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How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain)

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Abstract

Background: Bioassessment of rivers is a fundamental method to determine surface water quality. One of the groups most commonly employed as bioindicators of aquatic ecosystems are benthic macroinvertebrates. Their conventional assessment is based on morphological identification and entails several limitations, such as being time-consuming and requires trained experts for taxonomic identification. The use of genetic tools to solve these limitations offers an alternative way to evaluate rivers status. The use of environmental DNA (eDNA) metabarcoding has increased in recent years for different purposes, but its use in water quality evaluation is yet to be tested. Here, morphological and eDNA based inventories of macroinvertebrates were compared from the same seven sampling sites in the Upper Nalón River Basin (Asturias, Spain).

Materials & Methods: High-Throughput Sequencing (HTS) of the cytochrome oxidase subunit 1 (COI) gene was carried out on DNA from water samples using an Ion Torrent platform. Biotic water quality indices were calculated from morphological and molecular data and compared with independent physico-chemical habitat assessment to validate eDNA based approach.

Results: Highly positive and significant correlation was found between eDNA metabarcoding and morphological methods (Morphological and eDNA indices, $r = 0.798$, 5 degrees of freedom d.f., $P = 0.031$;) and a highly significant negative correlation was found between molecular and habitat quality indices (Stress score & eDNA, $\rho = -0.878$ and $P = 0.009$; Stress score & Visual, $\rho = -0.949$ and $P = 0.0002$).

Discussion: The similarity of results from the two approaches and the correlation of eDNA metabarcoding data with the habitat quality indices, suggest that eDNA performs as well as conventional methods for calculating biotic indices in this system, positioning eDNA metabarcoding of macroinvertebrate communities to transform how river bioassessment can be achieved.

Eva Garcia-Vazquez and Alba Ardura are co-senior authors, equal contribution to this article.

Conclusion: The usefulness of eDNA metabarcoding to assess rivers water quality based on macroinvertebrates assessment has been demonstrated in a dammed river basin.

KEYWORDS
bioassessment, conservation, genetics, invertebrates, rivers

1 | INTRODUCTION

Running waters provide a wide range of ecosystem services for human societies (Lim et al., 2016). Since they are the focus of human settlements, rivers are heavily exploited for diverse uses, such as water supply, irrigation, and electricity generation, thus being among the most impacted ecosystems on earth (Malmqvist & Rundle, 2002; Vörösmarty et al., 2010). Many restoration and conservation initiatives are focused on the aim of reaching a good ecological state in rivers, for a long-term sustainability of these essential ecosystems (AQEM Consortium, 2002). The preservation of aquatic ecosystems is legally binding for public administrations and private owners in most countries. In Europe, the main instrument for this purpose is the Water Framework Directive (WFD) (Directive 2000/60/EC) (Leese et al., 2016) that was established to achieve a good ecological status in all surface waters (Gabriels, Lock, De Pauw, & Goethals, 2010). The accomplishment of WFD requirements implies that regular river monitoring is conducted, and the water quality assessment is one of its main elements. Similarly, in the United States the National Water-Quality Assessment Program was implemented to support national, regional, and local information needs and decisions related to water quality management and policies (Moulton, Kennen, Goldstein, & Hambrook, 2002).

Several multimetric indices (MMIs) are employed across countries to measure water quality (Armitage et al., 1983; De Pauw & Vanhooren, 1983; Gabriels et al., 2010; Hawkes, 1997; Mondy, Villeneuve, Archaimbault, & Usseglio-Polatera, 2012; Moulton et al., 2002; Stark, 1993; Skriver, Friberg & Kirkegaard, 2000), and biological indicators are central in the panel of MMIs. Benthic macroinvertebrates are the most widely used species for bioassessment metrics, since they are key indicators of aquatic ecosystems' health (Carew et al., 2013), and as such, they are commonly used to identify impacted sites (Prat, Ríos, & Raúl Acosta, 2013). Water monitoring programs usually involve macroinvertebrate sampling in Europe, North America, and many other regions worldwide (Buss et al., 2015). Macroinvertebrates are collected from the river benthos and morphologically identified. Water quality indices are then calculated based on the presence, abundance, or proportion of indicator taxa (Birk, 2003).

Water quality indices often use different taxonomic levels. For example, the River Invertebrate Prediction and Classification System in the UK (Murphy, Davy-Bowker, McFarland, & Ormerod, 2013) uses the taxonomic rank of species or uses the rank of families instead as biotic indicators such as the Biological Monitoring Working Party (BMWP). The latter is one of the most widely employed indices in Europe (e.g., the British BMWP/ASPT: Average Score per Taxon (Birk, 2003)) and has been specifically adapted for the Iberian Peninsula (IBMWP; Ref; see also Figure S2). It applies different scores to macroinvertebrate families depending on their tolerance to organic pollution: The lower the tolerance, the higher the score (Spanish Regulation: Ministerio de Agricultura, Alimentación y Medio Ambiente, NIPO: 770-11-308-X, 2013; Based on BMWP index calculation (Hawkes, 1997)). The index value is the sum of the scores of the families present in a site.

These biomonitoring protocols based on macroinvertebrate species deal with logistic and financial limitations derived from sampling, based on the use of nets when the river is wadable, and taxonomic identification, depending on taxonomists, which is sometimes a difficult task because diagnostic characteristics of some species are not present in larval stages. Thus, morphological identification is both time-consuming and expertise-demanding (Lejzerowicz et al., 2015). Moreover, conventional sampling methods are invasive because the individuals are often removed from the river and killed for identification in the laboratory under a magnifying glass or the microscope (Clusa et al., 2017). Alternatively, the use of environmental DNA (eDNA), DNA that organisms expel or release in the environment (air, sediment, or water), could deal with these limitations of conventional assessments. eDNA can be amplified through molecular techniques to detect a species' presence, among other applications (Thomsen & Willerslev, 2015). The combination of high-throughput sequencing (HTS) with the taxonomic assignment of the obtained DNA sequences to reference sequence databases (Deiner et al., 2017) allows the noninvasive detection of many species from the same environmental sample (Borrell et al., 2017). This method has been called eDNA metabarcoding. The use of eDNA metabarcoding in ecological projects has increased over the last years, and many studies have successfully tested its use for different purposes, such as the detection of invasive (Borrell et al., 2017) and nuisance species (Zaiko et al., 2015), biodiversity monitoring (Deiner et al., 2017; Valentini et al., 2016).

The use of metabarcoding bulk samples for calculating water quality indices based on diatoms has been described (Apothéloz-Perret-Gentil et al., 2017), and also for calculating indices based on benthic macroinvertebrates as a key group for river water quality monitoring worldwide (Aylagas et al., 2018; Bista et al., 2017; Pawlowski et al., 2018). Molecular techniques (DNA barcoding and metabarcoding) have been compared with the morphological identification of benthic macroinvertebrates (i.e., identification based

on diagnostic morphological traits) (Elbrecht, Vamos, Meissner, Aroviita, & Leese, 2017; Emilson et al., 2017; Gibson et al., 2015; Stein, White, et al., 2014). Emilson et al. (2017) concluded from their results that DNA barcoding and morphological identification give the same key gradients of water quality in stream conditions. Stein, Martinez, Martinez, Stiles, Miller, and Zakharov (2014) found that DNA barcoding gives a deeper ecological signal than morphology, providing higher taxonomic richness as a result of the improvement of assignations in some groups (midges, mayflies, caddis flies, and black flies), since from DNA individuals from those groups were assigned to a species level. Elbrecht et al.(2017) used DNA extracted from bulk macroinvertebrate samples to demonstrate that metabarcoding represents a feasible method to identify these organisms, and if applied in streams, it would give results comparable to conventional protocols based on morphological identification for water quality assessment. The application of high-throughput sequencing to eDNA samples has shown to be useful for evaluating macroinvertebrate diversity in marine and freshwater ecosystems (Aylagas et al., 2014; Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Dowle, Pochon, Banks, Shearer, & Wood, 2016; Fernández et al., 2018; Lejzerowicz et al., 2015), but no studies have compared results obtained by eDNA metabarcoding and that of morphological assessments for water quality.

