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Taxonomic accuracy and complementarity between bulk and eDNA metabarcoding provides an alternative to morphology for biological assessment of freshwater macroinvertebrates

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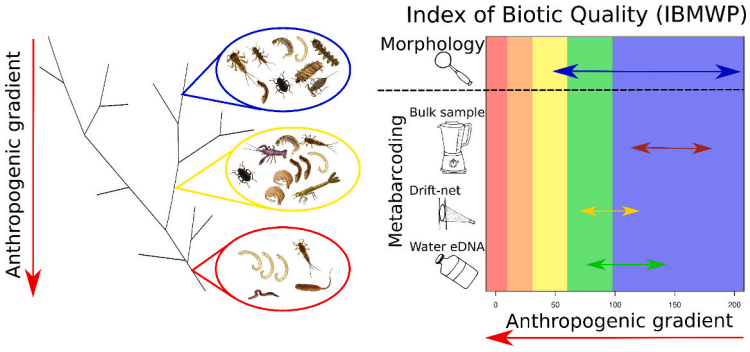
HIGHLIGHTS

- Among metabarcoding techniques, only bulk sampling showed congruence with morphology.
- Biological metrics in disturbed sites were higher using metabarcoding than morphology.
- Bulk benthic can already be use changing water-quality classification boundaries.
- Bulk benthic and eDNA metabarcoding detected complementary community composition.
- Water eDNA for biomonitoring challenged by low success in capturing benthic biota.

GRAPHICAL ABSTRACT



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ABSTRACT

Determining biological status of freshwater ecosystems is critical for ensuring ecosystem health and maintaining associated services to such ecosystems. Freshwater macroinvertebrates respond predictably to environmental disturbances and are widely used in biomonitoring programs. However, many freshwater species are difficult to capture and sort from debris or substrate and morphological identification is challenging, especially larval stages, damaged specimens, or hyperdiverse groups such as Diptera. The advent of high throughput sequencing technologies has enhanced DNA barcoding tools to automatise species identification for whole communities, as metabarcoding is increasingly used to monitor biodiversity. However, recent comparisons have revealed little congruence between morphological and molecular-based identifications. Using broad range universal primers for DNA barcode marker *cox1*, we compare community composition captured between morphological and

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molecular-based approaches from different sources — tissue-based (bulk benthic and bulk drift samples) and environmental DNA (eDNA, filtered water) metabarcoding — for samples collected along a gradient of anthropogenic disturbances. For comparability, metabarcoding taxonomic assignments were filtered by taxa included in the standardised national biological metric IBMWP. At the family level, bulk benthic metabarcoding showed the highest congruence with morphology, and the most abundant taxa were captured by all techniques. Richness captured by morphology and bulk benthic metabarcoding decreased along the gradient, whereas richness recorded by eDNA remained constant and increased downstream when sequencing bulk drift. Estimates of biological metrics were higher using molecular than morphological identification. At species level, diversity captured by bulk benthic samples were higher than the other techniques. Importantly, bulk benthic and eDNA metabarcoding captured different and complementary portions of the community — benthic versus water column, respectively — and their combined use is recommended. While bulk benthic metabarcoding can likely replace morphology using similar benthic biological indices, water eDNA will require new metrics because this technique sequences a different portion of the community.

1. Introduction

Human economic development of industrialised regions has generated an increasing number of anthropogenic disturbances that causes severe ecosystem degradation, but also impacts upon human health. In order to assess, detect, legislate, and restore ecosystem degradation, biomonitoring programs and management tools have been developed for ensuring ecosystem services and a cost-effective resource for management and conservation (i.e. EU's Water Framework Directive, WFD) (Friberg et al., 2011). For freshwater ecosystems, early biomonitoring schemes to track changes in the environment and to quantify anthropogenic impacts focused on bacteriological aspects and the presence of microorganisms (algae, fungi and protozoa) (Hynes, 1960; Bonada et al., 2006). By the turn of the 20th century, benthic macroinvertebrates were incorporated in freshwater biomonitoring because of their ubiquity, inexpensive sampling, high richness yet well-known taxonomy, and wide spectrum of biological responses in front of environmental stressors (Bonada et al., 2006). Since 1970, the community composition of freshwater macroinvertebrates has been assessed for determining the biological status of rivers and restore their ecosystem health (Rosenberg and Resh, 1993). The most widely used indices for routine biological assessments of freshwater macroinvertebrates are a combination of individual metrics, which capture some aspects of the structure, and function of biological metrics, which integrates a range of responses to human impacts (Bonada et al., 2006). However, these multimetric indices are based on taxonomic identification at the family level because the economic and time cost constraint associated to species level identification of challenging groups. Consequently, sensitivity of biota to multiple stressors at the genus or species level is neglected, which is especially critical in species-rich groups that show huge variability of tolerance to pollutants, such as Trichoptera (Insecta) (Bonada et al., 2004) or Chironomidae (Insecta, Diptera) (Puntí et al., 2009; Serra et al., 2016; Beermann et al., 2018).

Variation contained in a short fragment (few hundreds of nucleotides) of DNA has been shown to be able to assign unknown target sequences to species based on extensive and comprehensive DNA libraries (Tautz et al., 2003). In particular, a short section of the mitochondrial gene cytochrome C oxidase I (COI or *cox1*) has been established as a standard marker for the automatic identification of metazoan species, which is referred as the "DNA barcode" (Hebert et al., 2003). Once a significant DNA barcode reference library is available, DNA barcoding allows a rapid assessment and assignment of *cox1* to species taxonomic level of sequences from multiple sites and various life stages. Large scale sequencing of DNA barcodes has been applied in different studies for inferring population dynamics, phylogeographic and macroecological patterns, and evolutionary history of diverse groups (Hajibabaei et al., 2007; Baselga et al., 2013; Múrria et al., 2015, 2017). Despite the ability of DNA barcoding to accelerate biodiversity inventories, significant optimisation and standardization efforts are required for establishing an ecosystem-wide method for community level biodiversity assessment and developing routine biomonitoring schemes.

The advent of high throughput sequencing technologies has enhanced DNA barcoding approaches to efficiently tackle species identification from whole communities (Hajibabaei et al., 2011). As a result, DNA metabarcoding potentially provides a more cost and time-efficient, and finer taxonomic resolution technique for biomonitoring programs than traditional morphology-based surveys (Creer et al., 2016; Deiner et al., 2017; Pawlowski et al., 2018; Hering et al., 2018; Porter and Hajibabaei, 2018). The term Biomonitoring 2.0 (Baird and Hajibabaei, 2012) has been coined to refer to the use of DNA metabarcoding for ecosystem monitoring. Previous studies have mostly focused on the methodological optimisation, feasibility, and viability of molecular-based approaches. Therefore, there are few studies that directly compare the biological metrics derived from community composition of freshwater macroinvertebrates recorded using morphological and molecular-based approaches (Deiner et al., 2016; Macher et al., 2018). Moreover, only few studies assessed differences in freshwater macroinvertebrate community composition recovered from different media (benthic bulk samples or water environmental DNA, eDNA) (Elbrecht et al., 2017; Hajibabaei et al., 2019; Pereira-da-Conceicoa et al., 2021; Macher et al., 2018; Gleason et al., 2021), and thus none of them compared directly the values of biological status indices derived from different molecular approaches (bulk and eDNA) and morphology. To compare the relative performance and coherence of biological quality values obtained from morphology and molecular-based techniques, evaluating the reliability of the taxonomic assignments is needed for updating biological indices and harmonising further applications of both approaches. In this exercise to compare methods, the use of enhanced degenerate primer sets able to amplify all metazoans with low levels of primer bias and an optimized bioinformatic pipelines to retrieve reliable molecular inventories from *cox1* sequencing data is crucial.

The Llobregat river in the North-East Iberian Peninsula covers an anthropogenic gradient from almost pristine, unaffected reference conditions in the headwaters through agricultural and industrial/urbanised disturbed sites to highly polluted reaches at the lowlands (Fig. 1) (Munné et al., 2012). Since 1979, the macroinvertebrate fauna of the Llobregat river has been widely studied for assessing and improving biological quality metrics and morphology-based indices (Prat and Ward, 1994; Prat and Rieradevall, 2006). Notably, since 1994, a continuous long-term semestral sampling in spring and summer of macroinvertebrates has been conducted, which is when freshwater communities are prone to change associated with severe summer droughts that may result in community shifts between early and late summer (Resh et al., 2013; Huttunen et al., 2022). Moreover, an intercalibration exercise was made for the application of the WFD (Munné and Prat, 2011), because of the detailed knowledge of the inter-annual and intra-annual variation of macroinvertebrate fauna (Cañedo-Argüelles et al., 2020), including also complex lineages such as Chironomidae (Prat et al., 1983). The Llobregat river provides an ideal model system to compare morphology and molecular-based techniques to assess biological status of rivers and investigate potential discordances.

