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Review

# The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems

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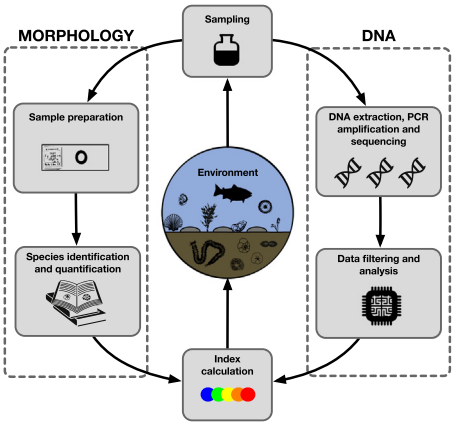
## HIGHLIGHTS

- Current biomonitoring approaches are widely used but have some limitations.
- DNA metabarcoding provides a new complementary tool for biomonitoring.
- Metabarcoding allows extending the range of taxa used as bioindicators.
- Metabarcoding data could be used to establish molecular metrics and indices.
- Future work should standardise procedures and improve data analysis.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The bioassessment of aquatic ecosystems is currently based on various biotic indices that use the occurrence and/or abundance of selected taxonomic groups to define ecological status. These conventional indices have some limitations, often related to difficulties in morphological identification of bioindicator taxa. Recent development of DNA barcoding and metabarcoding could potentially alleviate some of these limitations, by using DNA sequences instead of morphology to identify organisms and to characterize a given ecosystem. In this paper, we review the structure of conventional biotic indices, and we present the results of pilot metabarcoding studies using environmental DNA to infer biotic indices. We discuss the main advantages and pitfalls of metabarcoding approaches to assess parameters such as richness, abundance, taxonomic composition and species ecological values, to be used for calculation of biotic indices. We present some future developments to fully exploit the potential of metabarcoding data and improve the accuracy and precision of their analysis. We also propose some recommendations for the future integration of DNA metabarcoding to routine biomonitoring programs.



**Contents**



## 1. Introduction

A key global challenge in the 21st century is to maintain the supply of clean water and other aquatic ecosystem services or benefits to humans, without affecting the supporting biodiversity and ecosystem processes that underpin their sustainability. Accordingly, extensive national and international regulations have been adopted to protect water resources, including the European Union Water Framework

Directive (WFD, Directive 2000/60/EC) and Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC), the Swiss Water Protection Ordinance (WPO, Swiss Federal Council 1998), the Clean Water Act (CWA, 33 U.S.C. §1251 et seq. 1972) of the US Environmental Protection Agency, and the United Nations Convention on the Law of the Sea (UNCLOS, 1982). All of these regulations aim at protecting aquatic ecosystems from damage and restoring degraded systems to at least "good status", defined as conditions only slightly altered by anthropogenic activities. In order to achieve this aim, and assess recovery of the systems after restoration or rehabilitation measures, accurate assessment is needed and is part of all global environmental programmes. Since 2000, the status of aquatic ecosystems has been monitored in the European Union by characterising biological communities, and physicochemical and hydromorphological conditions. Among this, the occurrence and abundance of biological indicators have the heaviest weight in determining the ecological status of the different water bodies.

To address the requirements of the above-mentioned legislation, a large number of biotic metrics/indices, based on morphological identification of various groups of aquatic indicator organisms at different levels of organisation, has been developed in different countries (reviewed in Birk et al., 2012; Borja et al., 2013). For example, the WFD requires ecological status assessment of surface waters to be based on 'Biological Quality Elements' (BQEs), which depending on the water body type, include "phytoplankton", "diatoms", "aquatic flora", "macroalgae and angiosperms", "benthic invertebrate fauna", and "fish fauna". The resulting lists of taxa and their abundances are used to compute biotic metrics/indices and to define the ecological quality status. These biotic metrics/indices are usually defined as measures of the structure, function or some other characteristics of biological assemblages that show a predictable response to anthropogenic disturbances (Bonada et al., 2006). About 300 methods have been developed to assess the ecological status of aquatic ecosystems, including rivers, lakes, transitional and marine coastal waters across countries implementing the WFD (Birk et al., 2012). Regarding the MSFD, methods are still under development (Borja et al., 2013; European Commission, 2017). Similar to the WFD, the MSFD requires the description of aquatic habitats based on a set of so-called "qualitative descriptors". These descriptors require the assessment of various biological attributes such as "biodiversity", "non-indigenous species", "exploited fish and shellfish", "food webs", "eutrophication and sea-floor integrity", including plankton, benthic invertebrates, algae and macrophytes, marine mammals and reptiles, seabirds, fish and other groups of organisms.

All of these traditional biological monitoring and assessments methods are based on the direct observation of the organisms used to calculate biotic metrics/indices, which have been proven to be time and resource-intense. Recently, the new field of DNA-based bioassessment (called also Biomonitoring 2.0) emerged from advances in DNA barcoding and metabarcoding (Baird et al., 2012). It has been proposed to assess ecological status by detection of single species or characterization of whole communities through the sequencing of environmental DNA (eDNA) (Taberlet et al., 2012, 2018). We focus here on the use of DNA for community studies, which can be done either by analysis of DNA extracted from bulk samples of non-identified macroinvertebrates (Hajibabaei et al., 2012; Yu et al., 2012), or by analysing the total eDNA (and eRNA) extracted from water, sediment or biofilm samples (Pawlowski et al., 2014; Visco et al., 2015; Deiner et al., 2016; Valentini et al., 2016). In the latter case, dependent on the taxonomic group targeted, either only the DNA released from organisms into the environment (so called "extra-cellular" DNA) is analysed (e.g. to survey fish community) or, alternatively, the analyses include the totality of DNA present in environmental samples, isolated from living cells (e.g. diatoms), the entire specimens or tissue fragments (e.g. invertebrates) and including also the DNA molecules present in organelles and cellular debris (e.g. fish).

The development of DNA metabarcoding has been boosted by advances in high-throughput sequencing (HTS) technologies that overcome most of the limitations of classical cloning/Sanger sequencing approaches, and generate millions of sequences in a relatively rapid and inexpensive way (Shokralla et al., 2012). One major advance was the development of multiplexing protocols, which allowed many samples to be processed at the same time (Herbold et al., 2015). The number of HTS-based metabarcoding studies is growing exponentially, leading to spectacular advances in our knowledge of the global patterns of diversity in aquatic ecosystems, of both prokaryotic (e.g. Besemer et al., 2012; Yilmaz et al., 2016; Thompson et al., 2017) and eukaryotic organisms (e.g. Thomsen et al., 2012; de Vargas et al., 2015; Massana et al., 2015; Leray and Knowlton, 2015; Hänfling et al., 2016; Deiner et al., 2016; Debroas et al., 2017).

There is now a growing body of literature summarizing the potential of environmental DNA metabarcoding for biological monitoring (e.g. Bohmann et al., 2014; Cristescu, 2014; Valentini et al., 2016; Keck et al., 2017; Leese et al., 2018; Deiner et al., 2017; Darling et al., 2017) and highlighting its importance for environmental management (Kelly et al., 2014; Jackson et al., 2016; Hering et al., 2018). All of these papers present DNA metabarcoding as faster, cheaper and easier-to-use alternative to conventional biomonitoring. However, none of them focuses directly on inferring DNA-based biotic indices. Here, we review the opportunities, achievements and challenges of linking traditional WFD or MFSD metrics and indices with metabarcoding data. We begin with an overview of conventional biological monitoring focussing on the function, structure, application and limitations of the current metrics and indices. Then we present the state of the art of metabarcoding studies applied to biomonitoring and the potential for further developments in this field. We also highlight the opportunities offered by the metabarcoding approach to provide a new generation of biotic indices spanning across multiple levels of biological organisation. We conclude by discussing the role that metabarcoding could play in supporting and/or replacing traditional approaches to enhance bioassessment related to the two key European legislative frameworks (WFD and MSFD).