Nevertheless, the metabarcoding technique itself has limitations that should be addressed (Cristescu & Hebert, 2018; Shaw et al., 2016). One is the lack of universal primer sets (Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014). Although there are some tools available to find the most appropriate primer set for a range of organisms (Elbrecht & Leese, 2017), sometimes the universality of the primers is not enough to cover all groups of interest as they can be so different (e.g., from arthropods to cnidaria). The range of laboratory methods (Dopheide, Xie, Buckley, Drummond, & Newcomb, 2019) and different pipelines used for bioinformatics can determine the results (Pauvert et al., 2019). The still incomplete status of reference databases is other of the debated issues for application of metabarcoding in studies of aquatic biodiversity (Deiner et al., 2017). Weigand et al. (2019) did find large gaps in current Barcode databases for macroinvertebrates, and their coverage varies among aquatic taxonomic groups and regions in Europe. It is also worthy to mention eDNA degradation rate and transport in freshwater ecosystems need to be considered to interpret the results (Goldberg, Strickler, & Pilliod, 2015).

Water quality assessments based on macroinvertebrates,such as BMWP indices (Hawkes, 1997), require that at least the taxonomic identification at the family level could be easily implemented using eDNA metabarcoding. However, in practice the technique is still immature. Although it has been recently demonstrated that it is more sensitive than conventional morphological approaches for identifying macroinvertebrate families (Andújar et al., 2018; Fernández et al., 2018), the results may vary considerably depending on the specific genes and assignment criteria applied within bioinformatics pipelines (Fernández et al., 2018). In freshwater environmental samples, Fernández et al. (2018) found cytochrome oxidase subunit 1 (COI) gene as better suited than ribosomal 18S DNA for this purpose, at least partially due to the fact that there are more COI sequences of freshwater macroinvertebrates in reference databases. From the current state of the field (Andújar et al., 2018; Deiner et al., 2017; Fernández et al., 2018; Pawlowski et al., 2018), it is clear that further validation is needed for the application of eDNA metabarcoding in water quality surveys. Given the scarcity of river data, the validation should be focused on field studies, comparing eDNA-based biological indices with the same indices obtained from conventional (morphology-based) methodology. Comparisons with independent indicators of river water quality are also needed, in order to confirm the validity of the technique for river monitoring.

In this study, morphological and molecular approaches were used to calculate water quality indices based on benthic macroinvertebrates as bioindicators, in particular, IBMWP (the adaptation of the BMWP to the Iberian Peninsula). The results were compared between methods and with independent indices estimated from physical and chemical indicators of habitat quality. The upstream area of River Nalón Basin (south-central Bay of Biscay, northwest of Iberian Peninsula) was considered for field validation, as in previous studies (Fernández et al., 2018), because it contains locations of very different river water quality. Some samples were taken from pristine well-conserved streams inside the Biosphere Reserve of Redes (Natura 2000) (García-Ramos, Jiménez-Sánchez, Piñuela, Domínguez Cuesta, & López Fernández, 2006), and others from degraded river zones affected by dams; thus, different water quality scores were expected. The hypothesis of this study was that IBMWP indices obtained from eDNA metabarcoding and de visu conventional methods would be positively and significantly correlated with each other and that the more sensitive eDNA approach would provide stronger correlation with nonbiological indicators of water quality than the conventional biological method.

2 | METHODS

2.1 | eDNA and macroinvertebrate sample collection

Our study river was along the Upper Nalón River Basin (Figure 1), located in the central part of the region of Asturias (Bay of Biscay, Spain). Nalón-Narcea is the largest river basin in the area. The upper zone of the Nalón River belongs to the UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve and Natural Park of Redes. There are two big dams and associated reservoirs (Tanes and Rioseco) interrupting river connectivity, and it is expected to find cleaner waters upstream these barriers.

On 2 March, water samples were collected from seven sites (three 1-L replicates per site) from the bank of the river near the bottom of the water column. Each sample was collected in a separate decontaminated bottle that underwent UV irradiation for 30 min and sealed in laboratory conditions before use.

Immediately after taking the water samples, they were stored on ice until arriving to the laboratory where they were stored at

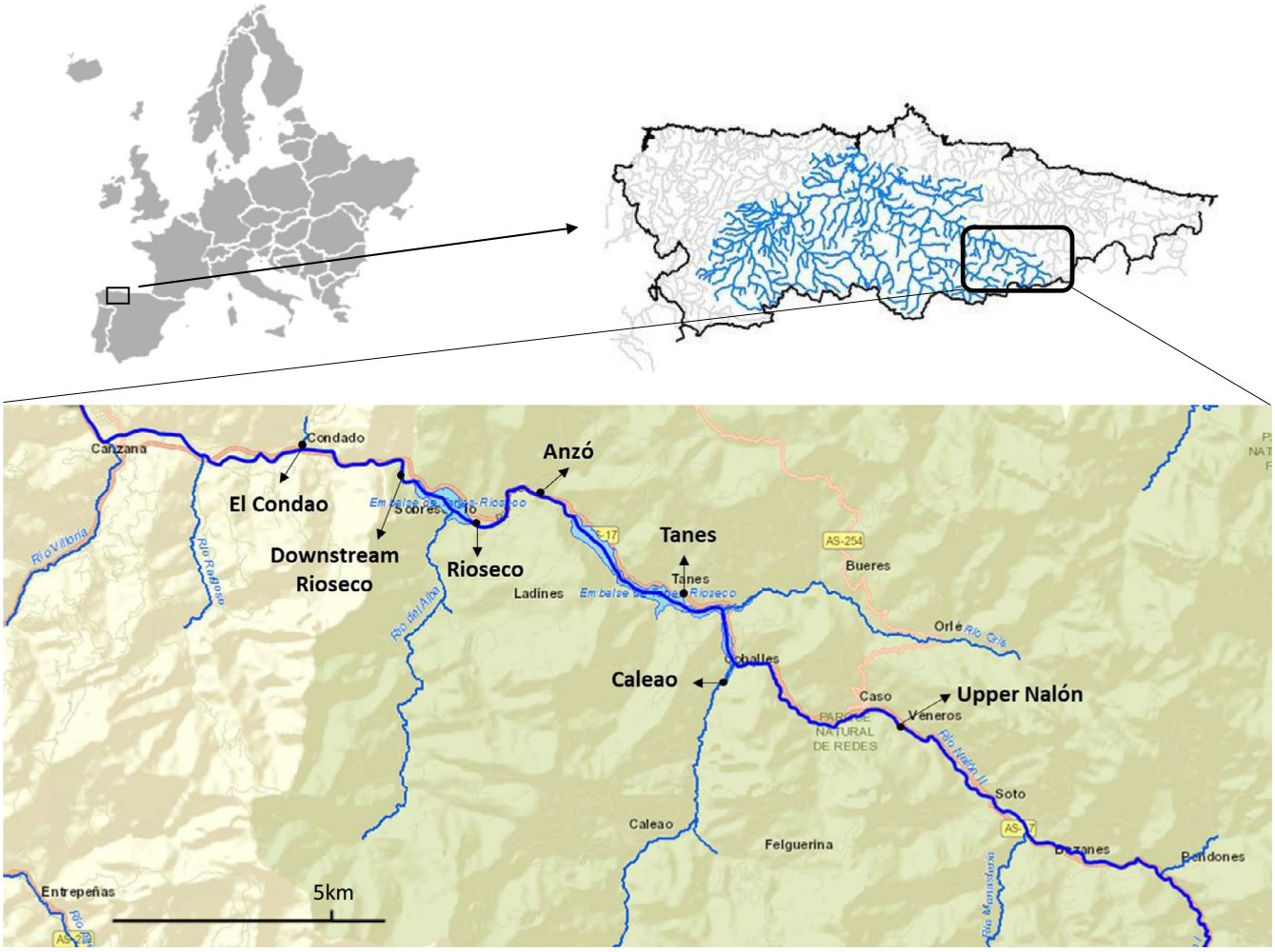

FIGURE 1 Sampling point location. Sampling points in Nalón River Basin in Asturias, northern Spain, marked with arrows. Two sites were located in the reservoirs present in the area (Tanes and Rioseco); two upstream of the impounded river (Upper Nalón and Caleao); one between the two reservoirs (Anzó); and two downstream the reservoirs (Downstream Rioseco and El Condao)