Here, we used metabarcoding of *cox1* gene with a broad-range

primer set for sequencing bulk benthic (i.e. pool of organisms that have been extracted from their habitat matrix), bulk drift (i.e. biota captured in a bulk drift) and filtered water (environment, eDNA) (i.e. DNA released from an organism into the environment) samples. The latter is important as an alternative to the time-consuming process of sample collection and individual sorting required in bulk benthic metabarcoding because eDNA can likely characterize macroinvertebrate communities from water (Deiner et al., 2016; Hajibabaei et al., 2019; Macher et al., 2018; Mächler et al., 2019; Gleason et al., 2021). Considering only species belonging to taxa used to estimate IBMWP (Iberian Biological Monitoring Working Party index, Alba-Tercedor et al., 2002), which is the most commonly used metrics by the Catalan Agency of Water, this study aims to determine (1) if morphology and molecular-based techniques capture comparable diversity of taxa and community composition of freshwater macroinvertebrates included in IBMWP, and (2) if these techniques can be interchangeably used for assessing biological status of rivers. Despite disparity in efficiency of capturing DNA among techniques used regarding differences in fraction of the community sampled (i.e., benthic versus drift versus eDNA), DNA amount in each sample, DNA extraction methods or numbers and types of replicates, we predict high concordance between morphology and bulk benthic metabarcoding, which, if true, will empower molecular-based techniques as a more efficient and informative alternative for routine biological assessment of rivers health. Given the variability in concordance of freshwater macroinvertebrate detections between morphological and molecular methods found in previous studies (Deiner et al., 2016; Elbrecht et al., 2017; Macher et al., 2018; Hajibabaei et al., 2019; Pereira-da-Conceicoa et al., 2021; Gleason et al., 2021), the degree of overlap found here, with improved degenerate primers and an optimized pipeline for the *cox1* marker, can determine potential future use of bulk drift and water eDNA to establish biomonitoring protocols under the WFD. For instance, high overlap in community composition would indicate that those techniques are interchangeable, whereas a high dissimilarity in community captured among techniques can suggest their simultaneous use to capture different signatures from the local and regional communities.

2. Material and methods

2.1. Study area and sampling methods

Five reaches that covered an entire anthropogenic disturbance gradient from headwaters to river mouth along the Llobregat river were sampled in 2016–2017. Macroinvertebrates were collected in December 2016 using a 20 × 20 cm 250 μm-mesh D-net sampling 20 times across all available microhabitats (i.e. different mineral and organic substrates), following the national standard quantitative sampling protocol (Pardo et al., 2010). Debris was removed in the field and samples were fixed with 96 % ethanol immediately after collection and kept at −20 °C until macroinvertebrates were sorted and identified in the laboratory. All equipment was sanitised between sites. Although protocols of WFD indicates that around 500 ± 20 % specimens must be identified at the family level under the stereo microscope (e.g., Munné et al., 2006), here all organisms captured were identified morphologically at the lowest

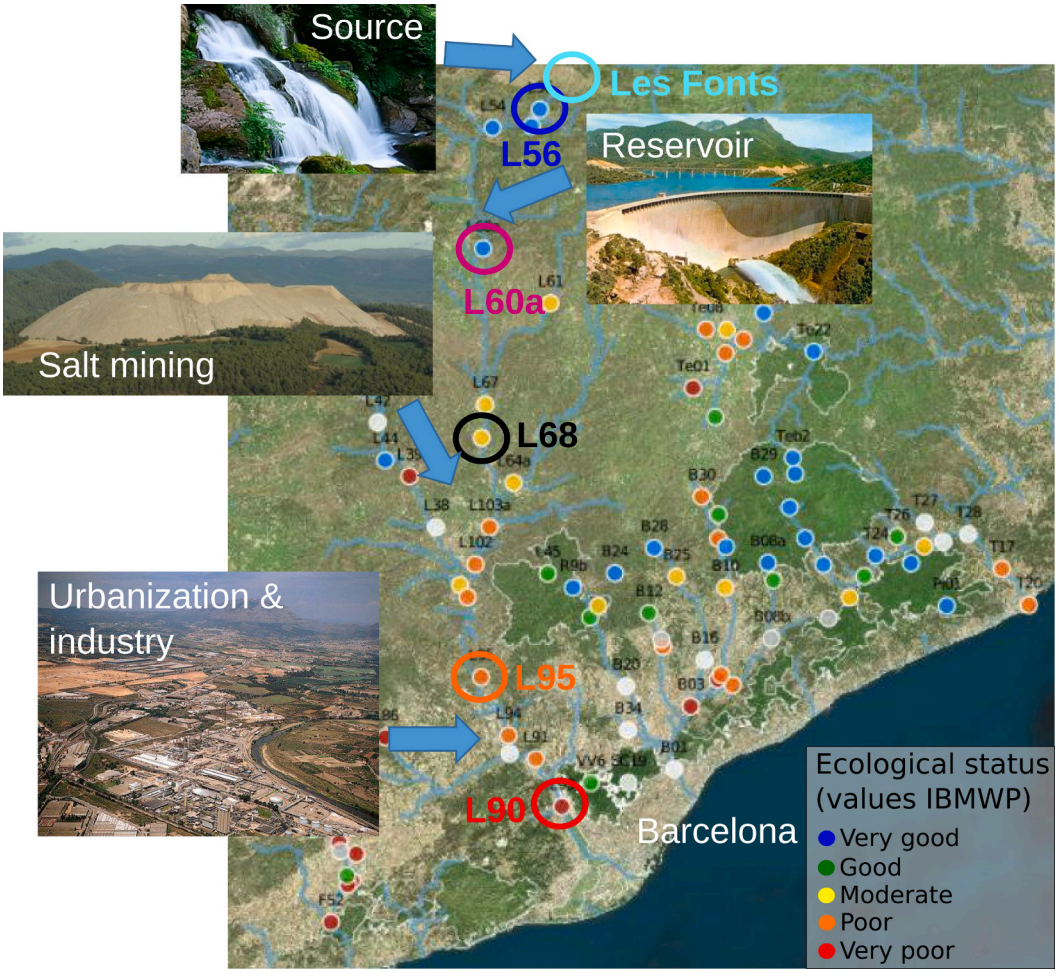

Fig. 1. Sites location along the Llobregat river. Dots indicate the ecological status of streams in Barcelona province in 2017. Blue: "very good", green: "good", yellow: "moderate", orange: "bad" and red: "very bad" (https://www.ub.edu/barcelonarius/web/index.php/informes-anteriors/informe-2017).

taxonomic level possible, usually genus, using the keys of Tachet et al. (2010), but with some Diptera identified to family, and annelids and acari to phylum and subclass level, respectively. Following morphological identification, all individuals from the same site were pooled together and stored overnight in an oven for ethanol evaporation before the DNA extraction. These samples are hereafter referred as "bulk benthic".

In parallel, 250 μm drift-net was hung vertically in the water for one hour, and the samples captured were fixed with 96 % ethanol immediately after collection. These samples were not morphologically identified and are hereafter referred as "bulk drift".

At each reach, eDNA was directly isolated from the water. Water eDNA contains breakdown of body parts from organisms together with faecal, mucus, skin cells, organelles, gametes, or even extracellular DNA (Taberlet et al., 2012). At each site, three samples of 500 ml of stream water were collected in a sterile bottle for a total of 1.5 l around 20–30 cm from the water surface. The three replicates were collected from the exact same location one minute apart. Each sample was filtered separately in the field through a GF 0.47 μm filter papers to capture DNA diluted in the water. The three filters were stored separately in a cooler during the field work and then stored at −20 °C in sterile plastic bags until DNA extraction from each filter. Unfortunately, filters collected in December 2016 defrosted during an electric cut, and eDNA from water was sampled again in June 2017, and therefore eDNA and macroinvertebrates were unfortunately collected in early winter and late spring. However, the main interannual change in community composition of Mediterranean streams is found from early to late summer and it is mainly driven by summer droughts (Resh et al., 2013). These samples are thereafter called "eDNA". Additionally, one eDNA sample was collected at the source of the Llobregat river, which was called Les Fonts. This eDNA sample was used to characterize the initial eDNA from the source and it was compared only with the other eDNA samples.

2.2. DNA extraction, PCR amplification and library preparation

For the bulk benthic, all individuals were homogenized with liquid nitrogen and four independent subsamples were transferred to sterile tubes for four separated DNA extraction, except L90 for which the amount of tissue was lower and only three DNA extractions were done. For bulk drift samples, the amount of sample was lower than bulk benthic samples, and therefore the entire sample was homogenized using liquid nitrogen for a unique DNA extraction. DNA was extracted using the Soil DNA isolation Plus Kit (Norgen, Biotek Corp., Thorold, ON, Canada), and following commercial instruction, 0.3 g of homogenized tissue was transferred to sterile tubes for each DNA extraction in a laminar flow cabinet sterilised with UV light between samples. Soil DNA isolation kit was preferred because it can eliminate the PCR inhibitors, which commonly cause low success during PCR amplification when Tissue kits or Phenol-chloroform protocols are used. In parallel, DNA retained in each GF 0.47 μm filter papers were extracted in the cabinet sterilised with UV light using PowerWater commercial kit (MoBio, Carlsbad, CA, US). Overall, for the bulk benthic and eDNA samples, DNA was extracted for respectively four and three subsamples per community, whereas only one DNA extraction was prepared for the bulk drift samples.

