlands to volcanic plateaus, piedmont zones and mountainous areas, spanning from the sea level to 2,458 m a.s.l. Large volcanic lakes are situated in the central and northern part of the region, while coastal lakes are found in the south. Additionally, three reservoirs are located along the Velino, Turano and Liri rivers. The Italian capital city, Rome, is centrally located within the region and likely serves as a source of several invasive alien species (Ferri et al., 2020; Petrassi et al., 2007; Spear et al., 2013).

The eight catchments included the two largest river basins of Central Italy, i.e. Tiber (405 km) and Liri (158 km), as well as six smaller catchments flowing close to the coast, i.e. Fiora, Marta, Mignone, Arrone, Astura and Amaseno. The sites were located in either protected areas (n = 5), the Natura 2000 network (n = 28), or the regional ecological network designed to connect the protected areas (Scalisi et al., 2011) (n = 20) (Fig. 1; Suppl. Mat. 1 S1).

Sampling sites were located in different water bodies according to habitat requirements of the target species (Tab. 2; Suppl. Mat. 1 S1). To perform cost effective analyses, each sampling site was selected based on species range, ecological requirements, and recent presence data. Native species not reported in the regional fauna but expected to return in the short term, like the Eurasian otter *L. lutra* (Giovacchini et al. 2018), were searched at sites located close to areas of ascertained presence in the neighboring regions (Ercole e t al., 2021; Petrassi et al., 2017; Suppl. Mat. 1 S2). To account for species phenology and sampling biases, we collected three sampling replicates, in summer 2021 (July-August, 52 sites), late spring 2022 (May-June, 35 sites), and autumn 2022 (October, 17 sites) (Fig. 1). Twelve sites found in drought conditions in 2021 were replaced by 12 new sites in spring and autumn 2022.

Table 2. Number (N) of sampling sites for each habitat type and suitability for target species (IDs are those as in Tab. 1).

Habitat type	N	Target species
Lowland Rivers	5	UE, VE, OC, LT, AF, TC, LI, EO, LL, PC, PP, LC, TS
Rivers	16	UE, VE, CC, OC, AI, AF, SL, BP, EO, MP, LL, DP, FL, PC, PP, PB, LC, TS
Creeks	13	UE, VE, CC, AI, SL, BP, MP, FL, PC, PB, BD
Channels	3	EO, MP, PC, PP, PB, TS
Ponds	5	CC, BP, TC, LI, EO, MP, TS, BD
Lakes	10	UE, VE, CC, OC, LT, TC, LI, EO, MP, DP, FL, PC, TS

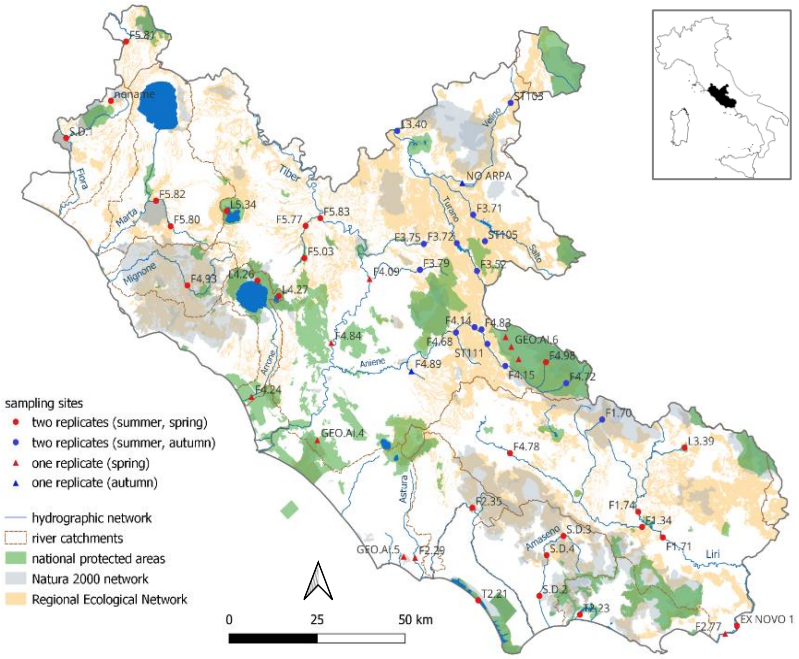


Figure 1. Location and codes of 52 sampling sites. Each site was sampled once (triangles) or twice (dots). Sites with two replicates were sampled during summer and in either autumn (blue) or spring (red). Sampling IDs are those reported in Suppl. Mat. 1 S6.

2.3 Water sampling

At each site, we collected 3 liters of water using sterile bottles. For running waters, we sampled across the channel's width whenever possible to include different microhabitats; otherwise we collected samples only from the shore. For standing waters, we walked along the shore for 300 m and collected 200 ml of water every 20 m. Water was filtered within 24 hours using an electric vacuum pump (6 l/min, final vacuum: 100 mbar, N86 KN.18, KNF Laboport, Neuberger GmbH, Germany). Samples were initially filtered through 8 µm mixed cellulose ester (MCE) filters and then through 0.22 µm MCE filters (diameter 47 mm, Merck-Millipore, Burlington, USA). For the highly-turbid waters, found at four sites, we filtered at least one liter of water, replacing the filters as soon as they became clogged. All filters were stored at -20°C until DNA extraction.

2.4 Environmental variables

To identify factors that could influence eDNA detection and/or species occurrence at each sampling site, we collected nine-fine scale and 16 large-scale environmental variables. Fine-scale variables were measured in 2022 and included air temperature, water temperature, river depth, river width, pH, nitrites and nitrates, water turbidity and water speed (Suppl. Mat. 1 S3). Large scale environmental parameters were derived from digital maps and converted to a uniform raster format with a 250 m resolution. These included six climatic 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) was sourced from the MODIS database (https://lpdaac.usgs.gov/products/mod13q1v006/). Additionally three 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 three land use features were derived from CORINE Land Cover dataset from the Copernicus database (https://www.copernicus.eu/it), i.e. Euclidean distance from agricultural areas, artificial surfaces, forests and semi-natural areas (Suppl. Mat.1 S3).

2.5 Assay design

For each target species, we first searched literature for already published primers and probes used in the species detection qPCR-based approach. Published primers were found for nine species, i.e. the pathogen *B. dendrobatidis* (Canessa et al., 2017), the bivalve *D. polymorpha* (Amberg et al., 2019), the crayfish *A. italicus* (Manfrin et al., 2022b) and *P. clarkii* (Mauvisseau et al., 2018), the fish *A. fallax* (Antognazza et al., 2019) and *P. parva* (Manfrin et al., 2022a), the amphibian *L. catesbeianus* (Sanz et al., 2023), the terrapin *T. scripta* (Kakuda et al., 2019), and the mammal *L. lutra* (Park et al., 2011) (Suppl. Mat. 1 S4). For the remaining 13 species, we designed new species-specific primers and probes. Assays were designed following Thalinger et al. (2021). All mitochondrial DNA deposited sequences of the target and closely related sympatric species were downloaded from BOLD Systems IDS (Ratnasingham and Hebert, 2007) and GenBank (https://www.ncbi.nlm.nih.gov/). For each marker, sequences were aligned using MAFFT 7 and visualized in BioEdit 7.2.5 (Hall, 1999; Katoh et al., 2009; Thomsen et al., 2012a). For each target species, we selected the genetic marker with the largest number of available deposited sequences (also considering congenerics of possible or ascertained syntopic occurrence at the regional investigation scale and across Europe), low intra-specific and high inter-specific polymorphisms. Primers were selected manually based on the following molecular characteristics: i) 40-60% GC content, ii) length between 18-24 bp, iii) melting temperature (TM) between 58-68 °C and within 2 °C of each other. Probes were selected with a length between 20-25 bp and TM 4 to 6 °C higher than the primers. Total length of the amplicon expected to be about 150 bp (Jo, 2023; Langlois et al., 2021).

2.6 Assay validation

As recommended by Thalinger et al. (2021), for the successful application of robust eDNA assays we followed three validation steps. We first checked *in silico* primers and probes of candidate assays using Oligo Analysis (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/) for the probability of generating hairpin, self- and hetero-dimer structures that could affect amplification reaction and lower assay sensitivity in detecting target species, thus increasing the probability of false negatives (Kibbe, 2007). Specificity of primers and probes was first evaluated on the alignments against possible effects of intraspecific genetic variability of Italian populations, that could increase false negative probability, and the possible detection of false positives due to co-amplification of phylogenetically closely related non-target species. For each assay, primer pairs were also blasted against GenBank nucleotide database with Primer BLAST (Altschul et al., 1997) to identify potential targets of unspecific amplification with taxa that

were not included in the alignment but represent common contamination sources (e.g., human and dog DNA).

Candidate primer pairs were synthesized (Eurofins Genomics srl, Hamburg, DE) and validated *in vitro* on tissue samples of target species kindly provided by experts of each taxon. Genomic DNA (gDNA) was extracted from tissue samples using DNeasy® Blood & Tissue kits (Qiagen, Venlo, NL). To evaluate amplification success and select optimal annealing temperature of each primer pair, we set up end-point PCR amplifications with gradients of annealing temperature in 20µL total volume reactions containing 11.75 µL of Milli-Q water, 4 µL of 10x Buffer solution (5x WonderTaq reaction Buffer EuroClone®, Milano, IT), 1 µL of each primer (10 µM), 0.25 µL Taq polymerase (WonderTaq EuroClone®) and 2 µL of DNA template. PCRs were run with the following thermal profile: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 60 seconds, annealing with 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. To assess species-specific amplification, amplicons corresponding to the expected target fragment size visualized with agarose gel electrophoresis (2%) were sequenced at Eurofins Genomics srl, Hamburg, DE (Wilcox et al., 2015) and generated sequences were compared to the reference databases available in BOLD Systems and GenBank.

For assays whose primer pairs amplified successfully and specifically on the target taxa, real-time quantitative PCR (qPCR) with TaqMan technology (StepOnePlus, Applied Biosystem, Waltham, USA) were run on DNA extracts of tissue samples of target species and confounding taxa in a total volume of 10 µL: 2.35 µl of Milli-Q water, 5 µL of buffer (SSoAdvanced™ Universal Probe Supermix), 0.25 µL of each primer (10 µM), 0.15 µL of dual-labelled (5'-FAM, 3'-MGBEQ) Taqman probe (10 µM; Eurofins Genomics srl, Hamburg, DE), 2 µL of extracted DNA. Each sample was run in two dilutions (1:1, and 1:10), together with a negative technical control, following standard thermal conditions with the annealing step varying depending on the target, in three technical replicates. Evaluation of eDNA assay accuracy was based on amplification efficiency and limit of detection (LOD) (Bustin et al., 2009; Klymus et al., 2020), to generate the standard curve tenfold serial dilutions of quantified positive tissue controls were used Samples were considered as positive when a sigmoidal signal was observed in at least two technical replicates for each run and above the LOD (e.g. 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 LOD but equal or more than five copies per reaction (Bustin et al., 2009).

Assays that passed *in vitro* validation were tested on water samples collected and filtered from two locations of ascertained presence of the target species, as well as from two locations confirmed to lack the species (see par. 2.2 and 2.3 for details). A total of 35 certain occurrence sites were sampled in both captive (Cavalcante et al., 2020; Tancioni et al., 2019) and wild conditions following expert recommendations, personal sightings, or recent literature data (Suppl. Mat. 1 S2). Absence sites were identified outside the species range and in unsuitable habitats (Suppl. Mat. 1 S5). We extracted eDNA from water filters using the DNeasy PowerSoil Pro kit (Qiagen, Venlo, NL). For each water sample, 8 µm and 0.22 µm filters were merged and processed to maximize DNA yield. eDNA extracts were finally diluted with 75 µL bidistilled Miili-Q sterile water and preserved at -20 °C.

We ran qPCR analyses on environmental samples with the same reaction mix and thermal profile described above for the *in vitro* validation step on tissue samples. Each qPCR included the following: i) PCR positive controls, using the gDNA extracts of target species; ii) environmental positive control, i.e. eDNA extracts from water samples collected in two sites of ascertained presence; iii) environmental

negative control, i.e. eDNA extracts from water samples from two sites of ascertained absence of the species; iv) PCR negative technical controls: no target species samples to verify the absence of contamination with DNA of target or untarget species during the reaction set-up steps (Karlsson et al., 2022; Klymus et al., 2020; Thalinger et al., 2021). 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 (Miya et al., 2015). Pre and post amplification phases were carried out in separate rooms to avoid contaminations, and each step was conducted in a laminar flow cabinet.

2.7 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. DR was computed considering the cumulative presences from both sampling replicates, and for each sampling replicate, habitat types, and sampling season. To finally test the reliability of eDNA, occurrence data were used to confirm species ranges reported on a 10x10 km grid cell (ETRS LAEA 52 10 projection). Species distribution was provided by HD reporting ex Art. 17 (Ercole et al., 2021) and the Regional Atlas of Invasive Species (Monaco, 2014) maps. Here, DR is calculated only on eDNA sites where target species were tested that fall over presence cells (i.e., positive sites/tested sites). Chi-Squared and Fisher's exact tests are performed to assess statistically significant differences among replicates, seasons, and habitat types. For species with more than five eDNA occurrences, a principal component analysis (PCA) was run to explore fine- and large-scale factors affecting eDNA presence or absence. PCA were run using the function *prcomp* from the *factoextra* package (Kassambara and Mundt, 2020) in the software R (R Core Team, 2013).

2.8 Costs of eDNA vs traditional surveys

We estimated times and costs of traditional and eDNA surveys for individual species or species clusters, following optimization methods (e.g., group-specific expert surveying more than one species), for a single-replicate survey undertaken within the scope of this specific case study. Costs and time of traditional surveys were based on the recommendation (survey technique, personnel involved, samples per day) by Stoch and Genovesi (2016) for the species listed in HD occurring in Italy. For IAS we referred to published guidelines for individual species (Bletz et al., 2015; Carosi et al., 2021; Gherardi et al., 2009; Kamoroff et al., 2020; Macchi et al., 2020; Mackie and Claudi, 2010; Weldon et al., 2013). We calculated the cost of personnel, materials, and transport, considering the number of samples obtained per day to reach the required final sample size. We also considered the costs gained by different census techniques required for habitat-specific sampling (i.e., lentic, and lotic waters). Moreover, we considered the costs of site revisitation when required, i.e. in case of trap setting and checking. Permanent material that is reusable for multiple sampling was distinguished from disposable material to be purchased for each sampling. Field materials arranged in arrays (e.g., traps) were compared among a similar number of instruments (n=5), overlooking the expected accuracy related with trap numbers. Transport costs were calculated with distance routes and national fuel inventory [https://www.mimit.gov.it/it/prezzo-medio-carburanti/regioni updated to 22/05/2024]. Personnel salaries were rounded from the national collective labor agreement. NHBS catalogue 2024 (https://www.nhbs.com/) was consulted to provide costs of materials for traditional surveys. Environmental DNA cost estimates, instead, were referred to the costs of our study. We specifically considered the costs (material, transport, personnel) for sampling, filtration, extraction, and amplification stage (including all laboratory equipment), including a pre-phase of assay design and validation.

3. Results

3.1 eDNA assays design and validation

Details of new primers and probes that successfully passed all validation steps for the 13 species are reported in Tab. 3. For six species primers and probes were derived from COI, for four species from CytB and for two species from 16S rRNA genes. Primer specificity was evaluated *in silico* aligning 1,220 accession numbers for all target species, and 3,953 sequences belonging to other species strictly related, downloaded between 8 April 2022 and 8 June 2022 (Suppl. mat. 2 S1). In the case of *Vertigo* sp., it was not possible to obtain species-specific primers due to the low genetic diversity among the species of the genus. Furthermore, initial attempts to use the mitochondrial COI and CytB genes for primer design proved unsuccessful as these regions are conserved in both species. Tab. 4 shows parameters (LOD, Amplification efficiency, DNA count) used to evaluate the accuracy of *in vitro* validations. LOD values ranged between $4{,}00*10^{-7}$ and $2{,}90*10^{-3}$ ng. Efficiency of amplification on tissue samples (*in vitro*) ranged from 82.2% to 191.9%. All assays showed no amplification in the field negative controls, i.e. 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 21 species tested. *B. dendrobatidis* was not tested *in situ*, as in Mirone et al. (under revision) (Suppl. mat. 1 S5). However, *in situ* validation was unsuccessful for the three odonate species. The assay for European polecat *M. putorius* and the topmouth gudgeon *P. parva* were validated only for one of the two environmental samples of ascertained presence (Suppl. Mat 1 S5). The assay for the red swamp crayfish *P. clarkii* and the pond slider *T. scripta* were tested over three samples and amplified for one (Suppl. mat. 1 S5).

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
		CGGAGGATATACTGTTCAACC	21	60		
		[FAM]AGTGATGCCAATAATAATTGGCGG[MGBEQ]	24	61.5		
Vertigo sp.	COI (mtDNA)	CAAGAATAGTTGAAGGCGGTGC	22	62.6	94	60
		GCCAAATCAACGGATGCTCC	20	62.4		
		[FAM]GAACAGGGTGGACTGTTTATCCC[MGBEQ]	23	64.5		
Faxonius limosus	COI (mtDNA)	ACAGGGTGAACAGTGTATCC	20	56.85	70	60
		TACCCAAATCCACTGATGCC	20	57.56		
		[FAM]TACCCAAATCCACTGATGCC [BHQ1]	22	63.55		
Coenagrion castellani	COI (mtDNA)	CTCATGCAGGAGGTTCAG	18	59.9	156	54
		ACCTGCTGGATCAAAGAATGAT	22	58.9		
		[FAM]ACCAGTATTAGCTGGTGCCATC[MGBEQ]	22	62.6		
Lindenia tetraphylla	COI (mtDNA)	CCTCCACTAGCAGGTGCTA	19	57.8	96	57
		GGCCCCTAGAATTGAGGATACT	22	62.6		
		[FAM]GCACACGCTGGAGGGTCG[MGBEQ]	18	66.7		

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

Table 4. Parameters used to assess accuracy for each species assay. CT: cycle threshold, LOD: Limit of Detection.

TARGET SPECIES	concentration tissue DNA (ng/µL)	CT	LOD (g/L)	Amplification efficiency (%)	DNA counts (copies)
Unio elongatulus	53.5	34.0301	$5.35*10^{-4}$	95.180	$6.48*10^{11}$
Vertigo sp.	98.5	34.552	$9.85*10^{-7}$	191.908	$2.74*10^{12}$
Faxonius limosus	8.04	36.98	$8.04*10^{-5}$	95.933	$9.67*10^{5}$
Coenagrion castellani	1.38	37.89	$1.38*10^{-7}$	101.319	$1.70*10^{4}$
Lindenia tetraphylla	45.7	33.445	$4.57*10^{-7}$	169.530	$4.09*10^{14}$
Oxygastra curtisii	50.5	35.795	5.0510^{-7}	161.486	$1.93*10^{9}$
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 v. 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

3.2 Species monitoring and detection

Environmental DNA allowed to detect 18 over the 22 target taxa, resulting in a total 101 occurrences, including 39 new occurrences of endangered native species and 10 new occurrences of IAS (Suppl. Mat 1 S6).

The highest detection rates (DR) for both sampling replicates were observed in fish (0.59 ± 0.2), amphibians (0.43 ± 0.3), molluscs (0.30 ± 0.12) and mammals (0.27 ± 0.29). In contrast, the lowest detection rates were recorded for terrapins (0.02, tested across 31 sites) and dragonflies (no detection for any of the three species) (Tab. 5). The DR for 10x10 km grid cells further confirmed fish (0.83), molluscs (0.72) and amphibians (0.61) as the taxa with the highest DR (Tab.5). For individual sampling replicates, positive occurrences increased in the second (spring and autumn) compared to the first (summer) replicate with 44 versus 73 total occurrences, respectively; however, this difference was not statistically significant (X=1.52, p=0.22). This trend was also confirmed for all taxa except for molluscs. The majority of occurrences were detected in spring (n=51), followed by summer (n=44) and autumn (n=21), although this difference was 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). The DR for fish and crustaceans were comparable for both spring and summer (p=0.67). Additionally, crustaceans exhibited a consistent DR across all seasons (p=0.93). The unique occurrence of the pathogenic fungus was recorded in autumn.

Table 5. eDNA species-specific 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
Osteichthyes	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

The highest detected native species (DR>0.6) included *T. carnifex, A. fallax* and *B. v. 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 a very low 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). 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 the 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 summer), *T. carnifex* (0.63 vs 0.33 in 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. v. pachypus* (0.64 vs 0.33 in spring), *B. dendrobatidis* (0.07). *A. italicus* showed a high DR both in summer and in autumn (0.2 vs 0.12). Finally, some species did not display any specific seasonality in DR, as for *F. limosus* and *M. putorius*. DR based on habitat types

(i.e., total occurrences over the total number of times that a specific habitat was tested) did not show any statistically significant difference, ranging from 0.20 for lakes to 0.30 for lowland rivers (X=2.7, p=0.73) (Tab. 6). In contrast, habitat types belonging to channels had the highest DR in spring and summer, and creeks during autumn.

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 sites	50	147	75	109	28	31
DR	**0.3**	**0.21**	**0.20**	**0.22**	**0.29**	**0.26**

One to four target species were detected at 45 over the 53 sampled sites (Fig. 2). Native species were found at 24 over 53 (45%) sites, mainly concentrated in mountain and headwaters sites. IAS were found at 5 sites (9%), mainly located along main water courses and in artificial lakes. Both native and invasive species co-occurred at 16 sites (30%). The highest number of native species (3 to 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 the lowland of Southern Latium and the area between the river Tiber and mountainous reservoirs along Velino tributaries. The Eurasian otter *L. lutra* was detected for the first time 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 in Italy through eDNA, co-occurring with the endangered endemic amphibian *B. v. pachypus* (Mirone et al., under revision).

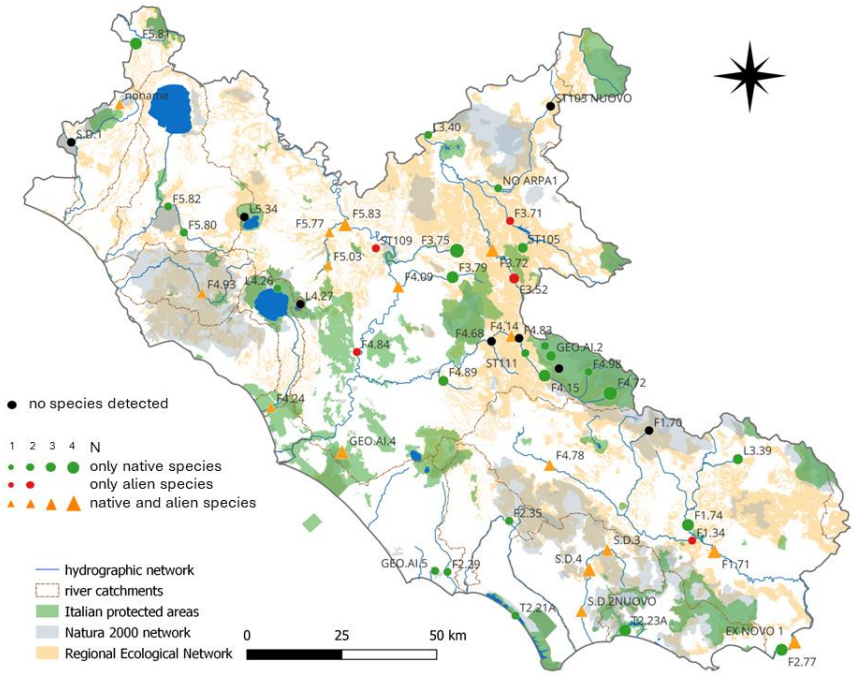


Figure 2. Map of the study area showing the sites with no detections (black circles), sites where only native species (green dots), only IAS (red dots), or both native and alien species (orange triangles) were detected through eDNA. Symbol size is proportional to the total number of species detected at the site (1 to 4).

Compared with species range reported as 10x10 km grid, the native *U. elongatulus* and *A. fallax*, and the alien D. *polymorpha, F. limosus,* and *P. bonelli* showed the highest DR in terms of positive/tested cells

(Tab. 7). After dragonflies and *T. scripta*, not detected in any cell of known presence, *E. orbicularis*, *P. clarkii*, and *M. putorius* were the less detected species (Tab. 7).

Table 7. Detection rate (DR) by species based on positive over tested sites, and by positive eDNA 10x10 km cells falling over cells of known species presence.

Species	eDNA		10x10 km presence grid cells	
	positive/tested sites	DR	positive eDNA /presence cells	DR
Batrachochytrium dendrobatidis	1/21	0.05	-	-
Unio elongatulus	7/28	0.25	2/2	1
Vertigo sp.	7/32	0.22	2/11	0.18
Dreissena polymorpha	4/9	0.44	2/2	1
Coenagrion castellani	0/26	0	0/10	0
Lindenia tetraphylla	0/23	0	0/1	0
Oxygastra curtisii	0/21	0	0/3	0
Austropotamobius pallipes	7/26	0.27	5/19	0.26
Faxonius limosus	2/15	0.13	2/2	1
Procambarus clarkii	3/34	0.09	3/14	0.21
Alosa fallax	15/20	0.75	2/2	1
Squalius lucumonis	5/10	0.50	3/5	0.60
Pseudorasbora parva	7/20	0.35	3/4	0.75
Padogobius bonelli	10/13	0.77	5/5	1
Bombina v. pachypus	12/20	0.60	10/15	0.67
Lithobates catesbeianus	1/10	0.10	-	-
Triturus carnifex	6/8	0.75	5/6	0.83
Lissotriton italicus	3/12	0.25	1/3	0.33
Trachemys scripta	0/21	0	0/7	0
Emys orbicularis	1/26	0.04	0/17	0.05
Mustela putorius	2/31	0.06	1/16	0.06
Lutra lutra	8/17	0.47	-	-

PCA performed on fine scale variables recorded at sampling sites on running waters allowed to distinguish between positive and negative sites for only one native (*T. carnifex*) and two alien (*D. polymorpha* and *P. parva*) species, out of 11 species tested (Fig. 3a). For *T. carnifex, negative* sites were primarily clustered in the upper part of the second PC axis (22% of cumulative variance), characterized by high water turbidity, low pH and reduced river width. For *D. polymorpha* negative sites were distinct along the first PC axis (47.8% of variance explained) and were primarily characterized by low ambient and water temperature, as well as to a lesser extent by low depth and width of the watercourse, 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 were characterized by high pH and low river width.

PCAs performed for large scale variables showed a clear distinction of positive versus negative sites only for *S. lucumonis* (Fig. 3b), 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 values of aspect.

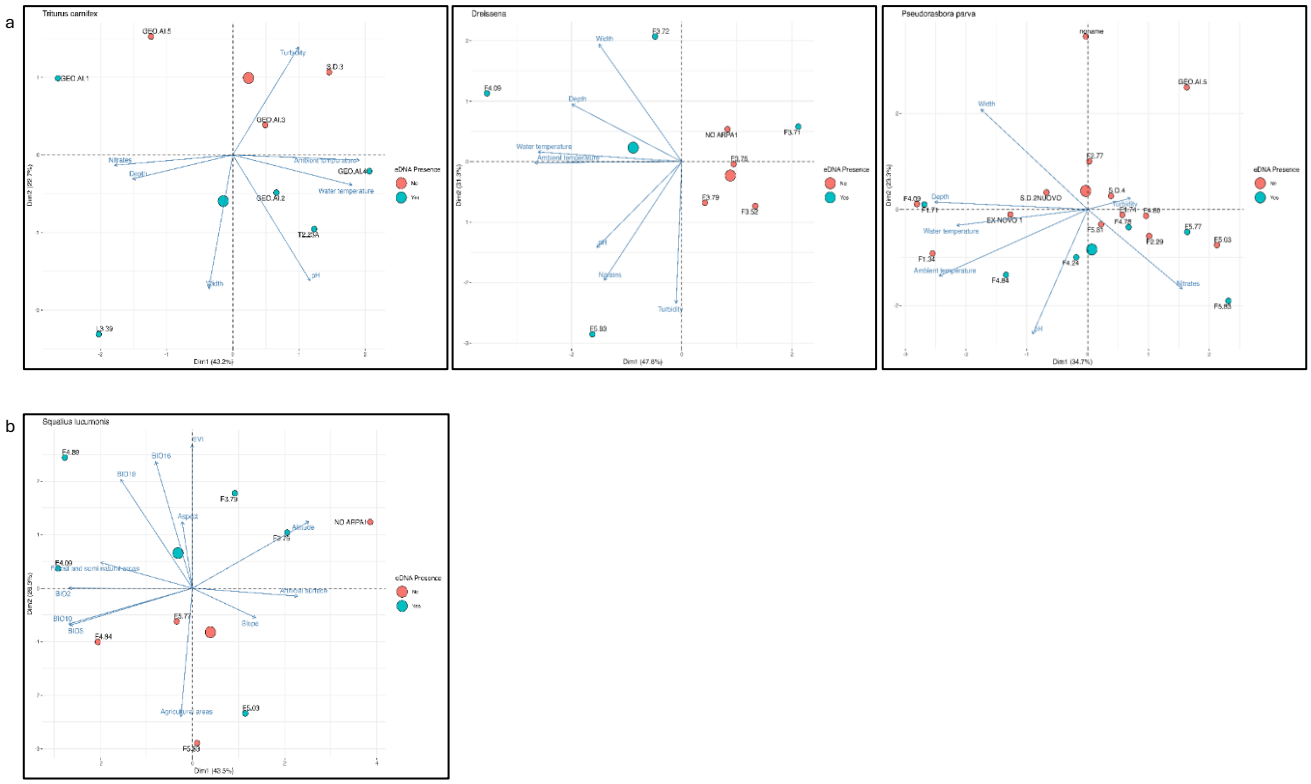


Figure 3. Results of PCA run on a) fine scale and on b) large scale variables, recorded at sampling sites, showing the distribution of positive and negative sites for two native (T. carnifex [a] and S. lucumonis [b]) and two alien (D. polymorpha [a] and P. parva [a]) species. Biplot superimposed to PCA axes indicate the direction and relative importance of the original variables.

3.3 Costs of eDNA vs traditional surveys

Costs for traditional survey techniques calculated on the same species/sites of this case study (n=440) amounted 195,914 €, involving 25 operators in 11 specialist groups and 246 working days (Suppl. Mat. 1 S7). Costs for environmental DNA were estimated at 44,960 €, involving 4 operators and 135 working days (Suppl. 1 Mat. S8).

4. Discussion

The main objective of this work was to develop a protocol for the detection of multiple taxa at the same site using the same technique. The protocol was addressed to monitor at large scale both endangered freshwater species and alien species that threaten them.

The new assays developed for molluscs, crustaceans, fish, amphibians and mammals were accurate and did not reveal any constraint during primer design and validation phases. Most of the primers developed reached level 4 out of 5 in the classification proposed by Thalinger et al. (2021) as they are supplied with LOD parameters, extensive field testing, and can be considered operational. Instead, red swamp crayfish *P. clarkii* and both pond terrapins remained almost undetected (Gherardi et al., 2009; Monaco, 2014; Scalici et al., 2009; Capizzi and Bellavita, 2014; Ferri et al., 2019; Vignoli et al., 2015).

Effectiveness in eDNA detection for organisms like fish and amphibians (Collins et al., 2018; Goldberg et al., 2016) is likely related to a significant shedding of living cells and mucus released in the water during all their life stages. 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) and showed high accuracy especially with the crested newt *T. cristatus* (Harper et al., 2018).This species indeed tends to be generalist and occupies numerous habitats and is quite common (Mori et al., 2017). Indeed, our occurrences of *T. carnifex* analyzed with PCAs showed a high variability of water parameters, such as turbidity and pH.

Furthermore, fish eDNA is well recognized as a particularly efficient survey method (Pont et al., 2023; Wozney and Wilson, 2017; Knudsen et al., 2019; Minegishi et al., 2023; Tsuji et al., 2018), to the extent that several initiatives are exploring the feasibility of inferring additional information from eDNA sampling such as biomass and population size (Lacoursière-Roussel et al., 2016; Rourke et al., 2022). Our results confirmed fish species as one of the most detected taxa in our study. The numerous occurrences of *S. lucumonis* subjected to PCAs indicated that species detection is influenced by the presence of forest and semi-natural areas as well as high values of vegetation, aspect, and altitude. These findings are consistent with the species' habitat requirements (Giannetto et al., 2013; Rossi et al., 2021; Tancioni et al., 2013). Thus, landscape complexity seems to support the survival of this endangered species in remnant patches of wild habitats. Instead, fish IAS occupied disturbed habitats of larger main rivers. *P. parva*, indeed, was positively influenced by high levels of nitrates (10-50 mg/l), pH (7.6-8), and water turbidity (34min-5h30min, x= 2h28min) like due to the species' preference for low valley rivers, with calm, shallow waters with fine-grained riverbed (Sunardi et al., 2005).

Molluscs are another freshwater taxonomic group easily detectable by qPCR-based assays in eDNA water samples, particularly unionids bivalves that are fully aquatic (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, despite the recent proposed taxonomic distinction between the two species (Marrone et al., 2019; Prie et al., 2012). This assay was designed with just one and two mismatches in the forward and reverse primer, leaving uncertainty whether our results indicate new translocations of the *U. elongatulus* in or acknowledge new populations of *U. mancus* (G. Manganelli, pers. comm.). qPCR technique also confirmed accurately the recent finding in the Turano lake of the IAS *D. polymorpha* (Monaco, 2014; Peñarrubia et al., 2016; Gingera et al., 2017; Grano et al., 2020), and detected three new occurrences in the Tiber river catchment, likely deriving from the source population in Turano lake (Grano, 2022). Negative detections are associated between this species (and all the others analyzed within this study) and low values of pH, confirming the general influence of this parameter to inhibit eDNA detections or to degrade DNA directly into the environment (B. C. Schmidt et al., 2021). Terrestrial pulmonated gastropods are rarely monitored with eDNA, as they are often found in riparian zones or in turbid marshes which complicate filtration procedures (Rees et al., 2023). Despite obtaining a species-specific assay for species of the genus *Vertigo* has been troublesome we were still able to detect the presence of *Vertigo sp.* at new locations. Traditional surveys will be necessary to confirm the presence of the protected species *V. angustior* and *V. moulinsiana* (Ghezzi, 2021; Manganelli et al., 2020), as well as the recently introduced alien species *V. pygmaea* and *V. pusilla* (M. Bodon, pers. comm.).

Regarding mammals, the findings regarding the European polecat (*Mustela putorius*) and the Eurasian otter (*Lutra lutra*) highlight the complexities involved in using eDNA methodologies for monitoring mammal populations. The polecat, despite being a common species, was likely underestimated in our study (Russo et al., 2020), while the Eurasian otter was detected at multiple sites for the first time after its extinction in1975 (Battisti et al., 2011; Giovacchini et al., 2021). Polecats show low detection probabilities in eDNA studies (i.e., p=0.01), suggesting challenges in detecting this species that do not have a strong aquatic association (Broadhurst et al., 2021). In contrast, the Eurasian otter's detection via eDNA has proven to be much more effective, particularly in environments where traditional survey methods are

logistically challenging (Jamwal et al., 2023; Padgett-Stet al., 2016). As otters spend considerable time in aquatic environments (Kruuk, 2006), the differential detection rates between these species can be attributed to their ecology. Overall, these observations underscore the importance to include species-specific behaviors when designing eDNA monitoring programs inform conservation strategies for elusive species.

Finally, the successful detection of the fungus *Batrachochytrium dendobatidis* underscores the utility of qPCR-based eDNA for monitoring pathogens that threaten amphibian populations globally (Kamoroff, 2017; Kirshtein et al., 2007; Lastra González et al., 2021; Porco et al., 2024). Given that *B. dendrobatidis* exhibits optimal growth at moderate temperatures (Stevenson et al., 2013), it is plausible that our detection in autumn correlates with opportune and suitable temperature conditions. Further research could explore the influence of climate variables on the dynamics of chytrid fungi in amphibian habitats to enhance our predictive understanding of pathogen outbreaks.

For those species that were not detected in positive control sites, such as odonates and *T. scripta*, we suggest a possible low release of target DNA into the environment. The exoskeleton of crayfish and the highly keratinised skin of terrapins may severely limit the exchanges of live cells with the environment. Consequently, at low population densities, the amount of DNA released might be insufficient for the detection. A preliminary confirmation was provided by Adams et al. (2019) who detected eDNA of *T. scripta* only at high density values (13 kg/ha). More sensitive assays would enhance detectability and control for false absences (Tréguier et al., 2014; Troth et al., 2020).

Differently, null detection rates of odonates during eDNA survey are likely due to challenges encountered at different stages of assay designed and execution failure of *in vitro* and *in situ* steps (Brocchieri et al., 2014; Corso et al., 2019; Crescia et al., 2020). Initial assays (i.e., COI) co-amplified non-target genomic fragments from different taxa (e.g., Pilgrim et al., 2021). Despite these setbacks, qPCR methodologies have demonstrated success in detecting odonate eDNA in both controlled microcosmos settings and natural field environments (K. J. Schmidt et al., 2021; Thomsen et al., 2012b), as well as other freshwater macroinvertebrates in wild conditions (Deiner et al., 2015; Mächler et al., 2014; Seymour et al., 2018; Leese et al., 2021). To enhance the sensitivity of eDNA detection methods in forthcoming surveys, the integration of novel techniques such as droplet digital PCR (ddPCR) presents a promising avenue (Mauvisseau et al., 2019).

In summary, our approach allowed the detection of multiple species for 18 out of 22 target species belonging to a wide range of taxonomic groups and across a large area, confirming the efficiency of multiple taxa detection (Thomsen et al.,2012b; Leblanc et al.,2020). In our case, the multiple species approach was revealed to be particularly efficient for amphibians, fish, molluscs, and mammals, confirming both the reliability of eDNA assays and the effectiveness of site selection, whereas it remains largely inadequate for Odonata, crayfish and terrapins.

This study emphasizes the effectiveness of a multiple species-specific qPCR approach for analyzing eDNA to identify areas with high diversity of endangered native species as well as areas dominated by alien species. This technique shows promise in distinguishing between regions where native, alien, or a mix of both types of species are present. Historically, studies employing qPCR to detect co-occurring native and alien species have been limited in scope, often focusing on only a few target species (Hernandez et al., 2020; Thomas et al., 2020) and centering on direct competition scenarios(i.e., three native vs three IAS species: Jo et al., 2020) or on host-parasite dynamics (Robinson et al., 2018; Rusch et al., 2020). Moreover, studies that have taken a broader geographical approach are still relatively rare (King et al., 2022). Overall, our findings contribute to a growing body of literature that advocates for the use of qPCR-based eDNA techniques in conservation biology, particularly in monitoring biodiversity and assessing the impacts of invasive species within various ecosystems. By identifying pristine biotopes and prioritizing areas for eradication and mitigation measures, such surveys can significantly enhance the preservation of

threatened native species (Caffrey et al., 2014, Giovacchini et al., in prep.). For instance, the detection of the pathogen fungus *B. dendrobatidis* at a site inhabited by the highly endangered endemic Apennine yellow-bellied toad (*B. variegata pachypus*) emphasizes the importance of ongoing surveillance and intervention to safeguard vulnerable populations (Canestrelli et al., 2013; Tessa et al., 2013; Mirone et al., under revision). Similarly, the co-occurrence of the invasive spiny-cheek crayfish (*F. limosus*) with the native endangered white-clawed crayfish (*A. italicus*) in Turano Lake presents another critical example of how invasive species can pose direct threats to native aquatic fauna, as IAS are not only competitors but also reservoirs for diseases, like the crayfish plague caused by *Aphanomyces astaci*. Additionally, the presence of the red swamp crayfish (*P. clarkii*) alongside endangered amphibian species, like *B. variegata pachypus*, *L. italicus* and *T. carnifex,* highlights the predation risks that IAS pose to the juvenile stages of native amphibians (Wilson and Williams, 2014). Our results also revealed the expanding range of certain species, such as the translocated fish *P. bonelli* (Sarrocco et al., 2012), which poses a threat to the native *P. nigricans* (Pompei et al., 2018, 2016a, 2016b, 2014), especially along the Tiber River and in artificial reservoirs where it was accidentally released. In conclusion, multispecific surveys are vital for understanding the ecological dynamics between native and invasive species, allowing also to address future wildlife conservation and management planning.

4.1 Time and cost of traditional techniques and eDNA

Multispecies monitoring of freshwater species conducted on a large scale is time and cost expedient taking advantage of the eDNA single-species approach. eDNA surveys are quick in field collection and laboratory procedures, require less personnel, and are incredibly informative for many species at the same time. We believe this new way of collecting non-invasive data can really lead in the future to new winning conservation strategies supporting endemic biodiversity. One of the main prospects that may expand the routine use of eDNA in monitoring is its cost. As it is often a central constraint to launch a project on wildlife conservation, we demonstrated that an eDNA survey carried out on multiple species is a low-cost method compared to traditional techniques, with a ratio ranging from 1:5 (this study) to 1:10 (Biggs et al., 2015). This is furthermore valorized by our valuation of eDNA costs as it took into account the cost of permanent laboratory equipment such as machines, a matter not fully considered in previous papers (Akre et al., 2019; Davy et al., 2015; Nathan et al., 2014; Sigsgaard et al., 2015). Although clearly renowned to be more cost-effective (Herder et al., 2014; Smart et al., 2016), eDNA surveys cannot substitute field occurrences retrieved with traditional methods by experts and specialists that provide higher quality information on wildlife populations. Nevertheless, eDNA has been confirmed to be a rapid, cost-effective way to get sound data for wildlife conservation, sparing also on human resources, such as personnel or taxonomists.

4.2 Setting a protocol for the monitoring of multi-taxa species through eDNA

These results satisfactorily met the needs of a cost-effective multi-species survey at large geographic scale spanning a wide range of taxonomic groups, although restricted to a smaller number compared to the initial target. These encouraging results pave the way for the implementation of an optimal protocol to address the many monitoring obligations for endangered species and IASs that involve numerous countries worldwide, including Europe (i.e., regulations 43/92/CE and 1143/2014). We can highlight several key points to maximize the outcomes of a multi taxa eDNA survey.

1. The selection of target species should be followed by a thoroughly accurate three-steps validations of eDNA assays. This validation process is essential to ensure their ability to unequivocally detect the target taxa in the study area, accounting for intraspecific genetic variation, biogeographic factors and local distribution information.

2. For the selection of sampling sites, it is crucial a deep knowledge of species habitat preference, as well as the overlap of species distribution maps, to maximize the number of taxa that could be searched at each site. Distribution modeling may significantly enhance the identification of potential occurrence sites and optimize the sampling design.
3. Given that eDNA detections likely encompass a larger area than the specific point at which water is sampled, especially in the case of running waters, we recommend extending the number of taxa searched to include those species that could potentially be found some distance upstream from sampling location.
4. Considering phenological stages greatly enhances the detection probability of eDNA. Most target species of this study were detected during spring (n=7), and others during summer (n=3), or autumn (n=3). Therefore, seasonal sampling replicates are recommended to maximize species detectability (Troth et al., 2021).

Such an eDNA protocol could be hardly implemented to specific taxa which likely release a low number of cells into the water, even though they either spend much of their time in this environment (i.e. pond terrapins) or have fully aquatic life stages (i.e. crayfish). New techniques for detection of highly diluted eDNA will likely overcome these limitations and allow the detection of a wider array of species in the future. The use of CRISPR-Cas13-based methods or ddPCR represents a significant advancement in the field of molecular biodiversity assessment (Abudayyeh et a., 2017; Yang et al., 2024). (). As these techniques continue to develop and become more accessible tools for academic research and environmental monitoring, they will enhance our ability to monitor several species in various environments, providing valuable data for biodiversity conservation and ecological studies (Bowers et al., 2021).

The application of eDNA analysis as a reliable wildlife monitoring tool can be finally refined through the investigation of the dynamics of DNA molecules in different ecosystems. eDNA is indeed influenced by a variety of environmental factors, which can affect both its degradation and detectability. Understanding these factors is crucial for the implementation of an eDNA protocol, as they are fundamental to be take into account during sample planning. For instance, it is imperative to avoid sampling activities when are occurring the environmental conditions that decrease the possibility to detect species, particularly at fine spatial scales. In this study, the efforts to investigate the relationship between environmental parameters and the concurrent presence of species represent just the beginning of a deeper exploration into this complex interplay. Future research should aim to identify the single environmental factors that contribute to variations in eDNA detectability, as well as establish standardized protocols that account for these influences that can be species-specific (Thalinger et al., 2021).

5. Conclusions

implementing eDNA methodologies is a powerful tool for meeting the surveillance obligations outlined in the Habitats Directive (92/43/EEC) about freshwater endangered species, and early warning procedures of IAS recommended by the EU Regulation on IAS 1143/2014. Our findings underscore the effectiveness of eDNA methods in simultaneously detecting multiple species, thus providing a promising avenue for large-scale surveillance of diverse organismal groups. The results of our multiple species eDNA surveys affirm the utility of this approach as a pivotal element in management plans targeting IAS, particularly for species identified as being of Union Concern for which eradication efforts are imperative according to Regulation 1143/2014. Furthermore, eDNA monitoring represents a cost-effective strategy for identifying rare and endangered species listed in Annexes II and IV of the Habitats Directive, helping State Members comply with periodically reporting species distribution and establishing protected areas. Our findings will give the opportunity for evaluating the effectiveness of protected areas in conserving native endangered

species. We can also identify priority areas for prevention against IAS introduction, prioritize sites for IAS eradication, and pinpoint highly invaded zones for mitigation strategies.

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Supplementary Information

The first chapter of this thesis contains supplementary material available online at the following link: https://acrobat.adobe.com/id/urn:aaid:sc:eu:68b9e95a-a65a-4132-9158-8efdef388b32

Supplementary materials 1

S1. Length of rivers and surface of lakes sampled.

S2. List of papers and gray literature used to assess habitat requirements and distribution of the target species in the study area.

S3. Details and source of data for environmental variables collected in the field and from digital map layers.

S4. List of published qPCR-eDNA primers and probes used for eDNA detection.

S5. Positive control (PCS) and negative control (NCS) sites sampled for in situ validation of probes. Target species are reported with their ID (see Tab. 1).

S6. Presence (P) and absence (A) of species at each site, irrespective of replicate. Coloured cells indicate the tested species at each site.

S7. Cost and time estimate for traditional surveys, conducted in the same sites of this eDNA case study. When survey techniques and sampling sites are distinguished by habitat type, values for lentic waters are displayed in italic.

S8. Cost and time estimate of this eDNA case study conducted in Central Italy across 53 survey sites and 44 assay validation sites.

Supplementary materials 2

S1. Accession numbers tested in silico for target species and species strictly related.

References

Abudayyeh, O., Gootenberg, J., Essletzbichler, P., Han, S., Joung, J., Belanto, J.J., Verdine, V., Cox, D.B.T., Kellner, M.J., Regev, A., Lander, E.S., Daniel F. Voytas, D.F.,Ting, A.Y., Zhang, F., 2017. RNA targeting with CRISPR–Cas13. *Nature* 550, 280–284. https://doi.org/10.1038/nature24049

Adams, C.I.M., Hoekstra, L.A., Muell, M.R., Janzen, F.J., 2019. A Brief Review of Non-Avian Reptile Environmental DNA (eDNA), with a Case Study of Painted Turtle (Chrysemys picta) eDNA Under Field Conditions. Diversity. https://doi.org/10.3390/d11040050

Akre, T.S., Parker, L.D., Ruther, E., Maldonado, J.E., Lemmon, L., McInerney, N.R., 2019. Concurrent visual encounter sampling validates eDNA selectivity and sensitivity for the endangered wood turtle (Glyptemys insculpta). PLoS One 14, 1–22. https://doi.org/10.1371/journal.pone.0215586

Amberg, J.J., Merkes, C.M., Stott, W., Rees, C.B., Erickson, R.A., 2019. Environmental DNA as a tool to help inform zebra mussel, Dreissena polymorpha, management in inland lakes. Manag. Biol. Invasions 10, 96–110. https://doi.org/10.3391/MBI.2019.10.1.06

Antognazza, C.M., Britton, J.R., Potter, C., Franklin, E., Hardouin, E.A., Gutmann Roberts, C., Aprahamian, M., Andreou, D., 2019. Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (Alosa spp.). Aquat. Conserv. Mar. Freshw. Ecosyst. 29, 148–152. https://doi.org/https://doi.org/10.1002/aqc.3010

Balletto, E., Bonelli, S., Borghesio, L., Casale, A., Brandmayr, P., Vigna Taglianti, A., 2010. Hotspots of biodiversity and conservation priorities: A methodological approach. Ital. J. Zool. 77, 2–13. https://doi.org/10.1080/11250000902758923

Battisti, C., Amori, G., Angelici, F.M., Luiselli, L., Zapparoli, M., 2011. Can the grey literature help us understand the decline and extinction of the Near Threatened Eurasian otter Lutra lutra in Latium, central Italy? Oryx 45, 281–287. https://doi.org/DOI:10.1017/S0030605310001055

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P., Williams, P., Dunn, F., 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). Biol. Conserv. 183, 19–28. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.029

Bletz, M.C., Rosa, G.M., Andreone, F., Courtois, E.A., Schmeller, D.S., Rabibisoa, N.H.C., Rabemananjara, F.C.E., Raharivololoniaina, L., Vences, M., Weldon, C., Edmonds, D., Raxworthy, C.J., Harris, R.N., Fisher, M.C., Crottini, A., 2015. Widespread presence of the pathogenic fungus Batrachochytrium dendrobatidis in wild amphibian communities in Madagascar. Sci. Rep. 5, 8633. https://doi.org/10.1038/srep08633

Bowers, H.A., Pochon, X., von Ammon, U., Gemmell, N., Stanton, J.-A.L., Jeunen, G.-J., Sherman, C.D.H., Zaiko, A., 2021. Towards the Optimization of eDNA/eRNA Sampling Technologies for Marine Biosecurity Surveillance. *Water*, 13(8):1113. https://doi.org/10.3390/w13081113

Broadhurst, H.A., Gregory, L.M., Bleakley, E.K., Perkins, J.C., Lavin, J. V, Bolton, P., Browett, S.S., Howe, C. V, Singleton, N., Tansley, D., Sales, N.G., McDevitt, A.D., 2021. Mapping differences in mammalian distributions and diversity using environmental DNA from rivers. Sci. Total Environ. 801, 149724. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.149724

Brocchieri, D., Castelluccio, P., Crucitti, P., 2014. Gli Odonati della Riserva Naturale "Macchia di Gattaceca e Macchia del Barco" (Lazio) (Odonata). Boll. della Soc. Entomol. Ital. 146, 31–40. https://doi.org/10.4081/bollettinosei.2014.31

Bruno, A., Sandionigi, A., Galimberti, A., Siani, E., Labra, M., Cocuzza, C., Ferri, E., Casiraghi, M., 2017. One step forwards for the routine use of high-throughput DNA sequencing in environmental monitoring. An efficient and standardizable method to maximize the detection of environmental bacteria. Microbiologyopen 6, e00421. https://doi.org/https://doi.org/10.1002/mbo3.421

Brusconi, S., Bertocchi, S., Renai, B., Scalici, M., Souty-Grosset, C., Gherardi, F., 2008. Conserving indigenous crayfish: Stock assessment and habitat requirements in the threatened Austropotamobius italicus. Aquat. Conserv. Mar. Freshw. Ecosyst. 18, 1227–1239. https://doi.org/10.1002/aqc.935

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Caffrey, J.M., Baars, J.R., Barbour, J.H., Boets, P., Boon, P., Davenport, K., Dick, J.T.A., Early, J., Edsman, L., Gallagher, C., Gross, J., Heinimaa, P., Horrill, C., Hudin, S., Hulme, P.E., Hynes, S., Macisaac, H.J., McLoone, P., Millane, M., Moen, T.L., Moore, N., Newman, J., O'Conchuir, R., O'Farrell, M., O'Flynn, C., Oidtmann, B., Renals, T., Ricciardi, A., Roy, H., Shaw, R., Van Valkenburg, J.L.C.H., Weyl, O., Williams, F., Lucy, F.E., 2014. Tackling invasive alien species in Europe: The top 20 issues. Manag. Biol. Invasions 5, 1–20. https://doi.org/10.3391/mbi.2014.5.1.01

Canessa, S., Maggesi, M., Salvidio, S., Grasselli, E., 2017. Validation and cost-effectiveness of an alternative method to quantify Batrachochytrium dendrobatidis infection in amphibian samples using real-time PCR. Rend. Lincei 28, 687–692. https://doi.org/10.1007/s12210-017-0640-0

Canestrelli, D., Zampiglia, M., Bisconti, R., Nascetti, G., 2014. Proposta di intervento per la conservazione ed il recupero delle popolazioni di ululone appenninico in Italia peninsulare. Minist. dell'Ambiente.

Canestrelli, D., Zampiglia, M., Nascetti, G., 2013. Widespread Occurrence of Batrachochytrium dendrobatidis in Contemporary and Historical Samples of the Endangered Bombina pachypus along the Italian Peninsula. PLoS One 8. https://doi.org/10.1371/journal.pone.0063349

Capizzi, D., Bellavita, M., 2014. Rete di monitoraggio della Testuggine palustre (Emys orbicularis) nel Lazio - Relazione attività 2012-2014 30.

Carosi, A., Ghetti, L., Lorenzoni, M., 2021. The role of climate changes in the spread of freshwater fishes: Implications for alien cool and warm-water species in a mediterranean basin. Water (Switzerland) 13. https://doi.org/10.3390/w13030347

Cavalcante, R., Seglie, D., Fiore, S., 2020. Il programma di conservazione ex-situ del Centro Emys Piemonte: risultati preliminari del primo triennio di lavoro. Atti II Congresso Nazionale Testuggini e Tartarughe (Albenga, 11-13 aprile 2019).

Corso, A., Janni, O., Fracasso, C., Biscaccianti, A., De Lisio, L., 2019. New data on the distribution of the endangered dragonflies Oxygastra curtisii (Dale, 1834), Somatochlora meridionalis (Vander Linden, 1825) and S. flavomaculata Nielsen, 1935 in Central-Southern Italy (Odonata Cordulidae). Biodivers. J. 10, 195–200. https://doi.org/10.31396/biodiv.jour.2019.10.3.195.200

Costa, M.J., Duarte, G., Segurado, P., Branco, P., 2021. Major threats to European freshwater fish species. Sci. Total Environ. 797, 149105. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.149105

Crescia, P., Soccini, C., Ferri, V., Celletti, S., 2020. Prima segnalazione di Lindenia tetraphylla (Vander Linden, 1825) per l'alto Lazio (Odonata: Gomphidae). Nat. Hist. Sci. 7, 79–82. https://doi.org/10.4081/nhs.2020.471

Davy, C.M., Kidd, A.G., Wilson, C.C., 2015. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0130965

Deiner, K., Walser, J.-C., Mächler, E., Altermatt, F., 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biol. Conserv. 183, 53–63. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.018

Dextrase, A.J., Mandrak, N.E., 2006. Impacts of alien invasive species on freshwater fauna at risk in Canada. Biol. Invasions 8, 13–24. https://doi.org/10.1007/s10530-005-0232-2

Duarte, S., Simões, L., Costa, F.O., 2023. Current status and topical issues on the use of eDNA-based targeted detection of rare animal species. Sci. Total Environ., 904, 166675.https://doi.org/10.1016/j.scitotenv.2023.166675.

Ercole, S, Angelini, P., Carnevali, L., Casella, L., Giacanelli, V., Grignetti, A., La Mesa, G., Nardelli, R., Serra, L., Stoch, F., Tunesi, L., Genovesi, P., 2021. Rapporti Direttive Natura (2013-2018). Sintesi dello stato di conservazione delle specie e degli habitat di interesse comunitario e delle azioni di contrasto alle specie esotiche di rilevanza unionale in Italia. (No. 394/2021), Serie Rapporti. ISPRA.

Ferri, V., Grano, M., Marini, D., Soccini, C., Filippi, E., Cervoni, F., Masulli, A., 2019. La biodiversità aliena dei Cheloni nel Lazio: monitoraggio dei nuclei di testuggini esotiche terrestri e palustri introdotti negli ambienti naturali della regione. Congr. Naz. Testuggini e Tartarughe.

Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biol. Lett. 4, 423–425. https://doi.org/10.1098/rsbl.2008.0118

Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G., Taberlet, P., 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Mol. Ecol. Resour. 15, 543–556. https://doi.org/https://doi.org/10.1111/1755-0998.12338

Francis, R.A., 2012. A Handbook of Global Freshwater Invasive Species, A Handbook of Global Freshwater Invasive Species. https://doi.org/10.4324/9780203127230

Galimberti, A., Assandri, G., Maggioni, D., Ramazzotti, F., Baroni, D., Bazzi, G., Chiandetti, I., Corso, A., Ferri, V., Galuppi, M., Ilahiane, L., La Porta, G., Laddaga, L., Landi, F., Mastropasqua, F., Ramellini, S., Santinelli, R., Soldato, G., Surdo, S., Casiraghi, M., 2021. Italian odonates in the Pandora's box: A comprehensive DNA barcoding inventory shows taxonomic warnings at the Holarctic scale. Mol. Ecol. Resour. 21, 183–200. https://doi.org/https://doi.org/10.1111/1755-0998.13235.

German Federal Agency for Nature Conservation, 1997. European Biodiversity. In: Biodiversity and Tourism. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-60689-2_8

Gherardi F., Aquiloni L., Bertocchi S., Brusconi S., Scalici M., Tricarico E., 2009. I gamberi alloctoni nel Lazio: distribuzione, impatti, costi, rischi e gestione. Rapporto tecnico.

Gherardi, F., Bertolino, S., Bodon, M., Casellato, S., Cianfanelli, S., Ferraguti, M., Lori, E., Mura, G., Nocita, A., Riccardi, N., Rossetti, G., Rota, E., Scalera, R., Zerunian, S., Tricarico, E., 2008. Animal xenodiversity in Italian inland waters: Distribution, modes of arrival, and pathways. Biol. Invasions 10, 435–454. https://doi.org/10.1007/s10530-007-9142-9

Ghezzi, D., 2021. Prima segnalazione di Vertigo moulinsiana Stylommatofora , Vertiginidae) in provincia di Cremona. Pianura 40, 130–136.

Giannetto, D., Carosi, A., Ghetti, L., Pedicillo, G., Pompei, L., Lorenzoni, M., 2013. Ecological traits of Squalius lucumonis (Actinopterygii, Cyprinidae) and main differences with those of Squalius squalus in the Tiber River Basin (Italy). Knowl. Manag. Aquat. Ecosyst.

Gingera, T.D., Bajno, R., Docker, M.F., Reist, J.D., 2017. Environmental DNA as a detection tool for zebra mussels Dreissena polymorpha (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. Manag. Biol. Invasions 8, 287–300. https://doi.org/10.3391/mbi.2017.8.3.03

Giovacchini, S., Antonucci, A., Bartolomei, R., Bandini, M., Caldarella, M., De Castro, G., ... Balestrieri, A., 2021. Conservation status of Eurasian otter Lutra lutra in Italy. Eurasian Otter Workshop, 26-28 February 2021.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7, 1299–1307. https://doi.org/10.1111/2041-210X.12595

Grano, M., 2022. Prima segnalazione di Dreissena polymorpha (Pallas , 1771) (Bivalvia : Dreissenidae) nel Lago del Turano e per la provincia di Rieti (Lazio). Alleryana 40, 12–15.

Grano, M., Nistri, R., Giuseppe, R. Di, 2020. Aggiornamento sui molluschi alloctoni nel fiume Tevere a Roma (Bivalvia). Allerya 38, 117–121.

Hall, A., 1999. BioEdit_a_user_friendly_biological_seque. Oxford Univ. Press.

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S., Hänfling, B., 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (Triturus cristatus). Ecol. Evol. 8, 6330–6341. https://doi.org/https://doi.org/10.1002/ece3.4013

Herder, J. E., Valentini, A., Bellemain, E., Dejean, T., Van Delft, J. J. C. W., Thomsen, P. F., & Taberlet, P., 2014. Environmental DNA-a review of the possible applications for the detection of (invasive) species. Stichting RAVON, Nijmegen. Report 2013-104. A Technical Report. https://doi.org/10.13140/RG.2.1.4002.1208.

Hernandez, C., Bougas, B., Perreault-Payette, A., Simard, A., Côté, G., Bernatchez, L., 2020. 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. Environ. DNA 2, 373–386. https://doi.org/https://doi.org/10.1002/edn3.89

Hii, K.S., Manaff, A.H.N.A., Gu, H., Lim, P.T., Leaw, C.P., 2024. A comparative analysis of real-time quantitative PCR and metabarcoding methods for eDNA-based detection of the toxic dinophyte Alexandrium tamiyavanichii (Dinophyceae). *Mar. Env. Res.,*106593. https://doi.org/10.1016/j.marenvres.2024.106593

Iannella, M., Cerasoli, F., Biondi, M., 2017. Unraveling climate influences on the distribution of the parapatric newts Lissotriton vulgaris meridionalis and L. italicus. Front. Zool. 14, 1–14. https://doi.org/10.1186/s12983-017-0239-4

IPBES, 2023. Summary for Policymakers of the Thematic Assessment Report on Invasive Alien Species and their Control of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, in: Roy, H.E., Pauchard, A., Stoett, P., Renard Truong, T., Bacher, S., Galil, B.S., Hulme, P.E., Ikeda, T., Sankaran, K. V, McGeoch, M.A., Meyerson, L.A., Nuñez, M.A., Ordonez, A., Rahlao, S.J., Schwindt, E., Seebens, H., Sheppard, A.W., Vandvik, V. (Eds.), . IPBES Secretariat, Bonn, Germany. https://doi.org/10.5281/zenodo.7430692

ISPRA, 2009. Key topics. Italian Environmental Data Yearbook. Chapter 2 – Biodiversity and Natural, Agricultural and Forest Areas. Italian Institute for Environmental Protection and Research.

Jamwal, P.S., Bruno, A., Galimberti, A., Magnani, D., Casiraghi, M., Loy, A., 2023. Environmental DNA revealed high accuracy in detection of the Eurasian otter in Himalaya. Aquat. Conserv. Mar. Freshw. Ecosyst. 33, 1309–1320. https://doi.org/https://doi.org/10.1002/aqc.4010

Jaureguiberry, P., Titeux, N., Wiemers, M., Bowler, D.E., Coscieme, L., Golden, A.S., Guerra, C.A., Jacob, U., Takahashi, Y., Settele, J., Díaz, S., Molnár, Z., Purvis, A., 2024. The direct drivers of recent global anthropogenic biodiversity loss. Sci. Adv. 8, eabm9982. https://doi.org/10.1126/sciadv.abm9982

Jo, T., Fukuoka, A., Uchida, K., Ushimaru, A., Minamoto, T., 2020. Multiplex real-time PCR enables the simultaneous detection of environmental DNA from freshwater fishes: a case study of three exotic

and three threatened native fishes in Japan. Biol. Invasions 22, 455–471. https://doi.org/10.1007/s10530-019-02102-w

Jo, T.S., 2023. Utilizing the state of environmental DNA (eDNA) to incorporate time-scale information into eDNA analysis. Proc. R. Soc. B Biol. Sci. 290, 20230979. https://doi.org/10.1098/rspb.2023.0979

Kakuda, A., Doi, H., Souma, R., Nagano, M., Minamoto, T., Katano, I., 2019. Environmental DNA detection and quantification of invasive red-eared sliders, Trachemy scripta elegans, in ponds and the influence of water quality. PeerJ 7, e8155. https://doi.org/10.7717/peerj.8155

Kamoroff, C., 2017. Using environmental DNA for early detection of amphibian chytrid fungus Batrachochytrium dendrobatidis prior to a ranid die-off . Dis. Aquat. Organ. 127, 75–79.

Kamoroff, C., Daniele, N., Grasso, R.L., Rising, R., Espinoza, T., Goldberg, C.S., 2020. Effective removal of the American bullfrog (Lithobates catesbeianus) on a landscape level: long term monitoring and removal efforts in Yosemite Valley, Yosemite National Park. Biol. Invasions 22, 617–626. https://doi.org/10.1007/s10530-019-02116-4

Karlsson, E., Ogonowski, M., Sundblad, G., Sundin, J., Svensson, O., Nousiainen, I., Vasemägi, A., 2022. Strong positive relationships between eDNA concentrations and biomass in juvenile and adult pike (Esox lucius) under controlled conditions: Implications for monitoring. Environ. DNA 4, 881–893. https://doi.org/https://doi.org/10.1002/edn3.298

Kassambara, A., & Mundt, F., 2020. factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7. Retrieved from https://CRAN.R-project.org/package=factoextra.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35, W43–W46. https://doi.org/10.1093/nar/gkm234

King, A.C., Krieg, R., Weston, A., Zenker, A.K., 2022. Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. J. Environ. Manage. 302, 113929. https://doi.org/https://doi.org/10.1016/j.jenvman.2021.113929

Kirshtein, J., Anderson, C., Wood, J., Longcore, J., 2007. Quantitative PCR detection of Batrachochytrium dendrobatidis DNA from sediments and water. Dis. Aquat. Organ. 77, 11–15.

Knudsen, S.W., Ebert, R.B., Hesselsøe, M., Kuntke, F., Hassingboe, J., Mortensen, P.B., Thomsen, P.F., Sigsgaard, E.E., Hansen, B.K., Nielsen, E.E., Møller, P.R., 2019. Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. J. Exp. Mar. Bio. Ecol. 510, 31–45. https://doi.org/https://doi.org/10.1016/j.jembe.2018.09.004

Kruuk, H., 2006. Otters: ecology, behaviour and conservation. Oxford University Press, USA.

Lacoursière-Roussel, A., Côté, G., Leclerc, V., Bernatchez, L., 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. J. Appl. Ecol. 53, 1148–1157. https://doi.org/https://doi.org/10.1111/1365-2664.12598

Langlois, V.S., Allison, M.J., Bergman, L.C., To, T.A., Helbing, C.C., 2021. The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. Environ. DNA 3, 519–527. https://doi.org/https://doi.org/10.1002/edn3.164

Lastra González, D., Baláž, V., Vojar, J., Chajma, P., 2021. Dual Detection of the Chytrid Fungi Batrachochytrium spp. with an Enhanced Environmental DNA Approach. J. Fungi. https://doi.org/10.3390/jof7040258

Leblanc, F., Belliveau, V., Watson, E., Coomber, C., Simard, N., Di Bacco, C., Bernier, R., Gagné, N., 2020. Environmental DNA (eDNA) detection of marine aquatic invasive species (AIS) in Eastern Canada using a targeted species-specific qPCR approach. Manag. Biol. Invasions 11, 201–217. https://doi.org/10.3391/mbi.2020.11.2.03

Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., Ekrem, T., Čiampor, F., Čiamporová-Zaťovičová, Z., Costa, F.O., Duarte, S., Elbrecht, V., Fontaneto, D., Franc, A., Geiger, M.F., Hering, D., Kahlert, M., Kalamujić Stroil, B., Kelly, M., Keskin, E., Liska, I., Mergen, P., Meissner, K., Pawlowski, J., Penev, L., Reyjol, Y., Rotter, A., Steinke, D., van der Wal, B., Vitecek, S., Zimmermann, J., Weigand, A.M., 2018. Chapter Two - Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action, in: Bohan, D.A., Dumbrell, A.J., Woodward, G., Jackson, M.B.T.-A. in E.R. (Eds.), Next Generation Biomonitoring: Part 1. Academic Press, pp. 63–99. https://doi.org/https://doi.org/10.1016/bs.aecr.2018.01.001

Lorenzoni, M., Borghesan, F., Carosi, A., Ciuffardi, L., De Curtis, O., Del Mastro, G.B., Di Tizio, L., Franzoi, P., Maio, G., Mojetta, A., Nonnis Marzano, F., Pizzul, E., Rossi, G., Scalici, M., Zanetti, M., 2019. Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane the Check-List of the Italian Freshwater Fish Fauna. Ital. J. Freshw. Ichthyol. 5, 239–254.

Lowe, S., Browne, M., Boudjelas, S., 2000. 100 of the World ' S Worst Invasive Alien Species 12, 12.

Macchi, S., Scali, S., Bisi, F., Martinoli, A., Alonzi, A., Carnevali, L., 2020. Piano nazionale per la gestione della testuggine palustre americana (Trachemys scripta) ISPRA-MA.

Mächler, E., Deiner, K., Steinmann, P., Altermatt, F., 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshw. Sci. 33, 1174–1183. https://doi.org/10.1086/678128

Mackie, G. L., & Claudi, R., 2010. Practical manual for zebra mussel monitoring and control. CRC Press, Boca Raton, FL.

Magliozzi, C., Tsiamis, K., Vigiak, O., Deriu, I., Gervasini, E., Cardoso, A.C., 2020. Assessing invasive alien species in European catchments: Distribution and impacts. Sci. Total Environ. 732, 138677. https://doi.org/10.1016/j.scitotenv.2020.138677

Manfrin, C., Mirimin, L., Zanetti, M., Pizzul, E., Giulianini, P.G., Pallavicini, A., 2022a. Highly sensitive environmental DNA detection of topmouth gudgeon, Pseudorasbora parva: a comparison of qPCR and microfluidic qdPCR. Biol. Invasions 24, 2121–2133. https://doi.org/10.1007/s10530-022-02761-2

Manfrin, C., Zanetti, M., Stanković, D., Fattori, U., Bertucci-Maresca, V., Giulianini, P.G., Pallavicini, A., 2022b. Detection of the Endangered Stone Crayfish Austropotamobius torrentium (Schrank, 1803) and Its Congeneric A. pallipes in Its Last Italian Biotope by eDNA Analysis. Diversity. https://doi.org/10.3390/d14030205

Manganelli, G., Favilli, L., Barbato, D., & Benocci, A., 2020. Distribuzione e conservazione di Vertigo angustior e Vertigo moulinsiana (Mollusca, Gastropoda, Vertiginidae) in Toscana: stato delle conoscenze. Atti della Società Toscana di Scienze Naturali, Memorie, Serie B, 127, 59-77.

Marrone, F., Nardi, G., Cianfanelli, S., Govedič, M., Barra, S.A., Arculeo, M., Bodon, M., 2019. Diversity and taxonomy of the genus Unio Philipsson in Italy, with the designation of a neotype for Unio elongatulus C. Pfeiffer (Mollusca, Bivalvia, Unionidae). Zootaxa 4545, 339–374. https://doi.org/10.11646/zootaxa.4545.3.2

Mattoccia, M., Marta, S., Romano, A., Sbordoni, V., 2011. Phylogeography of an Italian endemic salamander (genus Salamandrina): Glacial refugia, postglacial expansions, and secondary contact. Biol. J. Linn. Soc. 104, 903–992. https://doi.org/10.1111/j.1095-8312.2011.01747.x

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., Souty-Grosset, C., 2018. Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. Hydrobiologia 805, 163–175. https://doi.org/10.1007/s10750-017-3288-y

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., Sweet, M., 2019. Combining

ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. Sci. Rep. 9, 14064. https://doi.org/10.1038/s41598-019-50571-9

Meira, A., Lopes-Lima, M., Varandas, S., Teixeira, A., Arenas, F., Sousa, R., 2019. Invasive crayfishes as a threat to freshwater bivalves: Interspecific differences and conservation implications. Sci. Total Environ. 649, 938–948. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.08.341

Minegishi, Y., Wong, M.K.-S., Nakao, M., Nishibe, Y., Tachibana, A., Kim, Y.-J., Hyodo, S., 2023. Species-specific patterns in spatio-temporal dynamics of juvenile chum salmon and their zooplankton prey in Otsuchi Bay, Japan, revealed by simultaneous eDNA quantification of diverse taxa from the same water samples. Fish. Oceanogr. 32, 311–326. https://doi.org/https://doi.org/10.1111/fog.12631

Mirone, E., Giovacchini, S., Bologna, M. A., Bruno, A., Caprotti, L., Di Febbraro, M., Jamwal, P.S., Monaco, P., Ramazzotti, F., Vignoli, L., Galimberti, A., Loy, A., 2024. New eDNA-based insights for the detection of endemic and endangered Apennine yellow bellied toad (Bombina pachypus) and its plague Batrachochytrium dendrobatidis. Under review by Amphibia-Reptilia.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., Iwasaki, W., 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R. Soc. Open Sci. 2, 150088. https://doi.org/10.1098/rsos.150088

Monaco, A., 2014. Alieni: la minaccia delle specie alloctone per la biodiversità del Lazio. *Palombi Editori*.

Mori, E., Menchetti, M., Cantini, M., Bruni, G., Santini, G., Bertolino, S., 2017. Twenty years' monitoring of a population of Italian crested newts Triturus carnifex: strong site fidelity and shifting population structure in response to restoration. Ethol. Ecol. Evol. 29, 460–473. https://doi.org/10.1080/03949370.2016.1236040

Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L., Mahon, A.R., 2014. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. Environ. Sci. Technol. 48, 12800–12806. https://doi.org/10.1021/es5034052

Nicholson, A., McIsaac, D., MacDonald, C., Gec, P., Mason, B.E., Rein, W., Wrobel, J., de Boer, M., Milián-García, Y., Hanner, R.H., 2020. An analysis of metadata reporting in freshwater environmental DNA research calls for the development of best practice guidelines. Environ. DNA 2, 343–349. https://doi.org/https://doi.org/10.1002/edn3.81

Nocita, A., Tricarico, E., Bertolino, S., 2017. Fine-scale analysis of heavily invaded Italian freshwater fish assemblages. Integr. Zool. 12, 500–511. https://doi.org/https://doi.org/10.1111/1749-4877.12267

Osathanunkul, M., Minamoto, T., 2021. eDNA-based detection of a vulnerable crocodile newt (Tylototriton uyenoi) to influence government policy and raise public awareness. Divers. Distrib. 27, 1958–1965. https://doi.org/https://doi.org/10.1111/ddi.13236

Padgett-Stewart, T.M., Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., Schwartz, M.K., 2016. An eDNA assay for river otter detection: a tool for surveying a semi-aquatic mammal. Conserv. Genet. Resour. 8, 5–7. https://doi.org/10.1007/s12686-015-0511-x

Park, H.-C., Han, T.-Y., Kim, D.-C., Min, M.-S., Han, S.-Y., Kim, K.-S., Lee, H., 2011. Individual identification and sex determination of Eurasian otters (Lutra lutra) in Daegu city based on genetic analysis of otter spraint. Genes Genomics 33, 653–657. https://doi.org/10.1007/s13258-011-0051-z

Pawlowski, J., Bonin, A., Boyer, F., Cordier, T., Taberlet, P., 2021. Environmental DNA for biomonitoring 2931–2936. https://doi.org/10.1111/mec.16023

Peñarrubia, L., Alcaraz, C., Vaate, A. bij de, Sanz, N., Pla, C., Vidal, O., Viñas, J., 2016. Validated methodology for quantifying infestation levels of dreissenid mussels in environmental DNA (eDNA) samples. Sci. Rep. 6, 39067. https://doi.org/10.1038/srep39067

Petrassi, F., Cerfolli, F., Monaco, A., Sarrocco, S., 2017. La citizen science e il monitoraggio delle specie aliene. Regione Lazio, Roma, pp. 80.

Pilgrim, J., Thongprem, P., Davison, H.R., Siozios, S., Baylis, M., Zakharov, E. V, Ratnasingham, S., deWaard, J.R., Macadam, C.R., Smith, M.A., Hurst, G.D.D., 2021. Torix Rickettsia are widespread in arthropods and reflect a neglected symbiosis. Gigascience 10, giab021. https://doi.org/10.1093/gigascience/giab021

Pilliod, D.S., Goldberg, C.S., Arkle, R.S., Waits, L.P., 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Mol. Ecol. Resour. 14, 109–116. https://doi.org/https://doi.org/10.1111/1755-0998.12159

Pompei, L., Giannetto, D., Lorenzoni, M., 2018. Range expansion of an invasive goby in the river Tiber Basin (Central Italy): Effects on the abundance and shelter occupancy of a congeneric native goby. Aquat. Invasions 13, 277–288. https://doi.org/10.3391/ai.2018.13.2.08

Pompei, L., Giannetto, D., Lorenzoni, M., 2016a. The non-native goby Padogobius bonelli in the River Tiber, Italy and its effect on the reproductive potential of the native goby, P. nigricans. Aquat. Invasions 11, 83–92. https://doi.org/10.3391/ai.2016.11.1.09

Pompei, L., Giannetto, D., Lorenzoni, M., 2016b. Reproductive parameters in native and non-native areas of Padogobius bonelli and comparison with P. nigricans (Actynopterigii, Gobiidae). Hydrobiologia 779, 173–182. https://doi.org/10.1007/s10750-016-2812-9

Pompei, L., Giannetto, D., Lorenzoni, M., 2014. Feeding Ecology of Padogobius nigricans (Canestrini, 1867) and P. bonelli (Bonaparte, 1846) in Aggia River (Umbria, Italy) and Their Diet Overlap. Hydrobiologia 740, 101–113. https://doi.org/10.1007/s10750-014-1942-1

Pont, D., Meulenbroek, P., Bammer, V., Dejean, T., Erős, T., Jean, P., Lenhardt, M., Nagel, C., Pekarik, L., Schabuss, M., Stoeckle, B.C., Stoica, E., Zornig, H., Weigand, A., Valentini, A., 2023. Quantitative monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR. Mol. Ecol. Resour. 23, 396–409. https://doi.org/https://doi.org/10.1111/1755-0998.13715

Porco, D., Purnomo, C.A., Glesener, L., Proess, R., Lippert, S., Jans, K., Colling, G., Schneider, S., Stassen, R., Frantz, A.C., 2024. eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. BMC Ecol. Evol. 24, 1–16. https://doi.org/10.1186/s12862-023-02189-9

Prie, V., Puillandre, N., Bouchet, P., 2012. Bad taxonomy can kill : molecular reevaluation of Unio mancus Lamarck, 1819 (Bivalvia : Unionidae) and its accepted subspecies. Knowl. Manag. Aquat. Ecosyst.

Prié, V., Valentini, A., Lopes-Lima, M., Froufe, E., Rocle, M., Poulet, N., Taberlet, P., Dejean, T., 2021. Environmental DNA metabarcoding for freshwater bivalves biodiversity assessment: methods and results for the Western Palearctic (European sub-region). Hydrobiologia 848, 2931–2950. https://doi.org/10.1007/s10750-020-04260-8

R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Ratnasingham, S., Hebert, P.D.N., 2007. bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol. Ecol. Notes 7, 355–364. https://doi.org/https://doi.org/10.1111/j.1471-8286.2007.01678.x

Rees, H.C., Cousins, M.E., Baker, C.A., Maddison, B.C., 2023. A qPCR assay for the rapid and specific detection of Shining ram's-horn snail (Segmentina nitida) eDNA from Stodmarsh National Nature Reserve, UK. PLoS One 18, e0288267.

Reyne, M., Naaum, A.M., Marnell, F., Reid, N., Helyar, S.J., 2021. Development and validation of a quantitative qPCR assay for detecting Natterjack toad (Epidalea calamita) eDNA samples. Conserv.

Genet. Resour. 13, 319–322. https://doi.org/10.1007/s12686-021-01199-3

Robinson, C.V., Uren Webster, T.M., Cable, J., James, J., Consuegra, S., 2018. Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. Biol. Conserv. 222, 241–252. https://doi.org/https://doi.org/10.1016/j.biocon.2018.04.009

Rodgers, T.W., Lovich, R.E., Walton, J.A., Prince, D.J., Gonzalez, B.R., Shaffer, H.B., Mock, K.E., 2023. Multiplex qPCR assays for detection of 2 imperiled anuran species, Anaxyrus californicus and Spea hammondii, from environmental DNA. Freshw. Sci. 42, 315–322. https://doi.org/10.1086/726683

Rondinini, C., Battistoni, A., Teofili, C., 2022. Lista rossa 2022. List. Rossa IUCN dei Vertebr. Ital. 2022 Comitato I.

Rossi, A.R., Petrosino, G., Crescenzo, S., Milana, V., Talarico, L., Martinoli, M., Rakaj, A., Lorenzoni, M., Carosi, A., Ciuffardi, L., Tancioni, L., 2021. Phylogeography and population structure of Squalius lucumonis: A baseline for conservation of an Italian endangered freshwater fish. J. Nat. Conserv. 64, 126085. https://doi.org/https://doi.org/10.1016/j.jnc.2021.126085

Rourke, M.L., Fowler, A.M., Hughes, J.M., Broadhurst, M.K., DiBattista, J.D., Fielder, S., Wilkes Walburn, J., Furlan, E.M., 2022. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environ. DNA 4, 9–33. https://doi.org/https://doi.org/10.1002/edn3.185

Rund, H., Wanzenböck, J., Dobrovolny, S., & Kurmayer, R. (2024). Relating target fish DNA concentration to community composition analysis in freshwater fish via metabarcoding. Sci. Total Environ., *927*, 172281. https://doi.org/10.1016/j.scitotenv.2024.172281

Rusch, J.C., Mojžišová, M., Strand, D.A., Svobodová, J., Vrålstad, T., Petrusek, A., 2020. Simultaneous detection of native and invasive crayfish and Aphanomyces astaci from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58, 1–32. https://doi.org/10.3897/neobiota.58.49358

Sanders, E., Wassens, S., Michael, D.R., Nimmo, D.G., Turner, J.M., 2024. Extinction risk of the world's freshwater mammals. Conserv. Biol. 38, e14168. https://doi.org/https://doi.org/10.1111/cobi.14168

Sanz, N., Franch, N., Araguas, R.-M., Viñas, J., Vidal, O., 2023. Environmental DNA Assay for the Detection of the American Bullfrog (Lithobates catesbeianus) in the Early Stages of the Invasion in the Ebre Delta. Animals. https://doi.org/10.3390/ani13040683

Sarrocco S., Maio G., Celauro D., Tancioni L., 2012. Carta della Biodiversità ittica delle acque correnti del Lazio. Edizioni ARP, Roma, 194 pp [in Italian].

Scalici, M., Pitzalis, M., Gibertini, G., 2009. Crayfish distribution updating in central Italy. Knowl. Manag. Aquat. Ecosyst.

Scalisi, M., Monaco, A., Capizzi, D., Sinibaldi, I., Sarrocco, S., Pizzol I., 2011. Rete Ecologica Regionale (REcoRd Lazio): approccio metodologico e primi risultati. In: Bruschi M., Scalisi M., (a cura di), 2011. Verso un Piano per il Sistema delle aree protette del Lazio. Contributi ARP 2007-2010, Edizioni ARP, Roma; pp. 320.

Schmidt, B.C., Spear, S.F., Tomi, A., Bodinof Jachowski, C.M., 2021. Evaluating the efficacy of environmental DNA (eDNA) to detect an endangered freshwater mussel Lasmigona decorata (Bivalvia:Unionidae). Freshw. Sci. 40, 354–367. https://doi.org/10.1086/714411

Schmidt, K.J., Soluk, D.A., Maestas, S.E.M., Britten, H.B., 2021. Persistence and accumulation of environmental DNA from an endangered dragonfly. Sci. Rep. 11, 18987. https://doi.org/10.1038/s41598-021-98099-1

Sepulveda, A.J., Hutchins, P.R., Forstchen, M., Mckeefry, M.N., Swigris, A.M., 2020. The Elephant in the Lab (and Field): Contamination in Aquatic Environmental DNA Studies . Front. Ecol. Evol. .

Seymour, M., Durance, I., Cosby, B.J., Ransom-Jones, E., Deiner, K., Ormerod, S.J., Colbourne, J.K., Wilgar, G., Carvalho, G.R., de Bruyn, M., Edwards, F., Emmett, B.A., Bik, H.M., Creer, S., 2018. Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Commun. Biol. 1, 4. https://doi.org/10.1038/s42003-017-0005-3

Sigsgaard, E.E., Carl, H., Møller, P.R., Thomsen, P.F., 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biol. Conserv. 183, 46–52. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.023

Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: what's what and the way forward. Trends Ecol. Evol. 28, 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Smart, A.S., Weeks, A.R., van Rooyen, A.R., Moore, A., McCarthy, M.A., Tingley, R., 2016. Assessing the cost-efficiency of environmental DNA sampling. Methods Ecol. Evol. 7, 1291–1298. https://doi.org/https://doi.org/10.1111/2041-210X.12598

Solano, E., Hardersen, S., Audisio, P., Amorosi, V., Senczuk, G., Antonini, G., 2018. Asymmetric hybridization in Cordulegaster (Odonata: Cordulegastridae): Secondary postglacial contact and the possible role of mechanical constraints. Ecol. Evol. 8, 9657–9671. https://doi.org/10.1002/ece3.4368

Spear, D., Foxcroft, L.C., Bezuidenhout, H., McGeoch, M.A., 2013. Human population density explains alien species richness in protected areas. Biol. Conserv. 159, 137–147. https://doi.org/10.1016/j.biocon.2012.11.022

Stevenson, L.A., Alford, R.A., Bell, S.C., Roznik, E.A., Berger, L., Pike, D.A., 2013. Variation in Thermal Performance of a Widespread Pathogen, the Amphibian Chytrid Fungus Batrachochytrium dendrobatidis. PLoS One 8, e73830.

Stoch F., Genovesi P., (ed.), 2016. Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. ISPRA, Serie Manuali e linee guida, 141/2016.

Sunardi, Asaeda, T., Manatunge, J., 2005. Foraging of a Small Planktivore (Pseudorasbora parva: Cyprinidae) and its Behavioral Flexibility in an Artificial Stream. Hydrobiologia 549, 155–166. https://doi.org/10.1007/s10750-005-5442-1

Taberlet, P., Bonin, A., Zinger, L., Coissac, E., 2018. Environmental DNA: For biodiversity research and monitoring. Oxford University Press.

Tancioni, L., Martinoli, M., Olivo, P., Rakaj, A., De Lutiis, F., Martini, A., Boglione, C., 2019. Brook chub, Squalius lucumonis (Pisces, Cyprinidae) conservation aquaculture: First attempt at artificial reproduction and larval rearing. Aquaculture 499, 178–184. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.09.034

Tancioni, L., Russo, T., Cataudella, S., Milana, V., Hett, A.K., Corsi, E., Rossi, A.R., 2013. Testing Species Delimitations in Four Italian Sympatric Leuciscine Fishes in the Tiber River: A Combined Morphological and Molecular Approach. PLoS One 8, 1–10. https://doi.org/10.1371/journal.pone.0060392

Tessa, G., Angelini, C., Bielby, J., Bovero, S., Giacoma, C., Sotgiu, G., Garner, T.W.J., 2013. The pandemic pathogen of amphibians, Batrachochytrium dendrobatidis (Phylum Chytridiomycota), in Italy. Ital. J. Zool. 80, 1–11. https://doi.org/10.1080/11250003.2012.753473

Thalinger, B., Deiner, K., Harper, L.R., Rees, H.C., Blackman, R.C., Sint, D., Traugott, M., Goldberg, C.S., Bruce, K., 2021. A validation scale to determine the readiness of environmental DNA assays for

routine species monitoring. Environ. DNA 3, 823–836. https://doi.org/10.1002/edn3.189

Thomas, A.C., Tank, S., Nguyen, P.L., Ponce, J., Sinnesael, M., Goldberg, C.S., 2020. A system for rapid eDNA detection of aquatic invasive species. Environ. DNA 2, 261–270. https://doi.org/https://doi.org/10.1002/edn3.25

Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M., Willerslev, E., 2012a. Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. PLoS One 7, e41732.

Thomsen, P.F., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012b. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573. https://doi.org/https://doi.org/10.1111/j.1365-294X.2011.05418.x

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.-M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish Procambarus clarkii in freshwater ponds. J. Appl. Ecol. 51, 871–879. https://doi.org/https://doi.org/10.1111/1365-2664.12262

Troth, C. R., Burian, A., Mauvisseau, Q., Bulling, M., Nightingale, J., Mauvisseau, C., & Sweet, M. J., 2020. Development and application of eDNA-based tools for the conservation of white-clawed crayfish. Sci. Total Environ., 748, 141394. https://doi.org/10.1016/j.scitotenv.2020.141394

Troth, C. R., Sweet, M. J., Nightingale, J., & Burian, A. 2021. Seasonality, DNA degradation and spatial heterogeneity as drivers of eDNA detection dynamics. Sci. Total Environ., 768, 144466. https://doi.org/10.1016/j.scito tenv.2020.144466

Tsuji, S., Iguchi, Y., Shibata, N., Teramura, I., Kitagawa, T., Yamanaka, H., 2018. Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese medaka species. Sci. Rep. 8, 9138. https://doi.org/10.1038/s41598-018-27434-w

Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., Yamanaka, H., Minamoto, T., Kondoh, M., 2018. Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. Metabarcoding and Metagenomics 2. https://doi.org/10.3897/mbmg.2.23297

Vignoli, L., Bologna, M.A., Manzini, S., Rugiero, L., Luiselli, L., 2015. Attributes of basking sites of the European pond turtle (Emys orbicularis) in central Italy. Amphibia-Reptilia 36, 125–131. https://doi.org/https://doi.org/10.1163/15685381-00002988

Weldon, C., Crottini, A., Bollen, A., Rabemananjara, F.C.E., Copsey, J., Garcia, G., Andreone, F., 2013. Pre-emptive National Monitoring Plan for Detecting the Amphibian Chytrid Fungus in Madagascar. Ecohealth 10, 234–240. https://doi.org/10.1007/s10393-013-0869-8

Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., Schwartz, M.K., 2015. The Dual Challenges of Generality and Specificity When Developing Environmental DNA Markers for Species and Subspecies of Oncorhynchus. PLoS One 10, e0142008.

Wilson, N.J., Williams, C.R., 2014. A critical review of freshwater crayfish as amphibian predators: Capable consumers of toxic prey? Toxicon 82, 9–17. https://doi.org/https://doi.org/10.1016/j.toxicon.2014.02.005

Wozney, K.M., Wilson, C.C., 2017. Quantitative PCR multiplexes for simultaneous multispecies detection of Asian carp eDNA. J. Great Lakes Res. 43, 771–776. https://doi.org/https://doi.org/10.1016/j.jglr.2017.05.001

Xia, Z., Zhan, A., Johansson, M.L., DeRoy, E., Haffner, G.D., MacIsaac, H.J., 2021. Screening marker sensitivity: Optimizing eDNA-based rare species detection. Divers. Distrib. 27, 1981–1988. https://doi.org/https://doi.org/10.1111/ddi.13262

Yang, J., Matsushita, S., Xia, F., Yoshizawa, S., & Iwasaki, W., 2024). Rapid, easy, sensitive, low-cost and on-site detection of environmental DNA and RNA using CRISPR-Cas13. Methods in Ecology and

Evolution.15(8): 1275-1462

Chapter 3 – Conservation planning and reporting implications of qPCR eDNA multi-specific assessments.

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Abstract

Freshwater ecosystems are facing multiple anthropogenic threats, among which invasive alien species (IAS) are among the most impacting. European legislation supports the conservation of endangered freshwater species through the Habitats Directive (HD, 92/43/EEC) and the Water Framework Directive (WFD, 2000/60/EC), and the and the eradication of IAS through the Regulation 1143/2014. Quantitative PCR environmental DNA (qPCR eDNA) offers a new cost-effective and sensitive monitoring tool that could contribute to periodic reporting obligations and to spatial environmental planning. We analysed the results from qPCR eDNA simultaneous survey of 17 endangered species and IAS of EU concern occurring in central-Italian freshwaters to update the occurrence data and identify priority areas of conservation concern pursuant European regulations. Specifically, we developed indexes and prioritization scores for the 53 sampling sites according to the occurrence and prevalence of native species and IAS. As to species listed in Appendix II of HD occurring in running water sites, we interpolated the species presences 10 km-upstream the river network and identified gaps in the Natura 2000 Network, consisting in 808 km of unprotected river stretches. To prioritize areas of conservation concern in accomplishment of regulation obligations, for each site we computed a *Prioritizing Protection Score* (*PPS*), a *Prioritizing Eradication Score* (PES), and a *Freshwater Conservation Score (*FCS*)*. Results from qPCR eDNA survey revealed new occurrences of species listed in Annexes II, IV and V, and IAS, highlighting 53 new grid cells that will contribute to the next reporting for both HD and IAS Regulation. PPS, PES and FCS identified 39 priority sites claiming for Natura 2000 designation or extension, 22 sites for IAS eradication and management, and 33 sites in need of freshwater protection. This innovative approach enhances monitoring efficiency, fills data gaps, and can strengthen freshwater conservation and restoration across Europe.

1. Introduction

Freshwater ecosystems are among the most threatened at global scale, being impacted by multiple anthropogenic stressors (Sanders et al., 2024). Habitat loss, artificial barriers, water drainage, overexploitation and pollution have caused a severe decline of many freshwater species (Costa et al., 2021; Jaureguiberry et al., 2024). To halt the loss of biodiversity the international Convention on Biological Diversity (CBD), entered into force on 29 December 1993, committed all signatory countries to develop national strategies, plans or programs for the conservation and sustainable use of biological diversity. Art. 7 of the Convention (Identification and Monitoring) mandate each contracting party to a) identify components of biological diversity important for its conservation and sustainable use; b) monitor he components of biological diversity, paying particular attention to those requiring urgent conservation measures and those which offer the greatest potential for sustainable use; c) identify processes and categories of activities which have or are likely to have significant adverse impacts on the conservation and sustainable use of biological diversity, and monitor their effects.

To implement the CBD, the European Union adopted the Directive 92/43/EEC, also known as Habitats Directive (Council Directive 92/43/EEC). The Directive requires all Member States to be committed to preserve natural habitats and wild fauna and flora in a favorable conservation status through the implementation of conservation actions or restoration activities. The Habitats Directive is particularly addressed to the protection of a panel of habitats and wildlife species that are endangered, rare or endemic, listed in Annexes I (habitats), II, IV, and V (animal and plant species), and known as habitat and species of Community Interest (EU, 1992). Up to 91 freshwater species are recognized as species of Community interest and listed in the annexes of Habitats Directive. Moreover, for species listed in Annex II and habitats in Annex I, the Directive imposes the establishment of a network of protected areas (SAC – Special Areas of Conservation), known as the Natura 2000 Network (N2KN hereafter). The N2KN has been also recognized to provide a significant contribution towards achieving the EU Biodiversity Strategy to 2030 (https://environment.ec.europa.eu/strategy/biodiversity-strategy-2030_en), aimed to put Europe's biodiversity on the path to address the global biodiversity crisis and the successful adoption of an ambitious global biodiversity framework under the Kunming-Montreal Global Biodiversity Framework adopted during the 15 COP of the CBD (https://www.cbd.int/gbf). As part of this plan, the European Commission also adopted a Nature Restoration Law which includes an overarching restoration objective for the long-term recovery of nature in the EU's land and sea areas, with binding restoration targets for specific habitats and species. A total of 27,000 SACs are currently included in this network, covering 18.6% of EU's land area and 9% of marine territory (EEA, 2022). To monitor conservation effectiveness, each Member State should report on the status of EU protected species and habitats every six years. Thus, a surveillance monitoring of species of Community Importance is fundamental to assess population trends and effectiveness of conservation measures even beyond the N2KN (Ellwanger et al., 2018; Zettler and Jueg, 2007).

Also, recognizing that protected areas are among the most powerful tools for effective conservation of land, nature, and native wildlife (Rodrigues and Cazalis, 2020), the European Union declared the willingness to expand protected areas coverage up to the 30% of the European territory until 2030, a target in line with the Kunming-Montreal Global Biodiversity Framework. To this purpose, new protected areas can be indicated voluntarily by EU Member States, and several methods and criteria have been proposed to highlight potential areas that can satisfy suitable requirements for land protection (Cazzolla Gatti et al., 2024, 2023; Ricci et al., 2024; Spiliopoulou et al., 2023; Staccione et al., 2023; Tóthová and Heglasová, 2022).

Furthermore, the conservation of freshwater species that are endangered and listed in Habitats Directive is often dependent on the conservation of aquatic ecosystems and preservation of water quality. In this context, the European Water Framework Directive 2000/60/EC (WFD hereafter) represents a fundamental contribution to the conservation of freshwater habitats and species (Weigelhofer et al., 2020). In fact, the WFD aims to reach a sustainable use of water, reducing pollution, and enhancing the protection of aquatic environments. According to the WFD, all aims to achieve a good ecological status for all rivers, lakes and

transitional and coastal waters in the EU. River status is assessed with several parameters, most of them relying on biological surveys to understand the conditions of the communities of aquatic flora, benthic invertebrates, and fish (Hering et al., 2018). These freshwater components contribute to different parameters that should be measured by all member states. To this purpose, macrobenthic communities are often assessed through multimetric indexes (e.g., Buffagni and Erba, 2007; Buffagni et al., 2008), whereas fish communities are evaluated through the composition and structure of native species and IAS populations (e.g., Macchio et al., 2017). All these data are transferred to develop river basin management plans (Birk et al., 2012), where Habitats and species protection areas must be designed for the conservation of habitats and species directly depending on water (Art. 4, Art. 6, and Annex VII), also in synergy with other Directives, such as the Habitats Directive (Annex IV). Thus, purposes and goals of Habitats Directive can be integrated with the ones of the WFD to protect inland surface waters (Bolpagni et al., 2017).

Conservation measures under Habitats Directive and WFD are derived from specific threats acting on endangered habitats and species. Among the most impacting threats to biodiversity are the invasive alien species (IAS) (Dextrase and Mandrak, 2006; Francis, 2012). Specifically, IAS are considered among the most driving force of biodiversity loss worldwide (Dueñas et al., 2018, 2021; IPBES, 2023; Maxwell et al., 2016Roberts et al., 2013), especially in freshwater ecosystems (Meira et al., 2019; Simberloff et al., 2013). Biological invasions change ecosystem composition and structure, impoverishing local native communities, and this is reflected in a decrease of ecosystem functions and their resilience or resistance (IPBES, 2023). Currently, IAS invasions have been estimated to amount to a global cost of $ 423 billion per year (IPBES, 2023). Best practices in solving this threat to native biodiversity include a coordinated system of early warning of new colonization and a promptly activation of IAS eradication or control (Sepulveda et al., 2023). Compared to other continents, Europe has been invaded by the highest number of freshwater alien species (i.e. fish, crustaceans, and molluscs: IPBES, 2023). The increasing trend of invasive freshwater species recorded in Italian rivers and lakes makes this country one of the most impacted by IAS in Europe (Gherardi et al., 2008; Magliozzi et al., 2020), and in some river basins the number of species introduced is now equal to or even higher than the number of native species (Lorenzoni et al., 2019; Nocita et al., 2017).

To prevent new introductions and manage the spreading of IAS, the European Union adopted the Regulation 1143/2014 (Genovesi et al., 2015). A key role in this Regulation is the setting of a surveillance system (Pagad et al., 2015), and member states should establish a monitoring framework for the early detection of 88 IAS of Union concern (amended version 2022/1203) in their territory. Every six years, member states should update the distribution of IAS in their territory, as well as the efficiency of rapid eradications (art. 24 of regulation 1143/2014). At the same time, management measures should be applied on those IAS that are widely spread. To this purpose, a spatial prioritization to eradicate IAS where they have recently appeared, and to mitigate their impact in areas heavily colonized is highly recommended (EC, 2014). However, monitoring several species of different taxa is time-consuming and requires many experts and high costs, often hampering monitoring programs.

In this complex context of European monitoring obligations, environmental DNA (eDNA) provides a new tool for a cost-effective simultaneous monitoring of several species belonging to different taxa (Taberlet et al., 2018). This technique has also revealed accurate in detecting rare and elusive species that are difficult to detect through traditional field surveys, and particularly successful in monitoring freshwater communities (Huang et al., 2022). Accordingly, eDNA could represent an effective complementary tool to fulfill the monitoring obligations under the Habitats Directive, for the early detection of IAS of Union concern, as to the 1143/2014 EU Regulation, and to accomplish biological assessments about water quality under the Water Framework Directive (Giovacchini et al., submittes; Leese et al., 2018; Pawlowski et al., 2021; Thomsen et al. 2012).

Multiple species detection through eDNA usually follows a metabarcoding approach, implying generic genetic sequences are processed contemporary to identify a biotic community at a broad taxonomic scale (Taberlet

et al., 2018). Although in the last decade the eDNA metabarcoding approach boosted to a finer taxonomic level up to reach species detection, the single species approach based on quantitative PCR (qPCR) still represents a more sensitive alternative (Harper et al., 2018), especially for the detection of rare endangered species and invasive species at the initial stage of their colonization (Hernandez et al., 2020; Xia et al., 2021). A qPCR single species approach is currently expanding and specific protocols have been developed to quickly inform the presence of invasive species (Thomas et al., 2020). However, the qPCR eDNA approach is rarely used to simultaneously detect different species (King et al., 2022; Minegishi et al., 2023; Tsuji et al., 2018; Wozney and Wilson, 2017), and even more rarely applied to wide taxonomic inventories (Leblanc et al., 2020; Seymour et al., 2018; Thomsen et al., 2012).

In a recent study we designed a species-specific eDNA protocol to the simultaneous detection of 22 species of conservation interest occurring in freshwater bodies of Central Italy (Giovacchini et al., submitted). Taxonomic sampling spanned from Fungi and Mollusca, to Arthropoda and Chordata, and included 14 native endangered species listed in annexes II, IV and V of the Habitats Directive, six IAS of Union concern according to the EU Regulation 1143/2014, one transfaunate fish, and the pathogen fungus *Batrachochytrium dendrobatidis*. Out of the 22 target species, eight species existing eDNA essays were successfully used, whereas nine species-specific eDNA primers and probes were specifically developed and successfully validated within the project (Giovacchini et al., submitted). The protocol was tested at 53 sites on lentic and lotic freshwater bodies located both within and outside protected areas. These latter included Natura 2000 sites and a regional ecological network (Scalisi et al., 2011).

Here we present a follow up of the study aimed at exploring the conservation and management implications of new occurrence data obtained from the eDNA simultaneous detection of both endangered and alien taxa (Tab. 1). We specifically aimed at i) develop an index of conservation value for the sampled sites based on eDNA occurrence and co-occurrences of native endangered and alien species; ii) identify priority areas of conservation concern pursuant to the three European regulations (HD, IAS Regulation, and WFD) through a scoring system, iii) contribute to the next reporting cycle of the Habitats Directive by updating the distribution of species listed in Annexes II, IV and V; iv) early warning of new IAS of Union Concern and/or update their distribution in accordance with the Regulation 1143/2014; v) integrate eDNA to routine monitoring under the Water Framework Directive.

2. Methods

Species selection

The target species for which we obtained accurate records from the previous study (Giovacchini et al., submitted) belong to six taxonomic groups, i.e. Fungi, Mollusca, Crustacea, Osteichthyes, Amphibia, and Mammalia. Ten species are listed in Annexes II, IV, and V of the Habitats Directive (HD) 92/43/EEC (Tab. 1), five species are IAS of Union Concern (i.e., EU Regulation 1143/2014) and represent a direct threat to the first ones. Target species also included *Padogobius bonelli,* a fish translocated from North Italy, and the pathogen Chytridiomycete *Batrachochytrium dendrobatidis,* threatening native amphibian worldwide (Beranek et al., 2021) and listed in the IUCN 100 worst IAS impacting biodiversity at global scale (Lowe et al., 2000). Six of these species are also benthic invertebrates or fish species sensitive to water quality and included in Water Framework Directive (2000/60/EC) monitoring guidelines to evaluate the ecological status of freshwater. Two benthic invertebrats species contributing to the STAR_ICMi, the Italian index to evaluate macrobenthic communities under WFD (Buffagni and Erba, 2007; Buffagni et al., 2008). Specifically, target species are part of ASPT (Average Score Per Taxon), that is the most influential subindex of STAR_ICMi, measuring sensitivity and tolerance of river benthos based on the checklist of a wide range of Families belonging to different taxa, from Astacidae (Crustaceans) to Unionidae (Molluscs) (Archi et al. 2014; Armitage et al., 1983). Finally, four fish species are included of the Italian metric elaborated for fish assessments under WFD (i.e., NISECI index).

Two of them are expected among native fish stocks, as well as two are listed as harmful alien fish (Macchio et al., 2017).

Table 1. List of species used in the study and their legislative status. Alien species are in red.

Clade	Species	Synonym	Regulation
Bivalves	*Unio mancus*	*U. elongatulus*	Habitats Directive Annex V Water Framework Directive (benthos)
Gastropods	*Vertigo spp.*	*V. angustior* *V. moulinsiana*	Habitats Directive Annex II
Crustaceans	*Austropotamobius italicus*	*A. pallipes*	Habitats Directive Annex II, IV Water Framework Directive (benthos)
Osteichthyes	*Alosa fallax*	-	Habitats Directive Annex II, IV Water Framework Directive (fish)
Osteichthyes	*Squalius lucumonis*	-	Habitats Directive Annex II Water Framework Directive (fish)
Amphibians	*Bombina pachypus*	-	Habitats Directive Annex II, IV
Amphibians	*Triturus carnifex*	-	Habitats Directive Annex II, IV
Amphibians	*Lissotriton italicus*	-	Habitats Directive Annex IV
Mammals	*Mustela putorius*	-	Habitats Directive Annex V
Mammals	*Lutra lutra*	-	Habitats Directive Annex II, IV
Bivalves	*Dreissena polymorpha*	-	IUCN 100 of the World's Worst IAS
Crustaceans	*Faxonius limosus*	*Orconectes limosus*	IAS of Union concern
Crustaceans	*Procambarus clarkii*	-	IAS of Union concern
Osteichthyes	*Pseudorasbora parva*	-	IAS of Union concern Water Framework Directive (fish)
Osteichthyes	*Padogobius bonelli*	*P. martensii*	introduced outside its native range Water Framework Directive (fish)
Amphibians	*Lithobates catesbeianus*	-	IAS of Union concern IUCN 100 of the World's Worst IAS
Fungi	*Batrachochytrium dendrobatidis*	-	IUCN 100 of the World's Worst IAS

Study area

The study area consists of 53 sampling sites located in various lentic and lotic water bodies of the Latium region in Central Italy, located along the Tyrrhenian side. The region is characterized by highly heterogeneous landscapes, from hills to volcanic plateaus, from inland mountains to piedmont areas, lowlands and seacoast (Fig. 1). The region is intersected by the Tiber, the main river basin of Central Italy (405 km of length). The remaining river catchments are smaller and mainly located nearby the coast. Natural lakes are set in the northern part of the region and along the southern coasts, whereas two artificial reservoirs are found in the inland mountain ranges (Fig. 1). This high heterogeneity of landscapes hosts a high diversity of animal and plant communities (Balletto et al., 2010). The region represents also a biogeographic parapatric contact zone between species endemic to south-central Italy and more widely distributed vicariant species (Iannella et al., 2017). Species endemic to the Apennines include the Italian crayfish *Austropotamobius italicus,* the Apennine yellow-bellied toad *Bombina variegata pachypus*, and the Tuscany stream chub *Squalius lucumonis* (Brusconi et al., 2008; Canestrelli et al., 2013; Tancioni et al., 2013). At the center of the study area is located the large metropolitan city of Rome, hosting over 4 million of inhabitants, that contributed to the spread several IAS in the course of time (Li et al., 2024; Monaco, 2014; Spear et al., 2013).

Survey design

Environmental DNA was sampled at 53 sampling sites included in the routine monitoring network of the Water Framework Directive. Sampling sites cover a wide range of freshwater habitats, including rivers, creeks, channels, lakes, reservoirs, and ponds (Giovacchini et al., submitted). They are located both inside (n=23) and

outside (n=30) the Natura 2000 Network of the region. Those located outside fell within a regional ecological network (REN, n = 23), established to guarantee wildlife movements across the regional territory and the Nature 2000 sites (Scalisi et al., 2011) (Fig. 1).

Each site was sampled twice, in 2021 and 2022. Environmental samples consisted of 3 liters of water filtered through 8 µm MCE prefilters and 0.22 µm MCE filters (Merck-Millipore, Burlington, USA) using an electric vacuum pump (see Giovacchini et al, submitted for further details).

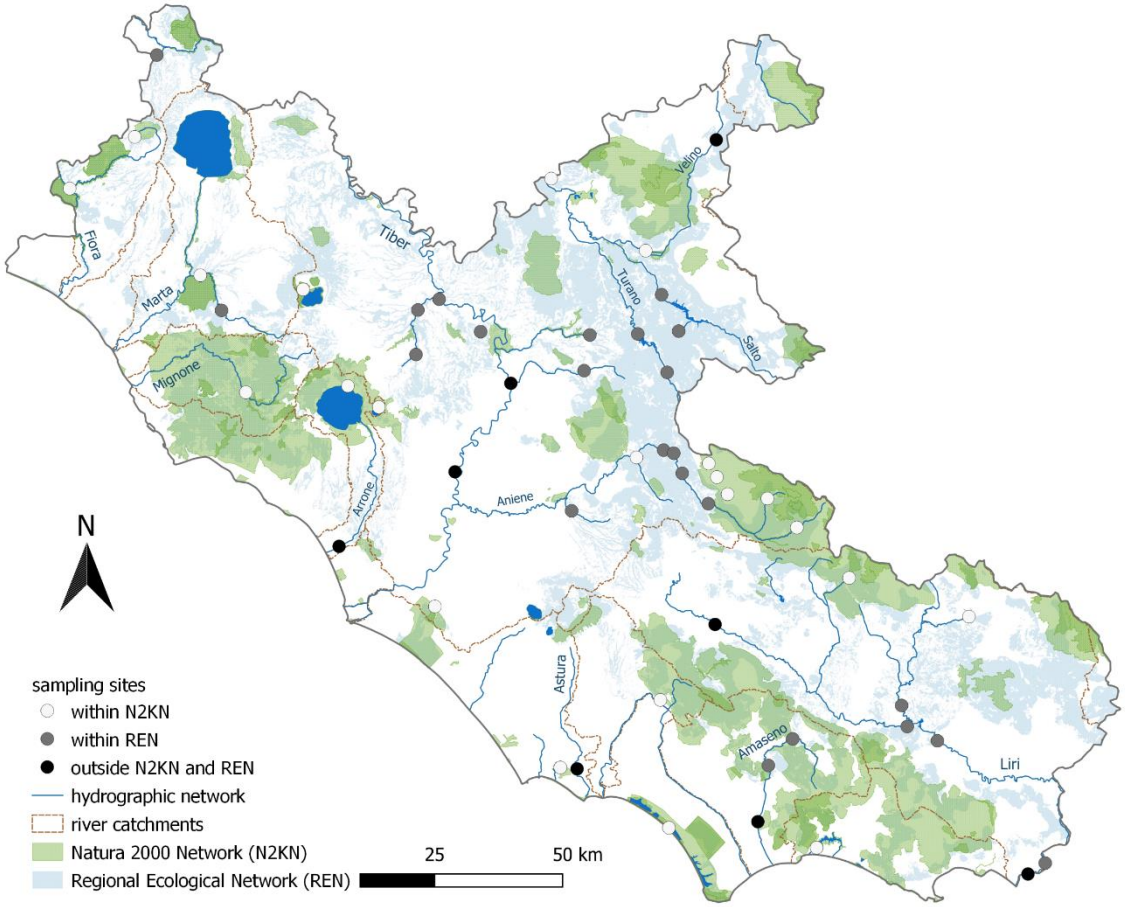


Figure 1. The study area (Latium region) showing sampling sites, river catchments, Natura 2000 sites and the Regional Ecological Network (REN).

Laboratory analyses

Laboratory procedures are reported in detail in Giovacchini et al. (submitted) and briefly summarized here. DNA was extracted from filters using the DNeasy PowerSoil Pro kit (Qiagen, Venlo, NL), diluted in 75 µL bidistilled Miili-Q sterile water and preserved at -21 °C. A subset of species-specific assays was selected based on the native or alien species potentially occurring at the site, ranging from 5 to 12 (Giovacchini et al., submitted). Generally, IAS were tested at a lower number of sites, thus sample size of IAS vs native species was unbalanced (see below how unbalance was accounted for in the computation of sites indexes). Assays of target species (Amberg et al., 2019; Antognazza et al., 2019; Canessa et al., 2017; Giovacchini et al. submitted; Manfrin et al., 2022a, 2022b; Mauvisseau et al., 2018; Park et al., 2011; Sanz et al., 2023) were provided by Eurofins Genomics srl (Hamburg, DE) and tested using real-time qPCR with TaqMan technology (StepOnePlus, Applied Biosystem, Waltham, USA). Reaction mix was composed by 2.35 µl of Milli-Q water, 5 µL of buffer (Eurofins Genomics srl, Hamburg, DE), 0.25 µL of each primer (10 µM), 0.15 µL of dual-labelled Taqman probe

(10 2 µM; Eurofins Genomics srl, Hamburg, DE), and 2 µL of extracted DNA. Samples were run in two dilutions (1:1, and 1:10) in three technical replicates, together with a PCR positive control with genomic DNA of target species, a PCR negative technical control to verify the absence of contamination using DNA of untargeted species, and an environmental positive and negative control (water samples collected in sites of ascertained presence and absence of target species) (Karlsson et al., 2022; Klymus et al., 2020; Thalinger et al., 2021). To verify the correct extraction of eDNA, samples were also tested with a MiFish set of universal 12S rRNA oligonucleotides for fish characterization using DNA metabarcoding (Miya et al., 2015). To avoid contaminations, we carried out pre and post amplification phases in separate rooms on a laminar flow cabinet. We adopted the following thermal profile: initial denaturation at 95 °C for 10 s, 40 cycles with a denaturation step at 95 °C for 15 s, annealing-elongation at 58 °C for 1 min. We considered positive samples when observed at least in two technical replicates, and above the limit of detection, or under it when the number of DNA copies were equal or more than five per reaction (Bruno et al., 2017; Bustin et al., 2009; Ficetola et al., 2015).

