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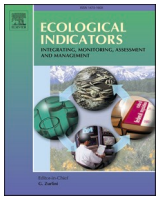

Assessing the conservation effectiveness of Natura 2000 areas for freshwater communities using environmental DNA

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ABSTRACT

Although covering less than 1% of the Earth's surface, freshwater habitats host a high proportion of global animal biodiversity and provide essential ecosystem services. These habitats, however, are experiencing some of the fastest rates of biodiversity decline and are among the most threatened ecosystems worldwide. To evaluate the effectiveness of Natura 2000 (N2K) protected areas in conserving both freshwater and terrestrial biodiversity, we compared protected and adjacent unprotected streams of the Northern Apennines hydro-ecoregion (Central Italy) through an integrated monitoring approach. We combined multi-marker eDNA metabarcoding to simultaneously record vertebrate and invertebrate species across terrestrial and aquatic biomes with the analysis of physico-chemical and geomorphological parameters of streams within and outside protected boundaries. We specifically assessed community richness and composition, taxonomic and phylogenetic diversity, environmental quality, and the occurrence of species of conservation concern. We detected over 1100 taxa and found that protected and unprotected sites exhibited comparable environmental conditions and did not differ significantly in overall species richness. However, protected streams hosted freshwater communities with significantly higher taxonomic diversity and a greater number of aquatic species of conservation interest. These results suggest that the targeted N2K sites effectively preserve freshwater-related communities. Furthermore, the good ecological status of adjacent unprotected sites highlights their role as functional buffer zones that bolster PA effectiveness. Finally, we discuss the application of eDNA metabarcoding and the use of taxonomically and phylogenetically weighted indices as robust, standardized ecological indicators which are critical for providing an in-depth evaluation of conservation effectiveness.

1. Introduction

During the last decades, multiple anthropogenic drivers have caused an extensive rate of biodiversity loss, ultimately undermining the provision of ecosystem goods and services that humankind depends upon (Langhammer et al., 2024). Inland waters and freshwater habitats cover less than 1% of the planet's surface but support almost 9.5% of all known animal species, often showing high levels of endemism (Williams-Subiza and Epele, 2021; Miller, 2021). Despite their socio-ecological importance, these habitats are experiencing the highest rates of biodiversity decline, even surpassing terrestrial ecosystems (Haase et al., 2023; Williams-Subiza and Epele, 2021).

The establishment of protected areas (PAs) is a primary tool for nature conservation (Dudley, 2008) and, when effective, can significantly reduce human-driven biodiversity loss (Pulido-Chadid et al., 2023; Langhammer et al., 2024). Under the Birds and the Habitats Directives, European Union (EU) Member States have designed the Natura 2000 (N2K) protected areas network which represents the world's largest coordinated system of PAs. In Italy, it covers about 19% of the terrestrial territory, often overlapping with national parks and regional reserves (SNPA, 2023).

In the last two decades, however, the efficacy of terrestrial PAs in preserving freshwater communities has come under scrutiny (Hermoso et al., 2016; Nogueira et al., 2021; Haase et al., 2023; Santangeli et al., 2023). Freshwater wildlife populations have declined globally by an average of 85% since 1970, the sharpest rate recorded across all systems (WWF, 2024). Indeed, a systematic review found that only 51% of PAs were effective for freshwater biodiversity (Acreman et al., 2019). This lack of conservation effectivenessis is attributed to PA design criteria that overlook the spatio-temporal complexity of aquatic systems, frequently treating rivers as mere administrative boundaries rather than ecological components of the protected landscape (Trochet and

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Schmeller, 2013; Santangeli et al., 2023; Moberg et al., 2024).

To address this lack of conservation effectiveness, robust monitoring tools are essential for developing reliable indicators of PA efficacy (Moberg et al., 2024). Such frameworks must integrate physical, chemical, and biological parameters to enable a comprehensive assessment of the ecological status of different taxa and allow for a comparison of trends between protected and unprotected sites, and/or within the same area across the pre- and post-designation phases (Acreman et al., 2019; Garg et al., 2022). Taxonomic richness and species assemblage composition serve as excellent proxies for ecosystem health as they reflect the collective condition of water quality and landscape connectivity (Moberg et al., 2024). In this context, environmental DNA (eDNA) metabarcoding offers a sensitive, non-invasive alternative to traditional biomonitoring, enabling a comprehensive characterization of community composition and taxonomic richness, including rare and elusive species (Taberlet et al., 2018; Chen et al., 2025). When applied to lotic ecosystems, eDNA effectively integrates information from the entire watershed, as waterways transport organic matter and connect diverse habitats (Yang et al., 2021). Therefore, eDNA sampling in freshwater bodies allows for the simultaneous monitoring of both aquatic and terrestrial diversity associated with the watershed (Deiner et al., 2016; Yang et al., 2021; Reji Chacko et al., 2023).

Building on these premises, the main objective of this study was to assess the effectiveness of PAs in conserving both freshwater and terrestrial biodiversity through an integrated monitoring approach. For this, we combined a multi-marker eDNA metabarcoding approach to simultaneously record vertebrate and invertebrate species across terrestrial and aquatic biomes with the analysis of physicochemical and geomorphological parameters of freshwater systems both within and outside protected boundaries. The study was conducted across twelve streams located within the same hydro-ecoregion (sensu Wasson et al., 2006) of the Northern Apennines (Tuscany, Italy). This experimental design allowed the comparison between protected and unprotected sites accounting for the possible confounding effects of different geo-climatic factors in shaping community composition (see Ricci et al., 2023). The Northern Apennines represent a vital transition zone between Alpine and Central Apennine biogeographic regions, and are characterized by high aquatic and terrestrial endemism, including species of conservation-priority, such as the Italian stream frog (*Rana italica*), the bullhead (*Cottus gobio*), the Mediterranean barbel (*Barbus meridionalis*), the European eel (*Anguilla anguilla*), the fire salamander (*Salamandra salamandra*), the white-clawed crayfish (*Austropotamobius italicus*), the European stag beetle (*Lucanus cervus*), the European pine marten (*Martes martes*), and the European badger (*Meles meles*). In this framework, we specifically aimed to assess the faunal diversity of N2K-protected sites against neighboring unprotected sites, evaluating the impact of protection on species richness, taxonomic and phylogenetic diversity, and overall community composition. We also aimed to verify whether protected areas showed better environmental conditions with respect to unprotected ones and, ultimately, to assess whether these sites hosted higher numbers of threatened species. We hypothesized that the protection status would significantly enhance ecological status, resulting in better chemical and geomorphological features. Consequently, we expected to find a significantly greater level of both freshwater and terrestrial biodiversity in protected sites, which would also be characterized by a higher species richness, a more complex community structures and a greater frequency of conservation-priority taxa compared to the surrounding unprotected landscape.

2. Materials and methods

2.1. Study area and sampling

The study area is located in the hydro-ecoregion of the Northern Apennines (Tuscany, Central Italy) (sensu Wasson et al., 2006, and in accordance with the Italian Ministerial Decree n. 131/2008), a foothill and mountainous zone characterized by both calcareous and siliceous sites and dominated by wooded areas (Fig. 1). This area is home to a high number of aquatic and terrestrial species, including regional endemism and conservation-priority species.

Within this area we selected 12 watercourses belonging to the Arno, Reno and Serchio basins, of which six protected and six unprotected (Fig. 1). These streams resembled each other in terms of size and hydrological regime and were placed in a piedmont belt between 147 and 733 m a.s.l. (Fig. 1 and Table 1). The selected N2K sites were Special Protection Areas (SPA) under the Habitats Directive (Tre Limentre-Reno, La Calvana, Monte Morello and Appennino Pratese) and a national park (Foreste Casentinesi National Park) (Fig. 1 and Table 1). At each watercourse, we delimited a 150-m-long sampling transect for which we recorded geographic coordinates and altitude using a Portable GPS eTrex 22× (Garmin Ltd.) (Table 1). At each transect we collected data on physico-chemical and geomorphological parameters as well as eDNA water samples, as detailed above.

Sampling transect were selected on the basis of previous studies showing that eDNA transport in lotic systems ranges from tens of metres in natural streams (Pilliod et al., 2013; Jane et al., 2015; Wilcox et al., 2016) up to 12 km in channelized rivers (Deiner and Altermatt, 2014; Civade et al., 2016; Wacker et al., 2019). Although our study focused on natural small streams, sampling transects representative of protected areas were still conservatively selected at least 12 km downstream from the upstream boundaries of their respective N2K sites (Fig. 1 and Table 1). Conversely, a stream was classified as unprotected, and sampled accordingly, only when both its upper section and drainage tributaries were located outside of N2K boundaries for at least 12 km.

Physico-chemical parameters were measured twice in summer (July–August 2022) and twice in winter (February–early March 2023) to account for seasonal variations. Geomorphological parameters and canopy cover were only evaluated during the first summer campaign (July 2022). eDNA water samples were collected once in summer and once in winter, in conjunction with the sampling of abiotic parameters. To prevent exogenous DNA contamination, eDNA sampling was strictly performed prior to any abiotic data collection.

2.2. Geo-morphological and physico-chemical parameters measurement

At each river transect, physico-chemical and geomorphological parameters of the streams were measured at three different spatial replicates, distant at least 25 m from each other and randomly selected. Each site was characterized in terms of substrate and riverbank morphology and canopy cover in July 2022 -the peak of the vegetative season in the area- following widely accepted standards in ecology and geology (Barbaresi et al., 2007). Furthermore, to minimize subjectivity and ensure consistency, data collection was always performed by two expert operators.

