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Assessment

Review DNA metabarcoding Macrozoöbenthos

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1 Introduction

Macrozoöbenthos (further: macrobenthos) is crucial to assess the ecological quality of marine environments. It is a target group for both the Marine Strategy Framework Directive (MSFD 2008/56/EC) and the Water Framework Directive (WFD 2000/60/EC). Monitoring macrobenthos is part of the three-yearly MWTL monitoring program assigned by Rijkswaterstaat. Furthermore, macrobenthos is used to assess anthropogenic impacts like oil drilling, aquafarming, wind farms and sand supplementations (Verduin en Engelberts 2017), to detect non-indigenous species (Gittenberger et al. 2014, Gittenberger et al. 2017), or to evaluate nature conservation or restoration measures in Natura 2000 areas (Stuijfzand et al. 2014) or ports like Rotterdam (Borst & Vellinga 2012).

Identification of macrobenthos species requires a high level of taxonomic expertise, especially for larval stages and damaged specimens. Because this kind of expertise is rare and even diminishing in the Netherlands, nucleated at just a few laboratories and research institutes, processing samples takes a long time and is rather costly. A possible solution might be offered by DNA metabarcoding, an emerging methodology which holds the premise to identify species more efficiently and with less observer bias (e.g. Taberlet et al. 2012, Valentini et al. 2016, Deiner et al. 2017). With DNA metabarcoding, many species are identified at the same time. Up to 400 samples can be processed in a single run on a sequencing machine in less than four hours. Subsequently, the raw output data is processed and matched with reference libraries to determine the final species lists.

Although studies of DNA metabarcoding in freshwater environments are conducted for some years now, applications in marine environments are relatively recent and still scarce (Taberlet et al. 2018). International networks are formed to boost the knowledge of metabarcoding freshwater indicator groups like fish, macrofauna and diatoms (Leese et al. 2016), which addresses topics like biological sampling, lab processing, bioinformatics, biotic indices and implementation in policy and legislation. For the marine environment the potential benefits are probably the same as for freshwater (e.g Bourlat et al. 2013, Leray & Knowlton 2016, Wangensteen & Turon 2016, Goodwin et al. 2017). This report presents the current state of affairs for metabarcoding macrobenthos based on an extensive literature study. This report provides the foundation for a research proposal which aims to fill the gaps in our knowledge in order to get the eDNA methodology implemented in monitoring and assessment activities in the marine environment.

2 Biomonitoring studies

One of the first applications of DNA-based methods to detect marine animals in their environment was done by Deagle et al. (2003). They used a PCR-based approach to detect the DNA of *Asterias* larvae (starfish) in Tasmanian waters and ballast water. Until 2010, metagenomic studies were limited to microbial diversity (Dinsdale et al. 2008). Fonseca et al. (2010) and Chariton et al. (2010) expanded the concept of DNA metabarcoding to eukaryotes and meiofauna communities in marine sediments in the UK and Australia. Since these first breakthrough publications, the number of metabarcoding studies targeting marine metazoa communities remain rather scarce until 2015, emphasizing the still emerging character of this research field.

Since 2010, at least fourteen studies have been published which are relevant for DNA metabarcoding of macrobenthos (table 1). These studies targeted eukaryotes, metazoans or more specific species groups in marine environments. Three of these focussed on hard substrates by processing scrape samples collected from artificial fouling plates (Leray & Knowlton 2015, Cahill et al. 2018) or from diving exercises with hammer and chisel (Wangensteen et al. 2018). One study exclusively used water samples (Stat et al. 2017). The remaining nine studies used estuarine and marine sediments for metabarcoding, both for extracellular and/or microbial DNA (Guardiola et al. 2015, Chariton et al. 2015, Lejzerowicz et al. 2015, Guardiola et al. 2016, Aylagas et al. 2016, Pearman et al. 2016) and for bulk samples including the organisms (Chariton et al. 2010, Fonseca et al. 2010, Aylagas et al. 2016, Aylagas et al. 2018).