## 2. Conventional biotic indices

As summarized in Birk et al. (2012), biological monitoring and assessment require standardized procedures to sample (1), process (2) and identify indicator organisms (3), followed by subsequent calculation of biotic metrics/indices (4), which are, in turn, compared with metric/index values derived from reference conditions, in order to assign an ecological status (5) (Fig. 1).

The terms biotic (or biological) metrics and indices are used interchangeably, because they are hard to separate conceptually. The WFD and MSFD do not specifically mention or define 'metric or index'. Nevertheless, they are implied in the text as "biological …factors" (MSFD, article 3.4) and "values of the biological elements" (WFD, article 1.4.1.).

There is also some variation in how metrics/indices are classified. For example, Birk et al. (2012) defined two major categories: (1) taxonomy-based metrics that do not account for ecological characteristics (e.g. richness, diversity, abundance and productivity metrics and multivariate approaches), and (2) autecology-based metrics that capture sensitivity to anthropogenic disturbances, traits, species health/condition and presence of non-native species. Furthermore, metrics/indices are classified by their structure, ranging from simple calculations of the number of certain organism groups to the combination of several individual metrics into a so-called multimetric index (Hering et al., 2006; Birk et al., 2012).

Here, we use the term "metric" only when referring to taxonomy-based metrics that do not account for ecological characteristics. We use the term "biotic index" (BI) when referring to those metrics and indices that aim at assessing water quality and degree of stressor impact. We have grouped them into three categories based on their structure: (1) simple BIs (univariate approach), (2) complex BIs such as multivariate or predictive models and multimetric indices, and (3) BIs using ecological function instead of/or additional to species composition.

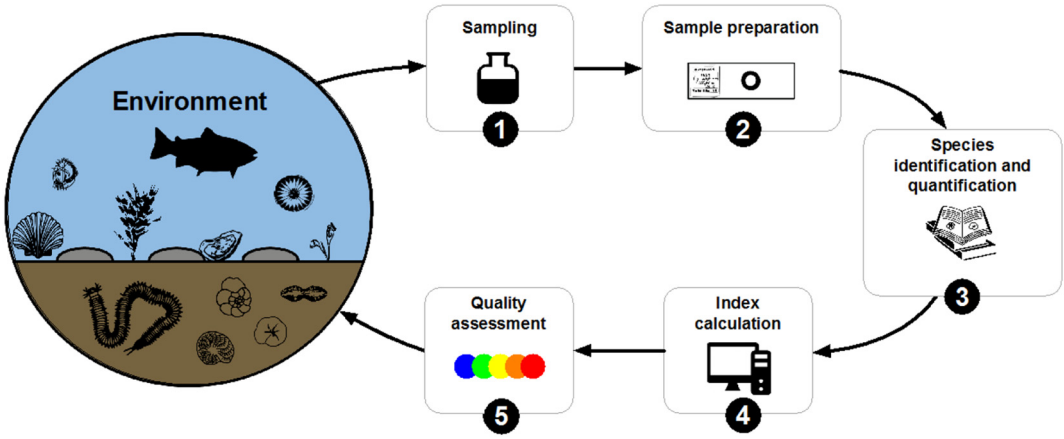


**Fig. 1.** Schema of key steps in traditional biological monitoring and assessment procedures.

A number of studies have reviewed BIs that assess the structural integrity of biological assemblages in different habitats and from various perspectives (Supplementary Table 1). Examples of the structure of a representative selection of metrics and indices are given in Supplementary Table 2. Here we present a short synthesis of the key components of BIs and their functions that need to be considered when linking conventional and DNA-based approaches.

### 2.1. Simple (univariate) indices

From the mid-20th century onwards, simple metrics, such as diversity, richness and evenness, have been used for water quality assessment. Examples include Shannon (diversity), Pielou (evenness), and Margalef (richness) indices, which are based on simple counts of individuals in a sample or the relative proportion across different taxa (Shannon and Weaver, 1949; Pielou, 1966; Margalef, 1980). Initially, the use and development of BIs was restricted to assessment of organic pollution, and early methods combined composition and abundance metrics with "ecological aspects" to provide an overall index value which is a measure of water quality (Sládeček, 1963). This led to the development of a range of BIs, which include in their calculation composition and/or abundance of indicator taxa, and pollution sensitivity. Most of these indices have been based on macroinvertebrates using a relatively limited number of indicator taxa, often only identified to family or genus level (two common and frequently used examples are presented in Supplementary Table 2).

### 2.2. Complex indices

Multivariate (predictive models) and multimetric approaches require more complex analyses, but the final result is always a single value of a BI, which in comparison to an expected value from a reference condition is used to define water quality or ecological status.

Predictive models are based on multivariate analyses that predict the expected community under reference conditions for a given site, from relevant environmental characteristics that drive community composition and structure (Wright et al., 1993; Reynoldson et al., 1997). Usually, predictive models in freshwater bioassessment are developed using linear combinations of predictors, from discriminant function analysis to logistic regressions and more recently on artificial neural networks and other machine learning tools (Feio et al., 2014). The first predictive models were based on invertebrate communities from rivers (e.g. RIVPACS) but more recently other biological elements and freshwater ecosystems have been considered, including lakes and wetlands (e.g., Reynoldson et al., 1995; Johnson and Sandin, 2001; Davis et al., 2006), fishes (Joy and Death, 2004; Kennard et al., 2006), diatoms and macrophytes (Chessman et al., 1999; Feio et al., 2012). Typically, these models are then used to derive an index value for the expected community, which in turn is compared to that from the observed community as a ratio, with deviation assumed to be due to disturbance. The BI based on a predictive model is thus calculated independently of stressor parameters (Clarke et al., 1996; Van Sickle, 2008).

Multimetric indices use a combination of several attributes and metrics of the communities (typically at least one metric representing richness/diversity, sensitivity/tolerance, composition/structure and function) to derive an overall index value (Hering et al., 2006; Feio and Poquet, 2011). The first multimetric index was the Index of Biological Integrity (IBI) developed for fishes in the USA (Karr, 1981) and still widely used in North America (Yoder and Kulik, 2003). The multimetric approach was later adapted to invertebrates by Barbour et al. (1996) but also to vertebrates (Miccachion, 2002), plants (e.g. Gernes and Helgen, 1999; Mack, 2002), terrestrial invertebrates (Kimberling et al., 2001), and even diatoms (Elias et al., 2016). The multimetric indices are now commonly used for macroinvertebrate- and fish-based bioassessment in Europe (Ofenböck et al., 2004; Hering et al., 2006; Feio et al., 2014, 2014).

