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

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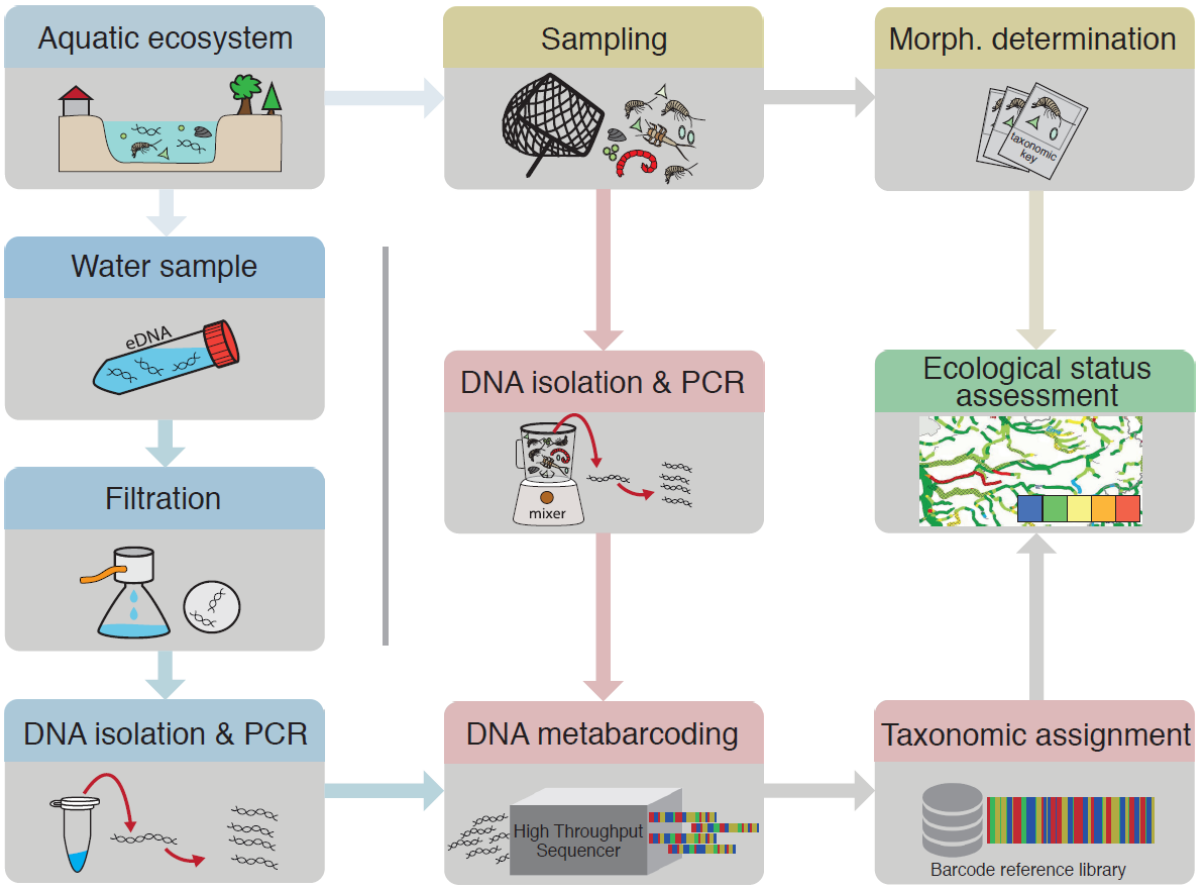
Graphical Abstract



Source: http://dnaqua.net.

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

Keywords: meta-barcoding, eDNA, Biological Quality Elements, rivers, lakes, transitional and coastal waters

Introduction

Worldwide, aquatic ecosystems are monitored using a range of organisms as indicators (Foden et al., 2008; Hallett et al., 2016; Patricio 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

87 best available sites) specific to each type of water body. The distance between the calculated value
88 and the value at reference conditions is termed the Ecological Quality Ratio (EQR), which is finally
89 translated into a quality class (high, good, moderate, poor and bad) on which management decisions
90 are based. The objective is to achieve at least "good status" for all water bodies in Europe by 2027: at
91 present, half of all water bodies do not meet this goal (EEA, 2012).
92 Most assessment methods for European freshwaters were developed in the 2000s, following
93 adoption of the WFD by EU Member States. In many cases, these methods were based on
94 approaches developed prior to adoption of the WFD with adjustments to translate assessment
95 results into ecological status classes. Whilst field and laboratory methods were largely left
96 unchanged, some Member States developed new assessment methods. Whatever the strategy
97 adopted, each biological method was then "intercalibrated" with the respective methods of other
98 Member States in the same broad ecoregion (termed "Geographical Intercalibration Groups", Birk et
99 al., 2013). Although the formal definition of ecological status encompasses both structure and
100 function (Article 2, definition 21, WFD), the assessment systems have been based primarily on
101 structure. Some assessment metrics do use species traits, such as size structure of fish assemblages
102 or feeding type composition of benthic invertebrates (Mondy et al., 2012; Pont et al., 2006) but most
103 methods neglect this aspect. Overall, despite the shortcomings of many of the methods, the process
104 of method development, adaptation and intercalibration have contributed to a better understanding
105 of reference conditions, responses of biota to stressors and the uncertainties associated with various
106 steps in the assessment of ecological status (Poikane et al., 2014).
107 Some aspects of monitoring procedures are time consuming and costly, requiring teams of skilled
108 individuals, for example the identification and counting of phytoplankton, phytobenthos and benthic
109 invertebrates (Ferraro et al., 1989; Haase et al., 2004; Nygård et al., 2016). Electrofishing and
110 gillnetting for fish are also costly and require teams of skilled staff. As budgets for such work are