The extent of canopy cover was assessed using a digital camera (Olympus Tough TG-3). Three photographs were taken at each site by placing the camera on a tripod in the middle of the stream and directing it toward the sky. The percentage of canopy cover was estimated for each picture using the image processing software CanopyCapture (Nikhil, 2018). The percentage cover of substrate types was visually estimated using a 80 × 80 cm grid divided into 16 squares of 20 × 20 cm. Substrate types were classified according to Wentworth Scale (Wentworth, 1922): silt (<0.063 mm), sand (<2 mm), pebbles (2–64 mm), cobbles (65–256 mm) and bedrock (fixed rock formations). At each spatial replicate, the riverbank morphology was also characterized within a 1-m-long segment along the banks calculating the percentage of bank length covered by roots, boulders and cobbles.

For the physico-chemical characterization of the watercourses, 16 abiotic variables were measured on four occasions (two in summer and two in winter) along the year at the three spatial replicates at each sampling occasion. Water conductivity (μScm^{-1}), pH, Temperature (°C), Oxidation-Reduction Potential (ORP, mV), Total Dissolved Solids (TDS,

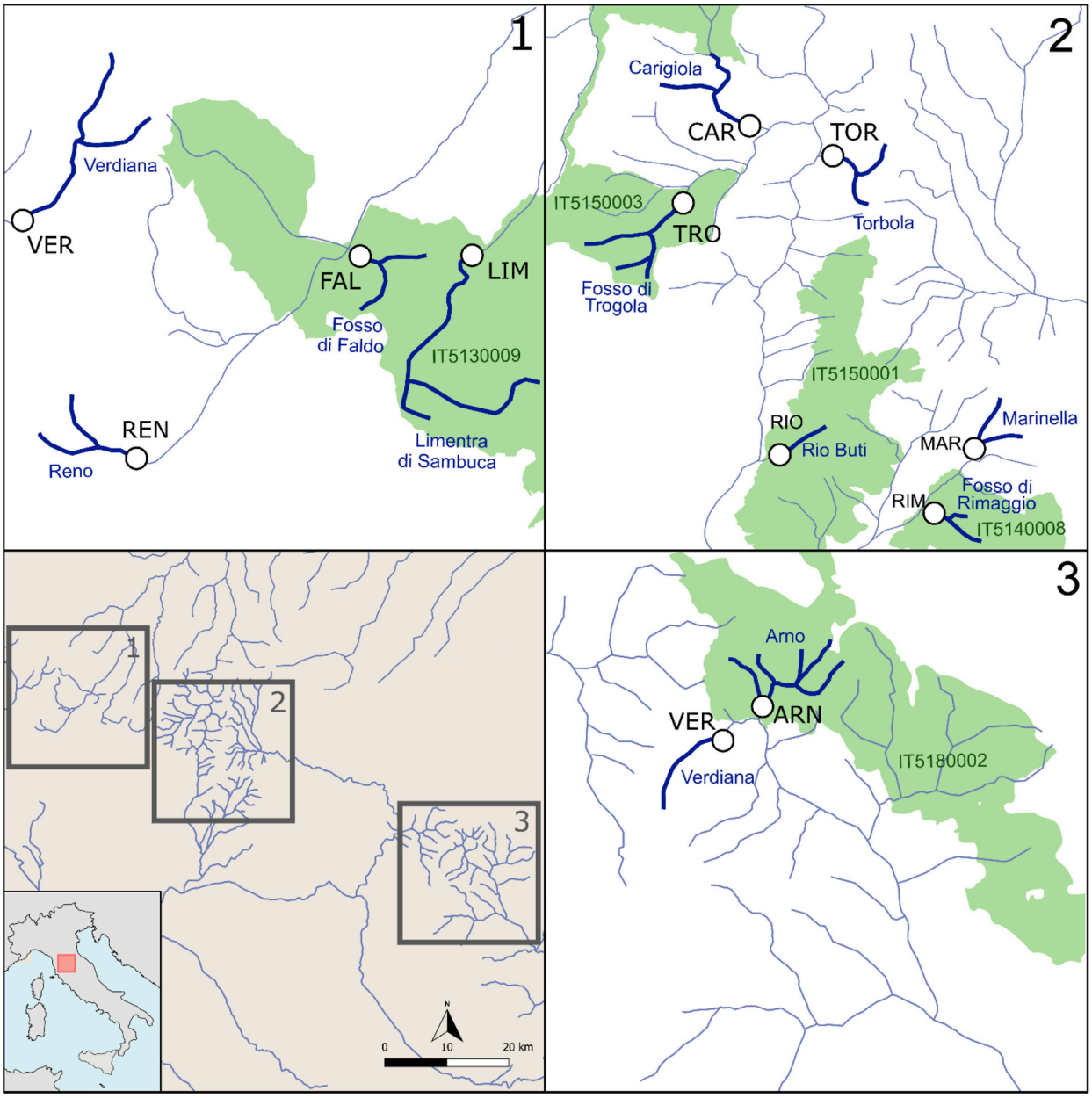

Fig. 1. Map of the study area. Dark blue lines represent watercourses sections covered by our sampling. White dots indicate the sampling points at each watercourse. Green shapes indicate the reference Natura 2000 areas (for details see Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mgL^{-1}), and dissolved oxygen (O_2, mgL^{-1}) were recorded using a multimeter (REVio Portable Multimeter, XS Instruments). The water depth of each stream at each spatial replicate was measured using a graduated rod. At each sampling event, a water sample was also collected from each spatial replicate using a clean plastic jar (~1 L) and transported to the laboratory. These water samples were analysed within 48 h using a Multiparameter Photometer HI83399 (Hanna instruments) to determine the concentrations for nitrate (NO_3^-, mgL^{-1}), phosphate (PO_4^{3-}, mgL^{-1}), phosphorus (P4, mgL^{-1}), sulfate (SO_4^{2-}, mgL^{-1}), calcium (Ca^{2+}, mgL^{-1}), ammonium (NH_4^+, mgL^{-1}), ammonia (NH_3, mgL^{-1}), nitrite (NO_2^-, μgL^{-1}) and carbon dioxide (CO_2, mgL^{-1}). Additionally, for each sampling site, we recorded maximum and minimum water temperatures (°C) using a maximum–minimum thermometer placed in the water for fifteen days during both summer and winter, in single spatial replicate.

2.3. Riverine environmental DNA sampling

Riverine eDNA samples were collected twice a year from each watercourse, once in summer and once in winter. Samplings were performed following procedures described in Ballini et al. (2024, 2025). To account for habitat heterogeneity within each site, we collected six randomly chosen spatial replicates per transect, distributed across both banks and the middle reach. Each sample consisted of one litre of water collected in a plastic jar previously sterilised with sodium hypochlorite. Water samples were filtered on-site using a portable hand vacuum pump connected to a polypropylene flask (Thermo Fisher Scientific Inc.). Sterile disposable filter units and nitrocellulose membranes with a pore size of 0.2 µm (ThermoFisher Scientific Inc.) were used. All filters were preserved individually in absolute ethanol immediately after collection, transported to the laboratories of the Department of Biology of the University of Florence and stored at −25 °C until DNA extraction.

Table 1
Protected (P) and unprotected (NP) streams sampled in this study. For each stream, the site abbreviations, the site name and the geographical coordinates and altitude of the sampling points (calculated as the mean value of the different sampling spatial replicates) are reported. For each P site, code number and name of the reference Natura2000 (N2K) area is also indicated.

	Site ID	Name	Coordinates (DD) and altitude	N2K site
Protected (P)	FAL	Fosso di Faldo	44.061421°, 10.918697° 631 amsl	IT5130009 Tre Limentre – Reno (Reno side)
	LIM	Limentra di Sambuca	44.058504°, 10.961709° 663 amsl	IT5130009 Tre Limentre – Reno (Limentre side)
	TRO	Fosso di Trogola	44.018198°, 11.093444° 386 amsl	IT5150003 Appennino Pratese
	RIO	Rio Buti	43.914844°, 11.134625° 147 amsl	IT5150001 La Calvana
	RIM	Fosso di Rimaggio	43.8907115°, 11.2109188° 243 amsl	IT5140008 Monte Morello
	ARN	Arno	43.841995°, 11.653205° 667 amsl	IT5180002 Foreste Alto Bacino dell'Arno
Unprotected (NP)	VER	Verdiana	44.065421°, 10.768486° 566 amsl	/
	REN	Reno	44.005877°, 10.837686° 733 amsl	/
	CAR	Carigiola	44.040925°, 11.131944° 273 amsl	/
	TOR	Torbola	44.031525°, 11.158651° 245 amsl	/
	MAR	Marinella	43.920042°, 11.227893° 217 amsl	/
	GRA	Gravina	43.838296°, 11.639240° 694 amsl	/

During both sampling campaigns, at each site, a jar containing one litre of DNA-free deionised sterile water was left open for two minutes. The water from such jars was then filtered on-site and used as field negative control.

2.4. DNA extraction, library preparation and sequencing

Environmental DNA purification, Polymerase Chain Reaction (PCR) amplification and library preparation were performed in a laminar flow cabinet using sterile equipment to avoid exogenous DNA contamination. Strict protocols for sterilisation and contamination control were used for all molecular work.