### 2.3. Biotic indices using functional metrics

According to Hering et al. (2006), a functional metric measures the ecological function of a taxon, and not only its sensitivity to a stressor. These functions, also called ecological or life-history traits, include for example feeding types for macroinvertebrates and fish (Usseglio-Polatera et al., 2000), substrate attachment preferences for diatoms (Rimet and Bouchez, 2012), lake habitat for phytoplankton (e.g. Reynolds et al., 2002), or the preferred spawning habitat for fish (EFI + CONSORTIUM, 2009). Functional metrics aim to represent robust stressor/impact relationships, and are intended to be more insensitive to biogeography than when using species occurrences (Poff et al., 2006; Menezes et al., 2010) and less prone to errors related to misidentification of species (Tapolczai et al., 2016). Several BIs are based on or include ecological function in diatoms (Tapolczai et al., 2017), phytoplankton (Padisák et al., 2006), macrophytes (Orfanidis et al., 2007; Wells et al., 2007), macroinvertebrates (Poff et al., 2006; Dolédec and Statzner, 2008; Borja et al., 2009) and fish (Pérez-Domínguez et al., 2012, Logez et al., 2013;). BIs based on body-size metrics have been also applied to macroinvertebrates and phytoplankton of transitional waters (Reizopoulou and Nicolaidou, 2007; Basset et al., 2012; Vadrucci et al., 2013).

### 2.4. Limitations of traditional BIs performance and sensitivity

Biological monitoring has had some notable successes resulting in significant improvements of the detection of multiple stressors in streams and rivers, as well as transitional and coastal waters, leading to ecological restoration or protection actions (Kenney et al., 2009; Jones et al., 2010; Pander and Geist, 2013; Parmar et al., 2016). Developed and used for over 100 years (e.g. Kolkowitz and Marsson, 1908), BIs are applied worldwide, and large intercalibration studies have been performed to harmonize BIs at national and international levels (e.g. Pont et al., 2011; Birk et al., 2012; Birk et al., 2013; Feio et al., 2014, 2014; Poikane et al., 2014; Poikane et al., 2016, 2016). However, traditional BIs have limitations that are related to their structure, general implementation and use in the assessment system (Birk et al., 2012, Borja et al., 2012, Reyjol et al., 2014, Table 1).

A well-known structural limitation is the taxonomic resolution. Identification to species level is considered the gold standard and the best reflection of the ecological community, although a lack of ecological understanding for rare taxa can constrain the benefits of increased resolution (Jones, 2008). Nevertheless, the taxonomic resolution used for bioassessment is often set without explicit justification and selected on subjective criteria, such as sample-processing, cost and time (Pinna et al., 2013). This clearly limits the spatial and temporal coverage that monitoring programmes can achieve. While broad taxonomic resolution appears to be well adapted for a quick and robust assessment of ecological quality (Rimet and Bouchez, 2012; Fornaroli et al., 2016), it is also well known that conclusions are not consistent across different levels of taxonomic resolution (e.g. Seymour et al., 2016).

Although low taxonomic resolution, as family or genus, is routinely accepted for certain groups, especially for macroinvertebrates, it severely limits assessment of the level of degradation and its cause, particularly in a multi-stressor environment. Furthermore, the range of taxa used in BIs is limited to those with distinctive morphological features, thus neglecting other potential indicator groups and morphologically inconspicuous taxa. Consequently, some taxonomic groups are used more frequently than others. Moreover, other factors affect the outcome of richness and composition estimates, such as sampling effort and techniques (Sangiorgio et al., 2014; Pinna et al., 2017), taxonomic expertise and training (Terlizzi et al., 2003; Haase et al., 2006; Kahlert et al., 2009), or the presence of life stages, which often cannot be assigned to species level (Darling and Mahon, 2011).

Another important limitation concerns the gaps in knowledge on the species-ecological coupling with stressors. Weighting taxa according to their sensitivity and tolerance to a stressor is a key component of many BIs. This requires testing and validating stressor-impact relationships. Sensitivity and indicator values have been set empirically for some BIs (e.g. in the saproby system, also many other indices such as Specific Polluosensitivity Index - IPS). However, we are still lacking knowledge of the ecology of many species, and their sensitivity to several relevant stressors. In fact, as highlighted by Birk et al. (2012), stressor-impact relationships have not been tested or documented for one-third of the 297 assessment methods they covered. Furthermore, the majority of studies tested the response to gradients of nutrient enrichment or organic pollution. Thus, many of the BIs can only provide a measure of general degradation, particularly in a multi-stressor environment. This limits the efforts to identify the most impacting stressors and target appropriate

**Table 1**
List of metabarcoding studies focused on freshwater biomonitoring, classified according to the indicator taxa, genetic marker, and main issues addressed. The correlation values between classical and DNA-based indices were added (in bold) whenever available.

| Taxon | Marker | Main issues | Reference |
|---|---|---|---|
| Bacteria | 16S | Taxonomic resolution | Salis et al. (2017) |
| Bacteria | 16S | Bioassays | Binh et al. (2014) |
| Bacteria | 16S | Lake diversity, ecotoxicology | Pascault et al. (2014) |
| Bacteria | 16S | Lake | Chen et al. (2016) |
| Bacteria | 16S | Faecal pollution | Vierheilig et al. (2015) |
| Bacteria/Fungi | 16S/ITS2 | Land-water interface | Veach et al. (2015) |
| Phytoplankton | 16S cpDNA | Lake diversity | Eiler et al. (2013) |
| Phytobenthos | 18S V4 | Metabarcoding vs morphology | Groendahl et al. (2017) |
| Diatoms | *rbc*L | Metabarcoding vs morphology, TDI5 index, **(Pearson's r = 0.9)** | Kelly et al. (2018) |
| Diatoms | 18S, *rbc*L, COI | Mock community, taxonomic assignment, | Kermarrec et al. (2013) |
| Diatoms | 18S, *rbc*L | SPI index, ref. database **(Spearman p < 0.05)** | Kermarrec et al. (2014) |
| Diatoms | *rbc*L | SPI index, DNA extraction (correlation not given) | Vasselon et al. (2017) |
| Diatoms | *rbc*L | SPI index, sequencing depth, reference database **Pearson correlation: r = 0.77, p-value < 0.05 ($R^2$ = 0.59)** | Vasselon et al. (2017), Vasselon et al. (2018) |
| Diatoms | 18S V4 | Reference database | Zimmermann et al. (2014) |
| Diatoms | 18S V4 | Metabarcoding vs morphology | Zimmermann et al. (2015) |
| Diatoms | 18S, *rbc*L | Reference database | Rimet et al. (2016) |
| Diatoms | 18S V4 | DI-CH index **($R^2$ = 0.58 DNA, $R^2$ = 0.85 RNA)** | Visco et al. (2015) |
| Diatoms | 18S V4 | DI-CH index, taxonomy-free approach **($R^2$ = 0.67 DNA)** | Apothéloz-Perret-Gentil et al. (2017) |
| Diatoms | *rbc*L | IPS index (**$R^2$ = 0.0042**), EPI-L index ($R^2$ = 0.0278), Sgro Index (**$R^2$ = 0.1342**), correlation values weak as calculated on lake samples, reference database | Rivera et al. (2018) |
| Chironomids | COI, CytB | Bulk samples, marker resolution | Carew et al. (2013) |
| Macroinvertebrates | COI | Shotgun sequencing | Zhou et al. (2013) |
| Macroinvertebrates | COI | Bulk samples | Hajibabaei et al. (2011) |
| Macroinvertebrates | COI | Bulk samples ethanol | Hajibabaei et al. (2012) |
| Macroinvertebrates | COI | Gene enrichment | Dowle et al. (2015) |
| Macroinvertebrates | COI | Primers bias | Elbrecht and Leese (2015) |
| Macroinvertebrates | COI | Primers design | Elbrecht et al. (2016) |
| Macroinvertebrates | 16S | Marker assessment | Elbrecht et al. (2016) |
| Macroinvertebrates | COI, 16S, 18S | Diversity metrics | Gibson et al. (2015) |
| Oligochaetes | COI | IOBS index (no test), abundance | Vivien et al. (2016) |
| Oligochaetes | COI | Formalin preservation | Vivien et al. (2016) |
| Fish/amphibians | 12S | HTS vs traditional surveys, marker assessment | Valentini et al. (2016) |
| Fish/amphibians | 12S/16S | Quantification | Evans et al. (2016) |
| Fish | 16S/CytB | HTS vs traditional surveys | Hänfling et al. (2016) |
| Fish | 12S/16S | Marker assessment; water column vs sediments sampling; water volume influence | Shaw et al. (2016) |

