Science of the Total Environment 946 (2024) 174106

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Comparative study of traditional and DNA-based methods for environmental impact assessment: A case study of marine aggregate extraction in the North Sea

Van den Bulcke Laure [a,*], De Backer Annelies [a], Hillewaert Hans [a], Maes Sara [a], Seghers Stephie [a], Waegeman Willem [b], Wittoeck Jan [a], Hostens Kris [a], Derycke Sofie [a,c]

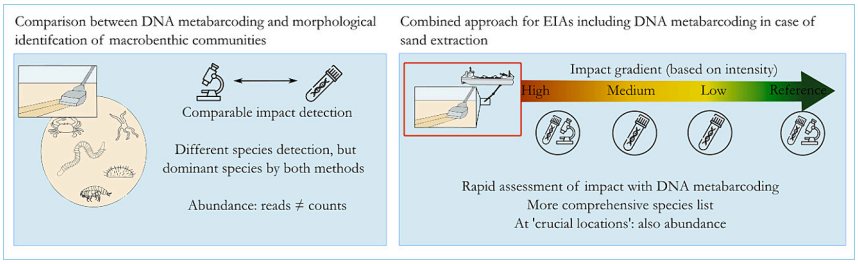
[a] Flanders Research Institute for Agriculture, Fisheries and Food – Animal Science Unit, Jacobsenstraat 1, 8400 Oostende, Belgium
[b] University of Ghent, Department of Data Analysis and Mathematical Modelling: Knowledge-based Systems Research Group, Coupure Links 653, 9000 Gent, Belgium
[c] University of Ghent, Department of Biology: Marine Biology Research Group, Krijgslaan 281, 9000 Gent, Belgium

HIGHLIGHTS

- Bulk DNA metabarcoding and morphology detect similar diversity patterns in macrobenthic communities related to sand extraction.
- Both methods show disparities in species detection.
- Bulk DNA metabarcoding is faster and cheaper than morphology-based identification of macrobenthos.
- An combined monitoring design for sand extraction maximizes the strengths of bulk DNA metabarcoding and morphological identification.

GRAPHICAL ABSTRACT

Bulk DNA metabarcoding and morphology can be seen as complementary methods. Both methods detect changes from sand extraction, but species identification differs. A combined monitoring design maximizes the strengths of both methods: a fast detection by bulk DNA metabarcoding, and quantitative data and detailed information on life stages and size by morphological identification.



ARTICLE INFO

Editor: Daniela Maria Pampanin

Keywords:
Genetics
COI
Macrobenthos
Metabarcoding
Monitoring, marine

ABSTRACT

Environmental impact assessments of marine aggregate extraction are traditionally conducted based on morphological characteristics of macrobenthos, which is time-consuming, labour-intensive and requires specific taxonomic expert knowledge. Bulk DNA metabarcoding is suggested as a promising alternative. This study compares the traditional morphological and the bulk DNA metabarcoding method to assess the impact of sand extraction activities on three sandbanks in the Belgian North Sea. Substantial differences in the detected species were observed between methods: Abundant and/or large macrobenthos species were detected by both methods, while small species or species with an exoskeleton were usually only detected by the morphological method. Taxa uniquely detected by bulk DNA metabarcoding could be explained by specimens identified at a higher taxonomic level by morphology, or by specimens with very low read numbers, probably representing species missed in the morphological sorting process, DNA traces on the specimens or false positives during PCR amplification efficiency. Despite the difference in detected species, comparable alpha and beta diversity patterns were observed by both methods, indicating that bulk DNA metabarcoding can effectively detect the overall ecological changes associated with sand extraction. We further demonstrate that bulk DNA metabarcoding

* Corresponding author.
E-mail address: laure.vandenbulcke@ilvo.vlaanderen.be (V. den Bulcke Laure).

https://doi.org/10.1016/j.scitotenv.2024.174106
Received 9 January 2024; Received in revised form 11 June 2024; Accepted 16 June 2024
Available online 21 June 2024

reduces sample processing both in time (44 % faster) and cost (26 % cheaper) compared to the morphology-based identification. However, biomass quantification remains challenging for bulk DNA metabarcoding since of the ten most abundant genera, only two genera (*Echinocardium* and *Ophelia*) showed a significant positive correlation between biomass and read numbers. Additionally, bulk DNA metabarcoding does not provide information on life stages or size of the identified specimens. As such, our results underpin the complementary nature of both methods, wherein DNA-based analyses allow for rapid detection of community changes (as similar patterns in alpha and beta diversity and biotic index were observed), while morphology-based analyses provide additional information on e.g. secondary production (biomass) and size composition. We show how the strengths of both methods can be combined to assess the impact of sand extraction.

1. Introduction

Aggregate extraction activities in the North-East Atlantic have expanded during the last 40 years (ICES, 2019) and are expected to further increase because of the higher demand for sand and gravel for concrete, offshore wind or other purposes. Extraction removes sediment, causing physical disturbance of the seabed by the draghead and redeposits material through screening and overflow, all potentially resulting in changes to the seabed morphology and sediment composition (Cooper et al., 2011). These sedimentary changes also affect benthic communities living in the seabed (Cooper, 2013; De Jong et al., 2015; Wyns et al., 2021). Therefore, macrobenthos is often used as an indicator to assess the environmental impact of human activities, such as sand extraction (Van Hoey et al., 2010).

The impact of extraction varies depending on site-specific characteristics such as seabed geology and hydrodynamics, as well as the resilience and recovery potential of the local fauna (Cooper et al., 2011; Foden et al., 2009). Moreover, extraction intensity and extraction frequency have also proven to be driving factors (Wyns et al., 2021). Some studies have reported a decrease in benthic diversity following sand extraction activities (Cooper et al., 2007), while others have observed an increase in diversity (De Backer et al., 2014b; Wyns et al., 2021). In addition to structural benthic biodiversity, sand extraction is also known to impact functional diversity (Festjens et al., 2023) and functional traits of the community (Goedefroo et al., 2023). To minimize the impact of sand extraction on the marine environment and ensure the sustainable use of aggregates, most countries have a legally obliged environmental monitoring programme in place to allow for environmental impact assessments (EIAs) in accordance with Europe's EIA Directive (Directive 2011/92/EU).

Traditionally, EIAs are based on morphological identification of the benthic species present in a grab sample, often preserved in formalin. This method is time-consuming and labor-intensive and requires specific taxonomic expert knowledge. Cryptic species, challenging taxonomic groups e.g. Nemertea and Oligochaeta, damaged specimens missing diagnostic parts and juveniles are often difficult to identify to species level based on morphological characteristics (Elbrecht and Leese, 2015). DNA metabarcoding is seen as a fast and cheaper alternative (Aylagas et al., 2016; Cowart et al., 2015; Leray and Knowlton, 2015). Provided that a good reference database is available, DNA metabarcoding may detect more species than the traditional monitoring method (Cahill et al., 2018). Several studies have already shown the applicability of metabarcoding to assess the impact of human activities for different anthropogenic pressures (Aylagas et al., 2018, 2014; Cowart et al., 2015; Duarte et al., 2021; Lobo et al., 2017). Moreover, a European ringtest has proven that bulk DNA metabarcoding of macrobenthos is reproducible and robust when inferring diversity patterns (Van den Bulcke et al., 2023). Despite these advantages, the implementation of DNA-based techniques into existing monitoring programmes of marine macrobenthos remains limited.

Remaining knowledge gaps could hamper the widespread adoption of bulk DNA metabarcoding. First, impact studies often rely on species abundance and biomass, but only relative abundance in terms of read numbers are available with DNA metabarcoding. As such, it is not known if a high read abundance is associated with a high number of individuals or due to a higher amount of extracted DNA (e.g. because of larger specimens) and/or as a consequence of PCR bias (e.g. higher primer efficiency for certain species) (Steyaert et al., 2020). To our knowledge, no discernible relationship between read abundance and the species count has been observed up to now (Hollatz et al., 2017; Lobo et al., 2017), while for biomass a (weak) positive correlation with relative read abundance has been reported (Elbrecht and Leese, 2015). However, Klunder et al. (2022) highlighted the lack of a consistent correlation between read abundance and biomass as only one of the six tested annelid taxa in the study showed a correlation between biomass and relative read abundance. This discrepancy underscores the complexity of the relationship between DNA metabarcoding read numbers and biomass in marine macrobenthos.

A second knowledge gap that may hamper adoption of DNA-based monitoring is linked to the absence of empirical data on the time and cost savings accompanied with metabarcoding or morphology-based identification of macrobenthos samples. Finally, a shift towards DNA-based monitoring would, for a number of countries, also require a shift from formalin, as suggested in the guidelines for quantitative sampling and sample processing of marine soft bottom macrofauna (ISO 16665:2014), to ethanol as a fixative. Ethanol dehydrates animals and makes them more fragile (Wittoeck, pers. comm.). Consequently, smaller species may dissolve more readily in ethanol and some taxa (e.g. amphipods) may become rigid and more susceptible to damage, which may complicate species identification and affect the resulting species composition.

In this study, DNA-based monitoring was run in parallel with the traditional morphology-based environmental monitoring for sand extraction in the Belgian part of the North Sea (BPNS). Three different sand banks with varying extraction volumes and frequencies were sampled. We investigated whether bulk DNA metabarcoding of macrobenthos samples detected comparable alpha diversity (species richness and Shannon index), beta diversity (assemblage patterns based on Bray-Curtis dissimilarity), and biotic index (BEQI) results as morphology-based analyses. Second, we studied the similarities and differences in detected species by both methods. Third, the effect of a different fixative (ethanol instead of formalin) on morphological species detection was studied and compared with bulk DNA metabarcoding identification. Fourth, we examined whether read abundance of the dominant genera correlated with biomass and density of morphology-based identifications of these genera. Fifth, we tracked the time and costs of processing benthic samples with both bulk DNA metabarcoding and traditional identification to investigate which of both methods was most time and cost-effective. Lastly, we discuss how the strengths of both methods can be combined, and we formulate recommendations for an optimal monitoring programme to assess the impact of sand extraction activities in the BPNS and beyond.

2. Material and methods

2.1. Study area, sample collection and sample impact allocation

Sand extraction in the Belgian Part of the North Sea (BPNS) is

currently restricted to five concession zones, some with different sectors (Royal Decree of May 22, 2019) (Fig. 1). This study focuses on three sand extraction areas situated on the Thorntonbank, the Oostdyck and the Hinderbanken (Fig. 1). The Thorntonbank experiences a high and continuous extraction rate, the Hinderbanken experiences a high extraction rate clustered in time, while a continuous but low extraction intensity occurs at the Oostdyck (see Wyns et al. (2021) for information on extraction history of these areas).

On each sandbank, macrobenthos was collected by means of a Van Veen grab with a sampled surface area of 0.1 m^2, both within the impact areas (subjected to extraction activity) and in nearby reference areas (where no extraction has taken place). In 2019, a total of 78 locations were sampled, and in 2021, 65 locations on the three different sandbanks were sampled (Fig. 1, Table 1). Sampling was performed in September, October or November using different research vessels (RV Belgica 1965, RV Simon Stevin, GeoSurveyor XI or Geo Ocean V) (Table 1). Real-time coordinates of all sampled locations were noted. In 2019, two Van Veen grabs were sampled per location (for bulk DNA metabarcoding and for traditional morphology-based processing separately). Each sample was sieved alive over a 1-mm sieve, and the residue with the species was preserved in absolute ethanol and stored at −20 °C (for bulk DNA metabarcoding) or fixed in an 8 % formaldehyde-seawater solution stained with eosin and stored at room temperature (for traditional morphology-based processing) (Table 1). In 2021, one Van Veen grab was sampled per location, sieved alive and the residue was preserved in absolute ethanol and stored at −20 °C for both bulk DNA metabarcoding and traditional morphology-based processing (Table 1).

