



Review

Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive



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ABSTRACT

Assessment of ecological status for the European Water Framework Directive (WFD) is based on “Biological Quality Elements” (BQEs), namely phytoplankton, benthic flora, benthic invertebrates and fish. Morphological identification of these organisms is a time-consuming and expensive procedure. Here, we assess the options for complementing and, perhaps, replacing morphological identification with procedures using eDNA, metabarcoding or similar approaches. We rate the applicability of DNA-based identification for the individual BQEs and water categories (rivers, lakes, transitional and coastal waters) against eleven criteria, summarised under the headlines representativeness (for example suitability of current sampling methods for DNA-based identification, errors from DNA-based species detection), sensitivity (for example capability to detect sensitive taxa, unassigned reads), precision of DNA-based identification (knowledge about uncertainty), comparability with conventional approaches (for example sensitivity of metrics to differences in DNA-based identification), cost effectiveness and environmental impact. Overall, suitability of DNA-based identification is particularly high for fish, as eDNA is a well-suited sampling approach which can replace expensive and potentially harmful methods such as gill-netting, trawling or electrofishing. Furthermore, there are attempts to replace absolute by relative abundance in metric calculations. For invertebrates and phytobenthos, the main challenges include the modification of indices and completing barcode libraries. For phytoplankton, the barcode libraries are even more problematic, due to the high taxonomic diversity in plankton samples. If current assessment concepts are kept, DNA-based identification is least appropriate for macrophytes (rivers, lakes) and angiosperms/macroalgae (transitional and coastal waters), which are surveyed rather than sampled. We discuss general implications of implementing DNA-based identification into standard ecological

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assessment, in particular considering any adaptations to the WFD that may be required to facilitate the transition to molecular data.

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Contents

1. Introduction	193
1.1. Assessment and monitoring methods under the WFD	195
1.2. DNA-based methods for species identification	195
1.3. Criteria to rate the potential for application of DNA-based identification	196
Representativeness	196
Sensitivity of species detection	198
Precision of DNA-based identifications	198
Comparability with conventional approaches	199
Cost-effectiveness	199
Criterion 6.1: Animal well-being, health and safety, environmental impact	199
1.4. Applicability of DNA-based identification for combinations of BQEs and water categories	200
Criterion 1.1 (Applicability of current sampling methods, and availability of alternative methods, for obtaining biological material for DNA-based identification)	200
Criterion 1.2 (Errors from DNA-based species detection and similarity of DNA-based and conventional taxon lists)	201
Criterion 1.3 (Need for abundance assessment and accuracy of abundance estimates with DNA-based methods)	201
Criterion 2.1 (Capability of DNA-based methods to sample sensitive taxa)	201
Criterion 2.2 (Unassigned reads)	201
Criterion 3.1 (Knowledge about uncertainty of DNA-based identification)	201
Criterion 4.1 (Sensitivity of EQRs to differences in DNA-based identification)	201
Criterion 4.2 (Intercalibration)	201
Criterion 5.1 (Costs compared with traditional methods)	201
Criterion 5.2 (Processing speed)	202
Criterion 6.1 (Animal well-being, health and safety, environmental impact)	202
2. Discussion and outlook	202
2.1. Suitability of DNA-based identification for different BQEs and water categories	202
2.2. Implications of implementing DNA-based identification	202
3. Conclusions	203
Acknowledgements	203
References	203

1. Introduction

Worldwide, aquatic ecosystems are monitored using a range of organisms as indicators (Foden et al., 2008; Hallett et al., 2016; Patrício et al., 2016). In the European Union, most freshwater monitoring is performed to fulfil the requirements of the EU Water Framework Directive (WFD, 2000/60/EC), which aims to improve the status of European freshwater resources and ecosystems. It requires Member States to assess the ecological status of all surface water bodies at regular intervals (De Jonge et al., 2006). Chemical status of surface and groundwater bodies is also assessed, but not discussed in this paper. The number of monitored river, lakes, transitional and coastal waters in Europe exceeds 100,000, and for most of them several organism groups (“Biological Quality Elements”, BQEs) are investigated. These include phytoplankton, phytobenthos and larger aquatic plants, as well as benthic invertebrates and fish (EEA, 2012). The Marine Strategy Framework Directive (MSFD, 2008/56/EC) also requires the use of several indicators including species diversity, seafloor integrity, food web structure, and non-indigenous and commercial species, but its implementation is currently not as advanced as for the WFD (Danovaro et al., 2016).

All monitoring and assessment methods applied under the WFD conform to the same conceptual framework, although the details differ among countries and regions (Birk et al., 2012). In short, organisms are sampled or surveyed following national or EU-wide

standard methods to produce lists of taxa present and (in most cases) estimates of abundance, processed in the laboratory (if necessary), and identified using morpho-taxonomic approaches. The resulting data are used to compute assessment metrics, which are compared against values for each metric expected at “reference conditions” (i.e. in a more-or-less unimpacted state derived from historical conditions or best available sites) specific to each type of water body. The distance between the calculated value and the value at reference conditions is termed the Ecological Quality Ratio (EQR), which is finally translated into a quality class (high, good, moderate, poor and bad) on which management decisions are based. The objective is to achieve at least “good status” for all water bodies in Europe by 2027: at present, half of all water bodies do not meet this goal (EEA, 2012).

Most assessment methods for European freshwaters were developed in the 2000s, following adoption of the WFD by EU Member States. In many cases, these methods were based on approaches developed prior to adoption of the WFD with adjustments to translate assessment results into ecological status classes. Whilst field and laboratory methods were largely left unchanged, some Member States developed new assessment methods. Whatever the strategy adopted, each biological method was then “intercalibrated” with the respective methods of other Member States in the same broad ecoregion (termed “Geographical Intercalibration Groups”, Birk et al., 2013). Although the formal definition of ecological status encompasses both structure and function (Article

2, definition 21, WFD), the assessment systems have been based primarily on structure. Some assessment metrics do use species traits, such as size structure of fish assemblages or feeding type composition of benthic invertebrates (Mondy et al., 2012; Pont et al., 2006) but most methods neglect this aspect. Overall, despite the shortcomings of many of the methods, the process of method development, adaptation and intercalibration have contributed to a better understanding of reference conditions, responses of biota to stressors and the uncertainties associated with various steps in the assessment of ecological status (Poikane et al., 2014).

Some aspects of monitoring procedures are time consuming and costly, requiring teams of skilled individuals, for example the identification and counting of phytoplankton, phyto-benthos and benthic invertebrates (Ferraro et al., 1989; Haase et al., 2004; Nygård et al., 2016). Electrofishing and gillnetting for fish are also costly and require teams of skilled staff. As budgets for such work are under pressure, there is a demand to simplify methods, to lower the costs and to speed up the monitoring process (Borja and Elliott, 2013), whilst maintaining quality, robustness and comparability. Recent technological advances could go some way towards alleviating these budget constraints.

New methods such as machine learning (Kiranyaz et al., 2011; Årje et al., 2017), and genetic methods such as metabarcoding of DNA obtained from organisms or simply by sampling environmental DNA (eDNA) from the water (for example Taberlet et al., 2012a; Ji et al., 2013) provide alternative tools for multiple species detection and identification. In the medium term, these new methods have the potential to fundamentally change ecological assessment. Although still in the development phase, genetic methods are already sufficiently well advanced for biodiversity assessment (for example Elbrecht et al., 2017). Thus, it is now possible to complement or even replace traditional sample processing and identification methods with DNA-based methods which are of equal or lower cost and which are able to detect species occurrences with a similar or higher level of precision (Stein et al., 2014; Smart et al., 2016; Aylagas, 2017; Elbrecht et al., 2017; Vasselton et al., 2017). DNA-based methods have some obvious advantages compared with traditional sampling and image recognition based identification schemes. Identification to species level is more precise and objective with DNA-based methods, particularly for cryptic taxa, microorganisms and difficult life stages (for example juveniles and pupae) while sample processing may be faster and cheaper than manual procedures (Hajibabaei et al., 2011; Kermarrec et al., 2014; Dafforn et al., 2014; Stein et al., 2014; Avó et al., 2017). An additional advantage of molecular techniques is the potential for assessing functional diversity based on gene expression (transcription), fulfilling an aim of the WFD that has yet to be addressed adequately with morpho-taxonomic approaches (Bourlat et al., 2013). On the other hand, molecular techniques are still developing and require standardisation and harmonization (Cristescu, 2014) before they can be used in national monitoring programmes. Furthermore, there is limited capability for the determination of species abundance, which is a prerequisite for many BQEs assessed for the WFD. Reference barcodes are not yet available for a considerable – although decreasing – proportion of species.

For a more general application of DNA-based techniques in WFD assessments, key questions regarding comparability with traditional methods need to be addressed, in particular the sensitivity of species detection and the precision of species identification (Leese et al., 2016). In principle, there are two options for including DNA-based methods into ecological status assessment:

Option 1: Under this option, specific steps of the conventional assessment procedure, particularly those leading to the

identification of organisms, could be replaced by DNA-based methods. Other elements, such as metrics, assessment system, interpretation and, in many cases, sampling, remain the same or are subject to minor adaptation, for example different preservatives, reassessment of taxa lists from reference water bodies, and replacement of electrofishing by water samples. This option could provide the same level of information as traditional methods, but may improve processing speed, comparability and cost efficiency. In the following, we refer to this method as “DNA-based identification”.

Option 2: This option combines different ways of using new assessment metrics, which take full advantage of the higher taxonomic resolution of DNA-based methods, producing typically more highly resolved taxa lists and possibly information on ecosystem functioning (Grossmann et al., 2016). This could, for example, enable the inclusion of species of currently widely ignored organism groups (such as Chironomidae) into biodiversity metrics, or development of metrics based on the expression of genes involved in osmoregulation to assess the impact of freshwater salinization. In cases where only scarce taxon information exist (for example protists), Operational Taxonomic Units (OTUs) can be assigned and used for index development. This option can only be implemented in the medium-to long-term and may require the complete redesign of assessment systems, including derivation of new reference condition values and the development of new assessment metrics. Functional metrics are currently underrepresented in WFD assessment systems, although trait-based data have been frequently derived from morphological criteria (Schmidt-Kloiber and Hering, 2015) and are used in several assessment methods. Molecular data, in particular transcriptomic data (Konopka and Wilkins, 2012; Creer et al., 2016) and placement into trait-informed phylogenies offer additional options for functional metrics, which would need to be developed from scratch, and their response to stressor gradients investigated. However, research in this field is still its infancy and implementation into practical ecological assessment is unlikely in the short and medium term.

Hybrid option: There is also the possibility of a hybrid between Options 1 and 2 where DNA-based methods are used to replace morphological identification whilst keeping metrics and reference conditions for assessment purposes (cf option 1). At the same time, additional information generated by DNA-based methods such as more highly resolved taxa lists or functional information derived from other approaches such as metagenomics and -transcriptomics would be used to better inform interpretation of assessment results, for example rating how stressors affect ecosystem functionality.