mitigation measures. Equally significant, most BIs lack a coupling to biological function, which makes ecological interpretation of change in BI values difficult, limiting our ability to define status boundaries based on ecological knowledge (Birk et al., 2012; Tapolczai et al., 2017). This also limits the potential link between ecosystem degradation and ecosystem function-service delivery impairment, which is needed to inform more efficient aquatic ecosystem management (Barquín et al., 2015).

The challenge for DNA-based assessment is to find a fit within current bioassessment frameworks that will enhance our ability to detect and identify stressor impacts. Therefore, we need to define technical and biological challenges, and consider how metabarcoding might influence the development of indices for biological monitoring and assessment.

## 3. Molecular biotic indices

### 3.1. Pilot studies

The first attempts to apply the metabarcoding approach to bioassessment aimed at testing the accuracy and precision of metabarcoding data to infer the same taxonomic composition of bulk samples or mock communities as morphotaxonomic inventories of bioindicator taxa (e.g. Hajibabaei et al., 2012; Carew et al., 2013; Zhou et al., 2013; Kermarrec et al., 2013). In parallel, other studies have been conducted to test the potential use of metabarcoding data to assess the ecological status of natural communities exposed to various anthropogenic pressures (e.g. Chariton et al., 2010; Bik et al., 2012; Pawlowski et al., 2014; Pascault et al., 2014). Since then, there has been a rapid increase in the number of applied metabarcoding studies focusing on various bioindicator groups in freshwaters (Table 1), and transitional and marine (Table 2) environments.

The pilot metabarcoding studies applied to bioassessment can be classified into three categories according to their scope: (1) studies that use metabarcoding data to infer existing morphotaxonomy-based biotic indices, (2) studies that explore the potential of new bioindicator taxa, and (3) studies that search for alternative analytical methods to develop new molecular indices. The challenges addressed by each of these categories are not the same. The first group of studies is mainly concerned with testing and improving the match between indices derived from morphological and molecular data. The key challenges of the second and third categories are to develop new analytical methods and indices based on metabarcoding data for the taxonomic groups that are not currently used in ecological quality assessment.

The greatest advances of the studies that compare the biotic indices inferred from morphological and molecular data have been made using diatoms (Kermarrec et al., 2014; Visco et al., 2015; Apothéloz-Perret-Gentil et al., 2017; Vasselon et al., 2017, 2017) and marine benthic invertebrates (Lejzerowicz et al., 2015; Aylagas et al., 2014, 2016). Some efforts have also been made to compare the assessment of ecological status based on freshwater benthic invertebrate communities derived from morphological and molecular data (Gibson et al., 2015; Elbrecht et al., 2017, 2017). Overall, the results of these studies indicate a relatively good correlation between conventional and molecular indices (averaging 70–80%). Yet, there are several issues that limit efforts to obtain higher correlation values, some of which are presented below.

**Table 2**
List of metabarcoding studies focused on marine biomonitoring, classified according to the indicator taxa, genetic marker, and main issues addressed. The correlation values between classical and DNA-based indices were added (in bold) whenever available.

| Taxon | Marker | Main issues | Reference |
|---|---|---|---|
| Bacteria | 16S V4 | microgAMBI index | Aylagas et al. (2017), Borja (2018) |
| Bacteria | 16S | Marine aquaculture | Dowle et al. (2015) |
| Bacteria | 16S | Offshore oil spill assessment | Smith et al. (2015) |
| Bacteria | 16S | Coastal pollution | Kisand et al. (2012) |
| Bact/Archaea/Euks | 16S, 18S | Marine picoplankton | Ferrera et al. (2016) |
| Bact/Archaea/Euks | 16S, 18S | Ocean acidification and oil pollution | Coelho et al. (2016) |
| Bact/Archaea/Euks | 16S, 18S | Offshore drilling | Laroche et al. (2017) |
| Bacteria/Euks (phytoplankton) | 23S cpDNA | Marker assessment | Yoon et al. (2016) |
| Eukaryotes | 18S | Offshore oil spill assessment | Bik et al. (2012) |
| Eukaroytes | 18S | Offshore drilling | Lanzén et al. (2016) |
| Eukaroytes | 18S | DNA extraction sediments | Lanzén et al. (2017) |
| Eukaryotes | 18S | Estuaries | Chariton et al. (2010, 2015) |
| Eukaryotes | 18S | Estuaries | Lallias et al. (2015) |
| Eukaryotes | 18S | Ballast water | Pagenkopp Lohan et al. (2016) |
| Eukaryotes | COI | Ballast water | Zaiko et al. (2015) |
| Eukaryotes | 18S | Pelagic times series | Brannock et al. (2016) |
| Eukaryotes | 18S | Marine canyons | Guardiola et al. (2015) |
| Eukaryotes | 18S V9 | Estuarine plankton | Abad et al. (2016) |
| Foraminifera | 18S | Marine aquaculture | Pawlowski et al. (2014) |
| Foraminifera | 18S | Marine aquaculture - index | Pochon et al. (2015) |
| Foraminifera | 18S | Marine aquaculture - index | Pawlowski et al. (2016, 2016) |
| Foraminifera | 18S | Offshore drilling | Laroche et al. (2016) |
| Foraminifera | 18S | Machine learning – index prediction (**NSI $R^2 = 0.83$, NQI $R^2 = 0.83$**) | Cordier et al. (2017) |
| Nematoda | 18S | Deep-sea biodiversity | Dell'Anno et al. (2015) |
| Nematoda | 18S, COI | Estuary benthos | Avó et al. (2017) |
| Meiofauna | 18S | DNA extraction, data analysis | Brannock and Halanych (2015) |
| Macroinvertebrates | COI, 18S | gAMBI, reference database | Aylagas et al. (2014) |
| Macroinvertebrates | COI | gAMBI, taxon composition | Aylagas et al. (2016) |
| Macroinvertebrates | 18S | aquaculture; AMBI (**$R^2 = 0.899$ DNA, $R^2 = 0.855$ RNA** ITI (**$R^2 = 0.866$ DNA, $R^2 = 0.974$ RNA**), | Lejzerowicz et al. (2015) |
| Macroinvertebrates | COI, 18S | Seagrass community | Cowart et al. (2015) |
| Macroinvertebrates | COI | Estuarine macrobenthos | Lobo et al. (2017) |
| Fish | 12S | NGS vs traditional surveys in deep ocean | Thomsen et al. (2016) |
| Fish | 12S | Marker assessment | Miya et al. (2015) |
| Fish | 12S | Marker assessment | Kelly et al. (2014) |
| Fish | Cytb | NGS vs traditional surveys in coastal waters | Thomsen et al. (2012) |
| Mammals | 12S | Genetic monitoring | Foote et al. (2012) |

### 3.2. Main biological and technical challenges

The standard metabarcoding approach consists of several steps that involve processing of eDNA samples (water, soil, sediment) or bulk samples to obtain DNA sequences of organisms present in those samples. These steps (illustrated in Fig. 2) include: (1) isolation of (environmental) DNA, (2) PCR amplification of a marker gene targeting the biotic community to be analysed, followed by (3) high-throughput sequencing of obtained amplicons. The sequence data are then filtered (4) to reduce the number of sequencing errors, and the identical sequences are dereplicated in order to obtain the Individual Sequence Units (ISU). The ISUs are clustered (5) into Molecular Operational Taxonomic Units (MOTUs) (further defined in Section 3.2.1). In the final step, the MOTUs are assigned to morphotaxa, whenever possible (6). The compiled taxa list based on assigned MOTUs can then be used to infer a set of biotic indices and to conduct the assessment of ecological quality of a given water body.