Sand extraction intensity at each sample location was calculated based on the Electronic Monitoring System (EMS)-derived extraction intensity data as the extracted volume in a 50 m buffer around the location (approximately 7850m^2 surface area) in the year prior to sampling (see Wyns et al. (2021) for a more detailed description). As such, samples could be divided into four impact groups: high intensity: > 2000 m^3 extracted, medium intensity: 500–2000 m^3, low intensity: < 500 m^3 and reference (without extraction activity): 0 m^3. These boundary thresholds are based on local expert judgement, as there is a long history of ecological impact evaluation for sand extraction in Belgium (De Backer et al., 2014b; Goedefroo et al., 2023; Wyns et al., 2021).

2.2. Sample processing and morphological identification

All samples (both formalin and ethanol preserved) were decanted 10 times using tap water and a 1 mm sieve in the lab. The specimens on the sieve were stored in absolute ethanol (for samples previously fixed in ethanol) or in disolol (=ethanol denaturated with eurodenaturant; for samples previously fixed in formalin). The remaining (mainly heavier) specimens in the decanted residue were manually picked and added to the decanted specimens.

All decanted specimens from the formalin preserved 2019 samples (n = 78), the 2019 ethanol preserved Thorntonbank samples (n = 24),

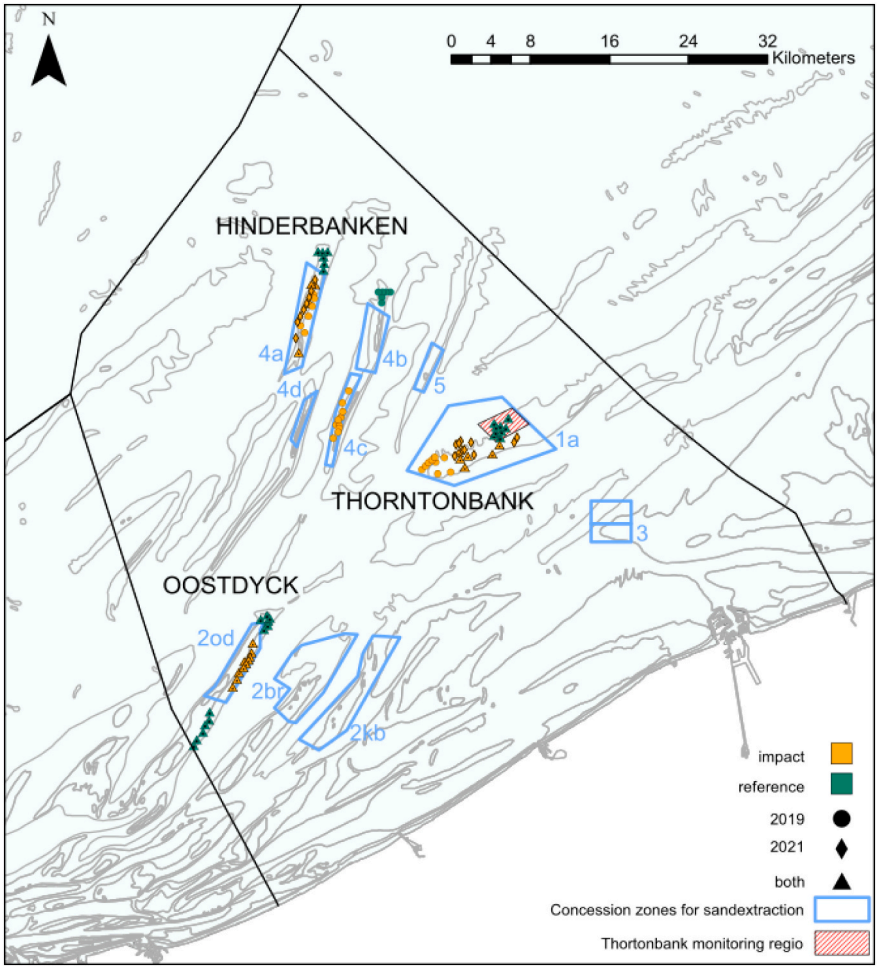


Fig. 1. Map of Belgian Part of the North Sea (BPNS) with the five sand extraction concession zones (blue lines) and the used sampling locations for this study in three sand extraction areas (Hinderbanken, Oostdyck, Thorntonbank) in 2019 and 2021. The sampling year was displayed by different symbols and the impacted and reference samples by a different color.

Table 1
Overview of the number of samples in each sandbank and impact group, with sampling information like sampling year, month and fixative.

Sandbank	Sampling year	Sampling month	Sampling locations for				Fixative of the grabs
			Reference (0 m^3)	Low intensity (<500 m^3)	Medium intensity (500–2000 m^3)	High intensity (>2000 m^3)	
Hinderbanken	2019	September	12	14	6	/	1 on formalin and 1 on ethanol
	2021	November	6	/	3	9	1 on ethanol
Oostdyck	2019	September	12	4	2	4	1 on formalin and 1 on ethanol
	2021	September	12	5	1	4	1 on ethanol
Thorntonbank	2019	September	9	5	4	6	1 on formalin and 1 on ethanol
	2021	October	9	5	5	6	1 on ethanol

and all 2021 ethanol preserved samples (n = 65) were identified morphologically under a stereomicroscope. Similar as for traditional monitoring, all individuals were identified to the lowest taxonomic level if possible (except for species belonging to Nemertea, Anthozoa and Oligochaeta) and counted. Biomass for each species per sample was measured by blotting all individuals of that species on absorbent paper before weighting to the nearest 0.01 mg (g Wet Weight). The density and biomass were calculated as counts and weight per m^2, respectively. The ethanol preserved samples were processed with caution to prevent DNA degradation (e.g. limit the time outside the ethanol fixative when weighting) and contamination (e.g. cleaning of material between samples with DNA Away; Thermo Scientific). Morphological identifications were conducted within maximum six months after sampling. The decanted specimens from the 2019 ethanol preserved samples from Oostdyck and Hinderbanken were not morphologically identified. A detailed overview of sampling processing for each sample can be found in ESM 1.

2.3. Library preparation for bulk metabarcoding DNA identification

All ethanol-fixed samples were immediately (for samples of Oostdyck and Hinderbanken from 2019) or after morphological identification (for samples of Thorntonbank from 2019 and all samples from 2021) grounded with a mortar and pestle for bulk DNA metabarcoding identification until homogeneous "soups" were obtained in all samples. The mixed bulk specimen samples were processed using the same GEANS laboratory protocol, developed for metabarcoding of soft sediment macrobenthos of the North Sea (GEANS 2021). In short, three subsamples of two mL were taken from the mixed bulk specimens samples for DNA extraction ($n = 234$ in 2019, $n = 195$ in 2021) and the resulting three DNA extractions were pooled per sample ($n = 78$ in 2019, $n = 65$ in 2021). UltraPure DNase/RNase-Free Distilled water (Thermo Fisher Scientific) instead of the mixed bulk specimen aliquot was added to the DNA negative control samples (six in 2019, four in 2021). PCR amplification was conducted in triplicate, using the miCOIintF/jgHCO2198 primer set which targets a 313 bp fragment of the mitochondrial COI gene (Leray and Knowlton, 2015). For each PCR plate, a negative control was conducted, adding UltraPure DNase/RNase-Free Distilled water (Thermo Fisher Scientific) instead of the DNA extract. The three PCR products were pooled for each sample (n = 78 in 2019, n = 65 in 2021), cleaned with CleanNGS beads (CleanNA) and an index PCR was performed with the Nextera kit set v2 (Illumina). The cleaned index PCR products were equimolarly pooled and the library was sent to a sequencing facility (Admera Health Biopharma Services). The two libraries (library one with 78 samples + seven negative controls in 2019 and library two with 65 samples + five negative controls in 2021) were sequenced on separate MiSeq runs (2 × 250 bp).

2.4. Processing of raw reads and taxonomic identification for the bulk DNA metabarcoding derived samples

The processing of raw reads was done separately for the 2019 and 2021 dataset in R v4.0.2 (R Core Team, 2020). After checking the quality of demulitplexed reads using MultiQC (Ewels et al., 2016), primers were removed with Trimmomatic (Bolger et al., 2014). The Dada2 pipeline was used to generate amplicon sequence variants (ASVs) with the Dada2 v1.26.0 package (Callahan et al., 2016). Reads with quality scores below 30 were trimmed, and unique reads in each sample were determined and merged. Chimeras were removed using the removeBimeraDenovo function and the resulting total number of reads in each filtering step was calculated for both datasets (2019 and 2021). With the decontam package v1.18.0 (Davis et al., 2018), contaminant ASVs were removed from the datasets using the "prevalence" method (threshold = 0.5), which flags an ASV as a contaminant when its occurrence is higher in the negative control samples than in the true samples. Using the assignTaxonomy function, based on the Ribosomal Database Project (RDP) (Wang et al., 2007), taxonomy was assigned to the reads with standard settings, except for the bootstrap confidence parameter, which was set to 80 instead of 50. A preliminary version (version 4) of the GEANS reference database was used, containing 1993 COI sequences from 565 marine invertebrates in the North Sea. The unassigned ASVs were blasted against the NCBI nucleotide database (release 254) to get taxonomic identification of species not present in the GEANS reference database. Taxonomic assignments were validated against the World Register of Marine Species (WoRMS) and the additional assignments were filtered for macrobenthos. In downstream analyses, only ASVs with a minimum taxonomic assignment on phylum level were retained, resulting in a taxonomy on phylum, class, order, family or genus level.

Samples were rarefied at 10000 reads for samples of 2019 and at 13000 reads for samples of 2021, obtaining a threshold between reaching the plateau of the rarefaction curve and limiting the number of removed samples (ESM 2). For 2019, 15 samples with <10,000 reads were removed (12 from Hinderbanken, two from Oostdyck and one from Thorntonbank), while only one sample of the Thorntonbank was removed in the dataset of 2021.

2.5. Alpha diversity analyses for morphologically and bulk DNA metabarcoding derived data

Based on both the bulk DNA metabarcoding and morphologically derived datasets, alpha diversity was assessed by calculating the species richness (number of taxa, based on lowest taxonomy available = S) and the Shannon index (H) using the diversity function of the vegan package v2.6.4 (Dixon, 2003). The morphologically derived dataset consisted of the morphologically identified samples fixed in formalin for 2019 ($n = 78$) and the morphological identified samples fixed in ethanol in 2021 ($n = 65$) (so the samples fixed in ethanol of the Thorntonbank in 2019 were not included). To lower the effect of high abundant reads and species in the Shannon index, a square root transformation was performed on the reads (bulk DNA metabarcoding) and counts (morphology) before calculating Shannon indices. The diversity was visualized in boxplots using the ggplot2 package v3.4.0 (Wickham, 2016).

To explore potential differences in S and H between bulk DNA metabarcoding and morphology across diverse aggregate extraction impact zones and sandbanks, a three way ANOVA was performed. The fixed factors included 'method' (two levels: bulk DNA metabarcoding and morphology), 'impact' (four levels: reference, low, medium and high) and 'sandbank' (three levels: Hinderbanken, Oostdyck and Thorntonbank). Sampling stations were added as random factors to the model and variation caused by sampling year was included as a fixed factor in the model because the number of levels (two: 2019 and 2021) was too low to be defined as a random factor. A non-significant interaction was excluded from the model before analyzing the outcomes of the model. Next, the homogeneity of the variances and normality of the data were checked based on plots of the residuals, and if violated, a log transformation was performed. Finally, post hoc tests using the package emmeans v1.8.7 (Searle et al., 1980) were conducted to further explore the significant interactions or main factors identified in the ANOVA analysis.