4°C until filtration (performed within 24 hr). Following water sampling, a macroinvertebrate sample was collected at each site using a Surber net following the official protocol of the Spanish Ministry of Agriculture for river water quality monitoring (Alba et al., 2005). Briefly, two Surber samples from different habitats on each location were taken from downstream to upstream sites. Before starting the netting protocol, the macroinvertebrates living on the water surface were first collected. Then, gravel, cobbles, blocks, and other materials within the sampling locations are sampled by keeping the bottom edge of the Surber net against the ground and dislodging organisms by removing them with hands for one minute from a length of 0.5 m of substrate upstream the net location. As the sampling units are completed, the net is emptied into the trays to avoid the loss of organisms. Then, the sample is observed, stones and large pieces of detritus are removed, and macroinvertebrates were conserved in 100% ethanol until further processing for identification.

To control possible contamination during the sampling, all the equipment, waders, and research gear that were in contact with the river water and banks were carefully cleaned with 10% bleach (5% of sodium hypochlorite concentration) before and after sampling each site. A closed bottle containing DI water (one per sampling point) was transported together with the sampling gear, opened in the field, and processed with the rest of eDNA water samples as a sampling negative control to monitor contaminations.

2.2 | Morphological identification and index calculation

Macroinvertebrate specimens were identified by an expert from the University of Oviedo, who categorized them down to a family level using an identification key (Tachet, Bournaud, & Richoux, 1987). For both morphological and molecular data, IBMWP index was calculated as described in the protocol (Alba et al., 2005), an adaptation of BMWP index (Hawkes, 1997) to Spanish Waters based on the tolerance of macroinvertebrate's families. IBMWP (Iberian Biological Monitoring Working Party) index was chosen for water quality bioassessment because it is the index employed in Spain, where the study took place. Briefly, each macroinvertebrate family has a score depending on its tolerance to organic pollution. The scores are from 1 to 10 points, 1 being the most and 10 the least tolerant. The final

value of the index is the sum of the scores of all the families present in a sample.

2.3 | Molecular analyses

2.3.1 | eDNA capture and extraction

Water samples were vacuum-filtered the same day of collection, immediately after arriving to the laboratory. A Supor® 200 Membrane Filter (Pall Corporation, Life Sciences) with 0.2 µm pore size and 47 mm diameter was used; one liter was filtered through one filter. The filtration process followed the protocol described in Clusa et al. (2017) to prevent contamination. Briefly, water samples were filtered in a room separated from the molecular laboratory in which only water samples are handled. The filtration apparatus was cleaned with 10% bleach (5% sodium hypochlorite concentration), triple-rinsed with DI water, and then exposed to 20 min of UV light in a PCR cabinet (normally utilized for pre-PCR experiments) between samples to prevent contaminations. The DI water carried to the field (negative field control) was filtered last, after the rest of river water samples. Finally, one liter of DI water was filtered as filtering negative control. Filters were manipulated with previously decontaminated (cleaned with bleach and UV as the filtration apparatus) forceps to place them in storage tubes. The filters were stored at −20°C until DNA extraction. Environmental DNA was extracted from filters (one extraction per filter) with the PowerWater® DNA Isolation Kit (MoBio Laboratories) under controlled airflow conditions using a laminar flow PCR cabinet. The extraction followed the manufacturer´s instructions. In total, three extractions per sampling point (one liter each) for each site (N = 21), extraction negative controls (N = 3; one per extraction round), one filtering negative control (N = 1), and the field negative controls (N = 7) were obtained at the end of the process.

2.3.2 | Positive control

A positive control was set up to verify that our laboratory methods and bioinformatics pipeline were able to correctly detect the taxa of interest. It was a known DNA mixture of nine species from different taxonomic groups and origin (one crustacean: *Caprella andreae*, one insect: *Rhithrogena* sp., two acorn barnacles: *Austrominius modestus* and *Chthamalus stellatus*, two goose barnacles: *Lepas anatifera* and *Lepas pectinata*, and three fish: *Oncorhynchus mykiss*, *Salmo salar*, and *Salmo trutta*) that may occur in aquatic environments at any life stage. This positive control was amplified together with the set of eDNA samples obtained from the field in order to have an assignment baseline (Table S1). The species from the Heptageniidae family (Rhithrogena sp.) can occur in our samples, as well as the salmonids. The presence of this species in the mock community was useful to inform chosen bioinformatics thresholds in the whole pipeline, as in other studies (i.e., Deiner et al., 2016). We took measures to avoid cross-contamination between the mock community used as positive control and the other eDNA samples, by adding the mock sample at the end of PCR preparation when all the other samples were sealed in the plate. We also used negative controls in all steps to monitor possible contamination.

2.3.3 | Library preparation

PCR and library preparation was done in the Scientific-Technique Services of the University of Oviedo (Spain). PCRs were carried out under controlled conditions inside a laminar flow cabinet. Negative controls from filtration, extraction, and PCRs were analyzed at the same conditions as the rest of the samples.

PCRs were carried out using the following primers for the mitochondrial region of COI gene: mlCOIintF (Leray et al., 2013) and jgHCO2198 (Geller, Meyer, Parker, & Hawk, 2013) modified with a PGM sequencing adaptor, the barcodes (one per sample) needed to differentiate the reads belonging to each water sample, and a "GAT" spacer (Table S2). Amplification was carried out in a total volume of 20 µl including Green GoTaq® Buffer 1X, 2.5 mM MgCl2, 0.25 mM dNTPs, 20 pmol of each primer, 4 µl of template DNA, 200 ng/µl of bovine serum albumin (Schrader, Schielke, Ellerbroek, & Johne, 2012), and 0.65 U of DNA Taq polymerase (Promega). PCR conditions in the Veriti Thermal Cycler (Applied Biosystems, Foster City, California) were 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 46°C for 15 s, 72°C for 10 s, and a final extension of 72°C for 3 min. Extraction (N = 3) and field (N = 1) negative controls were included in the PCRs, as well as the positive control. The amplification success was visually assessed on 2% agarose gel. PCR amplicons were purified from agarose gel using the Montage DNA Gel Extraction Kit (Millipore); quantified using the Qubit BR dsDNA Kit (Thermo Fisher Scientific); and double-checked in a Bioanalyzer 2100 (Agilent Technologies) to confirm the fragment size, the absence of by-products, and to do a more precise quantification.

2.3.4 | High-throughput sequencing

All samples that had positive amplification (i.e., no negative controls were sequenced) were diluted down to 26 pmol for preparing an equimolar pool with them. The pool was processed by liquid emulsion PCR in the One Touch System using the Ion PGM™ OT2 Supplies Kit (Life Technologies) following the manufacturer's instructions. The sample was loaded in the Ion "314" Chip (Life Technologies) and sequenced employing the Ion Torrent Personal Genome Machine (Life Technologies), following the specifications in the protocol Ion PGM™ Sequencing Kit. Low-quality and polyclonal sequences were filtered automatically, and the PGM adaptor was trimmed within the Ion Torrent Suite Server.

2.4 | Environmental stressors

Six physico-chemical variables (pH, conductivity, dissolved oxygen [O_2], temperature, oxygen saturation, and ammonium [NH_4]) were measured for each sampling point before taking the samples (both water and macroinvertebrates) to avoid disturbing the water.