Eight studies compared results from DNA metabarcoding with results from conventional morphological methods. Only three studies identified taxa to the taxonomic level of species. In general, lower number of macrobenthos species are retrieved from DNA metabarcoding than from morphological identifications. Aylagas et al. (2018) identified 208 species morphologically. Of these, on average 20% were detected with DNA metabarcoding of bulk samples, using a universal CO1 marker. Across sampling stations, the DNA based detection frequency relative to morphological detection ranged from 0 to 66,6%. Lejzerowicz et al. (2015) found less than 20% of the morphologically identified taxa, but they processed sediment samples with an 18S marker. These percentages are in concordance with the results of Van der Hoorn et al. (2018) for a biodiversity baseline survey for the Amelander sand supplementation, based on extracellular DNA in sediment samples. They retrieved 23% of species compared to morphological identifications. Cahill et al. (2018) who failed to detect all morhophyla from fouling plates conclude therefore that metabarcoding is not yet able to replace morphological identification as a monitoring tool. They recommend the combined use of morphological and molecular approaches.

Possible explanations for the low recovery rates of morphospecies are the coverage of reference libraries, primer choice and primer-bias, suboptimal lab protocols and bioinformatics. These topics will be addressed in more detail later in this report.

Interestingly, both Aylagas et al. (2018) and Lejzerowicsz et al. (2015) compared values of biotic indices (AZTI Marine Biotic indices and the Infaunal Trophic Index) based on morphological data with values based on metabarcoding data. Both found a strong correlation despite the low percentage of retrieved species with DNA metabarcoding. Furthermore, many studies used metabarcoding successfully to detect spatial patterns in general (Pearman et al. 2016, Guardiola et al. 2016) or along ecological gradients (Fonseca et al. 2010) or along disturbance gradients (Chariton et al. 2010, Lejzerowicz et al. 2015). So despite the fact that not all species are found with DNA metabarcoding, this approach can nevertheless be sufficient for calculating biotic indices for ecological quality.

Table 1: relevant metabarcoding studies

Reference	Comparison Morphology	DNA type	Sample type	Target group
Chariton et al. 2010	No	Bulk	Estuarine sediment	Eukaryotes
Fonseca et al. 2010	No	Bulk	Marine sediment	Meiofauna
Guardiola et al. 2015	No	Sediment	Marine sediment	Eukaryotes
Leray & Knowlton 2015	No	Bulk	Scrape sample	Metazoans
Chariton et al. 2015	No	Sediment	Estuarine sediment	Eukaryotes
Lejzerowicz et al. 2015	Yes	Sediment	Marine sediment	Metazoans
Guardiola et al. 2016	No	Sediment	Marine sediment	Eukaryotes
Aylagas et al. 2016	Yes	Bulk; sediment	Marine sediment	Macrobenthos
Pearman et al. 2016	Yes	Sediment	Marine sediment	Eukayotes
Kelly et al. 2017	Yes	Water	Water; marine sediment	Eukaryotes
Stat et al. 2017	No	Water	Water	Eukaryotes
Wangensteen et al. 2018	No	Bulk	Scrape sample	Metazoans
Aylagas et al. 2018	Yes	Bulk	Marine sediment	Macrobenthos
Cahill et al. 2018	Yes	Bulk	Scrape sample	Benthos

3 DNA reference libraries

A prerequisite for reliable identification of species through DNA metabarcoding is a complete and reliable DNA barcode reference database. The two major publicly available databases are the Barcode Of Life Datasystem BOLD (Ratnashingham and Hebert 2007) and GenBank (Benson et al. 2013). BOLD is set up as a workbench and uses strict criteria for the quality assurance of accepted DNA barcodes, such as specific marker choices and their minimum length (CO1 for animals), a proper taxonomic name and the availability of a voucher specimen. GenBank deposits all DNA sequences from research projects. In general, the quality assurance is better for BOLD than for GenBank. The latter is richer in sequences, covering more species and genes. DNA sequences in GenBank that meet the criteria of BOLD are included at BOLD. Still, BOLD also contains misidentifications. These are sometimes enhanced by applying reversed taxonomic assignments, when voucher specimen are not morphologically identified *a priori*, but genetically *a posteriori*. In this case, DNA sequences of unknown taxa are compared with DNA barcodes already stored at BOLD, and then assigninged with the taxon name of the matching sequences. A self-reinforcing circle for misidentified specimen.

