

Original Articles

DNA-based marine benthic assessment methods can perform as morphological ones, but an intercalibration is needed

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ABSTRACT

There is an increasing need for legislation worldwide to monitor and assess the ecological status of marine ecosystems, due to increasing pressures from human activities. The costs and time of traditional analyses are high, while methods based on molecular analysis could reduce these costs and shorten evaluation times significantly. Some biotic indices used to assess the status need reference conditions to be applied. Hence, our objective here is to develop reference conditions for a molecular-based benthic index (M-gAMBI), which can be compared with a morphological one (M-AMBI). Using 6 years of data from estuaries and coasts, we have been able to set reference conditions for five water types, including richness, diversity and AMBI. However, one problem is the absence of the whole human pressure gradient in all water types, making difficult to validate the reference conditions. Based on the results of this research, the M-gAMBI index could be considered suitable for ecological status assessment since it meets most of the criteria for considering a genomics-based index suitable. However, it is considered necessary to advance in (i) improving the detection of errors in genomic methods and similarity with morphological methods, and (ii) an intercalibration exercise, allowing adjusting quality class boundaries and determining the ecological status in an equivalent manner using both methods.

1. Introduction

The last few decades have seen an increasing need for legislation worldwide to monitor and assess the ecological status of marine ecosystems, due to increasing pressures from human activities (Borja and Elliott, 2021). One of the first such legislations was the Clean Water Act, in 1972, in USA (Tomczyk, et al., 2023). However, it was after the approval of the European Water Framework Directive (WFD; European Commission, 2000) that a plethora of ecological status assessment methods for various biological elements were developed (Birk et al., 2012), and started to be used routinely and massively in Europe and beyond (Borja et al., 2024).

In general, all these assessment methods have in common the need to evaluate the ecosystem condition of different biological elements, responding to multiple pressures. For doing that, they include single indicators, or a combination of several, selected based on criteria such as sensitivity to changes in the environment, representativity of different habitats, responsivity to human pressures, ecological meaningfulness, ease of measurement and ability to set reference conditions and quality thresholds, among others (Smit et al., 2021).

Among the various biological elements (e.g. phytoplankton, macroalgae, seagrasses, fish, etc.), macroinvertebrates are one of the most used groups of organisms, targeted by dozens of biotic indices developed to assess ecological status, mostly based on traditional morphological identification (Díaz et al., 2004; Pinto et al., 2009; Borja et al., 2024). However, in recent years, the use of DNA-based methods to identify species has opened the door for developing new ecological status assessment methods (Bourlat et al., 2013; Leese et al., 2016; Hering et al., 2018; Pawlowski et al., 2018).

Two of the most widely used benthic assessment methods in the world (see Anaisce et al., 2023), are AZTI's Marine Biotic Index (AMBI; Borja et al., 2000) and the Multivariate AMBI (M-AMBI; Muxika et al., 2007). M-AMBI includes AMBI in its calculation, as well as richness and Shannon diversity. It also requires regional specific reference conditions corresponding to high ecological status (i.e. absence or minimal human pressure), to determine the actual status of a water body (Muxika et al., 2007), needing large amounts of samples to be set (Borja et al., 2012; Santibañez-Aguascalientes et al., 2020)). However, the costs of traditional analyses are high, as is the time required for species identification, while methods based on molecular analysis could reduce these costs and

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shorten evaluation times significantly (Bourlat et al., 2013).

In the case of AMBI, it has already been demonstrated that the genomic AMBI (gAMBI) version provides results like those of the conventional index (Aylagas et al., 2014, 2018). However, genomic methods provide very different richness and diversity values for the calculation of M-AMBI, so the use of a genomic M-AMBI (M-gAMBI) requires the development of specific reference conditions (Aylagas et al., 2018), which, eventually, would allow the results to be intercalibrated with the traditional analysis, as required by the WFD (Poikane et al.,

2014). Intercalibration is the comparison and harmonization of assessment methods between countries, under the WFD, to ensure comparability in the final assessment of the ecological status in Europe (European Commission, 2000). Since M-AMBI is the method officially approved for its use in the north of Spain (European Commission, 2018, 2024) the eventual use of M-gAMBI in the future, to reduce the cost of analysis and obtain results more quickly, requires setting adequate reference conditions and a response to pressures or management measures like that of the official method.

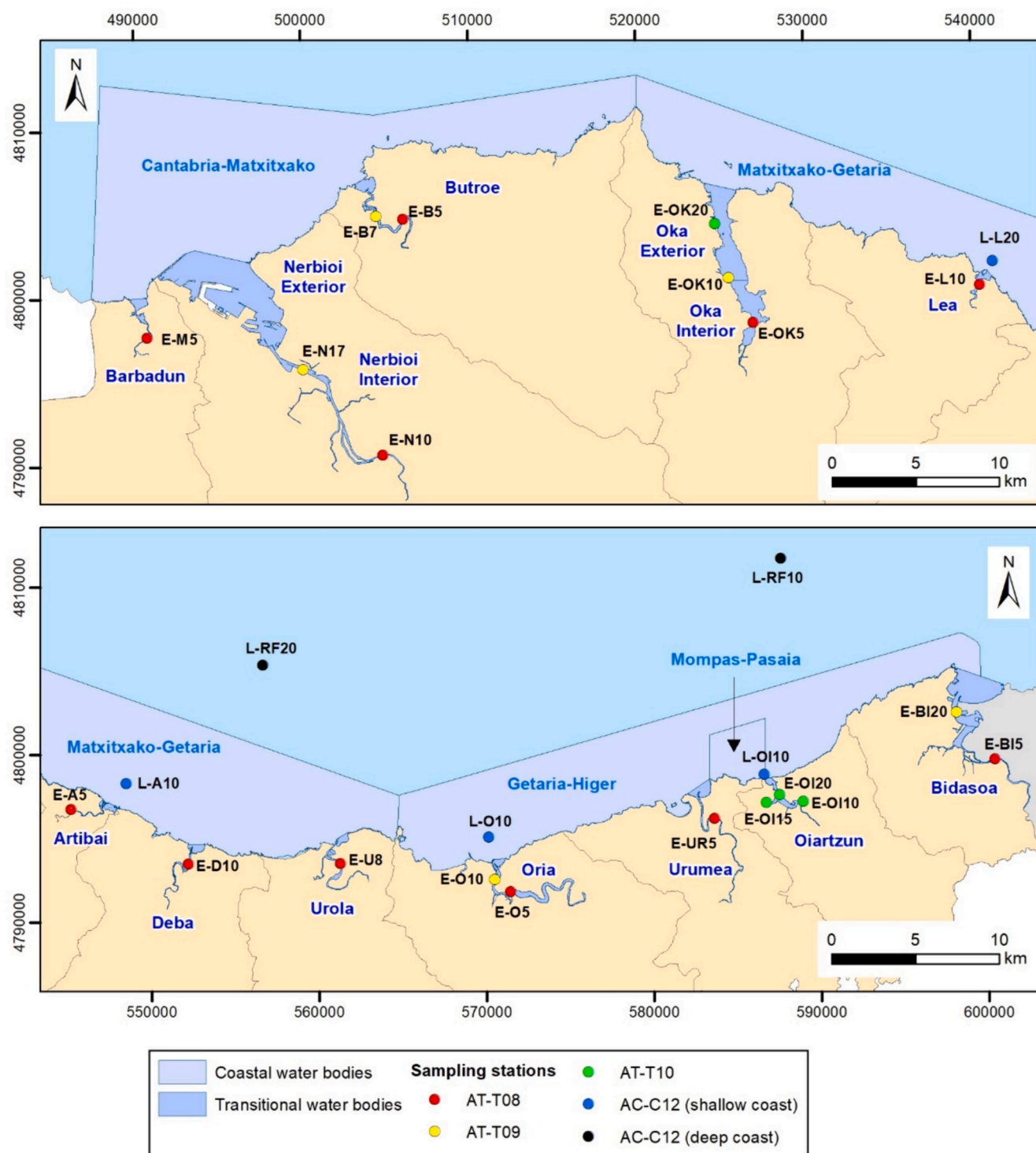


Fig. 1. Map showing the position of the sampling locations, within the “Monitoring network of transitional and coastal waters of the Basque Country” (North of Spain). The names and types of the associated water bodies are shown (see also Table 1).