At each sampling site, physical and chemical variables were recorded using a multiparametric probe (YSI Professional Plus Multiparameter Water Quality Instrument). Obtained values were categorized as "good/not good" based on the reference values defined by Pardo, Abraín, Gómez-Rodríguez, and García-Roselló (2010) for Cantabrian Confederation Rivers (Alba et al., 2005). In addition to the physico-chemical variables, three other stressors were considered: the number of inhabitants in the nearest villages (up to 5 km upstream) discharging wastewaters in the river: score 0–1, 1 for >300 inhabitants; degree of substrate modification: score 0–1, 1 for modifications such as excessive sediments caused from impounded waters or works, artificial river bed (e.g., concrete), etc.; and water regime disturbances caused by damming (i.e., water releases to control reservoir water levels): score 0–1. For each sampling point, the environmental stress was calculated from the scores obtained in the first principal component axis of a PCA including all the environmental measures (PC1) as a proxy for the environmental status.

2.5 | Bioinformatics analysis

Low-quality and polyclonal (sequence made by two different parents of DNA template) sequences were automatically filtered out, and the PGM adaptor was trimmed within the PGM software. Qiime software (Caporaso et al., 2011) 1.9.1 version was used to split the "fastq" files into constituent. fna and.qual files using "convert_fastqual_fastq. py" python script, and to filter sequences by quality and size (minimum and maximum size of 250–400 and quality score of 25) using "split_libraries.py" python script. Then, primer trimming was done with PRINSEQ v0.20.4 software (Schmieder & Edwards, 2011). Not specific chimera removal was performed. It was based on the threshold criteria for sequence alignment against the reference database.

COI gene reference database was constructed from NCBI COI sequences using the workflow developed by Baker (2017) using all COI sequences except the ones from environmental samples contained in the NCBI database. Then, BLAST alignment was done against this database with the settings described by Fernandez et al. (2018) as optimal for this gene and taxonomic groups (maximum *E*-value = 10^{-50}; minimum percent identity = 90.0 and 97 to assign at family and species level, respectively; and minimum percent query coverage = 0.9), employing "assign_taxonomy. py" python script without clustering or dereplication, taking into account all the sequences and haplotypes obtained. Finally, OTU (Operational Taxonomic Unit) tables with a 90% OTU threshold, a list of OTUs obtained in each sample and the number of sequences assigned to them (Table S3) were constructed with the algorithm "fromTaxassignments2OtuMap.py." In downstream analysis (index calculation and statistics), families represented by 1 sequence (singletons) were removed from the OTU table.

Operational Taxonomic Units corresponding to the taxonomic groups Annelida, Arthropoda, Mollusca, Cnidaria, and Platyhelminthes and considered in the IBMWP index were filtered from the OTU table in Microsoft Excel (2013), and then, they were given the corresponding family scores and IBMWP index was calculated as the sum of all the family scores, following IBMWP methodology (Table 2).

Each of the three replicates for the seven sites was processed separately. The detected taxa list used for each site to calculate indexes scores resulted from summing the taxa across each sample replicate, after OTU table construction (Table 2).

2.6 | Statistical analysis

All statistical analyses were implemented in PAST software (Hammer, Ryan, Hammer, & Harper, 2001).

To test for a correlation between sampling methods, data normality was first checked from the Shapiro–Wilk tests. According to the results, parametric (ANOVA, *t* tests of independent or paired groups) or nonparametric (Kruskal–Wallis, Mann–Whitney, Wilcoxon) tests were employed to compare groups of samples. Similarly, Pearson's *r* or Spearman's rho tests were employed for determining correlations between normal and non-normal datasets, respectively.

To test the similarity between sampling points regarding dams influence in macroinvertebrates' families, we have used nonmetric multidimensional scaling (nMDS). A general representation of the community similarity present in the different samples from eDNA metabarcoding and morphologic methods was obtained through a 2D scatter plot, employing the Bray–Curtis similarity index for the distances and 9,999 bootstraps. A Shepard plot assessing the goodness of fit of the scatter plot was also constructed, and the stress of the two axes were calculated (Figure S1).

Principal component analysis was performed for the environmental variables and stressors considered, after normalization of the vectors, using variance–covariance option. The scores of the locations in the first component PC1 were employed as a proxy of environmental stress.

3 | RESULTS

3.1 | High-throughput sequencing output

Raw sequencing data comprised a total of 2,650,693 sequences distributed across 21 water samples—three replicates per site, seven sites—and one positive control (Table 1). All the negative controls were below quantification limits and thus were assumed to be evidence that no substantial contamination occurred in the field and laboratory. They were not included in the sequencing pool because they had no quantifiable DNA. All the species included in the positive control were detected after our applied bioinformatics workflow, confirming the robustness of the sequencing and analytical pipeline. However, the number of reads per species in this positive control was variable and not related to the amount of DNA template employed. Less than 10 sequences were assigned to *Austrominius modestus*, *Caprella andreae*, *Chthamalus stellatus*, and *Salmo salar*. For *Caprella andreae* and *Salmo salar*, small number of reads was expected because only 0.05 ng of DNA was added in the control,

TABLE 1 High-throughput sequencing output. Number of raw, filtered (after quality and size filtering), and assigned sequences

Sampling point	Sample	Raw	Filtered	Assigned	Macroinvertebrates assigned
Caleao	C1	127,379	74,816	11,259	7,441
	C2	137,869	71,586	12,099	3,661
	C3	113,859	53,644	10,092	6,918
Upper Nalón	N1	133,220	85,908	30,348	24,568
	N2	124,524	70,749	6,934	2,562
	N3	70,068	35,737	7,002	708
Tanes	T1	125,211	74,247	2,916	419
	T2	55,575	28,054	1,331	59
	T3	112,924	68,646	1,946	58
Anzó	A1	119,638	69,257	24,045	14,764
	A2	81,893	46,929	20,334	16,916
	A3	55,250	29,982	6,433	5,478
Rioseco	R1	156,094	105,070	92,117	91,528
	R2	143,941	80,729	16,123	11,234
	R3	39,810	23,620	7,192	5,339
Downstream Rioseco	DR1	173,045	106,887	13,962	6,468
	DR2	165,446	99,494	6,409	2,222
	DR3	56,160	32,446	2,675	740
El Condao	EC1	153,362	81,701	4,465	1,018
	EC2	264,839	148,637	9,654	1,605
	EC3	47,683	28,271	2,243	425
Positive control	PC	192,903	132,026	131,825	126,857
Total		2,650,693	1,548,436	421,404	330,988

Note: Macroinvertebrates assigned: total of sequences assigned to target macroinvertebrate families (maximum *E*-value = 10^{-50} and minimum percent identity = 90.0 in BLAST alignment tool within Qiime pipeline (Caporaso et al., 2011)).

but 0.5 and 5 ng of DNA were added for *Austrominius modestus* and *Chthamalus stellatus*, respectively (Table S1). The result in these two species, both acorn barnacles, was probably due to primer bias that was confirmed in silico with 3 and 4 mismatches for *A. modestus* and *C. stellatus*, respectively.

The raw sequences are available on NCBI's Sequence Read Archive (SRA accession: SRP128681) with the BioSample number SAMN08295300. After application of quality and size filters, 58.42% of the sequences were recovered (Filtered). Out of these sequences, 27.21% were assigned down to a family level with the settings chosen for taxonomic assignation (Table S3). The three water samples taken from each point yielded similar but not identical information regarding the number of reads per species, although at family level, the information was generally consistent among replicates (Table S3). A considerably high number of sequences (70.5% of the assigned sequences) were from macroinvertebrate groups (Table 1).