The universal Leray-XT primer set introduced by Wangensteen et al. (2018) was optimized for amplifying a 313 bp fragment of the mitochondrial marker *cox1* for almost all Metazoan lineages covering all benthic freshwater macroinvertebrates. Forward and reverse primers were attached to identical 8-base specific tags per sample for further sample identification and to a variable number of leading Ns (two to four) for improving sequence diversity for Illumina processing. Two PCR (replicates) were run per each DNA extraction, moreover three negative controls (PCR mixture without DNA template) and one negative control for each DNA extraction method (distilled water was filtered for eDNA) were performed following standard conditions for *cox1* amplifications (Wangensteen et al., 2018). For the negative PCR controls, no band (no amplification) was observed on agarose gels.

PCR products were pooled, purified, and concentrated using MinElute PCR purification kit (Qiagen). A Qubit fluorometer was used to check DNA concentrations. A single Illumina library was built using the Nextflex PCR-free library preparation kit (Perkin-Elmer), which was sequenced in an Illumina MiSeq V3 run using 2 × 250 bp paired-end sequencing.

2.3. Bioinformatic analyses

Bioinformatic analyses were based on the OBITools package (Boyer et al., 2016). Following Antich et al. (2021a), "Illuminapairedend" was used to align paired-end reads with >40 alignment quality score and remove the primer sequences. Reads were demultiplexed using "ngsfilter" and only reads with the same primer tags at both extremes and no ambiguous bases were kept. "Obigrep" and "obiuniq" were used to retain reads between 310 and 317 bp of length filter and to dereplicate sequences. Chimeric amplicons were removed using "Uchime-denovo" algorithm from VSEARCH v2.7. SWARM v2.1.7 (Mahé et al., 2015) was used to cluster sequences into molecular operational taxonomic units (MOTUs) by setting the clustering distance threshold (*d* parameter) to 13 (Antich et al., 2021b). The *d* parameter is the distance between amplicons to be clustered together. MOTUs with less than five reads in a PCR and not detected in the two PCRs were removed to ensure quality of data. A custom reference library containing sequences from the EMBL nucleotide database, Barcode of Life Database (BOLD) and the Iberian DNA barcode reference library (Múrria et al., 2020) was used to assign MOTUs to nominal species. Species assignment was checked and improved when possible by querying the BOLD and GenBank database, and only MOTUs with an identity match to reference data base higher than 85 % were used in further analyses (e.g. weak taxonomic identification beyond family-level resolution). Finally, LULU (Frøslev et al., 2017) was used to remove supernumerary MOTUs possibly arising from pseudogenes (NUMTs) from the final dataset.

2.4. Taxa selection and statistical analyses

Given that this study aims to compare biological metrics calculated following the Catalan bioassessment metrics using morphological and molecular-based techniques, only taxa included in the IBMWP were considered, and DNA sequences belonging to other taxa were removed. Therefore, only families included in the IBMWP belonging to Turbillaria, Hirudinea, Mollusca, Crustacea and Insecta, together with all aquatic families belonging to class Arachnida and phylum Annelida were considered. IBMWP is a tolerance-based biological index commonly used in the Iberian Peninsula for freshwater macroinvertebrates (Prat and Munné, 2014). This biological metric is based on family richness and its tolerance. Values of IBMWP were estimated for each community and technique.

Fourth square root-transformed values of the relative read abundances of MOTUs in each sample were estimated for subsequent quantitative analyses (Ershova et al., 2021). All reads for each of the two PCR replicates and subsamples were summed up for obtaining a total number of reads per sample. To compare the obtained community composition among morphology and the molecular techniques, bar-plots were generated for visualizing the abundance and diversity of families, which is the taxonomic level used in morphology for estimating biological indices. To visualize differences among communities determined by bulk benthic, bulk drift and eDNA metabarcoding, the species level was used because it is the taxonomic level assigned during bioinformatic analyses. However, statistical differences between techniques were not directly tested because each technique captured different abundance of reads and any standardization was possible. The distribution of the family and species richness captured along the river for each technique separately was represented by violin plots, which are similar to a box

plot but also include the probability density of the data at different values smoothed by a kernel density estimator. Each violin plots included the rarefied taxa richness to minimum sampling abundance using 1000 permutations and were performed separately for each technique. Each plot shows the median, and interquartile range of the 1000 permutations (α-diversity). Complementarity of the taxonomic composition of communities across techniques was assessed by overlapping captured composition using Venn Diagrams, which show relations among taxa detected by different techniques. Moreover, similarity of community composition (β-diversity) at both the taxonomic family and species levels among subsamples and molecular techniques was explored using nMDS analysis based on Bray-Curtis dissimilarity matrices. This analysis also allowed to explore how similar are the different subsamples from the same site. Differences in centroids and dispersion of the groups captured by sites and techniques were tested using the non-parametric multivariate PERMANOVA test (Anderson, 2001).

Statistical analyses were performed in R (R Development Core Team 2012, version 3.4.0) using the packages "vegan" (Oksanen et al., 2020), "vioplot" (Adler and Kelly, 2021) and "Venn Diagram" (Hulsen, 2021).

3. Results

A total of 43,324 individuals belonging to 17 orders, 55 families and 54 genera of freshwater macroinvertebrates were sorted and morphologically identified (Supplementary Material Table S1). The most diverse orders were Diptera (12 genera), Coleoptera (10 genera), Ephemeroptera and Trichoptera (9 genera each), Plecoptera and Mollusca (7 genera each). At all sites, freshwater communities were mainly dominated by Baetidae and Caenidae (Ephemeroptera), Chironomidae and Simuliidae (Diptera), and Hydropsychidae (Trichoptera) (Fig. 2a). Family richness at each site decreased almost linearly from the headwaters to the river mouth in parallel to the increase of anthropogenic pressures. Total richness per site ranged from 25 families in the most pristine to 8 in the most altered river reaches (Fig. 3a).

After quality filtering, demultiplexing, dereplicating and chimera elimination, MiSeq sequencing resulted in 8,988,827 reads and a total of 815 MOTUs of Metazoa assigned to species (list available as Supplementary Material Table S2). The controls had a negligible number of reads. There were intrinsic differences in the fraction of the community sampled (tissue versus water), the quantity of DNA extracted, the number of subsamples processed and the number of PCR replicates across techniques, which resulted in disproportional number of reads among molecular techniques (Supplementary Material S2). For instance, the number of reads in one PCR for eDNA ranged from 200 to 20,000 reads, whereas for bulk drift or bulk samples, it ranged from 2000 to 250,000 and from 32,000 to 400,000, respectively. As a result, this disparity in the number of final reads obtained made difficult to compare abundance across techniques, and the analyses focus mainly on differences in the community composition captured by each technique. For comparing values of biological metrics among techniques, 8,323,320 reads for taxa included in the IBMWP metric were retained, which yielded 383 MOTUs belonging to 88 families of freshwater macroinvertebrates (list available as Supplementary Material Table S3).

The most represented families (>80,000 reads) considering all molecular techniques together were Simuliidae and Chironomidae (Diptera); Heptageniidae, Baetidae, Ephemeridae, Ephemerellidae and Caenidae (Ephemeroptera); Perlidae and Nemouridae (Plecoptera); Limnephilidae and Hydropsychidae (Trichoptera); Hydrobiidae and Corbiculidae (Mollusca); Elmidae (Coleoptera); and Naididae (Oligochaeta). In general, molecular techniques together detected more taxa than morphology. Patterns of relative abundances across sites were different between morphology and molecular techniques, and also among molecular techniques (Fig. 2). All methods detected 20 of the most common and abundant families (Fig. 4a; Supplementary Material Table S4). Morphology, eDNA and bulk benthic samples contributed the most with unique taxa for each of the techniques, whereas bulk drift samples captured the lowest number of those taxa. Morphology did not detect Oligochaeta (e.g., Enchytraeidae, Naididae and Tubificidae and Acari (e.g., Sperchontidae) at the family level, while they showed high diversity using the molecular techniques). Only morphology and bulk benthic metabarcoding detected common families belonging to the Insecta Hemiptera (Aphelocheiridae), Odonata (Calopterygidae, Gomphidae), Coleoptera (Gyrinidae, Hydraenidae), Diptera (Athericidae, Ceratopogonidae, Limoniidae), and the Clitellata (Erpobdellidae and Glossiphoniidae). Morphology was the only method to recover certain families of Mollusca (Planorbidae), Crustacea (Asellidae), Diptera (Anthomyiidae, Rhagionidae, Stratiomyidae), Megaloptera (Sialidae), Coleoptera (Haliplidae, Scirtidae), Trichoptera (Beraeidae, Leptoceridae) and Hemiptera (Corixidae). Similarly, bulk benthic samples were the only to detect some families of Plecoptera (Chloroperlidae, Perlodidae, Taeniopterygidae), Oligochaeta (Lumbriculidae, Ocnerodrilidae), Tricladida (Dugesiidae) and Acari (Lebertiidae, Libertiidae). In contrast, only the eDNA and bulk drift captured certain families of Acari (Acaridae, Achipteriidae, Aturidae, Compactozetidae, Eupodidae, Hydrozetidae, Phenopelopidae), although some of these families are not freshwater lineages (e.g., Tarsonemidae is terrestrial, and Carpoglyphidae grows on fruits).