2.6. Comparison of species identifications detected with morphology and bulk DNA metabarcoding

The taxa identified with the morphology-based method (similar as for alpha diversity, fixed on formalin in 2019 and fixed on ethanol in 2021) and with bulk DNA were studied in more detail. Samples that were removed by rarifying the bulk DNA dataset were also removed in the morphology dataset. Number of shared and unique taxa for each method was visualized in VennDiagrams per sandbank and year using the ggvenn package 0.1.9 (Linlin, 2021), and the taxonomic classification, abundance, size class, presence of exoskeleton and reference sequence of these species was listed in a table (ESM 3). Size classes and presence of exoskeleton were based on data from Derycke et al. (2021), and missing information was completed based on expert judgement and the World Register of Marine Species (WoRMS). This comparison was performed at the lowest taxonomic identification level feasible, aligning with current practices in traditional monitoring. Additionally, the VennDiagrams illustrating shared and unique taxa for each method were generated at the genus level.

To study the correlation between read numbers (DNA metabarcoding) and density or biomass (morphology-based identification), scatterplots were made for the 10 most abundant genera and for the 10 genera with the highest biomass in the morphology. For this analysis, only the ethanol-fixed samples identified by both bulk DNA metabarcoding and morphology (i.e. $n = 89$) were considered. This subset of samples allowed us to focus on the comparison of results obtained from identical samples using the two different methodologies. Initially, the correlations between read numbers and density or biomass were examined using all samples. Subsequently, the analysis was refined to include only samples for which the genera were detected by both methods. For each genus, a scatter plot was generated with the density or biomass on the x-axis and the read numbers on the y-axis. Next, a Pearson correlation between read numbers and biomass or density was calculated and added to the scatterplot with the stat_cor function from the ggpubr package (Kassambara, 2023). Only correlation coefficients with a significant p-value were considered, and following guidelines were used when interpreting the magnitude of the Pearson correlation coefficients: small ($0.1 < |r| < 0.29$), medium ($0.3 < |r| < 0.49$) and large ($0.5 < |r| < 1.0$) (Cohen, 1988).

2.7. Beta diversity analyses for morphologically and bulk DNA metabarcoding derived data

The variation in community composition between the different impact zones of aggregate extraction (reference, low, medium and high) and between the sampling years (2019 and 2021) was investigated for each methodology (bulk DNA metabarcoding and morphology) and each sandbank (Hinderbanken, Oostdyck, and Thorntonbank) separately. This allows investigating whether detected multivariate patterns are similar between both methodologies. Similar as for alpha diversity analyses, the morphologically identified samples fixed in ethanol of the Thorntonbank in 2019 were not included in this morphologically derived dataset. A square root transformation was performed on the morphology-based dataset, while a fourth root transformation in the DNA metabarcoding dataset was carried out to address the elevated read numbers of specific species (e.g. *Echinocardium cordatum*). Separate dissimilarity matrices were computed for each methodology and sandbank, based on Bray-Curtis dissimilarity (Bray and Curtis, 1957). Subsequently, for each methodology and sandbank, a principal coordinates analysis ordination (PCO) was constructed, followed by a two-way PERMANOVA with 9999 permutations and main effects 'impact' (four levels: reference, low, medium, high) and 'year' (two levels: 2019 and 2021) in PRIMER 7. Pairwise tests were conducted for significant effects, and homogeneity of dispersion of these samples was checked with a PERMDISP-test.

Next, a SIMPER analysis in both the bulk DNA and morphology-based dataset was performed for each sand bank separately in PRIMER 7 to assess the top 90 % species contribution to within-group similarity, here the different impact groups (reference, low, medium and high). These taxa were compared between the two methods.

2.8. Biotic index calculation

The morphology dataset and bulk DNA metabarcoding dataset with assigned taxonomy from sampling year 2021 (from the identical Van Veen grabs) were used for the calculation of the Benthic Ecosystem Quality Index (BEQI) (Van Hoey et al., 2008a), the index currently applied in Belgium for MSFD evaluations (ICES, 2024), next to assessing sand extraction impact (De Backer et al., 2014a). BEQI evaluates habitat health using four biological parameters: species richness, density, biomass and species composition based on Bray-Curtis similarity. This index assesses the habitat health per habitat, e.g. a group of samples with distinct environmental attributes such as the different sandbanks in the BPNS. For each habitat, this index compares impacted areas (based on the extraction intensity: low, medium, high) to reference areas that remain unaffected by direct human impact. The reference areas were used to calculate the expected reference values for each BEQI parameter (richness, density, biomass and similarity), using permutations. First, 2000 reference sample combinations were generated, each with summed surface area equivalent to the total assessment sample surface (i.e. impact group). Then, richness, density, biomass, and similarity were calculated for each reference sample combination and all combinations were visualized in a distribution per parameter. Next, based on this distribution, boundaries were determined by the median and percentiles to define scaled BEQI scores and the associated classes representing the ecological status: bad (0–0.2), poor (0–2-0.4), moderate (0.4–0.6), good (0.6–0.8) and high (0.8–1). Finally, the observed richness, density, biomass and similarity in the impacted samples were compared with these reference boundaries to determine the habitat health. The total BEQI score represents the mean of the four parameter BEQI scores. A BEQI score below 0.6, indicating bad, poor or moderate health, shows that the impacted habitat have significant differences from the reference location. BEQI scores and classes were automatically calculated using the calculation tool available at www.beqi.eu. Using R, the total BEQI scores calculated with the morphology dataset and bulk DNA metabarcoding dataset (with assigned taxonomy) were visualized in a scatter plot and a Pearson correlation was calculated.

2.9. Impact of the fixative on the species detection

The impact of the fixative (ethanol versus formalin) on the species detection of morphologically identified samples and their similarity with the species detection using bulk DNA metabarcoding was investigated in a subset of replicate Van Veen grab samples (24 samples, taken

in sampling year 2019 and at sandbank Thorntonbank). The number of detected species with the ethanol and formalin fixative was visualized with a boxplot and a two-way ANOVA was conducted with main factors methodology (three levels: bulkDNA metabarcoding, morphology fixed on ethanol, morphology fixed on formalin) and impact (four levels: reference, low, medium, high). The number of shared and unique species was visualized with a Venn diagram. We checked the body size and the presence of an exoskeleton for species uniquely detected with each method. To study the effect of the fixative on the beta diversity patterns, a dissimilarity matrix based on the Jaccard (presence/absence) dissimilarity index (Jaccard, 1912) was calculated. A PCO plot was conducted and two-way PERMANOVA with 9999 permutations and main effects impact (four levels: reference, low, medium, high) and method (three levels: bulk DNA metabarcoding, morphology fixed on ethanol, morphology fixed on formalin) were performed. All beta diversity analyses were conducted in PRIMER 7.

2.10. Time and cost calculation

The samples of Thorntonbank 2019 ($n = 24$) were used for detailed time and cost tracking of all the processing steps and consumables used for bulk DNA metabarcoding and morphological identification up to acquiring the species list with abundance and biomass or read numbers. For this comparison, the tracking of time and cost was not conducted for steps that were performed identically in both methods (e.g. sampling on the ship, decantation and the screening for heavier animals). Steps that were included for the morphology-based method involved the identification step, weighing, and input of the data in the database. Each sample was tracked individually for time, except for the data input, which was timed in batch. Steps included for the bulk DNA metabarcoding involved mixing the sample, lab analysis (DNA extraction, PCR amplification and library preparation) and the bio-informatics pipeline used to process the sequencing output. Tracked time for the mixing step for bulk DNA metabarcoding was timed per sample, but the other steps were tracked in batch. Next to the equipment cost, staff costs were included by multiplying the total time with the hourly wage, i.e. a taxonomic expert for morphology and a lab technician for bulk DNA metabarcoding.

3. Results

3.1. Processing of raw reads

Raw reads were processed and filtered, resulting in a mean of 112,572 and 185,997 reads per sample for the bulk DNA metabarcoding datasets in 2019 and 2021, respectively. ESM 4 provides the read numbers after each processing step for all samples. Taxonomic assignment to species level using the GEANS reference and blastn against Genbank showed only 1368 (25 %) assigned ASVs in 2019 and 1212 (16 %) in 2021. However, these assigned ASVs represented 95 % and 92 % of the total number of reads in the dataset of respectively 2019 and 2021, indicating that most of the reads showed a taxonomic assignment.

3.2. Alpha diversity analyses for morphology-derived data and bulk DNA metabarcoding derived data

For species richness (S) and Shannon index (H), the three-way ANOVAs with main factors 'methodology', 'impact' and 'sandbank' showed no significant effects of the interaction term 'methodology: impact:sandbank' and this interaction term was excluded from the model (Table 2). Crucially, the detection of the sand extraction impact was not significantly affected by the used method for S and H, as no significant interactions between the sand extraction impact and identification method were noted (Fig. 2, Table 2).

For S, a significant interaction between the method and sandbank was observed, indicating significant differences among the sandbanks based on the used method (Fig. 2A, Table 2). The post hoc test showed significant differences among the three sandbanks using morphology with the highest number of taxa found on the Thorntonbank (16.12 ± 10.69), followed by Oostdyck (8.91 ± 3.01) and Hinderbanken (7.32 ± 5.13), while for bulk DNA metabarcoding only a significant difference was observed between Thorntonbank (mean = 17.47 ± 6.08 SD) versus Oostdyck (mean = 13.10 ± 3.36 SD) and Hinderbanken (mean = 11.03 ± 3.00 SD) (Fig. 2A, Table 2). Next, a significant interaction between impact and sandbank was observed, indicating the impact varied across sandbanks. The post hoc test revealed only significant differences between reference and low impact group versus medium and high impact groups at the Thorntonbank (Fig. 2A, ESM 5).

For H, only a significant interaction between impact and sandbank was detected (Fig. 2B, Table 2). The post hoc test showed only significant differences between the reference versus medium impact group, and low impact group versus medium and high impact groups at the Thorntonbank (Fig. 2B, ESM 5).

3.3. Comparison of species identifications detected with morphology and bulk DNA metabarcoding

The same set of samples (samples excluded from the metabarcoding dataset because of low read numbers, were also excluded from the morphology dataset), were used to compare species identifications using bulk DNA metabarcoding and morphology. Overall, 34 % of the taxa were shared between both methods, with 26 % being unique to bulk DNA metabarcoding and 41 % unique to morphology-based identification (Fig. 3). Similar patterns were observed when analyzing the three sandbanks individually (ESM 6). However, when species identifications were compared at genus level, a higher percentage of identifications was shared between the methods (ESM 7).

Both methods detected a comparable number of species across different phyla, except for "Mollusca", which were more frequently detected using the morphology-based method (Table 3). Species uniquely found with the morphological method typically involved assignments at higher taxonomic levels (46 %), of which 37 % on genus level (ESM 8, Table 4). If the specimens were identified at species level, the lack of a reference sequence was only an explanation for a few

Table 2
Output of the mixed ANOVA for number of taxa and Shannon index, with fixed factors 'method', 'impact' and 'sandbank' and their interactions. Significant values are indicated in bold. Following number of samples were removed in the bulk DNA metabarcoding dataset in the rarefying step: 12 (Hinderbanken, 2019), two (Oostdyck, 2019), one (Thorntonbank, 2019) and one (Thorntonbank, 2021).