Until 2027, only Option 1 provides a realistic option for operational monitoring under the WFD. European countries have spent considerable resource developing WFD assessment systems and have used them in previous monitoring cycles: they will continue to apply them until the end of the fourth River Basin Management Cycle in 2027. Therefore, this paper focuses on DNA-based identification (Option 1), acknowledging that it is a straightforward, but rather conservative approach in comparison with Option 2, as it aims for maximum comparability with traditional methods.

In some circumstances, the inclusion of DNA-based techniques into WFD assessment has already been tested, for example for river phyto-benthos in Mayotte Island, France (Vasselton et al., 2017) and the UK (Kelly et al., 2017), and is likely to be used increasingly for a range of BQEs in other countries (Leese et al., 2018). However, for a variety of reasons the applicability of Option 1 differs between BQEs and water body types (river, lakes, transitional and coastal waters). Amongst others, there is the need to secure comparability with traditional identification, which may be more problematic for those BQEs where there are large discrepancies between morphological

and DNA-based species identification. In addition, the potential benefits in sample processing speed differ strongly between BQEs.

Here, we evaluate the potential of DNA-based identification (Option 1) for routine WFD assessment for different BQEs and water categories. Our aim is to rate the applicability of DNA-based identification methods, assuming that current WFD assessment metrics are kept or only slightly adapted. We use a variety of criteria related to the anticipated suitability (for example the expected increase in processing speed, lower costs) and the maturity of development (for example the extent to which assessment methods will need to be adapted). The paper is addressed at scientists and officials involved into the commissioning and development of DNA-based methods, stakeholders and consultants involved in WFD monitoring.

1.1. Assessment and monitoring methods under the WFD

Considerable research effort has been devoted to the development of methods for ecological assessment of waterbodies following implementation of the WFD (Birk et al., 2012). The primary focus has been to establish sensitive and precise methods capable of assessing the impact of a wide range of pressures on biota and, hence, guide management efforts to restore good ecological status. The reference condition approach is a central principle of the WFD: the biota observed are compared with those expected in the absence of environmental stress, resulting in an Ecological Quality Ratio (EQR), calculated as the observed score/expected score (Jones et al., 2010).

Although always based on the same principles, subsidiarity has led to diversity in the methods developed by Member States for each BQE-water category combination. This reflects the variety of methods and data existing prior to the WFD, and regional differences in stressors and taxonomic knowledge. Overall, more than 300 methods are in use across Europe (Birk et al., 2012), with comparability ensured by an obligatory intercalibration process (Birk et al., 2013; Poikane et al., 2014). At a first glance, the large number of methods is bewildering; however, all methods are based on the same chain of steps and many differ only in detail (Birk et al., 2013):

- Surveys are always stratified by water bodies, for example individual lakes or homogeneous river sections which may be several kilometres in length.
- Sampling is conducted using standardised approaches allowing for (semi)quantitative analysis. Identification is to species for those BQEs with a low number of species (fish, macrophytes, macroalgae, angiosperms), and varies between species and family level (for the remaining BQEs (phytoplankton, phyto-benthos and invertebrates), depending on feasibility, regional taxonomic knowledge, and bioindication potential.
- Metrics are calculated from the resulting taxon lists, reflecting either general degradation or individual stressors. The results are compared with metric values obtained at reference conditions, which are specific to each type of water body.

The deviation from reference conditions is expressed as the EQR (from 0 to 1) from which the biological status class (“high”, “good”, “moderate”, “poor” or “bad”) is derived, harmonised between EU member states through intercalibration. The status classes of the individual BQEs are finally combined with other quality elements into an ecological status class, using the “one-out-all-out” principle (the worst status class determines the overall ecological status class).

Three types of monitoring are specified by the WFD, each with a different objective, namely: (1) surveillance monitoring to classify

water bodies and assess large-scale, long-term change; (2) operational monitoring, focussed on water bodies unlikely to reach good status, in order to establish local management options, and (3) investigative monitoring to identify the causes of a water body not achieving environmental objectives, and to assess the magnitude and source of accidental pollution.

1.2. DNA-based methods for species identification

DNA-based methods for species identification cover a wide range of techniques and considerations. Before any molecular analysis can be applied, DNA must first be obtained either by collecting organisms directly or by sampling the environment (for example water) and extracting the genetic material present (environmental DNA or eDNA) without sorting organisms (Baird and Hajibabaei, 2012; Bohmann et al., 2014; Taberlet et al., 2012a). These two broad sources of DNA differ in some fundamental aspects. First, whereas large amounts of DNA can be extracted from community bulk samples (for example macroinvertebrates) and microorganisms such as diatoms in biofilms or water, aqueous eDNA from macroorganisms (for example fish, amphibians) is generally present at very low concentrations (Pilliod et al., 2013) and can be heterogeneously distributed throughout the environment, which has consequences for species detection.

Individually caught specimens can be identified using DNA barcoding, which uses short genetic markers (DNA barcodes) in an organism's DNA to assign it to a species using a pre-existing classification and reference database. Today, the public library of standardized DNA barcodes (<http://www.barcodeoflife.org>) allows the identification of a wide range of species based on the corresponding sequence reference for animals (COI gene), plants (rbcL, matk, 18S), cyanobacteria (16S) and fungi (ITS) (see Creer et al., 2016 for an overview of other markers currently in use). Single specimen DNA barcoding is widely used, for example in biodiversity conservation, environmental management, invasion biology, studies of trophic interactions and food safety (Cristescu, 2014) but is not yet a cost efficient method for most ecological assessment purposes (Stein et al., 2014).

More recently, high throughput sequencing (HTS) techniques have allowed the barcodes of multiple organisms to be obtained in a single reaction, enabling parallel sequence-based identification in an approach termed DNA metabarcoding (Taberlet et al., 2012b; Shokralla et al., 2012). This approach offers the opportunity for non-targeted (passive) detection of a wide range of rare and invasive species (for example Blackman et al., 2017; see Lawson Handley, 2015; for a review) and to assess the composition of whole communities. The application of DNA metabarcoding to community DNA extracted from organisms or environmental samples (eDNA) is the focus of this paper.

Most current sequencing protocols rely on rather short (i.e. about 70–500 base pair) metabarcoding markers and thus are capable of using the degraded DNA often found in eDNA samples (see Elbrecht and Leese, 2017, for an overview). Recent research has shown that DNA-based methods are effective at detecting aquatic species of microalgae and protists (Medinger et al., 2010; Kermarrec et al., 2014; Kelly et al., 2017), meiofauna (Carugati et al., 2015), macroinvertebrates (Hajibabaei et al., 2011; Sweeney et al., 2011; Aylagas et al., 2016), fish (Thomsen et al., 2012; Kelly et al., 2014; Civade et al., 2016; Hanfling et al., 2016; Shaw et al., 2016) and amphibians (Ficetola et al., 2008; Dejean et al., 2012). However, the protocols and workflows used for capture, extraction and identification of DNA are highly diverse even within BQEs. This makes comparison of results from different laboratories and studies difficult (Deiner et al., 2015) and will limit the use of DNA for aquatic biodiversity assessment until the biases associated with

different methods are fully understood and controlled. Probably the critical consideration is choosing the most appropriate primer, which determines the DNA marker used for identification, and its length. This in turn influences the taxonomic resolution that can be achieved and affects the extent to which species level identifications can be made; primer choice also affects the specificity of the analysis. In some cases, highly specific primers can be developed that will amplify the entire target organism group and little else (the 12S primers for fish are a good example). In other cases, primers that are general enough to capture the whole group will inevitably amplify non-target taxa as well. An example of this is the primers designed to amplify benthic invertebrates, which consistently amplify a wide range of non-metazoan taxa when used on environmental samples.

1.3. Criteria to rate the potential for application of DNA-based identification

Here we describe and justify a set of criteria, which will later be used to rate the applicability of DNA-based identification for incorporation into WFD assessment for different BQEs and water categories. As we limit the applicability check to DNA-based identification, and do not include more advanced approaches (i.e. Option 2 described in the introduction), the criteria are restricted to those rating the performance of WFD-related assessment methods. The criteria are categorised under six headings: 1) Representativeness, 2) Sensitivity, 3) Precision, 4) Comparability, 5) Cost-effectiveness and 6) Environmental impact, and are not always independent. For example, the cost of sample collection and processing will influence the sampling strategy undertaken (frequency and number of samples collected), which, in turn, will influence the representativeness and precision of the overall assessment of ecological status. Here, we will address each of these criteria separately, whilst considering those interactions relevant to DNA-based identification.

Representativeness

Criterion 1.1: Applicability of current sampling methods, and availability of alternative methods for obtaining biological material for DNA-based identification. This criterion addresses how samples are collected and processed prior to sequencing, to determine if current sampling methods are suitable for molecular methods, or if simple alternatives are available. The criterion is relevant to establish whether DNA-based identification can be used without changing current sampling strategies significantly, or if major changes in sampling methods are required.

For some taxa (microalgae, macroinvertebrates) entire unprocessed samples have been used for extraction and subsequent metabarcoding (Zimmermann et al., 2015; Elbrecht et al., 2017), which can be analysed in parallel with microscopy. However, for inventories of fish species, the current sampling methods (for example electrofishing) cannot be used for DNA-based assays. The proposed solution of sampling eDNA from water is a simple and effective alternative. Results from eDNA approaches are often very similar to those from traditional netting or electrofishing, although usually more effective (Takahara et al., 2012; Shaw et al., 2016; Hanfling et al., 2016; Stoeckle et al., 2017; Pont et al., submitted). However, the inference of temporal and spatial distribution of species through eDNA is complicated since detection is influenced by environmentally variable DNA degradation rates, transport and species specific behavioural patterns (Barnes and Turner, 2015; Stoeckle et al., 2017). The spatial scale of eDNA detectability is of particular importance in lotic ecosystems, as eDNA may only detect species present in upstream regions or tributaries. On the other hand, eDNA may better represent species composition across the

whole waterbody (from a few to several tens of kilometres; Cívade et al., 2016; Pont et al., submitted), as is required for surveillance monitoring. Understanding the spatial and temporal scales that eDNA represents is a hurdle to the deployment of this approach for WFD monitoring.

After the removal of an organism, DNA persistence under normal conditions in water is quite short (a few days to two weeks in mesocosms; Ficetola et al., 2008; Dejean et al., 2012; Pilliod et al., 2013). In rivers, eDNA concentration and detectability downstream from the point of production are dependent on production and degradation rates, dilution, transport through the river network, deposition, and resuspension (Thomsen et al., 2012). Detectable eDNA can be found at distances from a few hundred metres to a few kilometres downstream of its source (Deiner and Altermatt, 2014; Jane et al., 2015; Cívade et al., 2016; Wilcox et al., 2016). The detection distance of eDNA is important for defining the scale at which eDNA can reveal spatial and temporal differences in biological communities (Cívade et al., 2016; Deiner et al., 2016; Stæhr et al., 2016; Bista et al., 2017; Stoeckle et al., 2017; Yamamoto et al., 2017).