At each step of this metabarcoding pipeline, various factors can influence the value of inferred indices (e.g. Deiner et al., 2015; Zimmermann et al., 2015; Goldberg et al., 2015, 2016; Mächler et al., 2016). These factors can be related to biological, ecological and genomic characteristics of the analysed community (biological factors), to the sampling, processing of the samples, and to the data analysis (technical factors). The relationships between some of these factors and the main variables used in biodiversity metrics,richness, taxonomic composition, abundance and ecological values, are presented in Table 3.

#### 3.2.1. Richness: how many taxa are there?

Taxonomic richness appears to be the simplest parameter that can be assessed from metabarcoding data. The richness unit is a cluster of sequences that are grouped together according to their genetic similarity (or distance), called Operational Taxonomic Units (OTU) or MOTU (Blaxter, 2004). Although MOTUs are often treated as genetic substitutes for species, they do not necessarily correspond to the morphologically defined taxa used as quality elements in bioassessment. The estimation of richness based on MOTUs depends on the distance or similarity thresholds used to cluster sequences, as well as on the presence of cryptic diversity frequently observed within morphological units. Thus, both approaches may give quite different results, affecting BI computation when richness is part of the index.

**Table 3**
Different biological and technical factors that may impact biotic indices inferred from DNA metabarcoding data.

| Factors | BI variables | | | |
|---|---|---|---|---|
| | Richness | Taxonomic composition | Abundance | Sensitivity & indicator values |
| Biological factors: | | | | |
| Cryptic species | X | X | | |
| Genomic polymorphism | X | X | | |
| Introgression/hybridisation | X | X | | |
| Biomass | | X | X | |
| Gene copy number | | | X | |
| Life cycle | | | X | |
| Functional traits | | | | X |
| Technical factors: | | | | |
| Sampling: | | | | |
| Sampling methods (volume/size, filters, precipitation) | X | X | X | X |
| Sample preservation | X | X | X | X |
| Wet lab: | | | | |
| DNA/RNA extraction | X | X | X | X |
| Primer specificity | X | X | X | X |
| PCR & HTS errors | X | | | |
| Sequencing depth | X | X | | |
| Dry lab: | | | | |
| Quality filtering | X | X | | |
| OTU clustering | X | | X | |
| Taxonomic assignment | X | X | X | X |
| Reference database | X | X | X | X |
| Ecological database | | | | X |

The metabarcoding data are considered as reliable source of information about the richness of some taxonomic groups, e.g. fish (Olds et al., 2016). However, in many other groups, especially invertebrates and protists, the number of MOTUs generated by HTS deviates considerably from the number of taxa observed morphologically in the same environmental samples (e.g. Pawlowski et al., 2014; Deiner et al., 2016). There are several biological and technical factors that contribute to this over- or under-estimation of taxonomic richness, especially concerning the rare species (Zhan and MacIsaac, 2015).

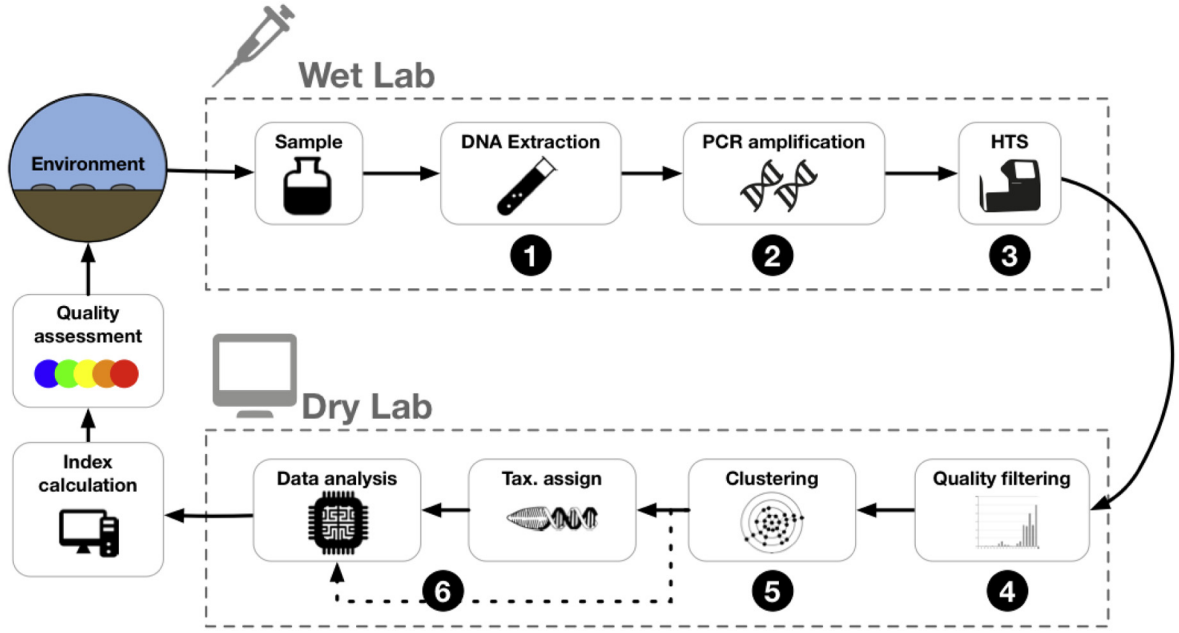


**Fig. 2.** Schema of key steps in DNA metabarcoding applied to bioassessment.

The most important biological factor that influences richness overestimation is the natural intraspecific and intragenomic variability. This is particularly problematic when a single traditionally recognized species or bioindicator taxon comprises a variety of different genotypes. Sequences corresponding to different genotypes within the same taxon may cluster into different MOTUs, and thus inflate taxonomic richness. High cryptic diversity is well documented in almost all groups of bioindicator taxa (e.g. aquatic insects, Pauls et al., 2006, Previšic et al., 2014; diatoms – Rimet et al., 2014; oligochaetes – Vivien et al., 2015). Moreover, some taxa show high intragenomic polymorphism (e.g. diatoms – Behnke et al., 2004; nematodes - Dell'Anno et al., 2015; foraminifera - Weber and Pawlowski, 2014), contributing further to the increased number of MOTUs. MOTU richness can also be artificially inflated through technical errors generated during PCR amplification and amplicon sequencing (Schirmer et al., 2015).