Considering all molecular techniques together, the species that most contributed in number of reads (>80.000 reads) were *Simulium lineatum*. *S. ornatum*, *Micropsecta* cf. *notescens* (Diptera); *Heptagenia flava*, *Ephemera danica*, *Baetis pavidus*, *B. rhodani*, *Caenis* sp., *Serratella ignita* (Ephemeroptera) *Potamopyrgus antipodarum*, *Corbicula leana* (Mollusca), *Potamophylax latipennis*, *Hydropsyche exocellata* (Trichoptera); *Dinocras cephalotes*, *Protonemura meyeri*, *Perla marginata*, *Nemoura uncinata* (Plecoptera), and *Elmis maugetii*, *Riolus subviolaceus* (Coleoptera). Because of the incompleteness of the Iberian DNA barcode reference library for Diptera and Chironomidae (Múrria et al., 2020), scientific names for sequences matches >97 % in Supplementary Material S3 (column "best_identity") should be treated with caution. The number of species captured by bulk benthic (259 MOTUs) was higher than both eDNA (135 MOTUs) and bulk drift (94 MOTUs), and only 59 MOTUs were detected by the three molecular techniques (Fig. 4b). Bulk benthic detected the highest number of unique species (i.e., species detected only by this technique) (139 MOTUs), which stands at sharp contrasts with the low number of unique species captured by eDNA and bulk drift (26 and 18 MOTUs, respectively).

Taxonomic richness captured at family and species levels varied among techniques (Fig. 3). Bulk benthic detected the highest richness, whereas eDNA retrieved the lowest diversity. Taxonomic richness decreased along the anthropogenic gradient only for bulk benthic. In contrast, the richness retrieved by eDNA was constant along the river zonation, whereas bulk drift increased downstream towards the river mouth at family level.

The similarity in community composition based on Bray-Curtis distances revealed overlapping in community composition among subsamples from the same site (i.e., four for bulk benthic and three for eDNA), which indicates subsamples were highly coherent across molecular techniques, especially for bulk benthic samples (Fig. 5). Community similarity across samples grouped by molecular techniques and sites were significantly different at both the family (F-statistic = 8.41, p-value > 0.01 and F-statistic = 4.02, p-value > 0.01, respectively) and species levels (F-statistic = 4.68, p-value > 0.01 and F-statistic = 5.21, p-value > 0.01, respectively). The highest F-statistic value was found by grouping by techniques at the family level.

In general, the IBMWP values decreased along the anthropogenic gradient of disturbances for all techniques except for bulk drift, however the estimated values of ecosystem health were inconsistent among

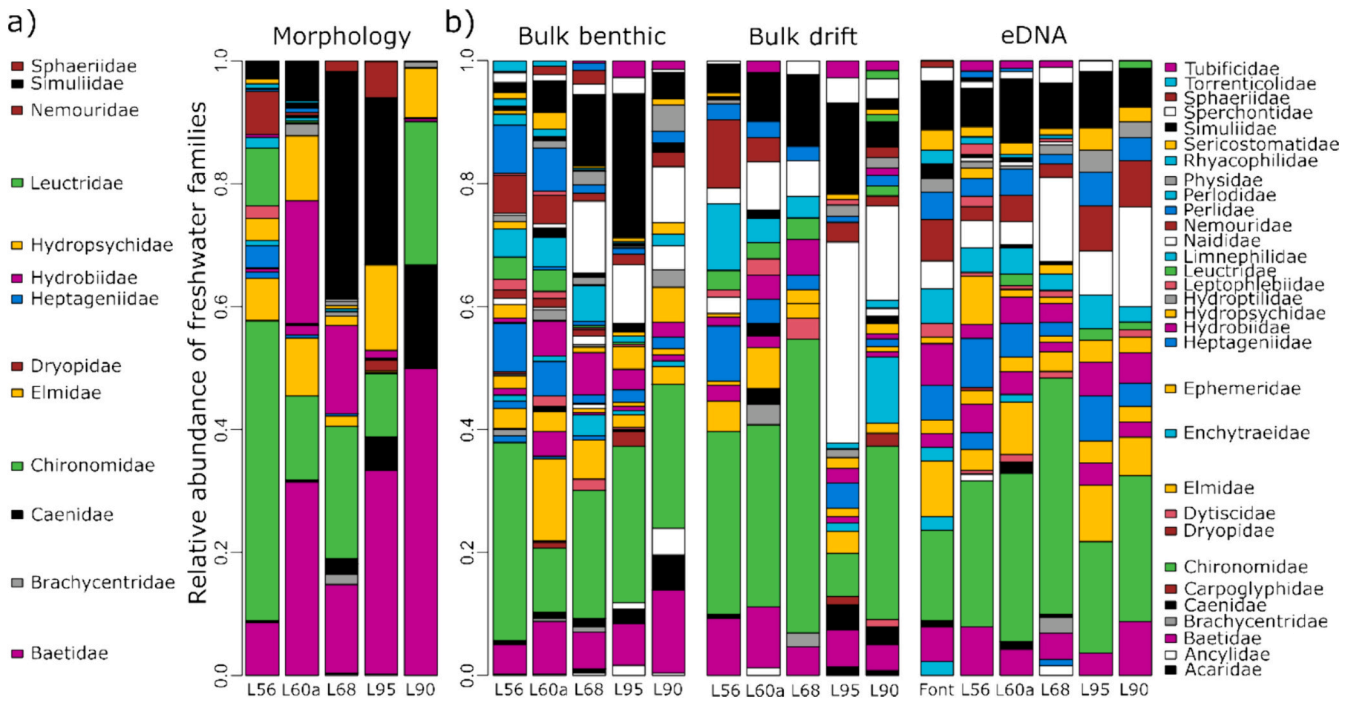

Fig. 2. Relative abundance of families recorded in each site for each of the morphological (a) and molecular-based (b) methods. The most abundant families are ordered alphabetically.

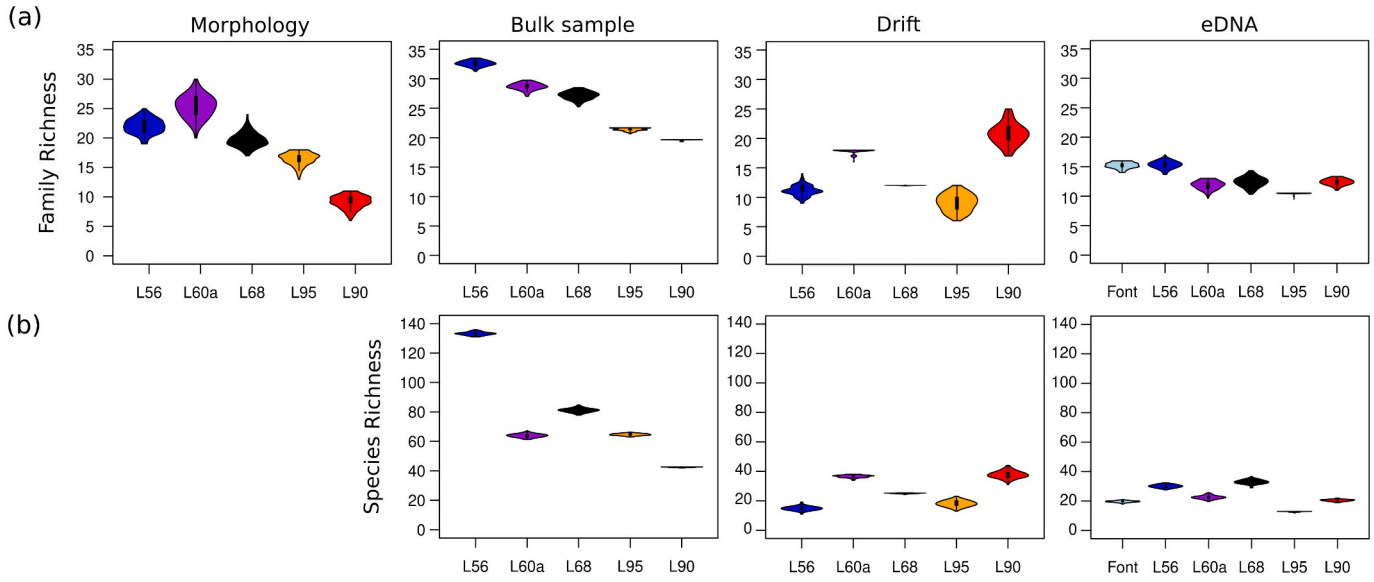

Fig. 3. Violin plots of rarefied diversity at (a) family and (b) species levels (number of taxa) per each site along the Llobregat river for the morphological and molecular methods.

morphological and molecular-based techniques (Fig. 6). IBMWP values based on morphology tend to decrease from headwaters to downstream reaches, ranging from "very good" biological status to "moderate" in the most disturbed sites. In contrast, molecular-based methods revealed mainly a "very good" and a "good" quality, except the bulk benthic that determined "very good" conditions across all sites with IBMWP values decreasing in parallel to the anthropogenic gradient of disturbances.