Because of the inaccuracies in these public databases many researchers create their own custom reference databases, applying strict quality control criteria on retrieved DNA barcodes. One drawback using this method is that you are only allowed to download DNA sequences which are public available, while there are many sequences not yet public available, depending on the choice made by the submitter. These are included in online search results for identification, but cannot be downloaded. For metazoa, a quality filtered subset for metazoa was published, the Midori database (Machida et al. 2017). The benefits of this initiative are not yet clear.

In some cases the publicly available DNA Barcodes in custom databases are supplemented with DNA Barcodes determined by the research institute which are not yet shared on BOLD or GenBank. In these cases, custom databases can be more complete and reliable than public databases for a specific field of research.

Focussing on the macrobenthos of the Dutch continental shelf, a survey of the availability of DNA Barcodes on BOLD for 1.100 marine species from the Dutch checklist of marine species (Bos et al. 2016) demonstrated that for 80% (885 species), one or more specimen were collected as voucher. For 73% percent (802 species), a reliable DNA barcode was submitted (Van der Hoorn et al. 2018). However, not all these barcodes are publicly available. A survey focussing on the 16.962 European species from the European Register of Marine Species (ERMS) demonstrated that 22% of these species have at least one DNA barcode present in BOLD (Fig. 2). Of these species, 26% have singletons and nearly 10% have five or more DNA Barcodes present (Weigand et al. 2019). Many authors stress the importance of a complete and reliable reference database for proper identifications in DNA metabarcoding studies (e.g. Günther et al 2018).

Naturalis is currently developing a custom database for marine species of the North Sea region, including recorded but not yet established species and expected non-indigenous species. Available sequences are downloaded from public databases and supplemented with DNA barcodes of specimen collected by naturalists on different expeditions (e.g Doggersbank expedition 2015, NICO expedition 2018), during specific projects (e.g. non-indigenous species surveys) and on dedicated diving events with amateur naturalists. Besides a database containing DNA barcodes from morphologically reliably and verifiably identified voucher specimen, the aim is to sequence the full mitogenome. These mitogenomic sequences will be used to develop custom made primers for specific groups of macrobenthos, and to study the deployment of mitogenomics in biomonitoring. Mitogenomics uses the full sequence of the mitochondria for comparisons with reference databases. The proposed custom database will be taxonomically curated at Naturalis and continually supplemented with new records from different research projects.

4 Primer choice

The choice for a marker (DNA metabarcode) and primer combination greatly influences the final number of detected species by metabarcoding. The most widely used marker to identify animals is the cytochrome c oxydase subunit 1 gene, in short CO1, located at the mitochondria. This is the original marker proposed at the very beginning of DNA barcoding activities (Hebert et al. 2003). There have been doubts on the accuracy and applicability of this marker for DNA metabarcoding studies with the years (Deagle et al. 2014). Still, some authors strongly advocate the use of CO1 as the community DNA metabarcode for animals because, among others, the unprecedented coverage of reference sequence databases for this gene (Andújar et al. 2018). They argue that the right primers for DNA metabarcoding CO1 have not yet been developed.

Focussing on macrobenthos species, the primers used for DNA barcoding (determining a DNA barcode from a single voucher specimen) and building up a DNA reference library were developed by Folmer et al. (1994). These primers amplify the total length of the CO1 marker of 658 base pairs. For DNA metabarcoding smaller markers are used under the assumption that extracellular DNA is highly degraded. However, most eDNA exists in at least intramembranous form (Turner et al. 2014), which means that using the complete CO1 gene for these kind of purposes shouldn't necessarily be a problem. Currently, the most widely used general primers for metazoa are the Leray primers (Leray et al. 2013) with a length of 313 base pairs, recently improved to Leray-XT primers (Wangensteen et al. 2018). The improvement consist of the addition of some degenerated base pairs, base pairs that match with every of the four complementary base pairs, with the result that these primers are less specific and amplify the DNA metabarcodes of more different macrobenthos species than the original Leray primers did (Wangensteen et al. 2018).