111 under pressure, there is a demand to simplify methods, to lower the costs and to speed up the
112 monitoring process (Borja and Elliott, 2013), whilst maintaining quality, robustness and
113 comparability. Recent technological advances could go some way towards alleviating these budget
114 constraints.
115 New methods such as machine learning (Kiranyaz et al., 2011; Ärje et al., 2017), and genetic methods
116 such as metabarcoding of DNA obtained from organisms or simply by sampling environmental DNA
117 (eDNA) from the water (for example Taberlet et al., 2012a; Ji et al., 2013) provide alternative tools
118 for multiple species detection and identification. In the medium term, these new methods have the
119 potential to fundamentally change ecological assessment. Although still in the development phase,
120 genetic methods are already sufficiently well advanced for biodiversity assessment (for example
121 Elbrecht et al., 2017). Thus, it is now possible to complement or even replace traditional sample
122 processing and identification methods with DNA-based methods which are of equal or lower cost and
123 which are able to detect species occurrences with a similar or higher level of precision (Stein et al.,
124 2014; Smart et al., 2016; Aylagas, 2017; Elbrecht et al., 2017; Vasselon et al., 2017). DNA-based
125 methods have some obvious advantages compared with traditional sampling and image recognition
126 based identification schemes. Identification to species level is more precise and objective with DNA-
127 based methods, particularly for cryptic taxa, microorganisms and difficult life stages (for example
128 juveniles and pupae) while sample processing may be faster and cheaper than manual procedures
129 (Hajibabaei et al., 2011; Kermarrec et al. 2014; Dafforn et al., 2014; Stein et al., 2014; Avó et al.,
130 2017). An additional advantage of molecular techniques is the potential for assessing functional
131 diversity based on gene expression (transcription), fulfilling an aim of the WFD that has yet to be
132 addressed adequately with morpho-taxonomic approaches (Bourlat et al., 2013). On the other hand,
133 molecular techniques are still developing and require standardisation and harmonization (Cristescu,
134 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

159 derivation of new reference condition values and the development of new assessment metrics.
160 Functional metrics are currently underrepresented in WFD assessment systems, although trait-based
161 data have been frequently derived from morphological criteria (Schmidt-Kloiber and Hering, 2015)
162 and are used in several assessment methods. Molecular data, in particular transcriptomic data
163 (Konopka and Wilkins, 2012; Creer et al. 2016) and placement into trait-informed phylogenies offer
164 additional options for functional metrics, which would need to be developed from scratch, and their
165 response to stressor gradients investigated. However, research in this field is still its infancy and
166 implementation into practical ecological assessment is unlikely in the short and medium term.
167 Hybrid option: There is also the possibility of a hybrid between Options 1 and 2 where DNA-based
168 methods are used to replace morphological identification whilst keeping metrics and reference
169 conditions for assessment purposes (cf option 1). At the same time, additional information generated
170 by DNA-based methods such as more highly resolved taxa lists or functional information derived
171 from other approaches such as metagenomics and -transcriptomics would be used to better inform
172 interpretation of assessment results, for example rating how stressors affect ecosystem functionality.
173 Until 2027, only Option 1 provides a realistic option for operational monitoring under the WFD.
174 European countries have spent considerable resource developing WFD assessment systems and have
175 used them in previous monitoring cycles : they will continue to apply them until the end of the fourth
176 River Basin Management Cycle in 2027. Therefore, this paper focuses on DNA-based identification
177 (Option 1), acknowledging that it is a straightforward, but rather conservative approach in
178 comparison with Option 2, as it aims for maximum comparability with traditional methods.
179 In some circumstances, the inclusion of DNA-based techniques into WFD assessment has already
180 been tested, for example for river phytobenthos in Mayotte Island, France (Vasselon et al., 2017) and
181 the UK (Kelly et al., 2017), and is likely to be used increasingly for a range of BQEs in other countries
182 (Leese et al., submitted). However, for a variety of reasons the applicability of Option 1 differs

183 between BQEs and water body types (river, lakes, transitional and coastal waters). Amongst others,
184 there is the need to secure comparability with traditional identification, which may be more
185 problematic for those BQEs where there are large discrepancies between morphological and DNA-
186 based species identification. In addition, the potential benefits in sample processing speed differ
187 strongly between BQEs.
188 Here, we evaluate the potential of DNA-based identification (Option 1) for routine WFD assessment
189 for different BQEs and water categories. Our aim is to rate the applicability of DNA-based
190 identification methods, assuming that current WFD assessment metrics are kept or only slightly
191 adapted. We use a variety of criteria related to the anticipated suitability (for example the expected
192 increase in processing speed, lower costs) and the maturity of development (for example the extent
193 to which assessment methods will need to be adapted). The paper is addressed at scientists and
194 officials involved into the commissioning and development of DNA-based methods, stakeholders and
195 consultants involved in WFD monitoring.
196
197
198 **Assessment and monitoring methods under the WFD**
199 Considerable research effort has been devoted to the development of methods for ecological
200 assessment of waterbodies following implementation of the WFD (Birk et al., 2012). The primary
201 focus has been to establish sensitive and precise methods capable of assessing the impact of a wide
202 range of pressures on biota and, hence, guide management efforts to restore good ecological status.
203 The reference condition approach is a central principle of the WFD: the biota observed are compared
204 with those expected in the absence of environmental stress, resulting in an Ecological Quality Ratio
205 (EQR), calculated as the observed score /expected score (Jones et al. 2010).

206 Although always based on the same principles, subsidiarity has led to diversity in the methods
207 developed by Member States for each BQE-water category combination. This reflects the variety of
208 methods and data existing prior to the WFD, and regional differences in stressors and taxonomic
209 knowledge. Overall, more than 300 methods are in use across Europe (Birk et al., 2012), with
210 comparability ensured by an obligatory intercalibration process (Birk et al., 2013; Poikane et al.,
211 2014). At a first glance, the large number of methods is bewildering; however, all methods are based
212 on the same chain of steps and many differ only in detail (Birk et al., 2013):

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

223 The deviation from reference conditions is expressed as the EQR (from 0 to 1) from which the
224 biological status class (“high”, “good”, “moderate”, “poor” or “bad”) is derived, harmonised between
225 EU member states through intercalibration. The status classes of the individual BQEs are finally
226 combined with other quality elements into an ecological status class, using the “one-out-all-out”
227 principle (the worst status class determines the overall ecological status class).
228 Three types of monitoring are specified by the WFD, each with a different objective, namely: (1)
229 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.