We used this criterion for rating the magnitude of alterations in sampling methods required to apply DNA-based identification.

Criterion 1.2: Errors from DNA-based species detection and similarity of DNA-based and conventional taxon lists. This criterion addresses the question of how comparable taxon lists obtained with DNA-based methods are to taxon lists obtained with traditional methods, in particular as a result of detection errors. The criterion is relevant to judge if current assessment indices and associated class boundaries can be applied to taxon lists generated with DNA-based methods.

In the production of taxon lists, two types of error occur, false negatives, where a taxon is recorded as absent yet is in fact present, and false positives, where a taxon is recorded as present yet is in fact absent: misidentifications comprise both type of error (the correct species is falsely recorded as absent, whilst the incorrect species is falsely recorded as present). Both error types affect index values and hence the accuracy of assessments (Criterion 2), and add uncertainty (Criterion 3). Both visual and DNA-based methods are prone to identification errors. Whilst it is known that errors can significantly affect the results of traditional assessments (Haase et al., 2006), much work remains to be done for DNA-based methods. If the DNA-based identification targets morphotaxa rather than OTUs, benchmarking against morpho-taxonomic approaches will be critical before molecular approaches can be implemented in regular assessment programs. This has been performed partly for fish (Hanfling et al., 2016), marine phytoplankton (Mohrbeck et al., 2015; Albaina et al., 2016), macroinvertebrates (for example Aylagas et al., 2016; Elbrecht and Leese, 2015) and diatoms (Zimmermann et al., 2015).

Direct comparison of detection rates from DNA surveys and traditional survey methods have found that the likelihood of species detection increases with the density of target organisms for both approaches, but at a higher rate for DNA based methods than for morpho-taxonomic methods (Darling and Mahon, 2011). Where they have been tested, false negative rates are either similar to those of established methods or lower (Deiner et al., 2017). Reasons for false negatives in DNA approaches include inefficiency of molecular assays (incomplete barcode libraries, primer bias, low sensitivity), low DNA quality (insufficient DNA, poor quality of eDNA due to environmental conditions or ineffective sample preservation; Darling and Mahon, 2011; Thomsen et al., 2016), the presence of PCR inhibitors (Jane et al., 2015), structural errors (for example errors in bioinformatics) and, in the case of eDNA studies, stochastic effects during sampling due to the low concentration and

heterogeneous distribution of DNA molecules (Ficetola et al., 2015). In order to ensure that rare species are detected, sampling effort needs to be high in terms of the number of replicates or volume of water filtered (Hanfling et al., 2016; Shaw et al., 2016; Valentini et al., 2016). The low target DNA concentration typical for eDNA samples also increases the risk of contamination during sampling and laboratory work. Similarly, the probability of species detection is dependent on sampling effort when using traditional methods, such as electrofishing (Lyon et al., 2014).

On the other hand, false positives (including “unexpected” detections) are an important problem especially in eDNA metabarcoding. False positive detections may arise through contamination during sampling and laboratory work, structural errors (for example errors in bioinformatics, chimeras), the presence of target DNA in samples where the organism in question is not present (Darling and Mahon, 2011; Stoeckle et al., 2017; Yamamoto et al., 2017) or only present in upstream sites (Hanfling et al., 2016), and dead organisms or life-stages (seeds, spores, eggs, early instars) associated with non-viable populations. The results of eDNA studies can be influenced strongly by single molecules. It is less likely to be a concern for whole community analyses where the majority of organisms present in the sample will be relevant and their abundant DNA reduce the influence of trace DNA. There is a clear need to relate DNA reads to the presence of viable populations within the water body. At some point the information gained from molecular methods will tip from “signal” to “noise”, and it will be important to learn to differentiate between an indication of a genuinely rare species and reads caused by DNA from non-viable organisms.

As a result, the taxa lists produced by DNA-based methods are different from those generated by traditional methods: additional taxa will be included that are not identifiable with morphometric methods, while other taxa will not be detected. In addition, detection limits will differ, dependent on the way specimens/DNA are extracted from the raw samples. DNA-based taxa lists will inevitably require some manipulation before they can be used in current assessment methods. This may involve filtering DNA-based lists against the operational taxon list used for that assessment system, thus eliminating those taxa which are not detected with traditional methods (Elbrecht et al., 2017) as well as indicating those that cannot (yet) be identified with DNA based methods (for example due to incomplete reference databases). Alternatively, assessment systems may need to be modified, by aligning (inter-calibrating) future indices suitable for DNA-based methods with existing indices if the full potential of genetic identification is to be realised.

We used this criterion to rate the suitability of DNA-based taxon lists for the calculation of the assessment indices applied in the current WFD assessment schemes.

Criterion 1.3: Need for assessment of abundance and accuracy of abundance estimates with DNA-based methods. This criterion addresses questions regarding the capability of DNA-based methods to estimate abundance alongside the relevance of abundance estimates is for current WFD assessment methods. The criterion is relevant to understand whether missing information on abundance will be a significant obstacle before DNA-based assessments can be applied to meet current WFD requirements.

The WFD specifies that abundance should be considered when determining ecological status; hence, current WFD approaches include estimates of abundance (often as abundance classes). For straightforward integration of DNA-based identification into these approaches, molecular methods also need to generate abundance estimates. Therefore, a key question is whether or not DNA-based

methods can provide reliable estimates of absolute or relative species abundance (see for review Bohmann et al., 2014; Rees et al., 2014; Lawson-Handley, 2015). While quantitative PCR approaches can be used to quantify target organisms (Takahara et al., 2012; Kelly et al., 2014; Nathan et al., 2014; Klymus et al., 2015; Baldigo et al., 2017), this becomes problematic for metabarcoding due to primer bias (Pinol et al., 2014; Elbrecht and Leese, 2015). Factors that influence DNA concentration and errors along the analytical pipeline can alter the relationship between the initial quantity of DNA in the sample and the final number of reads per species (see Bohmann et al., 2014; for a review). Nevertheless, recent results have tended to demonstrate a link between the initial amount of DNA and the number of reads (Elbrecht et al., 2017; Klymus et al., 2017), opening the possibility of estimating relative abundances of target taxa from high-throughput sequences of eDNA samples (Hanfling et al., 2016; Pont et al., submitted; Brys et al., 2017). Metagenomic approaches, where target DNA is sequenced without a PCR-amplification step, could potentially overcome or reduce taxa biases associated with some metabarcoding assays (Thomsen et al., 2016; Choo et al., 2017). Whilst correlations between metagenomic-approaches and PCR-based approaches are significant, their strength is moderate, and the first results have been a proof of concept rather than demonstration of quantitative [predictive?] relationships.

It is important to note that even if a strong relationship can be obtained between amount of DNA in a sample and the number of sequence reads, the relationship between the number (or biomass) of organisms and the amount of DNA released into the environment is not straightforward. Some organisms (for example fish) shed DNA continuously while others (for example crayfish) shed large amounts when they breed or moult but very little at other times of year. Even for fish, spawning introduces large amounts of DNA into the environment that does not reflect the size of the adult population. Thus, sampling campaigns need to take account of the ecology and life-histories of the target organisms before quantitative inferences can be made.

Correction factors can eliminate biases to an extent when DNA-based data are used in assessment systems. Furthermore, many assessment systems use relative rather than absolute abundance or summarise absolute abundance as broad categories (for example log categories), where small biases may not introduce much uncertainty (Birk et al., 2012). A number of studies have demonstrated that relative abundance estimates from eDNA metabarcoding of fish communities show good correlations with abundance estimates from established survey methods. A comparison of electrofishing and eDNA based methods along the Rhône River, for example, revealed a sufficient correlation between the two techniques to describe the structure of fish assemblages and their longitudinal change in terms of relative abundance (Pont et al., submitted). In Windermere, a large lake in the UK, rank abundance from long-term traditional survey data correlated well with eDNA based estimates of relative abundance (Hanfling et al., 2016) and a recent study in Belgian ponds showed strong correlations between sequence read counts and fish biomass (Brys et al., 2017). As the WFD assessment approach demands that comparison are made between observed and expected conditions, it may be possible to correct for consistent biases, particularly when the reference condition is based on new characterisation using molecular techniques.

We used this criterion to rate the degree of changes required in current WFD assessment schemes to account for the differences in abundance data generated by DNA-based identification methods compared with traditional identification methods.

Sensitivity of species detection

Criterion 2.1: Capability of DNA-based methods to sample sensitive taxa. This criterion addresses the question of whether or not DNA-based methods are suitable for the detection of sensitive taxa, which are an integral part of most WFD assessment methods. The criterion is relevant to rate if current assessment metrics can reasonably be applied with taxon lists generated with DNA-based methods.

Whilst some management objectives may require complete lists of taxa present (for example the conservation objectives of the Habitats Directive, which target species listed in Annexes II, IV and V; see http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm), the objective of the WFD is the sustainable development of water bodies. Hence, the principal role of biological monitoring is to determine the condition of the ecosystem and to detect impacts that could impede WFD objectives. Those taxa that are sensitive to human-induced stress are not necessarily those that contribute the most to structure and function, and assessments need to be aware of this. For example, several sensitive benthic invertebrate species with a long life cycle, whose occurrence indicates the absence of pollution events over a long time period, tend to occur at low abundance (e.g. large Plecoptera species). Whilst a complete list of taxa might not be required to determine stress effects, rare taxa are important components of some assessment metrics as they are typically most sensitive to water body deterioration (Clarke and Murphy, 2006). For those BQEs and water categories where this has been demonstrated, it is important to ensure that rare species are accurately characterised when developing techniques that involve bulk extraction of genetic material. For fish, the capacity of DNA based methods to detect rare species in rivers more effectively than traditional methods has been clearly demonstrated (Civade et al., 2016; Pont et al., submitted), whereas for invertebrate samples it may be necessary to transform or increase sequencing depth in order to ensure rare taxa are detected (Elbrecht et al., 2017). For phytobenthos, the main issue is the severe underrepresentation of rare species in existing reference databases (Kermarrec et al., 2014).

Another issue affecting sensitivity is sequencing depth relative to non-target DNA. For example, samples may have high concentrations of DNA from taxa that are not relevant for calculation of indices (e.g. fungi) and these high concentrations may reduce sensitivity to target or rare taxa.

We used this criterion to rate if current assessment indices can be applied with DNA-based taxon lists.