While these CO1 primers have proven themselves for many of the relevant marine taxa, some other taxa don't amplify equally well. For example, molluscs and tunicates are notoriously difficult to detect in metabarcoding studies (Cahill et al. 2018, Günther et al 2018). So CO1 might not always be the right marker of choice. Alternative 16S markers for dietary studies were developed for marine invertebrates such as cephalopods (Peters et al. 2014) and crustacea (Berry et al. 2017). Studies focussing on eukaryotes in general use different regions of 18S as DNA barcode or DNA metabarcode (Tsagkogeorga et al. 2009, Pochon et al. 2013, Guardiola et al. 2015, Stat et al. 2017). Although 18S seems to provide less accurate diversity estimates than CO1 (Tang et al. 2012) and provides less resolution at the species level (Wangensteen et al. 2018), a study on macrobenthos found species using 18S that were not detected by CO1 because reference barcodes of this gene were lacking (Lejzerowicz et al. 2015).

One universal primer to detect all macrobenthos species at once will be unlikely to be discovered. It is more likely that using a mix of primers directed towards specific macrobenthos groups ('primer cocktails') will yield the best results. Development and testing new and existing primers for specific species groups has the potential to increase metabarcoding efficiency for North Sea macrobenthos species. A recent example is a yet unpublished study at ILVO (Belgium) where 58% of macrobenthic species were recovered from bulk samples using CO1, while most of the unrecovered species were lacking in the reference database (pers. comm. Sofie Derycke, ILVO). This is considerably higher than the percentages reported so far from biomonitoring studies (this report) and therefore very promising.

Table 2: Overview of regularly used universal CO1 primers

Taxonomic group	Year	Marker	Length	FW name	RV name	Reference
Metazoa	1994	CO1	710	LCO1490	HCO2198	Folmer et al. 1994
Arthropods	2011	CO1	157	ZBJ-ArtF1c	ZBJ-ArtR2c	Zeale et al 2011
Metazoa	2013	CO1	313	mlCOIintF	dgHCO2198	Leray et al. 2013
Metazoa	2013	CO1	658	jgLCO1490	jgHCO2198	Geller et al. 2013
Macrofauna	2015	CO1	217	BF1	BR1	Elbrecht & Leese 2015
Macrofauna	2015	CO1	421	BF2	BR2	Elbrecht & Leese 2015
Metazoa	2018	CO1	313	mlCOIintF-XT	jgHCO2198	Wangensteen et al. 2018
Metazoa	2018	CO1	124	nsCOIFo	mlCOIintK	Günther et al. 2018

5 Sequencing platforms and depth

Currently, the Illumina MiSeq is widely used for DNA metabarcoding studies, with an output of 10M reads per sequencing run. However, this platform was developed for sequencing full genomes instead of amplified (and hence multiple occurring) DNA metabarcodes with attached primers. There is a risk of overclustering (merging) DNA metabarcodes caused by the limited genetic variation between them. To avoid overclustering, 30-50% control DNA has to be added to increase the variability. This decreases the effective output data to 5M reads, but increases the data quality.

Illumina also offers two alternative platforms, the Hiseq 4000 and the recently launched platform NovaSeq. These platforms yield a higher output than the MiSeq, resp. 10 billion and 20 billion reads. Having a higher output per sequencing run facilitates processing more samples in one run with the same sequencing depth (the number of reads per sample), or processing the same number of samples with a higher sequencing depth. In general, a higher sequencing depth leads to the detection of more rare species. In a very recently preliminary published study, a direct comparison was made between the MiSeq and the NovaSeq for analysing eDNA in seawater samples. With the NovaSeq, 40% more metazoan families were found than with the MiSeq (Singer et al. 2019). The authors advise a sequencing depth of 0.8 - 1 million reads per sample to detect all taxa, where the median from studies reviewed by them was only 60.000.

An exciting new development is the MinIon from Oxford Nanopore Technologies, a sequencer the size of a match box which is plugged into an USB-port of a computer. This device is already useful for sequencing single species for DNA Barcoding and whole genome sequencing in the field and has been deployed on expeditions in tropical rainforests (Pomerantz et al. 2018). The use for DNA metabarcoding is currently being explored. Critics mention that this device is not developed for amplicon sequencing (as used for DNA metabarcodes), just like the Illumina MiSeq. The error rate of determining basepairs is too high for reliable species identifications. However, the forthcoming R10 flow cell should reduce this to 1-2%, according to Nanopore (pers. comm. BaseClear). Furthermore, there are already workarounds that successfully reduce the errors by sequencing and clustering the same DNA metabarcode multiple times and merge them into a reliable consensus read. The MinIon has been applied in DNA metabarcoding projects to detect fish. The MinIon has the benefit that it can be easily taken on field trips, that results are generated real-time and ready in five hours (with a maximum of 48 hours) and that it is suitable to sequence longer reads (pers. comm. Reindert Nijland). The latter is especially useful for DNA metabarcoding of bulk samples where the DNA is not yet fragmented in small pieces.