3.2 | Detection of macroinvertebrate families in field samples

A total of 57 macroinvertebrate families listed in the Iberian version of the IBMWP index (Alba et al., 2005) (Figure 2) were detected from either molecular or morphological methods in the field samples (Table 2). Of these 57 families, 26 were identified from both molecular and visual methods, while 13 and 18 were detected only from visual and eDNA techniques, respectively (Table 2).

With the morphological method, between 6 and 17 families were found from each sampling site, and between 7 and 25 were detected using molecular methods (Table 2). The number of families detected by each method for each site was not significantly different (Wilcoxon test $W = 16$, normal approximate $t = 1.156$, $p = .247$).

3.3 | Biotic Indices and environmental stressors

The correlation between index values inferred from morphological and eDNA metabarcoding approaches was positive and statistically significant for both species and family level (family level: $r = .798$, 5 *df*, $p = .031$; species level: $r = .765$, 5 *df*, $p = .04$). The water quality obtained from molecular data was equal or higher than that obtained from conventional sampling (Figure S2).

Several physico-chemical parameters (Table 3) were out of the range considered as acceptable (good) in Spanish directives. In the PCA, the two first components composed more than 70%

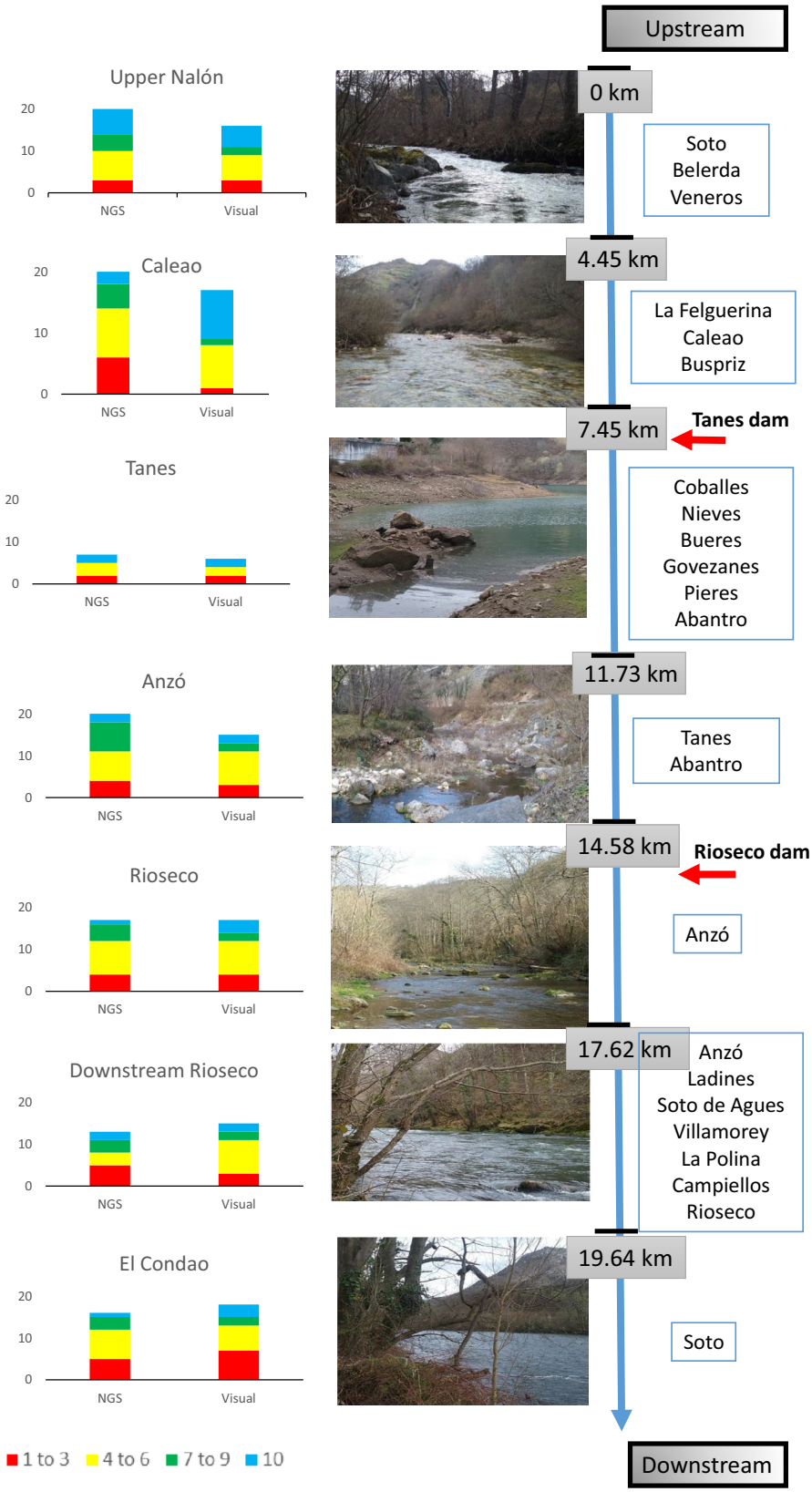

FIGURE 2 Macroinvertebrate families. Number of macroinvertebrate families in each sampling point, with their correspondent classifications from the IBMWP index (1–10, for most to least tolerant families so worst to best water quality) grouped in different colors based on the tolerance value. Results from eDNA (HTS) and morphological traits (visual) are presented. The villages discharging along the surveyed river sectors are shown at right. The situation of the dams is marked with red arrows. The distance (km) between the Upper Nalón point and the rest of the sampled points is shown

of the total variance. PC1 accounted for 44.2%, PC2 for 29.5%, and PC3 for 18.6% of the variance. The scores of each location in the PC1 (Table 3) were taken as a proxy of environmental stress. The correlations between that proxy and the biological water quality indices were negative and significant for eDNA and visual assessments (stress score and eDNA, family level: $\rho = -0.878$ and $p = .009$/species level: $\rho = -0.794$ and $p = .032$; stress score and visual, $\rho = -0.949$ and $p = .0002$).

The differences in ecological status among sampling points are evidenced in the MDS graph (Figure 3). The dataset included the