4. Discussion

Morphology and bulk benthic showed the highest congruence for capturing a similar inventory of taxa because identification was performed on the samples that were sequenced. As a result, ecological assessment was comparable between these two techniques, however values of IBMWP were higher for the bulk benthic metabarcoding

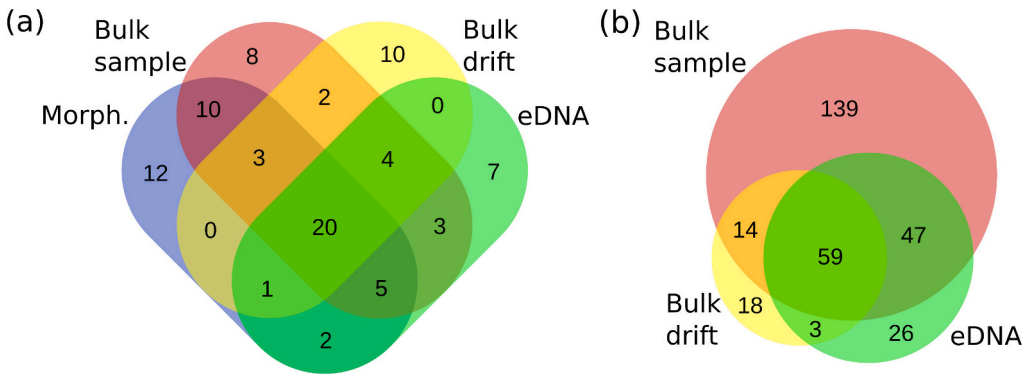

Fig. 4. Venn diagram showing the taxonomic overlap across techniques at the (a) family and (b) species levels comparing the morphological and molecular based tecniques.

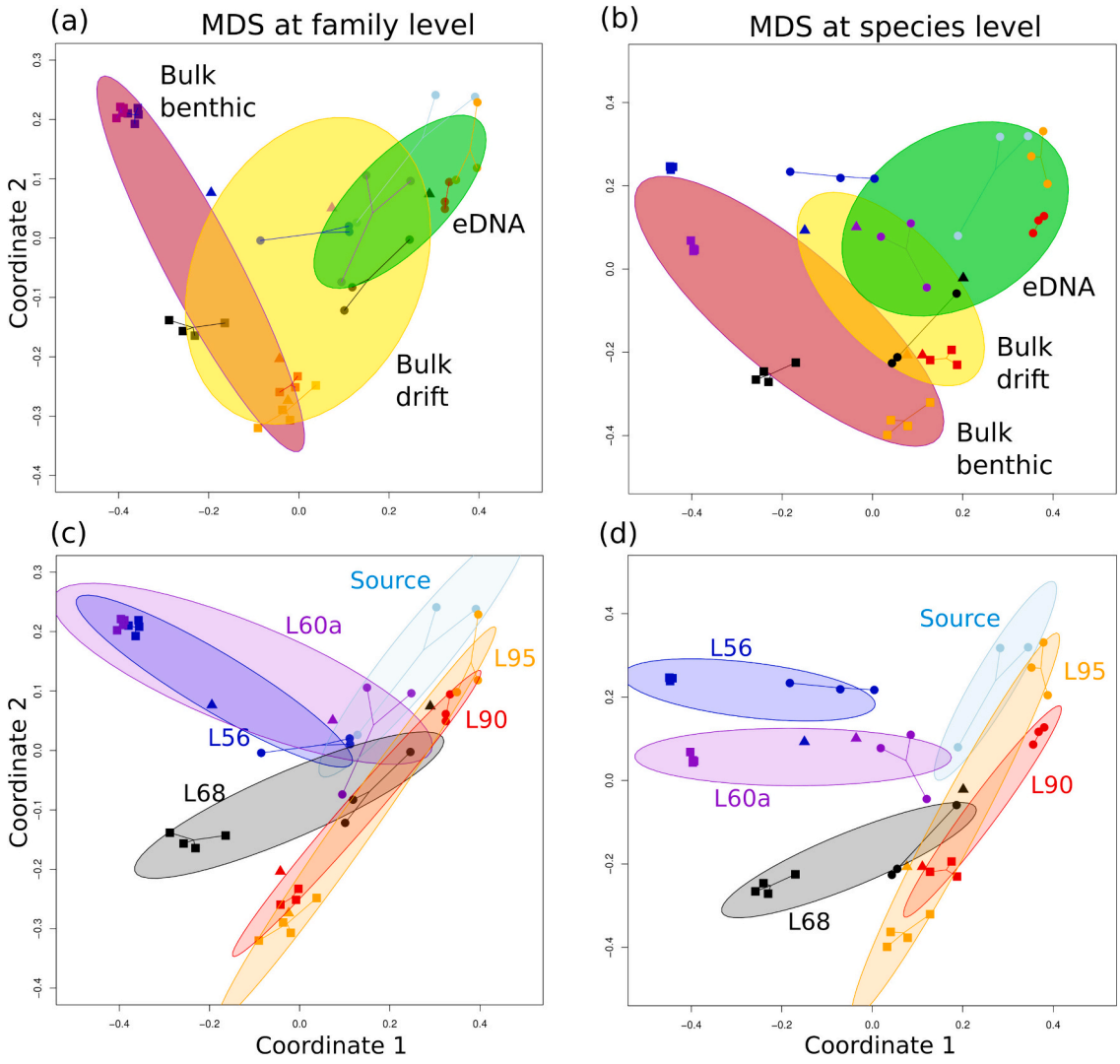

Fig. 5. Results of Multi-Dimensional Scaling (MDS) analysis showing the level of composition similarity across communities and molecular techniques using the Bray-Curtis distance of fourth root-transformed relative read abundances at the family (a, c) and species (b, d) levels. In (a) and (b) samples are grouped by technique, whereas in (c) and (d) samples are grouped by sites. Sites were coloured as in Fig. 1, and techniques were codified following: squares (bulk sample), triangles (drift net) and dots (water eDNA).

(Fig. 6). Also, these inventories were congruent with records available from the same sites since 1979 (Prat and Rieradevall, 2006; Prat and Munné, 2014; Cañedo-Argüelles et al., 2020). Discrepancies in IBMWP values between morphology and bulk benthic can be explained by either the presence of DNA traces from dead, predated and part of organisms in bulk benthic samples, or the low size and abundance of some taxa identified by morphology (e.g., Anthomyidae, Asellidae or Haliplidae), which likely failed to amplify in some replicates of the PCR and

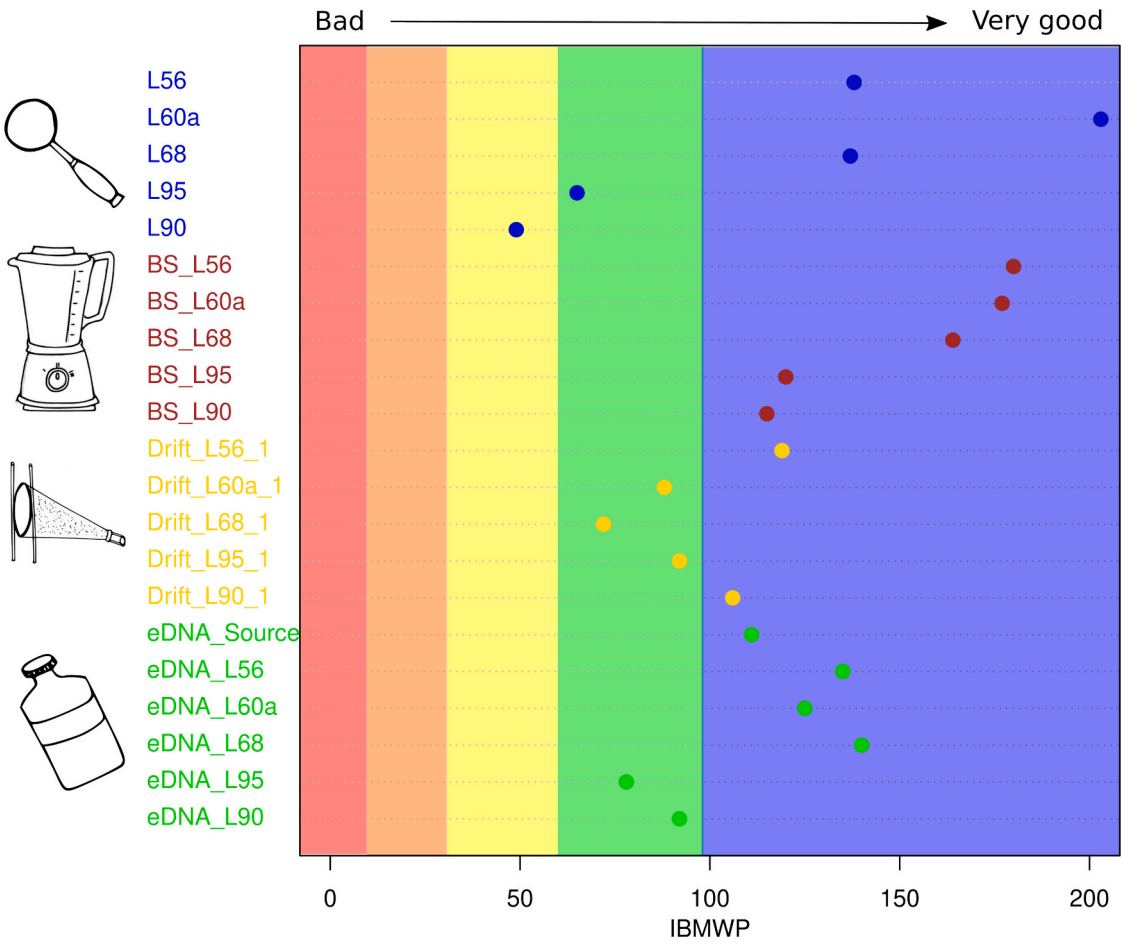

Fig. 6. Estimates of the IBMWP for each site using the morphological and molecular methods. The health of the ecosystem (i.e., value of IBMWP) is indicated from "bad" in red to "very good" in blue.

therefore were removed during cleaning. Additionally, early larval stages collected in winter (December 2016) could have been misidentified by morphology. Because IBMWP sums up the values for all families that have been collected, more recorded families equate to higher values of IBMWP, which resulted in all bulk benthic IBMWP values falling into the "very good" category. Our findings therefore suggest the utilisation of bulk benthic metabarcoding for assessing ecosystem health using currently available biological metrics at the family level such as IBMWP, but it will require further intercalibration exercises among institutions to harmonise new boundaries among the five water-quality categories (Poikane et al., 2014).