Environmental DNA was extracted from the membrane filters using the DNeasy Blood and Tissue Kit (Qiagen Inc.), following the manufacturer's instructions with minor modifications. Briefly, the filters and any associated precipitate were air-dried under a laminar flow cabinet for several minutes to ensure the removal of residual ethanol. The membranes and precipitates were then folded and placed directly into lysis tubes containing 600 µL of a custom lysis buffer (0.1 M Tris, 0.2 M NaCl, 5 mM EDTA, 0.4% SDS) and 20 µL of Proteinase K (20 mg/mL). Samples were incubated overnight at 56 °C for complete lysis. The resulting lysate was transferred to a new tube and then processed according to the standard DNeasy protocol, while the filter and residual solids were discarded. Finally, DNA was eluted in a volume of 40 µL of sterile water and stored at −25 °C. For eDNA amplification, we used four complementary primer sets, namely Vert01, Tele02, Inse01 and fwhF2/EPTDr2n (Leese et al., 2021; Riaz et al., 2011; Taberlet et al., 2018). All primers were synthesized using the original, unmodified sequences from the reference literature, appended with an Illumina adapter overhangs (Table 2). Vert01 and Tele02 target two different fragments of the mitochondrial DNA (mtDNA) 12S rRNA. Vert01 (~97 bp) was selected as a large-scale marker for general vertebrate detection. In contrast, Tele02 (~167 bp) was specifically chosen for its superior taxonomic resolution for fish species. The fwhF2/EPTDr2n primers (~142 bp of the mtDNA cytochrome C oxidase subunit I gene, COxI) were specifically designed for freshwater macroinvertebrates, allowing for high-quality resolution down to the species level. This was complemented by Inse01 (~155 bp of the 16S rRNA gene), which was included to expand the taxonomic range, enabling the detection of not strictly aquatic invertebrates.

Amplicon libraries were prepared using the two step PCR untagged methods for Illumina platforms with unique dual indexes (Bohmann et al., 2022; Bourlat et al., 2016), as in Ballini et al. (2025). In the first PCR step, barcode markers were amplified in a total volume of 25 µL using 1× Invitrogen™ Taq DNA Polymerase PCR Buffer, 1 U of Taq DNA Polymerase (Thermo Fisher Scientific Inc.), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 µM of each primer appended with an Illumina adapter overhang (Table 2) and 1 µL of template DNA. The following amplification conditions were used: 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 54 °C (Tele02) or 52 °C (Inse01) or 49 °C (Vert01) or 45 °C (fwhF2/EPTDr2n) for 30 s, 72 °C for 1 min, then 10 min at 72 °C. PCR products were resolved on a 1.7% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotum). Each eDNA sample (for a total of 72 samples, 6 samples per site × 12 sites) was amplified in three technical replicates, to account for analytical stochasticity. Negative field controls, an amplification negative control (containing ddH_2O DNA-free instead of template DNA) and an amplification positive control were included in the same PCR run in three replicates as well. The positive control was a mock community prepared from the mixture of DNA extracted from fresh tissue samples of non-European vertebrates and invertebrates (Komodo dragon and mangrove brachyuran crabs). Replicate PCR products were pooled for each sample and purified using KAPA™ Pure Beads (Kapa Biosystems Inc.), according to the manufacturer's sequencing library preparation protocol, with an elution volume of 40 µL. A second electrophoresis on a 1.7% agarose gel was run to confirm the retention of the target amplicons after purification.

A second PCR step was performed using 5 µL of purified amplicons, 1× KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc.), 10 µL of Illumina UD Indexes (Integrated DNA Technologies Inc.) and molecular biology grade water to reach a total volume of 50 µL. The following amplification conditions were used: 3 min at 95 °C, 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, then 5 min at 72 °C. A second purification was performed using KAPA™ Pure Beads (Kapa Biosystems Inc.). Purified products were resolved on a 1.7% agarose gel.

Then, the indexed libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) and pooled in equimolar concentrations in

Table 2
Primer pairs used in this study including Illumina overhang adapters. Locus-specific primers are highlighted in bold.

Vert01 (Riaz et al., 2011)	
Forward	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACA**TTAGATACCCCACTATGC** 3'
Reverse	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TAGAACAGGCTCCTCTAG** 3'
Tele02 (Taberlet et al., 2018)	
Forward	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AAACTCGTGCCAGCCACC** 3'
Reverse	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGGTATCTAATCCCAGTTTG** 3'
Inse01 (Taberlet et al., 2018)	
Forward	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**RGACGAGAAGACCCTATARA** 3'
Reverse	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACGCTGTTATCCCTAARGTA** 3'
COxI: fwhF2-EPTDr2n (Leese et al., 2021)	
Forward	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGDACWGGWTGAACWGTWTAYCCHCC** 3'
Reverse	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CAAACAAATARDGGTATTCGDTY** 3'

four different pools, one for each genetic marker. Finally, each pool was sequenced individually on an Illumina MiSeq System platform using Miseq™ Reagent Kit v2 (300 PE +10 + 10 cycles). The MiSeq runs were performed with a 25% PhiX v3 spike in.

2.5. Bioinformatic analyses and taxonomic assignment

We created four distinct reference databases, one for each amplified marker, by downloading the sequences available in public repositories of the 12S rRNA for vertebrate species (used to create Vert01 and Tele02 reference databases) and 16S rRNA and COxI sequences for invertebrate species (used to create Inse01 and fwhF2/EPTDr2n reference databases). As public repositories we selected GenBank (Benson et al., 2013) and BOLD (Ratnasingham and Hebert, 2007) and the sequences were downloaded using CRABS v0.1.8 (Jeunen et al., 2023).

In silico PCRs were conducted for each marker, allowing for the following mismatches in the primer-binding region: two for Vert01, Tele02 and fwhF2/EPTDr2n libraries and zero for Inse01. We then performed a Pairwise Global Alignment (PGA) to identify and extract sequences deposited without primer binding regions (settings: –speed medium –percid 0.90 –coverage 0.90 –filter_method relaxed). The remaining amplicon sequences were curated with an in-house generated script to remove doubtful records and simplify records (e.g. removing records containing aff./cf./sp. in the species name and formatting hybrids records). The databases were then dereplicated to contain unique sequences per species and environmental sequences were removed (−-enviro yes). Further filtering was applied based on length (−-minlen 30 –maxlen 150 for Vert01; −-minlen 100 –maxlen 230 for Tele02; −-minlen 30 –maxlen 162 for fwhF2/EPTDr2n; −-minlen 55 and –maxlen 285 for Inse01) and number of missing taxonomic information (−-nans 2). Vert01, Tele02, Inse01 and fwhF2/EPTDr2n final reference databases contained 41,294, 27,768, 31,255 and 254,058 sequences, respectively. The databases were finally converted into an idt-fasta format.

Raw sequence reads were demultiplexed using bcl2fastq v2.20 (Illumina) and quality-checked with FastQC. Sequences were then processed using the Barque pipeline v1.8.5 (https://github.com/enormandeau/barque). Before launching the pipeline, the previously generated reference databases were transformed into a Barque-friendly format (fasta header: >Family_Genus_Species). Two runs were then performed for each marker dataset. The first run was used to refine the database and generate denoised OTUs, along with their taxonomic assignment. In the second one, the OTUs and their taxonomic assignment were used as databases themselves to find read counts per sample.

In the first run, reads with lengths outside the expected range were removed (min_hit_length: 36 and crop_length: 152 for Vert01; min_hit_length: 109 and crop_length: 229 for Tele02; min_hit_length: 55 and crop_length: 285 for Inse01 and min_hit_length: 30 and crop_length: 162 for fwhF2/EPTDr2n). Primers were removed allowing for four mismatches (20% of the primer length). The retained reads were compared against the custom reference databases with a query coverage of 0.9 for all markers. OTUs were assigned at species level when top matches had a percent identity above 98% (Tele02 marker) or 97% (Vert01, Inse01 and fwhF2/EPTDr2n markers); at genus level when matches were between 98% and 95% (Tele02 marker), 97% and 95% (Vert01 marker) or 97% and 90% (Inse01 and fwhF2/EPTDr2n markers); and at family level if top matches had a percent identity lower than 95% (Vert01 and Tele02 markers) or 90% (Inse01 and fwhF2/EPTDr2n markers).

To minimize false positives and filter out background noise, a conservative threshold of 10 reads was applied at multiple stages of the pipeline (barque_config file: min_hits_sample: 10; min_hits_experiment: 10; min_size_for_otus: 10). To enhance accuracy in the taxonomic assignment and minimize erroneous sequence assignation, at the end of the first run the reference database was refined excluding species never reported for the European countries. The pipeline was subsequently re-run with the same settings and the depleted database. For the second run, the resulted OTU databases were used as reference databases for each marker following the pipeline's two-step workflow. The resulting OTUs were refined with the LULU R-package v0.1.0 with default parameter except for the min_seq_similarity which was raised to 87 for Vert01 and Tele02 data analysis (Frøslev et al., 2017). In the resulting curated datasets, OTUs assigned to the same taxon were merged, and multi-hits were resolved.

Decontamination was then performed using microDecon R-package v1.0.2 (McKnight et al., 2019) with default options. The replicates from the same site were then summed to obtain a single site-specific taxonomic list. Non-target species (humans and livestock) and possible contaminations were discarded. A threshold of >10 reads was set to declare a species as present in the watercourse.