Different solutions have been proposed to mitigate the impacts of these biological and technical biases. The intraspecific and intragenomic variation, and low-level sequence divergence can be efficiently removed through MOTU clustering. Fixed thresholds that arbitrarily define the level of genetic variation are commonly used. However, given large variation of divergence rates between taxa, group-specific thresholds can appear as a better solution in some taxa (Pawlowski et al., 2014; Brown et al., 2015). Alternative solutions to fixed thresholds are offered by algorithms that generate MOTUs based on a network of connected reads (e.g. Mahé et al., 2015) or take into account the distribution of sequences across samples (Preheim et al., 2013). Finally, different solutions exist to overcome technical biases (Morgan et al., 2013; Esling et al., 2015).

Analysis of metabarcoding data can also result in underestimation of the richness of particular taxonomic groups. For example, a wide range of taxa, including important BQEs, could not be detected in some eDNA-based freshwater biodiversity surveys (Mächler et al., 2014; Deiner et al., 2015). The same pattern was observed in metabarcoding assessments of WFD-compliant macroinvertebrate samples in stream ecosystems, where, on average, >30% of the occurring taxa were not revealed (Elbrecht et al., 2017). In general, primer specificity is considered the main factor controlling detection limits but also incomplete reference databases or biological processes such as recent divergence may lead to a reduced number of genetically identified taxa (e.g. Weiss et al., 2018 see further comments in Section 3.2.2).

*3.2.2. Taxonomic composition: how congruent are morphological and metabarcoding data?*

Several studies have compared the taxonomic composition of given communities estimated through metabarcoding and morphotaxonomic inventories. This has been done either on mock communities/bulk samples (Carew et al., 2013; Kermarrec et al., 2013; Vasselon et al., 2017) or on natural samples (Kermarrec et al., 2014; Visco et al., 2015; Lejzerowicz et al., 2015; Zimmermann et al., 2015; Valentini et al., 2016; Thomsen et al., 2016; Hänfling et al., 2016; Gibson et al., 2015; Rivera et al., 2018; Vasselon et al., 2017). Many studies show a disagreement between molecular and morphological datasets, both in terms of species presence and abundance (Kelly et al., 2017). Several possible reasons have been suggested to explain this discrepancy, but the most cited is the incompleteness and lack of accuracy of the molecular reference databases that impedes the correct taxonomic assignment of eDNA sequences. Taxa absent in the molecular databases could never be identified in eDNA datasets, while sequences linked to a wrong taxonomy in the databases will generate incorrect identifications.

The current status of existing DNA reference libraries depends on taxonomic group and molecular barcode. For example, the reference database of mitochondrial barcodes for European fishes is complete (Geiger et al., 2014; Leese et al., 2018), while the number of barcoded aquatic insects is much more limited. In diatoms, the proportion of European morphospecies present in DNA database averages 30% (Visco et al., 2015), but it drops to 18% in the case of tropical diatoms (Vasselon et al., 2017). In the case of the marine macroinvertebrates included in the AMBI list (Borja et al., 2000), only about 15% of species had COI and/or 18S rRNA gene sequences available in the reference database (Aylagas et al., 2014). A considerable effort has been made to complete and curate reference libraries for the principal groups of bioindicators such as diatoms (Zimmermann et al., 2014; Rimet et al., 2016) and macroinvertebrates (Ratnasingham and Hebert, 2007; Vitecek et al., 2017). In the case of diatoms, the comparison of metabarcoding data with morphological assemblages was proposed as an alternative source to complete the databases (Rimet et al., 2018).

Among other factors that interfere with the accurate assessment of taxonomic composition, sampling scale and size appear to be of paramount importance. Sample size is particularly relevant in the case of large organisms. For example, it is virtually impossible to obtain the same composition of marine benthic macroinvertebrate communities from a standard grab sample (>0.1 $m^3$ of sediment) and from the small sediment samples used for eDNA extractions (Cowart et al., 2015, Lejzerowicz et al., 2015,). Metabarcoding of bulk samples composed of sorted specimens is one of the possibilities to overcome this problem (Carew et al., 2013; Gibson et al., 2015). In the case of meiofauna, the elutriation (resuspension with decanting) of samples prior to DNA extraction provides a more consistent taxonomic composition compared with non-elutriated samples (Brannock and Halanych, 2015). Increasing the number of DNA extraction replicates has been proposed as another way to increase the reproducibility (Zhan et al., 2014) and improve the accuracy and precision of metabarcoding analyses (Lanzén et al., 2017).

Taxonomic composition can also be affected by the presence of so-called "ghost" MOTUs, corresponding to the taxa represented by "extracellular" DNA only. Indeed, the DNA can be preserved for a long time in aquatic ecosystems, either as "free" molecules or inside cellular organelles or cell debris. It can be bound to the sediments (Turner et al., 2015; Torti et al., 2015) or carried by water over large distances (Deiner and Altermatt, 2014). Extracellular DNA is commonly used to detect fish species (Valentini et al., 2016; Shaw et al., 2016, 2017; Stoeckle et al., 2017). In this case, the probability of detecting target DNA in aquatic systems depends on the concentration and dispersion of the extracellular DNA molecules at a site, the sampling method and the environmental conditions, e.g., UV exposure, pH, temperature, which affect the rate at which eDNA degrades or disperses through the environment (Barnes et al., 2014; Furlan et al., 2016; Seymour et al., 2018). Greater survey effort (e.g., collecting more field samples of larger volume at each site, and running more PCR replicates per sample) has been shown to increase the probability of detecting fish DNA, reducing the impact of false negatives and improving confidence in the eDNA metabarcoding approach (Ficetola et al., 2015). At the same time, the increasing controls for contamination at each step of laboratory work and stringent conditions of data analysis help detect and remove false positives (Ficetola et al., 2016).

During sample processing the taxonomic composition is mainly altered at the PCR step by differential primer efficiency and specificity. Considerable efforts have been made to develop PCR primers for DNA barcoding and metabarcoding targeting different taxonomic groups (Zimmermann et al., 2011; Leray et al., 2013; Hadziavdic et al., 2014; Elbrecht et al., 2016). Several studies comparing molecular and morphological taxonomic inventories in bulk samples have found primer bias to be the primary source of variation (Elbrecht and Leese, 2015; Elbrecht et al., 2017) and a common factor resulting in false negatives in metabarcoding data (e.g. Carew et al., 2013; Vivien et al., 2016, 2016). Although these PCR-induced incongruences could be circumvented by the use of direct sequencing of mitochondria-enriched samples (Zhou et al., 2013; Macher et al., 2017) or other PCR-free approaches, the high-throughput amplicon sequencing remains at this time the basic methodology for DNA metabarcoding.

*3.2.3. Abundance: what is the meaning of metabarcoding quantitative data?*

Relative or absolute abundance is used in most BIs, often as the key parameter (Diaz et al., 2004; Borja et al., 2015). Yet, the inference of abundance from metabarcoding data is considered as one of the most difficult issues (Shaw et al., 2016; Edgar et al., 2017). It has been demonstrated that the number of sequences generated by HTS does not directly correspond to the number of specimens or biomass (Carew et al., 2013; Stoeck et al., 2014; Elbrecht and Leese, 2015). Conversely, there are studies, indicating that the relative abundance of some taxa follows similar patterns in molecular and morphological data, e.g. in estuary plankton (Abad et al., 2016) or fish and amphibians (Hänfling et al., 2016; Evans et al., 2016; Kelly, 2016). Indeed, several studies have already successfully used relative abundance of reads for the calculation of BIs, e.g. for diatoms (Visco et al., 2015; Apothéloz-Perret-Gentil et al., 2017; Vasselon et al., 2017), foraminifera (Pawlowski et al., 2014, 2016, 2016; Pochon et al., 2015), and marine macro-invertebrates (Lejzerowicz et al., 2015; Aylagas and Rodríguez-Ezpeleta, 2016).