Higher taxonomic resolution captured by bulk benthic is evident in higher dissimilarity across sites along the anthropogenic gradient captured at species level (Fig. 5c–d). Taxonomic resolution is critical in biological assessments because related species or genus within a family can exhibit contrasting responses to multiple stressors (Gardham et al., 2014; Beermann et al., 2018; Prat and García-Roger, 2018), which is missed when using morphology-based indices at family level. The observation that bulk benthic recovers higher local diversity than morphology is not novel (e.g., Elbrecht et al., 2017; Macher et al., 2018; Hajibabaei et al., 2019; Gleason et al., 2021; Pereira-da-Conceicoa et al., 2021), and has been further revealed by recent meta-analyses (Fediajevaite et al., 2021; Keck et al., 2022). However, some caution should be exerted given the relatively low number of published studies that directly compared morphological and molecular-based techniques, and also because of the differences in molecular markers used, number of amplicons obtained across techniques and studies, sequencing depths, number of sites, and replicates across studies (Gleason et al., 2021; Keck et al., 2022). Therefore, further efforts are necessary to determine the abundance of individuals in metabarcoding, since abundance is a key parameter for assessment under the WFD. Here, we provide more evidence that only bulk benthic metabarcoding using *cox1* primers for a broad range of metazoans is accurate and provides higher taxonomic richness than morphological identification (Hajibabaei et al., 2019; Gleason et al., 2021; Pereira-da-Conceicoa et al., 2021). Therefore, as previously suggested, bulk benthic metabarcoding could replace morphology-based methods for routine biological assessment at species level (Elbrecht et al., 2017; Hajibabaei et al., 2019; Cordier et al., 2021; Gleason et al., 2021). However, it will first be necessary to determine species level responses to human impacts, to review biologic indices, to harmonise water-quality classification boundaries and to check bulk benthic sample results against historical data based on morphology. Currently, morphological-based indices for the application of the WFD are standardised and intercalibrated at the taxonomic family level across European countries (Furse et al., 2009; Poikane et al., 2014), and we advocate to move to species level identification by metabarcoding as previously discussed within the context of DNAqua.net EU-COST Action (Hering et al., 2018; Leese et al., 2018). Future intercalibration across countries at the species level represents a challenge since species show high replacement levels across Europe as a result of dispersal ability and adaptation to different ecological conditions (Múrria et al., 2017; Salinas-Ivanenko and Múrria, 2021; Grigoropoulou et al., 2022). Another issue to consider for the use of bulk benthic metabarcoding in biomonitoring under the WFD is that DNA barcode reference libraries remain incomplete (Weigand et al., 2019; Múrria et al., 2020).

This is the first study that implements the Leray-XT primers, which

are universal metazoan *cox1* primers, in combination with the laboratory protocols and bioinformatic pipelines originally designed and optimized for marine biota (Wangensteen et al., 2018; Antich et al., 2021a) to analyse freshwater macroinvertebrates communities. Former studies already validated their utility for terrestrial arthropods (Elbrecht et al., 2019). Our results revealed congruent community compositions, which validate the use of these primers and protocols for routine biological assessments of freshwater macroinvertebrates.

The ordination analyses and the low overlap between community composition recovered by bulk benthic and eDNA (Figs. 4 & 5), suggest a high degree of complementarity between these two techniques for capturing the composition of freshwater macroinvertebrates. Such complementarity can be explained by the different target species and by the spatial scale at which the DNA of a particular species can be transported (Macher et al., 2018; Gleason et al., 2021; Pereira-da-Conceicoa et al., 2021; Múrria et al., 2024). Bulk benthic recovered the macroinvertebrates sorted under the stereo microscope, which likely discarded the smallest organisms, whereas this fraction was probably retained in 0.47 μm filters used for eDNA. Moreover, eDNA probably detected small, swimmers or floating organisms, cells and free DNA from upstream communities at a sub-catchment scale, whereas bulk benthic recovered local and macroscopic organisms that inhabit a particular section of sampled riverbed (Deiner et al., 2017; Macher et al., 2018). Here, the low overlap between eDNA and bulk benthic can also be due to different sampling times, which is unlikely because in Mediterranean streams the community composition mainly change between early and late summer given the effects of summer droughts (Resh et al., 2013; Cañedo-Argüelles et al., 2020), especially in mid and lower Llobregat basin that holds an important concentration of industries, agricultural activities, and urban areas (Munné et al., 2012). Moreover, it was suggested that degenerate primers, such as Leray-XT, reduce the success of amplification of target DNA from freshwater macroinvertebrates by swamping by non-target eDNA (i.e., not derived from benthic invertebrate fauna), and therefore eDNA did not represent local communities (Gleason et al., 2021). This is critical here because only families used to estimate IBMWP were considered, and therefore it can explain the low diversity detected by eDNA (Fig. 3). Therefore, the use of optimized primers for freshwater macroinvertebrates should reduce nontarget amplification bias and increases the success of freshwater macroinvertebrates in eDNA samples (Leese et al., 2021). As a result, our finding also confirms the higher accuracy of bulk benthic than eDNA for detecting local benthic macroinvertebrates used as bioindicator taxa, such as Ephemeroptera, Plecoptera, and Trichoptera (Bista et al., 2017; Macher et al., 2018; Hajibabaei et al., 2019; Gleason et al., 2021; Múrria et al., 2024). However, eDNA performed better for small active swimmers such as Daphniidae and some Acari (e.g., Aturidae), which unfortunately are not fully included in benthic bioassessment protocols because they are not benthic or the challenging taxonomical identification of Acari (Goldschmidt, 2016).

Some taxa captured by eDNA such as Sericostamidae and Brachycentridae (Trichoptera) or Heptageniidae (Ephemeroptera) at the most polluted sites are known to be highly intolerant to pollution, which suggests that detection by eDNA may be the results of remnants of DNA from upstream or tributaries of higher biological quality (Deiner et al., 2016; Macher et al., 2018). However, we revealed high spatial turnover (β-diversity) of community composition along the anthropogenic gradient captured by eDNA (Fig. 5d), which indicates that a large part of eDNA from upstream communities did not persist at downstream sites (sites were separated by at least 25 km). Moreover, the complementarity in detecting site-specific community composition among molecular techniques is also visible in differences in the community composition across sites. Fig. 5d indicates that community composition detected for each molecular technique for the same site are in close proximity yet not overlap and each local community was significantly different from other communities, which means that each technique detected a different fraction of the community. This finding of site-specific communities can be explained because the sampled sites were spatially separated enough along the anthropogenic disturbance gradient, as was previously found (Macher et al., 2018), and because the stressors along the Llobregat river were different among sites (Barata et al., 2005; Prat and Rieradevall, 2006; Munné et al., 2012; Múrria et al., 2024). In contrast, other studies indicated that eDNA captures and integrates diversity at the catchment scale by water flow and downstream transportation, which results in similar community composition across sites located in proximity (Deiner and Altermatt, 2014; Deiner et al., 2016; Mächler et al., 2019). It should be borne in mind that our analyses of β-diversity considered only freshwater macroinvertebrates included in IBMWP and therefore excluded terrestrial biota and other taxa such as fungi, algae, and bacteria which can homogenize communities (Gleason et al., 2021; Leese et al., 2021; Pereira-da-Conceicoa et al., 2021).