	Number of taxa (S)				Shannon index (H)			
	F	Df	Df.res	Pr(>F)	F	Df	Df.res	Pr(>F)
(Intercept)	434.38	1	165.84	<2.2e−16	178.09	1	162.47	<2e−16
Method	27.60	1	182.20	**4.13e−07**	0.85	1	180.12	0.36
Impact	0.20	3	189.05	0.90	0.04	3	183.12	0.99
Sandbank	1.76	2	101.68	0.18	3.01	2	99.96	**5.37e−02**
Method:Impact	0.56	3	174.55	0.64	0.75	3	172.60	0.52
Impact:Sandbank	2.75	6	113.92	**1.58e−02**	2.41	6	116.02	**3.10e−02**
Method:Sandbank	3.85	2	174.55	**2.31e−02**	1.47	2	172.80	0.23
Method:Sandbank:Impact	No significant interaction, so removed from model				No significant interaction, so removed from model			

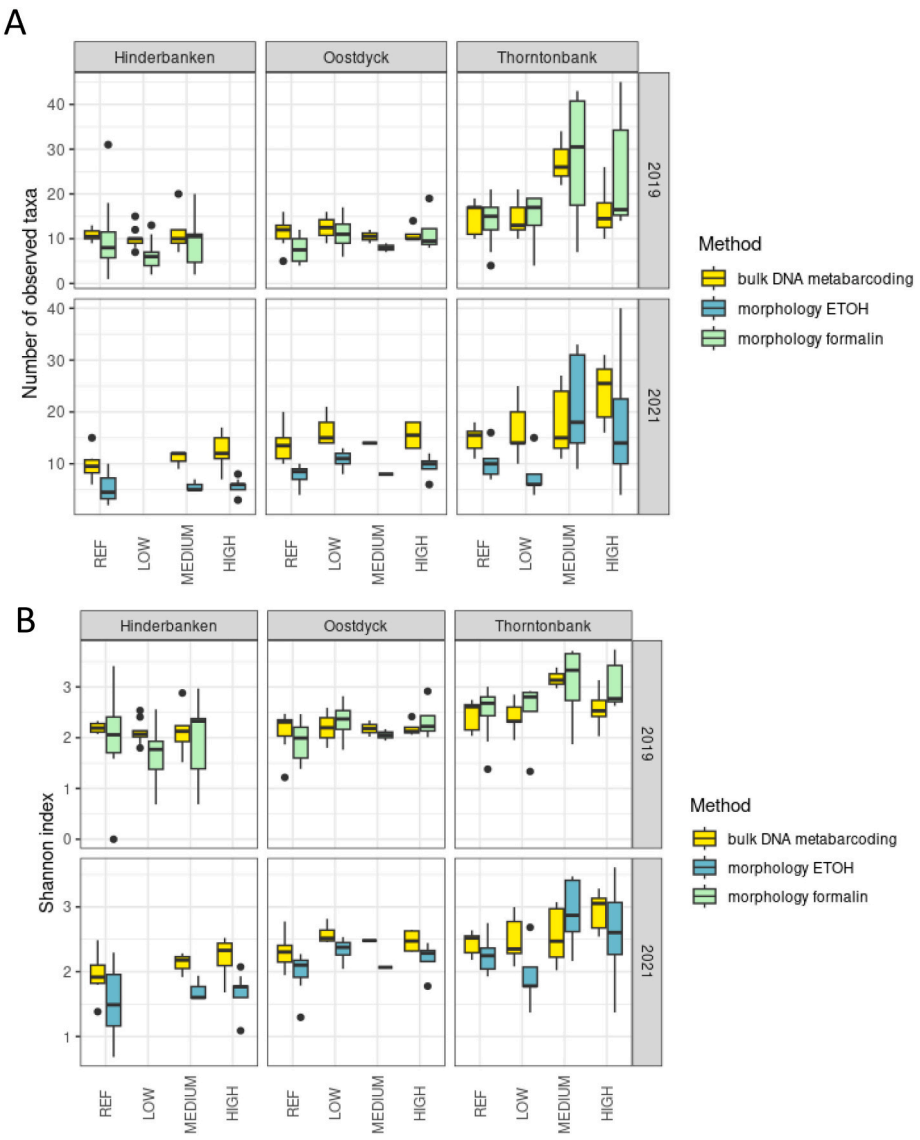


Fig. 2. Alpha diversity with the mean number of taxa (A) and mean Shannon index (B) per sand extraction zone and per year for each impact group (reference, low, medium and high) determined using bulk DNA metabarcoding (yellow), morphological identification from ethanol (blue) and from formalin fixed samples (green). In some impact groups, less samples were used for the bulk DNA datasets due to low sequencing depth.

species uniquely found with the morphology-based method (21 %) (ESM 8, Table 4). When a reference sequence was available, most species uniquely found with morphology were small (<21 mm) (58 %), had an exoskeleton (67 %), and/or were low abundant (1 ind/m^2, 39 %) (ESM 8, Table 4). For a few species, the lack of detection cannot be explained by these three factors (15 %). Unique taxa found with bulk DNA metabarcoding were nearly all identified at the species level and many showed very low read numbers (<50) (45 %) (ESM 8, Table 4). Some (49 %) of the unique taxa at the species level uniquely found with bulk DNA metabarcoding were identified at a higher taxonomic level with morphology.

3.4. Beta diversity analyses for morphologically and bulk DNA metabarcoding derived data

PERMANOVA results of sandbanks differed for both methods at the Hinderbanken (Fig. 4, Table 5). A significant effect was found only for the factor 'year' using bulk DNA metabarcoding (Table 5). In contrast, the morphology-based method demonstrated significant effects for both the main factors 'year' and 'impact' (Table 5), with pairwise tests showing significant differences between the references group and the low impact group (ESM 5). For the Oostdyck, results were similar for both methods, as significant effects on species assemblage were observed for the main factors 'year' and 'impact' with the bulk DNA metabarcoding and the morphology based method (Fig. 4, Table 5). Pairwise tests revealed for both methods significant differences in species assemblage between the reference group versus the high impact group (ESM 5), but morphology detected also significant differences between the low impact group versus high impact group and the reference versus medium impact group. For the Thorntonbank, clear and significant effects of sand extraction (factor 'impact') were observed by both methods (Fig. 4, Table 5). Using bulk DNA metabarcoding, a significant interaction effect 'impact:year' was observed: in 2019, significant differences were found between the reference group versus the low, medium and high impact groups, while in 2021, significant differences were detected between the high impact group versus reference, low and medium impact groups (ESM 5). The morphology-based method demonstrated a borderline significant interaction between 'year' and 'impact', and significant differences for the main factors 'year' and 'impact', with the reference and low impact groups significant

differences from medium and high impact group (ESM 5).

Next, SIMPER analysis showed that the species contributing to 90 % of the within-group similarity of reference, low, medium or high impact were largely similar in the three sandbanks, regardless of the method used. Shared taxa are marked with green in ESM 9. Only in the Thorntonbank, a higher number of species was required to meet the 90 % threshold with morphology, but also here, many species identified by SIMPER analysis were common to both methods. Furthermore, the species with the highest contribution (at the top of the list) were shared, except for the high impact Thorntonbank with morphology (ESM 9).

3.5. Abundance in morphology (biomass/density) and bulk DNA metabarcoding (reads)

Finally, the correlation between read numbers obtained from bulk DNA correlated with biomass or density from morphology was studied, focusing on the top 10 genera of the morphological dataset with the highest total biomass or density, respectively. For density, only eight of the 10 genera were plotted and correlation coefficients were calculated, as *Phoronis* (with the 10th highest density) was not identified by DNA metabarcoding and *Echinocyamus* was only detected by both methods in two samples. Among these eight genera, no significant relationship could be identified between density and read numbers (ESM 10 A). For biomass, *Ensis* showed the fifth highest biomass in the morphology (=12.45 gWW/m^2), but this genus was not detected by the bulk DNA metabarcoding approach, despite the availability of reference sequences. Again, *Echinocyamus* dropped out because it was only detected in two samples by both methods, but it generally showed low detection with bulk DNA metabarcoding (only in these two samples), in contrast to a total biomass of 4.12 gWW/m^2 spread over 25 samples for morphology. Among the remaining genera, only three genera showed a significant positive correlation between biomass and read numbers derived from metabarcoding: *Echinocaridum* ($R = 0.62$, p-value = 3.2e-04), *Ophelia* ($R = 0.59$, p-value = 4.9e-06) and *Thia* ($R = 0.47$, p-value = 0.013) (ESM 10B). These results were based on a dataset including samples in which the genus was detected by both methods. However, comparable results for the comparison between read numbers and biomass/density were observed when including all samples (ESM 11).

3.6. Biotic index calculation

A similar trend and large correlation ($R^2 = 0.87$, p-value = 0.0011) were observed when comparing the calculated BEQI values based on the bulk DNA metabarcoding dataset (with assigned taxonomy) and the morphology dataset. However, higher genetic BEQI scores were detected – with a single exception- when using the bulk DNA metabarcoding dataset, leading to an underestimation of the sand extraction impact (Fig. 5). Furthermore, not all points were positioned in the colored squares, showing a difference in associated health classes. Although a maximum difference of one class was observed in this study, six groups were misclassified, with a difference between moderate and good in two groups and a difference between good and high in four groups (Fig. 5).

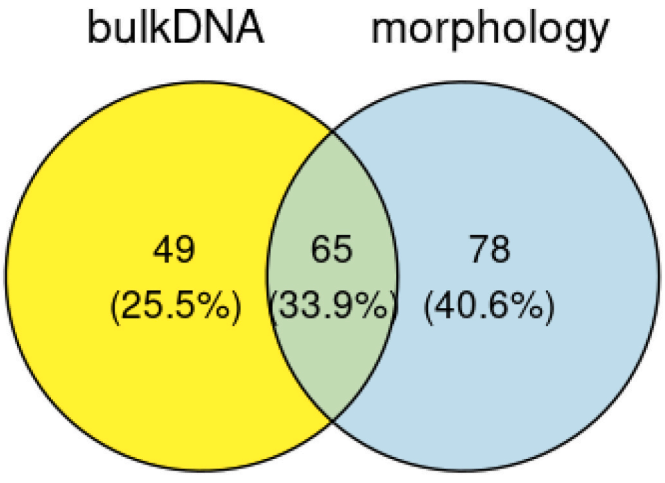


Fig. 3. Venn diagrams of the taxa detected with bulkDNA metabarcoding and morphological identification (in total: over the two years and all sandbanks).

3.7. Impact of the fixative on the species detection

To examine the influence of fixatives on species detection and their similarity with the species detection using bulk DNA metabarcoding, Van Veen grabs with two different fixatives (formalin and ethanol) were used at the Thorntonbank in 2019. The ethanol-fixed grab was used for both bulk DNA metabarcoding and morphology-based identification. Two-way ANOVA showed no significant interaction effect ('method: impact') on S and H. For H, both 'method' and 'impact' were significant (resp. $F = 5.40$, $p = 6.83e-03$ and $F = 5.95$, $p = 1.22e-03$). Post-hoc tests for factor 'method' revealed that H did not differ significantly between fixatives of the morphology-based method but that bulk DNA metabarcoding had a significantly lower average H than both morphological fixatives. Next, significant differences were observed between the medium impact group compared to the reference and low impact group. For S, no significant method effect was observed, while sand extraction impact was significant ($F = 9.29$, $p = 3.58e-05$). Post-hoc tests showed a significantly higher taxa richness in the medium impact group compared to the three other groups (ESM 12, Fig. 6A). Although S was not affected by the method, the three methods only shared 23.1 % of all taxa found. Additionally, both morphology-based methods showed 12 % and 20 % unique taxa for the formalin and ethanol fixative respectively, while bulk DNA metabarcoding detected 20 % (Fig. 6B). Between both fixatives and bulk DNA metabarcoding, a similar number of taxa were shared (formalin-bulk DNA: 23.1 % + 2.4 %, ethanol-bulk DNA: 23.1 % +1.8 %). For the unique taxa in the morphologically identified datasets, similar percentages of soft tissue taxa were observed for both fixatives (35 % for formalin and 32 % for ethanol fixed samples), while the ethanol fixative yielded a higher proportion of smaller species (59 %) compared to formalin (40 %) (ESM 13).