Site indexes and scores

For each species, positive locations were overlapped to the known distribution (10x10 km cell grids in ETRS LAEA 52 10 projection) provided by the last IV HD Reporting (Ercole et al., 2021) or by the last 2015-2018 IAS reporting under EU Regulation 1143/2014 (http://www.eea.europe.eu/en/datahub/), or derived from published surveys on IAS not listed in the aforementioned regulation (Canestrelli et al., 2013; Carosi et al., 2021; Grano, 2022; Grano et al., 2020; Sarrocco et al., 2012; Zampiglia et al., 2013).

For each sampled site we calculated the number of native and alien target species detected at each site (species richness), as well as the co-occurrence of IAS and the species they impacted, either through competition, predation, or disease transmission. To account for potential dispersal of IAS we extended the analysis of potential impacts at river catchment scale. As IAS were tested at a lower number of sites, we developed a *Native-Alien Prevalence* (NAP) index computed as the difference between the ratio of detected over tested native species (n. native detected/n. native tested = N_r), and the ratio of detected over tested IAS (n. IAS detected/n. IAS tested = A_r). The NAP index varies from -1 to 1, identifying pristine areas with prevailing native species (NAP = $N_r - A_r > 0$), vulnerable areas where protected species and IAS co-occur at the same rate (NAP = $N_r - A_r = 0$), and areas heavily colonized by IAS (NAP = $N_r - A_r < 0$). The index also accounted for unbalance due to testing only one category of target species (i.e., only protected species, or only IAS).

Occurrences of species listed in Annex II of Habitat Directive were also overlapped to the N2KN. The standard data form of each N2KN site was checked to assess the presence of Annex II species recorded at the site https://natura2000.eea.europa.eu/. However, as for running water sites DNA molecules may float from the upstream sectors of the river catchment (Deiner et al., 2016; Burian et al., 2021; Sepulveda et al., 2020), presence data were extended according to eDNA resilience evidences (Civade et al., 2016; Deiner et al., 2016; Deiner and Altermatt, 2014; Jane et al., 2015; Padgett-Stewart et al., 2016; Pont et al., 2018; Song et al., 2017; Villacorta-Rath et al., 2021; Wilcox et al., 2016). Most studies based on the collection of few liters of water report a resilience between 3 and 20 km (Civade et al., 2016; Deiner and Altermatt, 2014; Villacorta-Rath et al., 2021). Accordingly, occurrences were extended by the averaged value of 10 km upstream the sampling site, including the tributaries (Fig. 2). The resulting potential area of occurrence was overlapped to the N2KN to identify gaps and priority areas for N2KN extension. Three landscape metrics were then measured on the final maps of occurrence of species listed in Annex II of the Habitats Directive, to assess: i) the number of unprotected river stretches of potential occurrence of the species (i.e., patch number); ii) the length of those unprotected stretches over the total length of the river network (i.e., patch density); iii) the average Nearest Neighbor Distance (NND) of the nearest unprotected patch to the area of potential occurrence of the species (Rutledge, 2003). All analyses were performed using QGIS 3.28 (http://qgis.org).

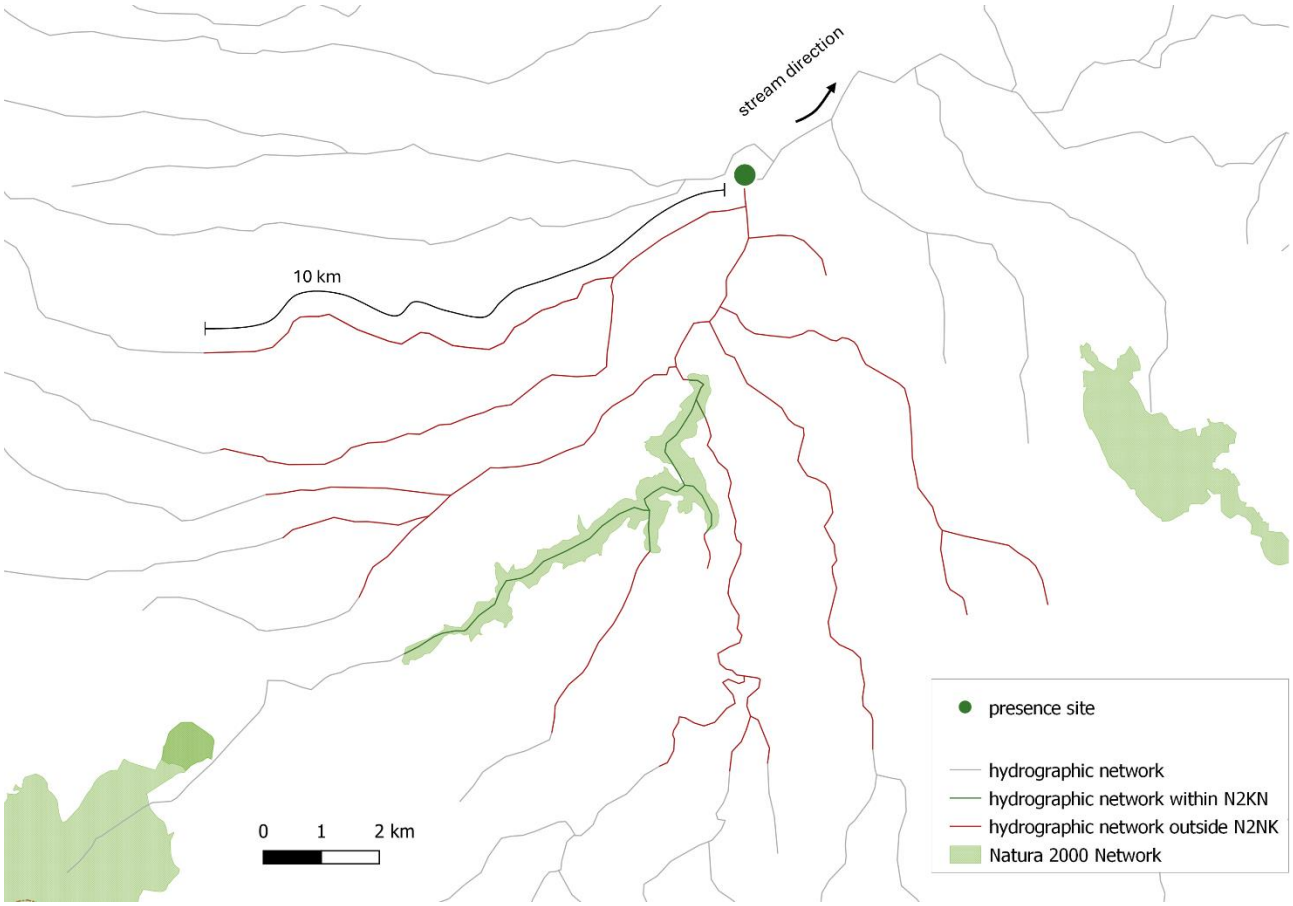


Figure 2. Accounting for eDNA floating from upstream to sampling sites. Starting from a new occurrence site (green circle) of a species listed in Annex II of Habitats Directive, we selected 10 km of the upstream river network, and overlapped the Natura 2000 Network along the resulting stream network.

Prioritizing Protection Score (PPS). To prioritize areas of conservation concern, we assigned a maximum score (score=2) to sampling sites located outside the N2KN where we detected new occurrences of species listed in Annex II, and a medium score (score=1) to sites within the N2KN but not reporting the species in the standard data form, or falling within the regional ecological network. Scores where then weighted for the patch density of unprotected upstream area (see Fig. 2). The final P*rioritizing Protection Score* was summed to index NAP, and then normalised from 0 to 1, followed the formula PPS = NAP + ($N2KN_{out\ (2)}$ + $N2KN_{in\ (1)}$ + $REL_{(1)}$) * patch density.

Prioritizing Eradication Score (PES). To prioritize areas for IAS eradication, sites were given a minimum score (score = 1) if a site with IAS was included in the N2KN, a medium score (score = 2) if IAS co-occurred at the same site with the native species they directly threat (e.g., *B. dendrobatidis* vs *B. pachypus*: Porco et al., 2024), and a final score equal to the number of native species detected in the river catchment directly threatened by IAS detected at the sampling site (e.g., *B. dendrobatidis* vs *T. carnifex* and *L. vulgaris*). All scores were subtracted to the index NAP, converted in absolute values, and finally normalised from 0 to 1, to get a final *Prioritizing Eradication Score*, following the formula PES = | NAP - ($N2KN_{in\ (1)}$ + $co\text{-}oc_{site\ (2)}$ + $co\text{-}oc_{river\ basin\ (x)}$) |.

Freshwater Conservation Score (FSC). A *Freshwater Conservation Score (FSC)* was developed to align to the WFD guidelines we considered only eDNA occurrences of benthic invertebrates and fish species contributing to WFD evaluations of freshwater ecological status. We overlaid the STAR_ICMi index (available for the study area for the years 2021 and 2023, see Fig. 7) and the NISECI index (available for 2018-2020) accessible at https://sira.arpalazio.it/web/guest/cartografia. Following the classification scores of WFD, we scored all rivers not reaching a Good status from 3 (Moderate) to 1 (Bad) separately for both STAR_ICMi (WFD_B) and NISECI evaluations (WFD_F). We assigned an average value of 1.5 to rivers classified as Not Evaluated. For each

sampling site, we computed a *benthic score* by multiplying the WFD_B score by the number of benthic invertebrates detected at site. A Native-Alien Prevalence index was calculated also on fish (NAP_F). We obtained a *fish* score according to the following formula NAP_F x (WFD_F score + 0.1), and we artificially converted all values < 0 of the *fish score* to be the specular values obtained > 0 in order to have a proportionate scale for sampling sites matching WFD_F and NAP_F (Tab. 2). A final index of prioritization for WFD Habitat and species protection areas (*Freshwater Conservation Score*) was obtained by summing the *benthic score* and the *fish score*, and finally normalised from 0 to 1.

Table 2. Scoring procedure for sampling sites in which we detected fish species of monitoring concern under the Water Framework Directive (see the text for details).

NAP_F \ score WFD	3	2	1
1	3,3	2,2	1,1
0,5	1,8	1,2	0,66
0	0,3	0,2	0,1
-0,5	-0,66	-1,2	-1,8
-1	-1,1	-2,2	-3,3

3. Results

New species occurrences

We obtained 101 species occurrences across 45 sites, including 58 new presence sites for 15 over the 17 target species. New presence data for species included in Annex II, IV and V corresponded to 36 new 10x10 km grid cells to be notified in the next V HD reporting cycle (Suppl. Mat. 1-7). We also detected 17 new cells hosting IAS that will contribute to the next 2019-2024 reporting under the EU IAS Regulation (Suppl. Mat. S1-S7). Moreover, we found from 1 to 3 (1.78 ± 0.67 per site) species listed in HD Annex II in 23 sites located outside the Natura 2000 network (Suppl. Mat. S8). Also, four species included in Annex II HD were not reported in the standard forms of nine Natura 2000 sites (Suppl. Mat. S9).

eDNA interpolation for running waters sites

New presence sites of target species listed in Annex II of HD found outside N2KN generated a cumulative upstream river network (defined as Annex II potential areas) of 808 km, most of which fall in areas without any form of protection (86.1%). We found 42 patches of unprotected river stretches dispersed across 23 Annex II potential areas, with an average value of 1.82 ± 1.8 patches per area (Fig. 3). Mean cumulative length of unprotected patches amounted to 30.3 ± 22.4 km (median = 21 km) for each Annex II potential area, with a mean patch density of 0.89 ± 0.2. Single patches had an average value of 16.3 ± 12.6 km (median = 6.1 km). The average NND value calculated on mean values for each Annex II potential area was 1.5 ± 3.5 km (Suppl. mat. S10).

New occurrences of Annex II species detected within Natura 2000 sites but not reported in the standard data forms corresponded to 316 km of river stretches. Almost half of them (54%) fell outside the N2KN. We found 21 unprotected patches over seven Annex II potential areas, with an average value of 3 ± 2.6 patches per area. Unprotected patches had a mean cumulative length of 24.6 ± 30.2 km (median = 12.4 km) for each

Annex II potential area, with a mean patch density of 0.48 ± 0.35. Accounting for patch density, the mean value amounted to 8.2 ± 11.5 km (median = 1.8 km), whereas the average value of NND was 5.2 ± 3.9 km (Suppl. mat. S11).

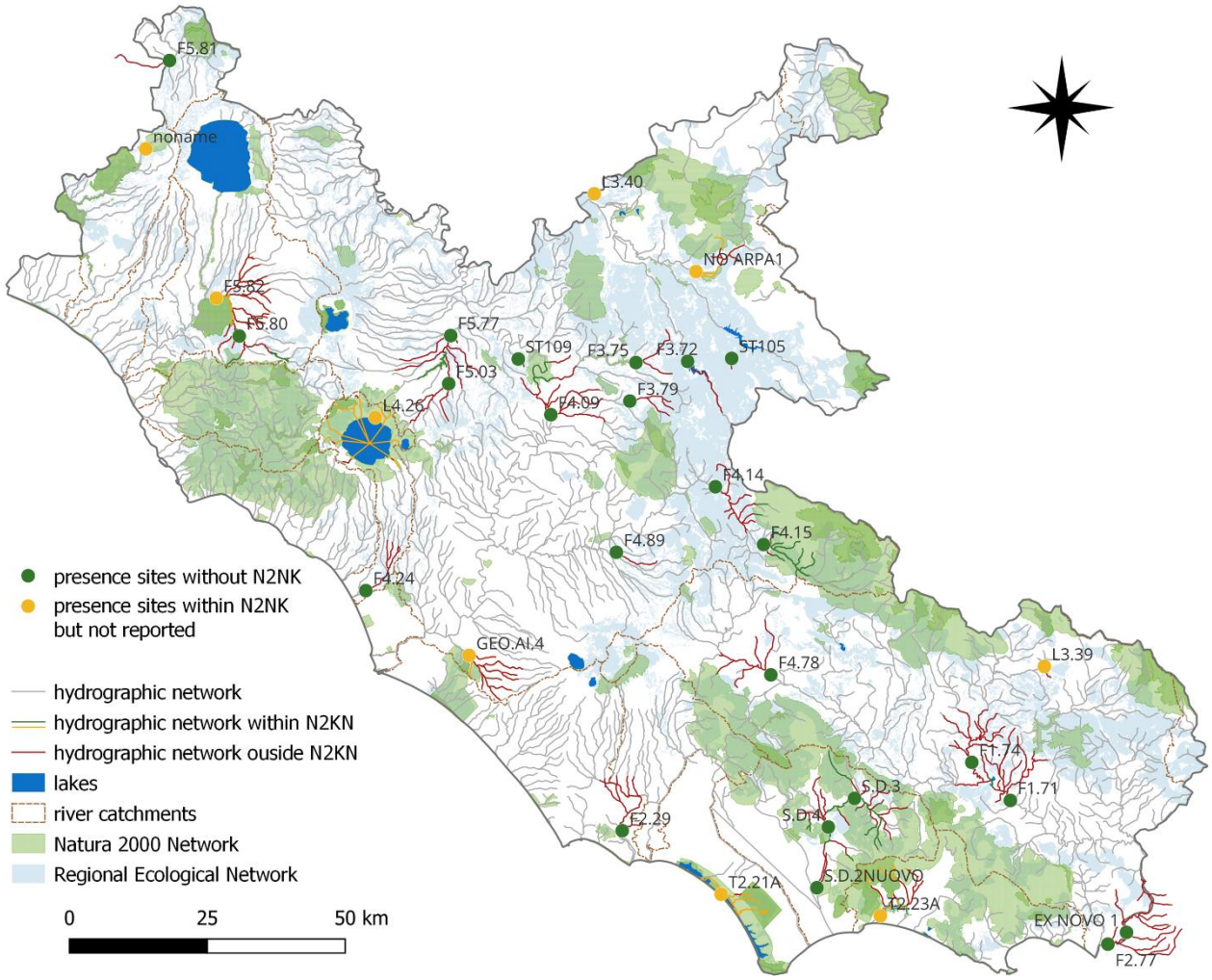


Figure 3. New presence sites of target species listed in Annex II of Habitats Directive for which the designation of a Natura 2000 site is required by law. New presence sites found outside Natura 2000 network (green dots) and new presence sites found within Natura 2000 network but not reported in the standard forms (orange dots).

Species richness and co-occurrence

Only native species were found at 24 sampling sites (45%), ranging from 1 to 4 species per site (Fig. 4). These sites were mainly distributed on mountains and headwaters areas. Conversely, only IAS were found at five sites (9%), along lowlands main rivers and channels and in artificial reservoirs, with a species richness from 1 to 2. Native species and IAS were found together at 16 sites (30%), where occurrences of native species were slightly more frequent than IAS (i.e., 27 vs 22) (Fig. 5).

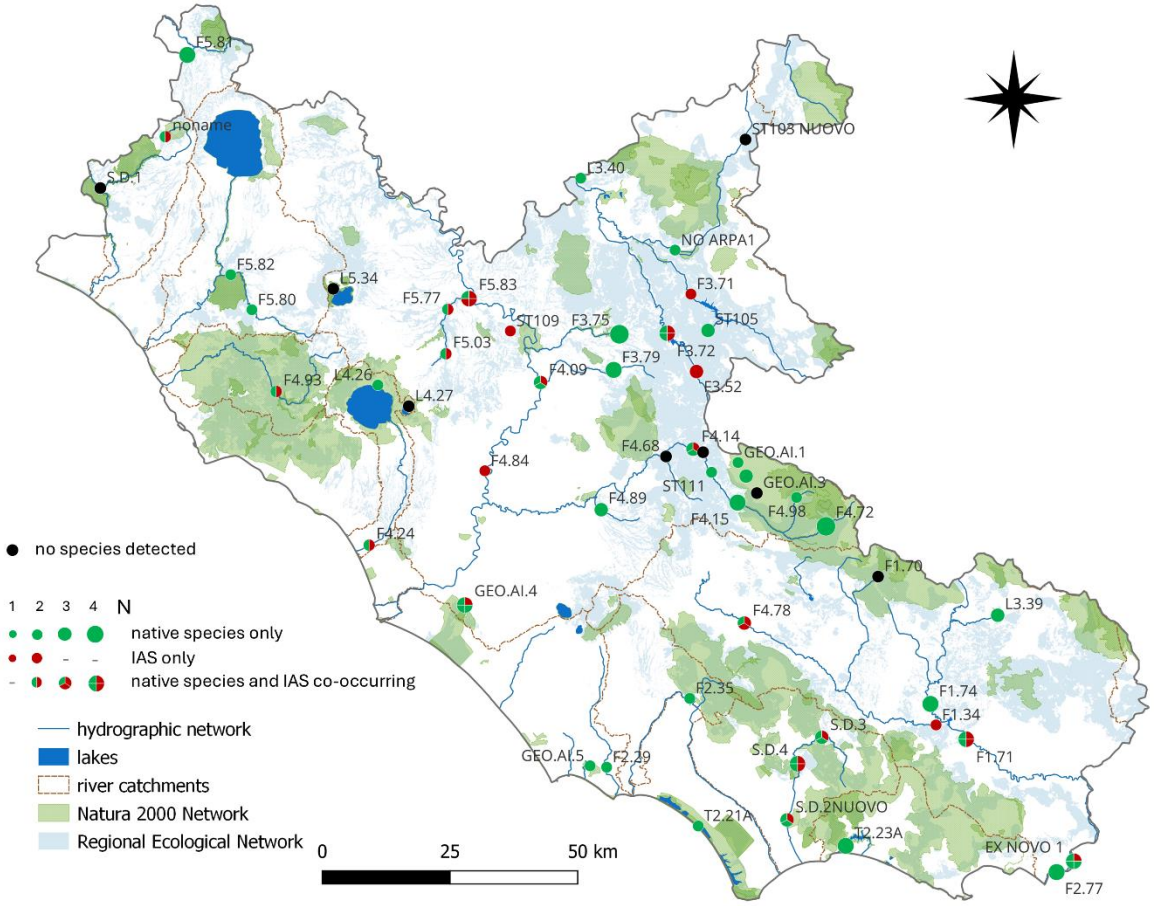


Figure 4. Native protected and alien species richness found within and outside the Natura 2000 network. Symbols are proportionate to the total number of species detected at the site (1 to 4). When protected species and IAS co-occur at the same site, a pie chart shows the proportions of protected species (green sub-sections) and IAS (red sub-sections).

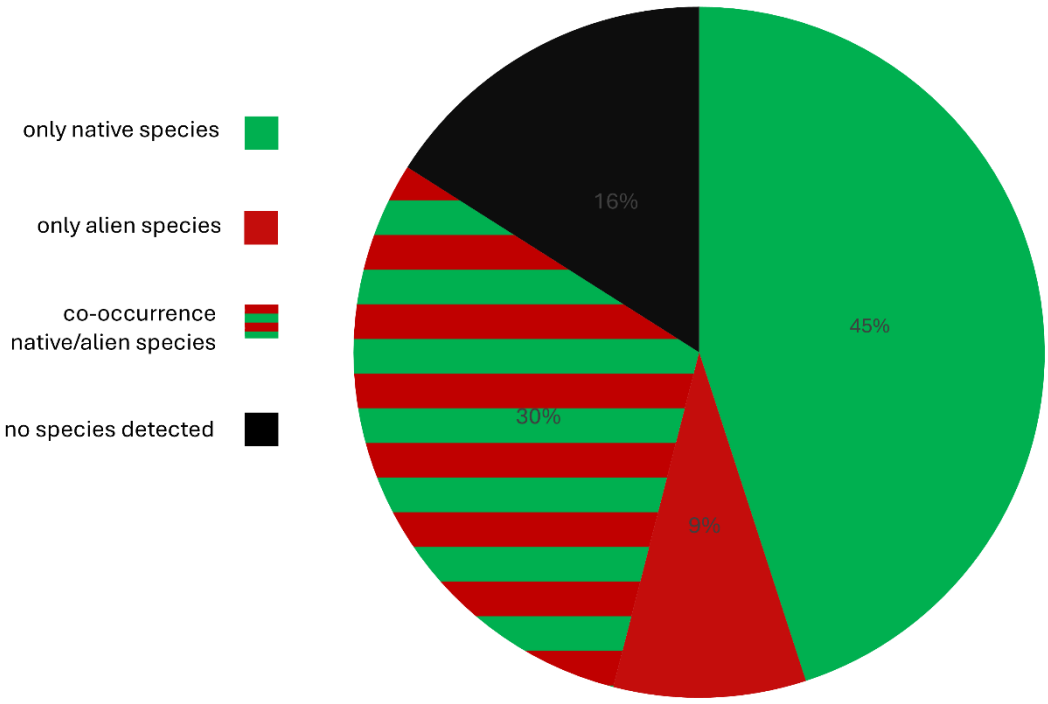


Figure 4. Piechart showing percentages of sampling sites in which native and alien species were detected.

At three sites we found IAS directly threatening native protected species (*B. dendrobatidis* vs *B. pachypus*, *F. limosus* vs *A. italicus*, *P. clarkii* vs *Vertigo* sp.) (codes F4.14, F3.72, and *noname* in Fig. 4). Moving to the river catchment scale six potential IAS-native species interactions were localized in the lowland portion of the Tiber river (*D. polymorpha* vs *U. elongatulus*, *P. clarkii* vs *A. italicus, F. limosus* vs *Vertigo* sp., *L. catesbeianus* vs *B. pachypus, B. dendrobatidis* vs *L. italicus, P. parva* vs *T. carnifex*). Another potential interaction (*B. dendrobatidis* vs *T. carnifex*) was revealed in the Tiber river basin along the main tributary Aniene. A last interaction was assessed in the Garigliano river basin (*P. parva* vs *L. italicus*).

The NAP index ranged from -0.83 to 1 (Fig. 6; Suppl. mat. S12). Specifically, 28 and 12 sites showed a NAP > 0 and NAP < 0 respectively. The remaining 13 sites had a value NAP = 0. In five of these sites protected species and IAS were detected at the same rate, whereas at eight sites no species were detected (Suppl. mat. S13). Pristine areas with NAP > 0 were concentrated in the interior hills and mountains, whereas areas heavily invaded by IAS were mainly located in the lower plains of central and southern Latium. Vulnerable areas with NAP = 0 were mainly located along tributaries and small rivers (Fig. 6).

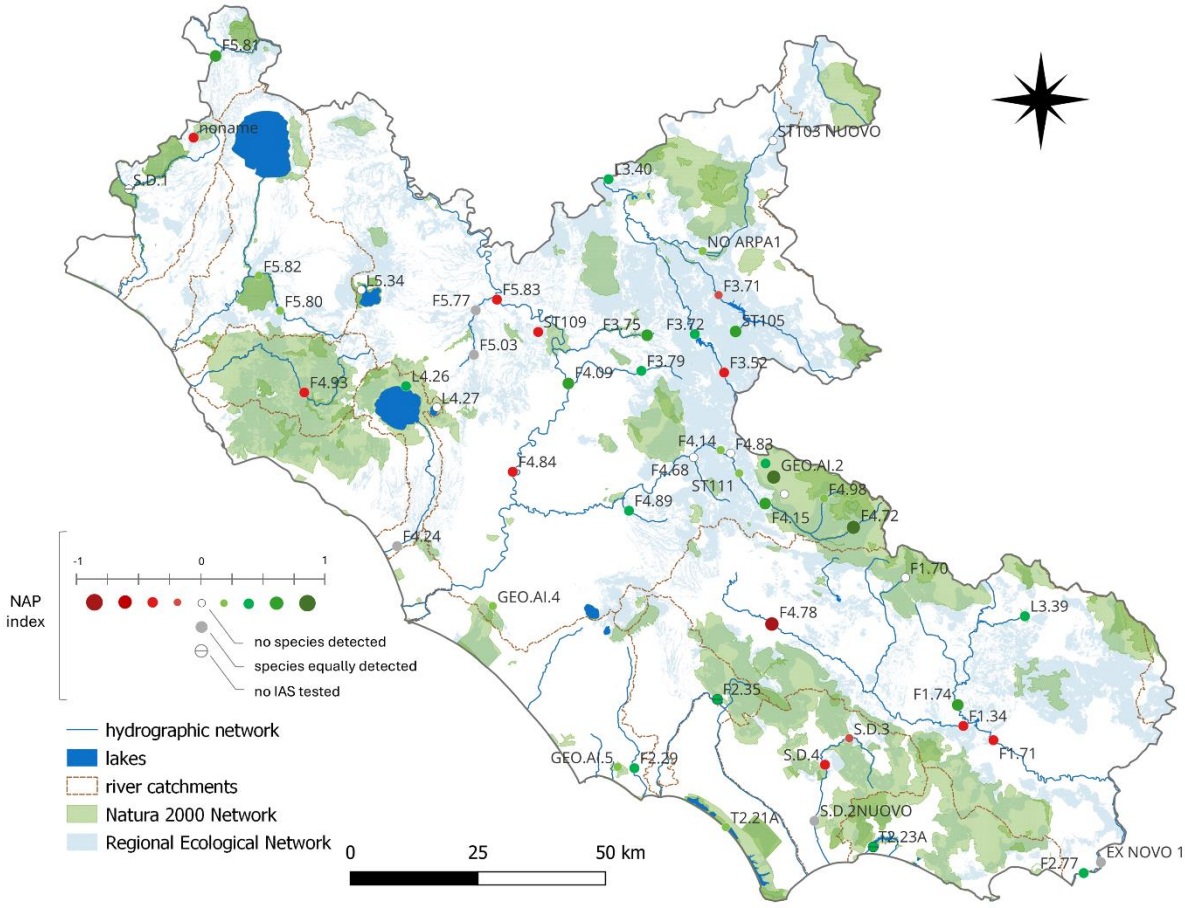


Figure 6. Native-Alien Prevalence values (NAP) ranging from -1 to 1, showing where native protected species (NAP > 0, green dots) and IAS (NAP < 0, red dots) were respectively more detected with eDNA compared to the number of tested species. When NAP = 0, native protected species and IAS ratios are equal due to either undetection (white dots) or equal detection rates (grey dots). Black circles with central dash indicate sites where IAS were not tested.

Two benthic invertebrates included in WFD monitoring occurred at 11 sites (total 14 records) (Fig. 7). Most of sites were located on rivers with a Good status for macrobenthic fauna, one on a river classified as Moderate, and 4 on rivers Not Evaluated. Locations of benthic invertebrates were mostly distributed in inland waters. Fish included in WFD monitoring were detected at 32 sites, for a total 37 occurrences. The NAP_F index for fish ranged from -1 to 1 (Fig. 7; Suppl. mat. S14). Specifically, 14 sites showed a $NAP_F > 0$, mostly located in the hills of the central region and along the southern coastline, and mostly on rivers evaluated as Good and Moderate status from WFD fish assessments (Fig. 7). A $NAP_F < 0$ was detected for five sites, mainly located in the lower plains and near reservoirs, mostly on rivers classified as Moderate or Poor. The remaining 13 sites results gave $NAP_F = 0$, with just one site with no species detected and seven with only IAS or native species tested, located along Moderate and Poor rivers (Suppl. mat. S14).

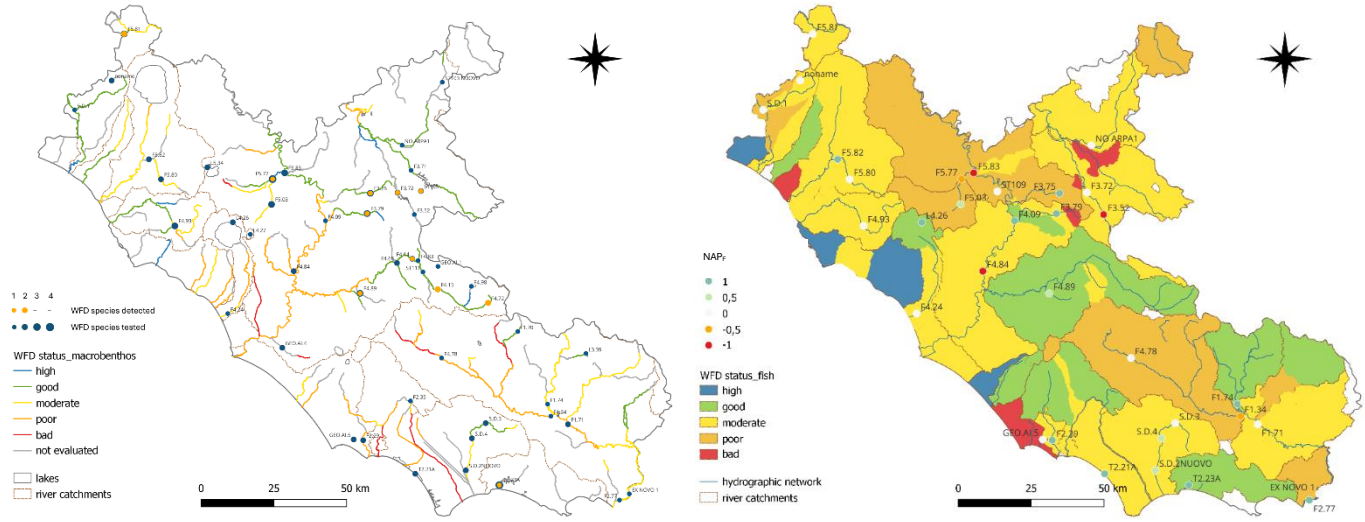


Figure 7. Details on locations of detections over tested sites for freshwater species used in Water Framework Directive monitoring. Benthic species and relative water quality index as evaluated by STAR_ICMi index of WFD 2021-2023 (left) and fish Native Alien Prevalence (NAP_F) with relative fish index NISECI as evaluated by WFD 2018-2020 (right).

Priority areas for species conservation, IAS eradication, and freshwater protection

Prioritizing Protection Score (*PPS*). We obtained 39 sites showing PPS ranging from 0.2 to 3.66 (x ± sd= 2.28 ± 1.02). These sites would require the establishment of new Natura 2000 sites (Suppl. mat. S15). The five sites with highest values are located along little tributaries in the inland mountains of the central portion of the region.

Prioritizing Eradication Score (PES). The scoring to prioritize eradication of IAS was computed on the 22 sites of IAS occurrence. Values ranged from 0 to 4.34 (1.49 ± 1.35) (Suppl. mat. S15). The five sites with highest values were in low valley sites, in the same areas where new Natura 2000 sites should be established (Fig. 8).

Freshwater Conservation Score (FSC). A *Freshwater Conservation Score (FSC)* to prioritize new Habitats and species protection areas pursuant to WFD was computed for 33 sites. FSC values ranged from -2.2 and 3.3 (1.01 ± 1.53) (Suppl. mat. S15). Five highest values were again in the same areas identified by two previous analyses, and along the southern coastline (Fig. 8).

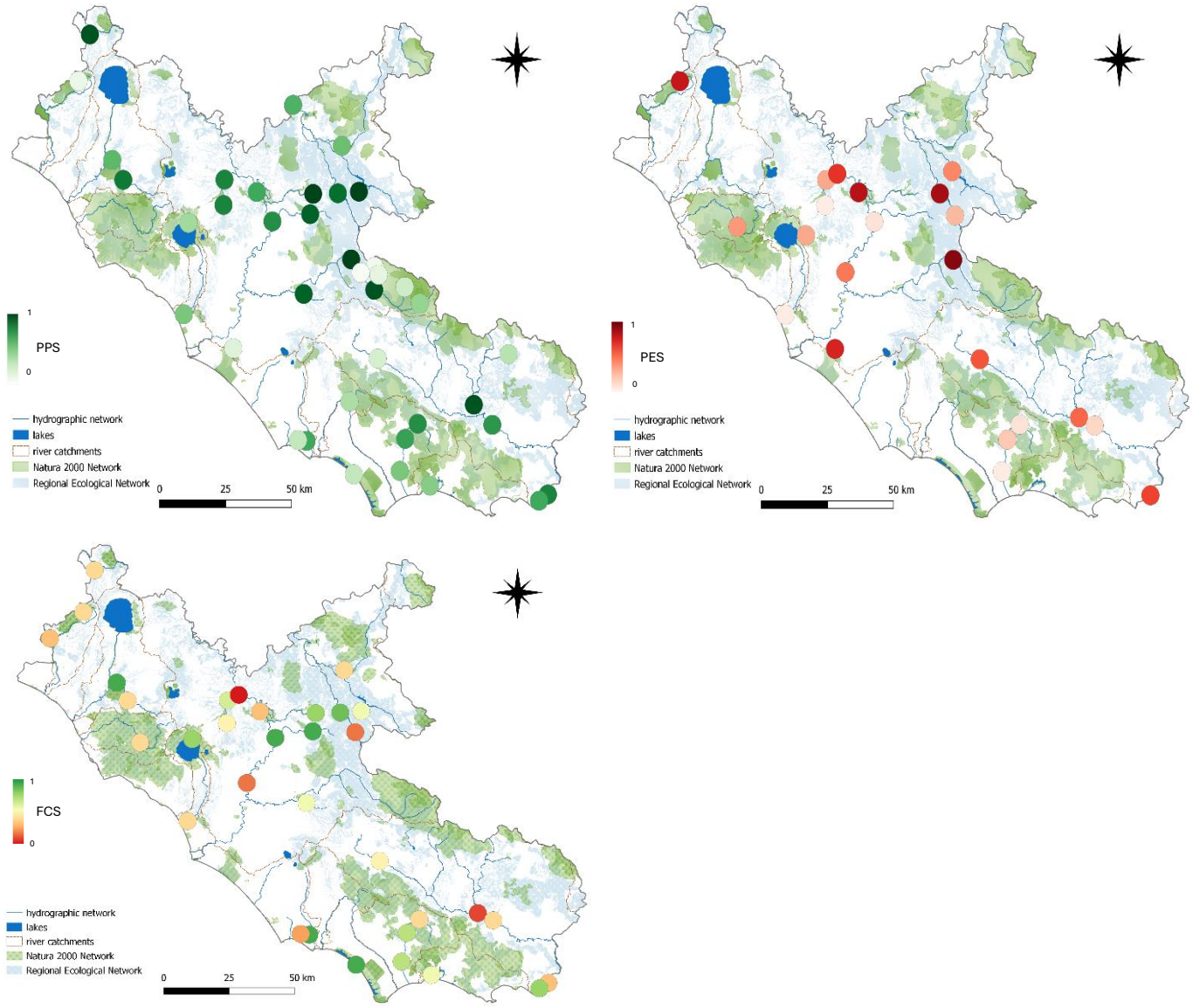


Figure 8. Priority areas for establishing new Natura 2000 sites (PPS, Prioritizing Protection Score, top left), launching eradication measures on IAS (PES, Prioritizing Eradication Score, top right), and proposing Habitats and species protection areas (FCS, Freshwater Conservation Score, bottom).

Discussion

Updating species distribution

We improved freshwater biodiversity monitoring with a rapid and cost-effective eDNA campaign covering both protected and unprotected areas (Capurso et al., 2023). Compared to the known distribution of target species, our qPCR-based eDNA survey revealed an accurate picture of new biotopes colonized by populations of both native and alien species. Our results confirmed species-specific eDNA approach as a rapid and cost effective tool to broadly depict range dynamics of cryptic and rare native species, and to alert about new IAS colonisations, thus suggesting to adopt this technique as a routine complementary technique to accomplish the monitoring obligations by the European Habitats Directive and Regulation 1143/2014 (Schmeller, 2008).

The Eurasian otter *Lutra lutra* was detected for the first time in the study area after its extinction in the last century (Giovacchini et al., 2023), confirming the ongoing range expansion in the Italian peninsula (Giovacchini et al., 2018). New occurrences of the endangered macrobenthic river mussel *Unio mancus* and white-clawed crayfish *Austropotamobius italicus* (Fuderer et al. 2010; Lopez-Lima et al. 2024) may potentially indicate high water quality in three water courses not evaluated by WFD monitoring, and contribute to reevaluate the ecological status of these rivers (i.e., S. Vittorino creek, Fondi outflowing channel, Fosso della Foresta). Also, we unveiled the endangered diadromous fish *A. fallax* to still swim on free-flowing rivers, in coastal lakes, and even upstream some dammed stretches, and confirmed no range expansion of the endangered Tuscanian chub *Squalius lucumonis* (Carosi et al., 2021; Pompei et al., 2018; Tancioni et al., 2013).

The IAS zebra mussel *Dreissena polymorpha* was found for the first time in the study area in Tiber river basin, probably following an anthropogenic introduction, as high density of individuals appeared in two close reservoirs managed for angling activities, and heavily invaded by IAS fish (Grano, 2022; Giovacchini S., Sarrocco S., and Carotenuto L., unpublished data). Our results also revealed the expansion of alien fish such as the padanian goby *Padogobius bonelli* and the topmouth gudgeon *Pseudorasbora parva*. These results confirm the tragic trend of invasion by alien fish in central-Italian rivers in the last 10 years, that pose serious threats to native fish communities (Carosi et al., 2021; Sarrocco et al., 2012). Consequentially, rivers struggle to meet standard conditions for fish population according to WFD classification.

Part of our outcomes were further confirmed by recent field observations. As an example, the endangered twait shad *A. fallax* was confirmed in the lower stretch of a river successfully assessed with eDNA (Ferri et al., 2024). New traditional field surveys also revealed an unexpected wider distribution for neglected species such as the river mussel *Unio mancus* (Giovacchini S., unpublished data).

Opportunities for new protected areas

Our results provided new occurrences of species requiring rigorous protection and the designation of Special Areas of Conservation under the EU Habitats Directive 43/92/EC that were found outside the Natura 2000 Network. These evidence claim either for the establishment of new Natura 2000 sites, or to extend preexistent Natura 2000 sites where new species are revealed within their boundaries for the first time. Our choice to infer species presence up to 10 km upstream is far from dispelling all doubts about the actual position of an endangered native population within the boundaries of a protected territory. Quite the opposite, these analyses often indicated potential presence outside N2KN. Land protection could also be improved through the identification of Key Biodiversity Areas (KBA) sponsored by the IUCN (Kullberg et al., 2019; Nania et al., 2024). KBAs ensure a sustainable use of terrestrial and freshwater natural resources, through a governance that involves governments, privates, and local communities as Other Effective area-based Conservation Measures (OECM) (Donald et al., 2019). Specifically, KBA include Important Sites for Freshwater Biodiversity, identified based on the occurrence of specific molluscs and fish.

Standing waters don't need to be treated with a spatial inference as eDNA molecules tend to settle into the water column, mobilized only by wind into the same lake biotope, and not through the river network as in flowing waters (Burian et al., 2021). Lentic systems provide direct insights for a protected area candidate (Chen et al., 2023). On the other side, running waters need to be treated for false presences due to the mobility of eDNA along the river (Burian et al., 2021; Sepulveda et al., 2023). As eDNA particles undergo to a fast degradation in aquatic environments, qPCR-based eDNA usually guarantees reliable assessments of species assemblages at each site location (Dejean et al., 2011; Maruyama et al., 2014). On the other side, for running water locations, inferring species presence at short distances offers a cost-effective way to meet recommendations produced by complex modeling integrating hydrology and river morphology to address eDNA sources within the river network (Carraro et al., 2021).

In our study area new occurrences were mainly located at headwaters, leading to use a short distances analysis to process homogenously with other locations of the study area. Most of endangered species assemblages found at headwaters may suggest relict populations are surviving in remote pristine areas such in the mountain areas of the region (Grantham et al., 2020). Our prioritization criteria for running water sites accounted for eDNA flowing downstream and revealed gaps in N2KN coverage which need to be filled in the short term to preserve remnant populations of endangered freshwater species, likely reflecting potential high qualities of habitats and low human disturbance (Foresta et al., 2016; Sallustio et al., 2017). Example of these gap areas are along Aniene river, Salto and Turano lakes, and along creeks flowing from Sabine hills. These sites are primarily characterized by a group of rare, endemic, and sensitive-to-pollution species like *U. mancus*, *A. italicus*, *B. pachypus*, and *S. lucumonis*, and secondarily by *Vertigo sp*. and *A. fallax*.

The establishment of protected areas is among the most effective conservation measures to protect biodiversity, especially in wetlands (Acreman et al., 2020; Chen et al., 2023). Designing new Special Areas of Conservation (Alexandridis et al., 2009; Hermoso et al., 2015; Kail et al., 2023) is also in line with the long-term ambition of the European Green Deal and the Biodiversity Strategy for 2030, aimed at protecting 30% of the European land surface and freshwaters (Hermoso et al., 2022; Spiliopoulou et al., 2023). As a main action, EU aspires at enlarging the actual coverage of 27,000 sites of N2KN. Most recommendations indicate to protect remaining natural areas, and to take advantage of land abandonment that is widely distributed and provides passive rewilding opportunities (Araújo and Alagador, 2024). Productive lands should also be included to reach the 30% EU target (Müller et al., 2020). This last suggestion is not particularly challenging for the protection of freshwater ecosystems crossing agricultural lands. Indeed, riverine networks and their vegetation belts are often not exploited by landowners, offering the opportunity for a sustainable land sharing that prevents conflicts between land exploitation and land protection.

A specific example in our study area is given by Garigliano river, crossing extensively used croplands, with only one river shoreline included in a Natura 2000 site (IT8010029). A coordinated effort among the regional administrations managing the riverbanks and the main river course that marks the boundary between two regions (Latium and Campania), is mandatory to protect valuable endangered species, such as the newly discovered Eurasian otter (Valentim et al., 2025). Similarly, many Natura 2000 sites should be extended to the creeks or streams that border the protected areas, where we found Annex II freshwater species. An example is the river Amaseno flowing on the boundary of the Ausoni Mountains Natura 2000 sites (IT6040043). In fact, river networks are often used as boundary lines, including the N2KN. These natural reserves are often devoted to the conservation of inland or mountain ecosystems. Extending all these protected areas to include the river channel could guarantee the preservation of entire new habitats with a relatively limited effort (Valentim et al., 2025).

Priority of intervention against IAS

Priority areas to eradicate or control IAS were widely overlapped to areas of conservation concern. In these areas we claim for immediate eradication to remove the IAS (Guerra et al., 2018). IAS-free refugia may be crucial for endangered species, especially under the synergetic threats introduced by climate changes, and the altitudinal location of these sites can play a strategic role (Johovic et al., 2020). Removal of IAS should be a prerogative objective also for preexistent Natura 2000 sites to make N2KN effective in nature conservation (Baquero et al., 2021). Examples of priority intervention outside N2KN are along Aniene river (where *B. dendrobatidis* was detected together with *B. pachypus* and *A. italicus*), Turano lake (where *F. limosus* and *D. polymorpha* occurred with *A. italicus* and *B. pachypus*), whereas among Natura 2000 sites Latera lake showed the cooccurrence of *P. clarkii* and *Vertigo sp*.

Also, field survey and population estimates are mandatory to evaluate if eradication may be successful or will be prevented by the existence of well-structured populations. In this case, mitigation interventions addressed to control populations under certain densities should be planned. Finally, prevention actions should be undertaken in areas where no IAS were detected. These include as stakeholder engagement,

sociological surveys, and controls on fish restocking, fish farms, and aquaculture facilities, including reptile pet shops (Hermoso et al., 2016). Monitoring, especially eDNA is also a fundamental part of the prevention process, as a rapid warning of the introduction of new IAS can promptly activate the eradication measures (Essl et al., 2020). Among the most concerning threats we detected in the study area is the pathogen *B. dendrobatidis* in the Tiber river catchment, the main river of Central Italy. Future monitoring should focus on the area of occurrence of this fungus and prevent its spreading across the river basin, to promote an effective implementation of both Regulation on IAS and WFD (Boon et al., 2020).

Areas for freshwater conservation under WFD

In this essay we underlined the importance of including new freshwater biotopes under the protection of a European legislation such as the WFD. The integration among different levels of nature protection (i.e., HD, IAS, and WFD) improves the efficacy of freshwater conservation as often regular protected areas do not have enough resources dedicated to riverine management (Thieme et al., 2012). Our multi species approach revealed the potential for the establishment of Habitat and species conservation areas under WFD articles in the inner portion of the study area. In many cases, rivers are just on the boundaries of pre-existent natural reserves. As for the N2KN, extending the boundaries of already existing protected areas to include the bounding rivers can be a very cost-effective option.

It has been demonstrated that even if there is not a direct management of surface water, the merely presence of a protected area dedicated to terrestrial ecosystems enhances the integrity of water environments (Valentim et al., 2025). The importance to include strategies of land protection under the governance of WFD also relies on the embracement offered by this legislation to the management of wide areas (Birk et al., 2012). WFD encourages the management at the river catchment scale with adequate planning, and thus it can create interconnections and synergies with other legislations addressing IAS removal, increasing native wilderness in WFD evaluations, and protecting the watershed not only on specific sites (Kail et al., 2023). This could especially benefit wide-ranging freshwater species such as the Eurasian otter, catadromous and anadromous fish, and species sensitive to water quality. The implementation of WFD commitments is therefore fostered in the EU Biodiversity Strategy for 2030, by promoting organic farming, soil remediation, IAS reduction, and river restoration. More fundings, the adoption of efficient river basin management plans, and the engagement of stakeholders will have a key role in boosting WFD improvements for all Member States that aim to rapidly reach the objective of a good water conservation (Hermoso et al., 2022, 2016).

Moreover, monitoring the water quality under WFD is an essential part of the process, from the achievement of base goals until the proposal of protection areas. Recent advancements on eDNA research shows that this method upscales the resolution of biotic indices of WFD and inform early warning systems for rapid changes in aquatic sensitive communities (Blackman et al., 2024). Including data gained through DNA-based assessments can help in upgrading the classification of river quality based on periodic surveys and using specific native species as bioindicator.

On the other side, highly accurate IAS occurrences recorded from eDNA suggest that alterations of fish communities and their integration into WFD metrics can negatively affect river status. In fact, eDNA multi species approach can highlight how much IAS are predominating in aquatic ecosystems and influence ecological evaluations of river status under WFD. River status under WFD classification indeed is shaped to determine the overall river status with the status of worst subordinate class (i.e., one-out-all-out rule). We hope this renewed awareness on fish IAS impacts will lead to implement effective eradications to fulfil WFD obligations and rehabilitate river ecosystems in all its components. Several experiences have already proven that DNA-based methods can serve WFD routine monitoring on the ecological assessment of freshwaters (Blancher et al., 2022; Macher et al., 2025; Pont et al., 2021). eDNA is actually an effective tool for the zoological evaluations under WFD, rather than those relating on plants, as this method is almost ready to

replace traditional samplings based on morphological identification for both macrobenthic invertebrates and fish (Hering et al., 2018). If on one side fish are very well tracked with eDNA due to their high production of DNA, invertebrates on the other hand lack of a barcode library for all species (Hering et al., 2018). Also, fish indices derived from eDNA sampling cannot inform on age structure, posing a limit to the use of this technique to evaluate the ecological status of rivers. Similarly, benthic invertebrates still require an enhancement of the method to provide also abundance parameters (Hering et al., 2018). However, abundance can be easily incorporated using quantitative PCR and count reads for metabarcoding (Huang et al., 2022; Pont et al., 2023), and the conversion of presence/absence data into abundances represents a goal within reach (Aylagas, 2017).

Conclusions

Our multi species eDNA approach based on quantitative PCR revealed a powerful tool to simultaneously detect the occurrence of conservation concerns and of species that pose serious threats to them. The results from such a multispecies approach have revealed to offer a cost-effective tool for spatial planning of conservation measures, and to rapidly respond to monitoring obligations by multiple EU biodiversity and ecosystem regulations, such as the Habitats and Freshwater Directives and the EU Regulation 1143/2014 to limit the impact of IAS. Quantitative PCR-based surveys can therefore outperform routine monitoring programmes that are mandatory for Habitats Directive and Regulation on IAS 1143/2014, standing the development of standardized monitoring system (Schmeller, 2008). Similarly, eDNA can inform also the Water Framework Directive (Ficetola et al., 2021; Leese et al., 2018). In this context, eDNA can be used also to fulfil other EU objective linked to river protection, such as re-establishing the river continuum along 25,000 km of rivers stated in the Biodiversity Strategy for 2030 (Fernandez et al., 2022).

The added value of a multispecies multitaxa detection also relies in the opportunity to cross data to obtain new indices and conservation score for the areas under analysis, also posing the base to establish regular monitoring across time and space that could evidence trends in the conservation status of areas. Our results allowed to identify critical gaps in freshwaters protected area coverage, providing an accurate mapping of risk of IAS invasions, and informing water conservation strategies at both site and river catchment scale (Hermoso et al., 2015; Niz et al., 2023)(Cortina and Boggia, 2014; Johovic et al., 2020). Our large-scale survey provided new data on rare and endangered species, claiming the need for species specific accurate surveys through traditional field techniques. in the next future, fine-scale survey could also use an eDNA quantification approach. The integration with traditional survey techniques can be a winning option to accurately recreate ranges and areas of occupancy of species suffering of poor data. Italy hosts the most biodiversity and endemism on freshwater fish among Europe, and all efforts toward their conservation are beneficial, from innovative monitoring to effective protection (Fliervoet et al., 2013; Hunting et al., 2017; Reyjol et al., 2007).

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Literature

Acreman, M., Hughes, K.A., Arthington, A.H., Tickner, D., Dueñas, M.-A., 2020. Protected areas and freshwater biodiversity: a novel systematic review distils eight lessons for effective conservation. Conserv. Lett. 13, e12684. https://doi.org/https://doi.org/10.1111/conl.12684

Alexandridis, T.K., Lazaridou, E., Tsirika, A., Zalidis, G.C., 2009. Using Earth Observation to update a Natura 2000 habitat map for a wetland in Greece. J. Environ. Manage. 90, 2243–2251. https://doi.org/https://doi.org/10.1016/j.jenvman.2007.06.024

Amberg, J.J., Merkes, C.M., Stott, W., Rees, C.B., Erickson, R.A., 2019. Environmental DNA as a tool to help inform zebra mussel, Dreissena polymorpha, management in inland lakes. Manag. Biol. Invasions 10, 96–110. https://doi.org/10.3391/MBI.2019.10.1.06

Antognazza, C.M., Britton, J.R., Potter, C., Franklin, E., Hardouin, E.A., Gutmann Roberts, C., Aprahamian, M., Andreou, D., 2019. Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (Alosa spp.). Aquat. Conserv. Mar. Freshw. Ecosyst. 29, 148–152. https://doi.org/https://doi.org/10.1002/aqc.3010

Araújo, M.B., Alagador, D., 2024. Expanding European protected areas through rewilding. Curr. Biol. 34, 3931-3940.e5. https://doi.org/10.1016/j.cub.2024.07.045

Archi F., Bussettini M., Piva F., Buffagni A., Erba S. (2014). Linee guida per la valutazione della componente macrobentonica fluviale ai sensi del DM 260/2010. ISPRA, Manuali e Linee Guida 107/2014.

Aylagas, E., 2017. DNA metabarcoding derived biotic indices for marine monitoring and assessment. PhD Thesis. University of the Basque Country, p. 248.

Armitage, P.D., Moss, D., Wright, J.F., Furse, M.T., 1983. The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites. Water Res. 17, 333–347. https://doi.org/https://doi.org/10.1016/0043-1354(83)90188-4

Balletto, E., Bonelli, S., Borghesio, L., Casale, A., Brandmayr, P., Vigna Taglianti, A., 2010. Hotspots of biodiversity and conservation priorities: A methodological approach. Ital. J. Zool. 77, 2–13. https://doi.org/10.1080/11250000902758923

Baquero, R.A., Ayllón, D., Oficialdegui, F.J., Nicola, G.G., 2021. Tackling biological invasions in natura 2000 network in the light of the new eu biodiversity strategy for 2030. Manag. Biol. Invasions 12, 776–791. https://doi.org/10.3391/MBI.2021.12.4.01

Beranek, C.T., Maynard, C., McHenry, C., Clulow, J., Mahony, M., 2021. Rapid population increase of the threatened Australian amphibian Litoria aurea in response to wetlands constructed as a refuge from chytrid-induced disease and introduced fish. J. Environ. Manage. 291, 112638. https://doi.org/https://doi.org/10.1016/j.jenvman.2021.112638

Birk, S., Bonne, W., Borja, A., Brucet, S., Courrat, A., Poikane, S., Solimini, A., van de Bund, W., Zampoukas, N., Hering, D., 2012. Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. Ecol. Indic. 18, 31–41. https://doi.org/https://doi.org/10.1016/j.ecolind.2011.10.009

Blackman, R.C., Carraro, L., Keck, F., Altermatt, F., 2024. Measuring the state of aquatic environments using eDNA—upscaling spatial resolution of biotic indices. Philos. Trans. R. Soc. B Biol. Sci. 379, 20230121. https://doi.org/10.1098/rstb.2023.0121

Blancher, P., Lefrançois, E., Rimet, F., Vasselon, V., Argillier, C., Arle, J., Beja, P., Boets, P., Boughaba, J.,

Chauvin, C., Deacon, M., Duncan, W., Ejdung, G., Erba, S., Ferrari, B., Fischer, H., Hänfling, B., Haldin, M., Hering, D., Hette-Tronquart, N., Hiley, A., Järvinen, M., Jeannot, B., Kahlert, M., Kelly, M., Kleinteich, J., Koyuncuoğlu, S., Krenek, S., Langhein-Winther, S., Leese, F., Mann, D., Marcel, R., Marcheggiani, S., Meissner, K., Mergen, P., Monnier, O., Narendja, F., Neu, D., Pinto, V.O., Pawlowska, A., Pawlowski, J., Petersen, M., Poikane, S., Pont, D., Renevier, M.S., Sandoy, S., Svensson, J., Trobajo, R., Zagyva, A.T., Tziortzis, I., Van Der Hoorn, B., Vasquez, M.I., Walsh, K., Weigand, A., Bouchez, A., 2022. A strategy for successful integration of DNA-based methods in aquatic monitoring. Metabarcoding and Metagenomics 6, 215–226. https://doi.org/10.3897/mbmg.6.85652

Bolpagni, R., Azzella, M.M., Agostinelli, C., Beghi, A., Bettoni, E., Brusa, G., De Molli, C., Formenti, R., Galimberti, F., Cerabolini, B.E.L., 2017. Integrating the water framework directive into the habitats directive: Analysis of distribution patterns of lacustrine EU habitats in lakes of Lombardy (Northern Italy). J. Limnol. 76, 75–83. https://doi.org/10.4081/jlimnol.2017.1627

Boon, P.J., Clarke, S.A., Copp, G.H., 2020. Alien species and the EU Water Framework Directive: a comparative assessment of European approaches. Biol. Invasions 22, 1497–1512. https://doi.org/10.1007/s10530-020-02201-z

Bruno, A., Sandionigi, A., Galimberti, A., Siani, E., Labra, M., Cocuzza, C., Ferri, E., Casiraghi, M., 2017. One step forwards for the routine use of high-throughput DNA sequencing in environmental monitoring. An efficient and standardizable method to maximize the detection of environmental bacteria. Microbiologyopen 6, e00421. https://doi.org/https://doi.org/10.1002/mbo3.421

Brusconi, S., Bertocchi, S., Renai, B., Scalici, M., Souty-Grosset, C., Gherardi, F., 2008. Conserving indigenous crayfish: Stock assessment and habitat requirements in the threatened Austropotamobius italicus. Aquat. Conserv. Mar. Freshw. Ecosyst. 18, 1227–1239. https://doi.org/10.1002/aqc.935

Buffagni A. & Erba S., (2007). Macroinvertebrati acquatici e direttiva 2000/60/EC (WFD). IRSA-CNR, Notiziario dei Metodi Analitici, (1): 94-100.