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; Bohman 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

253 management, invasion biology, studies of trophic interactions and food safety (Cristecu, 2014) but is
254 not yet a cost efficient method for most ecological assessment purposes (Stein et al., 2014).
255 More recently, high throughput sequencing (HTS) techniques have allowed the barcodes of multiple
256 organisms to be obtained in a single reaction, enabling parallel sequence-based identification in an
257 approach termed DNA metabarcoding (Taberlet et al., 2012b; Shokralla et al., 2012). This approach
258 offers the opportunity for non-targeted (passive) detection of a wide range of rare and invasive
259 species (for example Blackman et al., 2017; see Lawson Handley, 2015, for a review) and to assess
260 the composition of whole communities. The application of DNA metabarcoding to community DNA
261 extracted from organisms or environmental samples (eDNA) is the focus of this paper.
262 Most current sequencing protocols rely on rather short (i.e. about 70-500 base pair) metabarcoding
263 markers and thus are capable of using the degraded DNA often found in eDNA samples (see Elbrecht
264 and Leese, 2017, for an overview). Recent research has shown that DNA-based methods are effective
265 at detecting aquatic species of microalgae and protists (Medinger et al., 2010; Kermarrec et al., 2014;
266 Kelly et al. 2017), meiofauna (Carugati et al., 2015), macroinvertebrates (Hajibabaei et al., 2011;
267 Sweeney et al., 2011; Aylagas et al., 2016), fish (Thomsen et al., 2012; Kelly et al., 2014; Civade et al.,
268 2016; Hanfling et al., 2016; Shaw et al 2016) and amphibians (Ficetola et al., 2008; Dejean et al.,
269 2012). However, the protocols and workflows used for capture, extraction and identification of DNA
270 are highly diverse even within BQEs. This makes comparison of results from different laboratories
271 and studies difficult (Deiner et al., 2015) and will limit the use of DNA for aquatic biodiversity
272 assessment until the biases associated with different methods are fully understood and controlled.
273 Probably the critical consideration is choosing the most appropriate primer, which determines the
274 DNA marker used for identification, and its length. This in turn influences the taxonomic resolution
275 that can be achieved and affects the extent to which species level identifications can be made;
276 primer choice also affects the specificity of the analysis. In some cases, highly specific primers can be

277 developed that will amplify the entire target organism group and little else (the 12S primers for fish
278 are a good example). In other cases, primers that are general enough to capture the whole group will
279 inevitably amplify non-target taxa as well. An example of this is the primers designed to amplify
280 benthic invertebrates, which consistently amplify a wide range of non-metazoan taxa when used on
281 environmental samples.
282
283 **Criteria to rate the potential for application of DNA-based identification**
284 Here we describe and justify a set of criteria, which will later be used to rate the applicability of DNA-
285 based identification for incorporation into WFD assessment for different BQEs and water categories.
286 As we limit the applicability check to DNA-based identification, and do not include more advanced
287 approaches (i.e. Option 2 described in the introduction), the criteria are restricted to those rating the
288 performance of WFD-related assessment methods. The criteria are categorised under six headings: 1)
289 Representativeness, 2) Sensitivity, 3) Precision, 4) Comparability, 5) Cost-effectiveness and 6)
290 Environmental impact, and are not always independent. For example, the cost of sample collection
291 and processing will influence the sampling strategy undertaken (frequency and number of samples
292 collected), which, in turn, will influence the representativeness and precision of the overall
293 assessment of ecological status. Here, we will address each of these criteria separately, whilst
294 considering those interactions relevant to DNA-based identification.
295
296 <u>1) Representativeness</u>
297 <u>Criterion 1.1: Applicability of current sampling methods, and availability of alternative methods for</u>
298 <u>obtaining biological material for DNA-based identification</u>
299 This criterion addresses how samples are collected and processed prior to sequencing, to determine
300 if current sampling methods are suitable for molecular methods, or if simple alternatives are

301 available. The criterion is relevant to establish whether DNA-based identification can be used without
302 changing current sampling strategies significantly, or if major changes in sampling methods are
303 required.
304 For some taxa (microalgae, macroinvertebrates) entire unprocessed samples have been used for
305 extraction and subsequent metabarcoding (Zimmermann et al., 2015; Elbrecht et al., 2017), which
306 can be analysed in parallel with microscopy. However, for inventories of fish species, the current
307 sampling methods (for example electrofishing) cannot be used for DNA-based assays. The proposed
308 solution of sampling eDNA from water is a simple and effective alternative. Results from eDNA
309 approaches are often very similar to those from traditional netting or electrofishing, although usually
310 more effective (Takahara et al., 2012; Shaw et al., 2016; Hanfling et al. 2016; Stoeckle et al., 2017;
311 Pont et al. submitted). However, the inference of temporal and spatial distribution of species
312 through eDNA is complicated since detection is influenced by environmentally variable DNA
313 degradation rates, transport and species specific behavioural patterns (Barnes and Turner, 2015;
314 Stoeckle et al., 2017). The spatial scale of eDNA detectability is of particular importance in lotic
315 ecosystems, as eDNA may only detect species present in upstream regions or tributaries. On the
316 other hand, eDNA may better represent species composition across the whole waterbody (from a
317 few to several tens of kilometres; Civade et al., 2016; Pont et al. submitted), as is required for
318 surveillance monitoring. Understanding the spatial and temporal scales that eDNA represents is a
319 hurdle to the deployment of this approach for WFD monitoring.
320 After the removal of an organism, DNA persistence under normal conditions in water is quite short (a
321 few days to two weeks in mesocosms; Ficetola, 2008; Dejean et al., 2011; Pilliod et al., 2013). In
322 rivers, eDNA concentration and detectability downstream from the point of production are
323 dependent on production and degradation rates, dilution, transport through the river network,
324 deposition, and resuspension (Thomsen et al., 2012). Detectable eDNA can be found at distances