Several biological and technical factors have been considered as possible causes of differences in the abundance estimation between DNA-based and morphological studies. Among biological factors, taxon and developmental stage-specific variations in biomass, are the most commonly invoked as causes of quantitative biases especially among macro-organisms (Maruyama et al., 2014). In principle, taxa or individuals with high biovolume or body-surface should be over-represented in metabarcoding data compared to morphological counts. This factor seems particularly important with respect to fishes and macroinvertebrates, which vary by several orders of magnitude in biomass and in size depending on their developmental stage (Elbrecht et al., 2017). For example, biomass was strongly and positively correlated to the number of reads in the case of a single stonefly species studied (Elbrecht and Leese, 2015). However, in the same experiment, significant variation in sequence abundances was already observed despite using standardized amounts of biomass and only one species, suggesting that the biomass alone is not the only factor affecting abundance values.

Among technical factors, PCR primer bias is considered as the main source of quantitative biases. The final amount of sequences assigned to a given species is highly dependent on the number of amplicons generated during PCR reaction. PCR primer efficiency differs between species (Kermarrec et al., 2013; Elbrecht and Leese, 2015; Elbrecht et al., 2017, 2017; Piñol et al., 2015; Giner et al., 2016). Primer biases might also be responsible for preferential amplification of selected taxa that leads to a common situation when most of the sequence reads belong to few species that are easily amplified compared with others. The difference between highly abundant and rare taxa in molecular assessments can easily span several orders of magnitudes, impeding correct quantitative analysis. Moreover, PCR primer efficiency likely differs between samples in response to the sampled community, resulting in incomparable results of molecular biodiversity and abundance assessments. In case of highly diverse samples with low DNA template concentrations of individual taxa, PCR stochasticity might lead to deviations in the read abundance correlation given that less frequent templates might get unequally amplified and hence exponentially enriched during PCR cycles.

As of now, there is no simple solution to address the abundance issue. The most conservative approach is to use only presence/absence data, as proposed in the case of freshwater macrozoobenthos (Elbrecht and Leese, 2015). Alternative solutions consist in using corrections factors. Vasselon et al. (2017) successfully tested a correction factor based on species biomass to improve the quantification of diatoms species from read abundances. A correction factor based on PCR effectiveness was also proposed in the case of a freshwater oligochaetes index (Vivien et al., 2015).

*3.2.4. Ecology-based BIs: how to assign ecological values to MOTU?*

The ecological values (trophic, sensitivity, etc.) currently used have been established based on the autecology of single morphospecies or focal BQE taxa. Consequently, in order to use WFD-compliant BIs, the most straightforward solution is to relate metabarcoding data to these morphotaxonomic units. However, this would require a complete DNA barcoding reference database, which is far from being the case for many bioindicator groups (Leese et al., 2018). To overcome this problem, within some groups, it is common to use only the assigned MOTUs for BI calculation, which may provide good results but considerably reduces the amount of analysed metabarcoding data that is used.

An alternative solution proposed by some authors would be to reduce the taxonomic resolution of data used for biomonitoring. Some complex units have been introduced to reduce the complexity and size of metabarcoding datasets to a level that would better correspond to the phylogenetic species concept (Dunthorn et al., 2014; Mahé et al., 2017). Carew et al. (2011) showed that some phylogenetically closely related species have similar tolerance values and therefore there is no need to identify DNA sequences to species level. The use of phylogenetic signal for biomonitoring has also been positively tested with respect to the sensitivity of diatom species to different herbicides (Larras et al., 2014; Esteves et al., 2017) and applied to a wide range of river diatoms (Keck et al., 2016, 2016). Indeed, for different reasons, clustering of closely related phylotypes is often used in metabarcoding studies that infer biotic indices (Visco et al., 2015; Lejzerowicz et al., 2015). However, not all closely related taxa have the same autecological requirements (e.g. Murphy et al., 2015) and identification to the species level might be necessary for calculation of some indices (Aylagas et al., 2014).

Another issue related to sensitivity and trait values concerns the inference of metabolically active species. Most of metabarcoding studies are based on eDNA data. However, it has been shown that eRNA, which is more unstable and degrades more rapidly, could provide a better proxy of ecological changes (Laroche et al., 2016). Indeed, when both molecules are compared, the eRNA usually provides a slightly better (more robust) correlation with morphological indices (Pawlowski et al., 2014; Visco et al., 2015; Pochon et al., 2015; Lejzerowicz et al., 2015). The relative abundance inferred from eRNA data was also closer to the relative cell abundance compared with eDNA in marine picoeukaryotes (Giner et al., 2016). Some authors recommend using the combined eDNA/eRNA datasets advocating that MOTUs present in both datasets provide better insight into the environmental impacts on alpha and beta-diversity (Pawlowski et al., 2014; Laroche et al., 2017).

### 3.3. Perspectives

*3.3.1. New bioindicator groups*

Many taxonomic groups are not assessed in conventional biomonitoring mainly due to the difficulties with their morphological identification. Metabarcoding provides an effective approach to overcome this issue by using DNA-based identification, which opens the doors to a more holistic view of an entire ecosystem. The application of metabarcoding to biomonitoring allows the range of bioindicators to be extended to taxonomic groups known to be sensitive to environmental stressors, but largely ignored in routine biomonitoring (Dafforn et al., 2014; Caruso et al., 2015). These new potential bioindicator groups include prokaryotes, protists, and metazoan meiofauna.

Among various groups of prokaryotes, only cyanobacteria are routinely used for bioassessment (Mateo et al., 2015). The HTS-generated microbiome data open access to the composition of the whole bacterial and archaeal communities. The number of metabarcoding studies assessing environmental impacts on microbial diversity is rapidly increasing. Some studies are using the HTS approach to analyse the impact of pollutants on microbial communities (Dos Santos et al., 2011; Pascault et al., 2014; Smith et al., 2015). Other metabarcoding studies

show that the changes in bacterial communities can be used for the environmental impact assessment of anthropogenic activities (Dowle et al., 2015; Stoeck et al., 2018). Identification to order level was proposed as the best option to analyse the effects of multiple stressors on microbial communities (Salis et al., 2017). A new bacterial index (microgAMBI) has been developed to assess marine sediments quality using microbial diversity inferred from metabarcoding data (Aylagas et al., 2017) and its efficiency in detecting impacts has been tested around the world (Borja, 2018).

There are also increasing efforts to include the metabarcoding data from various groups of protists and meiofauna into routine biomonitoring (Pawlowski et al., 2016, 2016). Some of these groups are widely recognized as bioindicators, e.g. ciliates (Foissner and Berger, 1996), foraminifera (Alve et al., 2016) or nematodes (Fraschetti et al. 2015). Several metabarcoding studies confirm high environmental sensitivity of these taxa by successfully using them to assess the environmental impacts of marine aquaculture (Pawlowski et al., 2014, 2016, 2016; Pochon et al., 2015; Cordier et al., 2017; Stoeck et al., 2018). In addition to metabarcoding studies specifically targeting some taxonomic groups of protists (diatoms, foraminifera, ciliates), some authors have taken the opportunity to cover a broad range of potential bioindicators by analysing a large variety of taxa in the same metabarcoding dataset. This multi-taxon approach has been successfully applied to examine the impact of different environmental drivers on microbial eukaryotes diversity in estuarine (Chariton et al., 2010, 2014; Lallias et al., 2015) and freshwater (Capo et al., 2017) ecosystems, as well as to monitor offshore oil drilling activities (Lanzén et al., 2016; Coelho et al., 2016) and to demonstrate the impact of an oil spill on marine benthic communities (Bik et al., 2012).