Buffagni, A., Erba, S., & Pagnotta, R. (2008). Definizione dello stato ecologico dei fiumi sulla base dei macroinvertebrati bentonici per la 2000/60/EC (WFD): il sistema di classificazione MacrOper. *IRSA-CNR Notiziario dei Metodi Analitici*, *1*, 24-46.

Burian, A., Mauvisseau, Q., Bulling, M., Domisch, S., Qian, S., Sweet, M., 2021. Improving the reliability of eDNA data interpretation. Mol. Ecol. Resour. 21, 1422–1433. https://doi.org/10.1111/1755-0998.13367

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Canessa, S., Maggesi, M., Salvidio, S., Grasselli, E., 2017. Validation and cost-effectiveness of an alternative method to quantify Batrachochytrium dendrobatidis infection in amphibian samples using real-time PCR. Rend. Lincei 28, 687–692. https://doi.org/10.1007/s12210-017-0640-0

Canestrelli, D., Zampiglia, M., Nascetti, G., 2013. Widespread Occurrence of Batrachochytrium dendrobatidis in Contemporary and Historical Samples of the Endangered Bombina pachypus along the Italian Peninsula. PLoS One 8. https://doi.org/10.1371/journal.pone.0063349

Capurso, G., Carroll, B., Stewart, K.A., 2023. Transforming marine monitoring: Using eDNA metabarcoding to improve the monitoring of the Mediterranean Marine Protected Areas network. Mar. Policy 156, 105807. https://doi.org/https://doi.org/10.1016/j.marpol.2023.105807

Carosi, A., Ghetti, L., Lorenzoni, M., 2021. The role of climate changes in the spread of freshwater fishes: Implications for alien cool and warm-water species in a mediterranean basin. Water (Switzerland) 13. https://doi.org/10.3390/w13030347

Carraro, L., Stauffer, J.B., Altermatt, F., 2021. How to design optimal eDNA sampling strategies for biomonitoring in river networks. Environ. DNA 3, 157–172. https://doi.org/https://doi.org/10.1002/edn3.137

Cazzolla Gatti, R., Iaria, J., Moretti, G., Amendola, V., Cassola, F.M., Cerretti, P., Musciano, M. Di, Francesconi, L., Frattaroli, A.R., Livornese, M., Martini, M., Nania, D., Pacifici, M., Piovesan, G., Prandelli, S., Rocchini, D., Rondinini, C., Chiarucci, A., 2024. EU2030 biodiversity strategy: Unveiling gaps in the coverage of ecoregions and threatened species within the strictly protected areas of Italy. J. Nat. Conserv. 79, 126621. https://doi.org/https://doi.org/10.1016/j.jnc.2024.126621

Cazzolla Gatti, R., Zannini, P., Piovesan, G., Alessi, N., Basset, A., Beierkuhnlein, C., Di Musciano, M., Field, R., Halley, J.M., Hoffmann, S., Iaria, J., Kallimanis, A., Lövei, G.L., Morera, A., Provenzale, A., Rocchini, D., Vetaas, O.R., Chiarucci, A., 2023. Analysing the distribution of strictly protected areas toward the EU2030 target. Biodivers. Conserv. 32, 3157–3174. https://doi.org/10.1007/s10531-023-02644-5

Chen, P., Luo, J., Xiong, Z., Wan, N., Ma, J., Yuan, J., Duan, H., 2023. Can the establishment of a protected area improve the lacustrine environment? A case study of Lake Chaohu, China. J. Environ. Manage. 342, 118152. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.118152

Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J.-C., Bonin, A., Taberlet, P., Pont, D., 2016. Spatial Representativeness of Environmental DNA Metabarcoding Signal for Fish Biodiversity Assessment in a Natural Freshwater System. PLoS One 11, e0157366.

Cortina, C., Boggia, A., 2014. Development of policies for Natura 2000 sites: A multi-criteria approach to support decision makers. J. Environ. Manage. 141, 138–145. https://doi.org/https://doi.org/10.1016/j.jenvman.2014.02.039

Costa, M.J., Duarte, G., Segurado, P., Branco, P., 2021. Major threats to European freshwater fish species. Sci. Total Environ. 797, 149105. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.149105

Deiner, K., Altermatt, F., 2014. Transport Distance of Invertebrate Environmental DNA in a Natural River. PLoS One 9, e88786.

Deiner, K., Fronhofer, E.A., Mächler, E., Walser, J.-C., Altermatt, F., 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nat. Commun. 7, 12544. https://doi.org/10.1038/ncomms12544

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., Miaud, C., 2011. Persistence of Environmental DNA in Freshwater Ecosystems. PLoS One 6, e23398.

Dextrase, A.J., Mandrak, N.E., 2006. Impacts of alien invasive species on freshwater fauna at risk in Canada. Biol. Invasions 8, 13–24. https://doi.org/10.1007/s10530-005-0232-2

Dueñas, M.-A., Hemming, D.J., Roberts, A., Diaz-Soltero, H., 2021. The threat of invasive species to IUCN-listed critically endangered species: A systematic review. Glob. Ecol. Conserv. 26, e01476. https://doi.org/https://doi.org/10.1016/j.gecco.2021.e01476

EC, 2014. Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. Official Journal of European Union, L 317, 04.11.2014.

EEA, 2022. Natura 2000 barometer. *European Environment Agency.* https://www.eea.europa.eu/data-and-maps/dashboards/natura-2000-barometer [accessed November 22, 2022].

Ellwanger, G., Runge, S., Wagner, M., Ackermann, W., Neukirchen, M., Frederking, W., Müller, C., Ssymank, A., Sukopp, U., 2018. Current status of habitat monitoring in the European Union according to Article 17 of the Habitats Directive, with an emphasis on habitat structure and functions and on Germany. Nat. Conserv. 29, 57–78. https://doi.org/10.3897/natureconservation.29.27273

Ercole, S, Angelini, P., Carnevali, L., Casella, L., Giacanelli, V., Grignetti, A., La Mesa, G., Nardelli, R., Serra, L., Stoch, F., Tunesi, L., Genovesi, P., 2021. Rapporti Direttive Natura (2013-2018). Sintesi dello stato di conservazione delle specie e degli habitat di interesse comunitario e delle azioni di contrasto alle specie esotiche di rilevanza unionale in Italia. (No. 394/2021), Serie Rapporti. ISPRA.

Essl, F., Latombe, G., Lenzner, B., Wilson, J.R.U., Genovesi, P., Pagad, S., Seebens, H., Smith, K., 2020. The Convention on Biological Diversity (CBD)'s Post-2020 target on invasive alien species – what should it include and how should it be monitored? NeoBiota 62, 99–121. https://doi.org/10.3897/neobiota.62.53972

EU, 1992. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Official Journal of European Communities, L 206, 22.7.1992, p. 7-50.

Fernandez, S., Arboleya, E., Dopico, E., Garcia-Vazquez, E., 2022. Dams in South Europe: socio-environmental approach and eDNA-metabarcoding to study dam acceptance and ecosystem health. Wetl. Ecol. Manag. 30, 341–355. https://doi.org/10.1007/s11273-022-09864-6

Ferri, V., Crescia, P., Soccini, C., Celletti, S., 2024. A new possible breeding site of Alosa fallax (Lacépède 1803) (Actinopterygii: Clupeiformes: Alosidae) on the Tyrrhenian coast of Central Italy. Nat. Hist. Sci. 11, 9–14. https://doi.org/10.4081/nhs.2024.682

Ficetola, G.F., Boyer, F., Valentini, A., Bonin, A., Meyer, A., Dejean, T., Gaboriaud, C., Usseglio-Polatera, P., Taberlet, P., 2021. Comparison of markers for the monitoring of freshwater benthic biodiversity through DNA metabarcoding. Mol. Ecol. 30, 3189–3202. https://doi.org/https://doi.org/10.1111/mec.15632

Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G., Taberlet, P., 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Mol. Ecol. Resour. 15, 543–556. https://doi.org/https://doi.org/10.1111/1755-0998.12338

Fliervoet, J.M., Van den Born, R.J.G., Smits, A.J.M., Knippenberg, L., 2013. Combining safety and nature: A multi-stakeholder perspective on integrated floodplain management. J. Environ. Manage. 128, 1033–1042. https://doi.org/https://doi.org/10.1016/j.jenvman.2013.06.023

Foresta, M., Carranza, M.L., Garfì, V., Di Febbraro, M., Marchetti, M., Loy, A., 2016. A systematic conservation planning approach to fire risk management in Natura 2000 sites. J. Environ. Manage. 181, 574–581. https://doi.org/https://doi.org/10.1016/j.jenvman.2016.07.006

Francis, R.A., 2012. A Handbook of Global Freshwater Invasive Species, A Handbook of Global Freshwater Invasive Species. https://doi.org/10.4324/9780203127230

Füreder, L., Gherardi, F., Holdich, D., Reynolds, J., Sibley, P. & Souty-Grosset, C. 2010. *Austropotamobius pallipes*. *The IUCN Red List of Threatened Species* 2010: e.T2430A9438817. https://dx.doi.org/10.2305/IUCN.UK.2010-3.RLTS.T2430A9438817.en. Accessed on 27 December 2024.

Genovesi, P., Carboneras, C., Vilà, M., Walton, P., 2015. EU adopts innovative legislation on invasive species: a step towards a global response to biological invasions? Biol. Invasions 17, 1307–1311. https://doi.org/10.1007/s10530-014-0817-8

Gherardi, F., Bertolino, S., Bodon, M., Casellato, S., Cianfanelli, S., Ferraguti, M., Lori, E., Mura, G., Nocita, A., Riccardi, N., Rossetti, G., Rota, E., Scalera, R., Zerunian, S., Tricarico, E., 2008. Animal xenodiversity in Italian inland waters: Distribution, modes of arrival, and pathways. Biol. Invasions 10, 435–454. https://doi.org/10.1007/s10530-007-9142-9

Giovacchini, S., Marrese, M., & Loy, A. (2018). Good news from the south: filling the gap between two otter populations in Italy. *IUCN Otter Specialist Group Bulletin*, *35*(4), 212-221.

Giovacchini, S., Canu, A., Loy, A., & Di Febbraro, M. (2023). Otters reconquering Latium. The case of the re-expansion in Central Italy along the Tyrrhenian side. *First Conference of Conservation Biology for ECR. Book of Abstracts, Roma* (p. 88).

Giovacchini S, Mirone E, Bruno A, Ramazzotti F, Caprotti L, Monaco P, Belluardo F, Di Febbraro M, Jamwal PS, Manfrin C, Galimberti A, Loy A (subm). Using species-specific environmental DNA for multi-taxa detection of endangered and invasive alien species in Italian freshwater ecosystems. Implications for monitoring obligations under EU regulations. Biological Conservation, subm.

Giovacchini S., Mirone E., Monaco P., Ramazzotti F., Caprotti L., Galimberti A., Bruno A., Canu A., Galaverni M., Di Febbraro M., Loy A. (in prep.). Environmental DNA as a reliable monitoring tool. Otter (Lutra lutra) re-expansion in Central Italy along the Tyrrhenian side.

Grano, M., 2022. Prima segnalazione di Dreissena polymorpha (Pallas , 1771) (Bivalvia : Dreissenidae) nel Lago del Turano e per la provincia di Rieti (Lazio). Alleryana 40, 12–15.

Grano, M., Nistri, R., Giuseppe, R. Di, 2020. Aggiornamento sui molluschi alloctoni nel fiume Tevere a Roma (Bivalvia). Allerya 38, 117–121.

Grantham, H.S., Duncan, A., Evans, T.D., Jones, K.R., Beyer, H.L., Schuster, R., Walston, J., Ray, J.C., Robinson, J.G., Callow, M., Clements, T., Costa, H.M., DeGemmis, A., Elsen, P.R., Ervin, J., Franco, P., Goldman, E., Goetz, S., Hansen, A., Hofsvang, E., Jantz, P., Jupiter, S., Kang, A., Langhammer, P., Laurance, W.F., Lieberman, S., Linkie, M., Malhi, Y., Maxwell, S., Mendez, M., Mittermeier, R., Murray, N.J., Possingham, H., Radachowsky, J., Saatchi, S., Samper, C., Silverman, J., Shapiro, A., Strassburg, B., Stevens, T., Stokes, E., Taylor, R., Tear, T., Tizard, R., Venter, O., Visconti, P., Wang, S., Watson, J.E.M., 2020. Anthropogenic modification of forests means only 40% of remaining forests have high ecosystem integrity. Nat. Commun. 11, 5978. https://doi.org/10.1038/s41467-020-19493-3

Guerra, C., Baquero, R.A., Gutiérrez-Arellano, D., Nicola, G.G., 2018. Is the Natura 2000 network effective to prevent the biological invasions? Glob. Ecol. Conserv. 16, e00497. https://doi.org/https://doi.org/10.1016/j.gecco.2018.e00497

Harper, L.R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H.C., Gough, K.C., Lewis, E., Adams, I.P., Brotherton, P., Phillips, S., Hänfling, B., 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (Triturus cristatus). Ecol. Evol. 8, 6330–6341. https://doi.org/https://doi.org/10.1002/ece3.4013

Hering, D., Borja, A., Jones, J.I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., Kelly, M., 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Res. 138, 192–205. https://doi.org/https://doi.org/10.1016/j.watres.2018.03.003

Hermoso, V., Abell, R., Linke, S., Boon, P., 2016. The role of protected areas for freshwater biodiversity conservation: challenges and opportunities in a rapidly changing world. Aquat. Conserv. Mar. Freshw. Ecosyst. 26, 3–11. https://doi.org/https://doi.org/10.1002/aqc.2681

Hermoso, V., Carvalho, S.B., Giakoumi, S., Goldsborough, D., Katsanevakis, S., Leontiou, S., Markantonatou, V., Rumes, B., Vogiatzakis, I.N., Yates, K.L., 2022. The EU Biodiversity Strategy for 2030: Opportunities and challenges on the path towards biodiversity recovery. Environ. Sci. Policy 127, 263–271. https://doi.org/https://doi.org/10.1016/j.envsci.2021.10.028

Hermoso, V., Filipe, A.F., Segurado, P., Beja, P., 2015. Filling gaps in a large reserve network to address freshwater conservation needs. J. Environ. Manage. 161, 358–365. https://doi.org/https://doi.org/10.1016/j.jenvman.2015.07.023

Hernandez, C., Bougas, B., Perreault-Payette, A., Simard, A., Côté, G., Bernatchez, L., 2020. 60 specific eDNA

qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. Environ. DNA 2, 373–386. https://doi.org/https://doi.org/10.1002/edn3.89

Huang, S., Yoshitake, K., Watabe, S., Asakawa, S., 2022. Environmental DNA study on aquatic ecosystem monitoring and management: Recent advances and prospects. J. Environ. Manage. 323, 116310. https://doi.org/10.1016/j.jenvman.2022.116310

Hunting, E. R., de Jong, S., & Vijver, M. G. (2017). Assessment of monitoring tools and strategies safeguarding aquatic ecosystems within the European water framework directive. *Institute of Environmental Sciences Leiden*.

Iannella, M., Cerasoli, F., Biondi, M., 2017. Unraveling climate influences on the distribution of the parapatric newts Lissotriton vulgaris meridionalis and L. italicus. Front. Zool. 14, 1–14. https://doi.org/10.1186/s12983-017-0239-4

IPBES, 2023. Summary for Policymakers of the Thematic Assessment Report on Invasive Alien Species and their Control of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, in: Roy, H.E., Pauchard, A., Stoett, P., Renard Truong, T., Bacher, S., Galil, B.S., Hulme, P.E., Ikeda, T., Sankaran, K. V, McGeoch, M.A., Meyerson, L.A., Nuñez, M.A., Ordonez, A., Rahlao, S.J., Schwindt, E., Seebens, H., Sheppard, A.W., Vandvik, V. (Eds.), . IPBES Secretariat, Bonn, Germany. https://doi.org/10.5281/zenodo.7430692

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H., Whiteley, A.R., 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol. Ecol. Resour. 15, 216–227. https://doi.org/https://doi.org/10.1111/1755-0998.12285

Jaureguiberry, P., Titeux, N., Wiemers, M., Bowler, D.E., Coscieme, L., Golden, A.S., Guerra, C.A., Jacob, U., Takahashi, Y., Settele, J., Díaz, S., Molnár, Z., Purvis, A., 2024. The direct drivers of recent global anthropogenic biodiversity loss. Sci. Adv. 8, eabm9982. https://doi.org/10.1126/sciadv.abm9982

Johovic, I., Gama, M., Banha, F., Tricarico, E., Anastácio, P.M., 2020. A potential threat to amphibians in the European Natura 2000 network: Forecasting the distribution of the American bullfrog Lithobates catesbeianus. Biol. Conserv. 245, 108551. https://doi.org/https://doi.org/10.1016/j.biocon.2020.108551

Kail, J., Januschke, K., Hering, D., 2023. Freshwater-related species richness in Natura 2000 sites strongly depends on the surrounding land use besides local habitat conditions. J. Environ. Manage. 340, 118025. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.118025

Kakuda, A., Doi, H., Souma, R., Nagano, M., Minamoto, T., Katano, I., 2019. Environmental DNA detection and quantification of invasive red-eared sliders, Trachemy scripta elegans, in ponds and the influence of water quality. PeerJ 7, e8155. https://doi.org/10.7717/peerj.8155

Karlsson, E., Ogonowski, M., Sundblad, G., Sundin, J., Svensson, O., Nousiainen, I., Vasemägi, A., 2022. Strong positive relationships between eDNA concentrations and biomass in juvenile and adult pike (Esox lucius) under controlled conditions: Implications for monitoring. Environ. DNA 4, 881–893. https://doi.org/https://doi.org/10.1002/edn3.298

King, A.C., Krieg, R., Weston, A., Zenker, A.K., 2022. Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. J. Environ. Manage. 302, 113929. https://doi.org/https://doi.org/10.1016/j.jenvman.2021.113929

Klymus, K.E., Ruiz Ramos, D. V, Thompson, N.L., Richter, C.A., 2020. Development and Testing of Species-specific Quantitative PCR Assays for Environmental DNA Applications. JoVE e61825. https://doi.org/doi:10.3791/61825

Leblanc, F., Belliveau, V., Watson, E., Coomber, C., Simard, N., Di Bacco, C., Bernier, R., Gagné, N., 2020. Environmental DNA (eDNA) detection of marine aquatic invasive species (AIS) in Eastern Canada using a targeted species-specific qPCR approach. Manag. Biol. Invasions 11, 201–217.

https://doi.org/10.3391/mbi.2020.11.2.03

Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., Ekrem, T., Čiampor, F., Čiamporová-Zaťovičová, Z., Costa, F.O., Duarte, S., Elbrecht, V., Fontaneto, D., Franc, A., Geiger, M.F., Hering, D., Kahlert, M., Kalamujić Stroil, B., Kelly, M., Keskin, E., Liska, I., Mergen, P., Meissner, K., Pawlowski, J., Penev, L., Reyjol, Y., Rotter, A., Steinke, D., van der Wal, B., Vitecek, S., Zimmermann, J., Weigand, A.M., 2018. Chapter Two - Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action, in: Bohan, D.A., Dumbrell, A.J., Woodward, G., Jackson, M.B.T.-A. in E.R. (Eds.), Next Generation Biomonitoring: Part 1. Academic Press, pp. 63–99. https://doi.org/https://doi.org/10.1016/bs.aecr.2018.01.001

Li, Z., Li, F., Qin, S., Guo, F., Wang, S., Zhang, Y., 2024. Environmental DNA biomonitoring reveals the human impacts on native and non-native fish communities in subtropical river systems. J. Environ. Manage. 349, 119595. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.119595

Lopes-Lima, M., Prié, V. & Riccardi, N. 2024. *Unio mancus*. *The IUCN Red List of Threatened Species* 2024: e.T212975237A212995429. https://dx.doi.org/10.2305/IUCN.UK.2024-1.RLTS.T212975237A212995429.en. Accessed on 27 December 2024.

Lorenzoni, M., Borghesan, F., Carosi, A., Ciuffardi, L., De Curtis, O., Del Mastro, G.B., Di Tizio, L., Franzoi, P., Maio, G., Mojetta, A., Nonnis Marzano, F., Pizzul, E., Rossi, G., Scalici, M., Zanetti, M., 2019. Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane Check-List Dell ' Ittiofauna Delle Acque Dolci Italiane the Check-List of the Italian Freshwater Fish Fauna. Ital. J. Freshw. Ichthyol. 5, 239–254.

Macchio S, Rossi G L, Rossi G, De Bonis S, Balzamo S, Martone C (2017). Nuovo indice dello stato ecologico delle comunità ittiche (NISECI). ISPRA, Manuali e Linee Guida 159/2017.

Macher, T.-H., Beermann, A.J., Arle, J., Foerster, J., Greyer, M., Mora, D., Koschorreck, J., Rolauffs, P., Rother, A., Schüler, S., Zimmermann, J., Hering, D., Leese, F., 2025. Fit for purpose? Evaluating benthic invertebrate DNA metabarcoding for ecological status class assessment in streams under the Water Framework Directive. Water Res. 272, 122987. https://doi.org/https://doi.org/10.1016/j.watres.2024.122987

Magliozzi, C., Tsiamis, K., Vigiak, O., Deriu, I., Gervasini, E., Cardoso, A.C., 2020. Assessing invasive alien species in European catchments: Distribution and impacts. Sci. Total Environ. 732, 138677. https://doi.org/10.1016/j.scitotenv.2020.138677

Manfrin, C., Mirimin, L., Zanetti, M., Pizzul, E., Giulianini, P.G., Pallavicini, A., 2022a. Highly sensitive environmental DNA detection of topmouth gudgeon, Pseudorasbora parva: a comparison of qPCR and microfluidic qdPCR. Biol. Invasions 24, 2121–2133. https://doi.org/10.1007/s10530-022-02761-2

Manfrin, C., Zanetti, M., Stanković, D., Fattori, U., Bertucci-Maresca, V., Giulianini, P.G., Pallavicini, A., 2022b. Detection of the Endangered Stone Crayfish Austropotamobius torrentium (Schrank, 1803) and Its Congeneric A. pallipes in Its Last Italian Biotope by eDNA Analysis. Diversity. https://doi.org/10.3390/d14030205

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., Minamoto, T., 2014. The Release Rate of Environmental DNA from Juvenile and Adult Fish. PLoS One 9, e114639.

Mattoccia, M., Marta, S., Romano, A., Sbordoni, V., 2011. Phylogeography of an Italian endemic salamander (genus Salamandrina): Glacial refugia, postglacial expansions, and secondary contact. Biol. J. Linn. Soc. 104, 903–992. https://doi.org/10.1111/j.1095-8312.2011.01747.x

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., Souty-Grosset, C., 2018. Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. Hydrobiologia 805, 163–175. https://doi.org/10.1007/s10750-017-3288-y

Meira, A., Lopes-Lima, M., Varandas, S., Teixeira, A., Arenas, F., Sousa, R., 2019. Invasive crayfishes as a threat to freshwater bivalves: Interspecific differences and conservation implications. Sci. Total Environ. 649, 938–948. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.08.341

Minegishi, Y., Wong, M.K.-S., Nakao, M., Nishibe, Y., Tachibana, A., Kim, Y.-J., Hyodo, S., 2023. Species-specific patterns in spatio-temporal dynamics of juvenile chum salmon and their zooplankton prey in Otsuchi Bay, Japan, revealed by simultaneous eDNA quantification of diverse taxa from the same water samples. Fish. Oceanogr. 32, 311–326. https://doi.org/https://doi.org/10.1111/fog.12631

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., Iwasaki, W., 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R. Soc. Open Sci. 2, 150088. https://doi.org/10.1098/rsos.150088

Müller, A., Schneider, U.A., Jantke, K., 2020. Evaluating and expanding the European Union's protected-area network toward potential post-2020 coverage targets. Conserv. Biol. 34, 654–665. https://doi.org/https://doi.org/10.1111/cobi.13479

Niz, W.C., Laurino, I.R.A., Freitas, D.M. de, Rolim, F.A., Motta, F.S., Pereira-Filho, G.H., 2023. Modeling risks in marine protected areas: Mapping of habitats, biodiversity, and cultural ecosystem services in the southernmost atlantic coral reef. J. Environ. Manage. 345, 118855. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.118855

Nocita, A., Tricarico, E., Bertolino, S., 2017. Fine-scale analysis of heavily invaded Italian freshwater fish assemblages. Integr. Zool. 12, 500–511. https://doi.org/https://doi.org/10.1111/1749-4877.12267

Padgett-Stewart, T.M., Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., Schwartz, M.K., 2016. An eDNA assay for river otter detection: a tool for surveying a semi-aquatic mammal. Conserv. Genet. Resour. 8, 5–7. https://doi.org/10.1007/s12686-015-0511-x

Pagad, S., Genovesi, P., Carnevali, L., Scalera, R., Clout, M., 2015. IUCN SSC Invasive Species Specialist Group: invasive alien species information management supporting practitioners, policy makers and decision takers. Manag. Biol. Invasions 6(2), 127–135. https://doi.org/10.3391/mbi.2015.6.2.03

Park, H.-C., Han, T.-Y., Kim, D.-C., Min, M.-S., Han, S.-Y., Kim, K.-S., Lee, H., 2011. Individual identification and sex determination of Eurasian otters (Lutra lutra) in Daegu city based on genetic analysis of otter spraint. Genes Genomics 33, 653–657. https://doi.org/10.1007/s13258-011-0051-z

Pawlowski, J., Bonin, A., Boyer, F., Cordier, T., Taberlet, P., 2021. Environmental DNA for biomonitoring 2931–2936. https://doi.org/10.1111/mec.16023

Pompei, L., Giannetto, D., Lorenzoni, M., 2018. Range expansion of an invasive goby in the river Tiber Basin (Central Italy): Effects on the abundance and shelter occupancy of a congeneric native goby. Aquat. Invasions 13, 277–288. https://doi.org/10.3391/ai.2018.13.2.08

Pont, D., Meulenbroek, P., Bammer, V., Dejean, T., Erős, T., Jean, P., Lenhardt, M., Nagel, C., Pekarik, L., Schabuss, M., Stoeckle, B.C., Stoica, E., Zornig, H., Weigand, A., Valentini, A., 2023. Quantitative monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR. Mol. Ecol. Resour. 23, 396–409. https://doi.org/https://doi.org/10.1111/1755-0998.13715

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., Dejean, T., 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Sci. Rep. 8, 10361. https://doi.org/10.1038/s41598-018-28424-8

Pont, D., Valentini, A., Rocle, M., Maire, A., Delaigue, O., Jean, P., Dejean, T., 2021. The future of fish-based ecological assessment of European rivers: from traditional EU Water Framework Directive compliant methods to eDNA metabarcoding-based approaches. J. Fish Biol. 98, 354–366. https://doi.org/https://doi.org/10.1111/jfb.14176

Porco, D., Purnomo, C.A., Glesener, L., Proess, R., Lippert, S., Jans, K., Colling, G., Schneider, S., Stassen, R., Frantz, A.C., 2024. eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. BMC Ecol. Evol. 24, 1–16. https://doi.org/10.1186/s12862-023-02189-9

Reyjol, Y., Hugueny, B., Pont, D., Bianco, P.G., Beier, U., Caiola, N., Casals, F., Cowx, I., Economou, A., Ferreira, T., Haidvogl, G., Noble, R., De Sostoa, A., Vigneron, T., Virbickas, T., 2007. Patterns in species richness and endemism of European freshwater fish. Glob. Ecol. Biogeogr. 16, 65–75. https://doi.org/https://doi.org/10.1111/j.1466-8238.2006.00264.x

Ricci, L., Di Musciano, M., Sabatini, F.M., Chiarucci, A., Zannini, P., Gatti, R.C., Beierkuhnlein, C., Walentowitz, A., Lawrence, A., Frattaroli, A.R., Hoffmann, S., 2024. A multitaxonomic assessment of Natura 2000 effectiveness across European biogeographic regions. Conserv. Biol. 38, e14212. https://doi.org/https://doi.org/10.1111/cobi.14212

Roberts, P.D., Diaz-Soltero, H., Hemming, D.J., Parr, M.J., Wakefield, N.H., Wright, H.J., 2013. What is the evidence that invasive species are a significant contributor to the decline or loss of threatened species? A systematic review map. Environ. Evid. 2, 5. https://doi.org/10.1186/2047-2382-2-5

Robinson, C.V., Garcia de Leaniz, C., Rolla, M., Consuegra, S., 2019. Monitoring the eradication of the highly invasive topmouth gudgeon (Pseudorasbora parva) using a novel eDNA assay. Environ. DNA 1, 74–85. https://doi.org/https://doi.org/10.1002/edn3.12

Rodrigues, A.S.L., Cazalis, V., 2020. The multifaceted challenge of evaluating protected area effectiveness. Nat. Commun. 11, 5147. https://doi.org/10.1038/s41467-020-18989-2

Rutledge, D., 2003. Landscape indices as measures of the effects of fragmentation : can pattern reflect process ? DOC Sci. Intern. Ser. 98 1–27.

Sallustio, L., De Toni, A., Strollo, A., Di Febbraro, M., Gissi, E., Casella, L., Geneletti, D., Munafò, M., Vizzarri, M., Marchetti, M., 2017. Assessing habitat quality in relation to the spatial distribution of protected areas in Italy. J. Environ. Manage. 201, 129–137. https://doi.org/https://doi.org/10.1016/j.jenvman.2017.06.031

Sanders, E., Wassens, S., Michael, D.R., Nimmo, D.G., Turner, J.M., 2024. Extinction risk of the world's freshwater mammals. Conserv. Biol. 38, e14168. https://doi.org/https://doi.org/10.1111/cobi.14168

Sanz, N., Franch, N., Araguas, R.-M., Viñas, J., Vidal, O., 2023. Environmental DNA Assay for the Detection of the American Bullfrog (Lithobates catesbeianus) in the Early Stages of the Invasion in the Ebre Delta. Animals. https://doi.org/10.3390/ani13040683

Sarrocco S., Maio G., Celauro D., Tancioni L., 2012. Carta della Biodiversità ittica delle acque correnti del Lazio. Edizioni ARP, Roma, 194 pp.Scalisi, M., Monaco, A., Capizzi, D., Sinibaldi, I., Sarrocco, S., Pizzol I., 2011. Rete Ecologica Regionale (RecoRd Lazio): approccio metodologico e primi risultati. In: Bruschi M., Scalisi M., (a cura di), 2011. Verso un Piano per il Sistema delle aree protette del Lazio. Contributi ARP 2007-2010, Edizioni ARP, Roma; pp. 320.

Schmeller, D.S., 2008. European species and habitat monitoring: where are we now? Biodivers. Conserv. 17, 3321–3326. https://doi.org/10.1007/s10531-008-9514-1

Sepulveda, A.J., Dumoulin, C.E., Blanchette, D.L., McPhedran, J., Holme, C., Whalen, N., Hunter, M.E., Merkes, C.M., Richter, C.A., Neilson, M.E., Daniel, W.M., Jones, D.N., Smith, D.R., 2023. When are environmental DNA early detections of invasive species actionable? J. Environ. Manage. 343, 118216. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.118216

Seymour, M., Durance, I., Cosby, B.J., Ransom-Jones, E., Deiner, K., Ormerod, S.J., Colbourne, J.K., Wilgar, G., Carvalho, G.R., de Bruyn, M., Edwards, F., Emmett, B.A., Bik, H.M., Creer, S., 2018. Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Commun. Biol. 1, 4.

https://doi.org/10.1038/s42003-017-0005-3

Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: what's what and the way forward. Trends Ecol. Evol. 28, 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Solano, E., Hardersen, S., Audisio, P., Amorosi, V., Senczuk, G., Antonini, G., 2018. Asymmetric hybridization in Cordulegaster (Odonata: Cordulegastridae): Secondary postglacial contact and the possible role of mechanical constraints. Ecol. Evol. 8, 9657–9671. https://doi.org/10.1002/ece3.4368

Song, J.W., Small, M.J., Casman, E.A., 2017. Making sense of the noise: The effect of hydrology on silver carp eDNA detection in the Chicago area waterway system. Sci. Total Environ. 605–606, 713–720. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.06.255

Spear, D., Foxcroft, L.C., Bezuidenhout, H., McGeoch, M.A., 2013. Human population density explains alien species richness in protected areas. Biol. Conserv. 159, 137–147. https://doi.org/10.1016/j.biocon.2012.11.022

Spiliopoulou, K., Brooks, T.M., Dimitrakopoulos, P.G., Oikonomou, A., Karavatsou, F., Stoumboudi, M.T., Triantis, K.A., 2023. Protected areas and the ranges of threatened species: Towards the EU Biodiversity Strategy 2030. Biol. Conserv. 284, 110166. https://doi.org/https://doi.org/10.1016/j.biocon.2023.110166

Staccione, A., Brown, C., Arneth, A., Rounsevell, M., Hrast Essenfelder, A., Seo, B., Mysiak, J., 2023. Exploring the effects of protected area networks on the European land system. J. Environ. Manage. 337, 117741. https://doi.org/https://doi.org/10.1016/j.jenvman.2023.117741

Stoch F., Genovesi P., (ed.), 2016. Manuali per il monitoraggio di specie e habitat di interesse comunitario (Direttiva 92/43/CEE) in Italia: specie animali. ISPRA, Serie Manuali e linee guida, 141/2016.

Taberlet, P., Bonin, A., Zinger, L., Coissac, E. 2018. Environmental DNA: For biodiversity research and monitoring. Oxford University Press.

Tancioni, L., Russo, T., Cataudella, S., Milana, V., Hett, A.K., Corsi, E., Rossi, A.R., 2013. Testing Species Delimitations in Four Italian Sympatric Leuciscine Fishes in the Tiber River: A Combined Morphological and Molecular Approach. PLoS One 8, 1–10. https://doi.org/10.1371/journal.pone.0060392

Thalinger, B., Deiner, K., Harper, L.R., Rees, H.C., Blackman, R.C., Sint, D., Traugott, M., Goldberg, C.S., Bruce, K., 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environ. DNA 3, 823–836. https://doi.org/10.1002/edn3.189

Thieme, M.L., Rudulph, J., Higgins, J., Takats, J.A., 2012. Protected areas and freshwater conservation: A survey of protected area managers in the Tennessee and Cumberland River Basins, USA. J. Environ. Manage. 109, 189–199. https://doi.org/https://doi.org/10.1016/j.jenvman.2012.06.021

Thomas, A.C., Tank, S., Nguyen, P.L., Ponce, J., Sinnesael, M., Goldberg, C.S., 2020. A system for rapid eDNA detection of aquatic invasive species. Environ. DNA 2, 261–270. https://doi.org/https://doi.org/10.1002/edn3.25

Thomsen, P.F., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573. https://doi.org/https://doi.org/10.1111/j.1365-294X.2011.05418.x

Tóthová, D., Heglasová, M., 2022. Measuring the environmental sustainability of 2030 Agenda implementation in EU countries: How do different assessment methods affect results? J. Environ. Manage. 322, 116152. https://doi.org/https://doi.org/10.1016/j.jenvman.2022.116152

Tsuji, S., Iguchi, Y., Shibata, N., Teramura, I., Kitagawa, T., Yamanaka, H., 2018. Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese medaka species. Sci. Rep. 8, 9138. https://doi.org/10.1038/s41598-018-27434-w

Valentim, H.I.L., Feio, M.J., Almeida, S.F.P., 2025. Assessing the effectiveness of terrestrial protected areas towards riverine ecosystems. Sci. Total Environ. 958, 177878. https://doi.org/https://doi.org/10.1016/j.scitotenv.2024.177878

Villacorta-Rath, C., Hoskin, C.J., Strugnell, J.M., Burrows, D., 2021. Long distance (>20 km) downstream detection of endangered stream frogs suggests an important role for eDNA in surveying for remnant amphibian populations. PeerJ 9, e12013. https://doi.org/10.7717/peerj.12013

Weigelhofer, G., Feldbacher, E., Trauner, D., Pölz, E., Hein, T., Funk, A., 2020. Integrating Conflicting Goals of the EC Water Framework Directive and the EC Habitats Directives Into Floodplain Restoration Schemes. Front. Environ. Sci. 8, 1–14. https://doi.org/10.3389/fenvs.2020.538139

Wilcox, T.M., McKelvey, K.S., Young, M.K., Sepulveda, A.J., Shepard, B.B., Jane, S.F., Whiteley, A.R., Lowe, W.H., Schwartz, M.K., 2016. Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char Salvelinus fontinalis. Biol. Conserv. 194, 209–216. https://doi.org/https://doi.org/10.1016/j.biocon.2015.12.023

Wozney, K.M., Wilson, C.C., 2017. Quantitative PCR multiplexes for simultaneous multispecies detection of Asian carp eDNA. J. Great Lakes Res. 43, 771–776. https://doi.org/https://doi.org/10.1016/j.jglr.2017.05.001

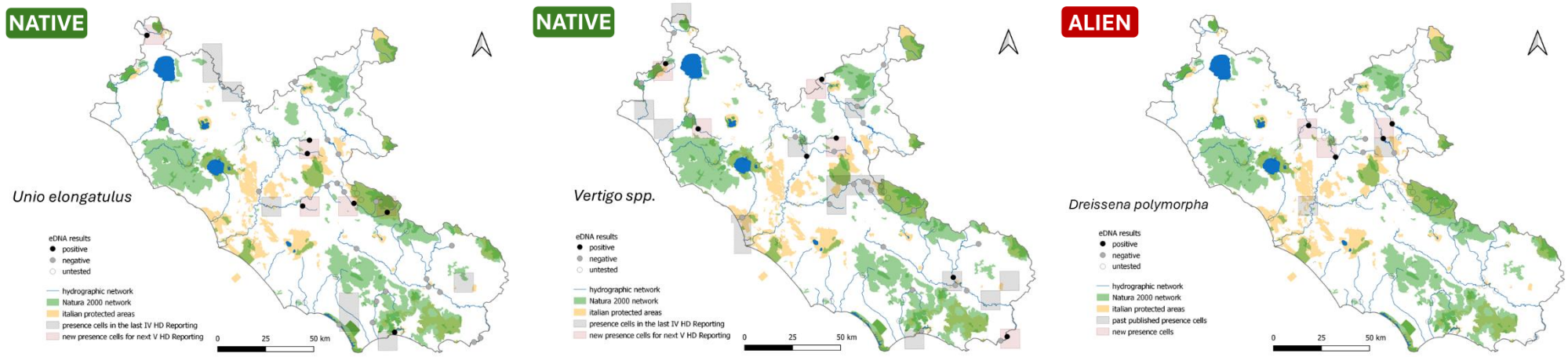
Xia, Z., Zhan, A., Johansson, M.L., DeRoy, E., Haffner, G.D., MacIsaac, H.J., 2021. Screening marker sensitivity: Optimizing eDNA-based rare species detection. Divers. Distrib. 27, 1981–1988. https://doi.org/https://doi.org/10.1111/ddi.13262

Zampiglia, M., Canestrelli, D., Chiocchio, A., Nascetti, G., 2013. Geographic distribution of the chytrid pathogen Batrachochytrium dendrobatidis among mountain amphibians along the Italian peninsula. Dis. Aquat. Organ. 107, 61–68. https://doi.org/10.3354/dao02655

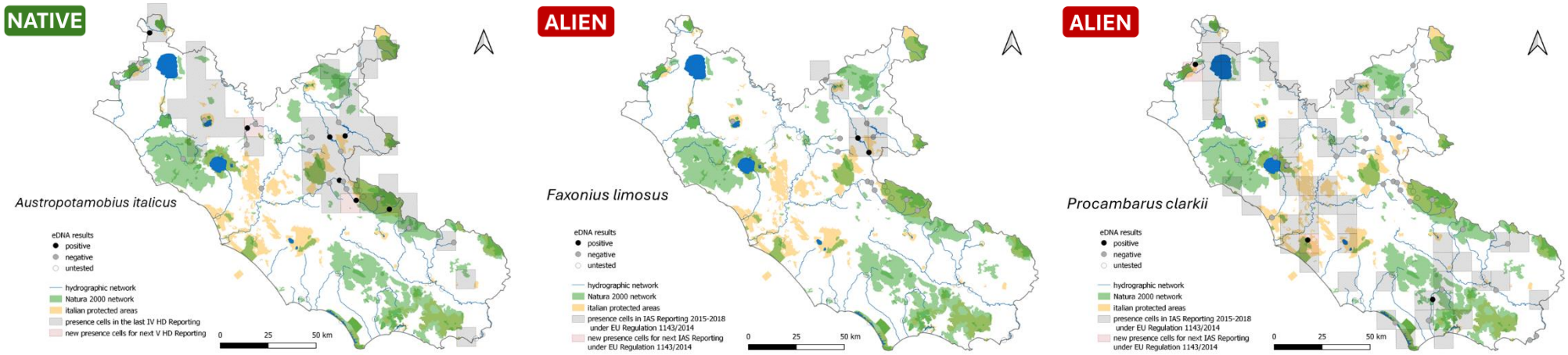
Zettler, M.L., Jueg, U., 2007. The situation of the freshwater mussel Unio crassus (Philipsson, 1788) in north-east Germany and its monitoring in terms of the EC Habitats Directive. Mollusca 25, 165–174.

Supplementary materials

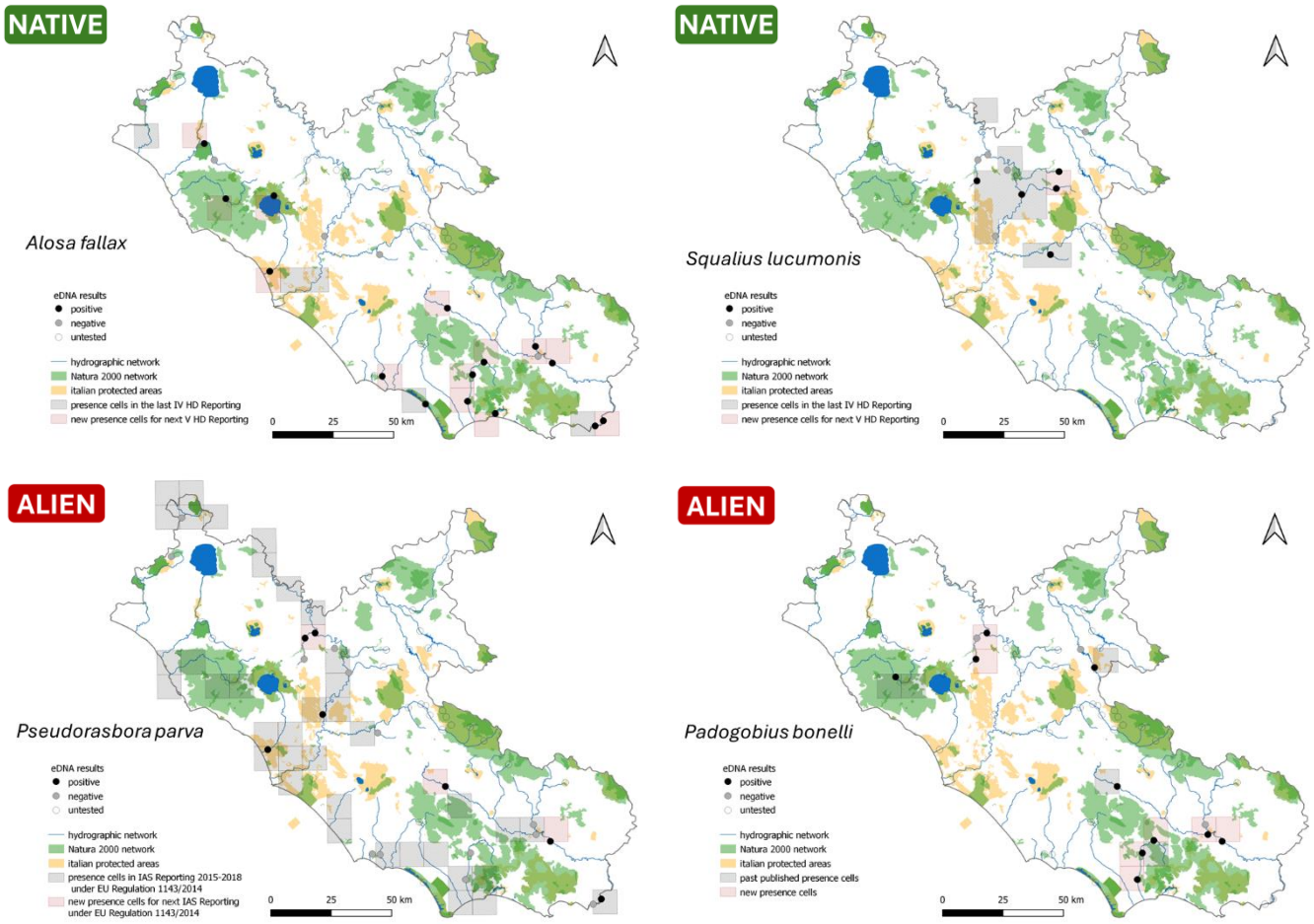
Supplementary material S1. Molluscs – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).



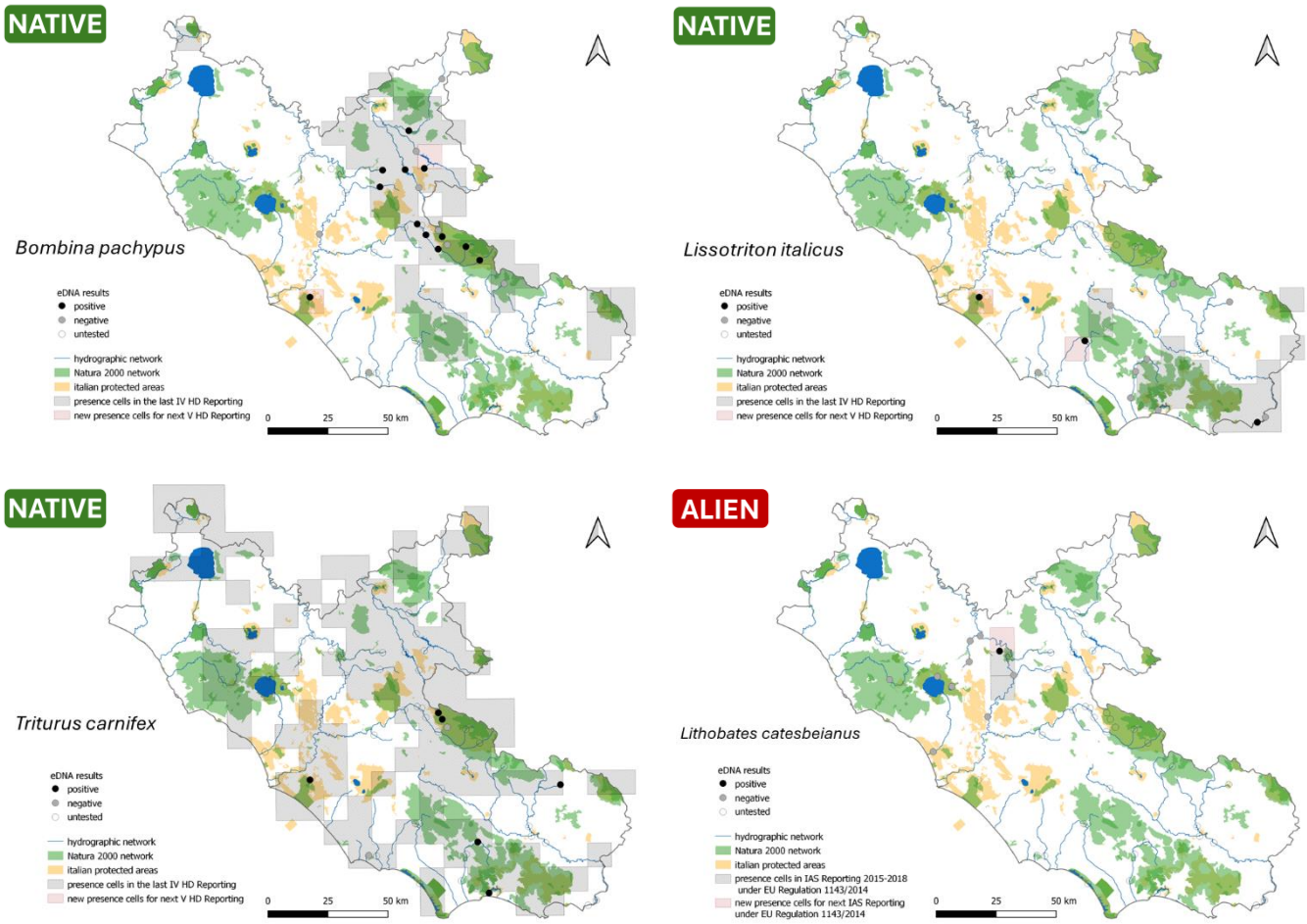
Supplementary material S2. Crustaceans – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).



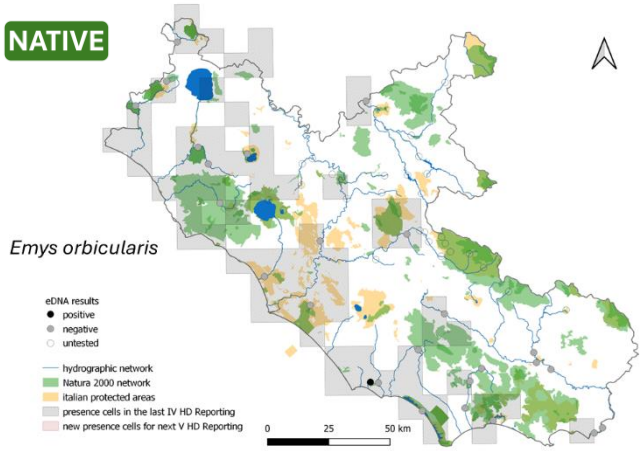
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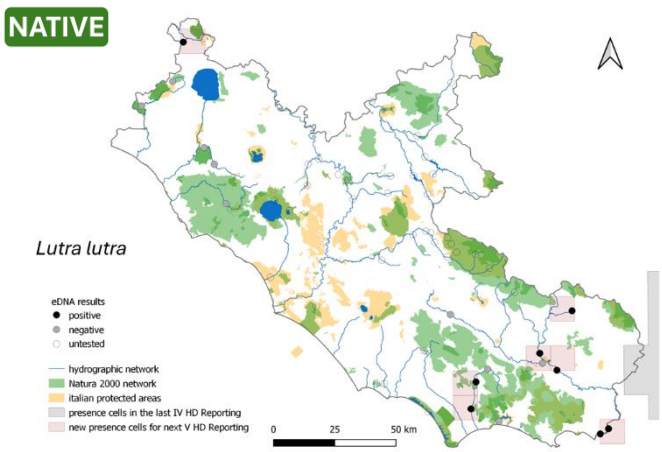
Supplementary material S4. Amphibians – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).

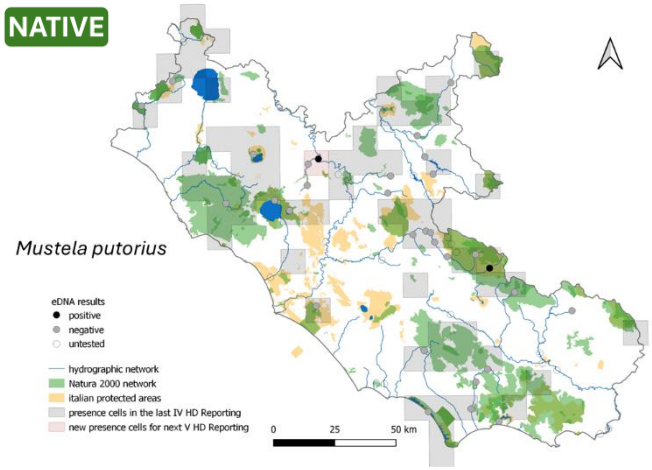


Supplementary material S5. Reptiles – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).

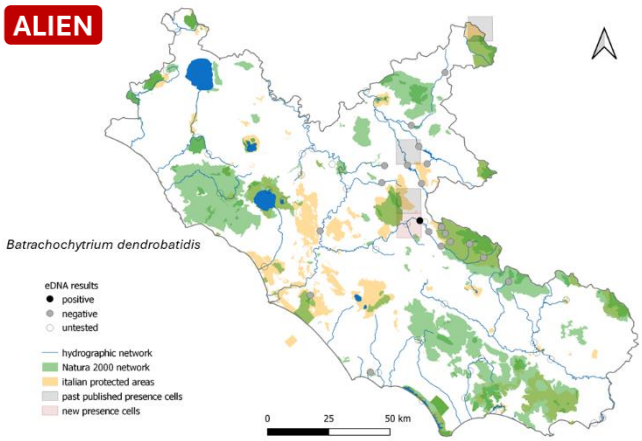


Supplementary material S6. Mammals – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).





Supplementary material S7. Fungi – Distribution of eDNA occurrences in the study area of Latium region, and new presence cells according to the last HD and EU IAS Regulation Reporting or other published surveys (see main document).



Supplementary material S8. Habitats Directive species found outside Natura 2000 Network. Coordinates provided in WGS84 EPSG: 4326 georeferenced system.

Longitude	Latitude	Locality	HD Annex II species (N)	HD species (N)
13.5979139	41.4875261	San Giovanni Incarico	2	2
13.5186841	41.5528575	Ceprano	3	3
12.7532441	41.4688748	Latina	1	1
13.8336374	41.2626197	Santi Cosma e Damiano	3	3
13.792098	41.245147	Santi Cosma e Damiano	2	3
12.9416922	42.2282542	Rocca Sinibalda	2	2
12.8282994	42.2302169	Monteleone Sabino	3	4
12.8109656	42.1679005	Scandriglia	2	3
12.636599	42.151386	Fiano Romano	2	2
12.9899387	42.0220295	Roviano	2	2
13.0891749	41.9241672	Subiaco	2	3
12.216322	41.876915	Fiumicino	1	1
13.091545	41.711616	Anagni	1	1
12.766916	41.922676	Roma	1	2
12.4153373	42.208807	Mazzano Romano	1	1
12.4239651	42.2869587	Civita Castellana	1	1
11.9590775	42.2992023	Vetralla	1	1
11.8259389	42.7520841	Proceno	2	3
13.1694668	41.3615092	Pontinia	2	2
13.2602414	41.5039698	Prossedi	2	2
13.2003796	41.4594335	Roccasecca dei Volsci	2	2
13.0387416	42.2296686	Marcetelli	2	2
13.031233	41.9797335	Agosta	1	1

Supplementary material S9. Habitats Directive species found within Natura 2000 Network sites but not reported in the relative Standard Data Forms. Coordinates provided in WGS84 EPSG: 4326 georeferenced system.