6 Bioinformatics

There are several software packages available for processing the raw sequencing data from IonTorrent and Illumina platforms, every one of those packages comes with their pros and cons. An example of currently used pipelines and packages are QIIME (Caporaso et al. 2010), MOTHUR (Schloss et al. 2009), OBITools (Boyer et al. 2016) and USEARCH (Edgar 2010).

Although the basic workflows of the pipelines are generally quite similar, they use different tools that require different criteria to process and filter the data files. The choice of settings are depending on the type of tools and goals of the project. Every setting can affect the final output. Changing the parameters in a pipeline could lead to twice as much Molecular Operational Taxonomic Units (Clare et al. 2016) (MOTU = cluster of DNA reads with similar sequences not yet assigned to species). For researchers, it is important to understand the bioinformatic pipeline to fully understand how different settings influences the final data for a correct interpretation.

To illustrate the workflow of a general bioinformatic process, the custom pipeline of Naturalis is described below:

1. The output from a sequencing machine (a FASTQ file) is imported into the pipeline
2. As sequences progresses during the sequencing process, the quality of the sequence diminishes. Bad quality ends of sequences are trimmed with either Sickle (Joshi & Fass 2011) or PRINSEQ (Schmieder & Edwards 2011)
3. Sequences come in two parts (forward and reverse) and are merged into DNA reads with FLASH (Magoč & Salzberg, 2011).
4. The primers for amplification, still attached to the targeted marker, are trimmed from the DNA reads with Cutadapt (Martin, 2011).
5. DNA reads that don't meet certain quality criteria or length criteria are removed with PRINSEQ (Schmieder & Edwards 2011).
6. DNA reads are dereplicated with VSEARCH (Rognes et al. 2016).
7. DNA reads are clustered to MOTUs, depending on the goal, with USEARCH UNOISE (Edgar 2016), VSEARCH (--cluster_size) or VSEARCH (--cluster_unoise)(Rognes et al. 2016), USEARCH UPARSE (Edgar 2013) or DADA 2 (Callahan et al. 2016) . Chimeras (wrong DNA reads as a result of recombinations) are removed at the same time.
8. DNA reads are mapped back on the MOTUs to create a MOTU table with VSEARCH (Rognes et al. 2016)
9. DNA reads are assigned to taxa using BLAST (Camacho et al. 2009), or with a custom developed 'lowest common ancestor' script (which determines the next taxonomic level when the highest level, like species level, cannot be determined).

Depending on the settings, in every step of the workflow DNA reads are removed and species might get lost. The output of any DNA metabarcoding pipeline is a matrix, with samples as columns and species or MOTUs as rows. The cells contain the number of reads per sample per species or MOTU. This is very similar as the abundance matrices from conventional sampling.

7 Filtering species matrices

The resulting species matrix contains species (or unidentified numbered MOTUs) and their DNA read abundances across different samples. Species with a relatively low number of reads in a sample could be genuine rare species. But their presence could also be caused by cross-contamination from other samples in the same PCR or sequencing run. To diminish the chance of false positives in samples, a threshold for a minimum number of DNA reads per species per sample is generally applied. There are several ways how to deal with this, which are recently thoroughly discussed with partners at Naturalis.

There is a general consensus to remove singletons from samples, which means species with an abundance of only one DNA read. Furthermore, there is an increasing number of studies where species that contribute less than 0,02% to the total number of reads of the sample are removed.

There are more complicated solutions and algorithms to apply. The incorporation of a positive control (adding an extra sample containing DNA of a foreign species) could be used to estimate the amount of cross-contamination between samples and hence the contamination threshold. One could argue for a minimum threshold relative to the total number of reads of the sample (a higher number of sample reads leads to higher read numbers for every individual species in that specific sample) or relative to the total number of reads per species (a higher number of species reads in a sample could lead to a higher number of contaminated reads in an adjacent sample). Ideally, both these amounts should be taken into account to determine a minimum threshold.