*3.3.2. Taxonomy-free approaches and machine learning predictive models*

To overcome the gaps in reference databases and different biases related to the taxonomic assignment of MOTUs, two different approaches have been proposed to compute biotic indices without any reference to morphotaxonomy. In a recent study relating to a benthic diatoms index, the MOTUs were given autecological values based on their occurrence in samples of known ecological status (Apothéloz-Perret-Gentil et al., 2017). The main advantage of this approach was that almost 95% of MOTUs could be used for index calculation, while only 35% of MOTUs have been used in traditional approach based on taxonomic assignment (Apothéloz-Perret-Gentil et al., 2017). This allows the exploitation of a dataset even if most morphospecies are not referenced in the barcoding database, for instance those belonging to taxonomically poorly known groups or less explored geographical regions. Another important advantage concerns the abundance issues. Biological and technical biases are usually reproducible and therefore, when biomass is constant, the relative abundance of specific phylotypes can be compared between samples even if they do not correspond exactly to the relative abundance of the morphospecies.

Another recently proposed taxonomy-free approach comprises the use of Supervised Machine Learning (SML) algorithms to predict BI values (Cordier et al., 2017). The SML methods allow developing predictive models based on the knowledge extracted from complex training datasets, which typically consist of a set of features and associated labels (classification) or continuous values (regression). The aim of SML is to fit the training data to some function (i.e. the model) that can be used to predict a label or a continuous value for the new input data (Knights et al., 2011). Until now, the application of SML to biomonitoring has been limited to the prediction of pollution levels based on a training dataset composed of bacterial 16S eDNA data (Smith et al., 2015) and to the prediction of biotic indices routinely used in benthic monitoring of marine aquaculture (Cordier et al., 2017). In both cases, the SML algorithms produced accurate predictions from metabarcoding data, confirming the applicability of the SML approaches for biomonitoring surveys. The main advantage of the SML compared with the correlative approach proposed in diatoms studies (Apothéloz-Perret-Gentil et al., 2017) is that it takes the communities as a whole, therefore accounting for MOTUs co-occurrence. However, this advantage means that MOTUs are not assigned to any specific ecological values, which makes it harder to compare molecular and morphological data.

Both of these approaches require a training dataset, consisting of samples from which both metabarcoding data and associated pressure data are known. Until now, the taxonomy-free studies have been using BI values inferred from specific taxonomic groups as proxy for ecological quality status. In the future, the taxonomy-free approaches should be calibrated directly on stressor values, if available. That would allow better untangling the effects of different stressors on particular MOTUs or the whole assemblage of MOTUs in the case of machine learning approaches.

## 4. Conclusions and recommendations

In summary, the traditional methods of environmental assessment are well established, accepted, harmonized, comprehensive, and widely used in Europe and elsewhere. The WFD and MSFD have ensured that the focus today is on the integrity of the ecosystem represented by its biology, and the sustainability of its use, and not as earlier on the chemical and physical characteristics alone. A huge amount of effort has been invested in the establishment of this assessment system, and we should be careful not to miss the benefits when introducing new methods.

As outlined above and suggested by Hering et al. (2018), DNA barcoding and metabarcoding can be used to establish molecular metrics and indices, which potentially provide conclusions broadly similar to those of the traditional approaches about the ecological and environmental status of aquatic ecosystems. The use of molecular methods can solve several technical issues faced by currently used BIs. In particular, DNA metabarcoding can increase the taxonomic resolution and comparability across geographic regions, which is often difficult using morphological characters only. Moreover, DNA-based identification allows including early life stages and partially destroyed or fragmented specimens impossible to identify morphologically in biotic indices. It also allows extending the range of potential bioindicators, including the inconspicuous taxonomic groups that could be highly sensitive or tolerant to particular stressors. Indirectly, the molecular methods can also help filling the gaps in knowledge of species ecology, by increasing the number of samples processed coupled with a decrease in processing time (cost-effectiveness), as well as by increasing the accuracy and precision of correlation between species/MOTUs occurrence and environmental factors. Finally, the monitoring of endemic, endangered and invasive alien species can immensely benefit of the easy detection of their DNA traces present in the water. In particular, in case of invasive species these methods help not only in detecting their presence, but also their persistence after the adoption of containment/eradication countermeasures.

However, we must remain cognisant of the limitations of the new methods. There are still several steps of the metabarcoding approach that are disputable, at different stages of the sample processing and of the data analysis. Currently there is no consensus concerning methods for DNA preservation and isolation, the choice of DNA barcodes and PCR primers, not to mention the debate concerning the parameters of MOTU clustering and their taxonomic assignment. Standardization of molecular protocols is urgently needed, taking into account a constant evolution and parallel development of new biotechnological tools for acquisition and analysis of DNA data. Moreover, the reference database of bioindicator taxa is far from complete despite the constant efforts of numerous national barcoding initiatives. Furthermore, most existing metabarcoding data are only locally available and geographically scattered, which is hindering the development of globally useful tools. A huge effort is still necessary to ensure coverage of a range of stressor values at least as broad as for the development of the traditional methods.

In view of these potential limitations, we recommend a two-step implementation of metabarcoding in routine biomonitoring. In the short term, we suggest the integration of metabarcoding data into the existing biotic indices. This could be easily done for diatoms, invertebrates and fish, which have been the focus of most metabarcoding studies. The use of metabarcoding data will provide considerable advantages for any BIs based on these BQEs, given that the adequate effort to complete comprehensive group specific databases is provided. In the case of diatoms, metabarcoding will enable a better harmonization of identification, which will improve the consistency of calculated biotic indices. The metabarcoding of invertebrates will increase the taxonomic resolution and will potentially improve the correctness of the taxa-ecology coupling, taking into account all specimens, including larval stages and juveniles that cannot be identified to species level. In the case of fish-based BIs, eDNA analyses offer the possibility to survey fish populations without killing or disturbing them, and to use genetic diversity as a new way to measure degradation. This first step integration could be done locally, with each country being able to use its own BIs to test and validate the use of molecular data, applied to the reference water bodies, as highlighted in Leese et al. (2018). In parallel, special efforts need to be provided in order to increase accuracy and precision of the biotic indices by ensuring that the databases are covering at least the important taxa for the biotic index calculations.

In the long term, we propose the new molecular indices should be developed based entirely on metabarcoding data. Such biotic indices could provide a more holistic view of biological community response to the anthropogenic stressors by including new potential BQEs, in particular various groups of prokaryotic and eukaryotic microbiota and meiofauna. They could be based on predictive models established using machine-learning and other algorithms capable of assessing ecological status and identifying ecologically meaningful MOTUs in the metabarcoding datasets. Last but not least, to comply with the WFD and MSFD, these new biotic indices should be benchmarked against both currently existing indices and directly against the pressure data in order to redefine the boundary settings, which will require large-scale intercalibration exercises. The final outcome of such exercises could be the development of pan-European or global molecular BIs, which will constitute a major advance towards a standardized and efficient assessment of the ecological quality of aquatic ecosystems.

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