To generate the final list of taxa, we integrated the results from taxonomically overlapping markers (Tele02 and Vert01 for vertebrate taxa, and Inse01 and fwhF2/EPTDr2n for invertebrates). This multi-marker approach is essential to improve taxonomic resolution, bridge gaps in specific groups, and minimize misassignment errors (Deagle et al., 2014; Furlan et al., 2020; Watts et al., 2026). This step involved expert ecological validation against official biodiversity databases (AIIAD, 2021; Audisio et al., 2014; Balletto et al., 2015; LifeWatch Italy, 2018; Regulation 1143, 2014; Riservato et al., 2014; Rondinini et al., 2022) to resolve ambiguities caused by incomplete reference libraries or limited discriminatory power of specific amplicons as discussed in Watts et al. (2026). Following established protocols (Aglieri et al., 2021; Juhel et al., 2022; Lynggaard et al., 2024), we applied two corrective criteria: (1) if an OTU was assigned to a species not native to the region or not ecologically expected within the specific study area due to the absence of congeneric local species in the reference database, the assignment was conservatively corrected to the most likely local species

or collapsed to the genus level; and (2) if one assignment achieved species-level identification while a second from the same or the overlapping marker reached only genus-level, the assignments were aggregated at the species level.

2.6. Statistical analyses

Univariate analyses were performed with Past v. 4.05 (Hammer et al., 2001) after having checked for data normality and homoscedasticity. Multivariate analyses were performed using PRIMER v. 7 (Clarke et al., 2014) and the PERMANOVA for PRIMER routines (Anderson et al., 2008).

2.6.1. Geo-morphological and physico-chemical data analysis

Environmental parameters were split in two distinct datasets due to differences in temporal replicates. The first dataset included the geomorphological variables and canopy cover data (nine variables, collected only during the summer) for a total of 36 measurements per variable (12 sites × 3 spatial replicates). The second dataset included the physico-chemical parameters and the water depth values (16 variables, collected twice in summer and twice in winter) for a total of 144 measures per variable (12 sites × 3 spatial replicates × 4 temporal replicates). For each dataset, we tested for multicollinearity by calculating the Pearson correlation coefficients between all pairs of variables. Given a threshold of $r > 0.90$, no variables were excluded since no pairwise correlations exceeded this value.

Principal Component Analysis was utilized as an unconstrained ordination method to visualize distribution patterns of the geomorphological variables and canopy cover data among protected and unprotected sites (Clarke et al., 2014). A two-way multivariate PERMANOVA was then applied to test for the occurrence of differences among protected and unprotected sites with protection status (PrS, fixed and orthogonal) and sampling site (Si, random and nested in protection status) as factors. The analysis was based on 9999 permutations of residuals within a reduced model (Anderson and Braak, 2003) and Type III sums of squares (Anderson et al., 2008). Canonical analysis of principal coordinates (CAP) (Anderson and Robinson, 2003; Anderson and Willis, 2003) was used as a constrained ordination method to visualize patterns of variation in the variables among protected and unprotected sites. PERMANOVA and CAP were computed using similarity matrices based on Euclidean distances on arcsine-transformed data, as suggested for percentage values (Underwood, 1997).

Considering the dataset of physical-chemical parameters and water depth values, a four-factor PERMANOVA (9999 permutations, Type III sum of squares) was performed to evaluate differences in variables collected at protected and unprotected sites during the summer and the winter, with Season (Se, fixed and orthogonal), Temporal Replicates (Tr, random and nested in Season), Protection Status (PrS, fixed and orthogonal) and Sampling Site (Si, random and nested in protection status) as factors. PCA and CAP were used to visualize patterns of environmental variations between protected and unprotected sites across both summer and winter seasons. PERMANOVA and CAP were computed using Euclidean similarity matrices of transformed data. All parameters were transformed by a $\log_{10}(x + 1)$ function, except pH values transformed by the arcsine$\sqrt{(x/p)}$ function.

2.6.2. Invertebrate and vertebrate community assessment

Vertebrate and invertebrate OTUs generated through the bioinformatic pipeline were merged into a single presence–absence matrix of recorded taxa for each site, integrating data from both winter and summer surveys. For statistical analysis, the total list of taxa (TOT) was split into two distinct datasets for each site: freshwater-dependent taxa (FW) and terrestrial taxa (T). Freshwater-dependent taxa are defined as organisms with an obligate ecological link to the lotic ecosystem, being strictly associated with streams for their entire life or for critical life stages, such as larval development or reproduction (e.g., fish, amphibians, freshwater macroinvertebrates, aquatic birds, and aquatic mammals). Consequently, their presence in the eDNA samples directly reflects the resident community inhabiting the streams. Conversely, terrestrial taxa encompass species belonging to the adjacent land-based communities. Their genetic signal is externally sourced, originating from the broader landscape rather than the stream itself. The assignment of taxa to these groups was based on the public database freshwaterecology.info (Schmidt-Kloiber and Hering, 2015) and expert judgement. This categorization allowed us to evaluate whether the conservation effect of PAs differed between organisms directly associated with the streams and those belonging to the surrounding catchments whose DNA is transported into the riverine water.

To provide a comprehensive evaluation of PA effectiveness at each site, we calculated a suite of diversity indicators from the three datasets (TOT, FW, T) using the DIVERSE function in PRIMER v.7. We selected indices covering both traditional species richness metrics and community taxonomic and phylogenetic structures to capture complementary dimensions of biodiversity, providing a multi-faceted assessment of how protection status influenced community assembly. Specifically, the following indicators were calculated: Total number of species (S), Shannon index (H′), Simpson index (λ), Gini-Simpson index (1-λ), Average Taxonomic distinctness (Δ+), Total Taxonomic distinctness (sΔ+), Variation in Taxonomic distinctness (Λ+), Average phylogenetic diversity (Φ+) and Total phylogenetic diversity (sΦ+). To assess the statistical significance of differences between protected and unprotected sites, we applied Kruskal-Wallis non-parametric tests, as this approach is robust for ecological datasets that do not meet normality assumptions. Boxplots of these indicators were drawn using the PLOTS function in PRIMER v. 7.

To assess whether the taxonomic complexity of the observed communities significantly deviated from the expected taxonomic structure (Clarke et al., 2014), the values of Average Taxonomic Distinctness were plotted in a funnel plot of confidence (95%) using the TAXDTEST function in PRIMER v. 7. This analysis was run for the three datasets.

For the FW, T and TOT datasets, Bray-Curtis dissimilarity distances were computed on $\log_{10}(x + 1)$ transformed data and analysed using one-way PERMANOVA tests to evaluate differences in community composition among protected and unprotected sites (factor protection status, PrS, fixed and orthogonal). The PERMANOVAs were based on 9999 permutations within a reduced model and Type III sums of squares. Patterns of community differentiation were visualized using non-metric multi-dimensional scaling (nMDS), applied as an unconstrained ordination method (Clarke, 1993; Field et al., 1982).

Finally, from the TOT dataset, we extracted the list of species of conservation interest mentioned in the Repertorio della Fauna Italiana Protetta (MASE, 2024), drawn up in accordance with the main international conservation conventions, EU directives, and IUCN criteria. This dataset was used as a direct indicator of conservation performance, testing the hypothesis that protected areas were home to a greater number of species of conservation interest compared with unprotected sites. The PERMANOVA test and the Similarity Percentage Analyses (SIMPER: Clarke et al., 2014) were run using the Bray-Curtis dissimilarity matrix to evaluate differences in species composition among protected and unprotected sites and evaluate the contribution of individual species of conservation interest to the dissimilarities between protected and unprotected sites. All biodiversity indices were then recalculated for each site based on the list of species of conservation interest using the DIVERSE function in PRIMER v. 7.

3. Results

3.1. Geo-morphological and physico-chemical evaluation

The values of the geo-morphological parameters measured at each stream are reported in Table S1. The two-way PERMANOVA revealed a significant effect of the factor sampling site whereas no significant effect

Table 3
a) Two-way PERMANOVA conducted on nine geo-morphological variables collected in three spatial replicates at each protected and unprotected site. b) Four-way PERMANOVA conducted on 16 physico-chemical parameters and water depth values recorded during summer and winter in three spatial and four temporal replicates at each protected and unprotected site. The following abbreviations are used: PrS, Protection status; Si, Sampling site; Tr, Temporal replicate; Se, Season; df, degree of freedom; SS, sum of squares; MS, mean squares; P, probability values calculated through 9999 permutations. Significant *P* values are in bold.

Source	df	SS	MS	Pseudo-F	P
a.					
PrS	1	2.072	2.072	1.136	0.361
Si(PrS)	10	18.240	1.824	1.879	**0.007**
Residual	24	23.294	0.971		
Total	35	43.607			
b.					
Se	1	68.066	68.066	2.429	**0.037**
PrS	1	20.025	20.025	1.075	0.402
Tr (Se)	2	48.526	24.263	4.773	**0.0012**
Si (PrS)	10	190.760	19.076	3.753	**0.0001**
Se × PrS	1	3.476	3.476	0.845	0.591
Se × Si (PrS)	10	58.498	5.850	1.1508	0.335
Tr (Se) × PrS	2	8.561	4.280	0.8420	0.506
Tr (Se) × Si (PrS)	20	101.670	5.083	3.522	**0.0001**
Residual	96	138.540	1.443		
Total	143	638.120			

was found for protection status (Table 3a). The first two axes of the PCA explained about 60% of the total variance; the resulting plot shows that sites did not cluster according to their protection status (Fig. 2a). Similarly, the CAP analysis confirmed that samples grouped by site rather than by protection status (Fig. 2b).