Criterion 2.2: Unassigned reads. This criterion addresses the separate but related question, of how the influence of f “unassigned” reads (i.e. those reads or OTUs that do not match a Linnaean taxon in DNA reference databases) is minimised. This criterion is relevant to judge if it is necessary to either generate more data for DNA reference databases or, alternatively, to generate data on ecological preferences for unassigned OTUs before they could be used in assessment systems.

The extent of this problem varies among BQEs and is particularly complex for taxa-rich BQEs. For microalgae, Linnaean nomenclature still needs to be reconciled with cryptic diversity and possibly the depth of coverage of each taxon needs to be reconsidered. Whilst chimeras and mistags occur for all BQEs, for most the frequency of unassigned reads is related to the completeness of barcode libraries. The COI gene, for example, is available for hundreds of thousands of species, yet many taxa have are still to be sequenced. Additional sequences are needed for adequate representation of intraspecific and geographic variation (Bergsten et al., 2012). For groups where other gene regions are preferred (for example 18S and rbcL for microalgae, 16S for Cyanobacteria) the

number of taxa sequenced is lower despite considerable sequencing effort (for example Rimet et al., 2016). For fish, a barcode library based on the 12S marker is still in development for Southern and Eastern Europe, but 90% of fish species encountered in Western European continental water bodies have already been sequenced (Valentini et al., 2016). For UK macroinvertebrates, most OTUs have been assigned to species based on COI data, although taxonomic problems resulting from cryptic species remain to be solved (Andujar et al., 2018).

Poor species representation in reference databases may lead to incorrect identifications and, thus, affect the assessments of ecological quality (Aylagas et al., 2014). In turn, this depends on the structure of the index. Four types of indices are used to assess ecological status for the WFD (Hering et al., 2006): Composition/abundances indices, richness/diversity indices, sensitivity/tolerance indices and functional indices. Incomplete barcode libraries may have little influence on diversity indices, as the number of OTUs overall or within broad classification groups (for example order) may be sufficient to derive index values. However, those indices that are calculated from species presence are more vulnerable, as they require correct species identification. Indices based on average scores are likely to be more robust to missing taxa, but efforts will be needed to benchmark indices derived through molecular methods against those derived using existing approaches (Ärje et al., 2017).

We used this criterion to rate how complete barcode libraries are for the individual BQEs and how incomplete barcode libraries will affect assessment results.

Precision of DNA-based identifications

Criterion 3.1: Knowledge about uncertainty of DNA-based identification. This criterion addresses the question of how well the uncertainty associated with DNA-based identification is known. The criterion is relevant as the WFD explicitly requires (Annex 1.3.4) that the uncertainty of assessments is reported.

As WFD assessments are used to guide management decisions and, hence, have both political and economic implications, there is considerable focus on the confidence in any assessment of ecological status made. The level of uncertainty can be estimated using specifically designed software (Clarke and Hering, 2006; Kelly et al., 2009) but differs between BQEs and associated assessment methods (Birk et al., 2012). As the use of molecular approaches does not result in directly equivalent data (see criteria 1.1 to 1.3), it will be necessary to quantify the uncertainty associated with the new methods and the impact on assessment metrics and classification. All steps in the identification and enumeration process will need to be considered, including processing (for example platform chosen, sequencing depth, pre-treatment), and data analysis (for example bioinformatics), as each has the potential to influence the resulting taxa list. Identification is only one step in the process and, at this stage, it is unclear whether or not uncertainty will increase or decrease if molecular methods are adopted. Leaving aside stochastic variability from sampling and biases associated with primer selectivity, representation and other processing errors, assessments are affected by the power of identification. Structural changes in the power of identification are likely to occur over time (for example infilling of barcode libraries, technological developments in platforms, better links between DNA-based and morpho-taxonomy). Robust quality assurance methods will be necessary in order to quantify such changes. Quality assurance procedures based on morpho-taxonomic approaches are also fundamental to account for any bias introduced by DNA contamination and chimeras, and their adoption would allow for continuous comparison with existing methods to demonstrate the effects of future advances in technology. Simulations can help to better

understand the effect of the differing taxonomic resolution on assessment indices and the degree of bias between morphology-based and DNA-based identification methods (for example [Arje et al., 2016](#)).

We used this criterion to roughly estimate the uncertainty associated with DNA-based identification of different BQEs.

Comparability with conventional approaches

Criterion 4.1: Sensitivity of EQRs to differences in DNA-based identification. This criterion addresses the question of whether or not current Ecological Quality Ratios can be used with assessment results generated with DNA-based identification methods. The criterion is relevant to estimate the degree to which EQRs need to be adapted, to achieve similar assessment results as traditional methods. It is a validation criterion integrating aspects of Criteria 1.1 to 1.3.

As the WFD approach requires the comparison of an observed assemblage to the assemblage expected under “reference conditions” (i.e. an EQR), anything which influences the observed or the expected score will affect the EQR. The adoption of molecular methods will alter the probability of detection of observed species. However, increased resolution will create a demand for data describing species tolerances to stressors. Currently we have little understanding of tolerances for many taxa at species level, a situation that will not be easy to resolve for species with limited distributions. Reducing the DNA-generated taxa list (see Criterion 1.2) to match current taxonomic resolution may resolve this issue, otherwise the expected reference condition and/or quality class boundaries will have to be adjusted. Differences in scores between existing and DNA-based methods could be converted using correction factors to ensure comparability between past and future monitoring results ([Vasselon et al., 2017](#)). Alternatively, molecular data can be treated at face value, an option for phytobenthos, for example, where the traditional approach itself has inherent biases ([Kelly et al., 2017](#)).

We used this criterion to rate if adaptations of EQRs are necessary and feasible.

Criterion 4.2: Intercalibration. This criterion addresses the question regarding whether or not an intercalibration of boundaries for ecological status classes is feasible for assessment methods that use DNA-based identification. Intercalibration is a requirement for all new or revised assessment methods to be applied under the WFD.

The statutory goal of Good Ecological Status requires that status class boundaries are harmonised between all Member States of the EU. Although each Member State is free to develop a method for a BQE that is most appropriate to its conditions, there is a practical need to have data that can be compared with that produced by neighbouring Member States in order to ensure consistent application of the WFD across the EU. Existing boundaries, in particular the high-good and good-moderate boundaries, have been harmonised through the process of intercalibration. New molecular methods will need to fit into this framework and procedures exist ([European Union, 2015](#)) to help Member States achieve this. However, this will inevitably entail comparisons with countries still using traditional approaches. This, however, will not be the first time that a Member State has proposed an approach that cannot be compared directly with those of nearby countries ([Poikane et al., 2014](#)). In such circumstances, it will be necessary to apply both methods in parallel at sites ranged along key environmental gradients such that the position of boundaries established using the new method can be compared with existing boundaries. In practice, this will concern the average position of boundaries established by those countries that have already taken part in the intercalibration exercise for a particular BQE and water body type. As such parallel

datasets are likely to be collected during the process of method development or testing in each country, intercalibration is unlikely to present a serious challenge.

It should be noted that intercalibrated standards do not just affect comparisons among Member States: the target of Good Ecological Status is a long-term policy goal and any change in methods within a country has implications for detection of long-term change and, hence, progress towards this target. Changes in the position of key status class boundaries will need to be justified to governments and stakeholders as these will have implications for regulation.

We used this criterion to rate if there are obstacles for intercalibrating indices that are calculated with DNA-based taxon lists.

Cost-effectiveness

Criterion 5.1: Costs compared to traditional methods. This criterion addresses the question of whether or not DNA-based methods have the potential to substantially lower the costs of monitoring. This is relevant as monitoring programmes are often subject to severe financial pressure.

In recent years, the cost of sequencing biological material has fallen sharply and is likely to fall further as technology develops. However, cost-effectiveness is not defined simply by the monetary cost of sample processing but includes factors such as cost and availability of facilities, training needs, speed of processing, sensitivity and precision. Here, molecular approaches could provide an advantage via low processing costs and rapid turn-round (“economies of scale”), potentially enabling increased sampling frequency, increasing precision of assessments and enabling more responsive monitoring of pollution events or restoration activities. Furthermore, sampling eDNA is often cheaper than traditional sampling methods, e.g. electrofishing, gillnetting or trawling. Again, we stress that the whole cycle should be considered when comparing approaches: advantages gained by mechanising one aspect can easily be offset by losses in other parts of the assessment process ([Stein et al., 2014](#); [Elbrecht et al., 2017](#)).

We used this criterion to rate the potential for cost reduction through the use of DNA-based methods for the individual BQEs.

Criterion 5.2: Processing speed. This criterion addresses the question of whether sample processing can be accelerated by DNA-based identification or not. The criterion is relevant as the time required for manual identification is often a bottleneck for processing biological samples for WFD monitoring, particularly those requiring trained experts for microscopic identification (i.e. phytoplankton and macroinvertebrates). The speed of processing could be enhanced by DNA-based methods ([Goodwin et al., 2017](#)). DNA based methods could also benefit those BQEs requiring time-consuming sampling (for example electrofishing, gill-netting). At present, however, sequencing and computer capacities are limited for such DNA-based methods in many countries. This can itself create a bottleneck, potentially exacerbated by the need to run sequencing machines at full capacity in order to access the economies of scale described in 5.1. Early experience in the UK is that the shift to DNA-based analysis of phytobenthos makes it harder for laboratories to respond to requests to prioritise particular samples. This situation should change over time, as capacity increases and technology advances, as well as through knowledge transfer ([Leese et al., 2018](#)).

We used this criterion to rate the potential for speeding up sample processing for individual BQEs.

Criterion 6.1: Animal well-being, health and safety, environmental impact

This criterion addresses the question of whether DNA-based

identification can reduce the environmental impact and safety risks of sampling methods.

“Hands-off” techniques, such as eDNA assessments of fish populations, provide benefits for the well-being of fish (and bycatches of non-target organisms such as mammals or birds) particularly when compared with destructive methods such as gill-netting. This also holds true for nationally or internationally protected or red-listed species. For endangered species, sampling is often limited during critical life stages (e.g. during breeding season) to reduce potential impacts on the species. However, that may be the best opportunity to document their presence or density. Use of eDNA provides an opportunity to sample during critical life history phases in a less intrusive manner.

Similarly, health and safety risks may be reduced when individuals do not have to enter the water or use heavy or potentially dangerous equipment (for example electrofishing apparatus) to collect samples or perform surveys.

We used this criterion to rate the potential for DNA-based methods to reduce the environmental and health and safety impacts of monitoring activities.