Hence, the objective of this research is to set reference conditions for M-gAMBI application, to replicate the results obtained using the assessment method based on morphological identification, and to explore the possibility of intercalibrating M-gAMBI with M-AMBI in the future.

2. Methods

2.1. Sampling stations and protocol

To establish a certain pressure gradient, 26 stations were selected for sampling in this study (Fig. 1), all of which were included in the framework of the monitoring program for transitional and coastal waters of the Basque Country. The sampling covered both transitional waters and coastal waters, from various water typologies, depending on the salinity in transitional and depth in coastal stations, as well as their associated benthic communities (Borja et al., 2004). Hence, there are three transitional types: (i) AT-T08, with 11 stations, has a salinity of 0–18, characterized by a community of *Cerastoderma edule-Scrobicularia plana*; (ii) AT-T09, with 5 stations, has a salinity of 18–30, with a community of *Venus fasciata*; and (iii) AT-T10, with 4 stations, has a salinity of 30–34, with a community of *Abra alba*; and two coastal types: (i) AC-T12, with 4 stations in shallow waters (20–50 m), characterized by a community of *Tellina tenuis-Venus fasciata*; and (ii) AC-T12, with 2 stations in deep waters (around 120 m), with a community of *Amphiuira* sp (Fig. 1).

To minimize biases when comparing M-AMBI to M-gAMBI, the same sampling protocol was followed for both morphology and DNA-based taxonomic identification: (i) the sampling season was in winter (January–March), between 2018 and 2023; (ii) sampling in the intertidal zone was carried out in the area closest to the zero-tide level, using a metal square of 0.5 x 0.5 x 0.15 m; (iii) sampling in the subtidal zone was carried out with a Van Veen dredge (0.1 m²); (iv) at each location, six replicates were taken, with three for traditional morphological analysis and three for genomic analysis; (v) all replicates were sieved through a 1 mm mesh; (vi) once the material was sieved, fixation was carried out in a formalin solution for samples for morphological identification, while samples for molecular analysis were kept in water and, once in the lab, stored at –20 °C until preprocessing.

2.2. Morphological identification

Samples were examined with a stereomicroscope, and identification was carried out to the species level whenever possible. The identification was always undertaken by the same taxonomy specialists from Insub society. Species identification was supported by the European Register of Marine Species (www.marbef.org/data/erms.php) and NODC or ITIS codes (<http://www.itis.usda.gov/>). All individuals of each taxon were counted, except for colonial organisms, which were noted as present.

Abundance data were transformed into density data (number of individuals per square meter) based on the sampled surface area, either with a dredge or square.

2.3. Genomic identification

For samples intended for genomic identification, there was a preprocessing step where the obtained samples were processed before DNA extraction depending on the sediment type. Samples were divided into two types: (i) in medium to coarse sediment (sand or stone), decantation was performed to separate the organic fraction from the sediment in several steps, and the sample was homogenized for extraction in a blender or mortar depending on its volume; and (ii) in muddy sediment, a complete homogenization of the sample was carried out using a large-volume blender and a representative sample of the sample (12–15 g) was collected. In both cases, a fraction of the sample was fixed in 96–100 % ethanol and stored at –20 °C for preservation until

subsequent DNA extraction.

For DNA extraction, samples preserved in ethanol were centrifuged at 2000 × g for 5 min, removing the liquid. DNA from solid material was extracted using the commercial DNeasy PowerMax Soil kit (Qiagen, Cat. No. / ID:12988–10), following the manufacturer's recommendations, with a modification during the lysis process; a sample fraction (up to 10 g) was digested by adding the Proteinase K enzyme and incubating for 16–18 h at 56 °C with agitation.

The next day, different purification steps were continued until obtaining DNA, specifically using the DNeasy PowerClean Pro Cleanup Kit (Qiagen, Cat. No. / ID: 12997–50) following the manufacturer's instructions, to eliminate PCR inhibitors. DNA extracts were quantified using the dsDNA BR assay kit used in a Qubit fluorometer.

In the end, normalized aliquots were prepared at a concentration of 10 ng µl^{–1}, diluted with Ultrapure water. The extracts were stored at –80 °C.

Once this step was completed, the genetic libraries were prepared for sequencing, following the Illumina “16S Metagenomic Sequencing Library Preparation” protocol. Briefly, aliquots of each sample were amplified by a first PCR using universal primers for the “Leray fragment” region of the mitochondrial gene Cytochrome Oxidase I (COI) (Leray et al., 2013) with the addition of a specific “linker” sequence that allowed a second amplification with Illumina adapters.

The amplification was carried out under the following conditions: 98 °C for 3 min; 30 cycles of 98 °C for 15 s, 46 °C for 30 s, and 72 °C for 45 s; and, finally, 72 °C for 5 min.

In the second amplification (98 °C 3 min. and 8 cycles of 98 °C 30 s, 55 °C 30 s, and 72 °C 30 s; plus 72 °C 5 min), each sample was labeled with a different combination of primers specific to the linker and with a specific index for each sample (“index” or “tag” sequence), allowing the sequencing of hundreds of samples at once and their subsequent separation in the sequence data analysis process.

After each amplification, the product was purified using magnetic beads (Ampure XP, Beckman Coulter) to remove any remaining primers. The result of the amplification of each sample was quantified using Qubit, and based on this quantification, an equimolar mixture was prepared, i.e., with an equal amount of DNA amplicons from each sample.

The generated libraries were sent to an external service (CNAG, National Center for Genomic Analysis, Barcelona) for sequencing on the MiSeq with v3 reagents with 300 cycles Paired-End reads.

From the raw data obtained from the sequencing facility (in FASTQ format), the following steps were taken to produce reads corresponding to the complete COI amplicon, eliminating problematic sequences, and grouping the remaining ones according to taxonomic classification:

- (i) The FASTQC tool was used to visualize quality along the reads and check for the presence of remaining linker or adapter sequences.
- (ii) Pair-read overlapping, primer removal, and initial quality filtering were performed using *vsearch* (v2.17.0; Rognes et al., 2016) and *cutadapt* (v3.5; Martin, 2011). A maximum of 40 discrepancies when overlapping paired reads was accepted. Sequences with incomplete primers or more than two bases difference between the sequence and the primers were discarded. Overlapping reads with a size of less than 274 or more than 333 bases were also discarded.
- (iii) Overlapped sequences were de-replicated using *vsearch* and subjected to clustering into operational taxonomic units (OTUs) using SWARM (v2.2.1; Mahé et al., 2015), according to default settings, followed by the removal of singletons (unique sequences with a single read) and filtering of chimeric sequences (both de novo and based on reference sequences) using *vsearch*. Additionally, a post-clustering correction was carried out using LULU (Froslev et al., 2017) with a similarity limit of 97 %.