The values of the physico-chemical parameters measured at each stream are reported in Table S2. The four-way PERMANOVA revealed a significant effect of the factors sampling site (Si), season (Se) and temporal replicate (Tr), but not of the protection status (PrS) (Table 3b). The first two axes of the PCA were able to explain 90% of variation showing a clear separation of the samples according to the factor season (Fig. 3a). This season-dependent pattern was mainly due to higher values of Ca^{2+} and ORP in the winter samples, and higher values of temperature and nitrates in the summer samples. The CAP further confirmed that a clearcut separation of samples occurred only in relation to the factor season (Fig. 3b).

3.2. Sequencing outputs, taxonomic assignment and community description

Amplicon sequencing yielded a high number of raw reads for all four genetic markers (Table 4). Sequencing outputs, including totals and averages per eDNA sample, field negative control, and PCR negative and positive control are reported in Table 4, alongside the number of sequences retained after Barque analysis and the final OTUs counts. Detailed read counts per site and season for Vert01, Tele02, Inse01, and fwhF2/EPTDr2n are provided in Tables S3–S6. Overall, summer eDNA samples produced higher sequencing yields and, consequently, a greater number of OTUs, revealing a biodiversity peak -mainly in invertebrates – across all sites. For each site, the overall list of taxa was obtained by merging the results of Tele02 and Vert01 for vertebrates and of Inse01 and fwhF2/EPTDr2n for invertebrates, for both seasons. After the manual curation step, we identified a total of 1193 taxa (69 vertebrates and 1124 invertebrates) categorized into 379 freshwater-dependent (FW) and 814 terrestrial (T) taxa (Table S7). Overall, vertebrates spanned five classes and 25 orders; Mammalia was the most prevalent class (26 taxa), followed by birds (19 taxa, dominated by Passeriformes). Fishes, amphibians, and reptiles were represented by 15, 5, and 4 taxa, respectively (Fig. 4 and Table S7). Invertebrates comprised 10 classes and 44 orders, with Insecta (notably Diptera) being the dominant group (Fig. 4, Table S7). Other recorded groups included Clitellata, Arachnida, Gastropoda, and Crustacea (Branchiopoda, Copepoda) (Fig. 4 and Table S7). The most frequent FW taxa included Actinopterygii, Amphibia, Gastropoda, Clitellata, and various aquatic insect orders (Coleoptera, Diptera, Ephemeroptera, Hemiptera, Odonata, Plecoptera, and Trichoptera) (Fig. 4 and Table S7). Terrestrial vertebrate records were dominated by birds, mammals, and reptiles, while terrestrial invertebrates were primarily represented by Insecta (Coleoptera, Hemiptera, Hymenoptera, Orthoptera, Neuroptera, and Lepidoptera) (Fig. 4 and Table S7).

Alpha-diversity indices and community structure indicators (taxonomic and phylogenetic indices) calculated for each site for FW, T and TOT datasets are reported in Table S8. The Kruskall-Wallis test revealed that Average Taxonomic distinctness was the only indicator showing a significant difference between the FW communities populating the protected versus unprotected sites ($p = 0.037$). Boxplots for all indices across datasets are provided in Figs. S1–S3.

The funnel plots generated via TAXDTEST for the FW, T, and TOT datasets are presented in Figs. 5 and S4. When considering the FW matrix, the plot revealed that many unprotected sites exhibited Average Taxonomic Distinctness values below the expected average value. Among the protected sites, only Fosso di Rimaggio (RIM) fell at the lower limit of the 95% confidence interval (Fig. 5a). Using the T matrix, one unprotected site (VER) and two unprotected ones (REN and TOR) had taxonomic distinctness values, respectively, lower and higher than the expected average value, while all the other sites fell within the 95% confidence intervals (Fig. 5b). When we run the analysis using the TOT matrix, the funnel plot showed that only the unprotected site (VER) fell outside the confidence boundaries (Fig. S4).

The community composition did not differ significantly between protected and unprotected sites across any of the analysed datasets. Specifically, PERMANOVA tests performed on the presence/absence matrices for FW, T, and the TOT dataset showed the absence of a protection effect (FW: Pseudo-F = 1.14, $P = 0.317$; T: Pseudo-F = 0.94, $P = 0.423$; TOT: Pseudo-F = 1.07, $P = 0.283$). This lack of differentiation was clearly corroborated by the nMDS plots which showed no distinct clustering based on protection status (Fig. S5 for FW and T; Fig. 6 for TOT).

Regarding species of conservation interest, we detected six insects and 38 vertebrates across all sites, with richness ranging from 6 to 19 species per site (Table S9). Fifteen species were FW taxa, occurring predominantly in protected sites, and 29 were T taxa (Table S10). The Kruskal-Wallis tests revealed no significant differences in diversity or taxonomic indices for this subset (Table S11). The PERMANOVA test based on the presence/absence of species of conservation interest did not reveal any difference between protected and unprotected sites (Pseudo-F = 0.67; df = 1; $P = 0.789$). The SIMPER analysis for TOT species of conservation concern, however, identified an average dissimilarity of 54.65% between protected and unprotected sites and listed eight FW species and 13 T species as the main contributors to this dissimilarity (Table 5).

4. Discussion

In this study we used an integrated approach combining extensive eDNA-based biodiversity monitoring with the assessment of physico-chemical and geomorphological variables to compare the faunal diversity of N2K-designated sites in the Northern Apennines (Tuscany, Italy) with adjacent unprotected sites from the same hydro-ecoregion. Our ultimate goal was to evaluate the effectiveness of N2K sites in preserving key species and habitats, since such designated zones represent the most important coordinated network of protected areas in the EU countries. Through the sampling of riverine eDNA and using a multi-marker metabarcoding approach, we efficiently and simultaneously monitored a very wide range of species (specifically 1193 taxa),

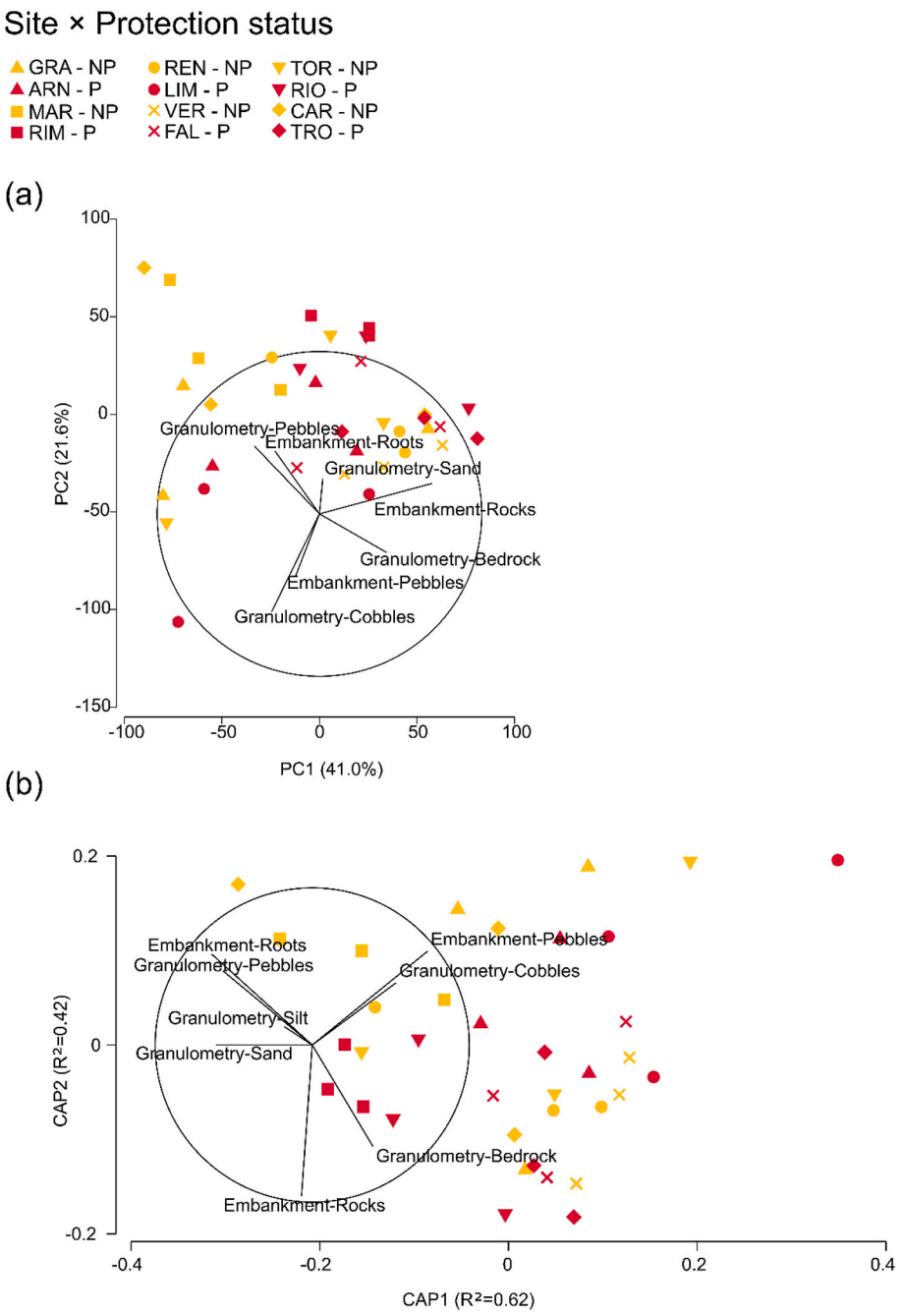

Fig. 2. a) Principal component analysis (PCA) and b) Canonical Analysis of Principal Coordinates (CAP) plots based on nine geo-morphological parameters recorded at protected and unprotected sites. Samples are reported according to the sampling site and their protection status. Vectors of the linear correlations (≥ 0.4) between individual variables are superimposed on the graphs. Site abbreviations as in Table 1.

encompassing both vertebrates and invertebrates and extending beyond obligate aquatic and amphibious taxa. We found that, although protected and unprotected streams exhibited largely comparable physico-chemical and geomorphological characteristics resulting in similar values for α-diversity indexes, the freshwater communities of watercourses placed under the protection of the N2K areas revealed a significantly higher phylogenetic diversity, expressed as Taxonomic Distinctness. Such protected water courses also hosted a larger number of aquatic species of conservation interest within the studied area.