1.4. Applicability of DNA-based identification for combinations of BQEs and water categories

We applied the criteria listed in the previous chapter to each combination of BQEs (phytoplankton, benthic flora, invertebrates, fish) and water categories (rivers, lakes, coastal and transitional waters) (Fig. 1). In the following, we provide justification for the values given in Fig. 1, where the applicability of the individual criteria is rated as:

- “high” (1), i.e. the criterion poses no obstacle to the implementation of DNA-based identification;

- “medium” (2), i.e. DNA-based identification could be applied but requires changes in the sampling scheme or the assessment system;
- “low” (3), i.e. DNA-based identification is currently not possible without substantial changes in the sampling scheme or the assessment system.

The ranking is based on the qualitative analysis of the literature given in the previous sections. As the criteria are not necessarily of equal relevance, the ranking of the individual criteria does not imply an overall ranking of the BQEs. In particular, Criteria 5.1, 5.2 and 6.1 do not address the technical feasibility of DNA-based identification, rather additional arguments for the use of DNA-based methods.

Criterion 1.1 (Applicability of current sampling methods, and availability of alternative methods, for obtaining biological material for DNA-based identification)

Applicability of sampling methods differs greatly between organism groups. For phytoplankton, phyto-benthos and invertebrates the traditional sampling methods can be used for DNA-based assessment (high), although some aspects such as use of ethanol as a fixative is problematic for cost and safety reasons in several European states. For fish, traditional electrofishing or gill-netting can be replaced by water samples for extraction of eDNA, which would be a simple and effective alternative (high). Macrophytes, macroalgae and angiosperms are surveyed rather than sampled; most species are identified in the field and their abundance is estimated directly. A different, and as yet not available, sampling method capable of detecting all relevant species adequately would need to be applied for DNA-based identification (low).

		1.1 sampling	1.2 errors	1.3 abundance	2.1 sensitive taxa	2.2 unassigned reads	3.1 uncertainty	4.1 EQR sensitivity	4.2 intercalibration	5.1 cost ratio	5.2 speed	6.1 animal well-being
phytoplankton	lakes, rivers	●	●	●	●	●	●	●	●	●	●	N/A
phytoplankton	TraC	●	●	●	●	●	●	●	●	●	●	N/A
phyto-benthos	rivers	●	●	●	●	●	●	●	●	●	●	N/A
phyto-benthos	lakes	●	●	●	●	●	●	●	●	●	●	N/A
macrophytes	rivers	●	●	●	?	●	●	●	●	●	●	N/A
macrophytes	lakes	●	●	●	?	●	●	●	●	●	●	N/A
macroalgae	TraC	●	●	●	?	●	●	●	●	●	●	N/A
angiosperms	TraC	●	●	●	?	●	●	●	●	●	●	N/A
invertebrates	rivers	●	●	●	●	●	●	●	●	●	●	●
invertebrates	lakes	●	●	●	●	●	●	●	●	●	●	●
invertebrates	TraC	●	●	●	●	●	●	●	●	●	●	●
fish	rivers	●	●	●	●	●	●	●	●	●	●	●
fish	lakes	●	●	●	●	●	●	●	●	●	●	●
fish	TraC	●	●	●	●	●	●	●	●	●	●	●

Fig. 1. Rating of the criteria for different BQEs and water categories. Large circles = high suitability of DNA-based identification; mid-sized circles = medium suitability; small circles = low suitability; N/A = not applicable. TraC: Transitional and Coastal waters.

Criterion 1.2 (Errors from DNA-based species detection and similarity of DNA-based and conventional taxon lists)

This criterion depends on the transferability of DNA-based taxon lists into taxon lists similar to those generated with morphology-based methods, and largely concerns taxa that are currently only identifiable with either morphology or DNA-based methods. In principle, additional taxa identified with DNA-based methods could be removed from a taxa list through use of filters (thus allowing the continuous use of the current assessment metrics; Elbrecht et al., 2017), while taxa not identified with DNA-based methods necessarily require changes in the assessment metrics. The number of the latter is low for fish and for invertebrates (Valentini et al., 2016; Aylagas, 2017) (high suitability), and despite a lower number of identifiable taxa, transferability has been demonstrated for phytobenthos (Kelly et al., 2017) (high). For phytoplankton, this is still to be demonstrated (medium). Combining directly identifiable taxa with known ecology, with those that are assigned to an OTU to give an ecological value should improve current assessment systems, without fundamentally changing their concept. For macrophytes, macroalgae and angiosperms most species can be identified, but as sampling methods associated with current assessment systems do not result in samples of all species (see 1.1), taxa lists generated with DNA-based identification may differ more than for other BQEs (medium).

Criterion 1.3 (Need for abundance assessment and accuracy of abundance estimates with DNA-based methods)

The relevance of this criterion depends on.

- the role of abundance-based metrics in assessment methods for the individual BQEs;
- options to measure relative abundance and to replace absolute by relative abundance;
- options to transform abundance-based metrics into presence/absence-based metrics.

Currently, the normative definitions for most BQEs specifies a need for abundance estimates. For phytoplankton, however, a measure of abundance is provided by chlorophyll concentration, resulting in a “medium” rating of this criterion. For phytobenthos and invertebrates, there are promising signs that presence/absence-based data and relative abundance estimates could be used (Vasselon et al., 2017) (medium). For fish, there are attempts to infer relative abundance from eDNA, while age classes cannot be detected (Hanfling et al., 2016; Pont et al., submitted) (medium). The species-poor groups of macrophytes, angiosperms and macroalgae are surveyed rather than sampled under the current assessment schemes; in its extreme form, an assessment system can be based on a single species (e.g. *Posidonia*) and the assessment system simply rates its abundance and density. This cannot be inferred from eDNA (low).

Criterion 2.1 (Capability of DNA-based methods to sample sensitive taxa)

For fish, DNA-based methods are clearly superior to electrofishing and gillnetting in terms of the detection of rare species (Hanfling et al., 2016) (high). For invertebrates and phytoplankton, there is good evidence that the relevant species are reliably captured with DNA-based methods (high), although unequal biomass still requires manual size adjustments especially for the biomass-rich specimens or great sequencing depths (Elbrecht et al., 2017). If a suitable sampling method could be found, this would also probably apply to macrophytes, but, in the absence of this, we rate it as “unknown”. For phytobenthos, the coverage of barcode libraries (see 2.2) limits this criterion (medium). There are currently

no papers on DNA-based methods for marine angiosperms and macroalgae (unknown). This does not, however, mean that DNA-based identification is unsuitable for detecting sensitive marine angiosperm and macroalgae taxa, only that more work is needed.

Criterion 2.2 (Unassigned reads)

This criterion is mainly associated with the completeness of barcode libraries (COI gene, 18S and rbcL for microalgae, 16S for Cyanobacteria) and cryptic diversity. Fish and macrophytes in rivers and lakes rate “high”, while barcode libraries for phytobenthos, invertebrates and fish in transitional and coastal waters are in an intermediate state of completeness (medium). For phytoplankton, cryptic diversity is an issue, as the number of taxa sequenced is lower (low), while for macroalgae and angiosperms cryptic diversity could be an issue only for small epiphytic species (low).

Criterion 3.1 (Knowledge about uncertainty of DNA-based identification)

For all BQEs, data on uncertainty associated with the different steps of the DNA-based processing chain have not been collected systematically or simulated (Årje et al., 2016). We rate this criterion as “low” for macrophytes, angiosperms and macroalgae, as sampling provides an additional - yet unquantified - source of uncertainty, while in the absence of more precise data the criterion is rated as “medium” for all other BQEs.

Criterion 4.1 (Sensitivity of EQRs to differences in DNA-based identification)

It is likely that approaches used to derive EQRs will need to be adapted for DNA-based identification, even if taxonomic issues (Criteria 1.2 and 2.2) have been solved. The feasibility of this procedure has already been demonstrated for phytobenthos (Kelly et al., 2017) and fish (Civade et al., 2016; Pont et al., submitted) (high), and we assume that this procedure will be possible for most other BQEs (medium). Exceptions are macrophytes in rivers and lakes, and angiosperms and macroalgae in coastal and transitional waters, for which we question the suitability of currently applied indices for use with DNA-based data, as most rely on measures of cover.

Criterion 4.2 (Intercalibration)

In principle, there are no obstacles preventing the WFD intercalibration procedure being performed to compare DNA-based methods against traditional methods. However, to date this process has not been undertaken, as few countries use DNA-based identification for formal WFD assessments. Promising examples, for which DNA-based and morpho-taxonomic approaches have been compared (although not yet intercalibrated) include phytobenthos in rivers, invertebrates in rivers and transitional and coastal waters, and fish in rivers and lakes (high), while we rate this criterion as “medium” for most other BQE-water type combinations. We expect more general problems for macrophytes, angiosperms and macroalgae (low), as the compatibility of these BQEs with DNA-based methods is generally questionable: These groups are species-poor, they are identified and their abundance estimated in the field; applying DNA-based identification would, therefore, require a different sampling strategy and different metrics, which limits comparability with traditional approaches.

Criterion 5.1 (Costs compared with traditional methods)

A comprehensive overview of the costs associated with DNA-based methods compared with traditional methods is not yet available (but see Stein et al., 2014; Sigsgaard et al., 2015; Smart et al., 2016; Aylagas, 2017). It is expected that the costs will be significantly lower for fish in rivers, lakes and transitional waters, as

sampling eDNA is much cheaper than electrofishing, gillnetting or trawling (high). For all other BQE-water category combinations, we expect a potential for cost reduction, which nevertheless still needs to be explored (medium).

Criterion 5.2 (Processing speed)

The potential for increased processing speed is particularly high for the labour-intensive identification of phytoplankton and invertebrates (high), while it is “low” for macrophytes, macroalgae and angiosperms, for which the field survey is the most time-consuming process. For all other BQEs, this criterion has been rated as “medium”.

Criterion 6.1 (Animal well-being, health and safety, environmental impact)

This criterion is only relevant for invertebrates and fish. For invertebrates, the same sampling methods are applied for traditional and DNA-based approaches. For traditional methods, the specimens are in most cases sacrificed for morphological identification, unless they are sorted and identified alive; however, rare and protected species (such as Odonata larvae and large mussels) are often identified in the field and placed back in the water body afterwards. Although this option is possible for DNA-based methods, there is generally a need to sacrifice specimens before DNA-based identification (low). For fish, the sampling of eDNA is non-invasive and offers advantages over gillnetting, trawling or electrofishing (high).

2. Discussion and outlook

2.1. Suitability of DNA-based identification for different BQEs and water categories

This paper is limited to the use of DNA-based identification for biological assessment systems in support of the WFD, although some of the issues discussed could be applicable to other directives (i.e. the Marine Strategy Framework Directive) and other geographical areas (for example in the USA, for the Clean Water Act; [Keck et al., 2017](#)). Clearly, DNA-based methods offer options, which can go beyond simple identification to a predefined taxonomic level. Therefore, DNA-based identification is likely to be a transition stage between conventional morpho-taxonomic approaches and DNA-based ecological assessment methods. However, even DNA-based identification poses many obstacles and cannot be implemented without adapting both the DNA-based identification procedure and the assessment methods to which they would be applied. These obstacles to implementation differ strongly among BQEs.