- (iv) Taxonomic classification was performed using CREST (v4.3.6; Lanzén et al., 2012) and the Midori v253 + DARN database (<https://github.com/xapple/crest4>) using default parameters (>97 % similarity with the reference sequence required for classification to species level and 95 % for genus). Probable contaminants were identified and removed based on abundance profiles and extraction blanks, sequence batch-wise, using decontam (Davis et al., 2018), removing 37 unique sequences representing 894,000 reads (1.8 % of the total). The remaining reads were grouped based on their taxonomic classification instead of single sequences corrected by SWARM and LULU.
- (v) All taxa without taxonomic classification or classified as another kingdom other than Metazoa were removed from the list (24 % of the reads). Additionally, a filtering based on taxonomic assignments was performed to remove taxa with a probable origin from pelagic, parasitic, terrestrial, or freshwater environments. Approximately 11 million additional reads (29 % of the total) were removed.
- (vi) Replicas with fewer than 1,000 reads after this filtering step (20 out of 401) were discarded for not having sufficient resolution to estimate the composition, structure, and diversity of the studied community.

Once the metabarcoding data were obtained, in terms of reads (as an approximation to abundance) per replicate and taxonomic level, tables of data were created to be more comparable with those existing for morphological analysis. This is because metabarcoding provides data for a multitude of taxa at different taxonomic levels, from species to phylum, reaching 1,289 taxa (26.9 million reads).

To achieve this, all taxonomic levels higher than the genus were removed from the list, except for the phyla Nematoda and Nemertea, the class Insecta, the subclass Oligochaeta, and the order Diptera. Also, all reads from taxa of each of the groups mentioned above were summed to make them comparable to the results of morphological identification, which does not achieve higher resolution for these groups. If there were duplicates (species or genera with any spelling error, etc.), the reads were summed, and one taxon was retained. Finally, all taxa that summed fewer than 10 reads in the total of samples and replicates were removed, as they were considered not representative of the fauna present in the samples.

After these corrections, the number of taxa remaining in the metabarcoding data were 310 (22.9 million reads).

2.4. Reference conditions and data evaluation

Both morphology-based data and metabarcoding data were used to calculate AMBI, richness, diversity, and M-AMBI, along with their respective genomic-based versions (g-). The reference conditions for M-AMBI for each typology were those intercalibrated in the WFD (Muxika et al., 2007; European Commission, 2018), included in Table 1. In the case of M-gAMBI, it is necessary to set adequate reference conditions. Several methods can be used to set those references (Borja et al.,

2012). It is well-known that benthic communities can show unimodal response – low richness at low pressures, increase of richness at intermediate pressure levels (decrease of richness at the further increase of pressure)–, after the model of Pearson and Rosenberg (1978). Hence, the most widely used methods to set reference conditions for those metrics are a spatial approach (choosing near-natural sites) or using modelling, or even a combination of both (Muxika et al., 2007). Despite this, in the case of M-gAMBI, they were determined based on the maximum values of richness and diversity and the minimum of AMBI corresponding to each type of the five investigated, as done in some places when there is not sufficient data (Borja et al., 2019). For the reference conditions of bad status, 6, 0 and 0 values were set for AMBI, richness and diversity, respectively.

In a few stations and years (E-N10 and E-U8 in 2019, E-L10 in 2020, and E-O10 in 2021), for various reasons, only one replicate remained available after filtering. As this may affect richness and diversity data, these samples were excluded from the analysis. Additionally, the metabarcoding data from E-OK20 in 2020, 2021, and 2023 were found to be inconsistent with morphological data (maybe due to contamination of the sample), so they were also excluded.

For statistical analysis, regressions, and correlation determination between morphological and metabarcoding analyses were performed, considering correlations with $p < 0.05$ as significant.

To assess whether differences in diversity and richness values were caused by differences in the determination of taxa present in samples, depending on whether determination is done by genomic methods or traditional morphological methods, cluster-type multivariate analyses were conducted. At the sample level, triangular matrices were constructed: (i) from presence/absence data of identified taxa (after applying the above-mentioned filters) based on the Jaccard similarity index (Jaccard, 1908); and (ii) from relative abundance data (number of individuals of each taxon divided by the total number of individuals identified in the respective sample, for data from traditional identification; and number of reads of each taxon divided by the total reads of the respective sample, for data from genomic analysis), based on the Bray-Curtis similarity index (Bray and Curtis, 1957).

The constructed triangular matrices were used to generate hierarchical dendrograms, using group average as the clustering method (Clarke and Warwick, 2001). The results of each matrix were subjected to SIMPROF Ltd routines (Clarke and Gorley, 2006), which verify which groupings were statistically significant.

Finally, SIMPER analyses were conducted to determine the taxa contributing most to the obtained ordination, either by their relative weight in (or contribution to) intragroup similarity or by their relative weight in intergroup dissimilarity (Clarke, 1993). This analysis identified taxa that were consistently identified by one of the techniques (morphology or taxonomy) and not by the other. It also identified taxa that may be characteristic of some of the groups.

All these analyses were carried out using Primer v6.1.12 software from Primer e-Ltd.

Table 1

Reference conditions for S (richness), H' (Shannon diversity) and AMBI for transitional and coastal water types, from the Basque Country (Muxika et al., 2007) and intercalibrated quality class boundaries for M-AMBI (European Commission, 2018). H: High, G: Good, M: Moderate, P: Poor, B: Bad ecological status.

| Category | Water Type | Salinity/ Depth | Associated community | Reference conditions | | | M-AMBI quality class boundaries | | | |
|---------------------|------------|---------------------|--|----------------------|-----|------|---------------------------------|-------|------|------|
| | | | | S | H' | AMBI | H/G | G/M | M/P | P/B |
| Transitional waters | AT-T08 | 0–18 | Cerastoderma edule-Scrobicularia plana | 13 | 2.5 | 2.8 | ≥0.77 | ≥0.53 | 0.38 | 0.20 |
| | AT-T09 | 18–30 | Venus fasciata | 32 | 3.8 | 2.0 | | | | |
| | AT-T10 | 30–34 | Abra alba | 40 | 3.5 | 2.1 | | | | |
| Coastal waters | AC-T12 | Shallow: 20–50 m | Tellina tenuis-Venus fasciata | 42 | 4 | 1 | ≥0.77 | ≥0.63 | 0.38 | 0.20 |
| | AC-T12 | Deep: 70–120 m | Amphiura | 130 | 5.7 | 1 | | | | |

3. Results

3.1. Comparison of single variables

In [Supplementary Material](#) (SM) Figure SM1, the results of comparing AMBI, diversity, and richness calculated using traditional and genomic methods can be observed. In all three cases, the correlation is highly significant ($p < 0.001$), with an r^2 of 0.6 in AMBI, 0.48 in richness, and 0.37 in diversity. Although these results are promising for calculating a genomic M-AMBI, it should be noted that these data

combine all the information from estuary and coast, whereas the comparison should be made by water types ([Fig. 2](#)).

In this case, AMBI, richness, and diversity of water types AT-T08 and AT-T10 show a significant correlation ($p < 0.05$) between morphological and metabarcoding data. In type AT-T09, only richness is significant, while in AC-T12 shallow water, diversity is significant. In the case of AC-T12 deep water, all three variables are close to being significant, but possibly the low number of data points prevents it.

One problem observed in [Fig. 2](#) is that the pressure gradient (if considering its measure with AMBI) is not complete in all types studied.