TABLE 2 Benthic macroinvertebrates

Consensus lineage				Caleao		Upper Nalón		Tanes		Anzó		Rioseco		Downstream Rioseco		El Condao		Family Score
Phylum	Class	Order	Family	G	V	G	V	G	V	G	V	G	V	G	V	G	V	
Annelida	Clitellata	Hirudinea	Glossiphoniidae	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3
Arthropoda	Insecta	Arachnida	Acariformes	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4
Arthropoda	Insecta	Coleoptera	Chrysomelidae	0	5	0	1	0	1	0	1	0	14	0	1	0	1	4
Arthropoda	Insecta	Coleoptera	**Elmidae**	0	0	0	1	0	0	94	18	0	46	0	9	0	2	5
Arthropoda	Insecta	Coleoptera	Haliplidae	0	0	0	0	0	0	0	1	0	0	0	0	0	0	4
Arthropoda	Insecta	Coleoptera	Gyrinidae	0	0	0	3	0	0	0	0	0	0	0	0	0	1	3
Arthropoda	Insecta	Coleoptera	Hydraenidae	102	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Arthropoda	Crustacea	Amphipoda	Gammaridae	0	0	0	0	0	0	0	0	0	2	0	2	0	0	2
Arthropoda	Insecta	Diptera	**Anthomyiidae**	26	0	10	0	0	0	0	0	0	0	0	1	0	0	4
Arthropoda	Insecta	Diptera	Athericidae	0	0	0	0	0	0	0	0	0	1	0	0	0	0	10
Arthropoda	Insecta	Diptera	**Ceratopogonidae**	0	5	0	0	0	0	0	0	10	0	10	0	10	0	4
Arthropoda	Insecta	Diptera	**Chironomidae**	3,143	10	17,103	33	269	4	4,268	62	93,585	125	5,710	9	154	232	2
Arthropoda	Insecta	Diptera	Culicidae	40	0	13	0	0	0	14	0	13	0	10	0	10	0	2
Arthropoda	Insecta	Diptera	Empididae	3,867	0	0	0	10	0	0	0	0	0	0	0	0	0	4
Arthropoda	Insecta	Diptera	Ephydridae	10	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Arthropoda	Insecta	Diptera	**Limoniidae**	109	3	0	1	0	0	0	1	0	5	0	1	0	1	4
Arthropoda	Insecta	Diptera	Psychodidae	0	0	14	0	0	0	10	0	10	0	0	0	0	0	4
Arthropoda	Insecta	Diptera	**Simuliidae**	1,169	0	323	15	10	1	10	21	10	0	25	0	11	0	5
Arthropoda	Insecta	Diptera	Thaumaleidae	10	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Arthropoda	Insecta	Diptera	**Tipulidae**	0	0	0	0	84	0	0	0	0	0	0	3	13	0	5
Arthropoda	Insecta	Ephemeroptera	**Baetidae**	10	101	6,424	68	0	0	44	185	45	375	0	7	0	7	4
Arthropoda	Insecta	Ephemeroptera	**Caenidae**	0	1	0	0	0	0	0	0	25	1	0	0	14	15	4
Arthropoda	Insecta	Ephemeroptera	**Ephemerellidae**	23	0	0	0	0	0	2,338	0	279	8	0	2	0	0	7
Arthropoda	Insecta	Ephemeroptera	Ephemeridae	0	1	0	0	0	0	0	0	0	1	0	0	0	26	10
Arthropoda	Insecta	Ephemeroptera	**Heptageniidae**	7,642	17	832	116	19	1	10	4	0	0	16	0	0	0	10
Arthropoda	Insecta	Ephemeroptera	Leptophlebiidae	24	0	13	0	11	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Odonata	**Calopterygidae**	48	0	0	0	0	0	25	0	10	0	0	0	12	2	8
Arthropoda	Insecta	Plecoptera	**Chloroperlidae**	0	3	14	25	0	1	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Plecoptera	**Leuctridae**	17	4	1,729	8	0	0	0	0	56	0	0	0	0	0	10
Arthropoda	Insecta	Plecoptera	**Nemouridae**	30	0	10	6	0	0	0	0	0	0	0	0	0	0	7
Arthropoda	Insecta	Plecoptera	**Perlidae**	15	4	10	3	0	0	0	0	0	0	0	0	0	0	10

(Continues)

TABLE 2 (Continued)

Consensus lineage				Caleao		Upper Nalón		Tanes		Anzó		Rioseco		Downstream Rioseco		El Condao		Family Score
Phylum	Class	Order	Family	G	V	G	V	G	V	G	V	G	V	G	V	G	V	
Arthropoda	Insecta	Plecoptera	Perlodidae	0	0	16	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	**Brachycentridae**	10	0	14	0	0	0	14	183	0	8	12	2	0	5	10
Arthropoda	Insecta	Trichoptera	Calamoceratidae	0	0	0	0	0	0	0	0	0	0	0	1	0	5	10
Arthropoda	Insecta	Trichoptera	Goeridae	13	0	11	0	0	0	0	0	0	0	0	0	13	0	10
Arthropoda	Insecta	Trichoptera	**Hydropsychidae**	0	1	240	15	0	0	15	44	0	9	0	3	0	0	5
Arthropoda	Insecta	Trichoptera	Hydroptilidae	0	0	0	0	0	0	15	0	32	0	0	0	0	0	6
Arthropoda	Insecta	Trichoptera	Lepidostomatidae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	Leptoceridae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	Limnephilidae	104	0	24	0	0	0	514	0	14	0	12	0	13	0	7
Arthropoda	Insecta	Trichoptera	Philopotamidae	0	0	10	0	0	0	22	0	0	0	0	0	0	0	8
Arthropoda	Insecta	Trichoptera	**Polycentropodidae**	0	0	0	0	0	0	13	1	0	8	0	0	0	21	7
Arthropoda	Insecta	Trichoptera	**Psychomyiidae**	0	1	12	0	0	0	16	0	11	0	12	0	0	0	8
Arthropoda	Insecta	Trichoptera	**Rhyacophilidae**	0	0	0	1	0	0	14	1	0	0	0	1	12	0	7
Arthropoda	Insecta	Trichoptera	**Sericostomatidae**	22	2	19	2	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Malacostraca	Decapoda	Astacidae	0	0	0	0	0	0	0	0	0	0	12	0	0	0	8
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	14	0	12	0	0	0	12	0	14	0	306	0	40	0	X
Mollusca			Ferrisia	0	4	0	0	0	0	0	1	0	11	0	10	0	0	6
Mollusca	Gastropoda	NA	Ancylidae	39	0	26	0	0	0	102	0	229	0	0	0	22	0	6
Mollusca	Gastropoda	NA	**Bithyniidae**	0	0	0	0	0	4	0	4	0	6	14	0	0	16	3
Mollusca	Gastropoda	NA	**Hydrobiidae**	13	0	0	0	0	0	1,913	1	205	0	10	0	24	0	3
Mollusca	Gastropoda	NA	Neritidae	0	0	0	0	0	0	0	0	0	0	0	0	12	0	6
Mollusca	Gastropoda	NA	**Physidae**	0	0	0	0	0	0	0	0	0	1	0	4	12	28	3
Platyhelminthes	NA	Tricladida	Dugesiidae	0	0	0	0	0	0	0	0	13	0	0	0	15	0	5
Mollusca	Gastropoda	NA	Planorbidae	0	0	0	1	0	0	0	0	0	0	0	0	0	3	3
Mollusca	Bivalvia	Veneroida	Sphaeriidae	0	0	0	0	0	0	0	0	0	0	0	0	0	6	3
Oligochaeta			**All families**	379	0	3,165	0	914	0	992	0	660	0	138	0	76	0	1
Number of families				25	17	23	16	7	6	21	15	18	17	13	15	17	17	
Index Value				149	121	144	99	39	34	109	79	80	97	69	78	78	94	

Note: Macroinvertebrate families found per sampling point with molecular (G: number of sequences amplified) and morphological (V: number of individuals visually assessed) approaches, and their punctuation following the official protocol for IBMWP index calculation. Bolded are the families detected by both techniques.

TABLE 3 Environmental stressors in the river sites considered

Sampling point	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Rioseco	El Condao
pH	7.38	**8.04**	**8.12**	**8.02**	**9**	**8.12**	**8.5**
Conductivity (µs)	125.7	127.1	168,6	123.6	134.2	120.5	121.9
Dissolved O_2 (mg/L)	8.9	9.2	8	8.76	10	9.3	8.9
Temperature (°C)	8.3	8.5	10.4	8.3	10.3	8.5	8.8
O_2 saturation (%)	80	86	76	77	94	83	80
NH_4 (mg/L)	**1.73**	0.39	0.39	0.27	**3.4**	**0.56**	0.26
Human population	293	203	**330**	187	8	**870**	51
Substrate modification	No	No	**Yes**	No	No	No	No
Water regime alteration	No	No	**Yes-high**	**Yes**	**Yes**	**Yes**	**Yes**
Environmental stress score, from PC1	−1	−0.357	1	0.191	−0.196	0.284	0.191

Note: Physico-chemical variables indicating in bold the values that do not fit within the reference values classified as "Good ecological state" for this type of river (Pardo et al., 2010). Human population pressure: number of inhabitants of the villages discharging in the river up to 5 km upstream a sampling site (in bold, the sites with >300 inhabitants); substrate modification and water regime alterations. Environmental stress score was estimated from the scores of each location in the first principal component of PCA.

macroinvertebrate families (1 presence, 0 absence) and five physico-chemical parameters: pH, conductivity, dissolved O_2 (mg/L), temperature, and NH_4 (mg/L). The R^2 values for axis 1 and axis 2 were .762 and .029, respectively (Figure 3a). The Shepard plot (Figure S1) had a stress of 0.089 (Figure 3a). The ecological values obtained from eDNA and visual methods for each sampling point were closely grouped together (Figure 3). The samples were roughly grouped according to their situation above or below the dams, being together the two upstream samples (Upper Nalón and Caleao) and relatively close to each other those located between and below dams, while the two samples directly affected by impounded waters (Tanes and Rioseco) were apart (Figure 3a). The analysis made without the environmental stressors gave a similar picture (Figure 3b; this analysis has a stress value of 0.092, axis 1 with R^2 = .499 and axis 2 with R^2 = .149), although only Tanes was clearly apart and Rioseco was grouped with the rest of points affected by dams.