The advantages of DNA-based identification are obvious for fish: eDNA offers a well-suited and reliable sampling method (although different from conventional methods), with a high probability of detecting species (compared to other organism groups), whilst avoiding cost-intensive and harmful sampling methods. But even for fish, assessment metrics will need to be adapted, in particular to account for the change from absolute to relative abundances. Furthermore, some criteria required by WFD legislation (for example age class) currently cannot be assessed using DNA-based methods but, on the other hand, several currently adopted (and intercalibrated) methods do not include age classes either.

For invertebrates and phytobenthos, DNA-based identification is close to being applicable in standard monitoring programmes. For invertebrates, the main challenges remaining include dealing with abundance and adaptation of EQRs for use with DNA-based methods. Furthermore, barcode libraries need to be completed, in particular for phytobenthos. For phytoplankton, the latter problem

is even more relevant, due to high taxonomic diversity in plankton samples. For phytoplankton, the problem of abundance can be circumvented, as chlorophyll concentration is also assessed. At present, risk of cyanobacterial blooms is inferred from the abundance estimates, and a future DNA-based approach would need to satisfy this requirement. For phytobenthos, most of the current methods assess relative abundance of taxa, and do not take total abundance into account.

DNA-based identification is currently least appropriate for macrophytes (rivers, lakes) and angiosperms/macroalgae (transitional and coastal waters), which are surveyed rather than sampled. Surveys require taxonomic knowledge to gain a representative sample, and most identification is carried out in the field. Furthermore, the indices rely on cover value, as a proxy for abundance.

Consequently, the applicability of DNA-based identification differs markedly among BQEs, while there are only minor differences between water categories, mainly due to differences in the completeness of barcode libraries and the translocation of eDNA in rivers.

2.2. Implications of implementing DNA-based identification

Even the relatively minor changes resulting from the replacement of morphological with DNA-based identification will have significant implications for WFD assessments. On the one hand, DNA-based identification will require flexibility in the interpretation of the WFD and in how regulators use data. On the other hand, it will pave the way for the development of a new generation of ecological assessment tools, beyond and in parallel to the current WFD approaches. The principal challenge is to solve the conflict between the inherent need for ecological assessment to be consistent over a long time period, and the opportunities provided by the new methods.

The options for dealing with abundance is a good example of this conflict. Annex V of the WFD stipulates that abundance must be recorded for most BQEs. The legislation is based on the assumption that abundance provides more information than taxa lists alone, as changes in abundance may occur long before human-induced pressures lead to the extinction of species. As a consequence, the calculation of most functional indices requires data on either the abundance of a taxon or, at the very least, the proportion of the whole sample or sub-sample that it represents. Therefore, before DNA-based identification can be implemented, two questions need to be addressed: (1) How best to fulfil the legal requirement of recording abundance? And (2) How can the information given by species' abundances best be provided? The answer to the first question differs between BQEs. For phytoplankton, there is the option of using chlorophyll concentration as a proxy for abundance or biomass. From a practical point of view, a filtered plankton sample can be divided, with one half being used to measure chlorophyll and the other half for DNA-based identification. The remaining quantitative indicators required for phytoplankton are algal bloom frequency and amplitude, which could be measured with frequent readings of pigments from satellites or continuous reading from an automated buoy placed within the water body ([Schluter et al., 2014](#)). Thus, a combination of DNA-based identification and other methods could fulfil the WFD's requirements. For fish, and probably other BQEs, there is the option to use relative rather than absolute abundance based on read count data, or frequency of occurrences in several eDNA samples as a proxy for abundance by analysing multiple eDNA replicates per site ([Pilliod et al., 2013](#)). In response to the second question, there are promising signs for various BQEs and metrics that presence-absence data give signals similar to abundance data and can be

translated between one another (Aylagas, 2017). However, questions remain, regarding the degree to which abundance data – whether traditional or molecular – reflects biomass or processes (for example related to the abundance of grazers or sediment feeders in the benthic invertebrate community). Currently applied measures of abundance do not discriminate between large and small specimens: a tiny chironomid larvae and a large stonefly larvae count the same, although the latter might have a 1000 times greater biomass. Clearly, there is room for improvement through DNA-based methods. Barcodes potentially represent the abundance of mitochondria and plastids and may, indeed, offer greater insights into which taxa are actually driving ecological processes within an ecosystem, by reflecting the intensity of metabolic processes.

More generally, there is the question of how to achieve compatibility in ecological assessments when replacing conventional by novel methods? The term “monitoring” implies recording of time series, and, inherently, the consistent use of standard methods. In case of the WFD, the monitoring intervals are very long: for River Basin Management Plans, for example, ecological status only needs to be reported at six-yearly intervals. It should be possible to change methods between these intervals in response to results and experience. DNA-based identification is only one, albeit significant, driver of changes to methods. The benefits of increased accuracy and performance of enhanced ecological assessment methods will always need to be carefully balanced against the potential loss of comparability. The implementation of new methods should, therefore, always be accompanied by a recalculation of indices from prior monitoring programmes, to ensure backward compatibility. This underlines the need to develop capacity to archive DNA samples, particularly from reference sites, so that as new technologies emerge, DNA from critical sites can be reanalysed using the new methods.

Closely related with the question of backward compatibility is the future evolution of methods. With DNA-based identification, there is a clear need to allow methods to evolve, which may require constant adaptation of indices and assessment methods. This is a potential paradigm shift in how to handle monitoring data. In future, a rolling comparison with existing methods will be needed to “buffer” monitoring results against the effects of advances in technology. However, provided there is sufficient storage capacity, sequence data can be stored and reanalysed more easily than traditional samples, ensuring a level of “forward compatibility” as bioinformatics and metrics improve, for as long as sampling, DNA extraction and the sequencing itself are robust. Most importantly, DNA extracts are relatively easy to store and this should be encouraged, as we do not know which barcodes and methods will be available in the future.

The expense of implementation is another consideration when introducing DNA-based methods into WFD assessments, since costs may be reduced compared with traditional assessment methods (Aylagas, 2017). Expenses are not solely related to the costs of processing individual samples, but encompass training, equipment purchase, administrative and maintenance costs, quality assurance and, importantly, the costs of initial method development and ongoing evaluations and upgrades. Any change in assessment methods and results needs to be communicated to policy makers and the general public, which is not necessarily a straightforward procedure and which will require education of stakeholder groups, including those from non-scientific backgrounds.

A general challenge for river basin management will be the breakdown of the assessment procedure into several smaller steps, which are performed by different people or units. While in many countries microscopic identification is still the responsibility of water boards, DNA-based identification is likely to induce a shift to

external service providers. Care must be taken that the individual steps of the assessment procedure stay connected and allow informed interpretation of the data. Data generated by DNA-based identification will need to be transferred to the responsible authorities in a way that allows for simple understanding of procedures, results and their uncertainties. Decisions based on assessment results precipitate significant investment by the private and public sectors, and it is essential that decision makers are provided with monitoring data that have been generated in a transparent way.

3. Conclusions

There is great potential for DNA-based identification to be used for assessment procedures to fulfil the requirements of the WFD. DNA-based identification can contribute to making assessment procedures more cost-effective, faster, more transparent and have greater reproducibility. There are, however, several practical obstacles, which will need to be overcome within the next years. We recommend that the potential benefits of DNA-based identification are quantified relative to existing traditional methods, together with the parallel application of morphometric and DNA-based identification in order to learn how comparable the approaches are and to increase compatibility where necessary. DNA-based identification will be a valuable step into more advanced methods of DNA-based monitoring, which may complement or even replace more traditional monitoring systems in the future.

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References

- Albaina, A., Aguirre, M., Abad, D., Santos, M., Estonba, A., 2016. 18S rRNA V9 metabarcoding for diet characterization: a critical evaluation with two sympatric zooplanktivorous fish species. *Ecol. Evol.* 6, 1809–1824.
- Andújar, C., Arribas, P., Gray, C., Bruce, K., Woodward, G., Yu, D.W., Vogler, A.P., 2018. Metabarcoding of freshwater invertebrates to detect the effects of a pesticide spill. *Mol. Ecol.* 27, 146–166. <https://doi.org/10.1111/mec.14410> accepted.
- Ärje, J., Choi, K.P., Divino, F., Meissner, K., Kärkkäinen, S., 2016. Understanding the statistical properties of the percent model affinity index can improve bio-monitoring related decision making. *Stoch. Environ. Res. Risk Assess.* 30, 1981–2008.
- Ärje, J., Kärkkäinen, S., Meissner, K., Iosifidis, A., Ince, T., Gabbouj, M., Kiranyaz, S., 2017. The effect of automated taxa identification errors on biological indices. *Expert Syst. Appl.* 72, 108–120. ISSN 0957-4174. <https://doi.org/10.1016/j.eswa.2016.12.015>.
- Avó, A.P., Daniell, T.J., Neilson, R., Oliveira, S., Branco, J., Adão, H., 2017. DNA barcoding and morphological identification of benthic nematodes assemblages of estuarine intertidal sediments: advances in molecular tools for biodiversity assessment. *Front. Mar. Sci.* 4.
- Aylagas, E., 2017. DNA Metabarcoding Derived Biotic Indices for Marine Monitoring and Assessment. PhD Thesis. University of the Basque Country, p. 248.
- Aylagas, E., Borja, A., Rodríguez-Ezpeleta, N., 2014. Environmental status assessment using DNA metabarcoding: towards a genetics based marine biotic index (gAMBI). *PLoS One* 9, e90529.
- Aylagas, E., Borja, A., Irigoien, X., Rodríguez-Ezpeleta, N., 2016. Benchmarking DNA metabarcoding for biodiversity-based monitoring and assessment. *Front. Mar. Sci.* 3 <https://doi.org/10.3389/fmars.2016.00096>.
- Baird, D.J., Hajibabaei, M., 2012. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. *Mol. Ecol.* 21, 2039–2044.
- Baldigo, P.B., Sporn, L.A., Scott, D.G., Ball, J.A., 2017. Efficacy of environmental DNA to detect and quantify brook trout populations in headwater streams of the Adirondack Mountains. *New York. Trans. Am. Fisheries Soc.* 146 (1), 99–111.
- Barnes, M.A., Turner, C.R., 2015. The ecology of environmental DNA and implications for conservation genetics. *Conserv. Genet.* 17, 1–17.