As expected, species richness was higher for bulk benthic than, respectively, eDNA and bulk drift. The last two also failed to capture the decrease in richness along the anthropogenic disturbance gradient revealed by morphology-based identifications. Consequently, a low congruence of the IBMWP values was obtained among morphology, bulk drift and eDNA, as previously found (Hajibabaei et al., 2019; Gleason et al., 2021; Pereira-da-Conceicoa et al., 2021). Our findings therefore advise against a direct implementation of morphology-based biological metrics for macroinvertebrates to communities exclusively detected by eDNA or bulk drift. Many factors may simultaneously affect eDNA composition, such as DNA source, local environment and DNA fragmentation, rates of DNA release, transport, and degradation, which can reflect other processes rather than local composition of benthic communities of macroinvertebrates (Barnes and Turner, 2016; Deiner et al., 2017; Gleason et al., 2021; Keck et al., 2022). Consequently, eDNA and bulk drift recover the diversity of organisms at a basin scale and the resulting biological indices should provide information at regional rather than at a specific site. Moreover, given the poor proportion of freshwater macroinvertebrates considered in the IBMWP captured by eDNA techniques, new biological metrics based on all taxa captured by eDNA rather than focus exclusively in freshwater macroinvertebrates are required (Hajibabaei et al., 2019; Gleason et al., 2021; Pereira-da-Conceicoa et al., 2021; Múrria et al., 2024). There is a recent trend to investigate the use of supervised machine-learning algorithms and OTUs with unknown taxonomy to develop new biological indices directly from eDNA data collected across a high number of communities located along a known gradient of anthropogenic disturbance level (Pawlowski et al., 2018; Cordier et al., 2019; Cordier et al., 2021). However, our results indicate that the origin of the DNA and its transportation can modify the ecological quality of local sites based on the human activities located upstream, and likely limiting its potential use for biomonitoring at the local scale.

4.1. Conclusions

DNA-based techniques provide finer taxonomic resolution, however only bulk benthic sample detected higher richness than the morphology-based approach and this method was the only one that retrieved similar community composition and were congruent with the biological quality provided by intercalibrated morphology methods. Before a routine application of bulk sample techniques, further efforts are required for accelerating the sorting of collected benthic macroinvertebrates before DNA extraction, which should reduce even more its economic cost and should allow its implementation at a broad scale. Importantly, water eDNA and bulk benthic metabarcoding recorded a complementary portion of the community composition because target taxa for the two techniques are different. Hence, a high proportion of the biota inhabiting the local community should be recovered by sequencing simultaneously both bulk benthic and eDNA, and therefore the combination of these two techniques is necessary to improve the accuracy of biological assessment and to retrieve a more complete biological assessment of freshwater ecosystem health. However, the high species turnover of

freshwater macroinvertebrates across Europe is a major challenge for the implementation of a routine biomonitoring program at the European scale using species-level DNA-based metrics. Future programs should consider developing region-specific metrics perhaps at genus or species level for ensuring intercalibration. Therefore, further progress in the use of DNA techniques as routine biological indicator requires to determine species level responses to human impacts, to review biologic indices, to harmonise water-quality classification boundaries and the intercalibration exercises between institutions and across border cooperation. In the meantime, since values of bulk benthic samples and morphology provide similar patterns of quality changes among sites, yet higher IBMWP values for bulk benthic samples, a change in water-quality classification boundaries will provide a comparable assessment that may be useful for water managers. Finally, urgent efforts to complete a DNA barcode reference database at the European level are required to fully exploit the capabilities of molecular techniques for characterizing freshwater ecosystems and ensure a more powerful assessment of the anthropogenic impacts.

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CRediT authorship contribution statement

Cesc Múrria: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Owen S. Wangensteen:** Formal analysis, Methodology, Writing – review & editing. **Simona Somma:** Data curation. **Leif Väisänen:** Data curation, Writing – review & editing. **Pau Fortuño:** Data curation. **Miquel A. Arnedo:** Conceptualization, Methodology, Writing – review & editing. **Narcís Prat:** Conceptualization, Funding acquisition, Writing – review & editing.

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.

Data availability

The raw sequencing datasets presented in this study are publicly available in the Sequence Read Archive (SRA) repository of NCBI. Bioproject ID: PRJNA1084784. The ngsfilter table needed for demultiplexing the sequence files, and the metadata of the samples (site and technique) can be found in Mendeley Data doi: 10.17632/tys336jdsd.

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References

Adler, D., Kelly, S.T., 2021. vioplot: violin plot. R package version. 0.3.7. https://github.com/TomKellyGenetics/vioplot.

Alba Tercedor, J., Jáimez-Cuéllar, P., Álvarez, M., Avilés, J., Bonada, N., Casas, J., Mellado, A., Ortega, M., Pardo, I., Prat, N., Rieradevall, M., Robles, S., Sáinz-Cantero, C.E., Sánchez-Ortega, A., Suárez, M.L., Toro, M., Vidal-Abarca, M.R., Vivas, S., Zamora-Muñoz, C., 2002. Caracterización del estado ecológico de ríos mediterráneos ibéricos mediante el índice IBMWP (antes BMWP'). Limnetica 21, 175–185.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral. Ecol. 26, 32–46.

Antich, A., Palacín, C., Cebrian, E., Golo, R., Wangensteen, O.S., Turon, X., 2021a. Marine biomonitoring with eDNA: can metabarcoding of water samples cut it as a tool for surveying benthic communities? Mol. Ecol. 30, 3175–3188.

Antich, A., Palacín, C., Wangensteen, O.S., Turon, X., 2021b. To denoise or to cluster? That is not the question. Optimizing pipelines for COI metabarcoding and metaphylogeography. BMC Bioinformatics 22, 177.

Baird, D.J., Hajibabaei, M., 2012. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Mol. Ecol. 21, 2039–2044.

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basin (NE Spain). Aquat. Toxicol. 74, 3–19.

Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv. Genet. 17, 1–17.

Baselga, A., Fujisawa, T., Crampton-Platt, A., Bergsten, J., Foster, P.G., Monaghan, M.T., Vogler, A.P., 2013. Whole-community DNA barcoding reveals a spatio-temporal continuum of biodiversity at species and genetic levels. Nat. Commun. 4, 1–7.

Beermann, A.J., Zizka, V., Elbrecht, V., Baranov, V., Leese, F., 2018. DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors. Environ. Sci. Eur. 30, 1–15.

Bista, I., Carvalho, G.R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M., Creer, S., 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nat. Commun. 8, 1–11.

Bonada, N., Zamora-Munoz, C., Rieradevall, M., Prat, N., 2004. Ecological profiles of caddisfly larvae in Mediterranean streams: implications for bioassessment methods. Environ. Pollut. 132, 509–521.

Bonada, N., Prat, N., Resh, V.H., Statzner, B., 2006. Developments in aquatic insect biomonitoring: a comparative analysis of recent approaches. Annu. Rev. Entomol. 51, 495–523.

Boyer, F., Mercier, C., Bonin, A., Taberlet, P., Coissac, E., 2016. OBITools: a Unix-inspired software package for DNA metabarcoding. Mol. Ecol. Resour. 16, 176–182.

Cañedo-Argüelles, M., Gutiérrez-Cánovas, C., Acosta, R., Castro-López, D., Cid, N., Fortuño, P., Bonada, N., 2020. As time goes by: 20 years of changes in the aquatic macroinvertebrate metacommunity of Mediterranean river networks. J. Biogeogr. 47, 1861–1874.

Cordier, T., Lanzén, A., Apothéloz-Perret-Gentil, L., Stoeck, T., Pawlowski, J., 2019. Embracing environmental genomics and machine learning for routine biomonitoring. Trends Microbiol. 27, 387–397.

Cordier, T., Alonso-Sáez, L., Apothéloz-Perret-Gentil, L., Aylagas, E., Bohan, D.A., Bouchez, A., Lanzén, A., 2021. Ecosystems monitoring powered by environmental genomics: a review of current strategies with an implementation roadmap. Mol. Ecol. 30, 2937–2958.

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Bik, H.M., 2016. The ecologist's field guide to sequence-based identification of biodiversity. Methods Ecol. Evol. 7, 1008–1018.

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, 1–9.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Bernatchez, L., 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. Mol. Ecol. 26, 5872–5895.

Elbrecht, V., Vamos, E.E., Meissner, K., Aroviita, J., Leese, F., 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods Ecol. Evol. 8, 1265–1275.

Elbrecht, V., Braukmann, T.W., Ivanova, N.V., Prosser, S.W., Hajibabaei, M., Wright, M., Zakharov, E.V., Hebert, P.D.N., Steinke, D., 2019. Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ 7, e7745.

Ershova, E.A., Wangensteen, O.S., Descoteaux, R., Barth-Jensen, C., Præbel, K., 2021. Metabarcoding as a quantitative tool for estimating biodiversity and relative biomass of marine zooplankton. ICES J. Mar. Sci. 78, 783342–783355.

Fediajevaite, J., Priestley, V., Arnold, R., Savolainen, V., 2021. Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. Ecol. Evol. 11, 4803–4815.

Friberg, N., Bonada, N., Bradley, D.C., Dunbar, M.J., Edwards, F.K., Grey, J., Woodward, G.U.Y., 2011. Biomonitoring of human impacts in freshwater ecosystems: the good, the bad and the ugly. In: Advances in ecological research, vol. 44. Academic press, pp. 1–68.

Frøslev, T.G., Kjøller, R., Bruun, H.H., Ejrnæs, R., Brunbjerg, A.K., Pietroni, C., Hansen, A. J., 2017. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. Nat. Commun. 8, 1188.

Furse, M.T., Hering, D., Brabec, K., Buffagni, A., Sandin, L., Verdonschot, P.F., 2009. The Ecological Status of European Rivers: Evaluation and Intercalibration of Assessment Methods, vol. 188. Springer Science & Business Media.

Gardham, S., Hose, G.C., Stephenson, S., Chariton, A.A., 2014. DNA metabarcoding meets experimental ecotoxicology: advancing knowledge on the ecological effects of copper in freshwater ecosystems. In: Advances in Ecological Research, vol. 51. Academic Press, pp. 79–104.