These results suggest that the selected N2K areas play a key role in preserving the diversity of freshwater-dependent communities. This protective effect, however, was less pronounced for selected endangered and vulnerable fish species, supporting the hypothesis that terrestrial PAs could be further optimised for freshwater systems conservation.

Although the number of sampled sites ($n = 12$) represents a potential limitation for broad generalizations, our multi-marker eDNA metabarcoding approach allowed for a comprehensive depth of taxonomic characterization. By providing a detailed, species-level description of local biodiversity -including invertebrate groups- this method offers a level of detail that compensates for the limited number of sites. This approach enabled us to successfully assess the taxonomic diversity of animal communities characterising the areas within and outside N2K protection. Protected and unprotected sites, selected in similar climatic and environmental areas, showed no significant differences in overall species richness or in any other α-diversity indexes. Our analyses, however, found a greater taxonomic diversity, measured as Taxonomic

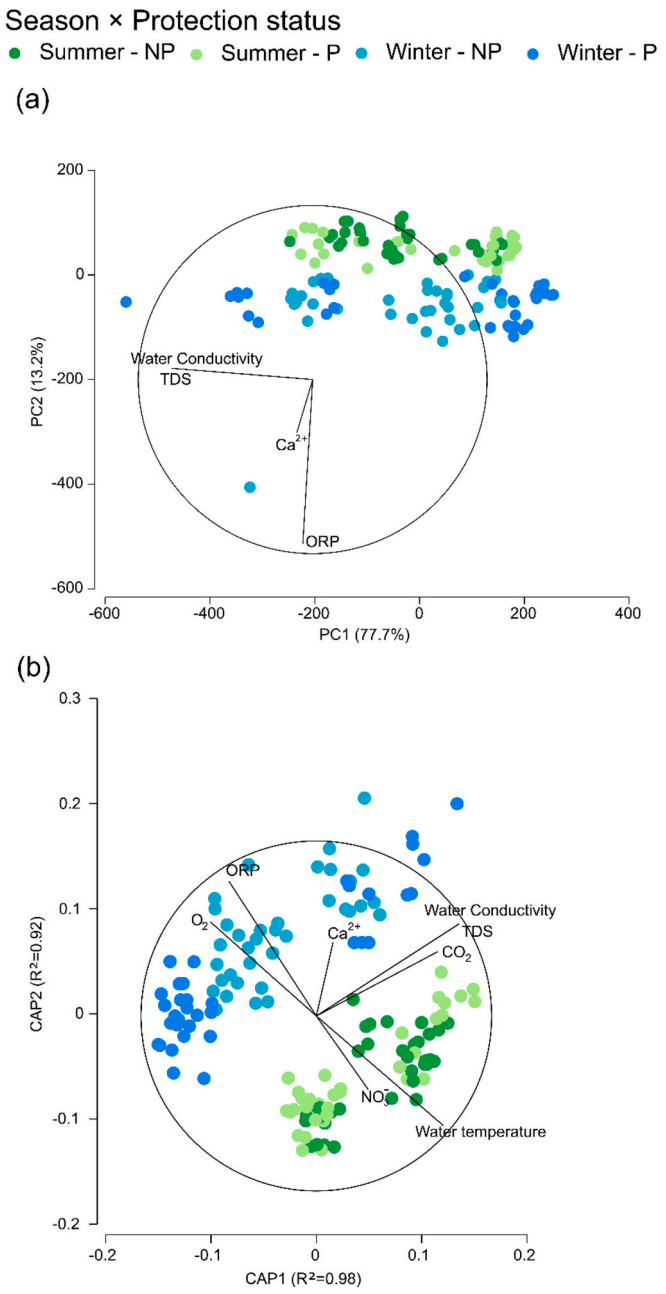

Fig. 3. a) Principal component analysis (PCA) and b) Canonical Analysis of Principal Coordinates (CAP) plots based on 16 physico-chemical parameters recorded at protected and unprotected sites during the winter and summer. Samples are reported according to the protection status and sampling season. Vectors of the linear correlations ($\geq$ 0.4) between individual variables are superimposed on the graphs.

Distinctness ($\Delta+$), of freshwater-dependent species at the protected sites. This indicates that N2K areas hosted a more diverse aquatic fauna at the supraspecific level, reflecting the fact that these communities encompass a broader range of evolutionary lineages compared with those at unprotected sites (Clarke and Warwick, 1999). From an ecological perspective, higher taxonomic distinctness often correlates with increased functional diversity, meaning that communities composed of distantly related taxa are more likely to show diverse functional traits (Clarke and Warwick, 1999; Tucker et al., 2018). As reviewed in Bevilacqua et al. (2021), $\Delta+$ is less influenced by sampling effort and natural variability and, therefore, should be more sensitive to human-driven impacts. The assumption underlying the effectiveness of taxonomic distinctness in impact assessment is that human disturbance may manifest at taxonomic levels higher than species by favouring closely related tolerant taxa, while removing more sensitive, distantly related groups (Marchant, 2007; Bevilacqua et al., 2021). This aligns with our results showing that the only sites characterized by values of taxonomic diversity lower than the expected average were, indeed, unprotected sites, where possible ecological degradation could be in place. Due to the sensitivity shown by our approach to detect subtle differences in community composition and possible cryptic ecological degradation, we advocate for the combined use of eDNA-based monitoring and taxonomy-weighted indices, possibly coupled with functional ones, to enhance sampling and monitoring strategies related to protection and conservation effectiveness, in agreement with other authors (see Liu et al., 2021).

Protected area designation criteria often cite one or more priority species, which receive particular attention under national and European conservation directives (Dudley, 2008). In this study we did not record a significantly higher total number of priority species in protected areas compared to unprotected ones. The richness of freshwater (FW) priority species, however, was generally higher in protected streams, whereas terrestrial priority species were more prevalent in unprotected areas. The differences between protected and unprotected sites in terms of aquatic species were attributable to nine species of conservation concern out of a total of 13 detected. Among these species, there were some critically endangered, endangered, or vulnerable ones, such as the European eel, *Anguilla anguilla*, the Italian barbel, *Barbus plebejus*, the Brook barbel, *Barbus caninus*, and the Arno goby, *Neogobius nigricans*. The higher prevalence of aquatic species of concern in the targeted N2K sites suggests that protection status—even when primarily designed for terrestrial habitats—may effectively confer a direct benefit to freshwater habitats through the mitigation of localized anthropogenic pressures. While the structural and chemical characteristics of the streams appear comparable, N2K designation likely buffers against direct human activities that impact sensitive freshwater taxa without necessarily altering the broader geomorphological and chemical framework. This suggests that the targeted N2K areas may provide critical protection from direct disturbance (e.g., localized harvesting or frequent human presence), which are essential for the persistence of vulnerable aquatic populations.

Assessing the effectiveness of protection plans for biodiversity conservation by comparing communities in protected and unprotected areas is a challenging task (Gaston et al., 2008; Rodrigues and Cazalis, 2020; Ricci et al., 2023). Ideally, the ecological performance of a PA should be assessed by comparing its biodiversity status against a counterfactual scenario—what would have occurred in the absence of protection (Coetzee et al., 2014). However, as such comparisons are rarely feasible, an alternative approach involves assessing biodiversity before and after establishment; yet, such studies can lead to misleading conclusions, and a general scarcity of historical data often precludes this (Coetzee et al., 2014; Wauchope et al., 2022). Consequently, evaluating PA efficiency almost invariably necessitates comparisons with unprotected areas that are similar in all respects except for their designation, an approach that nevertheless presents intrinsic difficulties (Ricci et al., 2023). Geomorphological and hydrological differences, for instance, can strongly influence the richness and composition of target animal communities at local scale and can impair the power of comparative analyses (Ricci et al., 2023). An unbiased, comprehensive evaluation therefore requires the integration of biological, physiochemical and geomorphological data, accounting for the possible confounding effects of different geo-climatic factors, as suggested by Ricci et al. (2023) and implemented in this study. In the present study, geomorphological and physicochemical analyses revealed that environmental characteristics and water quality were comparable across all investigated streams, regardless of protection status. Physicochemical data reflected only the expected seasonal patterns between summer and winter, consistent with the typical discharge fluctuations of temperate habitats (Dettinger and

Table 4
Sequencing output results for each mtDNA marker (Vert01, Tele02, Inse01 and fwhF2/EPTDr2n) used in the metabarcoding assay. The total number of raw reads and average values ± standard error (Av. ± SE) of reads obtained for environmental samples per site (reads per site), field negative control (field NC), PCR negative (PCR NC) and positive (PCR PC) controls are reported. The number of retained sequences, total OTUs, assigned OTUs and retained OTUs after the curation process are also reported.