Longitude	Latitude	Natura 2000 Network site	Annex II species (N)	HD species (N)	n. IAS species (N)	IAS species
11.9116101	42.3622037	IT6010021 - Monte Romano	1	1	0	-
12.4361753	41.7647898	IT6030084 - Castel Porziano (Tenuta presidenziale)	1	1	1	*P. clarkii*
13.6874171	41.7025594	IT6050015 - Lago di Posta Fibreno	1	1	0	-
12.7531269	42.5079872	IT6020010 - Lago di Ventina	1	1	0	-
12.2513676	42.1583449	IT6030010 - Lago di Bracciano	1	1	0	-
12.969484	42.373852	IT6020012 - Piana di S. Vittorino - Sorgenti del Peschiera	1	1	0	-
11.7668453	42.6093847	IT6010012 - Lago di Mezzano	1	1	1	*P. clarkii*

12.961372	41.358138	IT6040012 - Laghi Fogliano, Monaci, Caprolace e Pantani dell'Inferno	1	1	0	-
13.303556	41.310775	IT6040010 - Lago di Fondi	1	2	0	-

Supplementary material S10. Landscape metrics applied to 23 Annex II areas generated from the detections of Annex II species found outside Natura 2000 network.

ID Annex II area	number of patches	respective site ID	cumulative length of stretches out of N2KN [m]	cumulative length of stretches in the area [m]	patch density	mean NND	sd NND
1	1	F5.81	11408	11408	1	0	0
2	1	F5.80	11638	20531	0.57	0	0
3	2	F5.77	69040	80427	0.86	901	0
3.1	1	F5.03	15859	15859	1	0	0
4	1	F4.24	21097	21097	1	0	0
5	1	ST109	2425	2425	1	0	0
6	3	F4.09	63646	77311	0.82	15486	2463.56002
7	1	F3.75	16791	16791	1	0	0
8	1	F3.79	16912	16912	1	0	0
9	1	F3.72	13395	13395	1	0	0
10	1	ST105	2020	2020	1	0	0
11	1	F4.89	8606	8606	1	0	0
12	1	F4.14	38687	42419	0.91	0	0
13	3	F4.15	7053	39802	0.18	445	0
14	1	F4.78	39079	39393	0.99	0	0
15	1	F2.29	43705	43705	1	0	0
16	9	SD3	30652	57111	0.54	6162	609.663763
18	1	F2.77	48023	48023	1	0	0
19	1	EXNOVO1	48646	48646	1	0	0
20	2	F1.71	78416	81034	0.97	2231	584.777308
21	1	F1.74	59111	59111	1	0	0
22	3	SD2	20772	24481	0.85	2565	460.925156
23	4	SD4	29389	38325	0.77	6152	846.239524

x	1.82		30276.9565	35166.6087	0.88910866	1475.73913	-
sd	1.8		22422.8281	24299.1141	0.20603167	3555.47221	-
sum	42		696370	808832	-	-	-

Supplementary material S11. Landscape metrics applied to 7 Annex II areas generated from the detections of Annex II species found within Natura 2000 network but not already reported in respective standard data forms.

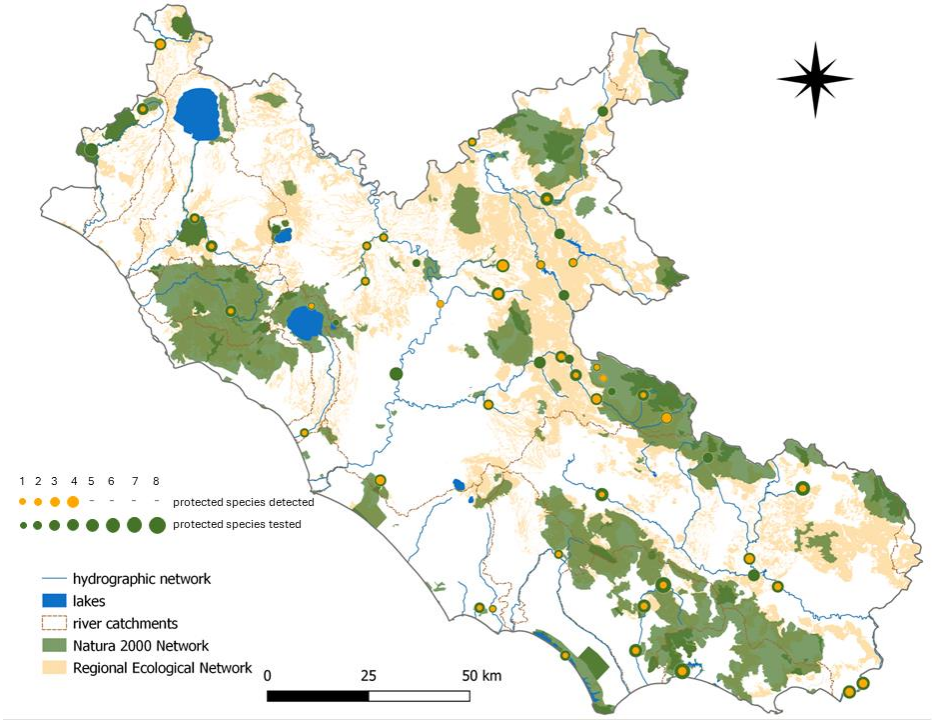
ID Annex II area	number of patches	respective site ID	cumulative length of stretches out of N2KN [m]	cumulative length of stretches in the area [m]	patch density	mean NND	sd NND
1	6	F5.82	82743	93395	0.89	2132	1372
2	7	L4.26	5329	71289	0.07	8986	3201
3	1	NOARPA1	12404	24342	0.51	5600	0
4	1	GEO.AI.4	46505	49586	0.94	-	0
5	1	T2.21A	1148	30153	0.04	8346	0
6	4	T2.23A	23110	44022	0.52	9692	1162
7	1	L3.39	1319	3421	0.39	1958	707
x	3		24651.14	45172.57	0.48	6119	-
sd	2.645751		30206.94	30085.9	0.352556	3905.515	-
sum	21		172558	316208	-	-	-

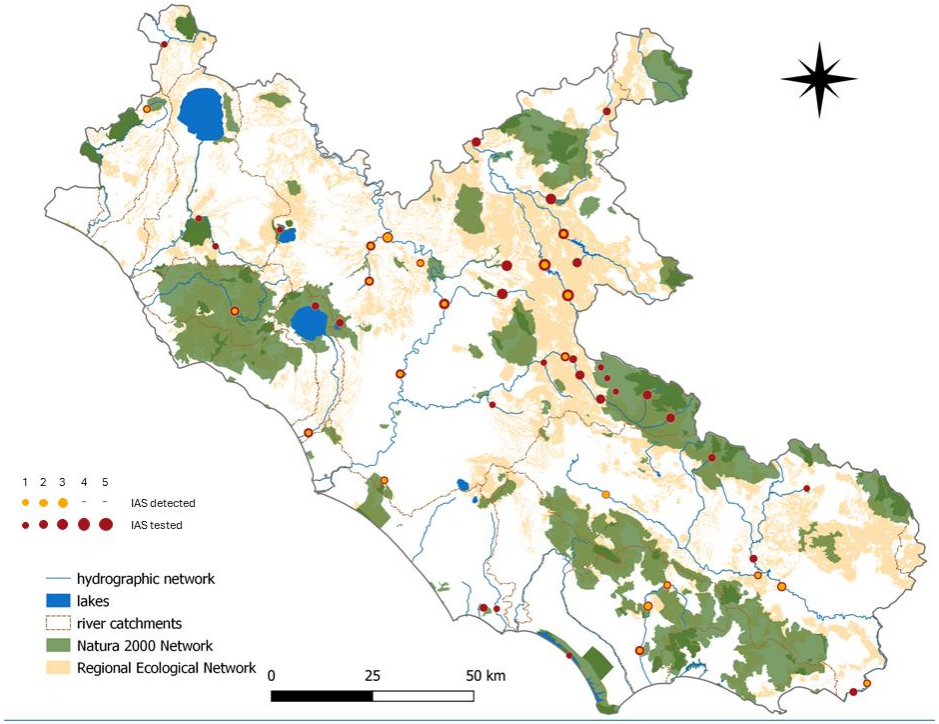
Supplementary material S12. Index NAP calculated to assess the difference between two ratios of detection over tested species among protected species and IAS every sampling site.

ID sampling site	protected species detected (PSD)	protected species tested (PST)	IAS detected (IASD)	IAS tested (IAST)	PSD/PST (N_r)	IASD/IAST (A_r)	$N_r - A_r$ (NAP)
EX NOVO 1	3	6	1	2	0.5	0.5	0
F1.34	0	5	1	2	0	0.5	-0.5
F1.70	0	4	0	2	0	0	0
F1.71	2	5	2	3	0.4	0.66	-0.26
F1.74	3	5	0	2	0.6	0	0.6
F2.29	1	2	0	1	0.5	0	0.5
F2.35	1	3	0	0	0.33	0	0.33
F2.77	3	6	0	2	0.5	0	0.5
F3.52	0	4	2	5	0	0.4	-0.4
F3.71	0	4	1	4	0	0.25	-0.25
F3.72	2	3	2	5	0.66	0.4	0.26
F3.75	4	6	0	4	0.66	0	0.66
F3.79	3	6	0	4	0.5	0	0.5
F4.09	2	2	1	4	1	0.25	0.75
F4.14	2	5	1	3	0.4	0.33	0.66
F4.15	3	5	0	3	0.6	0	0.6
F4.24	1	3	1	3	0.33	0.33	0
F4.68	0	5	0	1	0	0	0
F4.72	4	5	0	3	0.8	0	0.8
F4.78	1	6	2	2	0.16	1	-0.83
F4.83	0	3	0	2	0	0	0
F4.84	0	6	1	3	0	0.33	-0.33
F4.89	2	4	0	1	0.5	0	0.5
F4.93	1	5	1	3	0.2	0.33	-0.13
F4.98	1	5	0	3	0.2	0	0.2
F5.03	1	3	1	3	0.33	0.33	0
F5.77	1	3	1	3	0.33	0.33	0
F5.80	1	5	0	1	0.2	0	0.2
F5.81	3	5	0	1	0.6	0	0.6
F5.82	1	4	0	1	0.25	0	0.25
F5.83	1	3	3	4	0.33	0.75	-0.42
GEO.AI.1	1	2	0	1	0.5	0	0.5
GEO.AI.2	2	2	0	1	1	0	1
GEO.AI.3	0	2	0	1	0	0	0
GEO.AI.4	3	5	1	2	0.6	0.5	0.1
GEO.AI.5	1	4	0	2	0.25	0	0.25
L3.39	2	7	0	1	0.28	0	0.28
L3.40	1	3	0	3	0.33	0	0.33
L4.26	1	2	0	2	0.5	0	0.5

L4.27	0	1	0	2	0	0	0
L5.34	0	3	0	1	0	0	0
NO ARPA1	1	6	0	4	0.16	0	0.16
noname	1	5	1	2	0.2	0.5	-0.3
S.D.1	0	6	0	0	0	0	0
S.D.2NUOVO	2	6	1	3	0.33	0.33	0
S.D.3	2	8	1	2	0.25	0.5	-0.25
S.D.4	2	6	2	3	0.33	0.66	-0.33
ST103 NUOVO	0	4	0	2	0	0	0
ST105	2	3	0	3	0.66	0	0.66
ST109	0	2	1	2	0	0.5	-0.5
ST111	1	5	0	3	0.2	0	0.2
T2.21A	1	4	0	1	0.25	0	0.25
T2.23A	3	8	0	0	0.375	0	0.375

Supplementary material S13. Details on locations of detections over tested sites for native protected species according to Habitats Directive (left) and IAS of Union Concern (right). composing N_r and A_r parameters of the index NAP.





Supplementary material S14. Index of Native Alien Prevalence (NAP_f) calculated on fish species related to Water Framework Directive (WFD) to assess the difference between two ratios of detection over tested species among native fish (NF_r) and alien fish (AF_r) at each sampling site.

ID sampling site	native WFD fish species detected (NWFD)	native WFD fish species tested (NWFT)	alien WFD fish species detected (AWFD)	alien WFD fish species tested (AWFT)	NWFD / NWFT (NF_r)	AWFD / AWFT (AF_r)	$NF_r - AF_r$ (NAP_F)
EX NOVO 1	1	1	1	1	1	1	0
F1.34	0	1	1	2	0	0.5	- 0.5
F1.71	1	1	2	2	1	1	0
F1.74	1	1	0	2	1	0	1
F2.29	1	1	0	1	1	0	1
F2.77	1	1	0	1	1	0	1
F3.52	0	0	1	1	0	1	-1
F3.72	0	0	0	1	0	0	0
F3.75	1	1	0	0	1	0	1
F3.79	1	1	0	0	1	0	1
F4.09	1	1	0	1	1	0	1
F4.14	0	0	0	0	-	-	-
F4.15	0	0	0	0	-	-	-
F4.24	1	1	1	1	1	1	0
F4.72	0	0	0	0	-	-	-
F4.78	1	1	2	2	1	1	0
F4.84	0	2	1	1	0	1	-1
F4.89	1	2	0	1	0.5	0	0.5
F4.93	1	1	1	1	1	1	0
F5.03	1	1	1	2	1	0.5	0.5
F5.77	0	1	1	2	0	0.5	- 0.5
F5.80	0	1	0	0	0	0	0
F5.81	0	0	0	1	0	0	0
F5.82	1	1	0	0	1	0	1
F5.83	0	1	2	2	0	1	-1
GEO.AI.5	0	0	0	1	0	0	0
L4.26	1	1	0	0	1	0	1
NO ARPA1	0	1	0	0	0	0	0
noname	0	0	0	1	0	0	0
S.D.1	0	1	0	0	0	0	0
S.D.2NUOVO	1	1	1	2	1	0.5	0.5
S.D.3	1	1	1	1	1	1	0
S.D.4	1	1	1	2	1	0.5	0.5
ST105	0	0	0	0	-	-	-
ST109	0	1	0	1	0	0	0
T2.21A	1	1	0	0	1	0	1
T2.23A	1	1	0	0	1	0	1

Supplementary material S15. Table of scoring values for establishing new Natura 2000 sites. planning eradication strategies on IAS. and proposing new areas for species protection pursuant to WFD. HD= Habitats Directive. WFD= Water Framework Directive. N2KN= Natura 2000 network. REN= Regional Ecological Network. STAR_ICMi= index of WFD to assess microbenthic wildlife. NISECI= index of WFD to assess fish communities.

		new Natura 2000 sites (HD)				IAS eradication planning (Reg. on IAS 1143/2014)				new Species protection areas (WFD)						
sampling site ID	index NAP	outside/not reported in N2KN	patch density	REN	scoring for new N2KN sites (PPS – Prioritization Protection Score)	within N2KN	direct threat at site	Potential threat in river catchment	scoring for IAS eradication (PES – Prioritization Eradication Score)	WFD benthic species detected (A)	2021-23 WFD benthic classification and scoring (STAR_ICMi) (B)	NAP_F (C)	2028-20 WFD fish classification and scoring (NISECI) (D)	WFD macrobenthos score (A*B) (E)	WFD fish score (C*D+0.1) (F)	scoring for new WFD species protection areas (E+F) (FCS – Freshwater Conservation Score)
EX NOVO 1	0	2	1	1	3	1	0	1	2	0	-	0	2	-	0.2	0.2
F1.34	-0.5	-	-	-	-	0	0	1	1.5	0	-	-0.5	2	-	-1.2	-1.2
F1.70	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F1.71	-0.26	2	0.97	1	2.74	0	0	0	0.26	0	-	0	3	-	0.3	0.3
F1.74	0.6	2	1	1	3.6	-	-	-	-	0	-	1	-	-	-	-
F2.29	0.5	2	1	0	2.5	-	-	-	-	0	-	1	3	-	3.3	3.3
F2.35	0.33	0	-	1	1.33	-	-	-	-	-	-	-	-	-	-	-
F2.77	0.5	2	1	0	2.5	-	-	-	-	0	-	1	2	-	2.2	2.2
F3.52	-0.4	-	-	-	-	0	0	0	0.4	0	-	-1	3	-	-1.2	-1.2
F3.71	-0.25	-	-	-	-	0	0	1	1.25	-	-	-	-	-	-	-
F3.72	0.26	2	1	1	3.26	0	2	2	3.74	1	1.5	0	3	1.5	1.8	3.3
F3.75	0.66	2	1	1	3.66	-	-	-	-	1	-	1	2	-	2.2	2.2
F3.79	0.5	2	1	1	3.5	-	-	-	-	1	-	1	3	-	3.3	3.3
F4.09	0.75	2	0.82	0	2.75	0	0	1	0.25	0	-	1	3	-	3.3	3.3
F4.14	0.66	2	0.91	1	3.66	0	2	3	4.34	1	-	-	-	-	-	-
F4.15	0.6	2	0.18	1	3.6	-	-	-	-	2	-	-	-	-	-	-
F4.24	0	2	1	0	2	0	0	0	0	0	-	0	3	-	0.3	0.3
F4.68	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F4.72	0.8	0	-	1	1.8	-	-	-	-	2	-	-	-	-	-	-
F4.78	-0.83	2	0.99	0	1.17	0	0	1	1.83	0	-	0	2	-	1.2	1.2
F4.83	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F4.84	-0.33	-	-	-	-	0	0	1	1.33	0	-	-1	3	-	-1.2	-1.2

F4.89	0.5	2	1	1	3.5	-	-	-	-	1	1.5	0.5	-	1.5	-	1.5
F4.93	-0.13	0	-	1		1	0	0	1.13	0	-	0	3	-	0.3	0.3
F4.98	0.2	0	-	1	1.2	-	-	-	-	-	-	-	-	-	-	-
F5.03	0	2	1	1	3	0	0	0	0	0	-	0.5	2	-	1.2	1.2
F5.77	0	2	0.86	1	3	0	0	1	1	1	3	-0.5	2	3	-1.2	1.8
F5.80	0.2	2	0.57	1	3.2	-	-	-	-	0	-	0	3	-	0.3	0.3
F5.81	0.6	2	1	1	3.6	-	-	-	-	2	-	0	3	-	0.3	0.3
F5.82	0.25	1	0.89	1	2.25	-	-	-	-	0	-	1	3	-	3.3	3.3
F5.83	-0.42	-	-	-	-	0	0	2	2.42	0	-	-1	2	-	-2.2	-2.2
GEO.AI.1	0.5	0	-	0	0.5	-	-	-	-	-	-	-	-	-	-	-
GEO.AI.2	1	0	-	0	1	-	-	-	-	-	-	-	-	-	-	-
GEO.AI.3	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GEO.AI.4	0.1	1	0.94	0	1.1	1	0	2	2.9	-	-	-	-	-	-	-
GEO.AI.5	0.25	0	-	1	1.25	-	-	-	-	0	-	0	1	-	0.1	0.1
L3.39	0.28	1	0.39	0	1.28	-	-	-	-	-	-	-	-	-	-	-
L3.40	0.33	1	-	1	2.33	-	-	-	-	-	-	-	-	-	-	-
L4.26	0.5	1	0.07	0	1.5	-	-	-	-	0	-	1	2	-	2.2	2.2
L4.27	0	-	-	-	-	1	0	0	1	-	-	-	-	-	-	-
L5.34	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO ARPA1	0.16	1	0.51	1	2.16	-	-	-	-	0	-	0	3	-	0.3	0.3
noname	-0.3	1	-	0	0.7	1	2	0	3.3	0	-	0	3	-	0.3	0.3
S.D.1	0	-	-	-	-	-	-	-	-	0	-	0	2	-	0.2	0.2
S.D.2NUOVO	0	2	0.85	0	2	0	0	0	0	0	-	0.5	3	-	1.8	1.8
S.D.3	-0.25	2	0.54	1	2.75	0	0	0	0.25	0	-	0	3	-	0.3	0.3
S.D.4	-0.33	2	0.77	1	2.67	0	0	0	0.33	0	-	0.5	3	-	1.8	1.8
ST103 NUOVO	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ST105	0.66	2	1	1	3.66	-	-	-	-	1	1.5	-	-	1.5	-	1.5
ST109	-0.5	2	1	1	2.5	0	0	3	3.5	0	-	0	2	-	0.2	0.2
ST111	0.2	0	-	0	0.2	-	-	-	-	-	-	-	-	-	-	-
T2.21A	0.25	1	0.04	0	1.25	-	-	-	-	0	-	1	3	-	3.3	3.3
T2.23A	0.375	1	0.52	0	1.375	-	-	-	-	1	1.5	1	-	1.5	-	1.5

Chapter 4 – Comparing traditional vs environmental DNA monitoring protocols. The case of the Eurasian otter (*Lutra lutra*) re-expansion in Central Italy.

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Abstract

Once widespread throughout Italy, the Eurasian otter (*Lutra lutra*) went on the brink of extinction in the last century, surviving in few river basins in southern Italy and classified as Critically Endangered in the national Red List. Since the beginning of this century, legal protection and banning of pollutants allowed a slow but constant recovery of the species in Europe, where the species is protected by the Habitats Directive 92/43/EEC, making mandatory the surveillance of its conservation status over time. We compared the efficiency of environmental DNA (eDNA) and traditional field survey techniques in detecting otters at the border of its current expansion range in Central Italy. A systematic standard field survey was and quantitative PCR-based eDNA sampling were run in six river basins of the Latium region. Results from eDNA and traditional field survey were compared at the scale of i) sampling site, ii) 5 km and iii) 10 km radius upstream. Field survey allowed to detect otter signs at 23 sites in one river catchment in the southern part of the study area. Both eDNA and standard survey allowed to detect otters at seven sites in southern catchments and one located northward in a tributary of the river Tiber. Both techniques confirmed otter absence in northern Latium. eDNA showed a higher detection rate (0.41) compared to the standard field survey, both at sampling site (0.06), 5 km (0.23) or 10 km radius (0.35) scale. By excluding sites negative to both eDNA and standard survey, eDNA detection rate was significantly different at sampling site scale only (p=0.01). These results suggest eDNA a more sensitive method in revealing otter presence.

Introduction

Once spread all over Europe, the Eurasian otter (*Lutra lutra,* Linnaeus, 1758) suffered a severe decline in the last century due to direct persecution, habitat loss and water pollution (Loy et al., 2022). The adoption of legal protection of both the species and freshwater habitats fostered the otter recover in many European countries (Roos et al., 2012; Stockholm Convention 2001). Among these, as almost a unique exception, in Italy this recovering appeared to be attenuated as it happened very slowly and delayed (Panzacchi et al. 2011). In the '80s the species survived in southern Italy with a unique viable population, isolated from the other European populations, and showing a clear genetic distinctiveness as to be believed as an ESU (Evolutionarily Significant Unit) (Cassola 1986; S. M. Macdonald & Mason, 1983; Mucci et al., 2010; Randi et al., 2003). Recently Eurasian otters boosted its range in the southern parts of Italy and along the Adriatic side of the peninsula (Giovacchini et al. 2018; Loy et al. 2023; Marcelli et al., 2023) whereas we can also witness some colonisations in the northern part of Italy with individuals coming from other transboundary European

populations (Maltieux et al. 2020; Marcolin et al., 2020). This retrieval of the former extent of occurrence led to downgrade the conservation status of the otter in Italy to Vulnerable in the Italian Red List (Loy et al., in press; Rondinini et al., 2022). Still the core otter range is confined in the cul-de-sac of southern Italy at the point to define this ESU as the otter "southern-central population" of Italy (Giovacchini et al. 2021). As otters are reclaiming their ancient territories returning along the peninsula from south northward, there is currently a gap of knowledge on otter distribution along the Tyrrhenian side of Central regions. The expected expansion was not witnessed in the recent time (Giovacchini et al. 2019), maybe because of cryptical presence when at low densities or due to inaccessible harsh environments (Jamwal et al. 2023).

Otter distribution is typically surveyed using a standard field monitoring protocol (Kruuk 2006), involving the recording of presence signs like scats, anal jellies, and footprints, along riverbanks and lake shorelines. This technique is simple but highly time-consuming and requires sampling expertise, field skills, and appropriate condition to implement transect lines (Jeffress et al. 2011). Moreover, weather (i.e. rain in days before sampling) and local conditions like river morphology or dense vegetation along the banks prevent the access to the water body or affect the probability to detect tracks of the species. These limitations have been long debated for the degree they can affect survey results and lead to false-absence data, especially in areas where otters occur at very low density (Jefferies 1986; Kruuk et al., 1998; Kruuk & Conroy, 1987; Mason & Macdonald, 1987; Parry et al., 2013; Romanowski et al., 1996).

Environmental DNA (eDNA) represents a cost-effective novel technique that could support traditional species survey (Biggs et al., 2015; Keck et al., 2022), and is particularly accurate for revealing rare and elusive species (Beng and Corlett 2020; Mynhardt et al. 2024; Johnson et al. 2024). Specifically, the quantitative PCR (qPCR) approach to eDNA represents a high sensitive species-specific resolution, more accurate than other common approach like eDNA metabarcoding (Harper et al., 2018; McColl-Gausden et al., 2023). The use of quantitative PCR-based eDNA is particularly helpful in detecting endangered species outside their current range, as the low expected population densities make traditional methods time consuming and often unsuccessful, whereas an early detection allows the rapid adoption of protection measures to support the newly established populations (Duarte et al., 2023). A qPCR based eDNA has been already successfully applied to detect the occurrence of the Eurasian otter in Italy and in Himalaya (Ballini et al., 2024; Jamwal et al., 2021, 2023). However, these studies did not evaluate the relative efficiency of the eDNA vs traditional field survey.

To fill this gap, we run an integrative sampling using both eDNA and traditional field survey to detect the Eurasian otter at the boundary of its current range in south-central Italy, specifically aimed at i) assessing new expansion events along the Tyrrhenian side of the peninsula, ii) comprehend if eDNA is a profitable monitoring tool to detect the species in new areas of occurrence compared to the traditional techniques.

Methods

Study area

The study area includes seven river basins located in Central Italy, including the Tiber, the main river basin of Central Italy (405 km of length), the river Liri (158 km) and five smaller river catchments (Amaseno, Mignone, Marta, and Fiora), mainly located nearby the coast. To perform cost effective analyses, sampling sites were selected based upon the following criteria: i) areas adjacent to the current species range ii) high habitat suitability for the species iii) high accessibility and walkability of the river, iv) sites monitored over time by previous projects, v) sites with environmental features increasing the probability of detecting otters (e.g. river confluences).

Otter habitat suitability was defined through an expert based modelling approach (Loy et al., 2009) based on results from multiple inferential models produced at different scales on the Eurasian otter in Italy and Europe (Carone et al., 2012, 2014; Cianfrani et al., 2011, 2013; Weinberger et al., 2019). Accordingly, we considered river networks ranging from the first to the fourth order (Strahler 1974), a 10 m-scale digital elevation model with altitude and slope (Tarquini et al. 2023), and the riverine vegetation cover finely mapped at 10 m-scale

(De Fioravante et al., 2022). Each variable was reclassified following an expert based criterium (Suppl. Mat. S1). Results of the model is shown in Suppl. Mat. S2.

Environmental DNA sampling and laboratory procedures

Three lithers of water were collected at 17 sampling sites, with two replicates performed on July-August 2021 and May-June 2022. Water samples were prefiltered though a cellulose mixed esters 8 µm, and then 0.22 µm filters (Merck-Millipore, Burlington, USA) using an electric vacuum pump. eDNA was then extracted from filters using Power Soil Pro extraction kits (Qiagen, Venlo, NL), diluted in 75 µL bidistilled Miili-Q sterile water and preserved at -21 °C. Correct eDNA extraction from samples was verified with a MiFish set of universal 12S rRNA oligonucleotides for fish characterization using DNA metabarcoding (Miya et al., 2015). Finally, we amplified eDNA through quantitative real-time PCR (StepOnePlus, Applied Biosystem, Waltham, USA) using Eurasian otter species-specific primers (Park et al., 2011) with SybrGreen technology. We composed the reaction mix with 2.35 µl of Milli-Q water, 5 µL of buffer (Eurofins Genomics srl, Hamburg, DE), 0.25 µL of otter primers (10 µM), and 2 µL of extracted DNA. We run samples in two dilutions (1:1, and 1:10) and three technical replicates, with a PCR positive control using genomic DNA of Eurasian otter, a negative technical control using non-otter DNA to ensure absence of contamination, and an environmental positive and negative control (i.e., water samples collected in sites of verified presence and absence of Eurasian otter) (Karlsson et al., 2022; Klymus et al., 2020; Thalinger et al., 2021). Pre and post amplification phases were carried out in separate rooms on a laminar flow cabinet to avoid contamination. We adopted the thermal profile with an initial denaturation at 95 °C for 10 s, followed by 40 cycles with a denaturation step at 95 °C for 15 s, and an annealing-elongation phase at 58 °C for 1 min. Positive samples were considered as such when observed at least in two technical replicates, above the limit of detection, or when DNA copies were ≥ than 5 per reaction (Bruno et al., 2017; Bustin et al., 2009; Ficetola et al., 2015).

Traditional field survey

A systematic survey implementing traditional field survey was run in July 2022 at 192 sites following the standardized protocol recommended by the IUCN SSC Otter Specialist Group (Reuther et al. 2000). Up to four sites were selected on each 10x10 km cell grid (ETRS89 LAEA 5210 projection) overlapped to the study area. At each site otter signs, mainly scats (known as *spraints*), but also jellies and footprints, were searched by walking along 600 metres of riverbank. When it was not possible to implement transect lines because of riverbanks morphology, we inspected bridges only, as an accurate integrative methodology, up to reach the number of 6 bridges per grid cell (Schenekar et al. 2022; Weinberger et al. 2022). At 42 sampling sites along the river Liri where riverbanks were unaccessible the field survey was run using a kayak (Giovacchini et al. 2019).

Data analysis

Results from eDNA and traditional surveys were imported in a GIS environment (QGIS 3.28, http://qgis.org), detection rates were compared at respective sampling sites, and at 5 km and 10 km distance radius to account for potential DNA downstream transport (Burian et al., 2021; Pont et al., 2018; Wilcox et al., 2016). In these latters, results from traditional field survey were pooled at the scale of the analyses (5 km and 10 km), considering the pooled area positive if at least one of the sites was found positive (Fig. 1). The detection rates of eDNA and traditional survey were computed as the number of positive sites divided by the number of tested sites. Differences in detection rates between eDNA and traditional survey at all scales were compared through Fisher's exact tests on contingency tables (Freeman and Campbell 2007) implemented in the software R (R Core Team 2013). All analyses were repeated excluding negative sites from both eDNA and traditional surveys (i.e. potential true absences).

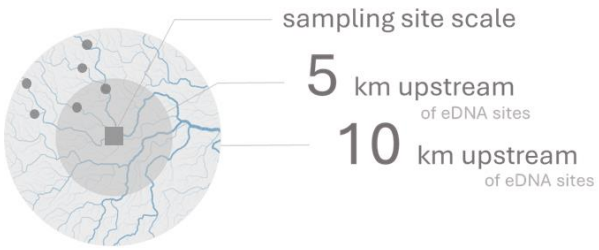


Figure 1. Environmental DNA (grey square) techniques are compared at sampling scale, at 5 km, and at 10 km scale on upstream watersheds with traditional field monitoring (grey dots).

Results

Environmental DNA allowed to detect otters at 8 over 17 sampling sites. Seven positive sites were located in the southern catchments of Liri river and Pontine Marshes (river Amaseno), and one in a tributary of the river Tiber in the northern portion of the study area (river Paglia) (Fig. 2). The traditional survey confirmed otter occurrence in the Liri river basin, where otter signs were found at 23 sites along several tributaries of the river network (rivers Gari, Rapido, Sacco, Liri, and Garigliano). No otter signs were found in the northern rivers of Latium (Paglia, Fiora, Marta, Mignone).

At sampling site scale, detection rate was higher for eDNA (DR=0.41) compared to traditional survey (DR=0.059). However, DR of traditional survey increased at 5 km scale (DR=0.23) and 10 km (DR=0.35) of distance upward the streamflow but remained lower compared to the DR of eDNA (Tab. 1). Excluding negative sites from both sampling techniques (i.e., potential true absences), eDNA detection rate (DR=0.8) was still higher compared to traditional survey. Differences decreased with the distance from eDNA sampling point (DR=0.1), and at 5 km (DR=0.4) and 10 km (DR=0.6) (Tab. 1).

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

	eDNA	TFM (sampling sites)	TFM (5 km)	TFM (10 km)
DR	0.412	0.059	0.235	0.353
DR without true absences	0.800	0.100	0.400	0.600

Fisher's extact tests showed a statistically significant difference between detections rates of eDNA and traditional monitoring only at the scale of sampling site (Tab. 2). Differences observed at a larger scale of 5 km and 10 km buffers from eDNA sampling sites were not significant.

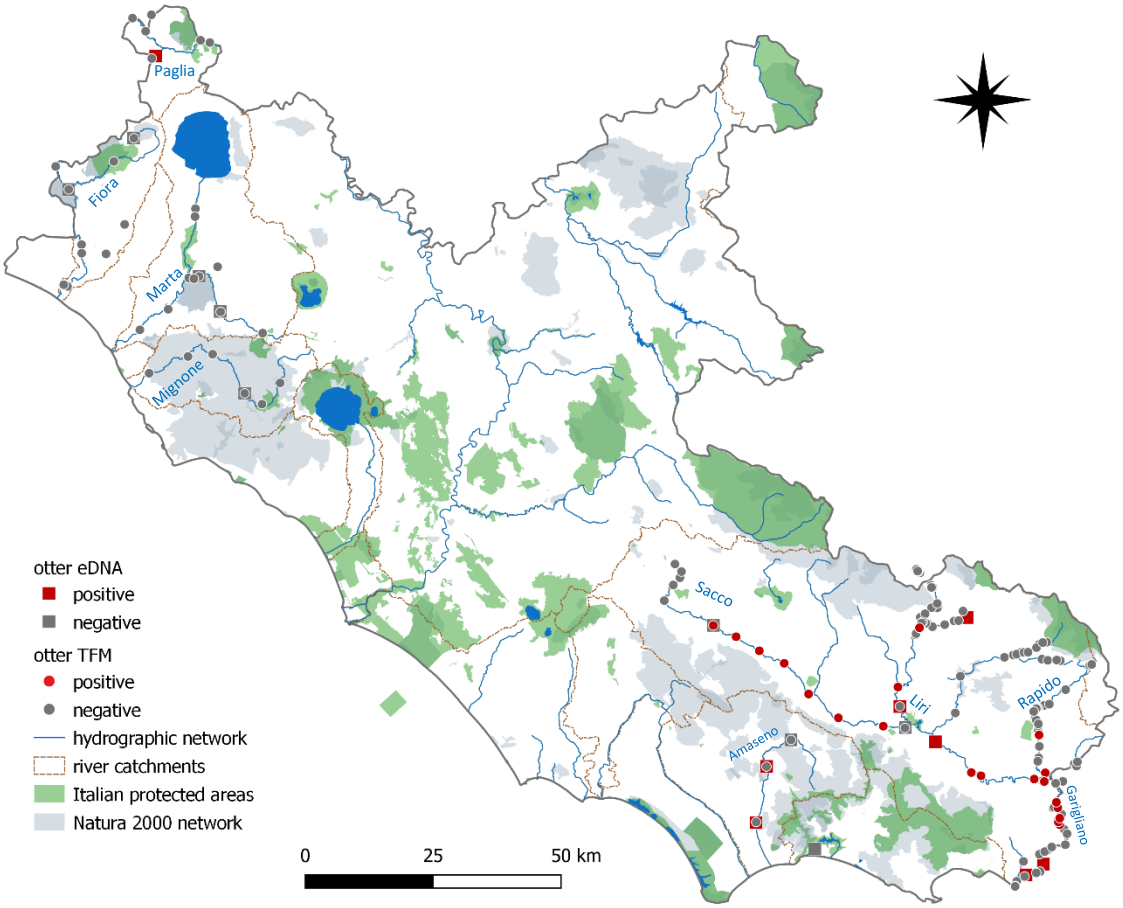


Figure 2. Otter occurrences in Latium region obtained with environmental DNA (eDNA) and traditional field monitoring (TFM) techniques.

Table 2. Statistical significance of Fisher's exact test results between environmental DNA (eDNA) and traditional field monitoring (TFM) techniques. Asterisk marks p-value < 0.05.

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

Discussion

This study attests to an expansion of the otter range in Central Italy along the Thyrrenian side. These new occurrences in southern Latium confirm the ongoing trend of reclaiming the ancient range by otters northward along the Italian peninsula (Marcelli et al., 2023; Giovacchini et al., 2021). The distribution of this new sub-population found along Liri river is extensive, and leads to estimate up to 30-35 individuals, according to reference values otter density found along Italian rivers (Lerone et al., 2014; Janssens, 2006; Prigioni et al., 2006). A population in this favourable condition can support from now the persisting colonization toward new rivers even further northward. However, traditional field techniques gained empirical observations questioning about carrying capacity is already achieved in the study area. Low marking frequencies, hidden marking sites, and spraints in advanced state of deterioration suggested low revisitation rates of marking sites that could be related to home ranges unstable over time. Furthermore, traditional techniques tracked several negative sites on many tributaries in the south-eastern part of the region, suggesting that the process of saturation of suitable habitats within Liri river catchment is still ongoing

(Chapman & Byron, 2018).

Differently, along these tributaries, qPCR eDNA unveiled the presence of some otters. As otter DNA is present but no spraints have been used by otters to mark their presence to other individuals, these occurrences may refer to wandering individual displaying dispersal behaviours and exploring new territories. Sprainting behaviour is known to be used in otters for marking their presence to other otters in well-established ranges and when home ranges overlap for communication purposes (Kruuk, 2006; Sittenthaler et al., 2020). qPCR eDNA, after all, is recognized as a powerful and sensitive technique to reveal species presence, able to catch also few individuals sustaining long dispersal movements (Richardson et al., 2016; Stewart & Taylor, 2020; Tesson & Edelaar, 2013). The sensitivity of qPCR eDNA to detect otter has been evidenced also in the Tiber river catchment, where otter occurrence was revealed along the tributary river Paglia very far from the range boundaries of the species. We believed that also this datum can be generated by dispersing individuals. A very recent finding collected after the study period with camera-traps along the river Tiber confirmed this supposition (Cervoni et al., 2024). Furthermore, a direct observation in the adjacent river catchment (i.e. Fiora river) may have tracked the same individual still dispersing in new territories (http://therio.unimol.it:8080/lontra/). eDNA indeed perfectly coupled traditional field techniques in the northern part of the study area, confirming the absence of otter where a remnant unviable population occurred until 2000 and was declared extinct (Reggiani et al. 2000; Panzacchi et al., 2011). Finally, eDNA revealed positive sites at river mouths where traditional field techniques failed, shedding light on the importance of coastline areas and estuarine environments in otter movements (Delibes-Mateos et al., 2014; Parry et al., 2011). As these areas are close to the range boundaries, we believed the colonization process may have been proceeded from the river mouth across the seacoast and the channels along the littoral zone.

Our statistical analysis underlined how a qPCR-based eDNA method is more sensitive in revealing otter presence. Specifically, compared to traditional field methods, our eDNA technique showed to particularly efficacy at sampling site scale. This can be explained by the concept of the river as a conveyor belt of molecules shred by organisms many kilometres upstream (Deiner et al., 2016). Hence, the probability to detect a species with eDNA is very high even where the species is not physically present onsite, and can also lead to a mismatch with presence/absence results gained with other techniques, such as the standard surveying (Eiler et al., 2018). Indeed, in our case, the reliability of traditional techniques increases when transect lines rise in number and are displaced at higher distances. This also suggests that standard surveys can be strongly influenced by false absences (Balestrieri et al., 2011; Janssens et al., 2006; Jeffress et al., 2011). Locally, accessible substrates for otter marking activities vary in numbers and availability due to riverbank morphology and hydrology conditions (Almeida et al., 2012; Balestrieri et al., 2011; Hong et al., 2022). Such observations confer to the site selection a primary importance to explain differences between eDNA and traditional techniques performances in water bodies where no substates for otter sprainting are surfacing, such as along artificial channels (Elmeros & Bussenius, 2002). eDNA was also more proficient to reveal otter presence at the mouth of the rivers where river sections are wide, emerged substrates are excessively numerous, and difficult to investigate even with a canoe. eDNA is completely independent, unconstrained by morphological variables of the river such as available substrates for marking behaviour, and gives also the opportunity to explore river sectors that are not walkable, without using cumbersome equipment such as a boat to navigate in deep waters. eDNA is not particularly hindered by topographical factor, offering great potential to monitor elusive species in harsh environments, but its results can be affected by other environmental parameters through inhibition mechanisms, mainly operated by chemical water variables and microbial communities (Barnes & Turner, 2016; Saito & Doi, 2021; Schmidt et al., 2021). Environmental DNA assessment on otters proved to be particularly efficient in otter detection without being influenced by no parameters related to water chemistry (Jamwal et al., 2023).

A quantitative approach to eDNA also proved to be more fast and cheap compared to traditional techniques for species detection, and for otter monitoring as well (Giovacchini et al., submitted; Sigsgaard et al., 2015; Thomas et al., 2020). An estimation of time and cost for otter monitoring undertaken on our 17 sampling sites in Central Italy revealed that qPCR eDNA requires 14 days and 1,800 € having a lab already set, and transect lines 6 days and 2,116 € (Giovacchini et al., submitted; Suppl. Mat. 3). qPCR eDNA is well known to

represent be a cost-effective alternative method to biological monitoring, and to save finances and time, especially when species-specific eDNA assay are already designed and ready to be used (Johnson et al., 2024; Thalinger et al., 2021). Cost-effectivity evaluations for wildlife sampling techniques often consider also the accuracy of the method. Transect lines conducted on 17 sites only turned out to be not efficient in determining otter presence. As a matter of fact, otter standard surveys require the check of a group of 4 sampling sites for each cell-grid with an extension of 10 km-side, turning out in the need for a considerable number of transect lines and making the traditional field technique highly time-demanding (Reuther et al., 2000). Indeed, in our experience, in the unique area where we found otters along Liri river, 7 sites sampled with eDNA outperformed 141 sites surveyed with traditional techniques. This is automatically translated in saving time for wildlife assessments, as the time we expended in water sampling was 2 days only against 35 days for transects looking for otter tracks.

The evidence of otter range expansion along the Tyrrhenian side of Central Italy draws attention to the urgent perimeter of new protected areas along the course of water bodies affected by the presence of a species of Community Interest, i.e. Liri river. Conservation measures directed to apex predators, such as the Eurasian otter defined as an umbrella species, can positively benefit enhance the freshwater biodiversity of river ecosystems (D. W. Macdonald et al., 2018). Ecological assessments can be undertaken along polluted rivers where otter conservation may be affected from biomagnification processes, from new contaminants to microplastics (O'Connor et al., 2022; Wainstein et al., 2022; Giovacchini et al., 2023). Alternative species-specific conservation actions involve community-based initiatives that can be launched to prevent the raising of human-otter conflict in vulnerable locations (Liordos et al., 2019; Sales-Luís et al., 2009).

Molecular approaches demonstrated once again the high sensitivity of eDNA in revealing species presence at low traces and the potentiality to guide wildlife conservation (Porco et al., 2022, 2024). Quantitative PCR-base investigations afford several advantages on tracking rare and elusive species, from unharmful procedures to quicky collection operations. The employment of eDNA techniques is therefore crucial to monitor expansion stages of highly wandering mammals, even in unusual environments difficult to explore in their integrity (Hobbs et al., 2019; Valsecchi et al., 2022). However, it is pivotal to adopt an integrated approach coupling Edna with traditional survey methods that rely on more robust data (e.g., having small spatial resolution) to provide sound decisions in wildlife conservation (Sales-Luís et al., 2009; Tolrà et al., 2024). Adopting a strategic view, eDNA can be used to optimize monitoring on large areas where the expansion of endangered species is expected. After species has been noticed, traditional techniques can be introduced to collect data and rapidly inform conservation actions to preserve new populations of endangered and rare species.

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Literature

Almeida, D., Barrientos, R., Merino-Aguirre, R., & Angeler, D. G. (2012). The role of prey abundance and flow regulation in the marking behaviour of Eurasian otters in a Mediterranean catchment. *Animal Behaviour*, *84*(6), 1475–1482. https://doi.org/https://doi.org/10.1016/j.anbehav.2012.09.020

Balestrieri, A., Remonti, L., & Prigioni, C. (2011). Detectability of the Eurasian otter by standard surveys: an approach using marking intensity to estimate false negative rates. *Naturwissenschaften*, *98*(1), 23–31. https://doi.org/10.1007/s00114-010-0737-0

Ballini, L., Ottonello, D., Repetto, V., Natali, C., Chini, G., Tolve, L., Ciofi, C., Fratini, S., & Iannucci, A. (2024). Early detection of rare and elusive endangered species using environmental DNA: a case study for the Eurasian otter and the white-clawed crayfish in northwestern Italy. *Conservation Genetics*, *25*(4), 999–1005. https://doi.org/10.1007/s10592-024-01619-5

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., Foster, J., Wilkinson, J. W., Arnell, A., Brotherton, P., Williams, P., & Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). *Biological Conservation*, *183*, 19–28. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.029

Bruno, A., Sandionigi, A., Galimberti, A., Siani, E., Labra, M., Cocuzza, C., Ferri, E., & Casiraghi, M. (2017). One step forwards for the routine use of high-throughput DNA sequencing in environmental monitoring. An efficient and standardizable method to maximize the detection of environmental bacteria. *MicrobiologyOpen*, *6*(1), e00421. https://doi.org/https://doi.org/10.1002/mbo3.421

Burian, A., Mauvisseau, Q., Bulling, M., Domisch, S., Qian, S., & Sweet, M. (2021). Improving the reliability of eDNA data interpretation. *Molecular Ecology Resources*, *21*(5), 1422–1433.

https://doi.org/10.1111/1755-0998.13367
Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797
Carone, M. T., Guisan, A., Cianfrani, C., Simoniello, T., Loy, A., & Carranza, M. L. (2014). A multi-temporal approach to model endangered species distribution in Europe. The case of the Eurasian otter in Italy. *Ecological Modelling*, *274*, 21–28. https://doi.org/https://doi.org/10.1016/j.ecolmodel.2013.11.027
Carone, M. T., Simoniello, T., Loy, A., & Carranza, M. L. (2012). 25 - Combining Habitat Suitability Models and Fluvial Functionality Data for a Multilayer Assessment of Riverine Vulnerability. In F. Jordán & S. E. B. T.-D. in E. M. Jørgensen (Eds.), *Models of the Ecological Hierarchy* (Vol. 25, pp. 431–444). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-444-59396-2.00025-0
Cassola F (ed) (1986) La lontra in Italia: censimento, distribuzione e problemi di conservazione di una specie minacciata. WWF Italia, Serie Atti e Studi, 5.
Cervoni, F., Giardini, M., D'Urbano, S., Marini, D., & Grispigni Manetti, C. (2024). A Eurasian otter *Lutra lutra* (Linnaeus 1758) (Carnivora: Mustelidae) in the Tiber basin near Rome: relict or range expansion? *Natural History Sciences*, *11*(2). https://doi.org/10.4081/nhs.2024.746
Chapman, E. J., & Byron, C. J. (2018). The flexible application of carrying capacity in ecology. *Global Ecology and Conservation*, *13*, e00365. https://doi.org/https://doi.org/10.1016/j.gecco.2017.e00365
Cianfrani, C., Lay, G. Le, Maiorano, L., Satizábal, H. F., Loy, A., & Guisan, A. (2011). Adapting global conservation strategies to climate change at the European scale: The otter as a flagship species. *Biological Conservation*, *144*(8), 2068–2080. https://doi.org/https://doi.org/10.1016/j.biocon.2011.03.027
Cianfrani, C., Maiorano, L., Loy, A., Kranz, A., Lehmann, A., Maggini, R., & Guisan, A. (2013). There and back again? Combining habitat suitability modelling and connectivity analyses to assess a potential return of the otter to Switzerland. *Animal Conservation*, *16*(5), 584–594. https://doi.org/https://doi.org/10.1111/acv.12033
De Fioravante, P., Strollo, A., Assennato, F., Marinosci, I., Congedo, L., & Munafò, M. (2022). High resolution land cover integrating copernicus products: A 2012–2020 map of Italy. *Land*, *11*(1), 1–30. https://doi.org/10.3390/land11010035
Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J.-C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications*, *7*(1), 12544. https://doi.org/10.1038/ncomms12544
Delibes-Mateos, M., Blázquez, M. C., Blanco-Garrido, F., Sánchez, J., Segura, A., & Delibes, M. (2014). Spraining sites and feeding habits of the otter (Lutra lutra) in the Douro River estuary, Portugal. *Galemys, Spanish Journal of Mammalogy*, *26*, 91–95. https://doi.org/10.7325/galemys.2014.n1
Duarte, S., Simões, L., & Costa, F. O. (2023). Current status and topical issues on the use of eDNA-based targeted detection of rare animal species. *Science of The Total Environment*, *904*, 166675. https://doi.org/https://doi.org/10.1016/j.scitotenv.2023.166675
Eiler, A., Löfgren, A., Hjerne, O., Nordén, S., & Saetre, P. (2018). Environmental DNA (eDNA) detects the pool frog (Pelophylax lessonae) at times when traditional monitoring methods are insensitive. *Scientific Reports*, *8*(1), 5452. https://doi.org/10.1038/s41598-018-23740-5
Elmeros, M., & Bussenius, N. (2002). Influence of Selection of Bank Side on the Standard. *IUCN Otter Spec. Group Bull.*, *19*(2), 67–74.
Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Rayé, G., & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, *15*(3), 543–556. https://doi.org/https://doi.org/10.1111/1755-0998.12338
Freeman JV, Julious SA (2007) The analysis of categorical data. Scope, 16(1): 18–21.
Giovacchini S, Marrese M, Loy A (2018) Good news from the south: filling the gap between two otter populations in Italy. IUCN Otter Spec. Group Bull., 35(4), 212-221.
Giovacchini S, Antonucci A, De Castro G, et al (2019) Recovery of the Eurasian otter (*Lutra lutra*) in central and southern Italy: a new hope. VIII European Congress of Mammalogy, 16-22 August 2019, Warsaw,

Poland. Book of abstracts.
Giovacchini S, Antonucci A, Bartolomei R, et al (2021) Conservation status of Eurasian otter Lutra lutra in Italy. Eurasian Otter Workshop 2021 (online). Available from https://www.otterspecialistgroup.org/osg-newsite/eurasian-otter-workshop/eow-2021-posters/ Accessed 18 November 2024.Giovacchini S., De Sanctis C., Di Febbraro M., Galaverni M., Canu A., Loy A. 2023. Il ritorno della lontra nel Lazio. Protected Areas and Conservation. Bridging the gap and beyond. Convegno nazionale WWF Italia. 15-17 maggio 2023, Belvedere San Leucio, Caserta.
Giovacchini S, Mirone E, Bruno A, Ramazzotti F, Caprotti L, Monaco P, Belluardo F, Di Febbraro M, Jamwal PS, Manfrin C, Galimberti A, Loy A (subm). Using species-specific environmental DNA for multi-taxa detection of endangered and invasive alien species in Italian freshwater ecosystems. Implications for monitoring obligations under EU regulations. Biological Conservation, subm.
Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S., & Hänfling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (Triturus cristatus). *Ecology and Evolution*, *8*(12), 6330–6341. https://doi.org/https://doi.org/10.1002/ece3.4013
Hobbs, J., Round, J. M., Allison, M. J., & Helbing, C. C. (2019). Expansion of the known distribution of the coastal tailed frog, Ascaphus truei, in British Columbia, Canada, using robust eDNA detection methods. *PLOS ONE*, *14*(3), e0213849. https://doi.org/10.1371/journal.pone.0213849
Hong, S., Di Febbraro, M., Kim, H. G., & Loy, A. (2022). Is scat marking a reliable tool for otter census and surveys at the landscape scale? *Journal of Environmental Management*, *315*, 115098. https://doi.org/https://doi.org/10.1016/j.jenvman.2022.115098
Jamwal P. S., Bruno A., Galimberti A., Casiraghi M., Loy A. (2021) Environmental DNA detection of the rare and endangered otter in Southern Italian waters: a preliminary study. Hystrix – The Italian Journal of Mammalogy 32 (2): 176–181.
Jamwal, P. S., Bruno, A., Galimberti, A., Magnani, D., Casiraghi, M., & Loy, A. (2023). Environmental DNA revealed high accuracy in detection of the Eurasian otter in Himalaya. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *33*(11), 1309–1320. https://doi.org/https://doi.org/10.1002/aqc.4010
Janssens, X., Defourny, P., Kermabon, J. De, & Baret, P. V. (2006). The recovery of the otter in the Cevennes (France): a GIS-based model. *Hystrix Italian Journal of Mammalogy*, *17*(1), 5–14.
Jefferies D.J. 1986. The value of otter *Lutra lutra* surveying using spraints: an analysis of its successes and problems in Britain. *Journal of the Otter Trust,* 1(9): 25-32.
Jeffress, M. R., Paukert, C. P., Sandercock, B. K., & Gipson, P. S. (2011). Factors affecting detectability of river otters during sign surveys. *The Journal of Wildlife Management*, *75*(1), 144–150. https://doi.org/https://doi.org/10.1002/jwmg.12
Johnson, M., Tetzlaff, S., Katz, A., & Sperry, J. (2024). Comparison of qPCR and metabarcoding for environmental DNA surveillance of a freshwater parasite. *Ecology and Evolution*, *14*(5), e11382. https://doi.org/https://doi.org/10.1002/ece3.11382
Karlsson, E., Ogonowski, M., Sundblad, G., Sundin, J., Svensson, O., Nousiainen, I., & Vasemägi, A. (2022). Strong positive relationships between eDNA concentrations and biomass in juvenile and adult pike (Esox lucius) under controlled conditions: Implications for monitoring. *Environmental DNA*, *4*(4), 881–893. https://doi.org/https://doi.org/10.1002/edn3.298
Keck, F., Blackman, R. C., Bossart, R., Brantschen, J., Couton, M., Hürlemann, S., Kirschner, D., Locher, N., Zhang, H., & Altermatt, F. (2022). Meta-analysis shows both congruence and complementarity of DNA and eDNA metabarcoding to traditional methods for biological community assessment. *Molecular Ecology*, *31*(6), 1820–1835. https://doi.org/https://doi.org/10.1111/mec.16364
Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282. https://doi.org/https://doi.org/10.1002/edn3.29
Kruuk, H., Carss, D. N., Conroy, J. W. H., & Gaywood, M. J. (1998). Habitat use and conservation of otters (Lutra lutra) in Britain: a review. In N. Dunstone & M. L. Gorman (Eds.), *Behaviour and Ecology of Riparian Mammals* (pp. 119–134). Cambridge University Press. https://doi.org/DOI: 10.1017/CBO9780511721830.009

Kruuk, H., & Conroy, J. W. H. (1987). Surveying otter Lutra lutra populations: A discussion of problems with spraints. *Biological Conservation*, *41*(3), 179–183. https://doi.org/https://doi.org/10.1016/0006-3207(87)90101-7

Lerone, L., Mengoni, C., Carpaneto, G. M., Randi, E., & Loy, A. (2014). Procedures to genotype problematic non-invasive otter (Lutra lutra) samples. *Acta Theriologica*, *59*(4), 511–520. https://doi.org/10.1007/s13364-014-0195-8

Liordos, V., Kontsiotis, V. J., Nevolianis, C., & Nikolopoulou, C. E. (2019). Stakeholder preferences and consensus associated with managing an endangered aquatic predator: the Eurasian otter (Lutra lutra). *Human Dimensions of Wildlife*, *24*(5), 446–462. https://doi.org/10.1080/10871209.2019.1622821

Loy A, Bucci L, Carranza ML, et al (2004) Survey and habitat evaluation for a peripheral population of the Eurasian otter in Italy. IUCN Otter Spec. Group Bull. 21A.

Loy, A., Carranza, M. L., Cianfrani, C., D'Alessandro, E., Bonesi, L., Di Marzio, P., Minotti, M., & Reggiani, G. (2009). Otter Lutra lutra population expansion: Assessing habitat suitability and connectivity in southern Italy. *Folia Zoologica*, *58*(3), 309–326.

Loy A, Kranz A, Oleynikov A, et al (2022) Lutra lutra (amended version of 2021 assessment). The IUCN Red List of Threatened Species 2022: e.T12419A218069689. https://dx.doi.org/10.2305/IUCN.UK.2022-2.RLTS.T12419A218069689.en.

Loy A, Giovacchini S, Agostini G, et al (2023) Genetica non invasiva, fototrappolaggio, analisi della dieta e censimento della metapopolazione di lontra (Lutra lutra) residente nel Parco Nazionale d'Abruzzo Lazio e Molise e nelle aree limitrofe. Research agreement between University of Molise, CNR-IRET and National Park of Abruzzo, Lazio e Molise.

Loy A, Baisero D, Bon M, et al (ed) in press. Atlante dei Mammiferi in Italia – Atlas of Italian mammals. Edizioni Belvedere.