325 from a few hundred metres to a few kilometres downstream of its source (Deiner and Altermatt,
326 2014; Jane et al., 2015; Civade et al., 2016; Wilcox et al., 2016). The detection distance of eDNA is
327 important for defining the scale at which eDNA can reveal spatial and temporal differences in
328 biological communities (Civade et al., 2016; Deiner et al., 2016; Staehr et al., 2016; Bista et al., 2017;
329 Stoeckle et al., 2017; Yamamoto et al., 2017).
330 We used this criterion for rating the magnitude of alterations in sampling methods required to apply
331 DNA-based identification.
332
333 Criterion 1.2: Errors from DNA-based species detection and similarity of DNA-based and conventional
334 taxon lists
335 This criterion addresses the question of how comparable taxon lists obtained with DNA-based
336 methods are to taxon lists obtained with traditional methods, in particular as a result of detection
337 errors. The criterion is relevant to judge if current assessment indices and associated class
338 boundaries can be applied to taxon lists generated with DNA-based methods.
339 In the production of taxon lists, two types of error occur, false negatives, where a taxon is recorded
340 as absent yet is in fact present, and false positives, where a taxon is recorded as present yet is in fact
341 absent: misidentifications comprise both type of error (the correct species is falsely recorded as
342 absent, whilst the incorrect species is falsely recorded as present). Both error types affect index
343 values and hence the accuracy of assessments (Criterion 2), and add uncertainty (Criterion 3). Both
344 visual and DNA-based methods are prone to identification errors. Whilst it is known that errors can
345 significantly affect the results of traditional assessments (Haase et al., 2006), much work remains to
346 be done for DNA-based methods. If the DNA-based identification targets morphotaxa rather than
347 OTUs, benchmarking against morpho-taxonomic approaches will be critical before molecular
348 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; Yamamato et al. 2017) or only present in upstream sites

373 (Hänfling et al., 2016), and dead organisms or life-stages (seeds, spores, eggs, early instars)
374 associated with non-viable populations. The results of eDNA studies can be influenced strongly by
375 single molecules. It is less likely to be a concern for whole community analyses where the majority of
376 organisms present in the sample will be relevant and their abundant DNA reduce the influence of
377 trace DNA. There is a clear need to relate DNA reads to the presence of viable populations within the
378 water body. At some point the information gained from molecular methods will tip from “signal” to
379 “noise”, and it will be important to learn to differentiate between an indication of a genuinely rare
380 species and reads caused by DNA from non-viable organisms.
381 As a result, the taxa lists produced by DNA-based methods are different from those generated by
382 traditional methods: additional taxa will be included that are not identifiable with morphometric
383 methods, while other taxa will not be detected. In addition, detection limits will differ, dependent on
384 the way specimens/DNA are extracted from the raw samples. DNA-based taxa lists will inevitably
385 require some manipulation before they can be used in current assessment methods. This may
386 involve filtering DNA-based lists against the operational taxon list used for that assessment system,
387 thus eliminating those taxa which are not detected with traditional methods (Elbrecht et al., 2017) as
388 well as indicating those that cannot (yet) be identified with DNA based methods (for example due to
389 incomplete reference databases). Alternatively, assessment systems may need to be modified, by
390 aligning (intercalibrating) future indices suitable for DNA-based methods with existing indices if the
391 full potential of genetic identification is to be realised.
392 We used this criterion to rate the suitability of DNA-based taxon lists for the calculation of the
393 assessment indices applied in the current WFD assessment schemes.
394
395 Criterion 1.3: Need for assessment of abundance and accuracy of abundance estimates with DNA-
396 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 Bohman 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, 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., submitted). 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

420 strength is moderate, and the first results have been a proof of concept rather than demonstration of
421 quantitative [predictive?] relationships.
422 It is important to note that even if a strong relationship can be obtained between amount of DNA in
423 a sample and the number of sequence reads, the relationship between the number (or biomass) of
424 organisms and the amount of DNA released into the environment is not straightforward. Some
425 organisms (for example fish) shed DNA continuously while others (for example crayfish) shed large
426 amounts when they breed or moult but very little at other times of year. Even for fish, spawning
427 introduces large amounts of DNA into the environment that does not reflect the size of the adult
428 population. Thus, sampling campaigns need to take account of the ecology and life-histories of the
429 target organisms before quantitative inferences can be made.
430 Correction factors can eliminate biases to an extent when DNA-based data are used in assessment
431 systems (Thomas et al., 2016). Furthermore, many assessment systems use relative rather than
432 absolute abundance or summarise absolute abundance as broad categories (for example log
433 categories), where small biases may not introduce much uncertainty (Birk et al., 2012). A number of
434 studies have demonstrated that relative abundance estimates from eDNA metabarcoding of fish
435 communities show good correlations with abundance estimates from established survey methods. A
436 comparison of electrofishing and eDNA based methods along the Rhône River, for example, revealed
437 a sufficient correlation between the two techniques to describe the structure of fish assemblages
438 and their longitudinal change in terms of relative abundance (Pont et al., submitted). In Windermere,
439 a large lake in the UK, rank abundance from long-term traditional survey data correlated well with
440 eDNA based estimates of relative abundance (Hanfling et al., 2016) and a recent study in Belgian
441 ponds showed strong correlations between sequence read counts and fish biomass (Brys et al.,
442 submitted). As the WFD assessment approach demands that comparison are made between

443 observed and expected conditions, it may be possible to correct for consistent biases, particularly
444 when the reference condition is based on new characterisation using molecular techniques.
445 We used this criterion to rate the degree of changes required in current WFD assessment schemes to
446 account for the differences in abundance data generated by DNA-based identification methods
447 compared with traditional identification methods.
448
449 2) Sensitivity of species detection
450 Criterion 2.1: Capability of DNA-based methods to sample sensitive taxa
451 This criterion addresses the question of whether or not DNA-based methods are suitable for the
452 detection of sensitive taxa, which are an integral part of most WFD assessment methods. The
453 criterion is relevant to rate if current assessment metrics can reasonably be applied with taxon lists
454 generated with DNA-based methods.
455 Whilst some management objectives may require complete lists of taxa present (for example the
456 conservation objectives of the Habitats Directive, which target species listed in Annexes II, IV and V;
457 see http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm), the
458 objective of the WFD is the sustainable development of water bodies. Hence, the principal role of
459 biological monitoring is to determine the condition of the ecosystem and to detect impacts that
460 could impede WFD objectives. Those taxa that are sensitive to human-induced stress are not
461 necessarily those that contribute the most to structure and function, and assessments need to be
462 aware of this. For example, several sensitive benthic invertebrate species with a long life cycle,
463 whose occurrence indicates the absence of pollution events over a long time period, tend to occur at
464 low abundance (e.g. large Plecoptera species). Whilst a complete list of taxa might not be required to
465 determine stress effects, rare taxa are important components of some assessment metrics as they
466 are typically most sensitive to water body deterioration (Clarke and Murphy, 2006). For those BQEs