Beta diversity patterns based on a Jaccard dissimilarity (presence/absence) illustrated clustering based on the methods, with the two morphological datasets clustering together and clearly separated from the bulk DNA metabarcoding (Fig. 6C). A significant interaction effect for the two-way Permanova was observed ($F = 1.76$, $p = 0.002$), but also a significant permdisp was detected ($p = 0.0001$). Pairwise tests demonstrated significant differences between the reference vs low, medium and high impact group for morphology fixed on ethanol, while differences between reference vs medium and high impact group and low vs medium impact group were observed for morphology fixed on formalin. For bulk DNA metabarcoding, significant differences were detected between reference vs low, medium and high impact group, next to medium vs low and high impact groups (ESM 12).

3.8. Time and cost calculations

Bulk DNA metabarcoding showed a 44 % reduction in time and a 26 % reduction in costs compared to the traditional morphology-based method (Table 6). For bulk DNA metabarcoding, most time was needed for the DNA extraction (11/37 h hands-on time). In contrast to morphological analysis, subsequent steps were performed on multiple samples simultaneously, resulting in a lower overall processing time (ESM 14). Additionally, bulk DNA metabarcoding demonstrated a lower processing cost compared to morphology, despite requiring more lab supplies. Approximately half of the total cost for bulk DNA metabarcoding was attributed to consumables and the MiSeq run (2553/4823 euro; 53 %), in contrast to the morphology-based method, where

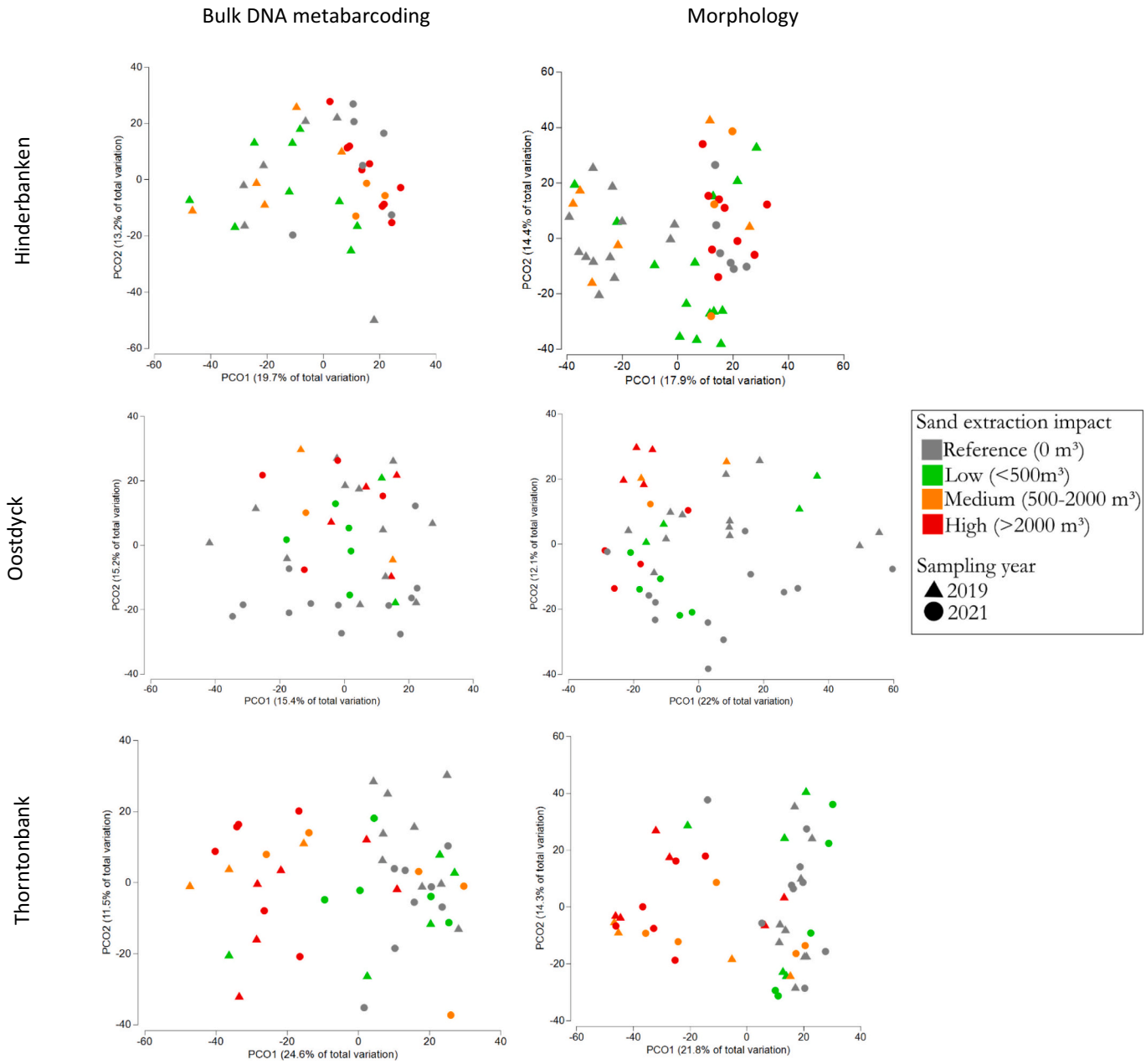


Fig. 4. PCO plots per sandbank (Hinderbanken, Oostdyck and Thorntonbank) and method (left: bulk DNA metabarcoding, right: morphology) based on Bray-Curtis similarity for square-root transformed densities and fourth-root transformed read abundance data. The different colors indicate the sand extraction impact (REF: grey, LOW: green, MEDIUM: orange, HIGH: red) and the two years are visualized by a different symbol (2019: triangle, 2021: circle). Some DNA metabarcoding datasets contained less samples as following samples with low sequencing depth were removed: 12 (Hinderbanken, 2019), two (Oostdyck, 2019), one (Thorntonbank, 2019) and one (Thorntonbank, 2021).

the staff costs were the highest (6434/6514 euro; 99 %) (ESM 15). While the morphology-based method requires the expertise of a taxonomic expert, typically commanding a higher hourly rate (96 euro/h in this study), bulk DNA metabarcoding can be performed by a laboratory technician at a comparatively lower rate (62 euro/h in this study). The detailed calculations can be found in ESM 14 (time) and ESM 15 (costs).

4. Discussion

Our results contribute to the growing body of evidence supporting the use of DNA metabarcoding in detecting the impact of anthropogenic pressures (Aylagas et al., 2018; Bik et al., 2012; Chariton et al., 2010; Pawlowski et al., 2014), and specifically, our study shows the potential of bulk DNA metabarcoding of macrobenthos for assessing the impact of sand extraction activities.

4.1. Bulk DNA metabarcoding and morphology detect similar diversity patterns of macrobenthos related to sand extraction

Although species detection differed between bulk DNA metabarcoding and morphology, our study showed that a similar impact of sand extraction activity on alpha and beta diversity was detected by both methods. First, increased species richness (S) and Shannon indices (H) (alpha diversity) were observed in highly impacted locations at the Thorntonbank. As we observed no significant interaction between the impact group and the identification method, this observation was

Table 3
Overview of the species, grouped in the different phyla, shared between or unique for bulk DNA metabarcoding and morphology. Species with an assignment at higher taxonomic levels are marked in light blue, possible correlated specimens with the other method in dark blue. For assignments on species level, species without a reference sequence are underscored and for the species with a reference sequence multiple features were described: Small specimens were marked with a *(abundance <50 reads for bulk DNA or abundance = 1 ind/m^2 for morphology).

Phylum	Unique for bulk DNA metabarcoding: 49		Shared between bulk DNA metabarcoding and morphology: 65		Unique for morphology: 78	
Annelida	Aricidea minuta	*Scolelepis neglecta	Aonides paucibranchiata	Poecilochaetus serpens	Aricidea	Nephtys longosetosa
	Capitellidae	Spio decorata	Eunereis longissima	Scolelepis bonnieri	Capitella	Notomastus latericeus
	Eumida mackiei	Spio goniocephala	Glycinde nordmanni	Scoloplos armiger	*Chaetopterus variopedatus	Oligochaeta
	Glycera alba	Streptosyllis websteri	Hesionura elongata	Spiophanes bombyx	Cirratulidae	Parougia eliasoni
	*Grania ovitheca	*Tubificoides diazi	Lagis koreni	Sthenelais boa	Eteone	Polydora
	*Grania postclitellochaeta		Lanice conchilega	Streptodonta pterochaeta	Eumida sanguinea	Polygordius
	*Grania variochaeta		Loimia ramzega		Exogone	Polynoinae
	*Lepidonotus squamatus		Magelona johnstoni		Fabriciinae	Protodriloides
	Magelona mirabilis		Malmgrenia		Glycera lapidum	Protodrilus
	*Microphthalmus listensis		Nephtys cirrosa		Glycera alba-tridactyla	Psammodrilus balanoglossoides
	Microphthalmus similis		Ophelia borealis		*Harmothoe antilopes	*Sabellaria spinulosa
	*Myrianida langerhansi		Owenia fusiformis		Macrochaeta helgolandica	Sipuncula
	*Mysta picta		Paraonis fulgens		*Mediomastus fragilis	Sphaerosyllis
	*Nephasoma rimicola		Phyllodoce rosea		Microphthalmus	Spio
	*Pholoe baltica		Pisione remota		Myrianida	Spirobranchus
	*Phyllodoce mucosa				Nephtys assimilis	Streptosyllis
	Polycirrus				Nephtys caeca	
	Polygordius appendiculatuS				*Nephtys hombergii	
Arthropoda	Anapagurus hyndmanni	Pilumnus hirtellus	Abludomelita obtusata	Monopseudocuma gilsoni	*Anoplodactylus petiolatus	Microdeutopus anomalus
	*Anapagurus laevis	Pinnotheres pisum	Aora gracilis	Nototropis falcatus	*Bathyporeia sarsi	*Nebalia reboredae
	*Carcinus maenas	Processa modica	Apolochus neapolitanus	Nototropis swammerdamei	Bodotria scorpioides	Perioculodes longimanus
	Cheirocratus intermedius		Bathyporeia elegans	Pagurus bernhardus	Cheirocratus	Pontocrates arenarius
	*Eurynome aspera		Bathyporeia guilliamsoniana	Pariambus typicus	Corophium	Processa
	Haustorius arenarius		Centraloecetes kroyeranus	Philocheras trispinosus	*Ebalia	Pseudocuma
	Liocarcinus depurator		Crangon crangon	Phtisica marina	*Eualus cranchii	*Pseudoprotella phasma
	*Liocarcinus holsatus		Diastylis bradyi	Pisidia longicornis	Eurydice spinigera	*Synchelidium maculatum
	Liocarcinus marmoreus		Diogenes pugilator	Pontocrates altamarinus	Hyas	Unciola planipes
	Liocarcinus pusillus		Galathea intermedia	Portumnus latipes	*Iphimedia minuta	
	Macropodia rostrata		Gastrosaccus spinifer	Pseudocuma simile	Liocarcinus	
	Monocorophium acherusicum		Gilvossius tyrrhenus	Stenothoe marina	Macropodia	
	Orchomene humilis		Iphinoe trispinosa	Thia scutellata	Maerella tenuimana	
	Philocheras bispinosus		Jassa herdmani	Urothoe brevicornis	Maja	
			Leucothoe incisa	Urothoe poseidonis	Megamphopus cornutus	
			Megaluropus agilis			
Chordata			Branchiostoma lanceolatum			
Cnidaria	Cylista troglodytes				Anthozoa	
Echinodermata	Amphipholis squamata		Asterias rubens	Ophiothrix fragilis	Amphiura	
	*Marthasterias glacialis		Echinocardium cordatum	Ophiura albida	*Ophiocten affinis	
	*Psammechinus miliaris		Echinocyamus pusillus	Ophiura ophiura		
Mollusca	*Epitonium clathrus		Donax vittatus		Abra alba	Kurtiella bidentata
	*Euspira catena		Epitonium clathratulum		*Abra prismatica	Mytilus edulis
	Euspira nitida		Spisula elliptica		Aequipecten opercularis	Parthenina indistincta
	*Macoma balthica		Spisula solida		Asbjornsenia pygmaea	Solenoidea
	*Tridacna gigas		Tellimya ferruginosa		Caecum glabrum	*Spisula subtruncata
			Tritia reticulata		Crepidula fornicata	Striarca lactea
					Ensis	*Thracia
					Euspira	
					Goodallia triangularis	
Phoronida					Phoronis	