Gleason, J.E., Elbrecht, V., Braukmann, T.W., Hanner, R.H., Cottenie, K., 2021. Assessment of stream macroinvertebrate communities with eDNA is not congruent with tissue-based metabarcoding. Mol. Ecol. 30, 3239–3251.

Goldschmidt, T., 2016. Water mites (Acari, Hydrachnidia): powerful but widely neglected bioindicators–a review. Neotrop. Biodivers. 2, 12–25.

Grigoropoulou, A., Schmidt-Kloiber, A., Múrria, C., 2022. Incongruent latitudinal patterns of taxonomic, phylogenetic and functional diversity reveal different drivers

of caddisfly community assembly across spatial scales. Glob. Ecol. Biogeogr. 31, 1006–1020.
Hajibabaei, M., Singer, G.A., Hebert, P.D.N., Hickey, D.A., 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 24, 167–172.
Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G.A.C., Baird, D.J., 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. PLoS One 6, e17497.
Hajibabaei, M., Porter, T.M., Robinson, C.V., Baird, D.J., Shokralla, S., Wright, M.T., 2019. Watered-down biodiversity? A comparison of metabarcoding results from DNA extracted from matched water and bulk tissue biomonitoring samples. PLoS One 14, e0225409.
Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B 270, 313–321.
Hering, D., Borja, A., Jones, J.I., Pont, D., Boets, P., Bouchez, 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.
Hulsen, T., 2021. BioVenn – an R and Python package for the comparison and visualization of biological lists using area-proportional Venn diagrams. Data Sci. 4, 51–61.
Huttunen, K.L., Väisänen, L.O., Paavola, R., Muotka, T., 2022. Partitioning of benthic biodiversity in boreal streams: contributions of spatial, inter-annual, and seasonal variability. Freshw. Biol. 67, 774–783.
Hynes, H.B.N., 1960. The Biology of Polluted Waters. Liverpool Univ. Press, Liverpool, UK (202 pp.).
Keck, F., Blackman, R.C., Bossart, R., Brantschen, J., Couton, M., Hürlemann, S., Altermatt, F., 2022. Meta-analysis shows both congruence and complementarity of DNA and eDNA metabarcoding to traditional methods for biological community assessment. Mol. Ecol. 31, 1820–1835.
Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., Weigand, A.M., 2018. 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: Advances in Ecological Research, vol. 58. Academic Press, pp. 63–99.
Leese, F., Sander, M., Buchner, D., Elbrecht, V., Haase, P., Zizka, V.M., 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. Environ. DNA 3, 261–276.
Macher, J.N., Vivancos, A., Piggott, J.J., Centeno, F.C., Matthaei, C.D., Leese, F., 2018. Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome c oxidase I primers. Mol. Ecol. Resour. 18, 1456–1468.
Mächler, E., Little, C.J., Wüthrich, R., Alther, R., Fronhofer, E.A., Gounand, I., Harvey, E., Hürlemann, S., Walser, J.-C., Altermatt, F., 2019. Assessing different components of diversity across a river network using eDNA. Environ. DNA 1, 290–301.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ 3, e1420.
Munné, A., Prat, N., 2011. Effects of Mediterranean climate annual variability on stream biological quality assessment using macroinvertebrate communities. Ecol. Indic. 11, 651–662.
Munné, A., Solà, C., Pagès, J., 2006. BIORI. Protocol d'avaluació de la qualitat biològica dels rius. Agència Catalana de l'Aigua (ACA), Departament de Medi Ambient i Habitatge, Generalitat de Catalunya. Barcelona.
Munné, A., Solà, C., Tirapu, L., Barata, C., Rieradevall, M., Prat, N., 2012. Human pressure and its effects on water quality and biota in the Llobregat River. In: The Llobregat: The Story of a Polluted Mediterranean River, pp. 297–325.
Múrria, C., Rugenski, A.T., Whiles, M.R., Vogler, A.P., 2015. Long-term isolation and endemicity of neotropical aquatic insects limit the community responses to recent amphibian decline. Divers. Distrib. 21, 938–949.
Múrria, C., Bonada, N., Vellend, M., Zamora-Muñoz, C., Alba-Tercedor, J., Elisa Sainz-Cantero, C., Vogler, A.P., 2017. Local environment rather than past climate determines community composition of mountain stream macroinvertebrates across Europe. Mol. Ecol. 26, 6085–6099.
Múrria, C., Väisänen, L.O., Somma, S., Wangensteen, O.S., Arnedo, M.A., Prat, N., 2020. Towards an Iberian DNA barcode reference library of freshwater macroinvertebrates and fishes. Limnetica 39, 73–92.
Múrria, C., Maceda-Veiga, A., Barata, C., Gomà, J., Faria, M., Antich, A., Arnedo, M.A., Bonada, N., Prat, N., 2024. From biomarkers to community composition: negative effects of UV/chlorine-treated reclaimed urban wastewater on freshwater biota. Sci. Total Environ. 912, 169561.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2020. vegan: community ecology package. R package version. 2.5-7. https://CRAN.R-project.org/package=vegan.
Pardo, I., García, L., Delgado, C., Costas, N., Abraín, R., 2010. Protocolos de muestreo de comunidades biológicas acuáticas fluviales en el ámbito de las Confederaciones Hidrográficas del Miño-Sil y Cantábrico. Convenio entre la Universidad de Vigo y las Confederaciones Hidrográficas del Miño-Sil y Cantábrico (65 pp.).
Pawlowski, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., Kahlert, M., 2018. The future of biotic indices in the ecogenomic era: integrating (e) DNA metabarcoding in biological assessment of aquatic ecosystems. Sci. Total Environ. 637, 1295–1310.
Pereira-da-Conceicoa, L., Elbrecht, V., Hall, A., Briscoe, A., Barber-James, H., Price, B., 2021. Metabarcoding unsorted kick-samples facilitates macroinvertebrate-based biomonitoring with increased taxonomic resolution, while outperforming environmental DNA. Environ. DNA 3, 353–371.
Poikane, S., Zampoukas, N., Borja, A., Davies, S.P., van de Bund, W., Birk, S., 2014. Intercalibration of aquatic ecological assessment methods in the European Union: lessons learned and way forward. Environ. Sci. Pol. 44, 237–246.
Porter, T.M., Hajibabaei, M., 2018. Scaling up: a guide to high-throughput genomic approaches for biodiversity analysis. Mol. Ecol. 27, 313–338.
Prat, N., García-Roger, E.M., 2018. Are there so many congeneric species of chironomid larvae in a small stream? J. Limnol. 77, 65–80.
Prat, N., Munné, A., 2014. Biomonitoreo de la calidad del agua en los ríos ibéricos: lecciones aprendidas. Limnetica 33, 47–64.
Prat, N., Rieradevall, M., 2006. 25-years of biomonitoring in two mediterranean streams (Llobregat and Besòs basins, NE Spain). Limnetica 25, 541–550.
Prat, N., Ward, J.V., 1994. The Tamed River.
Prat, N., Puig, M.A., Gonzalez, G., Millet, X., 1983. Chironomid longitudinal distribution and macroinvertebrate diversity along the Llobregat River (NE Spain). In: Memoirs of the American Entomological Society.
Puntí, T., Rieradevall, M., Prat, N., 2009. Environmental factors, spatial variation, and specific requirements of Chironomidae in Mediterranean reference streams. J. N. Am. Benthol. Soc. 28, 247–265.
Resh, V.H., Bêche, L.A., Lawrence, J.E., Mazor, R.D., McElravy, E.P., O'Dowd, A.P., Rudnick, D., Carlson, S.M., 2013. Long-term population and community patterns of benthic macroinvertebrates and fishes in Northern California Mediterranean-climate streams. Hydrobiologia 719, 93–118.
Rosenberg, D.M., Resh, V.H., 1993. Freshwater Biomonitoring and Benthic Macroinvertebrates (Eds). Chapman & Hall, New York (488 pp.).
Salinas-Ivanenko, S., Múrria, C., 2021. Macroecological trend of increasing values of intraspecific genetic diversity and population structure from temperate to tropical streams. Glob. Ecol. Biogeogr. 30, 1685–1697.
Serra, S.R., Cobo, F., Graça, M.A., Dolédec, S., Feio, M.J., 2016. Synthesising the trait information of European Chironomidae (Insecta: Diptera): towards a new database. Ecol. Indic. 61, 282–292.
Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050.
Tachet, H., Richoux, P., Bournaud, M., Usseglio-Polatera, P., 2010. Invertébrés d'eau douce. Systématique, biologie. CNRS Editions, Paris, France.
Tautz, D., Arctander, P., Minelli, A., Thomas, R.H., Vogler, A.P., 2003. A plea for DNA taxonomy. Trends Ecol. Evol. 18, 70–74.
Wangensteen, O.S., Palacín, C., Guardiola, M., Turon, X., 2018. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ 6, e4705.
Weigand, H., Beermann, A.J., Ciampor, F., Costa, F.O., Csabai, Z., Duarte, S., Ekrem, T., 2019. DNA barcode reference libraries for the monitoring of aquatic biota in Europe: gap-analysis and recommendations for future work. Sci. Total Environ. 678, 499–524.