467 and water categories where this has been demonstrated, it is important to ensure that rare species
468 are accurately characterised when developing techniques that involve bulk extraction of genetic
469 material. For fish, the capacity of DNA based methods to detect rare species in rivers more effectively
470 than traditional methods has been clearly demonstrated (Civade et al., 2016; Pont et al., submitted),
471 whereas for invertebrate samples it may be necessary to transform or increase sequencing depth in
472 order to ensure rare taxa are detected (Elbrecht et al., 2017). For phytobenthos, the main issue is the
473 severe underrepresentation of rare species in existing reference databases (Kermarrec et al. 2014).
474 Another issue affecting sensitivity is sequencing depth relative to non-target DNA. For example,
475 samples may have high concentrations of DNA from taxa that are not relevant for calculation of
476 indices (e.g. fungi) and these high concentrations may reduce sensitivity to target or rare taxa.
477 We used this criterion to rate if current assessment indices can be applied with DNA-based taxon
478 lists.
479
480 Criterion 2.2: Unassigned reads
481 This criterion addresses the separate but related question, of how the influence of f "unassigned"
482 reads (i.e. those reads or OTUs that do not match a Linnaean taxon in DNA reference databases) is
483 minimised. This criterion is relevant to judge if it is necessary to either generate more data for DNA
484 reference databases or, alternatively, to generate data on ecological preferences for unassigned
485 OTUs before they could be used in assessment systems.
486 The extent of this problem varies among BQEs and is particularly complex for taxa-rich BQEs. For
487 microalgae, Linnaean nomenclature still needs to be reconciled with cryptic diversity and possibly the
488 depth of coverage of each taxon needs to be reconsidered. Whilst chimeras and mistags occur for all
489 BQEs, for most the frequency of unassigned reads is related to the completeness of barcode libraries.
490 The COI gene, for example, is available for hundreds of thousands of species, yet many taxa have are

491 still to be sequenced. Additional sequences are needed for adequate representation of intraspecific
492 and geographic variation (Bergsten et al., 2012). For groups where other gene regions are preferred
493 (for example 18S and rbcL for microalgae, 16S for Cyanobacteria) the number of taxa sequenced is
494 lower despite considerable sequencing effort (for example Rimet et al., 2016). For fish, a barcode
495 library based on the 12S marker is still in development for Southern and Eastern Europe, but 90% of
496 fish species encountered in Western European continental water bodies have already been
497 sequenced (Valentini et al., 2016). For UK macroinvertebrates, most OTUs have been assigned to
498 species based on COI data, although taxonomic problems resulting from cryptic species remain to be
499 solved (Andujar et al., accepted).
500 Poor species representation in reference databases may lead to incorrect identifications and, thus,
501 affect the assessments of ecological quality (Aylagas et al., 2014). In turn, this depends on the
502 structure of the index. Four types of indices are used to assess ecological status for the WFD (Hering
503 et al., 2006): Composition / abundances indices, richness / diversity indices, sensitivity / tolerance
504 indices and functional indices. Incomplete barcode libraries may have little influence on diversity
505 indices, as the number of OTUs overall or within broad classification groups (for example order) may
506 be sufficient to derive index values. However, those indices that are calculated from species presence
507 are more vulnerable, as they require correct species identification. Indices based on average scores
508 are likely to be more robust to missing taxa, but efforts will be needed to benchmark indices derived
509 through molecular methods against those derived using existing approaches (Ärje et al., 2017).
510 We used this criterion to rate how complete barcode libraries are for the individual BQEs and how
511 incomplete barcode libraries will affect assessment results.
512
513 3) Precision of DNA-based identifications
514 Criterion 3.1: Knowledge about uncertainty of DNA-based identification

515 This criterion addresses the question of how well the uncertainty associated with DNA-based
516 identification is known. The criterion is relevant as the WFD explicitly requires (Annex 1.3.4) that the
517 uncertainty of assessments is reported.
518 As WFD assessments are used to guide management decisions and, hence, have both political and
519 economic implications, there is considerable focus on the confidence in any assessment of ecological
520 status made. The level of uncertainty can be estimated using specifically designed software (Clarke
521 and Hering, 2006, Kelly et al., 2009) but differs between BQEs and associated assessment methods
522 (Birk et al., 2012). As the use of molecular approaches does not result in directly equivalent data (see
523 criteria 1.1 to 1.3), it will be necessary to quantify the uncertainty associated with the new methods
524 and the impact on assessment metrics and classification. All steps in the identification and
525 enumeration process will need to be considered, including processing (for example platform chosen,
526 sequencing depth, pre-treatment), and data analysis (for example bioinformatics), as each has the
527 potential to influence the resulting taxa list. Identification is only one step in the process and, at this
528 stage, it is unclear whether or not uncertainty will increase or decrease if molecular methods are
529 adopted. Leaving aside stochastic variability from sampling and biases associated with primer
530 selectivity, representation and other processing errors, assessments are affected by the power of
531 identification. Structural changes in the power of identification are likely to occur over time (for
532 example infilling of barcode libraries, technological developments in platforms, better links between
533 DNA-based and morpho-taxonomy). Robust quality assurance methods will be necessary in order to
534 quantify such changes. Quality assurance procedures based on morpho-taxonomic approaches are
535 also fundamental to account for any bias introduced by DNA contamination and chimeras, and their
536 adoption would allow for continuous comparison with existing methods to demonstrate the effects
537 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 Ärje et al., 2016).

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

4) 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).