Table 4
Summary of the taxa uniquely found by one method (marked in bold) and possible explanations like identified at a higher taxonomic level in one dataset or availability of a sequence in the reference database, size, presence of exoskeleton and abundance.

	Hinderbanken		Oostdyck		Thorntonbank		Total (all sandbanks and years together)
	2019	2021	2019	2021	2019	2021	
Total number of detected taxa	74	56	71	65	142	116	192
Shared taxa	19	19	19	21	41	44	65
Unique for morphological method	**27**	**15**	**31**	**17**	**66**	**39**	**78**
→ Higher taxonomic level AND	12	6	13	9	29	17	36
• On genus level	10	6	11	7	24	16	29
• On higher taxonomic level	2	0	2	2	5	1	7
→ On species level AND	15	9	18	8	37	22	42
• No reference available in the used reference database	3	2	2	2	5	5	9
• Reference available in the used reference database AND	12	7	16	6	32	17	33
∘ Small size (<21 mm) and/or	6	5	10	3	19	8	19
∘ Exoskeleton and/or	7	5	13	3	20	10	22
∘ Low abundance (ind/m^2) < 20	5	6	8	5	14	5	13
∘ No clear reason (none of above)	3	1	1	1	5	5	5
Unique for BulkDNA metabarcoding method	**28**	**22**	**21**	**27**	**35**	**33**	**49**
→ Higher taxonomic level present in morphologically identified sample and/or	5	3	5	4	15	11	24
→ Abundance (number of reads) < 50	13	16	9	23	13	17	22
→ No clear reason (none of above)	12	4	9	3	14	8	11

irrelevant of the method used. Second, similar results in community composition (beta diversity) were observed at the Thorntonbank and Oostdyck regardless of the method used. However, differences in alpha and beta diversity were observed for the Hinderbanken between the two methods: 1/ significant differences in S (alpha diversity) between the three sandbanks with morphology, but no significant difference between the Hinderbanken and the other two sandbanks was observed with bulk DNA metabarcoding, and, 2/ a significant effect between the reference and low impact group of sand extraction in community composition (beta diversity) of the Hinderbanken with only morphology. During the bulk DNA metabarcoding bioinformatics pipeline, 12 samples from the Hinderbanken were excluded due to low read numbers, with 11 of these samples belonging to the reference or low impact group. The removal of these samples could potentially explain the lack of detection of an

Table 5
PERMANOVA results for bulk DNA metabarcoding and morphology, and for each sandbank separately. Each PERMANOVA had main factors Impact, Year and interaction, but DNA metabarcoding datasets contained less samples compared to morphology as following samples with low sequencing depth were removed: 12 (Hinderbanken, 2019), two (Oostdyck, 2019), one (Thorntonbank, 2019) and one (Thorntonbank, 2021). Significant values are marked in bold.

	Bulk DNA metabarcoding						Morphology					
	Fourth root transformation						Square root transformation					
	Permdisp	Df	SumOfSqs	MeanSqs	Pseudo−F	P(perm)	Permdisp	Df	SumOfSqs	MeanSqs	Pseudo−F	P(perm)
Hinderbanken												
Impact	**2.91e−02**	3	5945.50	1981.9	1.03	0.43	0.24	3	12,760	4253.2	1.77	**0.35e−02**
Year	**0.01e−02**	1	5169.80	5169.8	2.67	**0.2−e02**	**0.34e−02**	1	8743	8743	3.63	**0.01e−02**
Impact × Year	**1.66e−02**	1	1225.00	1225	0.63	0.83	0.44	1	1187.7	1187.7	0.49	0.93
Res		32	61,870	1933.4				44	1.06e+05	2408.4		
Total		37	78,544					49	1.33e+05			
Oostdyck												
Impact	0.15	3	7799.60	2599.9	1.50	**0.0372**	0.07	3	14,473	4824.3	2.44	**0.02e−02**
Year	0.12	1	4162.70	4162.7	2.40	**0.0045**	0.17	1	4209.2	4209.2	2.13	**2.32e−02**
Impact × Year	**8.98e−02**	3	4796.60	1598.9	0.92	0.59	0.07	3	4693.3	1564.4	0.79	0.77
Res		34	59,037	1736.4				36	71,132	1975.9		
Total		41	79,109					43	98,145			
Thorntonbank												
Impact	0.20	3	15,716	5238.7	2.98	**0.02e−02**	0.07	3	22,744	7581.2	3.30	**0.01e−02**
Year	0.51	1	5299.50	5299.5	23.02	**0.01e−02**	0.57	1	7151.4	7151.4	3.11	**0.06e−02**
Impact × Year	0.43	3	9254.30	3084.8	1.76	**0.35e−02**	0.88	3	9440.2	3146.7	1.37	5.63e−02
Res		39	68,530	1.76				41	94,177	2297		
Total		46	98,096					48	1.33e+05			

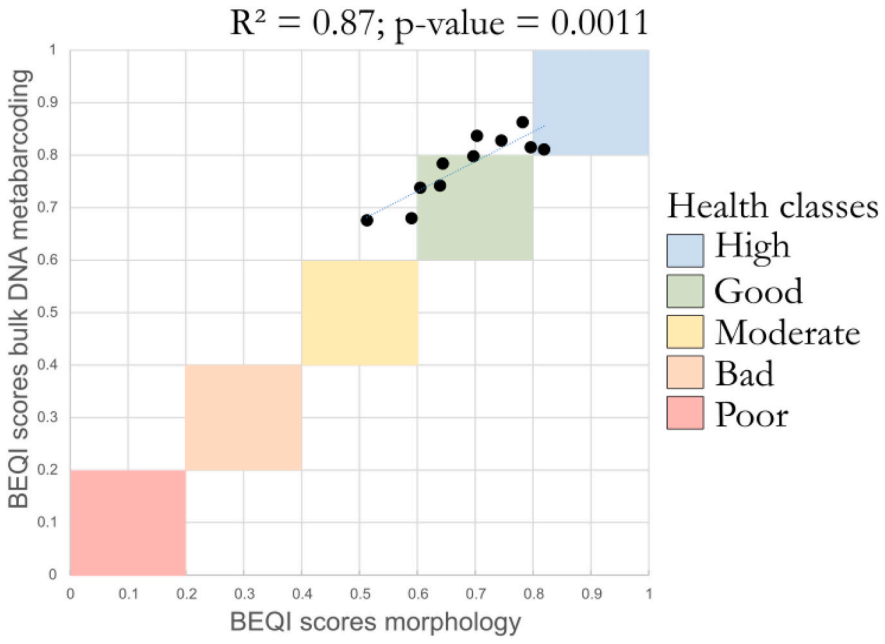


Fig. 5. Comparison of BEQI scores calculated with the Bulk DNA metabarcoding (y-as) and morphology (x-as) dataset. Each point is based on a number of samples in a habitat with a specific impact group (based on extraction intensity), 89 samples were divided over the 11 groups. The different health classes (associated with the BEQI scores) are marked with colors: poor (red), bad (orange), moderate (yellow), good (green) and high (blue). All points inside the colored squares are assigned to the same class.

Table 6
Summarized table of the cost and time calculation of Thorntonbank samples in 2019 (24 samples) for the bulk DNA metabarcoding and morphology based method.

	Number of samples	Total (hands-on) time (h)	Time/sample (h)	$\frac{(time_{morph} - time_{gen})}{time_{morph}}$	Total cost (€)	Cost/sample (€)	$\frac{(cost_{morph} - cost_{gen})}{cost_{morph}}$
Morphology	24	67	2.8	44 %	6514	271	26 %
Bulk DNA metabarcoding		37	1.5		4823	201	