For the investigation of the specific effect of dams on the water quality measured from macroinvertebrates, the IBMWP index was compared between the group of samples located upstream the dams (Caleao, Upper Nalón) and the rest of points affected by them. For the eDNA dataset, the difference in means was highly significant (t = 3.796 with 5 df and p = .012). For the visual dataset, the difference in medians was marginally significant with p < .10 (Mann–Whitney U with z = −1.74, p = .08).

4 | DISCUSSION

The results of the current study, in particular the correlation obtained between eDNA-BMWP values and independent indices based on environmental stressors in a dammed river basin, demonstrate the usefulness of eDNA metabarcoding to assess macroinvertebrate communities for river water quality assessment. The eDNA metabarcoding technique employed here, based on COI amplicons, gave a good taxonomic coverage with an overall 70.5% of the assigned sequences belonging to the targeted families of macroinvertebrates, and similar IBMWP indices were obtained from eDNA metabarcoding and morphological techniques. Given the correlation between methodologies, we show that eDNA metabarcoding is adequate for detecting water quality differences between points with different water quality values, from pristine to highly degraded areas. These results support that eDNA from macroinvertebrates can be used for water quality assessments within the same river continuum and confirm that site-level information is retrieved. Thus, the evidence here is that the spatial scale inferred using eDNA from macroinvertebrate communities is adequate for the aim of calculating family-based indices in this river system among our sampling localities.

Importantly, using eDNA metabarcoding of macroinvertebrate communities we could discern between clean and polluted waters, indicating that eDNA surveys of macroinvertebrate communities have the potential to comply with the requirements established by WFD to distinguish clean and highly degraded areas to determine their respective conservation and restoration management priorities. Our study revealed dams have a large effect on water quality estimated from macroinvertebrate communities, from both datasets (eDNA metabarcoding and morphological), with the site values grouped together with or without consideration of the environmental stressors (Figure 3). Using eDNA metabarcoding, significant differences were detected between the water quality indices found in the upstream and downstream groups of samples, while the morphological indices provided a lower t-value between them—as expected from less marked differences in morphological IBMWP indices between clean

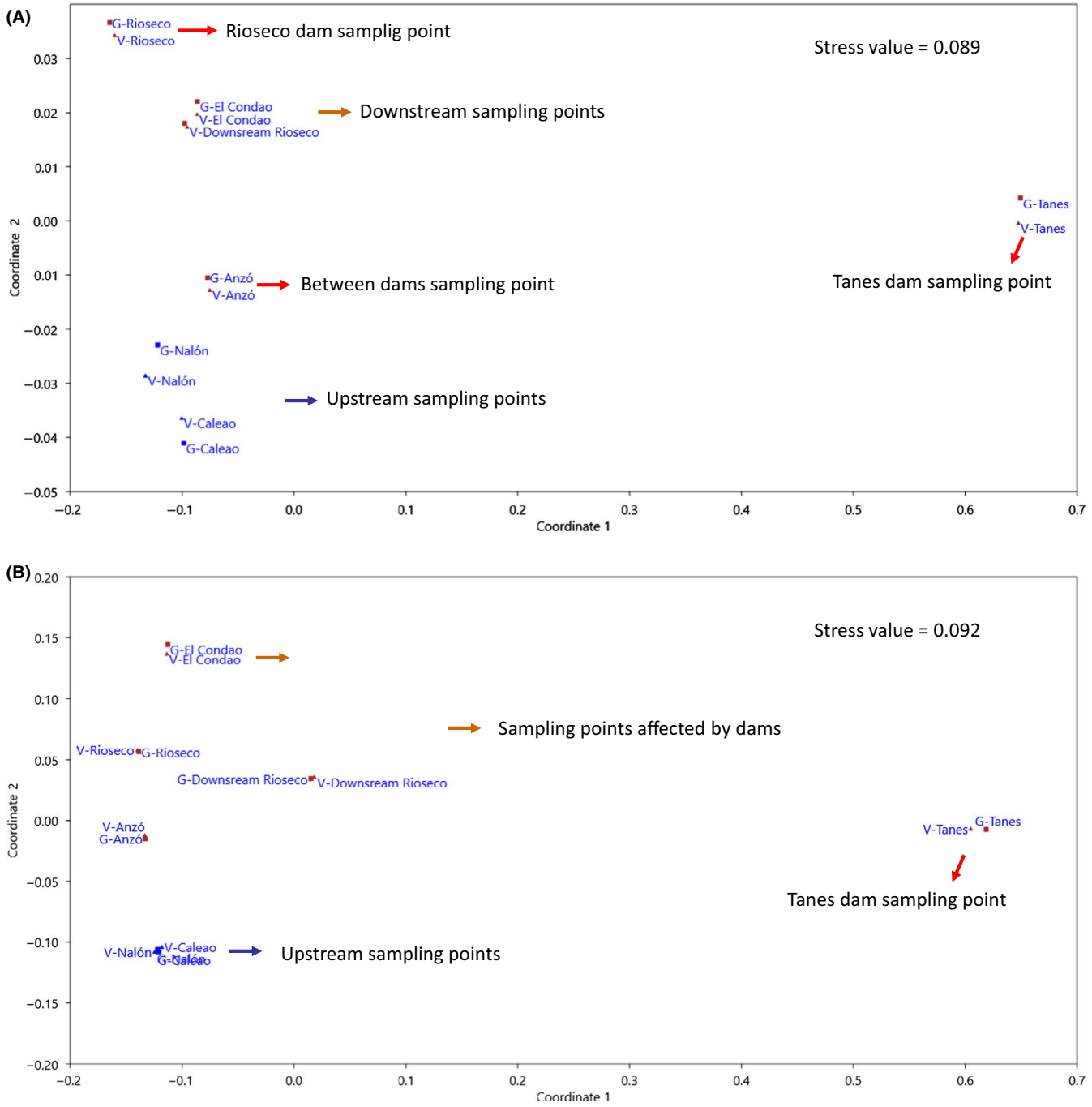

FIGURE 3 Scatter plot of the communities inhabiting the different samples obtained from multidimensional scaling analysis, with (a, above) and without (b, below) environmental stressors (V-sampling point: visual; G-sampling point: eDNA)

and disturbed sites. This supports the higher sensitivity of eDNA for detecting macroinvertebrate families revealed in other studies (Deiner et al., 2016; Fernández et al., 2018).

Some differences were found between the eDNA metabarcoding and morphological approaches in the estimated IBMWP assessment index. Indeed, part of them can be explained by the fact that the information obtained from each technique detected different taxa. While the visual assessment is based on evaluating macroinvertebrates inhabiting several square meters of river, the eDNA could, in theory, come from a broader spatial scale because it may be transported downstream suspended in the running water (Deiner & Altermatt, 2014). Using eDNA in water samples could thus be employed to bioassess longer river sections with lower sampling effort. Using eDNA metabarcoding for macroinvertebrate communities has other potential advantages. For example, conventional sampling has limitations in some sites where the access to the river bottom is difficult, or where trapping macroinvertebrates with a net is impractical due to low or inexistent current, but, in contrast, sampling eDNA only requires taking water samples, which is much easier and less invasive than kick-sampling macroinvertebrates. Finally, the

metabarcoding approach does not rely upon taxonomist expertise to assess each sample (Stein, Martinez, et al., 2014) and is life stage- and body size-independent.