- Bergsten, J., Bilton, D.T., Fujisawa, T., Elliott, M., Monaghan, M.T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, G.N., Ribera, I., Nilsson, A.N., Barracough, T.G., Vogler, A.P., 2012. The effect of geographical scale of sampling on DNA barcoding. *Syst. Biol.* 61, 851–869.
- Birk, S., Bonne, W., Borja, A., Brucet, S., Courrat, A., Poikane, S., Solimini, A., van de Bund, W., Zampoukas, N., Hering, D., 2012. Three hundred ways to assess Europe's surface waters: an almost complete overview of biological methods to implement the Water Framework Directive. *Ecol. Indic.* 18, 31–41.
- Birk, S., Willby, N.J., Kelly, M.G., Bonne, W., Borja, A., Poikane, S., van de Bund, W., 2013. Inter-calibrating classifications of ecological status: Europe's quest for common management objectives for aquatic ecosystems. *Sci. Total Environ.* 454–455, 490–499.
- Bista, I., Carvalho, G.R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M., Creer, S., 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nat. Commun.* 8, 14087.
- Blackman, R., Constable, D., Hahn, C., Sheard, A.M., Durkota, J., Hanfling, B., Lawson Handley, L., 2017. Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples - first record of *Gammarus fossarum* in the UK. *Aquat. Invasions* 12, 177–189.
- Bohmann, K., Evans, A., Gilbert, M.T., Carvalho, G.R., Creer, S., Knapp, M., Yu, D.W., de Bruyn, M., 2014. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol. Evol.* 29, 358–367.
- Borja, A., Elliott, M., 2013. Marine monitoring during an economic crisis: the cure is worse than the disease. *Mar. Pollut. Bull.* 68, 1–3.
- Bourlat, S.J., Borja, A., Gilbert, J., Taylor, M.I., Davies, N., Weisberg, S.B., Griffith, J.F., Lettieri, T., Field, D., Benzie, J., Glöckner, F.O., Rodríguez-Ezpeleta, N., Faith, D.P., Bean, T.B., Obst, M., 2013. Genomics in marine monitoring: new opportunities for assessing marine health status. *Mar. Pollut. Bull.* 74, 19–31.
- Brys, R., Bellemain, E., Halfmaerten, D., Dejean, T., Mergeay, M., 2017. Quantitatively Predicting Fish Community Composition Using Environmental DNA Metabarcoding (under revision).
- Carugati, L., Corinaldesi, C., Dell'Anno, A., Danovaro, R., 2015. Metagenetic tools for the census of marine meiofaunal biodiversity: an overview. *Mar. Genom.* 24, 11–20.
- Choo, L.Q., Crampton-Platt, A., Vogler, A.P., 2017. Shotgun mitogenomics across body size classes in a local assemblage of tropical Diptera: phylogeny, species diversity and mitochondrial abundance spectrum. *Mol. Ecol.* 26, 5086–5098.
- Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J.-C., Bonin, A., Taberlet, P., Pont, D., 2016. Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. *PLoS One*. <https://doi.org/10.1371/journal.pone.0157366>.
- Clarke, R.T., Hering, D., 2006. Errors and uncertainty in bioassessment methods – major results and conclusions from the STAR project and their application using STARBUGS. *Hydrobiologia* 566, 433–439.
- Clarke, R.T., Murphy, J.F., 2006. Effects of locally rare taxa on the precision and sensitivity of RIVPACS bioassessment of freshwaters. *Freshw. Biol.* 51, 1924–1940.
- Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Potter, C., Bik, H.M., 2016. The ecologist's field guide to sequence-based identification of biodiversity. *Meth. Ecol. Evol.* 7, 1008–1018.
- Cristescu, M.E., 2014. From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. *Trends Ecol. Evol.* 29, 566–571.
- Dafforn, K.A., Baird, D.J., Chariton, A.A., Sun, M.Y., Brown, M.V., Simpson, S.L., Kelaher, B.P., Johnston, E.L., 2014. Chapter one - faster, higher and Stronger? The pros and cons of molecular faunal data for assessing ecosystem condition. *Adv. Ecol. Res.* 51, 1–40.
- Danovaro, R.L., Carugati, L., Berzano, M., Cahill, A.E., Carvalho, S., Chenuil, A., Corinaldesi, C., Cristina, S., David, R., Dell'Anno, A., Dzhenbekova, N., Garcés, E., Gasol, J.M., Goela, P., Féral, J.-P., Ferrera, I., Forster, R.M., Kurekin, A.A., Rastelli, E., Marinova, V., Miller, P.L., Moncheva, S., Newton, A., Pearman, J.K., Pitois, S.G., Reñé, A., Rodríguez-Ezpeleta, N., Saggiomo, V., Simis, S.G.H., Stefanova, K., Wilson, C., Lo Martire, M., Greco, S., Cochrane, S.K.J.O., Mangoni, Borja, A., 2016. Implementing and innovating marine monitoring approaches for assessing marine environmental status. *Front. Mar. Sci.* 3 <https://doi.org/10.3389/fmars.2016.00213>.
- Darling, J.A., Mahon, A.R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environ. Res.* 111, 978–988.
- De Jonge, V.N., Elliott, M., Brauer, V.S., 2006. Marine monitoring: its shortcomings and mismatch with the EU Water Framework Directive's objectives. *Mar. Pollut. Bull.* 53, 5–19.
- Deiner, K., Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLoS One* 9 e88786.
- Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursiere-Rousse, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., de Vere, N., Pfrender, M.E., Bernatchez, L., 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Mol. Ecol.* 2017, 1–24.
- Deiner, K., Fronhofer, E.A., Mächler, E., Walser, J.-C., Altermatt, F., 2016. Environmental DNA reveals that rivers are conveyor belts of biodiversity information. *Nature Comm.* 7, 12544. DOI: 10.1038.
- Deiner, K., Walser, J.-C., Mächler, E., Altermatt, F., 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biol. Conserv.* 183, 53–63.
- Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., Miaud, C., 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *J. Appl. Ecol.* 49, 953–959.
- EEA, 2012. European waters – assessment of status and pressures. EEA Report 8, 100.
- Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species Abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS One* 10 e0130324.
- Elbrecht, V., Leese, F., 2017. Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Front. Environ. Sci.* <https://doi.org/10.3389/fenvs.2017.00011>.
- Elbrecht, V., Vamos, E.E., Meissner, K., Aroviita, J., Leese, F., 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. *Methods Ecol. Evol.* <https://doi.org/10.1111/2041-210X.12789>.
- European Union, 2015. Procedure to Fit New or Updated Classification Methods to the Results of a Completed Inter-calibration Exercise. CIS Guidance Document 30, p. 29. European Union, Luxembourg.
- Ferraro, S.P., Cole, F.A., DeBen, W.A., Swartz, R.C., 1989. Power-cost efficiency of eight macrobenthic sampling schemes in puget sound, Washington, USA. *Can. J. Fish. Aquat. Sci.* 46, 2157–2165.
- Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. *Biol. Lett.* 4, 423–425.
- Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguët-Covex, C., De Barba, M., Gelly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G., Taberlet, P., 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Mol. Ecol. Resour.* 15, 543–556.
- Foden, J., Rogers, S.I., Jones, A.P., 2008. A critical review of approaches to aquatic environmental assessment. *Mar. Pollut. Bull.* 56, 1825–1833.
- Goodwin, K.D., Thompson, L.R., Duarte, B., Kahlke, T., Thompson, A.R., Marques, J.C., Caçador, I., 2017. DNA sequencing as a tool to monitor marine ecological status. *Front. Mar. Sci.* <https://doi.org/10.3389/fmars.2017.00107>.
- Grossmann, L., Beisser, D., Bock, C., Chatzinotas, A., Jensen, M., Preisfeld, A., Psenner, R., Rahmann, S., Wodniok, S., Boenigk, J., 2016. Trade-off between taxon diversity and functional diversity in European lake ecosystems. *Mol. Ecol.* 25, 5876–5888.
- Haase, P., Lohse, S., Pauls, S., Schindehütte, K., Sundermann, A., Hering, D., 2004. Development of a practical standardized protocol for macroinvertebrate sampling and sorting in streams. *Limnologia* 34, 349–365.
- Haase, P., Murray-Bligh, J., Lohse, S., Pauls, S., Sundermann, A., Gunn, R., Clarke, R.T., 2006. Assessing the impact of errors in sorting and identifying macroinvertebrate samples. *Hydrobiologia* 566, 505–521.
- Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G.A.C., Baird, D.J., 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS One* 6 e17497. doi:17410.11371/journal.pone.0017497.
- Hallett, C.S., Valesini, F., Elliott, M., 2016. A review of Australian approaches for monitoring, assessing and reporting estuarine condition: I. International context and evaluation criteria. *Environ. Sci. Pol.* 66, 260–269.
- Hanfling, B., Handley, L.L., Read, D.S., Hahn, C., Li, J., Nichols, P., Blackman, R.C., Oliver, A., Winfield, I.J., 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Mol. Ecol.* 25, 3101–3119.
- Hering, D., Moog, O., Ofenböck, T., Feld, C.K., 2006. Cook book for the development of a Multimetric Index for biological condition of aquatic ecosystems: experiences from the European AQEM and STAR projects and related initiatives. *Hydrobiologia* 566, 311–324.
- Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H., Whiteley, A.R., 2015. Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams. *Mol. Ecol. Resour.* 15, 216–227.
- Ji, Y., Ashton, L., Pedley, S.S.M., Edwards, D.D.P., Tang, Y., Nakamura, A., Kitching, R., Dolman, P.M., Woodcock, P., Edwards, F.A., Larsen, T.H., Hsu, W.W., Benedick, S., Hamer, K.C., Wilcove, D.S., Bruce, C., Wang, X., Levi, T., Lott, M., Emerson, B.C., Yu, D.W., 2013. Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecol. Lett.* 16, 1245–1257.
- Jones, J.L., Davy-Bowker, J., Murphy, J.F., Pretty, J.L., 2010. Ecological monitoring and assessment of pollution in rivers. In: Batty, L.C., Hallberg, K.B. (Eds.), *Ecology of Industrial Pollution: Remediation, Restoration and Preservation*. CUP.
- Keck, F., Vasselon, V., Tapolczai, K., Rimet, F., Bouchez, A., 2017. Freshwater bio-monitoring in the information age. *Front. Ecol. Environ.* 15, 266–274.
- Kelly, M.G., Bennion, H., Burgess, A., Ellis, J., Juggins, S., Guthrie, R., Jamieson, B.J., Adriaenseens, V., Yallop, M.L., 2009. Uncertainty in ecological status assessments of lakes and rivers using diatoms. *Hydrobiologia* 633, 5–15.
- Kelly, M., Boonham, N., Juggins, S., Kille, P., Mann, D., Pass, D., Sapp, M., Sato, S., Glover, R., Walsh, K., 2017. A DNA Based Diatom Metabarcoding Approach for Water Framework Directive Classification of Rivers. *Science Report SC140024*. Environment Agency, Bristol, p. 151.
- Kelly, R.P., Port, J.A., Yamahara, K.M., Crowder, L.B., 2014. Using environmental DNA to census marine fishes in a large mesocosm. *PLoS One* 9 e86175.
- Kermarrec, L., Franc, A., Rimet, F., Chaumeil, P., Frigerio, J.M., Humbert, J.-F., Bouchez, A., 2014. A next-generation sequencing approach to river bio-monitoring using benthic diatoms. *Freshw. Sci.* 33, 349–363.
- Kiranyaz, S., Ince, T., Pulkkinen, J., Gabbouj, M., Ärje, J., Kärkkäinen, S., Tirronen, V., Juhola, M., Turpeinen, T., Meissner, K., 2011. Classification and retrieval on macroinvertebrate image databases. *Comput. Biol. Med.* 41, 463–472.