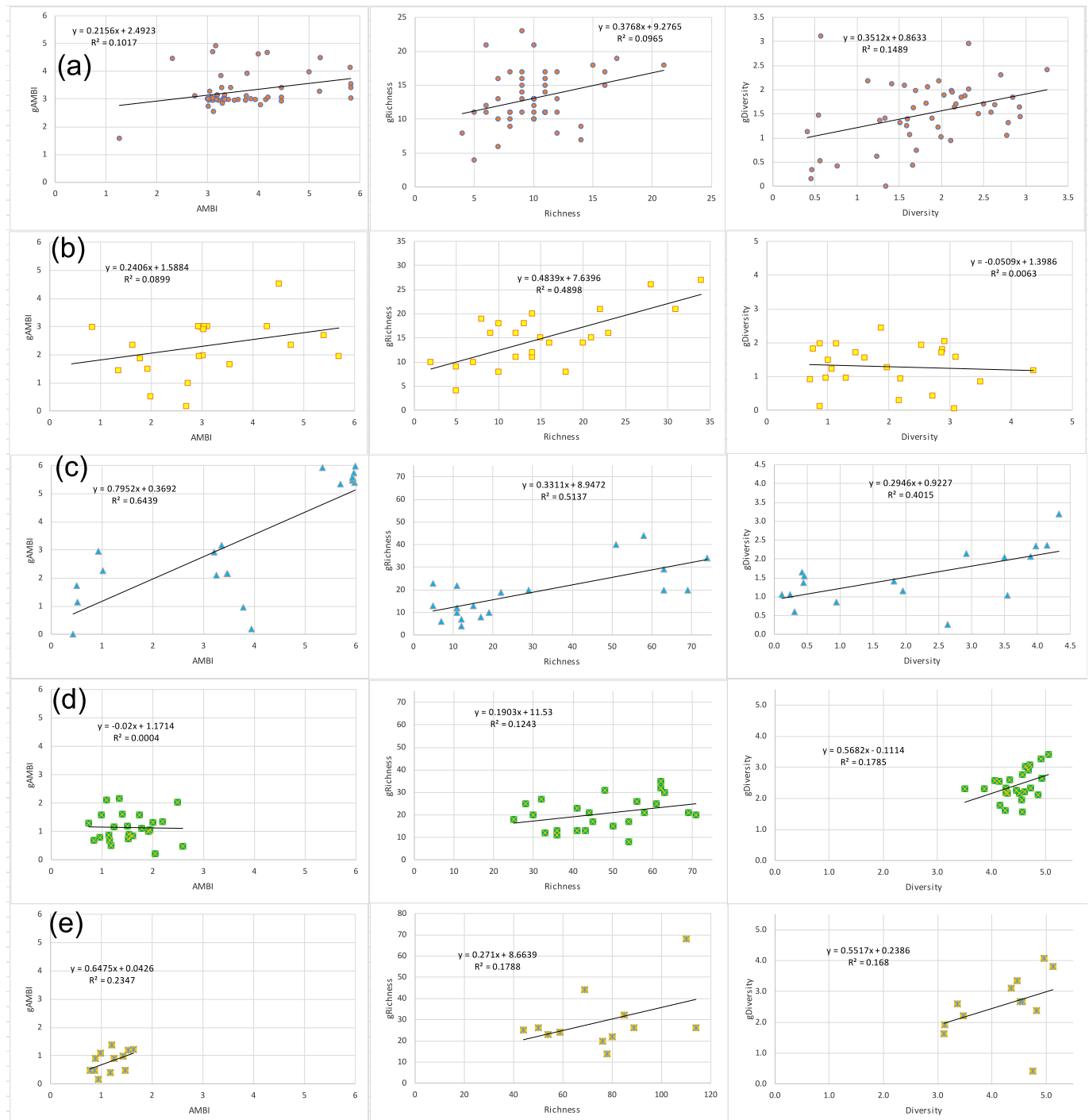


Fig. 2. Regression between AMBI, richness and Shannon diversity, calculated using the morphological and genomic (gAMBI, gRichness, gDiversity) methods, by water type including all data and samples obtained between 2018 and 2023, both in transitional and coastal waters, Water types: (a) AT-T08, *Cerastoderma edule-Scrobicularia plana*; (b) AT-T09, *Venus fasciata*; (c) AT-T10, *Abra alba*; (d) AC-T12, shallow coast, *Tellina tenuis-Venus fasciata*; (e) AC-T12, deep coast, *Amphiura*.

Thus, in AT-T08, which is typical of the interior of estuaries, there are almost no data with low AMBI values, while in the two coastal types (AC-T12 shallow and deep), there are no AMBI data with values indicating disturbance. This may explain the difficulty in finding a good regression between the variables in these types because their gradient is very short (e.g. Fig. 2e).

The range of richness seems shorter in metabarcoding compared to morphological values and tends to be narrower from the inner part of estuaries (AT-T08) towards the outer part (AT-T09, AT-T10) and the coastal zone (Fig. 2). For diversity, it appears that traditionally calculated values are higher than those calculated with metabarcoding in all types (Fig. 2). Something similar, but to a lesser extent, seems to occur in the case of AMBI (Fig. 2).

3.2. Reference conditions and comparison of M–AMBI and M–gAMBI

Since M–gAMBI requires reference conditions for each type, as mentioned in the methodology, the highest values of richness and diversity, and the lowest values of AMBI, have been used for each type (Table 2). When Tables 1 and 2 are compared, it can be observed that the reference conditions for richness in M–AMBI show an increasing gradient from the interior of estuaries (13 species) to the deepest part of the coast (130 species). In the case of metabarcoding, there is also an increasing gradient, which is broken in the euhaline area between the outer estuaries and the shallow coast (Table 2). In any case, the range is much smaller, starting with 23 species and ending at 68. Morphological diversity also increases from the interior of the estuary (2.5) to the deep zone (5.7), although with a small change between AT-T09 and AT-T10 (Table 1). In metabarcoding, the same happens, although with a smaller range, between 3.11 and 4.08 (Table 2). Finally, the reference conditions for morphological AMBI show a strong gradient from the interior of the estuary (2.8) to the deep zones (1), something that does not occur as much in metabarcoding (Table 2).

Once M–gAMBI has been calculated using the reference conditions from Table 2, Figure SM2 shows the regression between M–AMBI and M–gAMBI, using all the data together. The correlation is significant ($p < 0.001$), with an r^2 of 0.37, but the intercept in M–gAMBI is 0.34, indicating that M–gAMBI values are higher than those of M–AMBI, at least for lower values. This is well observed with the lines that determine the ecological status, although it should be noted that in this figure, estuary and coastal data are mixed, and the class limits for moderate-good are different in both.

Figure SM3 shows the same data but segregated according to whether they correspond to estuarine or coastal samples. In both cases, the correlation is significant ($p < 0.001$). In estuaries, the entire impact gradient is observed, with a slightly lower correlation than that obtained

with the non-segregated results. However, in the coastal area, the degree of correlation is lower ($r^2 = 0.15$), and the impact gradient is limited to the levels of good and high ecological status.

Finally, in Fig. 3, the regressions between M–AMBI and M–gAMBI are represented for each of the studied environment types. Types with a higher impact gradient (such as AT-T08, between moderate and high, and AT-T10, between poor and high) tend to have a better correlation, being the only significant cases ($p < 0.05$). As mentioned earlier, in coastal types, the gradient covered is shorter (only having good or high status), and the correlations are low.

3.3. Similarity analysis

Figure SM4 represents the ordination of samples conducted with the presence and absence data of taxa identified by traditional and genomic methods, based on the similarity between samples (Jaccard index). This ordination shows a clear segregation between replicates analyzed by both methods. In fact, only two statistically significant group simultaneously contain samples from the same station and survey in which taxonomic determination has been made by traditional methods and genomic methods.

In estuarine samples, the average dissimilarity between groups analyzed by traditional methods (two groups with only traditional techniques and one with 10 traditional and one genomic) and groups with genomic techniques (five groups with only genomic) is 65–91 % when sharing samples from the same stations and surveys. Among the species contributing most to this dissimilarity (>5% or a cumulative contribution of at least 10 %), it is noteworthy that in samples analyzed by genomic methods, some species considered characteristic of communities inhabiting estuaries in the Basque Country are absent, such as the arthropod *Cyathura carinata*, and the polychaete *Malacoceros fuliginosus*. On the contrary, the absence (or residual presence) in samples analyzed by traditional methods of some taxa identified at the genus level in samples analyzed by genomic methods, such as *Hediste* or *Peringia*, is remarkable. These genera are present in samples analyzed by traditional methods and reach significant abundance but were determined at the species level (*Hediste diversicolor* and *Peringia ulvae*).