The dissimilarities in macroinvertebrates' family detection depending on the technique can be explained by the differences in how the two methods sample macroinvertebrate communities. Some families´ eDNA amplified were not detected by the morphological assessment. This can be explained by the higher sensitivity of eDNA metabarcoding to detect scarce and low-density populations (Bohmann et al., 2014; Goldberg et al., 2015; Rees, Maddison, Middleditch, Patmore, & Gough, 2014). However, the IBMWP values obtained using eDNA metabarcoding were higher than those calculated by the morphological technique in the points located in clean waters (Caleao, Upper Nalón, and Anzó), but less so in disturbed points. This could be explained by eDNA being affected by the environmental quality and chemistry of the water affecting eDNA degradation (Collins et al., 2018; Eichmiller, Best, & Sorensen, 2016). On the other hand, some families physically found from the sampling sites were not detected by eDNA (Glossiphoniidae, Chrysomelidae, Haliplidae, Gyrinidae, Gammaridae, Athericidae, Ephemeridae, Calamoceratidae, Lepidostomatidae, Leptoceridae, Ferrisia, Planorbidae, Sphaeriidae). This could be explained by several reasons. For example, some families may shed less DNA into the water because they have hard exoskeletons/shells (e.g., Planorbidae and Sphaeriidae), or perhaps they have a low metabolic activity (i.e., less secretions) and leave less DNA traces. In addition to these biological reasons, some technical problems persist in HTS. COI primers used here are notorious for not performing well in mollusks in general. It is important to remark some limitations of the current technique's state of the art. Possible primer mismatches or template competition for primers affinity may limit amplification success. PCR inhibition could cause false negatives of scarce sequences. In this study, we used preventative measure to reduce inhibition, and this may still have caused issues in detection. Perhaps the most frequently reported problem is the scarcity of reference sequences in databases for some taxa (Dowle et al., 2016; Fernández et al., 2018; Zaiko et al., 2015). The use of COI as a barcode marker has also been contested in some studies (Deagle et al., 2014). However, for the taxonomic level (family) and groups (river macroinvertebrates including Annelida, Mollusca, Insecta) required for water quality indices, it seems that the molecular approach using a region of COI gene as barcoding marker is robust (Andújar et al., 2018; Aylagas et al., 2014; Carew et al., 2013; Deiner et al., 2016; Fernández et al., 2018; Lejzerowicz et al., 2015; Stein, Martinez, et al., 2014). This robustness is not substantially established for eDNA metabarcoding in general, but from the outcome of our study, it gives results comparable to the morphological approach. Thus, at least in this case, the reference database employed, barcode marker, and bioinformatics pipeline do not seem to limit water quality bioassessment using IBWMP index in water samples. The alternative use of shotgun sequencing technology, although more expensive, could improve the methodology avoiding biases associated with primers and aiming to do biomass estimations (Dowle et al., 2016).

Both eDNA and visual techniques may give false positives. False positives attributed to conventional methodologies such as IBMWP are mainly due to inaccurate taxonomic classification of juveniles and larvae, and the proportion of these false positives in morphological classification ranges between 22.1% (Stribling, Pavlik, Holdsworth, & Leppo, 2008) and 33.8% (Haase, Pauls, Schindehütte, & Sundermann, 2010). For eDNA, they could come from individuals inhabiting far upstream in cases of downstream transport of their eDNA, or from remains of dead individuals, although the average life of eDNA in freshwater has been estimated to be quite short (a few days to two weeks) (Hering et al., 2018). False positives may also occur in other steps of the eDNA analysis, for example, in the laboratory or in the bioinformatics pipeline (Ficetola, Taberlet, & Coissac, 2016). In this study, we used strict laboratory conditions and positive and negative controls to account for any potential contaminations. We did not sequence our negative controls as is now more commonly practiced but recommend that this is done in the future. Even though samples can fail to detect DNA based on limits of quantification, upon sequencing reads can still be detected (Deiner et al., 2018). To understand the impact of our bioinformatics pipeline, we used a positive control and filtered sequences by quality, as recommended for this type of studies (Ficetola et al., 2016). However, too strict filters could produce false negatives. The results from the positive control analysis showed that, although singletons should indeed be removed from OTU tables for downstream analysis, removing all the species represented by <10 sequences could produce false negatives, since we have obtained less than 10 sequences from four of the nine species of the positive control. As explained above, two of these species were represented in very small DNA quantity, and the other two were the acorn barnacles and the scarce sequences could be due to a primer bias (Deagle et al., 2014). For the particular purpose of this study, where freshwater insects, mollusks, and annelids are the targets, underrepresentation of marine acorn barnacles would not interfere with the results but could have attributed to missed detection of certain families as it is the case of the mollusks.

Considering together our study with previous works, a recalibration of molecular indices would be recommended for adapting biotic water quality indices to molecular data, as proposed for marine water quality indices (Lejzerowicz et al., 2015). Some international projects are already developing tools to apply metabarcoding in bioassessment, such as DNAqua-Net (Leese et al., 2018). However, this is a large undertaking and progress is being made (Blackman et al., 2019). While the techniques continue to be developed, the existing techniques based on morphological assessment could be complemented with eDNA metabarcoding assessments of macroinvertebrates, after their validation and implementation in different areas. Indeed, the specific characteristics of the eDNA in running waters should be considered, such as higher sensitivity and a broader spatial scale application, before applying the results in management actions.

As a technical remark, in the present study quality indices were calculated following the Spanish official protocol for water quality assessment (Ministerio de Agricultura & Alimentación y Medio Ambiente, 22013), based on presence/absence data, where family is the taxonomic level used. For this reason, 90% identity was the cut-off selected for sequence assignation against reference databases,

since Hebert, Cywinska, Ball, and Jeremy (2003) deemed it enough for family assignments through COI gene barcoding for most taxonomic groups. However, not all water quality indices are based on families, and the taxonomic level demanded for monitoring programs varies significantly among countries (Bonada et al., 2005). If the index requires species-level assignation, 97%–99% identity will be more suitable (Hebert et al., 2003). On the other hand, quantitative elements are also currently required within the WFD. Although some studies have tried to relate the sequence reads with individual abundance metrics (Klymus, Marshall, & Stepien, 2017; Ushio et al., 2018), it is still not possible to do it with the metabarcoding analysis carried out in this study. Nevertheless, the presence/absence of data seems to be sufficient for a precise assessment in many current indices (Aylagas et al., 2014; Eichmiller et al., 2016; Elbrecht & Leese, 2015; Lejzerowicz et al., 2015; Pawlowski et al., 2018); thus, the approach here employed has potential for application in the calculation of other indices employed in different regions of the world for river bioassessment (De Pauw & Vanhooren, 1983; Skriver et al., 2000; Wfd-Uktag, 2014). The method would be as informative as conventional methodology for many indices because it is possible to obtain other abundance data (i.e., total number of taxa, number of EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa, species richness). In the current study, estimating abundance was not an aim, because the macroinvertebrates' index IBMWP is calculated from the presence/absence data as it is officially carried out in Spain (Alba et al., 2005). However, if abundance-based indices are sought this eDNA method is not a solution because currently, it cannot estimate the number of individuals of different families and species.

In conclusion, eDNA metabarcoding of macroinvertebrate communities will expectedly transform river biomonitoring. Even though this new approach has limitations and its implementation still requires future development, it also has many advantages, such as minimum sampling effort, high sensitivity, species-level resolution that barcoding can often provide, and noninvasive sampling method. Implementing a quick standardized protocol that could be done routinely is a challenge, but evidence is mounting that we may in the future improve river biomonitoring with the use of eDNA-based tools.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw sequences are available on NCBI's Sequence Read Archive (SRA accession: SRP128681) with the BioSample number SAMN08295300.

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SUPPORTING INFORMATION

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