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

563

564 Criterion 4.2: Intercalibration

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

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

586 It should be noted that intercalibrated standards do not just affect comparisons among Member
587 States: the target of Good Ecological Status is a long-term policy goal and any change in methods
588 within a country has implications for detection of long-term change and, hence, progress towards
589 this target. Changes in the position of key status class boundaries will need to be justified to
590 governments and stakeholders as these will have implications for regulation.
591 We used this criterion to rate if there are obstacles for intercalibrating indices that are calculated
592 with DNA-based taxon lists.
593
594 5) Cost-effectiveness
595 Criterion 5.1: Costs compared to traditional methods
596 This criterion addresses the question of whether or not DNA-based methods have the potential to
597 substantially lower the costs of monitoring. This is relevant as monitoring programmes are often
598 subject to severe financial pressure.
599 In recent years, the cost of sequencing biological material has fallen sharply and is likely to fall
600 further as technology develops. However, cost-effectiveness is not defined simply by the monetary
601 cost of sample processing but includes factors such as cost and availability of facilities, training
602 needs, speed of processing, sensitivity and precision. Here, molecular approaches could provide an
603 advantage via low processing costs and rapid turn-round ("economies of scale"), potentially enabling
604 increased sampling frequency, increasing precision of assessments and enabling more responsive
605 monitoring of pollution events or restoration activities. Furthermore, sampling eDNA is often
606 cheaper than traditional sampling methods, e.g. electrofishing, gillnetting or trawling. Again, we
607 stress that the whole cycle should be considered when comparing approaches: advantages gained by
608 mechanising one aspect can easily be offset by losses in other parts of the assessment process (Stein
609 et al., 2014; Elbrecht et al., 2017).

610 We used this criterion to rate the potential for cost reduction through the use of DNA-based
611 methods for the individual BQEs.
612
613 Criterion 5.2: Processing speed
614 This criterion addresses the question of whether sample processing can be accelerated by DNA-
615 based identification or not. The criterion is relevant as the time required for manual identification is
616 often a bottleneck for processing biological samples for WFD monitoring, particularly those requiring
617 trained experts for microscopic identification (i.e. phytoplankton and macroinvertebrates). The speed
618 of processing could be enhanced by DNA-based methods (Goodwin et al., 2017). DNA based methods
619 could also benefit those BQEs requiring time-consuming sampling (for example electrofishing, gill-
620 netting). At present, however, sequencing and computer capacities are limited for such DNA-based
621 methods in many countries. This can itself create a bottleneck, potentially exacerbated by the need
622 to run sequencing machines at full capacity in order to access the economies of scale described in
623 5.1. Early experience in the UK is that the shift to DNA-based analysis of phytobenthos makes it
624 harder for laboratories to respond to requests to prioritise particular samples. This situation should
625 change over time, as capacity increases and technology advances, as well as through knowledge
626 transfer (Leese et al., submitted).
627 We used this criterion to rate the potential for speeding up sample processing for individual BQEs.
628
629 6) Criterion 6.1: Animal well-being, health and safety, environmental impact
630 This criterion addresses the question of whether DNA-based identification can reduce the
631 environmental impact and safety risks of sampling methods.
632 “Hands-off” techniques, such as eDNA assessments of fish populations, provide benefits for the well-
633 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.

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) (Figure 1). In the following, we provide justification for the values given in Figure 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

658 imply an overall ranking of the BQEs. In particular, Criteria 5.1, 5.2 and 6.1 do not address the
659 technical feasibility of DNA-based identification, rather additional arguments for the use of DNA-
660 based methods.
661
662 Criterion 1.1 (Applicability of current sampling methods, and availability of alternative methods, for
663 obtaining biological material for DNA-based identification): Applicability of sampling methods differs
664 greatly between organism groups. For phytoplankton, phytobenthos and invertebrates the
665 traditional sampling methods can be used for DNA-based assessment (high), although some aspects
666 such as use of ethanol as a fixative is problematic for cost and safety reasons in several European
667 states. For fish, traditional electrofishing or gill-netting can be replaced by water samples for
668 extraction of eDNA, which would be a simple and effective alternative (high). Macrophytes,
669 macroalgae and angiosperms are surveyed rather than sampled; most species are identified in the
670 field and their abundance is estimated directly. A different, and as yet not available, sampling
671 method capable of detecting all relevant species adequately would need to be applied for DNA-based
672 identification (low).
673
674 Criterion 1.2 (Errors from DNA-based species detection and similarity of DNA-based and conventional
675 taxon lists): This criterion depends on the transferability of DNA-based taxon lists into taxon lists
676 similar to those generated with morphology-based methods, and largely concerns taxa that are
677 currently only identifiable with either morphology or DNA-based methods. In principle, additional
678 taxa identified with DNA-based methods could be removed from a taxa list through use of filters
679 (thus allowing the continuous use of the current assessment metrics; Elbrecht et al., 2017), while
680 taxa not identified with DNA-based methods necessarily require changes in the assessment metrics.
681 The number of the latter is low for fish and for invertebrates (Valentini et al., 2016; Aylagas, 2017)

682 (high suitability), and despite a lower number of identifiable taxa, transferability has been
683 demonstrated for phytobenthos (Kelly et al., 2017) (high). For phytoplankton, this is still to be
684 demonstrated (medium). Combining directly identifiable taxa with known ecology, with those that
685 are assigned to an OTU to give an ecological value should improve current assessment systems,
686 without fundamentally changing their concept. For macrophytes, macroalgae and angiosperms most
687 species can be identified, but as sampling methods associated with current assessment systems do
688 not result in samples of all species (see 1.1), taxa lists generated with DNA-based identification may
689 differ more than for other BQEs (medium).
690
691 Criterion 1.3 (Need for abundance assessment and accuracy of abundance estimates with DNA-based
692 methods): The relevance of this criterion depends on
693 • the role of abundance-based metrics in assessment methods for the individual BQEs;
694 • options to measure relative abundance and to replace absolute by relative abundance;
695 • options to transform abundance-based metrics into presence/absence-based metrics.
696 Currently, the normative definitions for most BQEs specifies a need for abundance estimates. For
697 phytoplankton, however, a measure of abundance is provided by chlorophyll concentration, resulting
698 in a “medium” rating of this criterion. For phytobenthos and invertebrates, there are promising signs
699 that presence/absence-based data and relative abundance estimates could be used (Vasselon et al.,
700 2017) (medium). For fish, there are attempts to infer relative abundance from eDNA, while age
701 classes cannot be detected (Hanfling et al., 2016, Pont et al., submitted) (medium). The species-poor
702 groups of macrophytes, angiosperms and macroalgae are surveyed rather than sampled under the
703 current assessment schemes; in its extreme form, an assessment system can be based on a single
704 species (e.g. *Posidonia*) and the assessment system simply rates its abundance and density. This
705 cannot be inferred from eDNA (low).