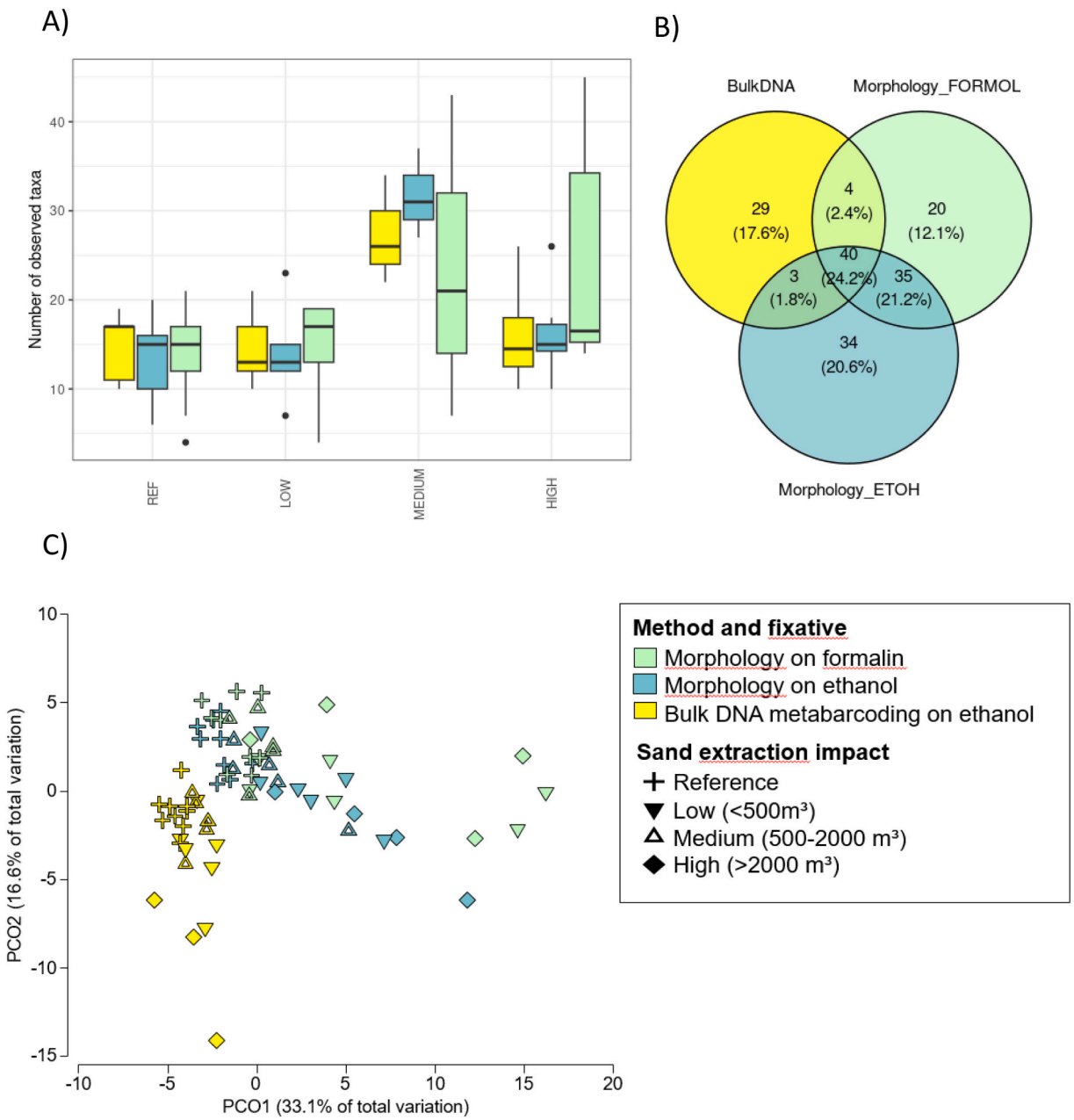


Fig. 6. Impact of the fixative on the species detection. (A) The detected number of observed taxa with detected with bulkDNA metabarcoding (yellow), morphological identification fixed on ethanol (blue) and morphological identification fixed on formalin (green), (B) Venn diagram showing the shared and unique taxa for the different methods and (C) a PCO plot showing the beta diversity based on a Jaccard dissimilarity (presence/absence).

impact with bulk DNA metabarcoding compared to morphology (beta diversity) or the lack of significant differences in S (alpha diversity) at the Hinderbanken.

The similar patterns in community composition of the Oostdyck and Thorntonbank with both methods can be explained by the most abundant species, that were detected by both methods. Differences in community composition between reference and impact groups were observed because these species respond to changes in sediment size resulting from sand extraction activities. First, the samples of the Oostdyck are mostly situated in the *Nephtys cirrosa* community, characterized by medium (250-500 μm) sands (Breine et al., 2018; Van Hoey et al., 2004). Both with bulk DNA metabarcoding and morphology, the species communities of the samples were mostly dominated by *Nephtys cirrosa*, except for the high impact locations. Here, *Urothoe brevicornis* showed highest species contribution for both methods. Similar results were described at the Oostdyck by Wyns et al. (2021), showing an increased presence of *Urothoe brevicornis* in the two high impacted samples. Second, sand extraction had a clear impact at the Thorntonbank regardless of the method used. While species composition in the reference samples was dominated by *Nephtys cirrosa*, the medium and high impacted samples on the Thorntonbank showed a higher contribution of *Lanice conchilega*. This species has a preference for finer sand fractions (Van Hoey et al., 2008b). A previous study demonstrated that the continuous, high extraction at the Thorntonbank increased seabed heterogeneity and exposed clay from underlying geological layers. Thereby causing a shift towards a more heterogeneous, macrobenthic community, including species typically associated with finer sediments such as *Lanice conchilega* (Wyns et al., 2021). So, the bulk DNA metabarcoding results corroborate these earlier findings. Earlier studies also observed that this increase in fine sand at high extraction locations could

increase biodiversity (De Backer et al., 2014b; Wyns et al., 2021). We indeed saw at medium and high impact locations of the Thorntonbank, the most intense extraction area, more species were needed to reach the 90 % SIMPER cutoff and that S and H had increased values, both for the bulk DNA metabarcoding and morphological dataset. This again underscores that DNA metabarcoding has the ability to detect changes caused by sand extraction.

For biotic indices, a comparable - but higher- pattern was observed in the calculated BEQI scores using the bulk DNA metabarcoding dataset (with assigned taxonomy) compared to the morphology dataset. These higher BEQI scores can result in different associated health classes. For example, we observed two groups (samples from medium impact in 2019 and high impact in 2021 at the Thorntonbank) classified as good ecological health using bulk DNA metabarcoding, while a moderate ecological health was assigned using the morphology-based method. This difference is relevant because habitats with a poor, bad or moderate ecological status require actions to improve the health status. Although the high correlation in BEQI scores is promising, the observed discrepancies in assigned health classes suggest further investigation before genetic BEQI scores can be reliably used in Environmental Impact Assessments (EIAs). For instance, incorporating a correction factor when determining the boundaries of health classes (e.g., 0.2, 0.4, 0.6, and 0.8) for bulk DNA metabarcoding datasets may be necessary to achieve comparable health classes.

4.2. Bulk DNA metabarcoding and morphological identification exhibit disparities in species detection

Although DNA metabarcoding demonstrated its ability to detect global patterns of sand extraction, it is important to note that a large share of the identified taxa differed between bulk DNA metabarcoding and the morphology-based method. First, species identification may vary between the two methods due to differences in taxonomic levels. Morphology-based identification often classifies taxa at the genus level, especially in the presence of juveniles or the absence of diagnostic features. In contrast, bulk DNA metabarcoding showed higher taxonomic resolution (mostly at species level). Comparison at the genus level reveals an increased percentage of shared genera, but each methods still identifies a notable number of unique genera.

Next, bulk DNA metabarcoding was not able to detect certain species identified by the morphology-based method. This could be linked to various potential factors. First, the lack of a reference sequence could explain the unique species for morphology. However, most unique taxa using morphology (79 %) that were identified at species level had a reference sequence available, which is consistent with findings from earlier studies (Derycke et al., 2021; Steyaert et al., 2020). Second, it is unlikely that morphological misidentifications can explain unique taxa for the morphology-based identification, as the taxonomic experts responsible for specimen identifications in this study were working in a NBN EN ISO/IEC 17025 regulated environment, certified for macrobenthos identification (BELAC T-315 certificate) and thus regularly undergo quality controls with low error detection. Third, some characteristics of taxa could explain the decreased chance to be identified by the bulk DNA metabarcoding method. Small specimens or the presence of a skeleton may hamper an efficient cell lysis step in the metabarcoding lab protocol. For example, lower number of Mollusca were detected by bulk DNA metabarcoding. Next to a low abundance, this results in a lower contribution of tissue (Deagle et al., 2018), which may increase the stochastic sampling of DNA molecules in the first cycles of the PCR (Steyaert et al., 2020). A combination of these three factors further reduce the likelihood of the detection through bulk DNA metabarcoding. Last, bulk DNA metabarcoding showed a lower detection of polychaetes (e.g. species of the genera *Glycera* and *Nephtys*), which are known to exhibit lower primer efficiency as a result of high variation in the COI gene (Carr et al., 2011) and as such challenges accurate detection using DNA metabarcoding.

In contrast, bulk DNA metabarcoding detected taxa that were not picked up by morphology. Many (45 %) of the taxa uniquely detected by bulk DNA metabarcoding showed very low read numbers per sample (<50). These taxa could possibly be missed specimens during the sorting process in the morphology-based identification. Another possibility for these observed low read numbers could be explained by for example traces of DNA attached on the surface or from the stomach of the bulk specimens or PCR amplification efficiency that could lead to false-positive detections.

4.3. Bulk DNA metabarcoding is faster and cheaper than morphology-based identification

DNA metabarcoding has been proposed as a time and cost-efficient method compared to traditional morphology-based identification of samples (Aylagas et al., 2014). The current study provides empirical evidence that processing time can be reduces by 44 % and costs by 26 % when using bulk DNA metabarcoding instead of morphological species identification. Previous studies estimated similar (Elbrecht and Leese, 2017) or higher (Aylagas et al., 2014) cost reductions for DNA metabarcoding compared to morphology-based method, but staff costs were not included. Within our study, we included both consumables and staff costs. The highest costs associated with bulk DNA metabarcoding were linked to the cost of the DNA extraction kit and the sequencing run (53 %), while for morphology highest costs were related to staff costs (99 %), as morphological identification is done by a taxonomic expert who is in general more expensive than a lab technician which can conduct the bulk DNA metabarcoding analyses. Although, staff costs used in our study are specific for Belgium, the ratio between different experience levels will proportionally be similar in other countries.

Moreover, the numbers provided by our study are based on relatively easy and fast to identify coarse sand samples taking on average under 3 h per sample. For other habitat types, morphological processing time can be much higher (even up to 2 days per sample), indicating that these percentages could even increase in favor of bulk DNA metabarcoding. Furthermore, unlike the morphology-based method, where processing time increases with the number of samples, bulk DNA metabarcoding requires only the mixing of samples to be performed on a per-sample basis. The other processing steps (i.e. DNA extraction, PCR amplification and library preparation) can be done in batch, and are optimized for sets of 96 samples. The hands-on processing time can even be further reduced through the implementation of pipetting robots, which have already demonstrated to produce consistent results (Buchner et al., 2021). Additionally, sequencing costs are expected to further decrease as well with new technological advancements and platforms offered, such as sequencing using the Novaseq instead of the Miseq. Cost and time of sample processing could be even further decrease when extracting DNA from the ethanol preservative. However, a previous study showed a more robust detection of macrobenthos when using bulk DNA (Derycke et al., 2021).

4.4. Limitation of bulk DNA metabarcoding for absolute quantification

When comparing both methods, our study showed no (for density) or little (for biomass) evidence supporting the use of read numbers as a proxy for biomass and density of the species, similar as in existing literature (Elbrecht and Leese, 2017; Hollatz et al., 2017; Klunder et al., 2022; Lobo et al., 2017). For three of the eight studied genera, a good correlation between reads and biomass was found. However, between the eight studied genera, we found differences between genera for the correlation. Three of the eight studied genera were polychaetes, but both good correlation (*Ophelia*), as no significant correlation (*Lanice* and *Nephtys*) were found. As high primer bias has been proven for Polychaeta (Carr et al., 2011), the observed difference was possibly caused by a more effective PCR amplification of *Ophelia* compared to *Lanice* and *Nephtys*. Morphology thus provides quantitative information that cannot

be achieved by bulk DNA metabarcoding. To reduce the bias introduced by the PCR process, primer-free approaches such as shotgun metagenomics sequencing have potential, as they eliminate the impact of PCR amplification bias. This method avoids targeting a specific locus for amplification, instead randomly fragmenting the extracted DNA into smaller pieces. Complex bioinformatics pipelines are then used to reassemble these fragments into longer contigs based on their overlap, resulting in fragmented (for less abundant organisms) or complete genomes. However, these techniques are currently still too expensive for routine monitoring use.

4.5. An optimized design for sand extraction using the complementary DNA-based and morphology-based methods

Our study showed DNA metabarcoding and morphology-based identification can be seen as complementary methods. On the one hand, impact assessment by DNA metabarcoding can detect changes by sand extraction on a fast and cost-efficient manner, while on the other hand, morphology-based identification can provide abundance measurements and detailed information like life stage and size. Furthermore, a combined approach includes a more comprehensive species list, as each method also detected unique species. Given the demonstrated complementarity of both methods, monitoring programmes can be optimized by combining the two methods in a manner that maximizes the strengths of both approaches.