Macdonald, D. W., Newman, C., & Harrington, L. A. (2018). Beneath the umbrella: Conservation out of the limelight. *Biology and Conservation of Musteloids*, 543–556. https://doi.org/10.1093/oso/9780198759805.003.0030

Macdonald, S. M., & Mason, C. F. (1983). The otter Lutra lutra in southern Italy. *Biological Conservation*, *25*(2), 95–101. https://doi.org/https://doi.org/10.1016/0006-3207(83)90054-X

Malthieux L (2020) La Loutre d' Europe Lutra lutra (Linnaeus, 1758) en Roya-Bévéra: relique ou retour? Prospections, état des lieux et implications. Faune PACA, Publication 98: 22 pp.

Marcelli, M., Striglioni, F., & Fusillo, R. (2023). Range reexpansion after long stasis: Italian otters (Lutra lutra) at their northern edge. *Ecology and Evolution*, *13*(1), e9726. https://doi.org/https://doi.org/10.1002/ece3.9726

Marcolin, F., Iordan, F., Pizzul, E., Pallavicini, A., Torboli, V., Manfrin, C., & Quaglietta, L. (2020). Otter diet and prey selection in a recently recolonized area assessed using microscope analysis and DNA barcoding. *Hystrix, the Italian Journal of Mammalogy*, *31*(1), 64–72. https://doi.org/10.4404/hystrix-00260-2019

Mason, C. F., & Macdonald, S. M. (1987). The use of spraints for surveying otter Lutra lutra populations: An evaluation. *Biological Conservation*, *41*(3), 167–177. https://doi.org/https://doi.org/10.1016/0006-3207(87)90100-5

McColl-Gausden, E. F., Weeks, A. R., Coleman, R., Song, S., & Tingley, R. (2023). Using hierarchical models to compare the sensitivity of metabarcoding and qPCR for eDNA detection. *Ecological Informatics*, *75*, 102072. https://doi.org/https://doi.org/10.1016/j.ecoinf.2023.102072

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(7), 150088. https://doi.org/10.1098/rsos.150088

Mucci, N., Arrendal, J., Ansorge, H., Bailey, M., Bodner, M., Delibes, M., Ferrando, A., Fournier, P., Fournier, C., Godoy, J. A., Hajkova, P., Hauer, S., Heggberget, T. M., Heidecke, D., Kirjavainen, H., Krueger, H.-H., Kvaloy, K., Lafontaine, L., Lanszki, J., … Randi, E. (2010). Genetic diversity and landscape genetic structure of otter (Lutra lutra) populations in Europe. *Conservation Genetics*, *11*(2), 583–599. https://doi.org/10.1007/s10592-010-0054-3

Mynhardt, S., Matthew, E., le Roux, J. P., Little, I., Bloomer, P., & Theron, C. (2024). Environmental DNA from soil reveals the presence of a "lost" Afrotherian species. *Biodiversity and Conservation*, *33*(1), 31–

50. https://doi.org/10.1007/s10531-023-02728-2
O'Connor, J. D., Lally, H. T., Mahon, A. M., O'Connor, I., Nash, R., O'Sullivan, J. J., Bruen, M., Heerey, L., Koelmans, A. A., Marnell, F., & Murphy, S. (2022). Microplastics in Eurasian otter (Lutra lutra) spraints and their potential as a biomonitoring tool in freshwater systems. *Ecosphere*, *13*(7), e3955. https://doi.org/https://doi.org/10.1002/ecs2.3955
Panzacchi M, Genovesi P, Loy A (2011) Piano d'azione per la conservazione della lontra. Ministero per la Tutela dell'Ambiente, del Territorio e del Mare e ISPRA. Quaderni 35/201. Available at : https://www.mase.gov.it/sites/default/files/archivio/biblioteca/protezione_natura/qcn_35_lontra_def.pdf Accessed 18 November 2024.
Park, H.-C., Han, T.-Y., Kim, D.-C., Min, M.-S., Han, S.-Y., Kim, K.-S., & Lee, H. (2011). Individual identification and sex determination of Eurasian otters (Lutra lutra) in Daegu city based on genetic analysis of otter spraint. *Genes & Genomics*, *33*(6), 653–657. https://doi.org/10.1007/s13258-011-0051-z
Parry, G. S., Bodger, O., McDonald, R. A., & Forman, D. W. (2013). A systematic re-sampling approach to assess the probability of detecting otters Lutra lutra using spraint surveys on small lowland rivers. *Ecological Informatics*, *14*, 64–70. https://doi.org/https://doi.org/10.1016/j.ecoinf.2012.11.002
Parry, G. S., Burton, S., Cox, B., & Forman, D. W. (2011). Diet of coastal foraging Eurasian otters (Lutra lutra L.) in Pembrokeshire south-west Wales. *European Journal of Wildlife Research*, *57*(3), 485–494. https://doi.org/10.1007/s10344-010-0457-y
Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., & Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, *8*(1), 10361. https://doi.org/10.1038/s41598-018-28424-8
Porco, D., Hermant, S., Purnomo, C. A., Horn, M., Marson, G., & Colling, G. (2022). eDNA-based detection of the invasive crayfish Pacifastacus leniusculus in streams with a LAMP assay using dependent replicates to gain higher sensitivity. *Scientific Reports*, *12*(1), 6553. https://doi.org/10.1038/s41598-022-10545-w
Porco, D., Purnomo, C. A., Glesener, L., Proess, R., Lippert, S., Jans, K., Colling, G., Schneider, S., Stassen, R., & Frantz, A. C. (2024). eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. *BMC Ecology and Evolution*, *24*(1), 1–16. https://doi.org/10.1186/s12862-023-02189-9
Prigioni, C., Remonti, L., Balestrieri, A., Sgrosso, S., Priore, G., Mucci, N., & Randi, E. (2006). Estimation of European Otter (Lutra lutra) Population Size by Fecal DNA Typing in Southern Italy. *Journal of Mammalogy*, *87*(5), 855–858. https://doi.org/10.1644/05-MAMM-A-294R1.1
R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
Randi, E., Davoli, F., Pierpaoli, M., Pertoldi, C., Madsen, A. B., & Loeschcke, V. (2003). Genetic structure in otter (Lutra lutra) populations in Europe: implications for conservation. *Animal Conservation*, *6*(2), 93–100. https://doi.org/DOI: 10.1017/S1367943003003123
Reuther C, Dolch D, Green R, et al (2000) Surveying and monitoring distribution and population trends of the Eurasian Otter (Lutra lutra): guidelines and evaluation of the standard method for surveys as recommended by the European Section of the IUCN/SSC Otter Specialist Group. Habitat, 12: 1–148.
Richardson, M. F., Sherman, C. D. H., Lee, R. S., Bott, N. J., & Hirst, A. J. (2016). Multiple dispersal vectors drive range expansion in an invasive marine species. *Molecular Ecology*, *25*(20), 5001–5014. https://doi.org/https://doi.org/10.1111/mec.13817
Romanowski, J., Brzeziński, M., & Cygan, J. P. (1996). Notes on the technique of the otter field survey. *Acta Theriologica*, *41*(2), 199–204. https://doi.org/10.4098/AT.arch.96-19
Rondinini, C., Battistoni, A., & Teofili, C. (2022). Lista rossa 2022. *Lista Rossa IUCN Dei Vertebrati Italiani 2022*, *Comitato I*.
Roos, A. M., Bäcklin, B.-M. V. M., Helander, B. O., Rigét, F. F., & Eriksson, U. C. (2012). Improved reproductive success in otters (Lutra lutra), grey seals (Halichoerus grypus) and sea eagles (Haliaeetus albicilla) from Sweden in relation to concentrations of organochlorine contaminants. *Environmental Pollution*, *170*, 268–275. https://doi.org/https://doi.org/10.1016/j.envpol.2012.07.017
Saito, T., & Doi, H. (2021). Degradation modeling of water environmental DNA: Experiments on multiple

DNA sources in pond and seawater. *Environmental DNA*, *3*(4), 850–860. https://doi.org/10.1002/edn3.192
Sales-Luís, T., Freitas, D., & Santos-Reis, M. (2009). Key landscape factors for Eurasian otter Lutra lutra visiting rates and fish loss in estuarine fish farms. *European Journal of Wildlife Research*, *55*(4), 345–355. https://doi.org/10.1007/s10344-009-0250-y
Schenekar, T., Clark, A., Holzinger, W. E., & Weiss, S. J. (2022). Presence of spraint at bridges as an effective monitoring tool to assess current Eurasian fish otter distribution in Austria. *European Journal of Wildlife Research*, *68*(5), 53. https://doi.org/10.1007/s10344-022-01604-8
Schmidt, B. C., Spear, S. F., Tomi, A., & Bodinof Jachowski, C. M. (2021). Evaluating the efficacy of environmental DNA (eDNA) to detect an endangered freshwater mussel Lasmigona decorata (Bivalvia:Unionidae). *Freshwater Science*, *40*(2), 354–367. https://doi.org/10.1086/714411
Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, *183*, 46–52. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.023
Sittenthaler, M., Schöll, E. M., Leeb, C., Haring, E., Parz-Gollner, R., & Hackländer, K. (2020). Marking behaviour and census of Eurasian otters (Lutra lutra) in riverine habitats: what can scat abundances and non-invasive genetic sampling tell us about otter numbers? *Mammal Research*, *65*(2), 191–202. https://doi.org/10.1007/s13364-020-00486-y
Stewart, K. A., & Taylor, S. A. (2020). Leveraging eDNA to expand the study of hybrid zones. *Molecular Ecology*, *29*(15), 2768–2776. https://doi.org/https://doi.org/10.1111/mec.15514
Stockholm Convention (2001) Stockholm Convention on persistent organic pollutants. Available at: https://www.pops.int/ Accessed 18 November 2024.
Strahler AN (1974) Geografia fisica. Omega, Barcelona.
Tarquini S, Isola I, Favalli M, Battistini A, Dotta G (2023) TINITALY, a digital elevation model of Italy with a 10 meters cell size (Version 1.1). Istituto Nazionale di Geofisica e Vulcanologia (INGV). https://doi.org/10.13127/tinitaly/1.1.
Tesson, S. V. M., & Edelaar, P. (2013). Dispersal in a changing world: opportunities, insights and challenges. *Movement Ecology*, *1*(1), 10. https://doi.org/10.1186/2051-3933-1-10
Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., Traugott, M., Goldberg, C. S., & Bruce, K. (2021). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA*, *3*(4), 823–836. https://doi.org/10.1002/edn3.189
Thomas, A. C., Tank, S., Nguyen, P. L., Ponce, J., Sinnesael, M., & Goldberg, C. S. (2020). A system for rapid eDNA detection of aquatic invasive species. *Environmental DNA*, *2*(3), 261–270. https://doi.org/https://doi.org/10.1002/edn3.25
Tolrà, A., Ruiz-Olmo, J., & Riera, J. L. (2024). Human disturbance and habitat structure drive eurasian otter habitat selection in heavily anthropized river basins. *Biodiversity and Conservation*, *33*(5), 1683–1710. https://doi.org/10.1007/s10531-024-02826-9
Valsecchi, E., Coppola, E., Pires, R., Parmegiani, A., Casiraghi, M., Galli, P., & Bruno, A. (2022). A species-specific qPCR assay provides novel insight into range expansion of the Mediterranean monk seal (Monachus monachus) by means of eDNA analysis. *Biodiversity and Conservation*, *31*(4), 1175–1196. https://doi.org/10.1007/s10531-022-02382-0
Wainstein, M., Harding, L. B., O'Neill, S. M., Boyd, D. T., Koontz, F., Miller, B., Klütsch, C. F. C., Thomas, P. J., & Ylitalo, G. M. (2022). Highly contaminated river otters (Lontra canadensis) are effective biomonitors of environmental pollutant exposure. *Environmental Monitoring and Assessment*, *194*(10), 670. https://doi.org/10.1007/s10661-022-10272-9
Weinberger, I. C., Muff, S., Kranz, A., & Bontadina, F. (2019). Riparian vegetation provides crucial shelter for resting otters in a human-dominated landscape. *Mammalian Biology*, *98*(1), 179–187. https://doi.org/10.1016/j.mambio.2019.09.001
Weinberger IC, Mausbach J, Kéry M, Karp D (2022) Searching for spraints. A comparison of finding efficiency between experts, volunteers and detection dogs. 15th IUCN/SSC OSG International Otter Congress "Communities and Otters", 19th-23rd September 2022 Sospel, Alpes-Maritimes, France. Book of Abstracts.
Wilcox, T. M., McKelvey, K. S., Young, M. K., Sepulveda, A. J., Shepard, B. B., Jane, S. F., Whiteley, A. R.,

Lowe, W. H., & Schwartz, M. K. (2016). Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char Salvelinus fontinalis. *Biological Conservation*, *194*, 209–216. https://doi.org/https://doi.org/10.1016/j.biocon.2015.12.023

Supplementary materials

Supplementary material S1. Layers, reclassification and source used to produce an expert-based habitat suitability model along the rivers of the Latium region.

Layer	Reclassification	Source
Altitude	> 2000 m.a.s.l. = 0 1500 – 2000 m.a.s.l.= 1 1000 – 1500 m.a.s.l. = 2 500 – 1000 m.a.s.l. = 3 0 – 500 m.a.s.l. = 4	Tarquini et al. 2023
Slope	>70° = 3	Tarquini et al. 2023
Land cover: artificial surfaces	Urban and industrial fabric = 0	De Fioravante et al. 2022
Land cover: agricultural areas	Arable lands = 1 Pastures = 1 Fruit trees = 1 Olive groves = 1 Vineyards = 1 Agro-forestry areas = 1	De Fioravante et al. 2022
Land cover: forest areas	Coniferous forest = 2 Broad-leaved forest = 3	De Fioravante et al. 2022
Land cover: semi-natural areas	Natural grasslands = 1 Shrubs = 3	De Fioravante et al. 2022
Land cover: open spaces	Beaches, dunes, sands = 1 Bare rocks and cliffs = 3 Glaciers and perpetual snow = 0	De Fioravante et al. 2022
Land cover: wetlands	Wetlands = 3	De Fioravante et al. 2022
Land cover: water bodies	Permanent water bodies = 3	De Fioravante et al. 2022

Supplementary material S2. Expert-based habitat suitability model results distinguished per suitability classes and relative amount of surface.

Suitability class	Sq-km
unsuitable	497,36
low	268,65
medium	7,93
high	183,83

Supplementary material 3. Cost and time estimate of this eDNA case study conducted in Central Italy across 17 survey sites and 2 validation sites.

		samples per day	days	cost	km	subtotal
personnel	assay design and in silico validation	0,33	1	80	-	1200
	in situ validation and survey sampling	4	6	480	-	
	eDNA filtration	3	5	400	-	
	DNA extraction (survey and validation)	8	2	160	-	
	DNA amplification (survey and in vitro and in situ validation)	2	1	80	-	
materials	disposable materials (per sample)	Blood and Tissue extraction kit (validation)	-	4	-	301
		filters (x2)	-	3	-	
		bottles (x3)	-	4,2	-	
		Power Soil extraction kit	-	7.6	-	
		qPCR reagents (amplification)	-	35	-	
		qPCR plastics (amplification)	-	10	-	
Transport	In situ validations (km)	-	-	25	200	299
	Survey (km)	-	-	274	2,268	

Chapter 5 - Guidelines for monitoring freshwater species in central Italy using environmental DNA PCR-based techniques

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Abstract

The quantitative PCR (qPCR) environmental DNA (eDNA) is a species-specific approach to environmental DNA monitoring that allows a higher sensitivity compared to eDNA metabarcoding. This approach allows the simultaneous detection of multiple species, thus providing a new accurate tool for large scale monitoring, especially of freshwater biodiversity. Here we present some guidelines to plan and implement a monitoring project on freshwater species based on qPCR eDNA, focusing on the sampling design, field sample collection, and DNA filtering operation. The guidelines do not go into detail of other phases of an eDNA project, such as the amplification of eDNA, or the design of genetic assays as these are largely available in the literature. The guidelines include an introduction to qPCR eDNA, practical issues related to sampling strategies and techniques, and advices on species and sampling sites selection. We then discuss pitfalls and limitations to be accounted when planning eDNA – based monitoring.

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1. Introduction

The vertiginous pace at which we are witnessing the continuous loss of biodiversity has become increasingly rapid over the past decades. Ecosystems around the globe and also the natural capital supporting human life on this planet are paying for it. However, the anthropogenic impacts that have led to these deleterious consequences have not affected the different natural environments in a uniform manner. Among these, indeed, freshwater ecosystems are probably the most threatened on the planet (Ahmed et al. 2022).

Understanding the state of environments is crucial for planning and implementing recovery programs that also consider biological components. Monitoring aquatic environments along the time, for example, allows to inform any changes on freshwater endangered species. However, monitoring efforts often don't focus on one of the most impactful threats to freshwater ecosystems, that is the invasion of non-native species, almost occurring worldwide. Tackling this issue is of vital importance to guarantee the health of inland water systems, as it has been widely demonstrated that a rapid eradication of the initial stages of a colonizing population is the most effective method to face the banalization of biological communities caused by the arrival of alien species (Kluever et al. 2023). For this aim, it is essential to carry out a sound framework of surveillance programs to monitor the arrival of those alien species that are most likely to occur in the habitats of interest. The complexity to quickly reveal new colonization events with a sampling campaign is mainly caused by technical-scientific challenges, particularly related to the sensitivity of the sampling method.

Conventionally, sampling techniques applied to freshwater fauna often prove insufficiently sensitivity in detecting low-density populations, a typical condition experienced during the early stages of a colonization process. These methods generally involve capturing individuals, using traps or nets in the case of reptiles, or electrofishing in the case of fish, or indirect contact by detecting vocalizations, in the case of amphibians. Capture, specifically, is a species-specific or taxon-specific method that rarely leads to an integrated assessment of the animal community. Moreover, it requires a very intensive sampling effort, and direct handling can also cause stress and potentially harm individuals.

Genomic techniques based on the detection of environmental DNA are a highly effective non-invasive method for investigating the presence of animal and plant species over large areas and in short periods of time. They can be applied to several environmental matrices: water, air, soil, snow, biofilms. Their potentiality lies in the fact that they don't require a direct contact with organisms or they don't imply slow tracking for indirect presence signs. This is particularly useful for detecting rare species that live at low population densities, exhibit elusive behaviours, or are difficult to locate due to their small size. Additionally, they also allow for the simultaneous analysis of the presence of multiple species, even if they belong to very different taxa, thereby providing insight somehow of the animal community within a specific area.

Environmental DNA offers two approaches to study the biodiversity: the species-specific approach and the metabarcoding. The species-specific approach allows studies targeting either the detection of a single species or the simultaneous detection of multiple species. As a result, presence/absence data are determined for all target species. This approach is often used for cryptic or rare species, or for invasive species in the early stages of invasion, in order to alert management authorities and attempt to contain or eradicate the population. The species-specific approach provides a focused understanding of few elements of the animal and plant community, but with a very high level of

taxonomic accuracy. This is justified because it has proven that this approach is more sensitive compared to metabarcoding, empowering its use to reveal species at very low densities. Finally, the use of PCR (Polymerase Chain Reaction) makes this method very easy and fast to be implemented both in the field and in the lab. These two criteria are of paramount importance for the local authorities that are in charge of environmental protection and aim at routinely control their territory. Monitoring, especially those that are mandatory (i.e., Habitats Directive 1992/43/EEC), are easier if they are faster, and the eDNA species-specific approach can punctually inform for rapid management responses.

The metabarcoding approach offers a completely different view of the community composition of a biotope. It's a methodology that provides a broader, more general picture of biodiversity, but generally applied with a less detail, as identification may generally reach only broad taxa (e.g. the Family level), but can also be pushed up to the Species level (Andres et al. 2023). The community of a specific biotope is thus described in its entirety, but from a taxonomic perspective. While it allows for a quantification of biological diversity, it is rarely used to determine which specific species make up the community.

Environmental DNA-based techniques provide a valuable tool for the rapid and simultaneous monitoring of many taxa, including elusive, cryptic, or phylogenetically distant species. They are useful for updating databases, reconstructing distribution ranges, defining species richness and abundance indices, studying communities, and the early detection of exotic species and wildlife pathogens (Porco et al. 2024).

This method is so powerful that it has been routinely adopted by local agencies of United States for water monitoring (USGS - United States Geological Survey). Aggregated biodiversity indices based on eDNA have also been proposed for monitoring required by water protection regulations (Apothéloz-Perret-Gentil et al. 2021; Curtis 2022). Europe is also recently experiencing a similar effort to incorporate environmental DNA among the techniques used for water monitoring as mandated by the Water Framework Directive (2000/60/EC), for the demographic monitoring of harvested species, and for environmental impact assessments (Leese et al. 2018). However, to obtain reliable results applying these techniques and to integrate them within the operational framework of monitoring, it is necessary to standardize the methodologies and calibrate them with traditional methods (Pilliod et al. 2019).

Recently, scientific community has been focusing on bridging the knowledge gaps regarding the ecological parameters that affect the environmental DNA, whose understanding is crucial for increasing the accuracy of the results. The role of scientific research also consists in developing and testing new technologies that will enable faster and more accurate results, with reduced costs. These include field sequencers, metagenomic approaches that do not involve the PCR phase, and tools for the rapid assessment of species. However, even today, the potential of techniques based on environmental DNA represents a ready-to-use tool ready for public agencies responsible for environmental protection and biodiversity monitoring, even more as supported by an incredible amount of scientific literature.

With this document, we aim to present guidelines that enable to plan and implement a monitoring project on freshwater species using a species-specific eDNA approach.

Setting up an environmental DNA study, may entail the arise of several strategic, logistical, scientific,

technological, and economic issues. A typical workflow of a project aiming at the detection of the environmental DNA is illustrated in Fig. 1. For each phase of the work, the necessary resources, timelines, and potential issues must be carefully assessed.

Before the collection of eDNA in the field with a sampling campaign, it is advised to choose a sampling design whose criteria depend on several factors. After collection, eDNA should be isolated from water, mainly by filtration, in order to be extracted with opportune procedures. At this stage, eDNA can be amplified through quantitative PCR to reveal species presence, and inform conservation strategies. eDNA amplification can be rapidly implemented if the genetic assays (i.e., primers and probes) of the species is already available in the scientific literature, otherwise an appropriate phase must be dedicated to design novel assays. New primers and probes have to be carefully selected on genetic databases and their efficacy has to be confirmed with three different validations check candidate sequences against genetic databases, using target species tissues, and finally using environmental samples collected in areas with ascertained species presence and species absence.

Each of these phases will be explained more in detail in the following chapters, documented by data derived from a study carried out by the University of Molise in central Itay, as part of a project funded by Latium Region through the Rural Development Program (RDP) 2014/2020 of Latium Region (Measure 7.6.1).

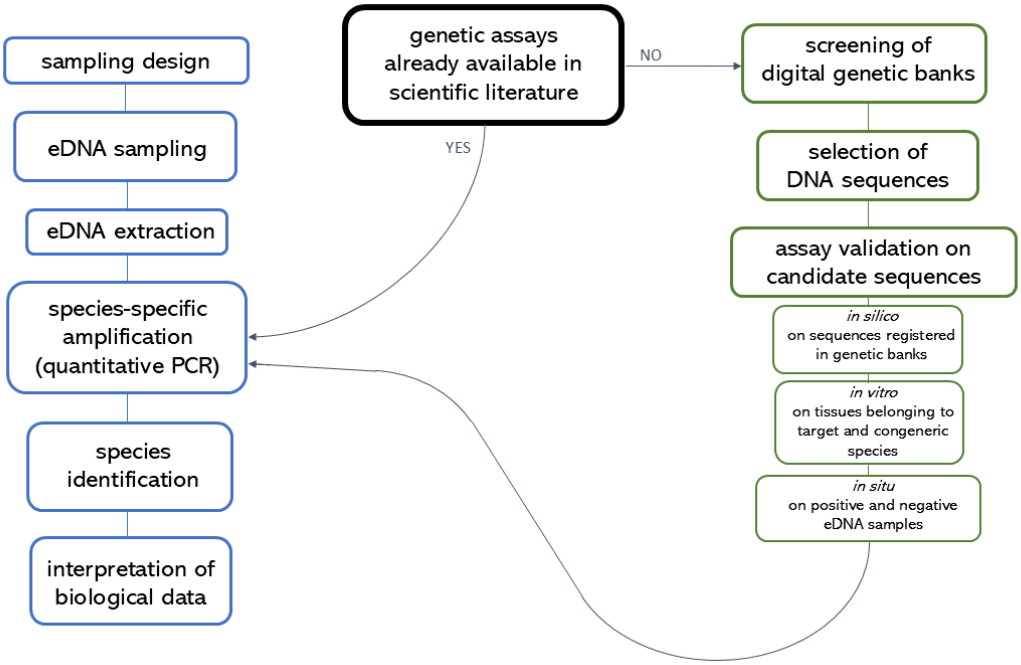


Figure 1. Workflow of a species detection study using environmental DNA-based techniques.

The ecology of environmental DNA. Consequences on sampling strategy and techniques.

Environmental DNA (eDNA) can appear in freshwaters in the form of DNA molecules released into the environment by an organism through its cellular exudates, such as eggs and sperms, blood, or skin, hair and other epithelial cells shed with faeces, urine, saliva, and tears (Rees et al. 2014). A second way in which eDNA can be present is in the form of DNA protected within subcellular organelles after the cells have undergone degradation, such as mitochondria, nucleus, and ribosomes for RNA. A third source of eDNA consists of free DNA dispersed directly into the environmental matrix. Environmental DNA, thus defined as *extracellular DNA* or *extra-organism DNA*, is distinguished from the DNA composed of cells or entire organisms that are sampled together with the environmental sample, for which we refer to as *organismic DNA*.

The probability of detecting the environmental DNA of a given species in freshwaters is influenced both by biotic and abiotic factors (Randall et al. 2023). The release of DNA into the environment can vary from species to species and is related to biomass, abundance, activity levels, life stages, and population density (Rees et al. 2014, Goldberg et al. 2016, Stewart 2019). Differences in the level of DNA dispersion in the environment often cause a variability in the detection probability over time and space (Randall et al. 2023). This is caused by factors including season (Goldberg et al. 2011, Buxton et al. 2017b, Franklin et al. 2018, Takahashi et al. 2018), watercourse type (lentic or lotic, Deiner et al. 2016, Hanfling et al. 2016), pH (Strickler et al. 2015, Seymour et al. 2018), turbidity (Wineland et al. 2019), water temperature (Takahara et al. 2012, Strickler et al. 2015, Buxton et al. 2017b, Saito and Doi 2021), substrate (Buxton et al. 2017a), and exposure to radiation (Pilliod 2014, Strickler et al. 2015).

These parameters describe a kind of "ecology" of environmental DNA, as they have a direct effect on:

- the production of environmental DNA
- the way with which DNA is transported and mobilized into the environment
- the speed at which DNA is degraded
- DNA inhibition.

These factors must be combined with the parameters that influence species presence, both in spatial terms (suitable habitats) and temporal terms (species phenology). Considering these factors is extremely important, as they can influence the interpretation of results.

Unlike methods that provide direct presence data of a species, through direct (i.e., trapping) or indirect observations (i.e., presence signs) during a specific period, environmental DNA involves a certain degree of uncertainty both about the precise location of the DNA's origin and the time frame in which the DNA was released (Randall et al. 2023).

In studies based on molecular data, there is also the possibility of fall into the so-called false absences (i.e., where a species is not detected despite being present) and false presences (i.e., where a species is detected in a location where it is not actually present). False absences can be caused by a premature degradation of eDNA or are due to a low concentration, whereas false presences are primarily caused by the movement of DNA between different environments, due to either anthropogenic or natural factors, or contamination between samples during the study (Kéry and Schmidt 2008).

Factors influencing the probability of detecting eDNA are summarized in Fig. 2. In the following sections, we will explore more deeply how to optimize a sampling campaign addressed on environmental DNA.

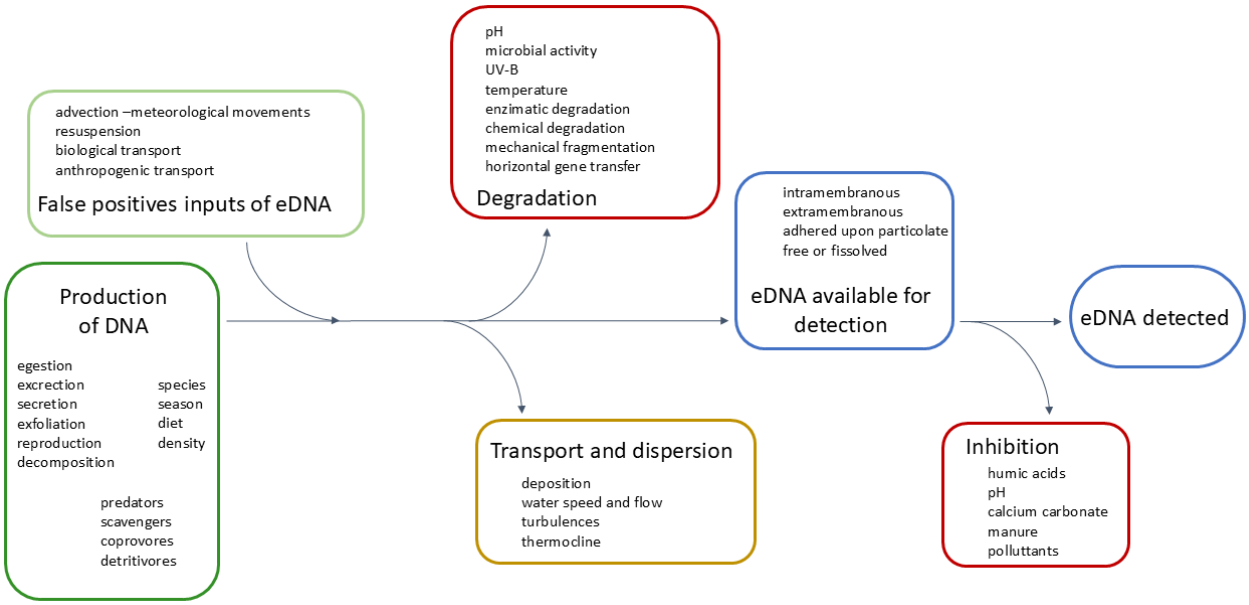


Figure 2. Biotic and abiotic factors influencing the probability of detection (detectability) of environmental DNA (modified from Strickler et al. 2015; Barnes and Turner 2016).

1.1 Production and detectability

Environmental DNA is primarily released into nature in the form of cells and tissues that later may also fragment and release DNA into the environment. In aquatic environments, cells and tissues are lost through faeces, which is an important mechanism for detecting terrestrial organisms living in surrounding habitats. Other sources include mucous secretions from the skin (e.g., amphibians, fish), decomposing carcasses, and eggs and sperms during mating seasons (Martellini et al. 2005; Ficetola et al. 2008; Jerde et al. 2011). This process defines what is known as the production of environmental DNA. The probability of detecting a species using environmental DNA increases in relation to the amount of DNA produced by the species. Higher concentrations of eDNA reduce the likelihood of false absences.

Among variables that are mostly correlated with an increase of eDNA production there is the population biomass of the target organism, and his proxy variable, the population size (Pilliod et al. 2014, Maruyama et al. 2014). The correlation between population size and the number of eDNA copies dispersed into the environmental matrix is so evident that eDNA has also been used to estimate population sizes (Thomsen et al. 2012; Takahara et al. 2012; Pilliod et al. 2013; Levi et al. 2019). However, the relationship between eDNA concentration and population density must be evaluated with caution, as the amount of DNA released into the environment may also be related to the source and not necessarily to the concentration of individuals (e.g., in the case of carcasses in water) (Randall et al. 2023).

eDNA detectability is also influenced by species metabolism. Changes in metabolism during the ontogeny of certain species result in growing juveniles shedding more tissue compared to other segments of the population, maximizing their contribution to DNA production and thus enhancing detectability despite lower biomass values (Maruyama et al. 2014; Iversen et al. 2015; Schmidt et al. 2021a). Metabolic changes can also be indirectly caused by variations in abiotic factors. For instance, increased eDNA production is associated with rising water temperatures. This is a clearly seasonal phenomenon due to fluctuations of air temperature and implies that during summer (in norther hemisphere), eDNA production by organisms significantly increases compared to winter (Schmidt et al. 2021; Lacoursiere-Roussel et al. 2016). A similar phenomenon has also been observed in plants (Potè et al. 2009).

eDNA detectability can also vary based on wildlife behaviour. Higher rates of eDNA production are associated with more pronounced feeding activities, that are individuals that spend more time searching and manipulating food (Klymus et al. 2014). In these cases, it is likely that the release of a greater amount of eDNA is due to increased metabolic rate resulting in a higher rate of turnover of cells and tissues, consequently lost into the environment. Additionally, the presence of stressors can lead to an increase of up to 100 times in the number of eDNA copies (Sassoubre et al. 2016; Jo et al. 2019; Maruyama et al. 2014; Thalinger et al. 2021). Therefore, organisms that are stressed, warm, and active tend to release larger quantities of eDNA.

The amount of eDNA varies according to the taxonomic group. Fish, amphibians, and molluscs release large quantities of DNA into freshwater environments. Mammals, both semi-aquatic and terrestrial (such as lagomorphs, ungulates, mustelids, and talpids), can also be efficiently detected, especially using the eDNA metabarcoding approach (Jamwal et al. 2021; Broadhurst et al. 2021). The results are highly heterogeneous among different species but highlight the potential applications of the phenomenon that describes rivers as conveyor belts of terrestrial wildlife DNA, likely due to the runoff of rainwater over the soil (Deiner et al. 2016; Sales et al. 2020a, b).

Conversely, arthropods and reptiles seem to be more difficult to detect using eDNA-based techniques, likely due to their exoskeleton or hyper-keratinized skin, especially when present at low densities (Treguier et al. 2014; Allan et al. 2020; Adams et al. 2019; Curtis 2020). It has also been hypothesized that ectothermic animals may have a lower detection probability due to their low metabolic rates, which result in reduced eDNA production (Bruce et al. 2021).

At high population densities and with the use of specific filtration and extraction protocols (Geerts et al. 2018, see Section 6 and 7), eDNA-based approaches have also been used to estimate the abundance of crustacean and freshwater reptile populations (Lam et al. 2019; Kakuda et al. 2019), and progress is being made even at very low densities (King et al. 2022).

1.2 Persistence and degradation

DNA has a scarce chemical stability and, depending on environmental conditions, begins to degrade immediately after its release into the environment (Lindahl 1993). Its persistence in the environment depends on the characteristics of the DNA (such as conformation, length, and association with other cellular organelles that have additional protective membranes, like mitochondria), the abiotic environment (temperature, light, pH, oxygen, salinity, substrate

composition), and the biotic components present in the environment (microbial community composition and activity, extracellular enzymes). Additional forms of DNA degradation include mechanical fragmentation (e.g., turbulence in water) and chemical degradation (Barnes and Turner 2016).

Once released, DNA starts fragmenting, first mechanically and then through the action of extracellular enzymes and spontaneous chemical reactions. An initial fragment of tissue reduces first into several cells, then it separates into various subcellular organelles, and finally it is reduced into free extracellular DNA (Harrison et al. 2019). The intensity of degradation in specific conditions allows researchers to select the types of molecules to be targeted during sampling phase, and thus to optimize eDNA collection strategies (Turner et al. 2014; see Section 2.4 on eDNA available for detection). Different cellular districts exhibit different decay rates and different time intervals in which their DNA is detectable (Shogren et al. 2018; Eichmiller et al. 2016b; Bylemans et al. 2018).

When detected through water, **DNA can persist from a few days to several weeks,** or even up to a month. In experimental conditions, 80% of the eDNA is degraded within the first three days and 90% within the first ten days, but a significant amount of eDNA (>5%) can remain detectable for up to 58 days (i.e., in the 4% of the sample; Strickler et al. 2015). Experiments conducted in natural settings confirm a persistence time for eDNA of 17 days, beyond which the detection probability drops below 5% (Dejean et al. 2011). In general, the average persistence time is one to two weeks. However, the time frame in which eDNA can remain detectable in aquatic environments depends on environmental contexts (Dejean et al. 2011; Piaggio et al. 2014; Dunker et al. 2016).

A significant difference arising from environmental contexts is the **type of habitat**. In flowing waters, DNA appears to persist less than in lentic systems, likely because it is physically transported away from the release point (see Section 2.3 on transport and dispersion of eDNA). Persistence in lotic environments is particularly low in smaller watercourses, ranging from 1-2 days (24 hours: Deiner et al. 2016; 43 hours: Seymour et al. 2018; 48 hours: Balasingham et al. 2016). Such persistence values are very similar across different taxa, suggesting that the dynamics of DNA degradation are comparable among various species, a phenomenon particularly relevant for the applied implications of eDNA-based techniques in community studies. However, in lotic environments, most of the DNA (>90%) is lost within much shorter time frames, even within the first three hours (Seymour et al. 2018) or within just a few minutes (Jerde et al. 2016; Wilcox et al. 2016).

Among the abiotic factors influencing DNA persistence in the environment is the penetration of sunlight in the water column. **Sunlight** can cause damage to DNA, especially from light falling within the ultraviolet radiation spectrum (Ravanat et al. 2001; Pilliod et al. 2014). UV-B radiation is the most energetic component of the solar radiation that reaches the surface of our planet, and it is considered by many to be the most harmful for aquatic environments. However, it penetrates the water column only few centimetres (Hader et al. 2003; Diffey et al. 2002). In contrast, UV-A rays, which have lower energy and a negligible effect on eDNA degradation, have a wavelength that allows them to penetrate water at greater depths. Depending on the suspended particulate matter absorbing this wavelength, UV-A can penetrate from few centimetres in eutrophic lakes to 10-20 metres in oligotrophic lakes (Kirk 1994). The impact of radiation is considered significant enough that algal cover, which can help reduce sunlight penetration, is a parameter often evaluated by eDNA experts in studies of eDNA ecology (Lance et al. 2017). Actually, many of these studies have shown that solar exposure has no direct effect on eDNA detectability, neither in terms of UV-A nor UV-B, but rather in terms of the indirect increase in the temperature of water subjected to solar radiation (Andruszkiewicz et al. 2017; Machler et al. 2018). In this way, solar radiation significantly

influences eDNA persistence only in aquatic ecosystems exposed to consistent radiation, such as those at high altitudes or closer to the equator (Godar 2005).

Water temperature indeed appears to be the most important environmental factor determining eDNA decay times. At higher temperatures, DNA degrades faster (He et al. 2015; Strickler et al. 2015). Numerous studies confirm this hypothesis, demonstrating that eDNA persists better in winter than in summer (Lacoursière-Roussel et al. 2016; Pilliod et al. 2014). However, it is not a matter of direct influence among temperature and eDNA. Temperature has a direct effect on eDNA denaturation only at very high values (e.g., >50 °C), which rarely occur in the natural contexts of intermediate latitudes and even more unlikely to influence waters and their buffering power in response to thermal stress. It remains unclear whether temperature *directly* causes the degradation of DNA released into the environment or if it rather has an *indirect* influence on other environmental factors responsible to causally promote eDNA degradation. Temperature has not been found to be significant in regulating eDNA persistence in many case studies (Andruszkiewicz et al. 2017; Seymour et al. 2018). Therefore, it is likely that key factors determining eDNA degradation over time are others, such as microbial activity or pH (Strickler et al. 2015; Tsuji et al. 2017; Machler et al. 2018; Seymour et al. 2018).

Microbial activity is directly influenced by temperature. High rates of microbial activity potentially lead to a significant degradation of eDNA due to the high metabolic activity of microorganisms and to the kinetic reactivity of extracellular enzymes belonging to the exonuclease family (Tsuji et al. 2017; Saito and Doi 2021). Recent studies have shown that microbial activity is likely the most important environmental factor influencing the persistence of eDNA. This conclusion is supported by research that has observed that fish eDNA decreases exponentially over time in both freshwater and saltwater environmental samples, but not in sterile water samples (Eichmiller et al. 2016a; Saito and Doi 2021). Several mechanisms illustrate how microbial activity reduces the persistence time of eDNA in water. Firstly, it directly contributes to enzymatic hydrolysis by producing degradation enzymes (exogenous nucleases or exonucleases) that break down DNA into smaller components (Lindahl 1993; Torti et al. 2015). These extracellular enzymes, such as nucleases, are found in a free form in aquatic ecosystems and can also be released by macroscopic organisms (Bochove et al. 2020; Nukuzawa et al. 2020). Secondly, DNA can be directly assimilated by microbes: under nutrient-poor conditions, such as scarcity of phosphorus, microbes can secrete DNA-degrading enzymes to convert DNA strands into phosphate and secondary waste products (purines and deoxyribose: Salter et al. 2019). Additionally, some waste products from bacterial activity, such as urea, can decrease the stability of eDNA molecules by weakening covalent bonds and facilitating their degradation (Yin et al. 2019). However, in some cases, particularly concerning plants, a positive influence of microbial activity has been observed, which increases the release of DNA from plant matter in river sediments and enhances detectability (Potè et al. 2009).

pH of waters has also a strong influence on eDNA detectability. It has been observed that eDNA retrieval decreases dramatically below a pH value of 6 (Seymour et al. 2018; Schmidt et al. 2021). The structure of DNA is indeed very stable under favourable conditions (anoxia and low humidity, half-life estimated about 500 years), it decays very quickly, even within a few minutes, in environments that have low pH values and high levels of oxygenation (due to oxidation and hydrolysis: Lindahl 1993; Allentoft et al. 2012; Torti et al. 2015). In other cases, eDNA has also been detected at low pH values (e.g., pH=4). This suggests that pH may influence the persistence of eDNA only in synergy with other environmental factors (Strickler et al. 2015). Evidence for this comes from a controlled environment study indicating that pH has no effect on DNA persistence (Bochove et al.

2020). Among the variables that may interact with pH are microorganisms. The most favourable environment for the explosion of microbial activity is observed at neutral pH values, which consequently leads to a decrease in DNA persistence in the environment (Strickler et al. 2015). Finally, there is also an interaction between pH and sediment type, as the adsorption of eDNA by the residual electrostatic charges of clay particles is facilitated at low pH values (Goldberg et al. 2015).

Suspended sediment indeed can protect eDNA and increase its persistence in the environment (Taberlet et al. 2018; Gutierrez-Cacciabue et al. 2016). For instance, adhesion to clay particles requires up to ten times the concentration of extracellular enzymes to achieve effective eDNA degradation because it prevents the double helix from opening or alters the conformation of the molecule, making the DNA unidentifiable to degrading enzymes and protecting it from chemical damage (Khanna and Stotzky 1992; Stewart and Taylor 2020, in Joseph, 80-81). Furthermore, clay and sand particulates also absorb extracellular enzymes, inhibiting or inactivating them and reducing the concentration of active enzymes in the environment (Khanna and Stotzky 1992). The presence of sediment thus makes eDNA more persistent and facilitates detections even outside the species' active period. For example, eDNA from migratory fish has been detected in water only for a few days after migration, while it has persisted in sediment for over four months (Sakata et al. 2020). However, this creates a series of disadvantages that reflect on the temporal resolution of presence data, as well as with the possibility of false positives in the case of mobilizing ancient well-preserved DNA from sediment.

eDNA molecules also bind to molecules in the **humic acid** family. In lakes with high concentrations of humic substances, the concentration of enzymes required for effective DNA degradation is 100-1000 times higher than that of free eDNA forms (Lance et al. 2017).

In conditions of high **oxygen concentration**, eDNA molecules degrade rapidly. Oxygenated waters, such as particularly turbulent flowing waters (high mountain creeks), could therefore represent unfavourable conditions for eDNA persistence. However, dissolved oxygen concentration is a factor that interacts with other environmental variables. Conditions of oxidative stress are observed, for example, in the presence of high temperatures. These conditions promote the decomposition of organic matter and the consequent production of reactive oxygen molecule species, decreasing eDNA persistence (Harrison et al. 2019).

Finally, the presence of specific **chemical compounds**, such as insecticides (diazinone, hexachlorocyclohexane), decreases eDNA persistence by causing direct degradation, deforming the structure of the molecule, and facilitating degradation by extracellular enzymes present in the environment (Eichmiller et al. 2014; Pourmoghadam et al. 2019). Antibiotics, which often spread in water through wastewater, form metal complexes that increase hydrolysis by $5.2x10^7$ times and rapidly degrade DNA (Sreedhara et al. 2000; Sreedhara et al. 2001).

1.3 Transport and dispersion

DNA released into aquatic environments is susceptible to the water movements and currents of water bodies. Therefore, the study of hydrology is of primary importance for understanding the transport dynamics of eDNA and for inferring the presence and spatial distribution of species from the point where their eDNA is detected.

In **lakes and lentic systems**, the spatial dispersion of eDNA is limited to the order of hundreds of meters (Ghosal et al. 2018; Goldberg et al. 2018; Dunker et al. 2016). Vertically in the water column, DNA has greater mobility since it tends to sink due to gravity and accumulate in deeper layers (MacIntyre et al. 1995; Turner et al. 2015). In particular, it has been suggested that faecal matter and the residual DNA it contains tend to accumulate in the lower layers of the water column. Therefore, to sample viable populations (i.e., non-ancient DNA), the optimal strategy is to focus on collecting water from the surface layers (Wotton and Malmqvist 2001; Kamoroff and Goldberg 2018). However, it should be noted that lentic systems in Mediterranean regions are often predominantly monomictic lakes, with water circulation and mixing of layers occurring once a year. This can lead to homogenization in the spatial distribution of eDNA, making it more easily detectable but also mobilizing ancient sediment from the bottom. In summer, on the other hand, waters stratify at different density values, and eDNA degradation in the surface layers of the epilimnion is much faster, while in the deeper layers (hypolimnion) it is almost nothing (Rawlence et al. 2014).

In **rivers and lotic systems**, by contrast, the spatial distribution of eDNA is made more homogeneous by water turbulences, which continuously mix water columns (Pont et al. 2018). eDNA tends to be transported downstream by the current, but its dynamics have not yet been accurately reconstructed. Since the particles of eDNA sampled often have a diameter between 0.2 µm and 10 µm, eDNA molecules can be categorized as fine particulate organic matter (0.5 µm – 1 mm). Some models of eDNA transport in river environments seem to describe results very similar to the transport dynamics of fine particulate organic matter (Sassoubre et al. 2013; Allan and Castillo 2007; Webster et al. 1987). These models estimate an average transport distance of eDNA between 100 m and 100 km (Jane et al. 2014; Wilcox et al. 2016; Deiner et al. 2016; Song et al. 2017; Pont et al. 2018). Within these models, it seems relatively easy to incorporate eDNA decay time or current velocity to estimate eDNA transport distances, while it is more difficult to consider other variables affecting eDNA transport, such as retention and resuspension, which are seen as more stochastic. In lotic bodies, DNA also tends to settle by gravity on the riverbed and bind to the substrate (Turner et al. 2015). Such molecules can persist in the substrate, be transported in the hyporheic water flow, infiltrate groundwater especially in karstic areas (thus exiting the river system and becoming undetectable in that environmental context), or be resuspended in the water column (Schogren et al. 2017; Turner et al. 2015). Some studies suggest that eDNA retention is a factor that predominantly limits eDNA dispersion compared to degradation (Shogren et al. 2016; Shogren et al. 2018; Shogren et al. 2019). Resuspension, on the other hand, leads to the presence of false positives regarding temporal resolution and may also be a possible explanation for unexpectedly long DNA persistence times (Tillotson et al. 2018).

Case studies suggest that the distance at which an eDNA signal can be detected is species-specific. For some fish, maximum eDNA detection distances have been found to be around 250 m, while for bivalves of the genus *Unio*, the spatial resolution of detection is greater (9 km), reaching even higher distances for amphibians (20 km) and fish (100 km) (Deiner et al. 2014; Jane et al. 2014; Villacorta-Rath et al. 2021; Pont et al. 2018). However, it is unrealistic to extrapolate information from case studies for accurately define the transport dynamics, at least since the use of different approaches provide incomparable data, such as in the case of technologies using a filtering water volume 30 times higher than the standard. In other cases, it has been suggested that the entire river community eDNA-detectable changes every kilometre or so (2-3 km: Civade et al. 2016).

Seasonality of stormwater also impacts the distance at which eDNA is detected. It decreases both during dry periods and during flood events, in the latter case likely due to significant dilution (Jane et al. 2014; Staley et al. 2018). During rains that do not constitute true floods, it is likely the best

time to sample flowing waters for terrestrial species as well (Denier et al. 2016).

1.4 eDNA available for detection

DNA can be detected in the form of cells or subcellular organelles, or it can exist as free DNA or adsorbed onto fine particulate matter. These different states in which eDNA appears have varying molecular sizes. Understanding the state with which DNA occurs in the environment can be crucial for developing an effective eDNA sampling strategy (Jo 2023).

The length of eDNA determines the surface area available for enzymatic attack and also influences the ability to bind to other particles in the environment, which in turn affects the probability of persistence, both positively and negatively (Wei et al. 2019). Finer sediments, such as clay, are the most efficient at retaining eDNA due to the conductivity between particles, a greater binding area offered by the surface of the particle compared to other grain sizes (e.g., sand), and the enrichment in iron hydroxides that allows for a maximum adsorption of 10 µg/g of eDNA (Pedersen et al. 2015; Buxton et al. 2017; Sakata et al. 2020).

eDNA collected using filters with larger pore sizes show a higher rate of genetic damage, implying that cell membranes do not necessarily protect intracellular DNA from degradation by DNases present within the cell (Jo 2023). This is why longer eDNA fragments allow for more accurate temporal inferences regarding living organisms. If the decay time of long DNA fragments is very short, they represent biological information collected almost in real-time (Jo et al. 2017; Jo et al. 2020). In contrast, shorter eDNA fragments (125 bp) have much slower decay times than longer eDNA fragments (716 bp). These have a low temporal resolution but a high spatial resolution, and are ideal for detecting rare species, which are sparsely distributed and thus more difficult to intercept spatially with sampling, or simply release small amounts of DNA (Bista et al. 2017; Harrison et al. 2019).

Sampling mitochondrial DNA molecules increases the capacity to detect target species. The DNA fragments selected for creating genetic primers often belong to mitochondrial gene coding for Cytochrome Oxidase I. Compared to nuclear DNA, which has 2 copies per cell, this type of genetic region has a greater DNA copy density, with 2 to 10 copies per cell (Barnes and Turner 2016). This increase in DNA copy density i.e., (eDNA production) also influences the persistence of eDNA in the environment, as various studies emphasize that mitochondrial DNA has a significantly lower decay rate than nuclear DNA (Jo et al. 2019a, 2019b; Harrison et al. 2019), maybe due to a double membrane envelope. For this reason, mitochondrial DNA have a more stable cellular structure, making it a more effective tool for assessing the fate of eDNA (Moushomi et al. 2019). Detectability therefore also heavily depends on the method of eDNA acquisition and the extraction protocol chosen (Geerts et al. 2017, see Section 6 and 7). For example, extracellular DNA can pass through filters with pore sizes larger than 0.2 µm (Matsui et al. 2001; Turner et al. 2014), while the more common filters, with 0.45 µm pores, likely only intercept DNA that persists within cells or mitochondria (Strickler et al. 2015).

1.4.1 Inhibition

It often happens that the eDNA that is in a sample becomes unavailable for detection during

laboratory phases (extraction, amplification, PCR: Tsai et al. 1992). This is due to masking effects from several compounds to which eDNA molecules can bind (e.g., sediment, algae, humic acids, dissolved organic compounds, carbonates, tannins) or the presence of soils contaminated with heavy metals or manure from domestic livestock (Nguyen et al. 2007a,b; Nguyen et al. 2010; Schrader et al. 2012; Pourmoghadam et al. 2019; Bruce et al. 2021).

The removal of inhibitors is an important step included in DNA extraction protocols, but its effectiveness is inhibitor-specific and depends on the type of extraction kit used. To assess its effectiveness, it is always advisable to conduct an inhibition test beforehand (Bruce et al. 2021). In this case, the interpretation of results should always be done responsibly in the light of the potential effects that inhibition can have on the occurrence of false negatives.

1.4.2 Contamination

Unlike the effects of inhibition, which generate false absence errors, there are some sources of error that can contaminate the environmental sample with allochthonous DNA, generating false presence data. This contamination can occur both spatially, in the case of DNA mobilized between different areas, and temporally, for example, due to the resuspension of ancient DNA.

Among the sources of input of allochthonous eDNA, there is the one related to the diet of predators. If the target species is classified as a prey within the food web, the DNA of that species can be released in the faeces of the predator at a great distance from where the species actually occurs, especially if the predator is a particularly vagile species (e.g., birds, apex carnivores).

The presence of human settlements also leads to false occurrences, mainly due to livestock, domestic animals, and pet species of allochthonous origin (i.e., if they have similar DNA to the target species), as well as species subject to trade for human consumption in supermarket products and the food supply chain, whose DNA is released into domestic and industrial waters (during processing and cleaning) as well as in sewage discharges (after consumption) (TES, 2019).

Considering the specificity of primers and probes used is a critical point that can be taken into consideration for an aware interpretation of results. The phenomenon of eDNA resuspension, on the other hand, is linked to stochastic factors, but it can somehow be predicted by monitoring environmental variables such as those related to hydrology, channel characteristics, sediment type, and the presence of biofilms that can retain or degrade eDNA (Wilcox et al. 2016; Shogren et al. 2017). To mitigate anthropogenic contamination between different samples during the study, using sterile materials is mandatory, and the use of intermediate control samples (*blank* or *blind samples*) to be filtered with sterile water between one sample and another can be considered.

1.5 Final considerations

In summary, a species detection campaign through eDNA sampling poses several technical challenges related to the low quantity of nucleotide sequences in the environment, the heterogeneous distribution in space, the risk of contamination, and the co-amplification of PCR inhibitors, which reflect in the sampling design and methodologies to be preferred.

It is important to emphasize that the detectability of eDNA is species-specific and state-specific, and that environmental variables act differently on the various DNA molecules present in the environmental matrix. Biotic and abiotic components of an ecosystem interact with each other almost at every stage of the process during which eDNA face the environment. This interconnection among driving forces generates a continuous influence on DNA production, its mobilization, and ultimately its persistence time (Fig. 3). This makes it difficult to predict the fate of eDNA in different study areas and poses interpretative challenges about the origin of the DNA itself. To counteract the troublesome interpretation of eDNA results related to the various conditions the environment can pose target species molecules, it is unavoidable to calibrate the eDNA assays with experimental tests assessing DNA yields given by known population size and structure under control conditions and with different parameters (e.g. Thalinger et al. 2021).

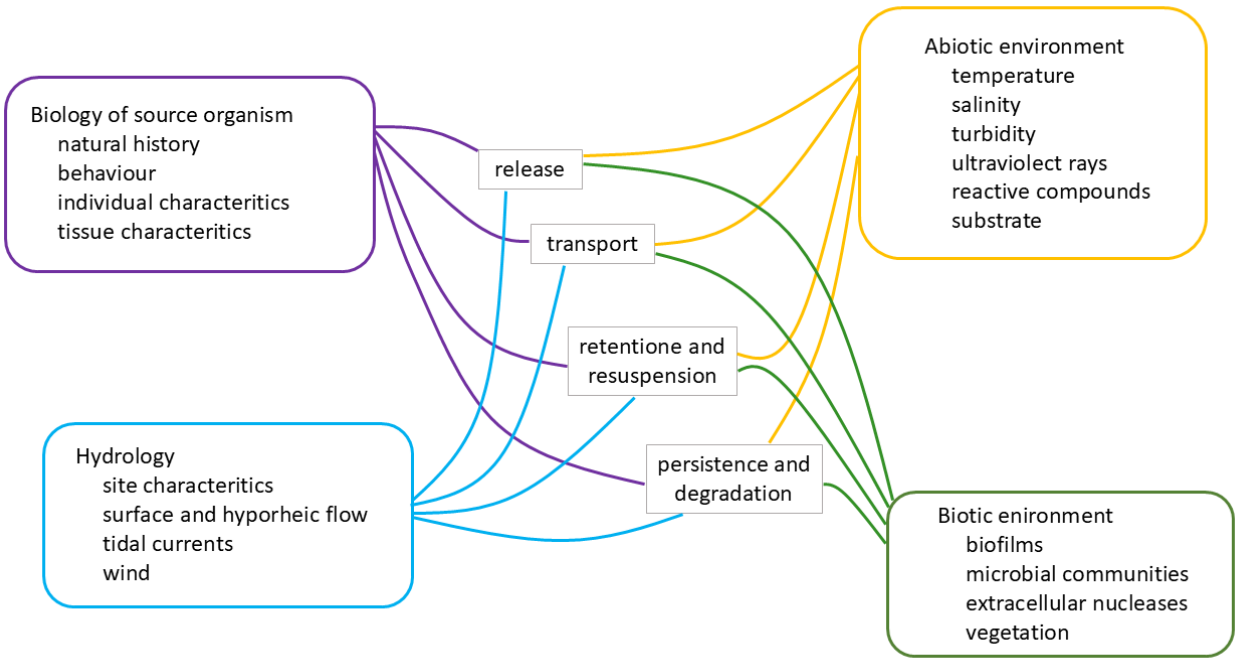


Figure 3. Mechanisms influencing eDNA concentration and fate in aquatic systems (adapted from Harrison et al. 2019).