706

707 Criterion 2.1 (Capability of DNA-based methods to sample sensitive taxa): For fish, DNA-based
708 methods are clearly superior to electrofishing and gillnetting in terms of the detection of rare species
709 (Hanfling et al., 2016) (high). For invertebrates and phytoplankton, there is good evidence that the
710 relevant species are reliably captured with DNA-based methods (high), although unequal biomass
711 still requires manual size adjustments especially for the biomass-rich specimens or great sequencing
712 depths (Elbrecht et al. 2017). If a suitable sampling method could be found, this would also probably
713 apply to macrophytes, but, in the absence of this, we rate it as “unknown”. For phytobenthos, the
714 coverage of barcode libraries (see 2.2) limits this criterion (medium). There are currently no papers
715 on DNA-based methods for marine angiosperms and macroalgae (unknown). This does not, however,
716 mean that DNA-based identification is unsuitable for detecting sensitive marine angiosperm and
717 macroalgae taxa, only that more work is needed.
718

719 Criterion 2.2 (Unassigned reads): This criterion is mainly associated with the completeness of
720 barcode libraries (COI gene, 18S and rbcL for microalgae, 16S for Cyanobacteria) and cryptic diversity.
721 Fish and macrophytes in rivers and lakes rate “high”, while barcode libraries for phytobenthos,
722 invertebrates and fish in transitional and coastal waters are in an intermediate state of completeness
723 (medium). For phytoplankton, cryptic diversity is an issue, as the number of taxa sequenced is lower
724 (low), while for macroalgae and angiosperms cryptic diversity could be an issue only for small
725 epiphytic species (low).
726

727 Criterion 3.1 (Knowledge about uncertainty of DNA-based identification): For all BQEs, data on
728 uncertainty associated with the different steps of the DNA-based processing chain have not been
729 collected systematically or simulated (Ärje et al., 2016). We rate this criterion as “low” for

730 macrophytes, angiosperms and macroalgae, as sampling provides an additional - yet unquantified -
731 source of uncertainty, while in the absence of more precise data the criterion is rated as “medium”
732 for all other BQEs.
733
734 Criterion 4.1 (Sensitivity of EQRs to differences in DNA-based identification): It is likely that
735 approaches used to derive EQRs will need to be adapted for DNA-based identification, even if
736 taxonomic issues (Criteria 1.2 and 2.2) have been solved. The feasibility of this procedure has already
737 been demonstrated for phytobenthos (Kelly et al. 2017) and fish (Civade et al., 2016; Pont et al.
738 submitted) (high), and we assume that this procedure will be possible for most other BQEs (medium).
739 Exceptions are macrophytes in rivers and lakes, and angiosperms and macroalgae in coastal and
740 transitional waters, for which we question the suitability of currently applied indices for use with
741 DNA-based data, as most rely on measures of cover.
742
743 Criterion 4.2 (Intercalibration): In principle, there are no obstacles preventing the WFD
744 intercalibration procedure being performed to compare DNA-based methods against traditional
745 methods. However, to date this process has not been undertaken, as few countries use DNA-based
746 identification for formal WFD assessments. Promising examples, for which DNA-based and morpho-
747 taxonomic approaches have been compared (although not yet intercalibrated) include phytobenthos
748 in rivers, invertebrates in rivers and transitional and coastal waters, and fish in rivers and lakes (high),
749 while we rate this criterion as “medium” for most other BQE-water type combinations. We expect
750 more general problems for macrophytes, angiosperms and macroalgae (low), as the compatibility of
751 these BQEs with DNA-based methods is generally questionable: These groups are species-poor, they
752 are identified and their abundance estimated in the field; applying DNA-based identification would,

753 therefore, require a different sampling strategy and different metrics, which limits comparability with
754 traditional approaches.
755
756 Criterion 5.1 (Costs compared with traditional methods): A comprehensive overview of the costs
757 associated with DNA-based methods compared with traditional methods is not yet available (but see
758 Stein et al., 2014; Sigsgaard et al., 2015; Smart et al., 2016; Aylagas, 2017). It is expected that the
759 costs will be significantly lower for fish in rivers, lakes and transitional waters, as sampling eDNA is
760 much cheaper than electrofishing, gillnetting or trawling (high). For all other BQE-water category
761 combinations, we expect a potential for cost reduction, which nevertheless still needs to be explored
762 (medium).
763
764 Criterion 5.2 (Processing speed): The potential for increased processing speed is particularly high for
765 the labour-intensive identification of phytoplankton and invertebrates (high), while it is “low” for
766 macrophytes, macroalgae and angiosperms, for which the field survey is the most time-consuming
767 process. For all other BQEs, this criterion has been rated as “medium”.
768
769 Criterion 6.1 (Animal well-being, health and safety, environmental impact): This criterion is only
770 relevant for invertebrates and fish. For invertebrates, the same sampling methods are applied for
771 traditional and DNA-based approaches. For traditional methods, the specimens are in most cases
772 sacrificed for morphological identification, unless they are sorted and identified alive; however, rare
773 and protected species (such as Odonata larvae and large mussels) are often identified in the field and
774 placed back in the water body afterwards. Although this option is possible for DNA-based methods,
775 there is generally a need to sacrifice specimens before DNA-based identification (low). For fish, the

776 sampling of eDNA is non-invasive and offers advantages over gillnetting, trawling or electrofishing
777 (high).
778
779 **Discussion and outlook**
780 Suitability of DNA-based identification for different BQEs and water categories
781 This paper is limited to the use of DNA-based identification for biological assessment systems in
782 support of the WFD, although some of the issues discussed could be applicable to other directives
783 (i.e. the Marine Strategy Framework Directive) and other geographical areas (for example in the USA,
784 for the Clean Water Act; Keck et al. 2017). Clearly, DNA-based methods offer options, which can go
785 beyond simple identification to a predefined taxonomic level. Therefore, DNA-based identification is
786 likely to be a transition stage between conventional morpho-taxonomic approaches and DNA-based
787 ecological assessment methods. However, even DNA-based identification poses many obstacles and
788 cannot be implemented without adapting both the DNA-based identification procedure and the
789 assessment methods to which they would be applied. These obstacles to implementation differ
790 strongly among BQEs.
791 The advantages of DNA-based identification are obvious for fish: eDNA offers a well-suited and
792 reliable sampling method (although different from conventional methods), with a high probability of
793 detecting species (compared to other organism groups), whilst avoiding cost-intensive and harmful
794 sampling methods. But even for fish, assessment metrics will need to be adapted, in particular to
795 account for the change from absolute to relative abundances. Furthermore, some criteria required
796 by WFD legislation (for example age class) currently cannot be assessed using DNA-based methods
797 but, on the other hand, several currently adopted (and intercalibrated) methods do not include age
798 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.