DNA marker	Raw reads	Av. ± SE Reads per site	Av. ± SE Reads field NC	Av. ± SE Reads PCR NC	Av. ± SE Reads PCR PC	Retained sequences	Total OTUs	Assigned OTUs	OTUs aft curation
Vert01	12,554,016	887,158 ± 90,450	109,615 ± 15,010	20,667 ± 5690	275,702 ± 227,381	10,677,959	229	191	55
Tele02	13,378,338	972,707 ± 46,765	124,906 ± 17,266	48,298 ± 10,582	55,193 ± 115	10,517,893	314	120	48
Inse01	8,880,525	720,176 ± 60,570	12,001 ± 3917	503 ± 356	46,700 ± 2373	7,725,642	4540	1549	563
fwhF2/ EPTDr2n	7,469,947	590,353 ± 58,610	30,187 ± 6141	2074 ± 514	9660 ± 6774	6,517,460	3597	2076	1083

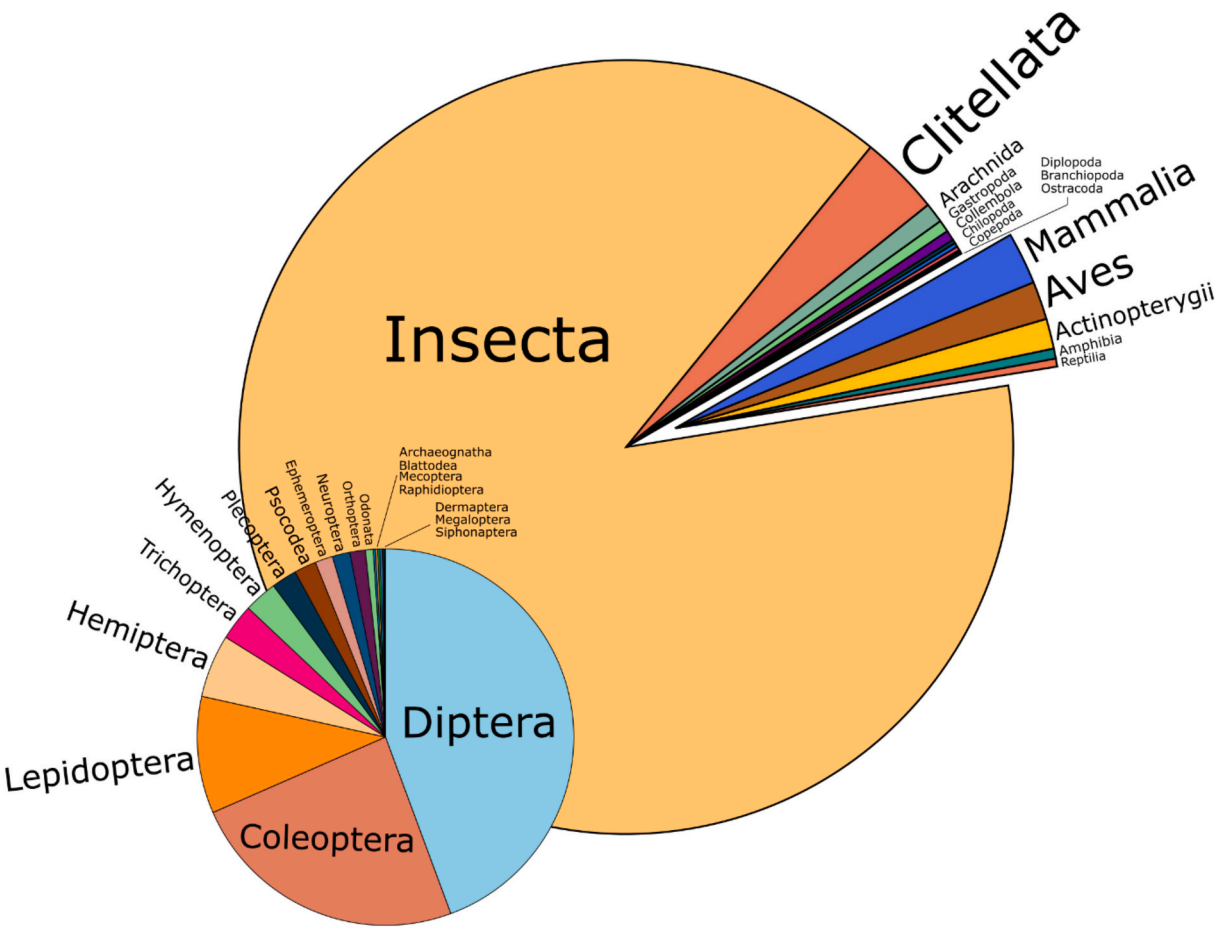

Fig. 4. Taxonomic diversity detected using environmental DNA metabarcoding across protected and unprotected sites. The plot is based on the number of taxa per Class/Order as calculated by the presence matrix of Table S7. Vertebrates are placed in a distinct section. The inset further breaks the Class Insecta (i.e., the most abundant one) into its Orders.

Diaz, 2000). These seasonal shifts are crucial ecological drivers, as changes in flow and temperature naturally regulate habitat availability and the life cycles of aquatic fauna (Hernández-Carrasco et al., 2025). Ultimately, the lack of significant abiotic differences between protected and unprotected sites confirms the robustness of our sampling design. By effectively controlling for environmental variability, we ensure that observed differences in community composition and diversity are attributable to protection status rather than underlying environmental heterogeneity. Overall, our data revealed a high ecological status and comparable α-diversity indices across the monitored protected areas and the unprotected adjacent ones, alongside higher taxonomic diversity and a greater number of freshwater priority species within N2K sites. These findings support the critical role that unprotected adjacent areas play in bolstering the effectiveness of protected areas for freshwater fauna conservation. The high ecological quality of these peripheral areas suggests they may function as functional buffer zones whose establishment is recognized as an effective strategy to enhance the success of conservation schemes by mitigating external anthropogenic pressures and expanding the available habitat for aquatic species (Mancini et al., 2005; Abell et al., 2007; Acreman et al., 2019). Furthermore, such adjacent zones can facilitate connectivity, acting as biological corridors that support metapopulation dynamics and recolonization processes