Similarly, the average dissimilarity between the main groups containing a minimum of five coastal samples analyzed by traditional methods (two groups formed exclusively by samples treated with traditional techniques) and groups containing five or more samples analyzed by genomic methods (three groups formed exclusively by samples treated with genomic techniques, but two of them including both estuarine and coastal samples) is 77–88 %. As indicated for samples from estuarine stations, among the species contributing most to dissimilarity between groups (a minimum cumulative contribution of 10 %), the absence in samples treated by genomic methods of some species characteristic of the infralittoral of the Basque Country is noteworthy: the polychaetes *Galathowenia oculata*, *Paradiopatra calliopae* and *Scoloplos armiger*, the sipunculid *Onchnesoma steenstrupii*, the arthropod *Hippomedon denticulatus*, and the bivalve *Fabulina fabula*, among others. On the other hand, the absence in samples treated by traditional methods of some taxa identified at the genus level in samples analyzed by genomic methods, such as *Diastylis*, is remarkable. This genus, however, is represented in samples analyzed by traditional techniques at the species level.

Finally, the identification by genomic methods of some taxa characteristic of estuarine areas in coastal samples, such as polychaetes of the genus *Hediste* and the bivalve *Scrobicularia plana*, is also noteworthy. These taxa were not identified in coastal samples treated by traditional methods. In fact, estuarine and coastal samples treated by traditional methods do not share any group, while, in the case of those treated by genomic methods, up to six groups include both estuarine and coastal samples.

To check whether differences in taxonomic resolution had a significant effect on the high dissimilarity between samples analyzed by

Table 2
Reference conditions set in this research to calculate M–gAMBI, based on genomic (g) methods, for gS (richness), gH' (Shannon diversity) and gAMBI for transitional and coastal water types, from the Basque Country.

| Category | Water Type | Salinity/Depth | Associated community | Reference conditions | | |
|---------------------|------------|------------------|--|----------------------|------|-------|
| | | | | gS | gH' | gAMBI |
| Transitional waters | AT-T08 | 0–18 | Cerastoderma edule-Scrobicularia plana | 23 | 3.11 | 1.58 |
| | AT-T09 | 18–30 | Venus fasciata | 27 | 2.44 | 0.18 |
| | AT-T10 | 30–34 | Abra alba | 44 | 3.20 | 0.00 |
| Coastal waters | AC-T12 | Shallow: 20–50 m | Tellina tenuis-Venus fasciata | 35 | 3.42 | 0.22 |
| | AC-T12 | Deep: 70–120 m | Amphiura | 68 | 4.08 | 0.16 |

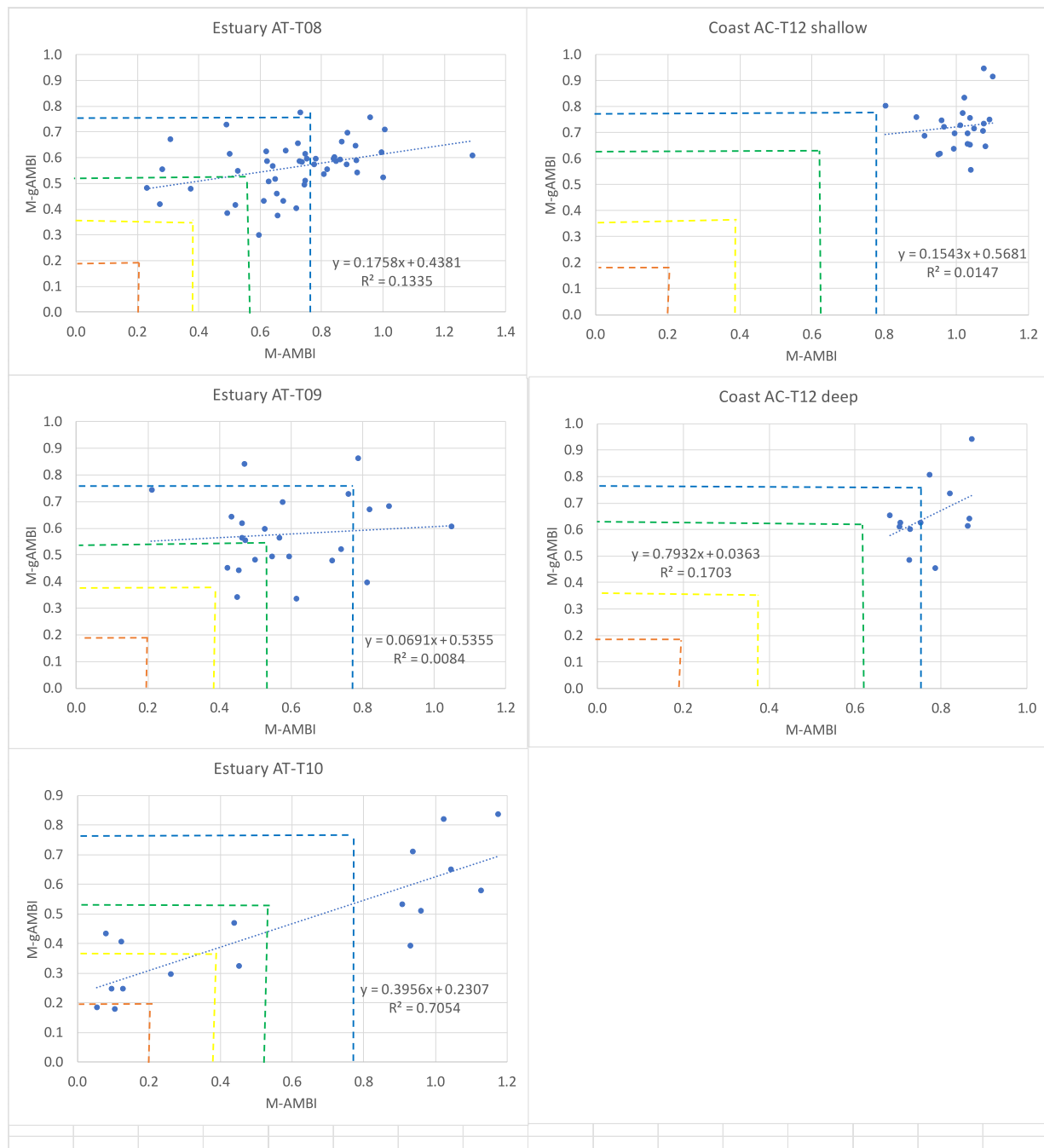


Fig. 3. Regression between M-AMBI and M-gAMBI, including all data and samples obtained between 2018 and 2023, but split into water types. Lines show boundary classes, as shown in Table 1: Orange: boundary between bad and poor ecological status, Yellow: between poor and moderate; Green: between moderate and good, and Blue: between good and high. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

traditional methods and those analyzed by genomic methods, taxa contributing more than 5 % individually or with a cumulative contribution of more than 10 % to the dissimilarity between the above-mentioned groups were aggregated at the genus level (i.e., *Ampelisca*, *Aricidea*, *Bathyporeia*, *Capitella*, *Chaetozone*, *Corophium*, *Diastylis*, *Eurydice*, *Gallardoneris*, *Glycera*, *Hediste*, *Malacoceros*, *Nephtys*, *Peringia* (along with *Hydrobia*), *Prionospio*, *Ruditapes*, *Scolecopsis* and *Urothoe*).