- Klymus, K.E., Marshall, N.T., Stepien, C.A., 2017. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. *PLoS One* 12 e0177643.
- Klymus, K.E., Richter, C.A., Chapman, D.C., Paukert, C., 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biol. Conserv.* 183, 77–84.
- Konopka, A., Wilkins, M., 2012. Application of meta-transcriptomics and -proteomics to analysis of in situ physiological state. *Front. Microbiol.* 18 (3), 184. <https://doi.org/10.3389/fmicb.2012.00184>.
- Lawson Handley, L., 2015. How will the 'molecular revolution' contribute to biological recording? *Biol. J. Linn. Soc.* 115, 750–766.
- Leese, F., Altermatt, F., Bouchez, A., Ekrem, T., Hering, D., Mergen, P., Pawlowski, J., Piggott, J., Abarenkov, K., Beja, P., Bervoets, L., Boets, P., Bones, A., Borja, A., Bruce, K., Carlsson, J., Coissac, E., Costa, F., Costache, M., Creer, S., Csabai, Z., Deiner, K., DelValls, A., Duarte, S., Fazi, S., Graf, W., Hershkovitz, Y., Japoshvili, B., Jones, I., Kahlert, M., Kalamujic Strojil, B., Kelly-Quinn, M., Keskin, E., Mächler, E., Mahon, A., Marečková, M., Mejdandzic, M., Montagna, M., Moritz, C., Mulk, V., Navodaru, I., Pålsson, S., Panksep, K., Penev, L., Petrusek, A., Pfannkuchen, M., Rinkevich, B., Schmidt-Kloiber, A., Segurado, P., Strand, M., Sulcius, S., Traugott, M., Turon, X., Valentini, A., van der Hoorn, B., Vasquez Hadjilyra, M., Viguri, J., Vogler, A., Zegura, B., 2016. DNAqua-Net: developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. *Res. Ideas and Outcomes* 2 e11321.
- Leese, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, A., Bruce, K., Ekrem, T., Ciampor Jr., F., Ciamporová-Začovičová, Z., Costa, F.O., Duarte, S., Elbrecht, V., Fontaneto, D., Geiger, M.F., Hering, D., Kahlert, M., Kalamujic Strojil, B., Kelly, M., Keskin, E., Liska, I., Mergen, P., Meissner, K., Pawlowski, J., Penev, L., Reyjol, Y., Rotter, A., Steinke, D., Vitecek, S., Zimmermann, J., Weigand, A.M., 2018. Chapter two - why we need sustainable networks bridging countries, disciplines, cultures and generations for aquatic biomonitoring 2.0: a perspective derived from the DNAqua-net COST action. *Adv. Ecol. Res.* 58, 63–99.
- Lyon, J.P., Bird, T., Nicol, S., Kearns, J., O'Mahony, J., Todd, C.R., Cowx, I.G., Bradshaw, C.J.A., 2014. Efficiency of electrofishing in turbid lowland rivers: implications for measuring temporal change in fish populations. *Can. J. Fish. Aquat. Sci.* 71, 878–886.
- Medinger, R., Nolte, V., Vinay Pandey, R., Jost, S., Ottenwälder, B., Schlötterer, C., Boenigk, J., 2010. Diversity in a hidden world: potential and limitation of next generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. *Mol. Ecol.* 19 (Suppl 1), 32–40.
- Mohrbeck, I., Raupach, M.J., Arbizu, P.M., Kneibelsberger, T., Laakmann, S., 2015. High-throughput sequencing-the key to rapid biodiversity assessment of marine metazoa? *PLoS One* 10 e0140342.
- Mondy, C.P., Villeneuve, B., Archambault, V., Usseglio-Polatera, P., 2012. A new macroinvertebrate-based multimetric index (I2M2) to evaluate ecological quality of French wadeable streams fulfilling the WFD demands: a taxonomical and trait approach. *Ecol. Indic.* 18, 452–467.
- Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L., Mahon, A.R., 2014. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. *Environ. Sci. Technol.* 48, 12800–12806.
- Nygård, H., Oinonen, S., Lehtiniemi, M., Hällfors, H., Rantajärvi, E., Uusitalo, L., 2016. Price versus value of marine monitoring. *Front. Mar. Sci.* 3 <https://doi.org/10.3389/fmars.2016.00205>.
- Patrício, J., Little, S., Mazik, K., Papadopoulou, K.-N., Smith, C., Teixeira, H., Hoffmann, H., Uyarra, M., Solaun, O., Zenetos, A., Kaboglu, G., Kryvenko, O., Churilova, T., Moncheva, S., Bučas, M., Borja, A., Hoepffner, N., Elliott, M., 2016. European marine biodiversity monitoring networks: strengths, weaknesses, opportunities and threats. *Front. Mar. Sci.* 3 <https://doi.org/10.3389/fmars.2016.00161>.
- Pilliod, D.S., Goldberg, C.S., Arkle, R.S., Waits, L.P., 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Can. J. Fish. Aquat. Sci.* 70, 1123–1130.
- Pinol, J., San Andres, V., Clare, E.L., Mir, G., Symondson, W.O.C., 2014. A pragmatic approach to the analysis of diets of generalist predators: the use of next-generation sequencing with no blocking probes. *Mol. Ecol. Resour.* 14, 18–26.
- Poikane, S., Zampoukas, N., Borja, A., Davies, S.P., van de Bund, W., Birk, S., 2014. Inter-calibration of aquatic ecological assessment methods in the European Union: lessons learned and way forward. *Environ. Sci. Pol.* 44, 237–246.
- Pont, D., Huguency, B., Beier, U., Goffaux, D., Melcher, A., Noble, R., Rogers, C., Roset, N., Schmutz, S., 2006. Assessing river biotic condition at the continental scale: a European approach using functional metrics and fish assemblages. *J. Appl. Ecol.* 43, 70–80.
- Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H. & Dejean, T., submitted. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Under review.
- Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C., 2014. The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. *J. Appl. Ecol.* 51, 1450–1459.
- Rimet, F., Chaumeil, P., Keck, F., Kermarrec, L., Vasselon, V., Kahlert, M., Franc, A., Bouchez, A., 2016. R-syst:diatom: an Open-access and Curated Barcode Database for Diatoms and Freshwater Monitoring. Database 2016, baw016.
- Schluter, L., Mohlenberg, F., Kaas, H., 2014. Temporal and spatial variability of phytoplankton monitored by a combination of monitoring buoys, pigment analysis and fast screening microscopy in the Fehmarn Belt Estuary. *Environ. Monit. Assess.* 186, 5167–5184.
- Schmidt-Kloiber, A., Hering, D., 2015. An online tool that unifies, standardises and codifies more than 20,000 European freshwater organisms and their ecological preferences. *Ecol. Indic.* 53, 271–282. www.freshwaterecology.info.
- Shaw, J.L.A., Clarke, L.J., Wedderburn, S.D., Barnes, T.C., Weyrich, L.S., Cooper, A., 2016. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biol. Conserv.* 197, 131–138.
- Shokralla, S., Spall, J.L., Gibson, J.F., Hajibabaei, M., 2012. Next-generation sequencing technologies for environmental DNA research. *Mol. Ecol.* 21, 1794–1805.
- Sigsgaard, E.E., Carl, H., Møller, P.R., Thomsen, P.F., 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biol. Conserv.* 183, 46–52.
- Smart, A.S., Weeks, A.R., van Rooyen, A.R., Moore, A., McCarthy, M.A., Tingley, R., 2016. Assessing the cost-efficiency of environmental DNA sampling. *Meth. Ecol. Evol.* 7, 1291–1298.
- Stæhr, P.A., Santos, S., Hansen, L.H., Lundsteen, S., Haraguchi, L., Dahl, K., Ávila, M.P., Winding, A., 2016. Comparison of EDNA and Conventional Techniques for Monitoring Species Diversity of Boulder Reefs in Danish Waters. DCE - Danish Centre for Environment and Energy. Aarhus University, p. 22.
- Stein, E.D., Martinez, M.C., Stiles, S., Miller, P.E., Zakharov, P.E., 2014. Is DNA barcoding actually cheaper and faster than traditional morphological methods: results from a survey of freshwater bioassessment efforts in the United States? *PLoS One* 9 e95525.
- Stoeckle, M.Y., Soboleva, L., Charlop-Powers, Z., 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PLoS One* 12 e0175186.
- Sweeney, B.W., Battle, J.M., Jackson, J.K., Dapkey, T., 2011. Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? *J. N. Am. Benthol. Soc.* 30 (1), 195–216.
- Taberlet, P., Coissac, E., Hajibabaei, M., Rieseberg, L.H., 2012a. Environmental DNA. *Mol. Ecol.* 21, 1789–1793.
- Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012b. Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol. Ecol.* 21, 2045–2050.
- Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., Kawabata, Z., 2012. Estimation of fish biomass using environmental DNA. *PLoS One* 7 e35868.
- Thomsen, P.T., Møller, P.R., Sigsgaard, E.E., Knudsen, S.W., Jørgensen, O.A., Willerslev, E., 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. *PLoS One* 11. <https://doi.org/10.1371/journal.pone.0165252>.
- Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M., Willerslev, E., 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One* 7 e4173.
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E., Dejean, T., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol. Ecol.* 25, 929–942.
- Vasselon, V., Rimet, F., Tapolczai, K., Bouchez, A., 2017. Assessing ecological status with diatom DNA metabarcoding: scaling-up on a WFD monitoring network (Mayotte Island, France). *Ecol. Indic.* 82, 1–12.
- Wilcox, T.M., McKelvey, K.S., Young, M.K., Sepulveda, A.J., Shepard, B.B., Jane, S.F., Schwartz, M.K., 2016. Understanding environmental DNA detection probabilities: a case study using a stream-dwelling char *Salvelinus fontinalis*. *Biol. Conserv.* 194, 209–216.
- Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., Minamoto, T., Miya, M., 2017. Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scie. Rep.* 7, 40368. <https://doi.org/10.1038/srep40368>.
- Zimmermann, J., Glockner, G., Jahn, R., Enke, N., Gemeinholzer, B., 2015. Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. *Mol. Ecol. Resour.* 15, 526–542.