Another solution is to develop a contamination model to correct for contamination. Naturalis analysed the cross-contamination and the biggest chance was, as expected, between adjacent samples. But this contamination only occured horizontal and vertical, diminishing from the source onwards. DNA is volatile and contamination arises during the pipetting process when sample specific labels (indexes) are added, to assign DNA reads to the correct samples during the downstream bioinformatic analysis. This results in ‘index-switching’, where a small amount of the index is added to a wrong sample, leading to assigning errors (species in sample A assigned to sample B) further downstream in the bioinformatic process. Larsson et al. (2018) developed a correction model for this issue based on positive controls. We adapted and implemented this model in our bioinformatic pipeline.

8 Abundance

The indices for the Marine Strategy Framework Directive and Water Framework Directive in the Netherlands to assess ecological quality of marine environments make use of abundance data (Van Loon et al. 2015, Wijnhoven & Bos 2017), see below. However, determining species abundance or biomass based on DNA read abundance is a hot topic in research. Only a few studies focus on marine benthic species and DNA abundance, so we included results from aquatic studies in general (table @@).

Most reviewed studies deal with eDNA from water samples to detect and quantify single species using a qPCR or ddPCR machine. For fish and amphibians there appear to be a positive correlation between DNA concentrations and abundance or biomass (e.g. Doi et al. 2016, Klymus et al. 2014, Pilliod et al. 2013). For crayfish the results are varying (Dougherty et al. 2016, Cai et al. 2017), the relation between DNA and abundance might only be measurable when females are ovigorous (Dunn et al. 2017). For insects, positive correlations with biomass were found for stoneflies (Elbrecht & Leese 2015) and with abundance for water fleas (Trimbos et al. 2019 submitted).

Also for DNA metabarcoding, detecting multiple species at the same time using eDNA from water samples and Next Generation Sequencing, positive correlations between DNA reads and (relative) abundance or biomass were found (Kelly et al. 2014, Thomsen et al. 2016, Hänfling et al. 2016). For marine benthic species and freshwater macroinvertebrates, DNA metabarcoding of bulk samples was applied instead of water samples. In a mock community of known composition, the number of DNA reads of species of equal biomass differed some orders of magnitude (Elbrecht & Leese 2015). However, for marine benthic species from natural samples, there was a positive correlation between DNA reads and relative abundance (Leray & Knowlton 2015) or biomass (Aylagas et al. 2018).

Although there are some promising results for estimating (relative) abundance and biomass based on DNA read numbers or DNA concentration, some of the current indices appear not even to be sensitive to abundance, and function almost as good with presence/absence (p/a) data instead. For freshwater indices used for the Water Framework Directive in the Netherlands, a simulation of data demonstrated there was a clear relationship between the water quality scores based on p/a compared to abundance (Beentjes et al. 2018). The same results were drawn for two marine indices used in Southern California, the Southern California Benthic Response Index (BRI) and the AZTI Marine Biotic Index (AMBI) (Ranasinge et al. 2012). The effect of a transition from abundance to p/a has not yet been tested on indices for transitional and marine waters in the Netherlands.