As relative abundances were used, the similarity matrix on which the clustering was based was constructed using Bray Curtis index. The aggregation at genus level did not significantly affect the previously obtained ordination, and once again, a clear segregation is observed between samples analyzed by traditional methods and genomic methods

(Fig. 4), although there are three groups that contain estuarine samples taken at the same station and survey but analyzed by both traditional and genomic methods (always along with other samples).

The SIMPER analysis conducted indicates that the group with higher similarity between samples (95 %) is characterized by the dominance of annelids of the genus *Capitella*, which contribution to the similarity is 99 %. The second group (83 % of similarity) is composed of samples characterized by the gastropods of the genus *Peringia* (84 % of contribution to intragroup similarity). Finally, the third group (72 % of similarity) encompasses samples characterized by amphipoda of the genus *Corophium* (93 % of contribution).

The average dissimilarity between groups containing at least five

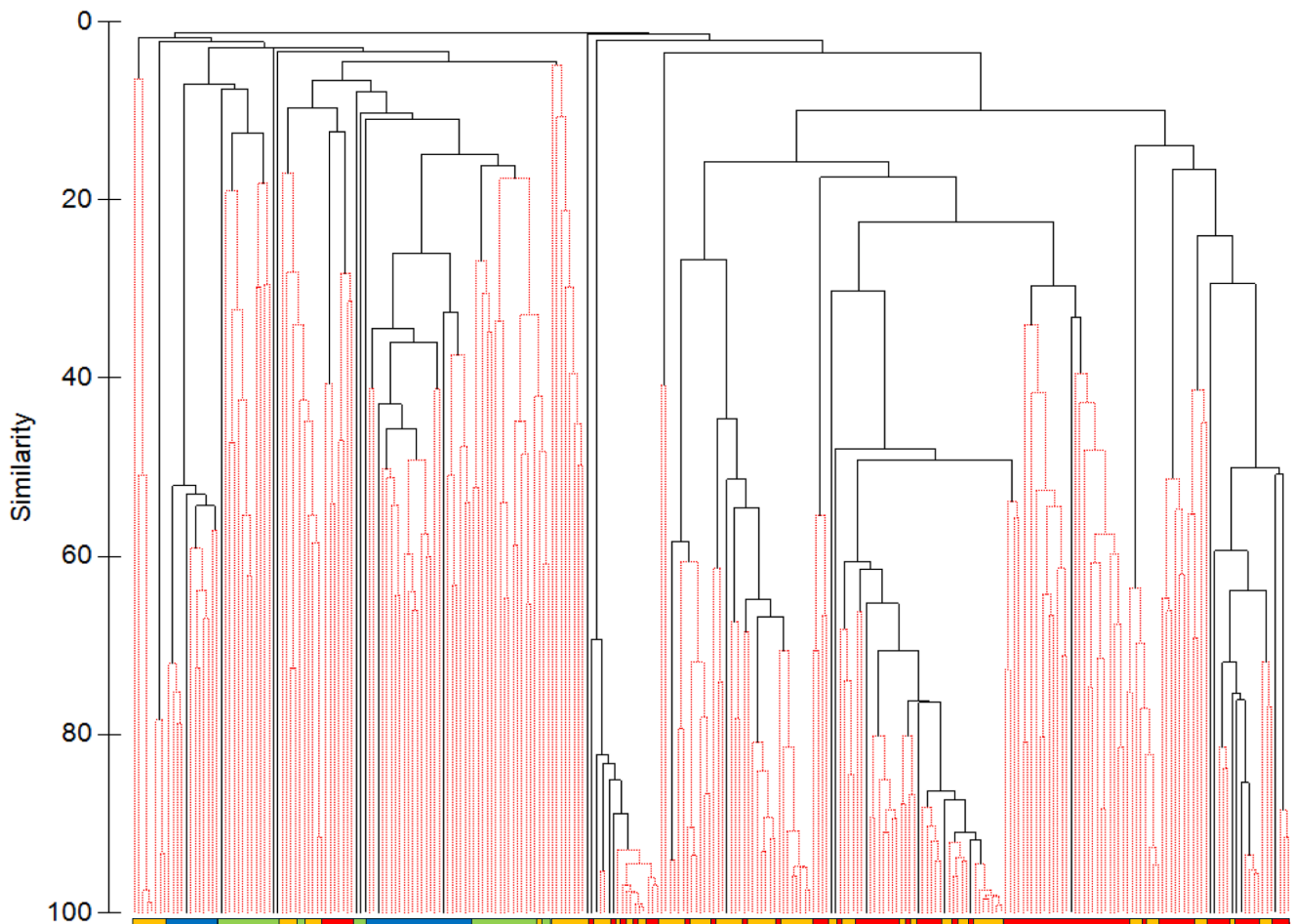


Fig. 4. Dendrogram obtained from the similarity analysis between samples, based on relative abundance or relative number of reads of the taxa identified by traditional and genomic methods, by grouping at genus level next taxa: *Ampelisca*, *Aricidea*, *Bathyporeia*, *Capitella*, *Chaetozone*, *Corophium*, *Diastylis*, *Eurydice*, *Gallardoneris*, *Glycera*, *Hediste*, *Malacoceros*, *Nephtys*, *Peringia* (with *Hydrobia*), *Prionospio*, *Ruditapes*, *Scolecopsis* and *Urothoe*. In red, estuarine samples analyzed by traditional methods; in orange the same using genomic; in blue, coastal analyzed by traditional methods and in green the same using genomic. Red groups correspond to significant different samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

estuarine samples analyzed by traditional or genomic methods (two groups containing exclusively samples treated with traditional techniques, two groups containing just samples treated by genomic techniques, one containing six samples analyzed by traditional methods and four by genomic techniques, and one containing seven samples treated by genomic methods and one by traditional methods), is 74–85 %. Among the taxa contributing most to this dissimilarity (>5%), the contribution of some taxa considered characteristic of estuaries in the Basque Country, identified in samples analyzed by traditional methods, but absent from samples worked by genomic methods, still stands out: *A. romijni*, *C. carinata* or *Streblospio eunataeae*. Besides, some of the taxa contributing most to the dissimilarity which were identified by both techniques, are systematically more abundant in the samples analyzed with traditional methods (i.e., *Peringia*), whereas other taxa are systematically more abundant in the samples analyzed by genomic methods (*S. plana* and *Hediste*). On the other hand, some of the taxa grouped for this analysis continue to contribute significantly to the dissimilarity between groups (e.g., *Hediste* or *Peringia*), due to their different average relative abundance in samples depending on whether they were treated with traditional or genomic methods.

Similarly, the average dissimilarity between the main groups containing at least five coastal samples analyzed by traditional or genomic methods (three groups formed exclusively by samples treated with

traditional methods and one group formed exclusively by samples treated with genomic techniques) remains high (82–91 %). As already indicated for samples from estuarine stations, among the species contributing most to the dissimilarity between groups (>5%), some of the taxa that have been grouped at the genus level (*Ampelisca* and *Prionospio*) still stand out.

It is also observed that estuarine and coastal samples treated by traditional methods are segregated into different groups. On the other hand, estuarine and coastal samples treated by genomic methods still coincide in two groups (one estuarine sample together with 12 coastal samples, and two coastal samples with eight estuarine samples).

A Mantel test based comparing the Bray-Curtis dissimilarity matrices of (1) COI metabarcoding profiles and (2) the corresponding morphology-based traditional taxon inventories, showed highly congruent dissimilarity patterns between the two methods ($R=0.48$, $p < 0.001$).

4. Discussion

4.1. Simple variables

In recent years, the scientific community has increasingly adopted genomics, especially metabarcoding, for monitoring and assessing the

status of aquatic environments, developing biotic indices that somehow replicate what traditional indices do (Bourlat et al., 2013; Pawlowski et al., 2018).