Table 1 summarizes the environmental variables that influence the ability to detect eDNA. The most important factor regulating the persistence of eDNA in the environment appears to be microbial activity, while the key factors to monitor in order to increase the likelihood of eDNA detection are temperature and pH, with the presence of inhibitors being a secondary consideration.

Microbial activity increases with temperature and is maximized at higher temperatures. As microbes we are considering live in freshwater ecosystems, we mainly refer to water temperature, and with air temperature that can be also considered as a proxy variable to broadly measure the microbial growth. At the same time, microbial communities proliferate exponentially at neutral pH levels, while they are less common in acidic and alkaline environments. Laboratory DNA preservation solutions are all at basic pH indeed (e.g., Longmire buffer). However, eDNA is difficult to detect at low pH.

To maximize detectability it is therefore evident that a sampling campaign should occur under conditions that are unfavourable for microbial proliferation, and specifically at low to moderate

temperatures and in alkaline pH conditions. However, these conditions are quite rare in freshwater systems, which typically have a neutral pH. Regarding temperature, DNA is better preserved at low winter temperatures, although most species reduce their activity during this time. The best times of year for sampling appear to be in summer or spring when the DNA is produced by species sufficiently high to counteract the effects of microbial degradation.

Table 1. Summary of environmental parameters and their influence on the probability of detecting eDNA (detectability).

parameter		increase detectability	decrease detectability	process affecting eDNA	cause-effect relationship
temperature	**low**	yes	no	degradation	interacts with microbial community and metabolic activity of target species
	high	no	yes		
microbial community		no	yes	degradation	interacts with temperature and pH
pH	**acid**	no	yes	degradation	interacts with microbial community
	neutral	yes	no		
	alcaline	yes	no		
substrate	**clay**	yes	yes	degradation inhibition	increase eDNA persistence but inhibits PCR ($CaCo_3$ inhibits only with large quantities)
	$CaCo_3$	no	yes		
population size		yes	no	production	positively correlated with detectability
metabolism		yes	no	production	positively correlated with detectability
ultraviolet light		no	may be	degradation	interaction with many other variables
dissolved oxygen		no	yes	degradation	correlated to litter presence
algal covering		yes	yes	inhibition	decrease temperature but inhibites PCR
humic acids		yes	yes	degradation inhibition	increase persistence but inhibites PCR

2. Species selection

For sampling campaign through a species-specific environmental DNA approach, the selection of target species is a process based on the goals and objectives of those undertaking an eDNA study (e.g., management objectives for protected areas), on technical-scientific assessments, and finally on practical aspects concerning logistics and finance.

Below, we illustrate some characteristics that can influence these selection criteria.

Management objectives

Monitoring obligations arising from European community or national regulations. Some European regulations require reporting on the presence, distribution, and conservation status of endangered species (Habitats Directive 92/43/EEC) or invasive alien species (Regulation 1143/2014). In relation to these obligations for periodic reporting, eDNA investigations can be very useful for obtaining a broad-scale simultaneous overview of many species, limiting the costs and time of species-specific assessments carried out by expert groups.

Monitoring of threatened species. Environmental DNA offers a rapid method for monitoring endangered species, especially in the case of rare, elusive, or hard-to-detect species (Thomsen et al. 2012). Local-scale projects can be directed toward assessing the conservation status of the most threatened species at local, national, or global scales (e.g., Rondinini et al. 2022).

Early warning of alien or problematic species. Invasive and alien species often compete for resources with native species, constituting one of the primary threats to the conservation of many endangered species. The list of the 100 Worst Invasive Alien Species in the World, compiled by the IUCN Invasive Species Specialist Group, can be used to identify which species to investigate using eDNA. In his way, it is possible to better understand the distribution of an alien species only partially spotted or to determine for the first time whether a species is present in the area by conducting an initial surveiiilance campaign (e.g., in the case of reports close to the borders of the study area). In addition to invasive species, transfaunated species can also be considered. These are species introduced outside their native range into geographically contiguous areas (*vicarious species*) due to human intervention, often belonging to the same political Country and therefore not subject to control and eradication laws, but functionally behaving as alien species in the biogeographic context in which they operate to threat native wildlife.

Estimates of population density. The size of a population is a crucial parameter for understanding the achievement of conservation goals or whether additional management measures are necessary. Environmental DNA provides the possibility to infer population size based on the number of DNA copies detected within the sample, although such estimates must be preceded by a rigorous calibration process that takes into account the ecology of species-specific environmental DNA (for an example, see Uthicke et al. 2022).

Practical aspects

Sometimes, environmental DNA underestimates the presence of certain species because the presence of hard cuticular or keratinous coverings, such as the exoskeletons of arthropods or the

multilayered stratum corneum of reptiles, prevents the release of sufficient concentrations of DNA into the environment (Gleason et al., 2020; Ficetola et al., 2021). Ectothermic animals may also have lower detectability since they have a slower metabolism that results in lower eDNA production (Ficetola et al., 2021). Although some technical adjustments, such as sampling during the correct phenology or implementing spatial and temporal replicates, can mitigate these detectability issues, the inclusion of these taxa should be carefully considered and potentially accompanied by field verification with traditional techniques, at least in random sites.

Contamination. In general, it is not advisable to detect species that are commercially exploited or to use generic primers shared with species commonly used for food consumption, as their DNA can be released downstream from any human settlement.

Costs. The expense of an eDNA project is primarily affected by the number of target species. The more the target species must be assessed within a survey, the more the funding is needed. An additional aspect to consider is the availability of the genetic assays. Species lacking an assay that is accurate and ready-to-use for an eDNA survey require time and can be uneconomic. Designing an assay for the first time involves multiple validation steps to ensure precise identification of the species without any mistake of false- absence and presence (Thalinger et al. 2021). Moreover, as previously mentioned, some genetic markers have particularly high development costs. This is especially true for assays of species that may undergo hybridization with non-target species. In these cases, developing a species-specific primers and probes requires several steps to identify and validate the unique sequences that allow for distinguishing pure individuals from hybrids (see after).

Another factor to consider is related to the limited **number of target species** that can be analysed with an environmental sample, which generally stands at about a dozen species (A. Bruno, pers. comm.). To increase the number of species analysed per sample, it is necessary to collect an additional environmental water sample. This affects the working time (filtration, extraction, analysis) and laboratory costs.

Scientific evaluations (assessment)

Availability of genetic assays. Using genetic primers and probes already published in scientific articles and tested in the field can be an easy solution to select which species can be included in a multi-specific eDNA project. The availability of assays that have already been developed and published in the literature allows for reduced project time and costs and can represent a main selection criterion for species.

Feasibility of developing genetic assays (taxonomic assays: species, subspecies, cryptic species, species complexes). If one wishes to proceed with the detection of a species for which no published genetic primers and probes are available, assays must be developed. This may be the case, for example, for many endemic species or species distributed in very limited areas. The process of designing an assay for the first time requires several validation steps to develop accurate primers and probes that can precisely identify the target species, also considering any geographical variability of the populations (Thalinger et al. 2021). Moreover, some species do not yet have a well-defined taxonomic placement, and therefore the use of small DNA sequences does not allow for accurate discrimination between species, often only enabling genus identification. This is the case, for instance, for river shrimps of the genus *Austropotamobius* (Manfrin et al. 2022).

Interspecific hybridization. Similarly, as mentioned above, the simultaneous presence of hybridizing species in a biotope results in a significant difficulty in detecting parental genotypes. For example, in an area representing the so-called *hybrid zone* between the ranges of two different species that can hybridize (e.g., *Cordulogaster trinacriae* and *C. boltoni* in Latium, Italy), detecting one species or the other using eDNA techniques is very difficult unless one has a financial resource twenty times greater than that required for developing standard primers and probes. This issue is particularly critical for taxa subject to illegal introductions, as in the case of fish species released for fishing purposes, which facilitate hybridization between native and translocated species belonging to the same genus, such as *Barbus tiberinus*, which hybridizes in Latium with translocated species such as *B. barbus* and *B. plebejus*.

Sympatry. Sympatry with species belonging to the same Genus can also limit the development of specific assays for a particular target species, especially in cases of taxonomic placement uncertainties. If there are no congeners in the study area, as confirmed by previous studies, then it is also possible to take advantage of genetic assays that allow for identification at the Genus level.

2.1 Single or multi-specific survey

The species-specific eDNA approach allows targeted studies aimed at detecting either a single species or multiple species simultaneously. Below, we discuss the advantages and disadvantages facing this choice.

Single Species Detection

Advantages

- Ability to sample in the habitats preferred by the species and in the appropriate season based on its phenology, ensuring homogeneity in the types of environments represented in the sample and maximizing the probability of detection
- Lower costs for purchasing genetic assays
- Standardization of the volume of water sampled and filtration methods

Disadvantages

- Lack of information on the composition of the community in which the species is inserted, including the presence of species directly connected to it within the food web (prey, competitors, predators)
- Absence of data on the presence of potential species that could interfere negatively in terms of competition and pose a direct threat to conservation, such as invasive alien species

Multi-species Detection

Advantages

- Ability to lift the sampling campaign to monitor the biotic communities of a particular habitat
- Gather information on species that are related to each other within the food web (predation or competition interactions), such as the relationship between alien species and native species (Wilcox et al. 2018)

- Meet European monitoring and reporting obligations for multiple species simultaneously, without the need for a large number of experts across different taxa
- Early detection of problematic or invasive alien species on a large scale

Disadvantages

- Need for a trade-off on sampling strategies (habitat type and season) and filtration techniques that maximize the probability of detecting many species simultaneously but not all
- Variability in the detectability of different target species
- Requirement for temporal replication to address detectability issues related to a different seasonality of several species-specific eDNA
- Increased costs to purchase (and design) genetic assays and for the necessary temporal replications,

Considerations listed above were also evaluated during the first experimental multi-species study conducted in Italy for the detection of 22 freshwater species of Community or Union interest. This study was developed by the University of Molise and funded by the Latium Region (Loy et al. 2023) and helps the regional environmental authority (Management Office of Natural Capital, Parks and Protected areas) implementing the mandatory monitoring on wildlife species to assess their status and distribution.

During the species selection process, several issues emerged, leading to the exclusion of some species initially included in the list of target species. In particular, due to the risk of hybridization, the following species were excluded from the surveys: *Hirudo verbana*, *Cordulogaster trinacriae* (hybridization with *C. boltonii*), *Lampetra fluviatilis* (difficult to distinguish from *L. planeri* without more advanced genomic approaches), *Barbus plebejus* and *Barbus barbus* (hybridization with *B. tiberinus*), and *Petromyzon marinus* (not detected for many years). In the case of the twaite shad (*Alosa fallax*), we were able to circumvent the issue of congeneric species presence due to their allopatric distribution, allowing identification at the genus level (*Alosa sp.*). For gastropod molluscs of the genus *Vertigo*, the choice of identification at the genus level was justified by the fact that all species of the Genus potentially present in the study area (Latium Region) are subject to periodic reporting under the Habitat Directive. Table 2 presents the final result of species selection for that project.

Table 2: List of species selected for a multi-species survey in 52 freshwater sites of the Lazio region as part of the project 'Implementation of a multi-species detection and surveillance protocol for species of Union interest in the freshwater bodies of Lazio using environmental DNA and connectivity models,' funded by the Lazio Region (Loy et al. 2023). HD: Habitats directive. 100 WW IAS: IUCN Blacklist of '100 of the World's Worst Invasive Alien Species.

Phylum	Clade	Species	Synonym	Regulation	IUCN national Red List/100 WWIAS
Fungi	Chytridiomycota	*Batrachochytrium dendrobatidis*	-	-	100 WWIAS
Mollusca	Bivalva	*Unio elongatulus*	*U. mancus*	HD, Ann. V	EN
Mollusca	Gasteropoda	*Vertigo spp.*	*V. angustior, V. moulinsiana*	HD, Ann. II	NT-VU
Mollusca	Bivalva	*Dreissena polymorpha*	-	-	100 WWIAS
Arthropoda	Crustacea	*Austropotamobius italicus*	*A. pallipes*	HD, Ann. II, IV	EN
Arthropoda	Crustacea	*Faxonius limosus*	*Orconectes limosus*	UC (2016)	-
Arthropoda	Crustaceans	*Procambarus clarkii*	-	UC (2016)	-
Arthropoda	Insecta	*Coenagrion castellani*	*C. mercuriale*	HD, Ann. II	NT
Arthropoda	Insecta	*Oxygastra curtisii*	-	HD, Ann. II, IV	NT
Arthropoda	Insecta	*Lindenia tetraphylla*	-	HD, Ann. II, IV	VU
Chordata	Osteichthyes	*Alosa fallax*	-	HD, Ann. II, IV	EN
Chordata	Osteichthyes	*Squalius lucumonis*	-	HD, Ann. II	CR
Chordata	Osteichthyes	*Pseudorasbora parva*	-	UC (2016)	-
Chordata	Osteichthyes	*Padogobius bonelli*	*P. martensii*	introduced out of range	-
Chordata	Amphibia	*Bombina pachypus*	-	HD, Ann. II, IV	EN
Chordata	Amphibia	*Triturus carnifex*	-	HD, Ann. II, IV	NT
Chordata	Amphibia	*Lissotriton italicus*	-	HD, Ann. IV	LC
Chordata	Amphibia	*Lithobates catesbeianus*	-	UC (2016)	100 WWIAS
Chordata	Chelona	*Emys orbicularis*	-	HD, Ann. II	EN
Chordata	Chelona	*Trachemys scripta*	-	UC (2016)	100 WWIAS
Chordata	Mammalia	*Mustela putorius*	-	HD, Ann. V	LC
Chordata	Mammalia	*Lutra lutra*	-	HD, Ann. II, IV	VU

3. Sampling sites selection

To undertake an eDNA project, the selection of sampling sites is determined by the objectives of the project itself. This section considers the factors that may influence site selection criteria for large-scale monitoring, such as for an entire regional area covering more than 10,000 km².

With the number of target species to be monitored (see Section 3), sample size is the second major source of economic burden for an eDNA project. Essentially, more samples to analyse means more expenditure for laboratory materials. Once decided the trade off and figured out the sample size, a correct understanding on where to place sampling sites is fundamental to guarantee the representativeness of the sample size.

Generally, a sampling strategy on freshwaters based on eDNA must take into account two key aspects: the ecology of the target species, and the chemical and physical properties of the water.

Species ecology

Understanding the ecology of the species is essential for the selection of sampling sites. As previously mentioned, the objective of the study, for instance monitoring a species within or outside its range, can influence the criteria adopted to select sampling sites (see Section 1).

A first selection criterion involves choosing biotopes that feature the favourite habitats of the target species. An alternative approach is to conduct a preliminary study and develop predictive or expert-based habitat suitability models to determine which areas may constitute potential suitable habitats within the study area. If models are developed at a highly detailed spatial scale, sampling sites can be selected in areas with the highest probability of species presence. This allows the identification of areas where sampling efforts should be focused (e.g., Erickson et al., 2019) or at least prioritized.

However, it is important to conduct a preliminary check on site of the water bodies and aquatic habitats designed for sampling. Torrential watercourses and ephemeral ponds are characterized by a strong seasonality and must undergo a careful selection to determine if their sampling feet the study objectives. The location of sampling sites also requires preliminary site visits or checks during the first sampling to ensure that they are truly accessible (e.g., impenetrable riparian strips) and suitable for eDNA collection (e.g., absence of sewage discharges).

Scientific literature can guide evidence-based eDNA sampling strategies. For the detection of common species, it is suggested to sample upstream tributaries flowing in the upper part of the watershed. Whereas in case of rare species or species patchily distributed in few hotspots, it has been shown that sampling the main river, in the lower part of the watershed along the valley floor or near the confluences with tributaries, is more effective (Carraro et al., 2021).

Chemical and physical water parameters

Physical and chemical parameters of water can influence the probability of eDNA persistence and detection, potentially leading to false absences or false presences. To avoid the risk of false presences, it is necessary to discard sampling sites in waters with very low pH or those rich in humic

acids or algae, as these conditions limit the persistence of eDNA in the environment or inhibit its extraction (unless specific extraction protocols are applied, see Section 7: eDNA Extraction).

To avoid the risk of false presences, it is essential not to sample near sewage collectors, water treatment plants, agricultural runoff, or close to cities. These discharge points are sources of allochthonous DNA, as well as chemical compounds that can inhibit or degrade eDNA prematurely.

Two strategies can be adopted for planning a sampling strategy.

Pre-sampling site selection. Sites that are suitable for eDNA sampling can be selected before sampling begins. This knowledge can be acquired by consulting water quality databases typically maintained by agencies responsible for environmental monitoring and tracking potential pollution sources.

On-site water quality monitoring. Another approach is to select sites collecting data during the sampling process without a knowledge of water quality (see Section 6.2: Collecting environmental variables). This approach allows for post-hoc evaluations of the results and helps controlling the risk of false occurrences during data analysis and interpretation.

During the multispecies experimental study for detecting 22 freshwater species of Community or Union interest in the Latium region, 53 sites were selected across several types of natural or artificial freshwater bodies. The selection criteria for these sites included:

- presence of suitable habitats for the target species
- sites located within protected areas (national parks, regional parks, and the Natura 2000 network according to Habitats Directive 92/43/EEC)
- sites monitored for water quality, particularly within the monitoring network established under the Water Framework Directive (2000/60/EC).

In addition to these criteria, where species presence data are available and georeferenced, to optimize a multispecies sampling, sites can be selected through the overlay of several species' distribution models. These maps are useful to determine the number of primers to test at each site.

4. When to sample: seasonality and phenologies

Within a sampling strategy, it is crucial to account for both water chemistry and species biology, including their potential seasonal variability. One of the essential aspects of a sound study design is performing sampling replicates. Implementing at least one temporal replicate is always recommended to address issues like false presences caused by stochastic factors, thereby increasing confidence levels.

Species phenology

The phenology of target species is a critical factor in designing appropriate sampling strategies. Species that use wetlands only for reproduction or those that undergo migrations (e.g., diadromous species) exhibit different life stages whose seasonality must be considered. This is especially important for species with low DNA production rates, such as arthropods and reptiles (Rush et al., 2020). For such species, a best practice is to conduct samplings during the molting or mating season, when DNA molecules are more likely to be released from soft tissues, gametes, larvae, or due to the increased metabolic activity of tissue growth. Diapause or hibernation periods should be avoided for any species exhibiting seasonal activity rhythms.

When sampling several species having different phenologies, it may be necessary to perform replicates across multiple seasons. This approach differs from single-species sampling, where the sampling is carried out during the optimal season for the species, making it easier to control for external sources of seasonal variability. In multispecies sampling, results will inevitably reflect the animal community that can be sampled during a specific season, meaning that only species with a similar phenology will be detected. In this case, species-specific protocols can be used to optimize the detection of different target taxonomic groups (Geerts et al., 2017).

For a single-species study, it is necessary to consider the variability of phenology across different environmental contexts. For instance, species whose phenology is linked to a specific temperature threshold are more likely to be detectable earlier in the year at lower altitudes (e.g., in plains). A simultaneous sampling over a large study area may lead the species undetected in mountainous regions, where this temperature threshold has not yet been reached. Therefore, temporal replicates are crucial. In their absence, monitoring specific parameters (e.g., air or water temperature) can guide a temporal differentiation of sampling as the season progresses.

Finally, avoiding period with large floods and meteorological events is always suggested to prevent dilution and avoid sediment loads in the water samples, as they reduce spatial inference, the first, and temporal inference, the second (Goldberg et al. 2015).

Physical and chemical water properties

The best time for sampling freshwater bodies seems to be during intermediate seasons like spring and autumn. eDNA is better preserved at lower temperatures, which are common in winter. However, during winter, many species are inactive, and water bodies are often subject to floods or heavy rains, which increase sediment loads. Moreover, the increased water volume in rivers causes eDNA dilution, making detection more difficult. A high sediment input also makes the water more turbid, complicating filtration (i.e., clogging filters, see after in Section 6) and potentially increasing the concentration of DNA inhibitors within the sample (Randall et al., 2023). Resuspended

sediments due to high flow events lead to false presences due to DNA bound and preserved to sediments. Resuspended eDNA can be etected up to 14-60 days (Goldberg et al. 2015).

During spring and summer, as temperatures rise, microbial loads and algal blooms also increase. Microorganisms degrade eDNA, and algal blooms inhibit its detection. However, during these seasons, the increased DNA production by target species, due to heightened activity levels and higher metabolic rates, is so high to compensate all negative effects related to eDNA degradation and possible invalidation of species detection.

Autumn and spring are seasons also ideal for eDNA sampling in lentic systems (lakes and ponds) at intermediate latitudes. At these times of the year, water circulation promotes vertical and horizontal mixing of eDNA. However, the choice of sampling season must align the objectives of the study, and with the phenology of all target species (see after). Sampling during seasons without a thermal stratification along the water column eliminates the need to collect water at different depths, and simplify logistics and reduce required materials. On the other hand, for those species whose optimal sampling season coincides with a thermal stratification, sampling at different depths is necessary. This is especially relevant for species behaving vertical migrations, which are usually linked to cooler water, that tend to concentrate at the bottom of lakes during the summer (Lawson Handley et al., 2019; Littlefair et al., 2020).

Other eDNA inhibitors or contaminant molecules, such coming from sources such as sewage discharges, may also be subject to seasonal factors, like the use of phytosanitary treatments in heavily agricultural areas or similar types of spread pollution. It is advisable to avoid periods when such phenomena are ongoing in the study area, as well as the following periods, when most pollutants are mobilized from land to water bodies by rain and peak during river flooding periods (Anderson et al., 2018). On-site checks of water conditions during field sampling are therefore essential. A list of chemical elements present in the area can also be helpful for understanding which pollutants require attention, identifying pollution sources, and reconstructing the plausible season for their spread. Consulting water quality monitoring reports (e.g., Water Framework Directive), which are periodically available, can be incredibly useful for gaining a preliminary overview of local pollutants that may interfere with an eDNA collection campaign.

In the multispecies experimental study for detecting 22 freshwater species of Community or Union interest in Latium region, the average frequency of positive sites sampled without considering any phenology (summer) was lower (16.5%) compared to sites where species phenology was considered (20.7%) and sampled during spring and autumn (Loy et al., 2023). However, it is also interesting to note that for some species like *Unio elongatulus*, *Austropotamobius italicus*, and *Alosa fallax*, the frequency of positive sites was higher during summer samplings, highlighting, as already mentioned, that seasonality is species- specific. The results also showed that temporal replicates improve species detection, correcting for absence data potentially gained by variations in the physical-chemical parameters of the water.

5. Sampling and filtering eDNA

5.1 Environmental water sampling

The implementation of a sampling strategy can be achieved through an appropriate management of field operations. Therefore, some technical and practical precautions must be taken into consideration to properly undertake the phases of sampling and filtering water from water bodies and to avoid the risk of contamination between environmental samples.

If the project aims at detecting species at the scale of a water body (e.g., a specific lake), the number of environmental samples should reflect the spatial complexity of the biotope desired to sample. Additional factors affecting the sampling size include the extent of the biotope and the accessibility of the water body. A greater number of environmental samples is advisable for particularly large water bodies, as eDNA tends to be less concentrated, thus influencing the probability of detection (McClenaghan et al., 2020). Furthermore, more samples are needed to cover the entire study area. In this way, to maximize the detection of a species for presence/absence data, it is preferable to carry out a sub-sampling only in those parts of the biotope covered by actual suitable habitats for the target species of the project (e.g., gained by species distribution modelling, for instance).

To prevent that field equipment physically contact water samples (especially boots) and potentially cause contamination across different sites with allochthonous eDNA, it is advisable to assess the direction of the water flow before collecting the water sample. The operator must always position downstream the sampling point to ensure that the water sample is not contaminated by boots or any other part of the operator itself. In case of sites located in lentic waters, pools, or ponds, the operator should never immerse boots or other equipment used in other sites that have not been adequately sterilized at the sampling point. If the topography of the shore is particularly complex, it is preferable to adopt a sub-sampling to ensure greater representativeness of water sample. It will be up to the operator and the aims of the study, to assess whether the sub-samples should be analysed separately or combined into a single sample, based on the distance and/or differences in microhabitat.

5.1.1 Location of environmental water sampling sites

The spatial distribution of DNA molecules in the environment is one of the most important aspects to consider when deciding to collect water samples. The distribution of eDNA across space is particularly influenced by the transport of eDNA and the distance over which it disperses from the source of release. Hydrological factors of single water bodies are particularly important to take into account to understand the dynamics of water mixing. The presence of currents and turbulence tends to homogenize the distribution of eDNA both horizontally and vertically, whereas their absence leads to compartmentalization of eDNA in specific zones of the water body and a very heterogeneous distribution (Deiner and Altermatt 2014; Deiner et al. 2016; Hänfling et al. 2016; Jerde et al. 2016; Shogren et al. 2017; Macher and Leese 2018; Pont et al. 2018).

In this section, we will examine factors that can influence eDNA collection at very small spatial scales, in order to provide useful guidelines to environmental water collection and to establish exact locations of sampling points. Paying attention to these aspects leads to the definition of a phase that is slightly distinct from that in which you define water bodies to be sampled (i.e., site selection).

Although the latter is typically related to the definition of the sampling strategy (see Section 4 on where to sample eDNA), the separation of these two phases is not particularly clear.

Lentic systems

Within lentic systems, such as lakes and ponds, eDNA is distributed in a non-homogeneous manner, and the specific composition of the ecological community can change even at distances of a few hundred meters (Brys et al. 2020). This means that it is necessary to sample water from multiple points to both maximize detectability and ensure sufficient accuracy in results. To achieve this goal, as previously specified (see Sections 4 and 5 on where and when to sample eDNA), it is necessary to increase the number of sampling sites by conducting spatial replicates (i.e., adding more samples, that means a sample per site) or using sub-sampling (i.e., partitioning the sample to be taken in different spots, for instance half of the sample in one site and another half in a second site). Sites should therefore be positioned in several points on the surface of the lake, both along the shores and at different distances from the shoreline toward the central portions of the lake. Sampling conducted exclusively from the shore in lentic bodies has shown to be subject to various sources of error that lead to an underestimation of the biodiversity of the sampled biotope (Lawson Handley et al. 2019). When the lake shows evidence of thermal stratification during specific seasons, it is also necessary to collect samples at different depths.

Lotic systems

In lotic systems like rivers and streams, eDNA is more thoroughly mixed within the water column, but there is still a general tendency for it to settle on the riverbed and along banks. Sampling that involves collecting sub-samples along the entire river section can increase detectability compared to those where eDNA is only collected from the banks, and may avoid various sources of error generating false absences. An additional factor not to be overlooked is the differential input of eDNA from tributary streams that flow into the water body of interest. Sampling upstream a tributary confluence allows the exclusion of eDNA contributions from that respective watershed. Conversely, sampling downstream of a confluence maximizes the ability to detect the occurrence of target species, as the eDNA collected theoretically represents the community of a larger study area, considering the decay rates of eDNA and the distance at which it can be detected. However, sampling downstream of a tributary exposes the sample to the risk of potential contamination from commercial species (e.g., restaurant or urban wastewater), which can lead to false presences or increase the concentration of pollutants that inhibit the eDNA of the main water body, consequently distorting species detection (increasing false absences). The resolution of site-specific biodiversity can also be enhanced by sampling lentic water bodies present in the riverbed that are disconnected from the main water body. Additionally, conducting sampling upstream and downstream of river sections that represent a discontinuity in the river, such as waterfalls or dams, can provide a better definition of species checklists present in an area. Finally, to maximize detectability, conducting a repeated sampling at regular distance intervals can be a method to counter early degradation of eDNA and understand the transport dynamics of eDNA from a wild population. This solution allows a more effective identification of rare species and is also a valid method for tracking changes in communities along the river gradient (Pont et al. 2018).

Lastly, we reiterate that sampling strategies should select sampling sites that are consistent with the suitability characteristics of an eDNA sampling. In the field phase, it is advisable to pay close

attention to the location of sampling sites in order to validate the sampling strategy criteria with which they were selected remotely. For example, it is necessary to avoid proximity to treatment plants or sewage discharges, as well as periods of drought or phytosanitary treatments in agriculture (see Sections 4 and 5 on where and when to sample eDNA).

5.1.2 Water quantity

The number of litres of water to collect at each site is related to the water filtration phase and therefore depends on the pore size of the filter, the type of pump used, and the turbidity of the water, which tends to clog the filter. The choice of these factors directly impacts the amount of working hours. Such technical aspects are generally integrated into the objectives that can be achieved by a sampling strategy. Alternatively, for example, we may have sampling strategies that involve the collection of a standard number of litres to compare different sites, or, in other cases, it is possible to adopt a sampling approach that collects a different number of litres depending on the complexity and extent of the biotope. A larger water body therefore corresponds to a greater sampling effort.

Similar issues arise at the site scale, and it can be resolved essentially in two ways. In case of turbid waters, one can either collect and filter water until the filter is clogged with sediment, or opt for a standard number of litres, changing filters as soon as they become clogged. Standardize the water quantity help reducing confounding factors in understanding eDNA results. A third way is to reach a standard water quantity and to not change filters, or to force the filtration up to the maximum capacity of the membrane filter. This last option saves money and time at the extraction stage (see Section 7) but increases tremendously working time at the filtration stage.

The choice of the amount of water to collect is also linked to the number of field replicates desired or the decision to opt for sub-sampling. It is possible to collect the same volume of water at multiple points (spatial replicates of a sample) or to collect a fractional amount at different points (e.g., around the sampling site or along a stretch of shoreline or across the entire lake) to reach the desired volume (pseudo-replicates) (see Sections 4 and 6.1.2).

Another issue is related to the number of target species to be analysed at the sampling site. Often, when the number of species exceeds the dozen, it is not possible to proceed to the further steps. In these cases, the quantity of the eDNA extract given by only one filter does not allow the PCR amplification for other target species (A. Bruno, pers. comm.). So, it is needed to sample more water at the same sampling site to provide another filter of eDNA. As if it is not a proper increase of water quantity *within* the sample in the strictest sense of the word, we remark this process in this section as the acquisition of another replicate is translated in an increased effort in filtration that influence the working time at this specific stage of an eDNA study.

There is little consensus in the scientific community regarding the minimum volume of environmental water to ensure the representativeness of the entire community. Generally, the larger the volume of water sampled, the greater the probability of detecting rare species and accurately identifying the composition of the animal community (Pont et al. 2018; McClenaghan et al. 2020). However, the relationship between the volume of filtered water and the number of species detected does not seem to be linear. In fact, small volumes (less than 1-2 litres) have proven to be sufficient to correctly detect all species present in the study area, especially when paired with

specific extraction protocols (Machler et al. 2016; Muha et al. 2019). Case studies conducted so far have demonstrated the validity of filtering an extremely heterogeneous amount of water, ranging from a few millilitres (15-100-300 ml) to tens of litres (30-100 l), and therefore there are currently no strict guidelines that can serve as standards, although most studies have produced data that can be used for comparisons by filtering 1-5 litres (Deiner et al. 2016; Civade et al. 2016; Valentini et al. 2016). Filtering 3 litres has, in several cases, represented the suitable volume to detect all species present in a given area (Santas et al. 2013; Sun et al. 2022).

One of the variables mostly affecting the filtration capacity is related to the characteristics of the filtration membranes, particularly the pore size and the filtration area that the membrane offers passing through to the liquid. Filters with smaller pores (0.2 µm - 0.45 µm) take longer time to filter a known quantity of water than those with larger pores (1.2 µm - 8 µm), but the choice of which type of filter to use is not only related to the expected filtration times. The pore size used in an eDNA campaign mostly depends on the state in which DNA molecules are present in the environment and their sizes (see Sections 2.4 and 6.3.1). To reduce filtration times, it is advisable to adopt a pre-filtration phase using filters with large pores, and then filter the liquid again through the selected pore size.

The filtration area of the membranes is generally linked to the type of filter used. There are three main types of filters (Peixoto et al. 2020). Encapsulated filters, the most common disposable models from the Sterivex brand, are very compact and filter up to 1 litre or slightly more. Disk membranes have a surface area of 17.35 cm^2 (47 mm of diameter) and have greater filtration capacity, especially when combined with a pre-filtering system for larger particles, and achieve from 1 to 5 litres. This type of filter can be used with active filtration similar to other filter types, that is, forcing the water to pass through the filter, or passively, by placing the filters directly in water for some time to capture the eDNA carried by currents and waves (Bessey et al. 2021; Takahashi et al. 2020). Capsule filters (e.g., Waterra model), finally, have an external cylindrical casing in which a pleated cylindrical membrane is housed and increased the filtration area up to 1300 cm^2. They can filter larger volumes up to 30-100 litres, but their cost ranges from 10 to 25 times that of disk filters. The choice of which filtration system to adopt depends on the study objectives, as previously mentioned, and on the material costs.

There are several techniques and technologies applicable for filtering environmental water (see Section 6.3.3: Filtering in the field and in the lab). One of the most commonly used involves the use of a vacuum pump. Different models can have different characteristics and therefore more or less efficient performances. The power of the pump, for example, is inversely correlated with filtration times. By increasing the power of the pump, therefore, one may expect a faster filtration saving working time, or one might expect the filtration of a larger volume of water. However, it has been shown that membranes subjected to high pressures have a reduced retention of eDNA since the molecules are forced to pass through them with excessive pressure (Thomas et al. 2018). This effect, however, seems to be balanced by the larger amount of eDNA that is collected from a greater volume of filtered water.

Passive collection of eDNA has been generally practiced in marine ecosystem suspending membranes in the water column (Bessey et al. 2021), but it has been recently demonstrated to be particularly efficient also in freshwater ecosystems (Cananzi et al., 2024). If used in running waters and placed for a night, the stream flow passing through the filters amounts to several hundreds of litres. Thus, detectability increases immensely, revealing a higher biodiversity richness compared to the one inferred by active filtration. Up to know, only metabarcoding experiences are known to be

applied on biodiversity assessments, with qPCR approaches used very rarely (Chen et al., 2024). We believe that passive filtering outperforms active filtering and it is particularly indicated for biodiversity checklists.

5.1.3 Practical aspects and technical observations

Sampling stagnant and flowing waters requires some precautions that, in summary, fall under simple guidelines to avoid the risk of eDNA contamination between different samples. The fundamental principle is that the same material should not come in contact with different environmental samples (and different sites).

It is essential to avoid entering the water to prevent contamination of the water body with allochthonous eDNA deposited on boots, shoe soles, or clothing, and also to avoid transferring any pathogens between different sites. Entering the water also alters the substrate of the water body, mobilizing sediment from the bottom and may increase water turbidity in the sample. This is a factor to consider depending on what is stated in the sampling strategy, as sediment tends to increase filtration and even extraction times, and can either decrease or increase the detectability of eDNA molecules. In flowing waters, it is possible to enter in the water, if necessary, but sampling should be done from downstream to upstream. Therefore, care should be taken regarding the point of access to the water, ensuring that one always walks upstream and strictly conducts the sampling stage in a downstream-to-upstream sequence. This attention should undertake especially if one decides to carry out subsampling of the various microhabitats present along the river section from the bank to the centre of the riverbed. Finally, the environmental sample must also be collected against the current, submerging collection bottles upstream the operator.

Materials that come into contact with multiple different samples must be properly sterilized or, if not possible, replaced with sterile single-use equipment. For effective sterilization, one can use diluted bleach at 10%, which has been shown to be efficient in several studies (Prince and Andrus 1992). A deeper sterilization can be implemented using diluted bleach at 50% (Kemp and Smith 2005; Champlot et al. 2010; Goldberg et al. 2016; Wilcox et al. 2016). When submerging sampling bottles below the water surface, the use of gloves is mandatory to avoid introducing allochthonous DNA belonging other sites (and to the operator). It is important to immerse the bottle only to the depth at which the glove still offers sterile protection, which is approximately 20 cm of maximum depth from the water surface.

Sampling from the bank without entering the water can introduce some biases in the representation of the sampled habitats. It may also present some operational difficulties. For example, from low sloping banks, it is difficult to fill the water into collection bottle to reach its full volume, as the bottle cannot be tilted. Additional equipment that can be used for sampling directly from riverbanks includes extendable grabbing sticks, small tubes that draws directly in the water and facilitates in situ filtration (with a peristaltic pump), or the use of small boats. The latter is essential if one wants to conduct sampling in deep water that involves both the surface of the water body and the deeper layers. In this case, it is preferable to use only local boats. Otherwise, to avoid contamination, one must sterilize the hull of the boat or must collect water exclusively from the front of the boat while the boat is moving. For sampling the deep layers, appropriate equipment must be used (e.g., Niskin deep water samplers).

Finally, the adoption of negative controls allows for the evaluation of whether the sterilization of

the material has been carried out correctly and has effectively prevented contamination between samples. In this way, it is possible to increase the reliability of the results and consequently facilitate the biological interpretation of the data by excluding the risk of false positives.

5.2 Collecting environmental parameters

Measuring environmental variables that can influence the detectability of eDNA is crucial to maintain experimental conditions during a study. The use of professional instruments, typical of hydrological disciplines, allows for accurate measurements of environmental parameters, such as the flowmeter for water velocity or surveying probes to measure the chemical-physical parameters of water (pH, temperature, etc.). However, using such equipment requires specific skills, more time for fieldwork, and often, an increase in the number of personnel involved.

The environmental variables to consider include weather conditions during sampling, season and sampling date, depth of sampling, pH, temperature (of air and water), salinity of the water, water velocity or flow rate, features of the riverbed (width and depth), type of habitat. These basic parameters are essential for making data comparable with other eDNA studies conducted worldwide (Harrison et al., 2019; Minamoto et al., 2020; Bruce et al., 2021).

In addition, other variables may be considered, such as turbidity, concentration of nitrites and nitrates, microbial community, measured through chlorophyll A or organic matter as proxy values, nutrient load, and type of substrate. Finally, it is important to document the specific conditions encountered at the sampling sites with final notes, such as the presence of litter, leaves in the water, livestock, or sources of chemical pollution, as these factors can influence the detectability of eDNA, and must be reported to better inform data interpretation.

5.3 Filtering water samples

Over the years, two main methods have been developed to collect eDNA from water: filtration and precipitation. Although eDNA detection techniques were initially applied using the precipitation method to obtain DNA from water (Ficetola et al. 2008), over time studies employing filtration methods have increasingly grown in number. Filtration has proven highly effective, offering greater sensitivity, reducing the risk of contamination, and facilitating logistical organization by eliminating additional issues related to proper disposal of materials and the need to adhere to specific safety regulations (Deiner et al. 2015; Spens et al. 2017; Tsuji et al. 2019; Muha et al. 2019).

Therefore, we will focus only on aspects related to filtration, particularly the various technical aspects affecting its efficiency in terms of DNA yield (e.g., material characteristics). However, the different phases (filtration, preservation, extraction) have so many combinations of materials and technological solutions, as demonstrated in several experimental eDNA case studies over the years, that it is very difficult to determine the ideal workflow for maximizing the detectability of eDNA. For instance, the choice of different combinations of materials may also be influenced by external factors, that are often case-specific, such as the target species and the characteristics of the species-specific DNA molecule (i.e., size, electrostatic bonds).

5.3.1 Filter material

Filters available for sale are made of cellulose nitrate (CN) or mixed cellulose esters (MCE), polyester sulfonate (PES), polyvinylidene fluoride (PVDF), glass fibres (GF), and polycarbonate (PC). Membranes made of hydrophilic material can facilitate the passage of water through the filter, and some types of membranes can be purchased from the manufacturer as either hydrophilic or hydrophobic. When comparing different materials used for filters with the smallest available pore sizes, nitrocellulose has been the most commonly used so far (Tsuji et al. 2019), but some studies suggest that mixed cellulose esters are superior (Turner et al. 2014; Li et al. 2018; Hinlo et al. 2017; Majaneva et al. 2018; Capo et al. 2019; Di Muri et al. 2020), while in other experiments, polyester sulfonate yielded the best results (Spens et al. 2017). For larger pore sizes, glass fibres, with their chemical properties that adsorb DNA and proteins onto the filter surface, could result in a higher quantity of eDNA being collected. The variable capacity of different materials to capture eDNA may be defined by the residual electrostatic charges of the species-specific eDNA molecules targeted in different studies or may be influenced by other confounding factors (pore size, extraction kit, experimental design, microbial load).

5.3.2 Pore size

The choice of filter pore size is closely linked to two factors: the type of molecules to be intercepted on the filter, and the amount of water to be filtered.

Mitochondrial DNA fragments (COI gene, 12S, and 16S) persist in the environment much longer than ribosomal DNA fragments (18S) or nuclear DNA, thanks to the greater protection provided by mitochondria in countering degradation once cellular lysis begins. However, the high abundance of ribosomal genes can offer a viable alternative for monitoring in certain conditions (Jo et al. 2019b; Moushomi et al. 2019; Barnes and Turner 2016).

For DNA fragment collection, sterile filters (supplied sterile or autoclaved in lab: Unnithan et al. 2014) with pore sizes of 0.22 µm or 0.45 µm are recommended (Strickler et al. 2015; Eichmiller et al. 2016a; Spens et al. 2017, Capo et al. 2019), although larger pore sizes have also been indicated as optimal (0.8 µm: Li et al. 2018). Generally, filters with pore sizes smaller than 1 µm can be used, with a minimal expected loss of eDNA, likely because this size corresponds to that of subcellular organelles (mitochondria, ribosomes) (Deiner et al. 2018). Larger pore sizes have also proven promising tools in capturing eDNA from macrofauna (e.g., fish: 1-10 µm, even up to 64 µm) and may therefore be a viable alternative during an eDNA sampling project (Turner et al. 2014; Wilcox et al. 2015; Schabacker et al. 2020).

The choice of pore size must balance the volume of water to be filtered (see section 6.1.3: Water quantity). Using a filter that is too fine increases the risk of clogging due to suspended sediment in the water. On the other hand, using a filter that is too coarse may limit its ability to capture smaller eDNA-containing particles. Some differential filtration methods have tested how this pore size-to-water volume ratio can influence detectability. Filtering a larger volume of water with larger pore size filters has proven more effective than filtering small volumes of environmental water with smaller pore size filters (Hosler 2017; Sepulveda et al. 2019).

If pore size does not allow to reach a desired water volume due to filter clogging by sediment, it is

possible to replace the clogged filter with a new one and increase the number of filters that will be analysed. This choice, however, impacts following phases of the eDNA workflow, particularly increasing working time during the extraction phase in the lab. Alternatively, a preliminary pre-filtration phase can be adopted before filtration, using a coarser filter that retains only suspended particulate matter (Xing et al. 2022). This pre-filtration is almost mandatory when facing a filtration on highly turbid waters to prevent filter clogging and reach the desired water volume. Including a pre-filtration phase also improves DNA amplification when PCR inhibitors are contained in the sample such as humic compounds (Takasaki et al. 2021). To mitigate these issues, it is often recommended to submit pre-filters to the DNA extraction phase as well. However, the sediment, which pre-filters are rich in, can increase the concentrations of inhibitors of the DNA extraction of interest.

Whether to extract pre-filters together with filters rich in eDNA or to conduct separate extractions for the two filter types or to completely discard the pre-filter and the sediment from the final analysis is a matter of experimental design and the constraints imposed by the budget and the cost of materials and staff employed (see Section 7: Extraction of eDNA). However, some authors discourage prefiltration due to the decrease in accuracy and replicability of results (Li et al. 2018; Takasaki et al. 2021). Including sediments for instance doesn't allow for a short timeframe inference on river community, as sediments can preserve DNA for a long time and lead to false positives (Turner et al. 2015).

DNA molecules bind to solid substrate particles, so the sediment substrate can act as both an inhibitor during the DNA extraction phase, and as an enhancer of DNA detectability. Avoiding inhibition during DNA extraction depends largely on the type of extraction kit used and the nature of the molecules that sequester DNA (see Section 7: Extraction of eDNA). Understanding in advance whether to include sediment in the environmental water sample is important in guiding operations downstream the collection stage. For example, if the environmental sample is collected with bottles that are temporarily stored at low temperatures (e.g., in a refrigerator), the sediment collected along with the water sample will tend to settle at the bottom of the bottles. This allows for the exclusion of sediment during filtration, speeding up work time. A compromise to reduce work time while including sediment in the filtrate is to add the sediment sank at the bottom during the last minutes during the filtration.

5.3.3 Filtering in the field and in the lab

For eDNA sampling campaigns covering study areas particularly wide, environmental water filtration can be carried out directly in the field using various methods. Manual pumps or syringes with pistons are a first available method, easy to use, applicable in all environmental contexts, and easily obtainable from common stores (Jamwal et al., 2021). Vacuum pumps and peristaltic pumps offer a more feasible solution for processing large volumes of water but require electricity or batteries. Finally, some innovative techniques are gaining popularity in the form of backpack systems, where sampling is conducted by an operator who can comfortably sample along a transect using a suction rod, reducing the risk of contamination by the operator. Some of these tools can be highly effective in preventing eDNA degradation, as they reduce the time the target DNA is exposed to ambient temperatures. Rapid water filtration, and the availability of portable refrigerators allow a quick preservation of DNA at low temperatures (see Section 6.4: eDNA storage).

However, techniques outlined here have some drawbacks. Manual pumps are slow, especially when

filtering many litres of water, and can be physically exhausting. Electric pumps can be heavy and unsuitable for transportation to remote areas without electricity to power them. Innovative systems, on the other hand, can be expensive or not yet commercially available. Field filtration, especially when using non-encapsulated filters, can expose the filter to exogenous DNA from equipment that has not been properly sterilized, from other objects that come into contact with multiple environmental samples (e.g., the vehicle used for transport), or because the filtration is conducted in a natural environment rich of eDNA that could be carried to the sample by wind. Moreover, when filtration becomes particularly slow, sudden changes in weather conditions can make this phase particularly difficult, exposing the sample to direct sunlight, wind, rain conditions, or contaminating the sample with particulate dropping.

Laboratory filtration can speed up field operations since these are limited to just water collection. However, DNA can degrade quickly, and thus to enable off-site water filtration, a portable refrigerator is first needed to lower the temperature during transport. Another option is to use a transport vehicle equipped with climate control. Filtration should occur the same day or shortly afterward (within less than 12 or 24 hours: Hinlo et al. 2017). Therefore, the filtration facility should be located in a location easily reachable. A lab can be set up centrally in the study area. Alternatively, a mobile filtration unit can be used, or multiple filtration units can be organized throughout the study area to work simultaneously. The distance between a filtration site and sampling locations is a key factor in large-scale eDNA collection campaigns because if the distance is too great, the number of sites that can be sampled per day decreases, slowing down the work. This makes it particularly difficult to conduct sampling campaigns within short time frames limited by the phenology of the eDNA of certain species.

In the project conducted to detect 22 species in the Latium region, water samples were filtered in the laboratory the same day or the following day, allowing at least 12 hours for sediment to settle, especially in the case of very turbid waters. To filter, a KNF Laboport vacuum pump (N86 KN.18, 6l/min, final vacuum: 100 mbar) was used, inserted into a 1-liter vacuum flask equipped with a single cylinder (CRAMI srl, KRS47/50). To prevent supernatant material (algae, insects, etc.) from clogging the filters, a metal strainer. A prefiltration was then performed with mixed cellulose ester filters with 8 µm pores (Millipore, 47 mm diameter), followed by filtration with 0.22 µm filters. The prefiltered water was temporarily stored in the same sampling bottles. Filters were sterilized in an autoclave, and all individual elements were sterilized with alcohol, flame, or a 10% bleach solution. Prefilters were combined in a single Petri dish, as well as filters in another separate dish, both placed in a freezer at -21°C. Every five filtrations, one litre of sterile water (blind sample) was filtered to assess potential contamination, and the sequence of filtrations was recorded.

5.3.4 eDNA storage

It is possible to preserve eDNA on filters by freezing them at -20 °C, dehydrating them, or adding liquid additives that prevent degradation. Refrigeration is likely the most accessible method, but it is important to note that undergoing DNA to repeated freeze-and-thaw cycles is detrimental, as it causes loss of DNA collected (Spens et al. 2017). Conversely, an increase in the amount of DNA extracted has been observed in environmental samples underwent to a single freeze-thaw cycle compared to samples from which extraction occurred on the same day as water filtration. This is likely because freezing promotes cell lysis and increases the likelihood of "accessing" DNA molecules by extraction agents (Mauvisseau et al. 2021). Dehydration is also quite easy to achieve, using silica gel and a desiccator or absorbent paper, but it is less commonly used. It is a particularly convenient

method for preservation at room temperature, but it is only efficient for a few months at most (Thomas et al. 2019). The use of ethanol (96% alcohol) is a readily available solution for preserving DNA in its entirety. The adoption of lysis agents, such as Longmire's buffer or Sarkosyl buffer, releases DNA into the solution, which in turn allows for preservation (Longmire et al. 1997; Wegleitner et al. 2015; Civade et al. 2016).

Several studies have demonstrated the positive effects of using preserving liquids, even compared to the freezing method (Minamoto et al. 2016; Spens et al. 2017; Kumar et al. 2020). However, freezing filters remains the method most commonly used in at least two-thirds of eDNA studies conducted so far. The use of freezers that reach -20 °C has been shown to have degradation rates of DNA in the first two weeks, although these were found to be negligible and statistically insignificant (Spens et al. 2017). Other experiences indicate that freezing should been preferred when combined with specific extraction kits, and this preservation method does not damage the ability to detect DNA when extracted 28 days after the filtration phase (Hinlo et al. 2017). There is not a particularly abundant literature on how preservation times influence DNA degradation. In many cases, filtration is performed immediately, without the need for a preservation phase, thus avoiding potential degradation. Some types of liquid preservatives, for example, do not prevent bacterial growth within the sample, leading to direct degradation of the DNA (Bruce et al. 2021). Freezing filters, in any case, demonstrates that even after 8 months it is possible to recover eDNA, proving that freezing is the most effective method guaranteeing such a long time interval between filtration and extraction (Mauvisseau et al. 2021).

During the first multi-specific experimental study conducted in Italy for the detection of 22 species of freshwater of Community or Union interest developed by the University of Molise and funded by the Latium Region, we tested several sampling and filtration methodologies. Where possible, 3 litres of water were collected using non-sterile single-use plastic bottles. Bottles were reused after a sterilization with a 10% bleach solution (then responsibly dispose), washed with tap water, and finally rinsed three times with environmental water before collection directly at the sampling site. Samples belonging lentic waters were collected at a depth of 20 cm using sterile single-use gloves, taking a sub-sample of approximately 200 ml of water every 20 m along approximately 300 m of the bank starting from the sampling point. Samples belonging to running waters were collected along transects on the river section when wadable, we sampled from riverbank taking different sub-samples in different points nearby the sampling site, with a maximum distance of 50 meters. Sampling bottles were then placed in thermal bags along with freezer packs inserted within a rigid portable cooler, and then stored in the refrigerator at +2 °C until filtration performed within 24 hours.

5.3.5 Surveying environmental parameters

During field surveys, it is useful to measure some environmental parameters that allow for a critical interpretation of results in terms of physicochemical parameters that may influence the probability of detecting eDNA. Below is a list of parameters that can be measured at low cost and by non-specialized operators with the aid of simple equipment, when professional tools are not allowed:

- air temperature, with a simple thermometer for outdoor measurements
- water temperature, measurable with the Deeper Pro sonar

- water depth, measured as the average of repeated measurements along a linear transect using a Deeper Pro sonar and launched with a fishing rod
- width of the riverbed, measurable through satellite images (remote sensing with Google Earth Pro)
- pH, presence of nitrites and nitrates, measurable using test strips for aquariums and often categorized, for instance as following: pH (6.4, 6.8, 7.2, 7.6, 8.0, 8.4), nitrites (0, 1, 5, 10, 25, 50), nitrates (0, 10, 25, 50, 100, 250, 500)
- water turbidity, visually assessed (low, medium, high) or measured with filtering time
- water velocity, measured by throwing a plastic ball attached to a 10 m string into the centre of the watercourse and measuring the distance three times in terms of seconds over metres, calculating the average of the three values, and converting in the inverse formula of the final value (m/s).

6 Extraction of environmental DNA

The extraction of eDNA collected on filters can occur in several ways depending on the type of filter, the preservation method used, and the environment from which the sample is collected. Generally, commercial extraction kits can be used, which are specific for different types of reactions and molecular substrates, or can be employed alternative methods involving a liquid phase.

Commercial kits come in different types, mainly based on the substrate from which the DNA has to be extracted. The company Qiagen, a global leader in this field, offers kits for DNA extraction from blood and tissues (DNeasy Blood and Tissue Extraction Kit), from water (PowerWater DNA Extraction Kit), and from soil (DNeasy PowerSoil Extraction Kit, PowerMax Soil DNA Extraction Kit, DNeasy PowerSoil Pro Extraction Kit). The DNeasy Blood and Tissue Kit (Qiagen) is the most commonly used commercial kit, although in comparisons with FastDNA SPIN kits from Biomedicals, the latter has been found to be the best. In presence of inhibitors, however, the best performance has been noted with the Power Soil Kit (Qiagen) (Deiner et al. 2015; Eichmiller et al., 2016a; Mahmoudi et al. 2011; Smith et al. 2012; Brannelly et al. 2020; Tumolo et al. 2021).

Commercial kits are easy to obtain and allow for standardized extractions. This condition is particularly advantageous for repeated routine monitoring and to enable comparisons between different studies.

The extraction method that involves separating a liquid phase, on the other hand, uses phenol-chloroform-isoamyl and effectively extracts a larger amount of DNA compared to that obtained from kits, but poses serious health and safety risks for the laboratory and requires well specialized and trained staff (Deiner et al. 2015; Bruce et al. 2021).

The type of filter chosen during the filtration phase affects the ability to perform cell lysis necessary to release DNA according to specific methods. Encapsulated filters, for example, do not allow for mechanical lysis, as the filter cannot be removed from the containment capsule. In this case, lysis

occurs exclusively through chemical methods via the addition of additives. Mechanical lysis takes advantage of microbeads that are particularly necessary for squashing cell walls with their reciprocal collision in organisms such as diatoms. For these species, it is recommended to use disc membranes or filters contained in cases that can be opened.

During the extraction phase, it is important to know whether the eDNA preservation method has already caused a cell lysis. In the case of ethanol, which acts as an inhibitor of DNA extraction, all the alcohol can be removed from the filter by complete evaporation (Spens et al. 2017). Alternatively, ethanol can be precipitated (Bruce et al. 2021). When using other liquid preservation methods, such as lysis buffers, the conditions lead to the liquid in which the filter is immersed being rich in DNA (P. Colangelo, pers. comm.). In these cases, however, care must be taken regarding the compatibility between the type of buffer used and the chosen extraction method.

The presence of inhibitors in the sampling environment (e.g., clay sediment, humic acids, chemical pollutants, and faeces also) must be considered when choosing the extraction method since inhibition is one of the major causes of false negatives (Jane et al. 2014; McKee et al. 2015; Capo et al. 2021). The amount of DNA bound to specific molecules or particles is likely very high in any sample, and without using the most appropriate extraction method, such eDNA molecules will not be detected in the following stage of the eDNA study (i.e., amplification by PCR). In case of turbid waters and with presence of clay, for example, using DNA extraction kits designed for soil and sediment is essential for releasing adsorbed molecules (i.e., PowerSoil kit). Other authors proposed the use of trisodium phosphate in the case of lysis buffers (Ogram et al. 1987; Sellers et al. 2018; Diaz et al. 2020; Pearman et al. 2020). Other DNA inhibitors such as organic compounds can be found in high concentrations in case of waters collected from smaller streams rich in litter and plant material under decomposition, or in eutrophic habitats, or along river stretches where livestock waste is discharged (Braid et al. 2003). Generally, commercial kits for DNA extraction from soil or faeces already include a phase for removing inhibitors. Alternatively, an additional laboratory step is required to remove inhibitors from the sample, using an appropriate kit.

Diluting the DNA extract is another common strategy used to reduce the concentration of inhibitors. However, this operation has the negative effect of reducing the concentration of target DNA, thus decreasing detectability. As a common approach, serial dilution is often performed to determine the minimum dilution level that can counteract inhibition.