Table 3: overview abundance studies

Reference	Realm	Method	Species (group)	Substratum	Conclusion
Takahara et al. 2012	Freshwater	Single species	Fish *Cyprinus carpio*	Water samples	Positive correlation DNA reads and biomass
Thomsen et al. 2011	Freshwater	Single species	Amphibians *Pelobates fuscus* and *Triturus cristatus*	Water samples	Positive correlation DNA concentration and population density
Pilliod et al. 2013	Freshwater	Single species	Amphibians *Ascaphus montanus* and *Dicamptodon aterrimus*	Water samples	Positive correlation DNA concentration, population density and biomass
Goldberg et al. 2013	Freshwater	Single species	Mollusk *Potamopyrgus antipodarum*	Water samples	Positive correlation DNA concentration and population density
Kelly et al. 2014	Marine	Multiple species	Fish	Water samples	Positive correlation rank abundance DNA reads and rank abundance biomass
Klymus et al. 2014	Freshwater	Single species	Fish *Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*	Water samples	Positive correlation DNA concentration and biomass
Elbrecht & Leese 2015	Freshwater	Single species	Stonefly *Dinocras cephalotes*	Bulk samples	Positive correlation DNA reads and biomass specimen
Leray & Knowlton 2015	Marine	Multiple species	Benthos	Bulk samples	Positive correlation DNA reads and relative abundance
Elbrecht & Leese 2015	Freshwater	Multiple species	Macroinvertebrates	Bulk samples	No correlation between DNA reads and biomass
Thomsen et al. 2016	Marine	Multiple species	Fish	Water samples	Positive correlation DNA reads, abundance and biomass
Yamatoto et al. 2016	Marine	Single species	Fish *Trachurus japonicus*	Water samples	Positive correlation DNA concentration and biomass
Evans et al. 2016	Freshwater	Multiple species	Fish and amphibian	Water samples	Positive correlation between DNA reads and abundance
Lacoursière-Roussel et al. 2016	Freshwater	Single species	Fish *Salvelinus namaycush*	Water samples	Positive correlation DNA concentration and relative abudance
Hänfling et al. 2016	Freshwater	Multiple species	Fish	Water samples	Positive correlation DNA reads and rank abundance
Dougherty et al. 2016	Freshwater	Single species	Crayfish *Orconectes rusticus*	Water samples	Poor correspondence DNA reads and relative abundance
Doi et al. 2017	Freshwater	Single species	Fish *Plecoglossus altivelis*	Water samples	Positive correlations DNA concentration, abundance and biomass
Dunn et al. 2017	Freshwater	Single species	Crayfish *Pacifastacus leniusculus*	Water samples	Positive correlation DNA concentration and biomass of ovigerous females
Cai et al. 2017	Freshwater	Single species	Crayfish *Procambarus clarkii*	Water samples	Positive correlation DNA concentration and abundance
Aylagas et al. 2018	Marine	Multiple species	Macrobenthos	Bulk samples	Positive correlation DNA reads and biomass
Günther et al. 2018	Marine	Multiple species	Metazoa	Water samples	No correlation number of DNA reads and DNA concentration
Trimbos et al. 2019 submitted	Freshwater	Single species	Water flea *Daphnia magna*	Water samples	Positive correlation DNA reads and abundance

9 Ecological indices

To implement metabarcoding in routine biomonitoring, the first step might be the integration of DNA metabarcoding data into existing biotic indices (Pawlowski et al. 2018). For the Netherlands, the most relevant indices for biomonitoring and impact assessments based on macrobenthos are the Bentic Indicator Species Index (BISI) (Wijnhoven & Bos 2017) and the Benthic Ecosystem Quality Index 2 (BEQI-2) (Van Loon et al. 2015).

The BISI is developed for implementing the Marine Strategy Framework Directive (MSFD 2008/56/EC) in the Netherlands. It is recently applied for the ecological quality assessment of seafloor habitats of the Dutch continental shelf (Wijnhoven 2018). It makes use of observed abundance data of indicator species compared to a reference. No efforts have been made to apply genetic data and/or test the actual influence of (relative) abundance data, which is, arguably, available from a metabarcoding approach.

BEQI-2 is developed for implementing the EU Water Framework Directive (WFD 2000/60/EC) in the Netherlands, which includes transitional and coastal waters. The output is an Ecological Quality Ratio Score (EQR-score). At first, a Benthic Ecosystem Quality Index (BEQI) was developed for the Netherlands and Belgium (Van Hoey et al. 2007). After intercalibration, this index appeared quite different from other EU countries and did not fulfil the WFD criteria (Boon et al. 2011). The index was modified to BEQI-2 and further applied for assessments of benthic invertebrates for the WFD (Van Loon et al. 2015). BEQI-2 combines three indicators: species richness (S), Shannon diversity (H') and AMBI. Shannon diversity and AMBI both use abundance data. Just like BISI, this indicator makes use of reference data.

For freshwater (lentic waters, lotic waters and artificial waters), the influence of abundance data on the outcomes of the EQR-scores have been tested. Replacing abundance data with presence/absence data in the index formulas for the three different water types (Evers et al. 2012, Van der Molen et al. 2016) resulted in almost 90% of the waters being assigned to the same quality class as with abundance data, and less than 10% of the waters shifted to a higher quality class (Beentjes et al. 2018). This indicates that the EQR-scores might still be applicable when p/a data from metabarcoding is used. But this has not yet been tested for transitional and coastal waters which uses a different formula to calculate the BEQI-2 index.