Initially, the studied variables were richness and diversity, but the first attempts to develop univariate indices for the assessment of environmental status, such as AMBI (Ranasinghe et al., 2012), soon followed. This led to a genomic-based index, gAMBI, although calculated only using presence-absence and not abundance (Aylagas et al., 2014). However, it was quickly established that abundance, based on the number of reads of gAMBI, could be related to the abundance of individuals in AMBI. Aylagas et al. (2018) found a correlation between both indices very similar to the one found in the present study (0.84 and 0.77, respectively), although in our case, the number of samples is much larger.

These results demonstrate that this relationship is robust, as shown by other studies (Lejzerowicz et al., 2015; Lanzén et al., 2021; Dias and Sukumaran, 2023), although some authors (Willassen et al., 2022; Duarte et al., 2023) have found some differences but using only presence-absence and not abundance and the number of reads. Additionally, Cordier et al. (2020) have reviewed studies comparing metabarcoding monitoring and morphology-based identification in various environments, summarizing the fundamental differences between the two methodologies, advocating for the use of *de novo* methods such as machine learning for the construction of novel biotic indices or classification tools, able to compensate for the fundamental differences between these approaches and utilize organisms that cannot or are not typically taken into account in morphology-based identification surveys.

The promising results presented here include joint data from the five types of water bodies in the Basque Country, with aggregated estuarine and coastal data. However, when the data are separated by types, the relationships are only significant in the inner and outer estuarine types (AT-T08 and AT-T10), although in the case of AT-T08, the slope is not very steep. This is likely explained by the absence of a complete pressure or disturbance gradient in our data from AT-T08 sites, an important factor when assessing ecological status (Borja et al., 2009). In coastal types (AC-T12 shallow and deep), the pressure gradient is also small, and the ecological status is generally good due to the status recovery of the coast in the Basque Country in recent years, after management measures were implemented (Borja et al., 2016). This results in a weak correlation, but a similar classification of undisturbed or slightly disturbed status measured with both methods.

Regarding richness, it is known that molecular methods can overestimate the number of identified species, due to intra-specific sequence variation and the fact that it can distinguish additional taxa that are difficult to identify morphologically to species level (e.g., Nematodes, Nemertean, Oligochaetes), as well as cryptic species (Gibson et al., 2015; Carugati et al., 2015; Lobo et al., 2017; Duarte et al., 2023). However, sieving the sediment and obtaining the species before extracting DNA can also lead to an underestimation of the richness of organisms with more delicate structures (Carugati et al., 2015). We have attempted to overcome these differences by removing the least abundant taxa and grouping organism groups such as nematodes, that cannot typically be resolved to species rank using morphology, resulting in a decrease from 1289 to 310 unique taxa, a number similar to that found in morphological analysis. However, these numbers differ depending on the water typology, with relatively higher richness found with molecular methods inside estuaries and lower in coastal areas. Perhaps this could be because species with shells or carapaces, which tend to be more abundant on the coast, are more challenging to identify with metabarcoding (Hajibabaei et al., 2019). Other authors, using standardized protocols, find similar richness between different laboratories (van den Bulcke et al., 2023), using total samples sieved through a 1 mm mesh, as done in our study.

Chariton et al. (2015), studying Australian estuaries with molecular methods, suggest that total richness is not a sensitive indicator of ecological condition, considering that the richness of key taxonomic

groups seems more sensitive to environmental conditions. This reinforces the role and response found with AMBI since its ecological groups (equivalent to functional groups) respond to human pressures (Borja et al., 2019).

Despite this, the response found when comparing total richness with both methods is highly significant ($p < 0.001$), even for each of the three estuarine types. This means that possibly its use in calculating M-gAMBI can be considered appropriate.

Of the three individual variables studied, diversity has the lowest correlation, although it is still highly significant ($p < 0.001$). In this case, it is also significant for two estuarine types (AT-T08 and AT-T10) and for one coastal type (AC-T12 shallow). Van den Bulcke et al. (2023) also found good agreement between laboratories for genomic Shannon's diversity, ensuring that this variable is reproducible and robust, especially when abundant and large-sized species are present, although in our case, this has not been the case for all types studied.

The need to use standardized protocols to ensure the reproducibility of results is emphasized by numerous researchers (Ransome et al., 2017; van den Bulcke et al., 2023), although it is not always possible due to continuous changes in molecular methodologies, with improvements, different extractions, updates in data libraries, etc., making long-term comparisons challenging.

In fact, it has been shown that changes in protocols can lead to significant changes in species composition and, therefore, changes in richness and diversity (Deiner et al., 2015). To minimize the changes that this can produce, it would be convenient to follow the recommendations proposed by van der Loos and Nijland (2021).

4.2. Reference conditions and comparison between M-AMBI and M-gAMBI

From the univariate indices studied above, there was an early transition to trying to replicate the multivariate methods that have been intercalibrated in Europe for the WFD (European Commission, 2018). Among them, one of the first to be developed was M-gAMBI using the number of reads in the calculation (Aylagas et al., 2018). However, the correlation found by these authors between M-AMBI and M-gAMBI was not significant ($p > 0.05$), attributing this result to the differences found in diversity and the need to set appropriate reference conditions for M-gAMBI.

Setting reference conditions for these types of indices is crucial for obtaining a robust assessment of ecological status, and there are various methods for their determination (Borja et al., 2012). As commented above, after the model of Pearson and Rosenberg (1978), the response of some metrics to human pressures could be unimodal, meaning that it might be not adequate to choose highest values of richness or diversity as reference conditions. Hence, in the case of M-AMBI, a mixture of historical data, expert judgment, and modeling has typically been used in determining these conditions (Muxika et al., 2007), requiring a good amount of available data (Santibañez-Aguascalientes et al., 2020).

For genomic indices, historical data is not available, so efforts must be made to obtain sufficient information that allows an approximation to the natural conditions of the studied typologies. This is the motivation for the research presented here, although it is true that it is difficult to obtain enough information for some typologies, covering the whole human pressure gradient. Although we have COI metabarcoding data from 125 samples, these are not evenly distributed between the five studied typologies AT-T08 ($n = 49$), AT-T09 ($n = 24$), AT-T10 ($n = 17$), AC-T12 Shallow ($n = 24$) and AC-T12 Deep ($n = 12$).

The method chosen to establish reference conditions, using the highest values of richness and diversity and the lowest values of AMBI in the studied dataset, is not considered the most suitable after the model of Pearson and Rosenberg (1978), although it can be used especially when it is known that the places under study are not affected by significant pressures (Borja et al., 2019). In the case of the Basque Country, the fact that the quality of the benthic environment has noticeably improved in

the last 20 years (Borja et al., 2016) makes it impossible to find the entire pressure or disturbance gradient in all typologies. Hence, in this particular case, this method to set reference conditions has been selected as an initial compromise, which should be confirmed during further intercalibration exercises.

The most striking results are the differences between reference conditions for morphological and DNA-based methods. Some of the differences come from the individual variables, as discussed in the previous section. In the case of richness, the larger differences are in the deep type (AC-T12 deep), while in the case of diversity, the larger differences are found in AT-T09 and again in AC-T12 deep. In the case of AMBI, the differences are important in all types. Although the gradient with salinity and depth, is maintained in the values of the reference conditions in both methods, the differences can be originated in the composition based in both methods, as discussed in the next section.

In this context, coastal stations, both referring to shallow and deep areas, are in a good or high ecological status, according to M-AMBI measurements (Borja et al., 2023), making it very plausible that the established reference conditions are close to those that could be determined in long-term monitoring. For the three estuary types, this is possibly also the case since some of the stations used present M-AMBI values corresponding to a high status (even with values exceeding 1). Therefore, the reference conditions associated with these stations must also be close to those determined in the long term.