It is useful within this phase to include a final test to understand whether the eDNA extraction performances were negatively influenced by DNA loss during the preservation process or by inhibition effects (Hoorfar et al. 2004; Furlan et al. 2016). This can be done using the so-called internal positive controls. A known amount of DNA primer can be added to the sample at the end of the filtration process, called also as internal positive controls (IPC). Quantitative PCR will allow to verify whether this DNA is detected as the same initial amount of IPC or if it has been rarefied during the various steps of the study workflow, for instance due to a strong inhibition process within the sample. Therefore, the DNA used as a positive control must belong to a non-target species or to a species not occurring in the natural system analysed and must not interfere with the target species' DNA.

7 Final comments on sampling methodologies

Here is a summary of the key aspects regarding how a sampling strategy can be modified based on study objectives and available resources (research time, personnel, funding) and what are the necessary tools to arrange a field survey using eDNA approaches.

eDNA surveys can be designed in several ways, depending on a range of factors. The choice is a compromise between study objectives, costs, selected methodologies, available personnel, and materials. The primary aspect in managing such projects regards the identification of the major constraints. Specifically, the available budget, logistical aspects related to the morphology of the study area (e.g., accessibility), and project timelines. Sanches and Schreier (2020) provide a useful overview of how these variables may influence an eDNA project. In particular, this study focuses on the choice of filtration and extraction materials to be preferred, depending on whether priority is given to the accuracy of the data acquired (e.g., nitrocellulose filters and Qiagen's DNeasy Blood and Tissue Kit), at a more affordable cost (e.g., glass fiber filters and qPCR reactive strips or sodium hydroxide NaOH), or to saving time (e.g., glass fibers and magnetic beads). Using fine mesh filters and extraction kits is the most effective but also the most expensive and time-consuming approach for eDNA detection.

It’s essential to balance these factors with the characteristics of the natural system, methodologies suitable for that natural system, and the constraints of costs and time. Once this balance is defined, you can focus on optimizing each stage of the typical eDNA workflow (Minamoto et al. 2020; Bruce et al. 2021).

Sampling strategy considerations. When dealing with large water bodies or rivers with wide riverbeds, it is possible to maximize detectability by taking multiple water samples along the entire water body. Alternatively, to reduce sampling time, a smaller number of sampling sites can be selected, though this will also decrease the accuracy of detection.

Logistics. Regional-scale monitoring campaigns involving a sampling on several large lakes and large rivers require an extensive water collection across the entire water bodies. However, this approach tends to be particularly demanding in terms of sampling effort (e.g., days of work) and can only be implemented effectively if multiple teams of operators are working simultaneously. Alternatively, two teams may be employed: one dedicated to sampling and the other to filtration in the laboratory. Additionally, sampling across different microhabitats within water bodies presents logistical and organizational challenges, often requiring a boat to access the centre of rivers or lakes.

Filtration. The timing of filtration can be a key factor in preventing the dilution or degradation of eDNA available for detection. The accuracy of the study can be improved by preventing post-sampling eDNA degradation by collecting only one sample per day, allowing filtration to occur either in the field or in the laboratory within 12 hours. This approach allows for the successful collection and filtration of environmental water samples from 30 sites within a month. If more sites are involved, it is important during the study design phase to ensure that work timelines allow for temporal consistency across the data, particularly for species that exhibit strong seasonality and need to be sampled within a narrow time window.

Depending on the materials used, performing filtration in a dedicated room for this phase is preferable to ensure proper sterilization of all materials. However, this solution requires more time

to account for the distance between the filtration room and the various sampling sites, given the same number of sampling locations. The alternative strategy is to prioritize logistical efficiency by sampling multiple sites per day. However, this compromises eDNA preservation, as the samples may wait up to 24 hours before being filtered.

Materials and resources. Depending on sampling and filtration techniques selected, certain specific tools may be more useful than others. Here is a list of equipment commonly used in the sampling phases of eDNA studies:

- sterile bottles, sterile gloves, permanent markers to label bottles with a unique site ID code
- rigid portable cooler made of polyurethane foam, with an internal cooler bag to increase thermal capacity, and ice packs to maintain a temperature well below ambient
- disposable bags to avoid contamination between samples within the portable cooler
- waders to sample with transect lines across a river section, or a boat for sampling from the centre of larger water bodies (optional)
- sterile filters (minimum pore size), sterile pre-filters (larger pore size), 50% diluted bleach, sterile containers for pre-filtered liquid, vacuum flask, filtration ramp, vacuum pump, sterile Petri dishes, parafilm, permanent markers to label site ID code and date, denatured alcohol, flame torch sterilizer, various tweezers, absorbent paper, a sterilized work surface (e.g., portable table), and fridges/freezers that can maintain 4 °C and -20 °C, respectively.

Finally, to approach new species-specific monitoring surveys, it is advisable to use occupancy models and probability of detection models (MacKenzie et al. 2002, 2004, 2017), employing multi-method approaches (multi-method occupancy models, Nichols et al. 2008) to account for non-independence in detections within each sampling session.

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References

Adams, C. I., Hoekstra, L. A., Muell, M. R., & Janzen, F. J. (2019). A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA under field conditions. Diversity, 11(4), 50.

Ahmed, S. F., Kumar, P. S., Kabir, M., Zuhara, F. T., Mehjabin, A., Tasannum, N., ... & Mofijur, M. (2022). Threats, challenges and sustainable conservation strategies for freshwater biodiversity. *Environmental Research*, *214*, 113808.

Allan, J. D., & Castillo, M. M. (2007). Stream Ecology: Structure and Function of Running Waters. 2nd (ed.) Springer. *Dordrecht, The Netherlands. 436p*.

Allan, E.A., Zhang, W. G., C Lavery, A., & F Govindarajan, A. (2021). Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environmental DNA*, *3*(2), 492-514.

Allentoft, M. E., Collins, M., Harker, D., Haile, J., Oskam, C. L., Hale, M. L., ... & Bunce, M. (2012). The half-life of DNA in bone: measuring decay kinetics in 158 dated fossils. Proceedings of the Royal Society B: Biological Sciences, 279(1748), 4724-4733.

Anderson, B. S., Hunt, J. W., Phillips, B. M., Nicely, P. A., Gilbert, K. D., de Vlaming, V., ... & Tjeerdema, R. S. (2003). Ecotoxicologic impacts of agricultural drain water in the Salinas River, California, USA. Environmental Toxicology and Chemistry: An International Journal, 22(10), 2375-2384.

Andres, K. J., Lambert, T. D., Lodge, D. M., Andrés, J., & Jackson, J. R. (2023). Combining sampling gear to optimally inventory species highlights the efficiency of eDNA metabarcoding. *Environmental DNA*, *5*(1), 146-157.

Andruszkiewicz EA, Sassoubre LM, Boehm AB. (2017). Persistence of marine fish environmental DNA and the influence of sunlight. PLoS One; 12(9): e0185043. https://doi.org/10.1371/journal.pone.0185043 PMID: 28915253; PubMed Central PMCID: PMCPMC5600408.)

Apothéloz-Perret-Gentil, L., Bouchez, A., Cordier, T., Cordonier, A., Guéguen, J., Rimet, F., ... & Pawlowski, J. (2021). Monitoring the ecological status of rivers with diatom eDNA metabarcoding: A comparison of taxonomic markers and analytical approaches for the inference of a molecular diatom index. Molecular Ecology, 30(13), 2959-2968.

Balasingham, K. D., Walter, R. P., & Heath, D. D. (2017). Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. Molecular Ecology Resources, 17(3), 523-532.

Basso, A., Paolini, V., Ghia, D., Fea, G., Toson, M., & Pretto, T. (2023). Cuticular Swabs and eDNA as Non-Invasive Sampling Techniques to Monitor Aphanomyces astaci in Endangered White-Clawed Crayfish (Austropotamobius pallipes Complex). Diversity, 15(2), 279.

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation genetics*, *17*(1), 1-17.

Bessey, C., Jarman, S. N., Simpson, T., Miller, H., Stewart, T., Keesing, J. K., & Berry, O. (2021). Passive eDNA collection enhances aquatic biodiversity analysis. *Communications biology*, *4*(1), 1-12.

Bista, I., Carvalho, G. R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., ... & Creer, S. (2017). Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nature communications*, *8*(1), 14087. 40

Bochove, van K., Bakker, F. T., Beentjes, K. K., Hemerik, L., Vos, R. A., & Gravendeel, B. (2020). Organic matter reduces the amount of detectable environmental DNA in freshwater. *Ecology and Evolution*, *10*(8), 3647-3654.

Bohara, K., Yadav, A. K., & Joshi, P. (2022). Detection of fish pathogens in freshwater aquaculture using eDNA methods. Diversity, 14(12), 1015.

Braid, M. D., Daniels, L. M., & Kitts, C. L. (2003). Removal of PCR inhibitors from soil DNA by chemical

flocculation. *Journal of microbiological methods*, *52*(3), 389-393.
Brannelly, L.A., Wetzel, D.P., Ohmer, M.E.B. et al. Evaluating environmental DNA as a tool for detecting an amphibian pathogen using an optimized extraction method. Oecologia 194, 267–281 (2020). https://doi.org/10.1007/s00442-020-04743-4
Broadhurst, H. A., Gregory, L. M., Bleakley, E. K., Perkins, J. C., Lavin, J. V., Bolton, P., ... & McDevitt, A. D. (2021). Mapping differences in mammalian distributions and diversity using environmental DNA from rivers. Science of the Total Environment, 801, 149724.
Bruce, K., Blackman, R. C., Bourlat, S. J., Hellström, M., Bakker, J., Bista, I., ... & Deiner, K. (2021). *A practical guide to DNA-based methods for biodiversity assessment*. Pensoft Advanced Books.
Brys, R., Haegeman, A., Halfmaerten, D., Neyrinck, S., Staelens, A., Auwerx, J., & Ruttink, T. (2021). Monitoring of spatiotemporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA. *Molecular ecology*, *30*(13), 3097-3110.
Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2017). Is the detection of aquatic environmental DNA influenced by substrate type?. *PLoS One*, *12*(8), e0183371.
Bylemans, J., Furlan, E. M., Gleeson, D. M., Hardy, C. M., & Duncan, R. P. (2018). Does size matter? An experimental evaluation of the relative abundance and decay rates of aquatic environmental DNA. *Environmental Science & Technology*, *52*(11), 6408-6416.
Cananzi, G., Tatini, I., Li, T., Petroni, G. (2024). Biodiversity gradients trough a river basin: markers, methods and tributaries in an eDNA case of study. 7th European Congress of Conservation Biology "Biodiversity positive by 2030", 17-21 june 2024, Bologna, Italy. Book of abstracts.
Capo, E., Spong, G., Königsson, H., & Byström, P. (2020). Effects of filtration methods and water volume on the quantification of brown trout (Salmo trutta) and Arctic char (Salvelinus alpinus) eDNA concentrations via droplet digital PCR. *Environmental DNA*, *2*(2), 152-160.
Capo, E., Spong, G., Koizumi, S., Puts, I., Olajos, F., Königsson, H., ... & Byström, P. (2021). Droplet digital PCR applied to environmental DNA, a promising method to estimate fish population abundance from humic-rich aquatic ecosystems. *Environmental DNA*, *3*(2), 343-352.
Carraro, L., Stauffer, J. B., & Altermatt, F. (2021). How to design optimal eDNA sampling strategies for biomonitoring in river networks. *Environmental DNA*, *3*(1), 157-172.
Champlot, S., Berthelot, C., Pruvost, M., Bennett, E. A., Grange, T., & Geigl, E. M. (2010). An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. PloS one, 5(9), e13042.
Chen, X., Li, S., Zhao, J., & Yao, M. (2024). Passive eDNA sampling facilitates biodiversity monitoring and rare species detection. *Environment International*, *187*, 108706.
Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J. C., Bonin, A., ... & Pont, D. (2016). Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. PloS one, 11(6), e0157366.
Curtis, A. N., & Larson, E. R. (2020). No evidence that crayfish carcasses produce detectable environmental DNA (eDNA) in a stream enclosure experiment. PeerJ, 8, e9333.
Curtis, A. N. (2022). Rockin'in the eDNA world: Testing and applying environmental DNA (eDNA) for applied ecology and conservation (Doctoral dissertation).
Curtis, A. N., Tiemann, J. S., Douglass, S. A., Davis, M. A., & Larson, E. R. (2021). High stream flows dilute environmental DNA (eDNA) concentrations and reduce detectability. Diversity and Distributions, 27(10), 1918-1931.
Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PloS one*, *9*(2), e88786. 41
Deiner, K., Walser, J. C., Mächler, E., & Altermatt, F. (2015). Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biological conservation, 183, 53-63.

Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J. C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nature communications, 7(1), 12544.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. PloS one, 6(8), e23398.

Díaz, C., Wege, F. F., Tang, C. Q., Crampton-Platt, A., Rüdel, H., Eilebrecht, E., & Koschorreck, J. (2020). Aquatic suspended particulate matter as source of eDNA for fish metabarcoding. Scientific Reports, 10(1), 1-8.

Diffey, B. L. (2002). Sources and measurement of ultraviolet radiation. Methods, 28(1), 4-13.

Di Muri, C., Handley, L. L., Bean, C. W., Li, J., Peirson, G., Sellers, G. S., ... & Hänfling, B. (2020). Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. *BioRvix*, 2020-07.

Dunker, K. J., Sepulveda, A. J., Massengill, R. L., Olsen, J. B., Russ, O. L., Wenburg, J. K., & Antonovich, A. (2016). Potential of environmental DNA to evaluate northern pike (Esox lucius) eradication efforts: an experimental test and case study. PloS one, 11(9), e0162277.

Eichmiller, J. J., Bajer, P. G., & Sorensen, P. W. (2014). The relationship between the distribution of common carp and their environmental DNA in a small lake. *PloS one*, *9*(11), e112611.

Eichmiller, J. J., Best, S. E., & Sorensen, P. W. (2016a). Effects of temperature and trophic state on degradation of environmental DNA in lake water. *Environmental science & technology*, *50*(4), 1859-1867.

Eichmiller, J. J., Miller, L. M., & Sorensen, P. W. (2016b). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular ecology resources*, *16*(1), 56-68.

Erickson, R. A., Merkes, C. M., & Mize, E. L. (2019). Sampling designs for landscape-level eDNA monitoring programs. *Integrated Environmental Assessment and Management*, *15*(5), 760-771.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology letters*, *4*(4), 423-425.

Furlan, E. M., Gleeson, D., Hardy, C. M., & Duncan, R. P. (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular ecology resources*, *16*(3), 641-654.

Geerts, A. N., Boets, P., Van den Heede, S., & Goethals, P. (2018). A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): A lab and field evaluation. Ecological Indicators, 84, 564-572.

Ghosal, R., Eichmiller, J. J., Witthuhn, B. A., & Sorensen, P. W. (2018). Attracting Common Carp to a bait site with food reveals strong positive relationships between fish density, feeding activity, environmental DNA, and sex pheromone release that could be used in invasive fish management. *Ecology and Evolution*, *8*(13), 6714-6727.

Godar, D. E. (2005). UV doses worldwide. *Photochemistry and photobiology*, *81*(4), 736-749.

Goldberg, C. S., Strickler, K. M., & Pilliod, D. S. (2015). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation*, *183*, 1-3. 42

Goldberg, C. S., C. R. Turner, K. Deiner, K. E. Klymus, P. F. Thomsen, M. A. Murphy, S. F. Spear, A. McKee, S. J. Oyler-McCance, and R. S. Cornman (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 11: 1299–1307.

Goldberg, C. S., Strickler, K. M., & Fremier, A. K. (2018). Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: Increasing efficacy of sampling designs. *Science of the Total Environment*, *633*, 695-703.

Gutiérrez-Cacciabue, D., Cid, A. G., & Rajal, V. B. (2016). How long can culturable bacteria and total DNA persist in environmental waters? The role of sunlight and solid particles. *Science of the Total*

Environment, *539*, 494-502.
Häder, D. P., Kumar, H. D., Smith, R. C., & Worrest, R. C. (2003). Aquatic ecosystems: effects of solar ultraviolet radiation and interactions with other climatic change factors. Photochemical & Photobiological Sciences, 2(1), 39-50.
Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., ... & Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular ecology*, *25*(13), 3101-3119.
Harrison, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B*, *286*(1915), 20191409.
He, X., Chen, H., Shi, W., Cui, Y., & Zhang, X. X. (2015). Persistence of mitochondrial DNA markers as fecal indicators in water environments. Science of The Total Environment, 533, 383-390.
Hinlo, R., Gleeson, D., Lintermans, M., & Furlan, E. (2017). Methods to maximise recovery of environmental DNA from water samples. PloS one, 12(6), e0179251.
Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., & Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of clinical microbiology*, *42*(5), 1863-1868.
Hosler, D. M. (2017). Where is the body? Dreissenid mussels, raw water testing, and the real value of environmental DNA. *Management of Biological Invasions*, *8*(3).
Iversen, L. L., Kielgast, J., & Sand-Jensen, K. (2015). Monitoring of animal abundance by environmental DNA—An increasingly obscure perspective: A reply to Klymus et al., 2015. Biological Conservation, 100(192), 479-480.
Jamwal, P. S., Bruno, A., Galimberti, A., Magnani, D., Krupa, H., Casiraghi, M., & Loy, A. (2021). First assessment of eDNA-based detection approach to monitor the presence of Eurasian otter in southern Italy. Hystrix, the Italian Journal of Mammalogy, 32(2), 176-181.
Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H. & Whiteley A. R. (2014). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources, 15(1), 216-227.
Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation letters, 4(2), 150-157.
Jerde, C. L., Olds, B. P., Shogren, A. J., Andruszkiewicz, E. A., Mahon, A. R., Bolster, D., & Tank, J. L. (2016). Influence of stream bottom substrate on retention and transport of vertebrate environmental DNA. Environmental science & technology, 50(16), 8770-8779.
Jo, T., Murakami, H., Masuda, R., Sakata, M. K., Yamamoto, S., & Minamoto, T. (2017). Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. *Molecular Ecology Resources*, *17*(6), e25-e33.
Jo, T., Arimoto, M., Murakami, H., Masuda, R., & Minamoto, T. (2019a). Particle size distribution of environmental DNA from the nuclei of marine fish. *Environmental science & technology*, *53*(16), 9947-9956.
Jo, T., Murakami, H., Yamamoto, S., Masuda, R., & Minamoto, T. (2019b). Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. *Ecology and evolution*, *9*(3), 1135-1146.
Jo, T., Murakami, H., Masuda, R., & Minamoto, T. (2020). Selective collection of long fragments of environmental DNA using larger pore size filter. *Science of the Total Environment*, *735*, 139462.
Jo, T., Arimoto, M., Murakami, H., Masuda, R., & Minamoto, T. (2020). Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass. *Environmental DNA*, *2*(2), 140-151.
Jo, T.S. (2023). A higher DNA damage rate in aqueous eDNA particles suggests intra-cellular eDNA

degradation in cellular environments. Environmental DNA, 5(2), 343-349.
Joseph, C., Faiq, M. E., Li, Z., & Chen, G. (2022). Persistence and degradation dynamics of eDNA affected by environmental factors in aquatic ecosystems. Hydrobiologia, 849(19), 4119-4133.
Kakuda, A., Doi, H., Souma, R., Nagano, M., Minamoto, T., & Katano, I. (2019). Environmental DNA detection and quantification of invasive red-eared sliders, Trachemy scripta elegans, in ponds and the influence of water quality. PeerJ, 7, e8155.
Kamoroff, C., & Goldberg, C. S. (2018). An issue of life or death: using eDNA to detect viable individuals in wilderness restoration. *Freshwater Science*, *37*(3), 685-696.
Kemp, B. M., & Smith, D. G. (2005). Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. Forensic science international, 154(1), 53-61.
Kéry, M., and B. Schmidt (2008) Imperfect detection and its consequences for monitoring in conservation. Community Ecology 9: 207–216.
Khanna, M., & Stotzky, G. (1992). Transformation of Bacillus subtilis by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. *Applied and Environmental Microbiology*, *58*(6), 1930-1939.
King, A. C., Krieg, R., Weston, A., & Zenker, A. K. (2022). Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. Journal of Environmental Management, 302, 113929.
Kirk, J. T. O. (1994). Optics of UV-B radiation in natural waters. *Ergeb. Limnol. Oceanogr.*, *43*, 1-16.
Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp Hypophthalmichthys nobilis and silver carp Hypophthalmichthys molitrix. *Biological conservation*, *183*, 77-84.
Kluever, B. M., Avery, M. L., Gawlik, D. E., Hall, P., Humphrey, J. S., Pernas, T., & Ridgley, F. (2023). Eradication of African sacred ibis (Threskiornis aethiopicus) from South Florida, USA: a collaborative early detection and rapid response case study. Management of Biological Invasions, 14(1), 123.
Kumar, G., Eble, J. E., & Gaither, M. R. (2020). A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. Molecular ecology resources, 20(1), 29-39.
Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. Molecular ecology resources, 16(6), 1401-1414.
Lam, I. P., Sung, Y. H., Lin, L., & Fong, J. J. (2020). Developing quantitative PCR assays to detect threatened and invasive freshwater turtles in Hong Kong using environmental DNA. Conservation genetics resources, 12, 293-300.
Lance, R. F., Klymus, K. E., Richter, C. A., Guan, X., Farrington, H. L., Carr, M. R., ... & Baerwaldt, K. L. (2017). Experimental observations on the decay of environmental DNA from bighead and silver carps. *Management of Biological Invasions*, *8*(3).
Lawson Handley, L., Read, D. S., Winfield, I. J., Kimbell, H., Johnson, H., Li, J., ... & Hänfling, B. (2019). Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, *1*(1), 26-39.
Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., Ekrem, T., Čiampor, F., Čiamporová-Zaťovičová, Z., Costa, F.O., Duarte, S., Elbrecht, V., Fontaneto, D., Franc, A., Geiger, M.F., Hering, D., Kahlert, M., Kalamujić Stroil, B., Kelly, M., Keskin, E., Liska, I., Mergen, P., Meissner, K., Pawlowski, J., Penev, L., Reyjol, Y., Rotter, A., Steinke, D., van der Wal, B., Vitecek, S., Zimmermann, J., Weigand, A.M., 2018. Chapter Two - Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action, in: Bohan, D.A., Dumbrell, A.J., Woodward, G., Jackson, M.B.T.-A. in E.R. (Eds.), Next Generation Biomonitoring: Part 1. Academic

Press, pp. 63–99. https://doi.org/https://doi.org/10.1016/bs.aecr.2018.01.001
Levi, T., Allen, J. M., Bell, D., Joyce, J., Russell, J. R., Tallmon, D. A., … Yu, D. W. (2019). Environmental DNA for the enumeration and management of Pacific salmon. Molecular Ecology Resources, 19, 597– 608.
Li, J., Lawson Handley, L. J., Read, D. S., & Hänfling, B. (2018). The effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding. *Molecular Ecology Resources*, *18*(5), 1102-1114.
Lindahl, T. (1993). Instability and decay of the primary structure of DNA. nature, 362(6422), 709-715.
Littlefair, J. E., Hrenchuk, L. E., Blanchfield, P. J., Rennie, M. D., & Cristescu, M. E. (2021). Thermal stratification and fish thermal preference explain vertical eDNA distributions in lakes. *Molecular Ecology*, *30*(13), 3083-3096.
Longmire, J. L., Baker, R. J., & Maltbie, M. (1997). Use of" lysis buffer" in DNA isolation and its implication for museum collections. *(No Title)*.
Loy A., Giovacchini S., Bruno A., Ramazzotti F., Di Febbraro M., Jamwal P., Krupa H., Melchionna M., Mirone E., Monaco P., Caprotti L., Galimberti A. 2023. Implementazione di un protocollo di rilevamento multispecifico e sorveglianza di specie di interesse unionale nelle acque dolci del Lazio attraverso DNA ambientale e modelli di connettività. Rapporto intermedio. P.S.R. 2014/2020 – Operazione 7.6.1 Studi e investimenti finalizzati alla tutela dell'ambiente e del patrimonio culturale e alla conservazione della biodiversità. Final report.
Macher, J. N., & Leese, F. (2018). eDNA metabarcoding of rivers: Is all eDNA everywhere, all the time?. In *ECCB2018: 5th European Congress of Conservation Biology. 12th-15th of June 2018, Jyväskylä, Finland*. Open Science Centre, University of Jyväskylä.
Mächler, E., Deiner, K., Spahn, F., & Altermatt, F. (2016). Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. *Environmental science & technology*, *50*(1), 305-312.
Mächler, E., Osathanunkul, M., & Altermatt, F. (2018). Shedding light on eDNA: neither natural levels of UV radiation nor the presence of a filter feeder affect eDNA-based detection of aquatic organisms. PloS one, 13(4), e0195529.
MacKenzie, D. I., J. D. Nichols, B. L. Gideon, S. Droege, J. A. Royle, and C. A. Langtimm (2002) Estimating site occupancy rates when detection probabilities are less than one. Ecology 83: 2248–2255.
MacKenzie, D. I., and L. L. Bailey (2004) Assessing the fit of site-occupancy models. Journal of Agricultural, Biological, and Environmental Statistics 9: 300–318.
MacKenzie, D. I., J. D. Nichols, J. A. Royle, K. H. Pollock, L. Bailey, and J. E. Hines (2017) Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence. Second edition. Academic Press, Boston, Massachusetts, USA.
MacIntyre, S., & Melack, J. M. (1995). Vertical and horizontal transport in lakes: linking littoral, benthic, and pelagic habitats. *Journal of the North American Benthological Society*, *14*(4), 599-615.
Mahmoudi, N., Slater, G. F., & Fulthorpe, R. R. (2011). Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils. *Canadian journal of microbiology*, *57*(8), 623-628.
Majaneva, M., Diserud, O. H., Eagle, S. H., Boström, E., Hajibabaei, M., & Ekrem, T. (2018). Environmental DNA filtration techniques affect recovered biodiversity. Scientific reports, 8(1), 4682.
Manfrin, C., Zanetti, M., Stanković, D., Fattori, U., Bertucci-Maresca, V., Giulianini, P. G., & Pallavicini, A. (2022). Detection of the endangered stone crayfish Austropotamobius torrentium (Schrank,

1803) and its congeneric A. pallipes in its last italian biotope by eDNA analysis. *Diversity*, *14*(3), 205.

Martellini, A., Payment, P., & Villemur, R. (2005). Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water research*, *39*(4), 541-548.

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., & Minamoto, T. (2014). The release rate of environmental DNA from juvenile and adult fish. *PloS one*, *9*(12), e114639.

Mauvisseau, Q., Halfmaerten, D., Neyrinck, S., Burian, A., & Brys, R. (2021). Effects of preservation strategies on environmental DNA detection and quantification using ddPCR. *Environmental DNA*, *3*(4), 815-822.

McClenaghan, B., Fahner, N., Cote, D., Chawarski, J., McCarthy, A., Rajabi, H., ... & Hajibabaei, M. (2020). Harnessing the power of eDNA metabarcoding for the detection of deep-sea fishes. *PLoS One*, *15*(11), e0236540.

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation*, *183*, 70-76.

Matsui, K., Honjo, M., & Kawabata, Z. (2001). Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. Aquatic Microbial Ecology, 26(1), 95-102.

Minamoto, T., Naka, T., Moji, K., & Maruyama, A. (2016). Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. *Limnology*, *17*, 23-32.

Minamoto, T., Miya, M., Sado, T., Seino, S., Doi, H., Kondoh, M., ... & Uchii, K. (2021). An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. *Environmental DNA*, *3*(1), 8-13.

Moushomi, R., Wilgar, G., Carvalho, G., Creer, S., & Seymour, M. (2019). Environmental DNA size sorting and degradation experiment indicates the state of Daphnia magna mitochondrial and nuclear eDNA is subcellular. *Scientific Reports*, *9*(1), 12500.

Muha, T. P., Robinson, C. V., Garcia de Leaniz, C., & Consuegra, S. (2019). An optimised eDNA protocol for detecting fish in lentic and lotic freshwaters using a small water volume. *PLoS One*, *14*(7), e0219218.

Nguyen, T. H.; Elimelech, M. Plasmid DNA adsorption on silica: Kinetics and conformational changes in monovalent and divalent salts. Biomacromolecules 2007, 8 (1), 24−32.

Nguyen, T. H.; Elimelech, M. Adsorption of plasmid DNA to a natural organic matter-coated silica surface: Kinetics, conformation, and reversibility. Langmuir 2007, 23 (6), 3273−3279. Environmental Science & Technology Article 9331

Nguyen, T. H.; Chen, K. L.; Elimelech, M. Adsorption kinetics and reversibility of linear plasmid DNA on silica surfaces: Influence of alkaline earth and transition metal ions. Biomacromolecules 2010, 11 (5), 1225−1230.

Nichols, J. D., L. L. Bailey, A. F. O'Connell, Jr., N. W. Talancy, E. H. Campbell Grant, A. T. Gilbert, E. M. Annand, T. P. Husband, and J. E. Hines (2008) Multi-scale occupancy estimation and modelling using multiple detection methods. Journal of Applied Ecology 45: 1321–1329.

Nukazawa, K., Akahoshi, K., & Suzuki, Y. (2020). Are bacteria potential sources of fish environmental DNA?. *Plos one*, *15*(3), e0230174.

Ogram, A., Sayler, G. S., & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Journal of microbiological methods*, *7*(2-3), 57-66.

Osman, O. A., Andersson, J., Martin Sanchez, P. M., & Eiler, A. (2022). National eDNA-based monitoring of Batrachochytrium dendrobatidis and amphibian species in Norway. Metabarcoding and Metagenomics, 6, 305-317.

Pearman, J. K., Keeley, N. B., Wood, S. A., Laroche, O., Zaiko, A., Thomson-Laing, G., ... & Pochon, X. (2020). Comparing sediment DNA extraction methods for assessing organic enrichment associated with marine aquaculture. PeerJ, 8, e10231.

Pedersen, M. W., Overballe-Petersen, S., Ermini, L., Sarkissian, C. D., Haile, J., Hellstrom, M., ... & Willerslev, E. (2015). Ancient and modern environmental DNA. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1660), 20130383.

Peixoto, S., Chaves, C., Velo-Antón, G., Beja, P., & Egeter, B. (2021). Species detection from aquatic eDNA: Assessing the importance of capture methods. *Environmental DNA*, *3*(2), 435-448.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., & Avery, M. L. (2014). Detecting an elusive invasive species: A diagnostic PCR to detect B urmese python in F lorida waters and an assessment of persistence of environmental DNA. Molecular ecology resources, 14(2), 374-380.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences, 70(8), 1123-1130.

Pilliod DS, Goldberg CS, Arkle RS, Waits (2014). Factors influencing detection of eDNA from a streamdwelling amphibian. Mol Ecol Resour.; 14(1): 109-16. Epub 2013/09/17. https://doi.org/10.1111/1755-0998.12159 PMID: 24034561.).

Pilliod, D. S., Laramie, M. B., MacCoy, D., & Maclean, S. (2019). Integration of eDNA-based biological monitoring within the US Geological Survey's National Streamgage Network. JAWRA Journal of the American Water Resources Association, 55(6), 1505-1518.

Pont, D., Valentini, A., Rocle, M., Maire, A., Delaigue, O., Jean, P., & Dejean, T. (2021). The future of fish-based ecological assessment of European rivers: from traditional EU Water Framework Directive compliant methods to eDNA metabarcoding-based approaches. *Journal of fish biology*, *98*(2), 354-366.

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., ... & Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Scientific reports, 8(1), 10361.

Porco, D., Purnomo, C. A., Glesener, L., Proess, R., Lippert, S., Jans, K., ... & Frantz, A. C. (2024). eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. *BMC Ecology and Evolution*, *24*(1), 4.

Poté, J., Ackermann, R., & Wildi, W. (2009). Plant leaf mass loss and DNA release in freshwater sediments. *Ecotoxicology and environmental safety*, *72*(5), 1378-1383.

Pourmoghadam, M. N., Poorbagher, H., de Oliveira Fernandes, J. M., & Jafari, O. (2019). Diazinon negatively affects the integrity of environmental DNA stability: a case study with common carp (Cyprinus carpio). Environmental monitoring and assessment, *191*, 1-6.

Randall, L. A., Goldberg, C. S., & Moehenschlager, A. (2023). Environmental DNA surveys can underestimate amphibian occupancy and overestimate detection probability: implications for practice. The Journal of Wildlife Management, 87(7), e22463.

Ravanat, J. L., Douki, T., & Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. Journal of Photochemistry and Photobiology B: Biology, 63(1-3), 88-102.

Rawlence, N. J., Lowe, D. J., Wood, J. R., Young, J. M., Churchman, G. J., HUANG, Y. T., & Cooper, A. (2014). Using palaeoenvironmental DNA to reconstruct past environments: progress and prospects. *Journal of Quaternary Science*, *29*(7), 610-626.

Rees, H. C., B. C. Maddison, D. J. Middleditch, J. R. Patmore, and K. C. Gough. 2014. The detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. Journal of Applied Ecology 51: 1450–1459.

Rondinini, C., Battistoni, A., Teofili, C. (compilatori) 2022. Lista Rossa IUCN dei vertebrati italiani 2022. Comitato Italiano IUCN e Ministero dell'Ambiente e della Sicurezza Energetica, Roma.

Rusch, J., Mojžišová, M., Strand, D., Svobodová, J., Vrålstad, T., & Petrusek, A. (2020). Simultaneous detection of native and invasive crayfish and Aphanomyces astaci from environmental DNA samples in a wide range of habitats in Central Europe.

Saito, T., & Doi, H. (2021). A model and simulation of the influence of temperature and amplicon length on environmental DNA degradation rates: a meta-analysis approach. *Frontiers in Ecology and Evolution*, *9*, 623831.

Sakata, M. K., Yamamoto, S., Gotoh, R. O., Miya, M., Yamanaka, H., & Minamoto, T. (2020). Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. *Environmental DNA*, *2*(4), 505-518.

Sales, N. G., McKenzie, M. B., Drake, J., Harper, L. R., Browett, S. S., Coscia, I., ... & McDevitt, A. D. (2020a). Fishing for mammals: Landscape-level monitoring of terrestrial and semi-aquatic communities using eDNA from riverine systems. Journal of Applied Ecology, 57(4), 707-716.

Sales, N. G., Kaizer, M. D. C., Coscia, I., Perkins, J. C., Highlands, A., Boubli, J. P., ... & Mcdevitt, A. D. (2020b). Assessing the potential of environmental DNA metabarcoding for monitoring Neotropical mammals: a case study in the Amazon and Atlantic Forest, Brazil. Mammal Review, 50(3), 221-225.

Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., & Vestergaard, P. (2019). Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Communications Biology*, *2*(1), 461.

Sanches, T. M., & Schreier, A. D. (2020). Optimizing an eDNA protocol for estuarine environments: Balancing sensitivity, cost and time. Plos one, 15(5), e0233522.

Santas, A. J., Persaud, T., Wolfe, B. A., & Bauman, J. M. (2013). Noninvasive method for a statewide survey of eastern hellbenders Cryptobranchus alleganiensis using environmental DNA. International Journal of Zoology, 2013.

Sassoubre, L. M., Yamahara, K. M., Gardner, L. D., Block, B. A., & Boehm, A. B. (2016). Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental science & technology*, *50*(19), 10456-10464.

Schabacker, J. C., Amish, S. J., Ellis, B. K., Gardner, B., Miller, D. L., Rutledge, E. A., ... & Luikart, G. (2020). Increased eDNA detection sensitivity using a novel high-volume water sampling method. *Environmental DNA*, *2*(2), 244-251.

Schmidt, K. J., Soluk, D. A., Maestas, S. E. M., & Britten, H. B. (2021a). Persistence and accumulation of environmental DNA from an endangered dragonfly. *Scientific Reports*, *11*(1), 1-8.

Schmidt, B. C., Spear, S. F., Tomi, A., & Bodinof Jachowski, C. M. (2021b). Evaluating the efficacy of environmental DNA (eDNA) to detect an endangered freshwater mussel Lasmigona decorata (Bivalvia: Unionidae). *Freshwater Science*, *40*(2), 354-367.

Sellers, G. S., Di Muri, C., Gómez, A., & Hänfling, B. (2018). Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*, *2*, e24556.

Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., ... & Creer, S. (2018). Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Communications biology, 1(1), 4.

Sepulveda, A. J., Schabacker, J., Smith, S., Al-Chokhachy, R., Luikart, G., & Amish, S. J. (2019). Improved detection of rare, endangered and invasive trout in using a new large-volume sampling method for eDNA capture. *Environmental DNA*, *1*(3), 227-237.

Shogren, A. J., Tank, J. L., Andruszkiewicz, E.A., Olds, B., Jerde, C., Bolster, D. (2016). Modelling the transport of environmental DNA through a porous substrate using continuous flow-through

column experiments. J R Soc Interface. 13(119):20160290. doi: 10.1098/rsif.2016.0290. PMID: 27251680; PMCID: PMC4938091.

Shogren, A. J., Tank, J. L., Andruszkiewicz, E., Olds, B., Mahon, A. R., Jerde, C. L., & Bolster, D. (2017). Controls on eDNA movement in streams: Transport, retention, and resuspension. *Scientific reports*, *7*(1), 5065.

Shogren, A. J., Tank, J. L., Egan, S. P., August, O., Rosi, E. J., Hanrahan, B. R., ... & Bolster, D. (2018). Water flow and biofilm cover influence environmental DNA detection in recirculating streams. *Environmental science & technology*, *52*(15), 8530-8537.

Shogren, A. J., Tank, J. L., Egan, S. P., Bolster, D., & Riis, T. (2019). Riverine distribution of mussel environmental DNA reflects a balance among density, transport, and removal processes. *Freshwater Biology*, *64*(8), 1467-1479.

Smith, K. F., Wood, S. A., Mountfort, D. O., & Cary, S. C. (2012). Development of a real-time PCR assay for the detection of the invasive clam, Corbula amurensis, in environmental samples. *Journal of Experimental Marine Biology and Ecology*, *412*, 52-57.

Song, J. W., Small, M. J., & Casman, E. A. (2017). Making sense of the noise: the effect of hydrology on silver carp eDNA detection in the Chicago area waterway system. *Science of the Total Environment*, *605*, 713-720.

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S., ... & Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635-645.

Sreedhara, A., Freed, J. D., & Cowan, J. A. (2000). Efficient inorganic deoxyribonucleases. Greater than 50-million-fold rate enhancement in enzyme-like DNA cleavage. *Journal of the American Chemical Society*, *122*(37), 8814-8824.

Sreedhara, A., & Cowan, J. A. (2001). Catalytic hydrolysis of DNA by metal ions and complexes. *JBIC Journal of Biological Inorganic Chemistry*, *6*, 337-347.

Staley, Z. R., Chuong, J. D., Hill, S. J., Grabuski, J., Shokralla, S., Hajibabaei, M., & Edge, T. A. (2018). Fecal source tracking and eDNA profiling in an urban creek following an extreme rain event. *Scientific reports*, *8*(1), 14390.

Stewart, K. A., & Taylor, S. A. (2020). Leveraging eDNA to expand the study of hybrid zones. *Molecular Ecology*, *29*(15), 2768-2776.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85-92.

Stewart, K. A. (2019) Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. Biodiversity and Conservation 28: 983–1001.

Sun, M., Guo, Y., Zhao, N., Zhang, S., Pei, K., & Qin, C. (2022). Fish eDNA detection and its technical optimization: A case study of Acanthopagrus latus. Marine Environmental Research, 176, 105588.

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For biodiversity research and monitoring*. Oxford University Press.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. I. (2012). Estimation of fish biomass using environmental DNA. PloS one, 7(4), e35868.

Takasaki K, Aihara H, Imanaka T, Matsudaira T, Tsukahara K, Usui A, et al. (2021). Water pre-filtration methods to improve environmental DNA detection by real-time PCR and metabarcoding. PLoS ONE 16(5): e0250162. https://doi.org/10.1371/journal.pone.0250162

Takahashi, S., Sakata, M. K., Minamoto, T., & Masuda, R. (2020). Comparing the efficiency of open and enclosed filtration systems in environmental DNA quantification for fish and jellyfish. *PloS one*, *15*(4), e0231718

TES (2019). Environmental DNA Sampling and Experiment Manual Version 2.1 (published April 25,

2019). The eDNA Society, Edna Methods Standardization Committee.
Thalinger, B., Rieder, A., Teuffenbach, A., Pütz, Y., Schwerte, T., Wanzenböck, J., & Traugott, M. (2021). The effect of activity, energy use, and species identity on environmental DNA shedding of freshwater fish. *Frontiers in Ecology and Evolution*, *9*, 623718.
Thomas, A. C., Howard, J., Nguyen, P. L., Seimon, T. A., & Goldberg, C. S. (2018). eDNA Sampler: A fully integrated environmental DNA sampling system. *Methods in ecology and evolution*, *9*(6), 1379-1385.
Thomas, A. C., Nguyen, P. L., Howard, J., & Goldberg, C. S. (2019). A self-preserving, partially biodegradable eDNA filter. *Methods in Ecology and Evolution*, *10*(8), 1136-1141.
Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. Molecular ecology, 21(11), 2565-2573.
Tillotson, M. D., Kelly, R. P., Duda, J. J., Hoy, M., Kralj, J., & Quinn, T. P. (2018). Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. *Biological Conservation*, *220*, 1-11.
Torti, A., Lever, M. A., & Jørgensen, B. B. (2015). Origin, dynamics, and implications of extracellular DNA pools in marine sediments. Marine genomics, 24, 185-196.
Tréguier, A., Paillisson, J. M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J. M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish P rocambarus clarkii in freshwater ponds. *Journal of Applied Ecology*, *51*(4), 871-879.
Tsai, Y. L., & Olson, B. H. (1992). Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Applied and environmental microbiology, 58(2), 754-757.
Tsuji, S., Ushio, M., Sakurai, S., Minamoto, T., & Yamanaka, H. (2017). Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance. PLoS One, 12(4), e0176608.
Tsuji, S., Takahara, T., Doi, H., Shibata, N., & Yamanaka, H. (2019). The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. Environmental DNA, 1(2), 99-108.
Tumolo M, Ancona V, Losacco D, Uricchio VF, De Paola D (2021) Optimized protocol proposal to extract eDNA from oligotrophic and degraded water samples. ARPHA Conference Abstracts 4: e64814.
Turner, C. R., Barnes, M. A., Xu, C. C., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods in Ecology and Evolution, 5(7), 676-684.
Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, *183*, 93-102.
Unnithan, V. V., Unc, A., Joe, V., & Smith, G. B. (2014). Short RNA indicator sequences are not completely degraded by autoclaving. Scientific Reports, 4(1), 4070.
Uthicke, S., Robson, B., Doyle, J. R., Logan, M., Pratchett, M. S., & Lamare, M. (2022). Developing an effective marine eDNA monitoring: eDNA detection at pre-outbreak densities of corallivorous seastar (Acanthaster cf. solaris). Science of the Total Environment, 851, 158143.
Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular ecology*, *25*(4), 929-942.
Villacorta-Rath, C., Hoskin, C. J., Strugnell, J. M., & Burrows, D. (2021). Long distance (> 20 km) downstream detection of endangered stream frogs suggests an important role for eDNA in surveying for remnant amphibian populations. PeerJ, 9, e12013.

Xing, Y., Gao, W., Shen, Z., Zhang, Y., Bai, J., Cai, X., ... & Zhao, Y. (2022). A review of environmental DNA field and laboratory protocols applied in fish ecology and environmental health. Frontiers in Environmental Science, 10, 725360.

Webster, J. R., Benfield, E. F., Golladay, S. W., Hill, B. H., Hornick, L. E., Kazmierczak Jr, R. F., & Perry, W. B. (1987). Experimental studies of physical factors affecting seston transport in streams 1. *Limnology and oceanography*, *32*(4), 848-863.

Wegleitner, B. J., Jerde, C. L., Tucker, A., Chadderton, W. L., & Mahon, A. R. (2015). Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources*, *7*, 789-791.

Wei, N., Nakajima, F., & Tobino, T. (2019). Variation of environmental DNA in sediment at different temporal scales in nearshore area of Tokyo Bay. Journal of Water and Environment Technology, 17(3), 153-162.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Lowe, W. H., & Schwartz, M. K. (2015). Environmental DNA particle size distribution from Brook Trout (Salvelinus fontinalis). *Conservation Genetics Resources*, *7*, 639-641.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Sepulveda, A. J., Shepard, B. B., Jane, S. F., ... & Schwartz, M. K. (2016). Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char Salvelinus fontinalis. Biological Conservation, 194, 209-216.

Wilcox, T. M., Young, M. K., McKelvey, K. S., Isaak, D. J., Horan, D. L., & Schwartz, M. K. (2018). Fine-scale environmental DNA sampling reveals climate-mediated interactions between native and invasive trout species. *Ecosphere*, *9*(11), e02500.

Wotton, R. S., & Malmqvist, B. (2001). Feces in aquatic ecosystems: feeding animals transform organic matter into fecal pellets, which sink or are transported horizontally by currents; these fluxes relocate organic matter in aquatic ecosystems. *BioScience*, *51*(7), 537-544.

Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: the microbial “protective clothing” in extreme environments. *International journal of molecular sciences*, *20*(14), 3423.

Chapter 6 – Concluding remarks

Freshwater ecosystems are characterized by unique biodiversity and provide essential contributions to humankind. Nevertheless, rivers, lakes and other type of inland waters are globally poorly protected and their biodiversity demonstrated a continuous decline in past years (Moilanen et al., 2008). With this study we provided potential applications to easily reveal species presence and quickly act with conservation measures toward native biodiversity in the hope of promoting sustainability, particularly in human-dominated environments (Bagheriyan et al., 2023).

The quantitative PCR approach of eDNA has demonstrated successful applications in detecting multiple species simultaneously, offering significant potential for future large-scale monitoring of freshwater wildlife belonging to several organismal groups (Thomsen et al., 2012). The high sensitivity of this method is a powerful tool in revealing the presence of elusive and cryptic species (Biggs et al., 2015). With this study, we showed how the implementation of this methodology can be fundamental to routinely fulfill the obligations requested by the Habitat Directive surveillance (92/43/EEC) regarding freshwater species. Additionally, eDNA is also helpful to constantly look after biological invasions as required by law for the European countries with the Invasive Alien Species Regulation (1143/2014). eDNA techniques are also broadly recognized to be particularly useful in integrating monitoring procedures pursuant to the Water Framework Directive 2000/60/EC (Hering et al., 2018). Several freshwater species are used as biomonitors to assess the quality of European waters. We showed how benthic invertebrates, and native and alien fish can benefit the use of a sensitive qPCR eDNA method to inform the ecological indexes created for the Water Framework Directive.

This study provided several new qPCR assays that can be used in the future for monitoring endangered cryptic species and IAS in freshwaters of Central Italy. These new tools are a valuable source of ecological information as they are rigorously designed through the standard criteria of assay validation (Klymus et al., 2020). They guarantee high accuracy data, and are ready to be adopted in other geographical areas to reveal unknown presences, co-occurrences between species at risk and IAS, and keep tracking on range expansions (Thalinger et al., 2021). This experience identified a monitoring protocol that optimize freshwater multi-species detection in Central Italy. Ecological requirements and range distribution are enough to unveil the real distribution of unnoticed taxa. Species with low detectability need refinement, especially those that release minimal DNA. Adjustments in sampling procedures may be needed to accurately capture the distribution of armored invertebrates and reptiles (Akre et al., 2019). Accounting for phenological stages showed to sufficiently inform a planning strategy on freshwater wildlife monitoring with eDNA. One of the main prospects that may expand the routinely use of eDNA in monitoring is its cost. We demonstrated that an eDNA survey carried out on multiple species is a more cheap and low-cost method compared to traditional techniques. This is furthermore valorised by considering the cost of permanent laboratory equipment such as machines, a matter not fully considered in previous papers (Akre et al., 2019). eDNA surveys are quick in field collection and laboratory procedures, allow to sample large areas, require less personnel, and are incredibly informative for many species at the same time. We believe this new way of collecting non-invasive data can really lead new winning conservation strategies to support endemic biodiversity in the future.

The different application of eDNA can serve European regulations emanated to protect native biodiversity to fulfil their purposes. We demonstrated how a qPCR approach is convenient to evaluate the effectiveness of European protected areas, such as Natura 2000 Network, and identify priority areas of intervention to manage IAS (Capurso et al., 2023). Conserving native species habitats and controlling alien species invasions

through spatial planning approaches is particularly demanded by the Habitats and the Water Framework Directives, as well as by the IAS Regulation. As the European Community decided to boost the implementation of these regulations, eDNA showed also to be an ideal candidate to accomplish some of the European Strategy on Biodiversity objectives.

A qPCR eDNA approach is also practical for identifying conservation measures directed to apex predators and umbrella species, that can positively benefit all freshwater biodiversity of river ecosystems (Macdonald et al., 2018). The employment of eDNA techniques is therefore crucial to monitor expansion stages of and highly elusive and wandering mammals, such as Eurasian otters, even in unusual environments difficult to explore in their integrity (Valsecchi et al., 2022). However, eDNA surveys cannot substitute field occurrences retrieved by experts and specialists that provide a higher quality information on wildlife populations. It is a primary importance to integrated different approaches to provide robust data and inform sound decisions in wildlife conservation (Tolrà et al., 2024). Strategically, eDNA can be used to optimize monitoring on large areas where the expansion of endangered species is expected. After species has been noticed, fine-scale surveying could also be implemented to find the source that spread the targeted DNA into the environment. A beneficial strategy to map where species populations really occur is to return upstream the river starting from the sampling site where target species is initially detected and using an eDNA quantification. The more you get closer to the source of eDNA, the more the quantity of eDNA might be retrieved (Seymour et al., 2018). Alternatively, traditional techniques can be introduced, with the same aim and adopting the same strategy, to collect data on new populations of endangered species and rapidly inform conservation actions to preserve them.

Using spatial replicates along the river with these two approaches (i.e., of qPCR-based sampling, and traditional survey techniques) are fundamental to confirm the results retrieved by large-scale eDNA campaigns. Indeed, the main disadvantages of such a sensitive method as eDNA rely on the risk of false-presences and on-site contamination. Thus, appropriate validation of early-warning results is needed. Quantification though eDNA may help in locating the source of target species eDNA, but only traditional technique, such as trapping or visual census, are able to certify real species occurrences and remove the risk of false presences. For instance, prey species such as crayfish can be transported into the gut of several predators and displaced through several water catchments through the faeces of predators, releasing the target eDNA far from the original area of their populations (Merkes et al., 2014). Another source of false presence is the resuspension in the water of old eDNA stored in sediments (Barnes and Turner, 2016). Thus, traditional sampling techniques still remain the main solid method in proving the existence of viable populations in wild conditions.

Nevertheless, eDNA represents a powerful technique to optimize monitoring efforts on large scales. Species distribution modeling offers great potential to further optimize the sampling strategies of eDNA campaigns. Connectivity models enhance the identification of possible paths of colonization, and allow to determine all suitable patches in the expansion areas that are firstly occupied by colonization events. eDNA sampling efforts can be restricted to these patches that are both well connected with the species range and characterized by high habitat quality. This framework can be used also to keep track of expansion progresses of endangered species and monitoring conservation successes of restorations or range recovery, as well as to adapt a surveillance monitoring of the possible spread of several pathogen affecting freshwater fauna (Porco et al., 2024).

Once selected sampling sites, practical aspects of sampling eDNA into the field are essential to carry out accurate results. Field procedures in water collection are listed in specific guidelines. Consultation and adoption of the guidelines we provided for Central Italy are strongly suggested to correctly implement eDNA as a proficient method to detect species in wild environments. Setting up an eDNA study also entails the arise of several strategic, logistical, scientific, technological, and economic issues. Guidelines typically offer a workflow of an eDNA project aiding at develop projects on species detection through the use of

environmental DNA. We also propose the use of eDNA in captive conditions, especially in fish farms used for fish restocking in wild conditions. eDNA analysis promise as a solution to unveil both accidental alien fish species and fish pathogen harming native freshwater fauna, and to prevent their spreading in wild environments among fish restocking, e.g., for recreational purposes (Svoboda et al., 2017).

References

Akre, T.S., Parker, L.D., Ruther, E., Maldonado, J.E., Lemmon, L., McInerney, N.R., 2019. Concurrent visual encounter sampling validates eDNA selectivity and sensitivity for the endangered wood turtle (Glyptemys insculpta). PLoS One 14, 1–22. https://doi.org/10.1371/journal.pone.0215586

Bagheriyan, E., Karimi, A., Yazdandad, H., 2023. Assessing spatio-temporal patterns of human-wildlife conflicts in a human-dominated landscape: a case study from Iran. Biodivers. Conserv. 32, 4239–4257. https://doi.org/10.1007/s10531-023-02685-w

Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv. Genet. 17, 1–17. https://doi.org/10.1007/s10592-015-0775-4

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P., Williams, P., Dunn, F., 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). Biol. Conserv. 183, 19–28. https://doi.org/https://doi.org/10.1016/j.biocon.2014.11.029

Capurso, G., Carroll, B., Stewart, K.A., 2023. Transforming marine monitoring: Using eDNA metabarcoding to improve the monitoring of the Mediterranean Marine Protected Areas network. Mar. Policy 156, 105807. https://doi.org/https://doi.org/10.1016/j.marpol.2023.105807

Hering, D., Borja, A., Jones, J.I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., Kelly, M., 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Res. 138, 192–205. https://doi.org/https://doi.org/10.1016/j.watres.2018.03.003

Klymus, K.E., Ruiz Ramos, D. V, Thompson, N.L., Richter, C.A., 2020. Development and Testing of Species-specific Quantitative PCR Assays for Environmental DNA Applications. JoVE e61825. https://doi.org/doi:10.3791/61825

Macdonald, D.W., Newman, C., Harrington, L.A., 2018. Beneath the umbrella: Conservation out of the limelight. Biol. Conserv. Musteloids 543–556. https://doi.org/10.1093/oso/9780198759805.003.0030

Merkes, C.M., McCalla, S.G., Jensen, N.R., Gaikowski, M.P., Amberg, J.J., 2014. Persistence of DNA in Carcasses, Slime and Avian Feces May Affect Interpretation of Environmental DNA Data. PLoS One 9, e113346.

Moilanen, A., Leathwick, J., Elith, J., 2008. A method for spatial freshwater conservation prioritization. Freshw. Biol. 53, 577–592. https://doi.org/https://doi.org/10.1111/j.1365-2427.2007.01906.x

Porco, D., Purnomo, C.A., Glesener, L., Proess, R., Lippert, S., Jans, K., Colling, G., Schneider, S., Stassen, R., Frantz, A.C., 2024. eDNA-based monitoring of Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans with ddPCR in Luxembourg ponds: taking signals below the Limit of Detection (LOD) into account. BMC Ecol. Evol. 24, 1–16. https://doi.org/10.1186/s12862-023-02189-9

Seymour, M., Durance, I., Cosby, B.J., Ransom-Jones, E., Deiner, K., Ormerod, S.J., Colbourne, J.K., Wilgar, G., Carvalho, G.R., de Bruyn, M., Edwards, F., Emmett, B.A., Bik, H.M., Creer, S., 2018. Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Commun. Biol. 1, 4. https://doi.org/10.1038/s42003-017-0005-3

Svoboda, J., Mrugała, A., Kozubíková-Balcarová, E., Petrusek, A., 2017. Hosts and transmission of the crayfish plague pathogen Aphanomyces astaci: a review. J. Fish Dis. 40, 127–140. https://doi.org/https://doi.org/10.1111/jfd.12472

Thalinger, B., Deiner, K., Harper, L.R., Rees, H.C., Blackman, R.C., Sint, D., Traugott, M., Goldberg, C.S., Bruce, K., 2021. A validation scale to determine the readiness of environmental DNA assays for

routine species monitoring. Environ. DNA 3, 823–836. https://doi.org/10.1002/edn3.189

Thomsen, P.F., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573. https://doi.org/https://doi.org/10.1111/j.1365-294X.2011.05418.x

Tolrà, A., Ruiz-Olmo, J., Riera, J.L., 2024. Human disturbance and habitat structure drive eurasian otter habitat selection in heavily anthropized river basins. Biodivers. Conserv. 33, 1683–1710. https://doi.org/10.1007/s10531-024-02826-9

Valsecchi, E., Coppola, E., Pires, R., Parmegiani, A., Casiraghi, M., Galli, P., Bruno, A., 2022. A species-specific qPCR assay provides novel insight into range expansion of the Mediterranean monk seal (Monachus monachus) by means of eDNA analysis. Biodivers. Conserv. 31, 1175–1196. https://doi.org/10.1007/s10531-022-02382-0