For DNA-based species lists, another relevant index is the genetic version of AZTI's Marine Biotic Index (gAMBI) (Aylagas et al. 2014). The gAMBI is a modified version of the original AMBI (Borja et al. 2000) that makes use of two derived indices: the p/a AMBI based on presence/absence data (Warwick et al. 2010) and the (B)AMBI based on biomass (Muxica et al. 2012). The original AMBI is a widely applied index which uses benthic indicator species and their sensitivity or tolerance to an environmental stress gradient (Borja et al. 2000). Because it is part of the BEQI-2 index to calculate EQR-scores for Dutch transitional and coastal waters, the sensitivity of benthic indicator species have been determined for local environmental and anthropogenic pressures (Gittenberger en Van Loon 2013). A recent study on adapting AMBI to metabarcoding-based monitoring with the p/a AMBI and (B)AMBI (Aylagas et al. 2018) resulted in a strong correlation between the original AMBI based on morphological data and abundances, p/a AMBI based on presence/absence from metabarcoding data, and (B)AMBI based on read abundances from metabarcoding data. These first results are in support of the application of metabarcoding to monitor ecological quality based on macrobenthos.

10 Conclusions and recommendations

This review demonstrates that DNA metabarcoding is a promising methodology for the detection and identification of macrobenthos species. However, despite some scientific studies, it has not yet been widely applied and validated for macrobenthos monitoring, neither national nor international. Whether the methodology is already operational depends on the research goals. It does identify many species, but still less than conventional monitoring. The species assemblages are different, yet representative. Even these different species assemblages could be sufficient to analyse the ecological quality of an ecosystem, or the impact of environmental change. The accuracy of detecting macrobenthos species could be improved by conducting some laboratory and field tests, and some further developments of primers. Therefore, we do have the following recommendations:

- DNA reference library. Genetic identification is only possible when a reliable reference is available for the marker used in the respective study. Markers are in flux so the best option would be to develop a reference with full mitogenomic sequences, covering all current popular marker regions as CO1, 12S and 16S, and future markers from genes on the mitogenome. An additional benefit would be that the mitogenomes could be used for functional trait analysis, explaining the role of species in their environment. Naturalis is currently collecting as many macrobenthos species as possible from the North Sea region to build this reference.

- Primer development. Although current primers have proven to cover a broad range of marine macrobenthos species in several studies, they should be tested specifically for North Sea species for two reasons: 1) to ensure that these primers match the DNA of all North Sea species, ii) to ensure that the targeted marker is able to identify all species, in other words, is sufficiently species specific for all North Sea species. Since the genetic identification of at least some groups seem to lack behind traditional morphological identifications, we expect to improve the success ratio by developing primers for specific species groups instead of using more universal primers. This could also lead to less bycatch (non-target species) and a higher data efficiency.

- Lab protocols and bioinformatics. Especially rare species can be hard to detect, both in conventional monitoring and in genetic monitoring. It is not always necessary to find all species if one is interested in discovering ecological patterns. However, most indices rely on species lists and detecting as many species as possible often strengthen the outcomes of indices or inferences on ecological quality. Detecting rare species could be improved by 1) increasing the number of PCR replicates, 2) increasing the sequencing depth, aiming at more DNA reads per samples (less samples on a sequencing run), 3) the choice of sequencing machine (NovaSeq instead of MiSeq), and 4) adequate settings of bioinformatics including optimized contamination models.

- Field test. If the previous bullets have been (partly) fulfilled by DNA (meta)barcoding projects, and some simple ring tests and lab tests with mock communities (samples with known species composition) have been conducted, the final step would be to set up field pilots. In this field pilots the results of conventional and genetic biomonitoring should be compared. Preferably, this would be a real monitoring or ecological impact assessment programme, where both the species lists are compared as the outcomes of ecological indices used.

- Ecological indices. Both BISI and BEQI-2 use abundance data and reference data which is lacking for DNA-based methods. One option is to analyse the incorporation of abundance data and transform these current indices, e.g. by using presence/absence data or rank abundance data. Another option is to use newly developed DNA-based indices and analyse how the outcomes relate to the conventional indices. The gAMBI might be a serious alternative to consider, on its own or incorporated in multi metric indices with species richness and/or rank abundances. This should be further explored.

11 Literature

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