Assuming that these reference conditions are adequate, when there is a complete or nearly complete pressure gradient, as in the AT-T08 and AT-T10 types, there are significant correlations between the data obtained by M-AMBI and M-gAMBI, which considerably improves the results obtained by Aylagas et al. (2018). To our best knowledge, there is no other publication attempting to apply M-gAMBI, possibly due to the lack of reference conditions, so our results cannot be placed into a broader context.

Here, we have undertaken a comparison and harmonization of a traditional morphological and a DNA method within the country. However, the intercalibration between countries is mandatory by the WFD (European Commission, 2000, 2024); if all countries develop DNA methods, the countries can intercalibrate these methods among them, following the same procedures as in the previous intercalibration exercise (European Commission, 2018, 2024). The intercalibration within a single country is necessary only if country aims to substitute the old (traditional) method with the new DNA one. In this case the WFD guidance (European Union, 2015) has to be followed, and the r^2 between the old and new method should be > 0.8 . In the case of the Basque Country, none of the types show a value higher than that, being the closest the estuary AT-T10, with a r^2 of 0.71. However, it is also possible to use both methods in parallel, if they are deemed to capture different aspects of biodiversity, with two methods complementing each other. Actually, some countries do have several methods, for the same biological element and water category, addressing different pressures (Poikane et al., 2016).

4.3. Similarity analysis

The similarity analyses conducted yield results indicating differences between samples analyzed by genomic methods and traditional methods, as expected considering their inherent differences. Overall, samples group consistently based on the method used (COI metabarcoding v morphology-based identification). In other words, the main groupings obtained almost exclusively contain samples analyzed by one method or the other, and secondarily group by different sites. Partly, this segregation of samples based on the methodology used for their treatment seems to be due to the absence, or more often failure to classify sequences to species level, in the data obtained by metabarcoding. This is also the case for many species characteristic of the communities described for estuaries and the coast of the Basque Country (Borja et al., 2004), such as the polychaetes *A. romijni*, *M. fuliginosus* and *S. eunatae*,

and the crustacean *C. carinata*, characteristic of the *Scobicularia plana*-*Cerastoderma edule* community. These differences are also caused by the significant presence in samples treated by genomic methods of taxa that could not be identified or had practically negligible abundances in those treated by traditional methods, such as nematodes.

Despite the low comparability between the two data sets (traditional vs. genomic method), the pattern in the data sets, i.e., the shared similarity or dissimilarity between samples and individual replicas, was highly congruent between the two data sets, according to a Mantel test. Further, many abundant species appear to be correctly classified in metabarcoding data to genus or family rank,

A large part of the observed dissimilarity between traditional methods and the metabarcoding results can be explained by the lack of taxonomic reference sequences. For example, there are no taxonomic reference sequences in the databases for *Alkmaria*, while there are COI sequences for species of the genera *Cyathura* and *Streblospio*, both unidentified in the present study. In total, among the species identified by traditional methods, 152 sequences would be absent in the database used (Midori v253). In total, of the 733 taxa identified using the traditional morphology-based method, 311 were also found using metabarcoding, as well as a rich diversity of taxa beyond the reach of traditional taxonomic identification, not considered in this study. Therefore, future efforts should be directed towards incorporating new reference sequences into the database that include at least some of the most characteristic species cited in this work, as well as species that are characteristic of communities that are characteristic elsewhere.

4.4. Adequacy of the method and conclusions

Regarding the proposed objective of whether genomic methods can be used in ecological status assessment, various reviews advocate the use of metabarcoding in monitoring and assessing status (Bourlat et al., 2013; Leese et al., 2016; Hering et al., 2018; Pawlowski et al., 2018), but few develop complex indices to do so (Keeley et al., 2018; Lanzén et al., 2021).

Among the criteria to consider whether a genomics-based index is suitable, Hering et al. (2018) proposed the following:

- **Representativeness:** This criterion involves evaluating the applicability of currently available media and alternative methods to obtain biological material; detecting errors in genomic methods and similarity to morphological methods; and assessing abundance in genomic methods. In this case, the applicability criterion is met since the same methods were used in both morphological and genomic sampling. Regarding error detection, this study has been able to detect errors in species assignment and correct them. However, a similarity study between genomic and morphological samples has revealed significant differences in composition, potentially due, at least in part, to the absence of valid reference sequences in the employed database. This could be addressed by sequencing the most frequent and abundant species and incorporating them into the database. Lastly, in this study, it has been demonstrated that the number of reads can be assimilated to abundance in morphological methods, as shown in Aylagas et al. (2018).
- **Sensitivity:** This criterion refers to the ability of genomic methods to sample sensitive species. In both this study and previous ones (Aylagas et al., 2014, 2018), species from all five ecological groups of AMBI, including the I group, which are sensitive species to pressure, have been presented.
- **Precision:** This criterion involves knowledge about the uncertainty of genomic method identification. The primers used also amplify non-targeted sequences, including bacteria and non-metazoans, so 24 % of the reads unrelated to macroinvertebrates were removed. One limitation of metabarcoding precision is the possibility of introducing contaminant DNA into the studied samples from external sources or between samples (cross-contamination), especially

biasing richness calculations and potentially affecting samples with low biomass. Here, a technology less affected by this issue (Illumina MiSeq) was used, and negative controls were included to compensate for these problems, revealing that it did not significantly influence the results. Additionally, due to insufficient reference sequence coverage (see Sensitivity above), false positives may be generated, or sequences may be classified as species absent in the dataset (Keck et al., 2022).

- **Comparability:** Considered fulfilled using an Ecological Quality Ratio based on the data found with M-gAMBI, although there are differences with the traditional method that could be improved through changes in class limits.
- **Cost-Benefit:** Not studied in this research compared to the traditional method or a comparison of processing speed, but Aylagas et al. (2018) did so and found it to be approximately 50 % of the traditional method.
- **Environmental Impact:** Whether the genomic method reduces impact. In this case, it must be considered that the impact is similar, as it uses destructive samples like the morphological method. No other study was undertaken on the cycle of life of the whole process (e.g. energy, reagents, etc.)

Based on the results of this research, the M-gAMBI index could be considered suitable for ecological status assessment since it meets most of the criteria for considering a genomics-based index suitable (Hering et al., 2018). However, in future surveys, it is considered necessary to advance in at least the following aspects:

- Concerning the representativeness criterion, it is proposed to improve the detection of errors in genomic methods and similarity with morphological methods. To achieve this, the currently available lists in GenBank and others should be studied to see which species present in the Basque Country are not on the lists and which species in the lists are the closest to those that do appear in the Basque Country. This will reduce the number of errors and, eventually, increase the similarity between samples treated by traditional methods and those treated by genomic methods.
- Regarding the comparability criterion, it is considered necessary to perform an intercalibration that allows adjusting quality class boundaries and determining the ecological status in an equivalent manner using both methods.

Despite the results obtained here, an increasing number of papers is addressing implementation of DNA methods and intercalibration in aquatic environments (Blancher et al., 2022), and our results can contribute to share knowledge on DNA-based methods, explain what can be done with these methods, and develop a strategy to compare and harmonise DNA-based methods, i.e., to intercalibrate them to ensure comparable results across Europe.

5. Data accessibility statement

Genetic data: Raw sequence data are available from the Sequence Read Archive of the International Nucleotide Sequence Database Collaboration under Bioproject accession PRJEB76912.

Morphological data: Raw data are available at the Basque Water Agency web site: <https://www.uragentzia.euskadi.eus/y76baWar/fillFilters.do>.

CRediT authorship contribution statement

Angel Borja: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anders Lanzén:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software,

Methodology, Investigation, Formal analysis, Data curation. **Inigo Muxika:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

We have shared the codes and links to the data

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2024.112638>.

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