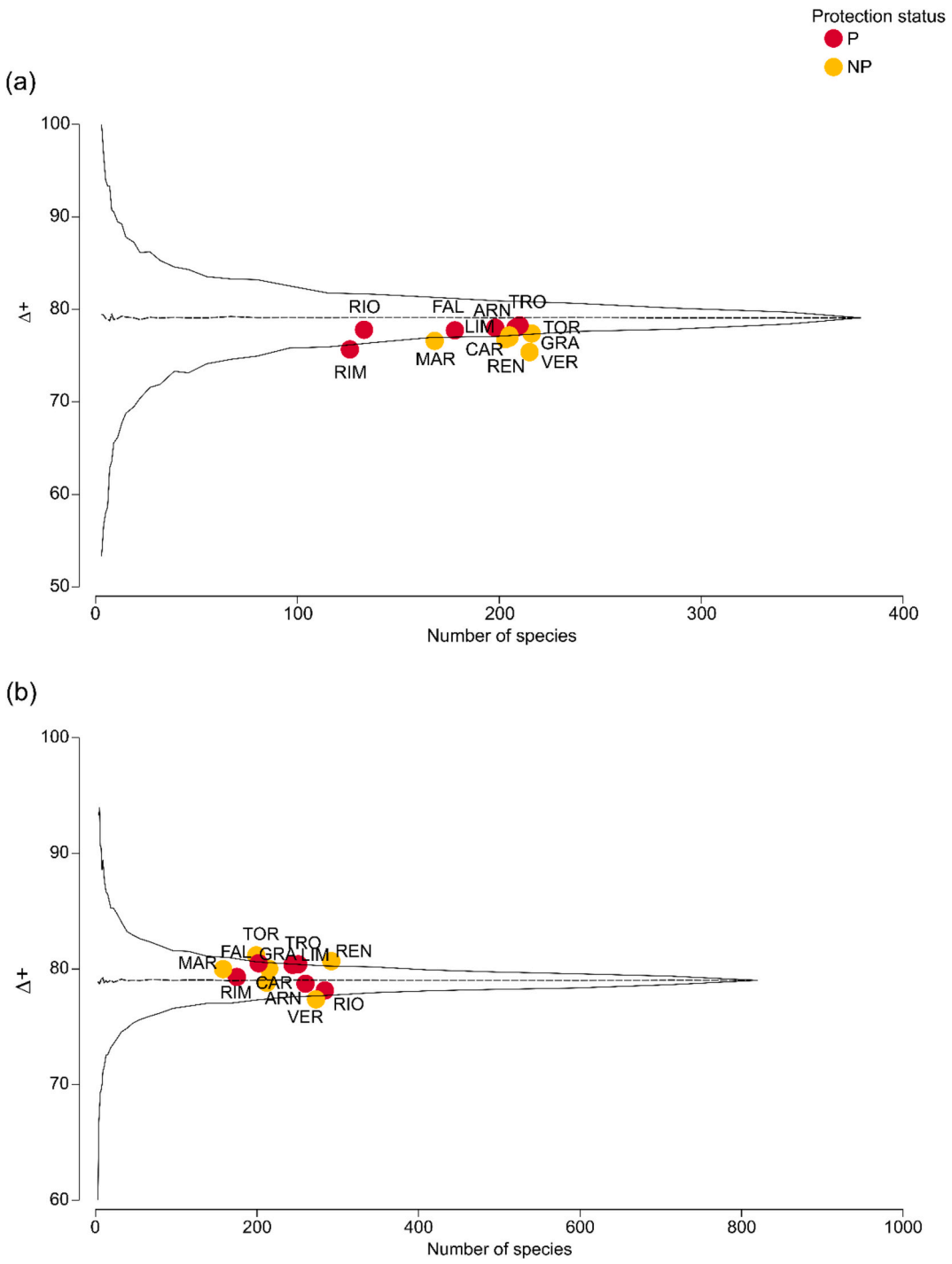

Fig. 5. Confidence funnel plot of Average Taxonomic distinctness (Δ+) of sampling sites, based on the presence matrix of freshwater dependent taxa (a) and terrestrial taxa (b). Red and yellow dots indicate protected (P) and unprotected (NP) sites, respectively. The central line shows the mean Taxonomic distinctness value, while the continuous lines are the distribution of probability at 95%. Site abbreviations as in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Gaston et al., 2008). Crucially, protected areas themselves may act as population sinks for certain species, whose persistence within PA boundaries depends on a steady flow of individuals from the surrounding areas (Hansen and Rotella, 2002). If habitat quality is degraded outside the protected sites, these species may face local extinction even within the PA. An ecologically healthy surrounding area is therefore essential for the effectiveness of a protected site, acting as a critical buffer that ensures the long-term viability of biodiversity and ecological processes within its boundaries. Since protected areas are rarely isolated, they must function as part of larger ecological networks where external environmental health significantly impacts internal conservation outcomes. Our results ultimately suggest that natural areas belonging to the same riverine basin and adjacent to the protected sites should be formally considered in the designation and management criteria of N2K areas to ensure the long-term conservation and resilience of European freshwater habitats.

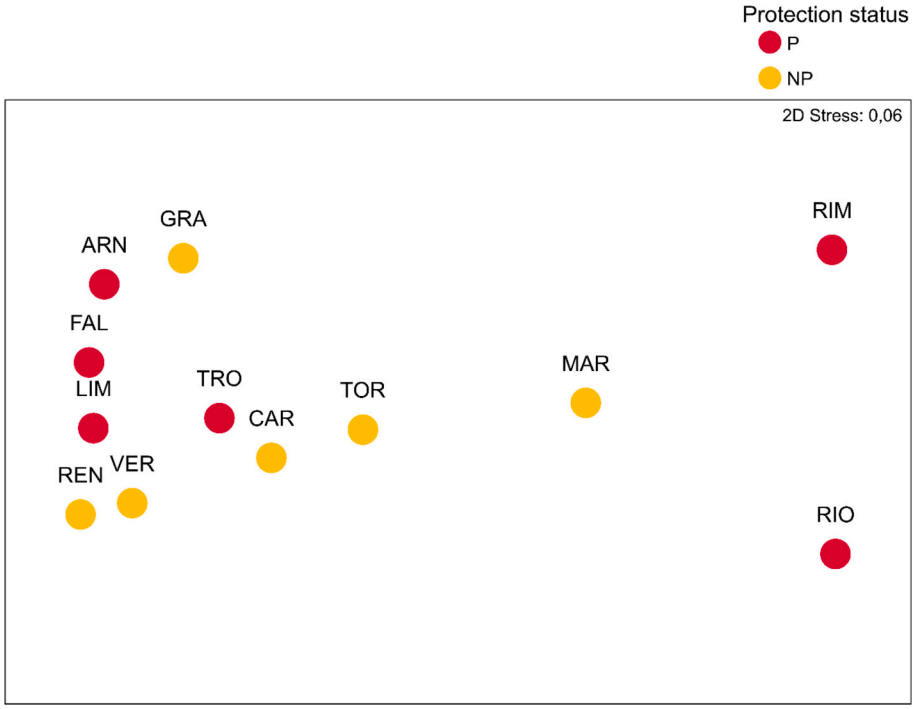

Fig. 6. Non-metric multi-dimensional scaling (nMDS) plots based on the total community composition of protected (red dots) and unprotected (yellow dots) sites. Site abbreviations as in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Results of SIMPER function: list of taxa affecting for more than 70% dissimilarities for the pairwise comparison between Species of conservation interest of Protected (P) and Unprotected (NP) sites. Reported values for each taxon are: Average abundance (Av.Abund), Average dissimilarity (Av.Diss), Ratio between the average contribution divided by the Standard Deviation of those contributions across all pairs of samples (Diss/SD), the percentage contribution to the total dissimilarity (Contrib%) and the Cumulative Percentage dissimilarity to the total (Cum.%). The category of each species is reported: Freshwater-dependent ("FW") and Terrestrial ("T").

Groups P and NP							
Average dissimilarity: 54.65%							
		Group P	**Group NP**				
Category	**Species**	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
T	*Turdus merula*	0.17	0.67	2.36	1.19	4.32	4.32
T	*Anaspis costai*	0.67	0.33	2.31	1.03	4.23	8.54
T	*Sus scrofa*	0.67	0.33	2.29	1.07	4.19	12.73
FW	*Anguilla anguilla*	0.67	0.33	2.23	1.06	4.07	16.81
T	*Capreolus capreolus*	0.33	0.67	2.22	1.03	4.07	20.87
FW	*Neomys fodiens*	0.50	0.50	2.06	0.93	3.77	24.64
FW	*Cottus gobio*	0.67	0.50	2.06	0.94	3.76	28.40
FW	*Neogobius nigricans*	0.5	0.50	2.04	0.95	3.74	32.14
T	*Muscardinus avellanarius*	0.00	0.50	2.04	0.91	3.73	35.88
FW	*Cinclus cinclus*	0.50	0.83	2.01	0.92	3.68	39.56
T	*Lucanus cervus*	0.50	0.67	2.01	0.94	3.68	43.23
T	*Cervus elaphus*	0.67	0.50	2.00	0.91	3.66	46.89
FW	*Barbus meridionalis*	0.33	0.50	1.98	0.95	3.63	50.52
FW	*Barbus plebejus*	0.50	0.33	1.98	0.96	3.62	54.14
T	*Canis lupus*	0.83	0.67	1.80	0.78	3.29	57.43
T	*Pseudocistela ceramboides*	0.33	0.17	1.48	0.77	2.70	60.13
FW	*Barbus caninus*	0.33	0.00	1.47	0.66	2.70	62.83
T	*Meles meles*	0.17	0.33	1.43	0.76	2.62	65.46
T	*Martes martes*	0.00	0.33	1.22	0.68	2.23	67.68
T	*Picus viridis*	0.00	0.33	1.16	0.69	2.13	69.82
T	*Columba palumbus*	0.17	0.17	1.07	0.60	1.96	71.78

5. Conclusions

In recent years, the effectiveness of protected areas (PAs) in conserving biodiversity has been challenged by numerous studies (Kerbiriou et al., 2018; Rosso et al., 2017; Princè et al., 2021; Gavioli et al., 2023), which have highlighted discrepancies in their actual success. While evidence exists for positive impacts on taxonomic richness (see Gray et al., 2016), other studies have reported biodiversity declines driven by resource exploitation, hunting, and habitat degradation within and around reserves (see Gaston et al., 2008; Ricci et al., 2023). Even the N2K network, which was specifically designed to protect priority species and habitats in Europe, has yielded inconsistent

results (Hermoso et al., 2016; Ricci et al., 2023; Santangeli et al., 2023). In particular, this network proved to be ineffective in increasing freshwater fish diversity (Gavioli et al., 2023; Ricci et al., 2023), reinforcing the need for better integration of freshwater-specific strategies in the designation criteria of N2K areas encompassing watercourses. Although our data indicate that the targeted N2K sites in the Northern Apennines host freshwater communities with higher taxonomic distinctness, these findings should not be over-generalized to the entire N2K network without considering broader geographical scales and a higher number of sites. Nevertheless, we support the call for a reassessment of the principles and criteria for PAs selection, toward the direction of a basin-scale approach (Trochet and Schmeller, 2013; Gavioli et al., 2023). We also emphasize the need to move beyond simple biodiversity richness metrics in monitoring schemes. We propose the integration of eDNA metabarcoding to simultaneously assess aquatic and terrestrial biodiversity, and the use of taxonomically and phylogenetically weighted indices as robust, standardized ecological indicators. Such indicators are critical for the development of modern monitoring frameworks, as required by the EU Biodiversity Strategy for 2030, providing a more nuanced evaluation of conservation effectiveness across different scales.

CRediT authorship contribution statement

Lorenzo Ballini: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Giorgia Staffoni:** Writing – original draft, Software, Formal analysis, Data curation. **Stefano Cannicci:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Alessio Iannucci:** Writing – review & editing, Validation, Methodology, Investigation. **Sara Fratini:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2026.115395.

Data availability

Raw sequencing reads and associated metadata have been deposited in the NCBI Sequence Read Archive (BioProject ID PRJNA1400304).

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Further reading

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