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

822 to solve the conflict between the inherent need for ecological assessment to be consistent over a
823 long time period, and the opportunities provided by the new methods.
824 The options for dealing with abundance is a good example of this conflict. Annex V of the WFD
825 stipulates that abundance must be recorded for most BQEs. The legislation is based on the
826 assumption that abundance provides more information than taxa lists alone, as changes in
827 abundance may occur long before human-induced pressures lead to the extinction of species. As a
828 consequence, the calculation of most functional indices requires data on either the abundance of a
829 taxon or, at the very least, the proportion of the whole sample or sub-sample that it represents.
830 Therefore, before DNA-based identification can be implemented, two questions need to be
831 addressed: (1) How best to fulfil the legal requirement of recording abundance? And (2) How can the
832 information given by species' abundances best be provided? The answer to the first question differs
833 between BQEs. For phytoplankton, there is the option of using chlorophyll concentration as a proxy
834 for abundance or biomass. From a practical point of view, a filtered plankton sample can be divided,
835 with one half being used to measure chlorophyll and the other half for DNA-based identification. The
836 remaining quantitative indicators required for phytoplankton are algal bloom frequency and
837 amplitude, which could be measured with frequent readings of pigments from satellites or
838 continuous reading from an automated buoy placed within the water body (Schluter et al., 2014).
839 Thus, a combination of DNA-based identification and other methods could fulfil the WFD's
840 requirements. For fish, and probably other BQEs, there is the option to use relative rather than
841 absolute abundance based on read count data, or frequency of occurrences in several eDNA samples
842 as a proxy for abundance by analysing multiple eDNA replicates per site (Pilliod et al., 2013). In
843 response to the second question, there are promising signs for various BQEs and metrics that
844 presence-absence data give signals similar to abundance data and can be translated between one
845 another (Aylagas, 2017). However, questions remain, regarding the degree to which abundance data

846 – whether traditional or molecular – reflects biomass or processes (for example related to the
847 abundance of grazers or sediment feeders in the benthic invertebrate community. Currently applied
848 measures of abundance do not discriminate between large and small specimens: a tiny chironomid
849 larvae and a large stonefly larvae count the same, although the latter might have a 1,000 times
850 greater biomass. Clearly, there is room for improvement through DNA-based methods. Barcodes
851 potentially represent the abundance of mitochondria and plastids and may, indeed, offer greater
852 insights into which taxa are actually driving ecological processes within an ecosystem, by reflecting
853 the intensity of metabolic processes.
854 More generally, there is the question of how to achieve compatibility in ecological assessments when
855 replacing conventional by novel methods? The term “monitoring” implies recording of time series,
856 and, inherently, the consistent use of standard methods. In case of the WFD, the monitoring intervals
857 are very long: for River Basin Management Plans, for example, ecological status only needs to be
858 reported at six-yearly intervals. It should be possible to change methods between these intervals in
859 response to results and experience. DNA-based identification is only one, albeit significant, driver of
860 changes to methods. The benefits of increased accuracy and performance of enhanced ecological
861 assessment methods will always need to be carefully balanced against the potential loss of
862 compatrability. The implementation of new methods should, therefore, always be accompanied by a
863 re-calculation of indices from prior monitoring programmes, to ensure backward compatibility. This
864 underlines the need to develop capacity to archive DNA samples, particularly from reference sites, so
865 that as new technologies emerge, DNA from critical sites can be reanalysed using the new methods.
866 Closely related with the question of backward compatibility is the future evolution of methods. With
867 DNA-based identification, there is a clear need to allow methods to evolve, which may require
868 constant adaptation of indices and assessment methods. This is a potential paradigm shift in how to
869 handle monitoring data. In future, a rolling comparison with existing methods will be needed to

870 “buffer” monitoring results against the effects of advances in technology. However, provided there is
871 sufficient storage capacity, sequence data can be stored and reanalysed more easily than traditional
872 samples, ensuring a level of “forward compatibility” as bioinformatics and metrics improve, for as
873 long as sampling, DNA extraction and the sequencing itself are robust. Most importantly, DNA
874 extracts are relatively easy to store and this should be encouraged, as we do not know which
875 barcodes and methods will be available in the future.
876 The expense of implementation is another consideration when introducing DNA-based methods into
877 WFD assessments, since costs may be reduced compared with traditional assessment methods
878 (Aylagas, 2017). Expenses are not solely related to the costs of processing individual samples, but
879 encompass training, equipment purchase, administrative and maintenance costs, quality assurance
880 and, importantly, the costs of initial method development and ongoing evaluations and upgrades.
881 Any change in assessment methods and results needs to be communicated to policy makers and the
882 general public, which is not necessarily a straightforward procedure and which will require education
883 of stakeholder groups, including those from non-scientific backgrounds.
884 A general challenge for river basin management will be the breakdown of the assessment procedure
885 into several smaller steps, which are performed by different people or units. While in many countries
886 microscopic identification is still the responsibility of water boards, DNA-based identification is likely
887 to induce a shift to external service providers. Care must be taken that the individual steps of the
888 assessment procedure stay connected and allow informed interpretation of the data. Data generated
889 by DNA-based identification will need to be transferred to the responsible authorities in a way that
890 allows for simple understanding of procedures, results and their uncertainties. Decisions based on
891 assessment results precipitate significant investment by the private and public sectors, and it is
892 essential that decision makers are provided with monitoring data that have been generated in a
893 transparent way.

894

895 **Conclusions**

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

906

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913

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		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
phytobenthos	rivers											N/A
phytobenthos	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											

1222

1223 Figure 1: Rating of the criteria for different BQEs and water categories. Large circles = high suitability

1224 of DNA-based identification; mid-sized circles = medium suitability; small circles = low suitability; N/A

1225 = not applicable. TRaC: Transitional and Coastal waters.

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