This combined approach involves molecular monitoring along the entire sand extraction gradient (Fig. 7). The gradient is defined by intensity data, ranging from the areas with high volumes of extracted sand to reference locations where no extraction occurs. Given the sensitivity of genetics to changes, there is a rapid assessment to detect changes, providing a continuous monitoring mechanism. Quantitative and detailed information, such as size and life stage, can be affected by sand extraction (Newell et al., 2004). Therefore, collecting this data is important for accurate impact assessments. As bulk DNA metabarcoding currently lacks quantitative information, a smaller subset of the samples will also be used for morphological identification. Previous studies have consistently shown that the highest ecological impact is expected at the locations with highest intensity (Wyns et al., 2021), so these morphological samples will be taken at the extremes (locations with the high intensity and reference locations). This strategy ensures the collection of the most detailed information at these critical points, enabling comprehensive mapping of changes.

The combined approach demands a shift of fixing macrobenthos in pure, nondenaturated ethanol (final concentration should be higher than 70 %), so that the same grab samples can be processed with both methods. Our study demonstrated that the diversity patterns for morphological identified samples fixed on ethanol or formalin were similar. First, while the number of detected taxa was comparable between both fixatives, differences in the identity of taxa were observed. These samples were taken from different sediment grabs (biological replicates), known to show variation between species detections and this could potentially explain the observed differences. Other reasons could be the effect of ethanol preservation, as ethanol could dissolve small soft-bodied taxa, or make specimens stiff, losing their diagnostic parts, both resulting in a lower detection. However, the ethanol-fixed samples did not show a lower number of small or soft-bodied taxa. Second, some studies indicated higher weight loss in ethanol fixed specimens than formalin preserved ones (Dermott and Paterson, 1974; Howmiller, 1972; Landahl and Nagell, 1978; Leuven et al., 1985), while other studies found no differences (Gaston et al., 1996; Mason et al., 1983; Wetzel et al., 2005). Although our study did not study biomass comparison between the two fixatives, Stein et al. (2013) demonstrated the preservation of specimens in ethanol for up to six months without significant effects on biomass. Therefore, considering the potential impact of ethanol on biomass, especially for specimens preserved over six months, is important. However, this seemed to have no effect on the observed diversity patterns, as these were similar between the two fixatives in our study.

By implementing DNA metabarcoding in monitoring programmes, advantages of both methods are combined. This comprehensive approach offers the potential for improved ecological assessments and a deeper understanding of the impact of sand extraction activities, since species level detection will be maximized and at the same time absolute quantitative information (biomass and density) are maintained. Additional work is now required for merging both morphological and DNA metabarcoding datasets in data analysis. A key challenge, here, lies in addressing the quantitative difference between the two types of data (counts/biomass vs reads). Also quality control procedures and harmonizing the bioinformatics pipelines will require careful consideration, underscoring the importance of collaboration between taxonomists and molecular biologists.

5. Conclusion

This study first demonstrated the potential of DNA metabarcoding as a valuable tool for marine environmental monitoring, e.g. sand extraction. While the same diversity patterns after sand extraction activity were detected with bulk DNA metabarcoding and morphology-based identification, this study also showed there are some differences between the two methods. Bulk DNA metabarcoding excels in rapidly detecting changes in benthic communities, offering insights into alpha and beta diversity. However, its primary limitation lies in the lack of a clear correlation between DNA reads and density or biomass, which are critical metrics in many monitoring programs. In contrast, morphology-based analysis can give quantitative data and detailed information on life stage and size that possibly drive observed changes. This level of detail enriches our understanding of the biological and ecological processes at play. Furthermore, the current underestimation of sand extraction by the BEQI using genetic data necessitates further investigation to refine this tool for use in Environmental Impact Assessments (EIAs). Lastly, the combination of the two methods resulted in a higher species detection. Therefore, integrating both bulk DNA metabarcoding and morphology-based methods provides a robust and comprehensive toolkit for assessing the impacts of sand extraction. By leveraging the

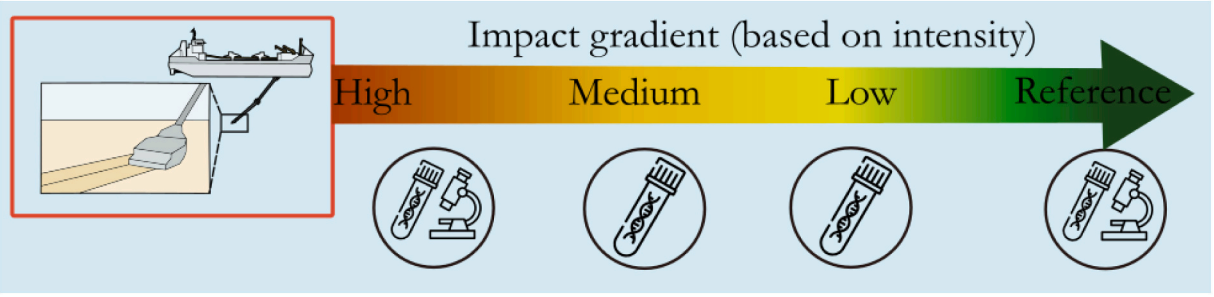


Fig. 7. Visual representation of the suggested monitoring approach, combining bulk DNA metabarcoding and morphological identification.

strengths of both techniques, we can achieve a more nuanced and holistic understanding of marine ecosystem dynamics, ultimately leading to more effective conservation and management strategies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174106.

CRediT authorship contribution statement

Van den Bulcke Laure: Writing – original draft, Visualization, Formal analysis, Conceptualization. **De Backer Annelies:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Hillewaert Hans:** Investigation. **Maes Sara:** Investigation. **Seghers Stephie:** Investigation. **Waegeman Willem:** Writing – review & editing. **Wittoeck Jan:** Investigation. **Hostens Kris:** Writing – review & editing. **Derycke Sofie:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

The sequencing datasets and corresponding metadata generated for this study are available as BioProject in the online NCBI repository under accession number PRJNA1126610.

Acknowledgements

We would like to thank our former colleague Charles Lefranc for his role in morphologically identifying the specimens within the samples of this study. Your efforts are truly appreciated. Funding for this research was received through the Sand Fund of the Federal Public Service Economy and GEANS – Genetic tools for Ecosystem health Assessment in the North Sea region, an Interreg project supported by the North Sea Programme of the European Regional Development Fund of the European Union. Ship Time at RV Belgica, GeoSurveyor XI and Geo Ocean V (in absence of RV Belgica during the sampling campaign of 2021) was provided by BELSPO and RBINS-OD Nature. Also the support of VLIZ for providing ship time at RV Simon Stevin is greatly appreciated. We thank all crews for the logistic support during the sampling campaigns. Willem Waegeman received funding from the "Onderzoeksprogramma Artificiële Intelligentie (AI) Vlaanderen Fund" of the Flemish Government.

References

Aylagas, E., Borja, Á., Rodríguez-Ezpeleta, N., 2014. Environmental status assessment using DNA metabarcoding: towards a Genetics Based Marine Biotic Index (gAMBI). PLoS One 9, e90529. https://doi.org/10.1371/journal.pone.0090529.

Aylagas, E., Borja, Á., Irigoien, X., Rodríguez-Ezpeleta, N., 2016. Benchmarking DNA metabarcoding for biodiversity-based monitoring and assessment. Front. Mar. Sci. 3 https://doi.org/10.3389/fmars.2016.00096.

Aylagas, E., Borja, A., Muxika, I., Rodriguez-Ezpeleta, N., 2018. Adapting metabarcoding-based benthic biomonitoring into routine marine ecological status assessment networks. Ecol. Indic. 95, 194–202. https://doi.org/10.1016/j.ecolind.2018.07.044.

Bik, H.M., Halanych, K.M., Sharma, J., Thomas, W.K., 2012. Dramatic shifts in benthic microbial eukaryote communities following the Deepwater Horizon oil spill. PLoS One 7. https://doi.org/10.1371/journal.pone.0038550.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 325–349. https://doi.org/10.2307/1942268.

Breine, N.T., De Backer, A., Van Colen, C., Moens, T., Hostens, K., Van Hoey, G., 2018. Structural and functional diversity of soft-bottom macrobenthic communities in the Southern North Sea. Estuar. Coast. Shelf Sci. 214, 173–184. https://doi.org/10.1016/j.ecss.2018.09.012.

Buchner, D., Macher, T.H., Beermann, A.J., Werner, M.T., Leese, F., 2021. Standardized high-throughput biomonitoring using DNA metabarcoding: strategies for the adoption of automated liquid handlers. Environ Sci Ecotechnol 8, 100122. https://doi.org/10.1016/j.ese.2021.100122.

Cahill, A.E., Pearman, J.K., Borja, A., Carugati, L., Carvalho, S., Danovaro, R., Dashfield, S., David, R., Féral, J.-P., Olenin, S., Šiaulys, A., Somerfield, P.J., Trayanova, A., Uyarra, M.C., Chenuil, A., 2018. A comparative analysis of metabarcoding and morphology-based identification of benthic communities across different regional seas. Ecol. Evol. 8, 8908–8920. https://doi.org/10.1002/ece3.4283.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581. https://doi.org/10.1038/nmeth.3869.

Carr, C.M., Hardy, S.M., Brown, T.M., Macdonald, T.A., Hebert, P.D., 2011. A tri-oceanic perspective: DNA barcoding reveals geographic structure and cryptic diversity in Canadian polychaetes. PLoS One 6, e22232. https://doi.org/10.1371/journal.pone.0022232.

Chariton, A.A., Court, L.N., Hartley, D.M., Colloff, M.J., Hardy, C.M., 2010. Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. Front. Ecol. Environ. 8, 233–238. https://doi.org/10.1890/090115.

Cohen, J., 1988. Statistical Power Analysis for the Behavioral Sciences. Lawrence Erlbaum Associates, New York, p. 567. https://doi.org/10.4324/9780203771587.

Cooper, K.M., 2013. Setting limits for acceptable change in sediment particle size composition: testing a new approach to managing marine aggregate dredging. Mar. Pollut. Bull. 73, 86–97. https://doi.org/10.1016/j.marpolbul.2013.05.034.

Cooper, K., Boyd, S., Aldridge, J., Rees, H., 2007. Cumulative impacts of aggregate extraction on seabed macro-invertebrate communities in an area off the east coast of the United Kingdom. J. Sea Res. 57, 288–302. https://doi.org/10.1016/j.seares.2006.11.001.

Cooper, K.M., Curtis, M., W.M.R., C.R.S., Defew, E.C., Nye, V., Paterson, D.M., 2011. Implications of dredging induced changes in sediment particle size composition for the structure and function of marine benthic macrofaunal communities. Mar. Pollut. Bull. 62, 2087–2094. https://doi.org/10.1016/j.marpolbul.2011.07.021.

Cowart, D.A., Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Mine, J., Arnaud-Haond, S., 2015. Metabarcoding is powerful yet still blind: a comparative analysis of morphological and molecular surveys of seagrass communities. PLoS One 10, e0117562. https://doi.org/10.1371/journal.pone.0117562.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. bioRxiv, 221499. https://doi.org/10.1101/221499.

De Backer, A., Hillewaert, H., Van Hoey, G., Wittoeck, J., Hostens, K., 2014a. Structural and functional biological assessment of aggregate dredging intensity on the Belgian part of the North Sea. In: De Mol, L., Vandenreyken, H. (Eds.), Which future for the sand extraction in the Belgian part of the North Sea?, pp. 29–58. https://api.semanticscholar.org/CorpusID:164208447.

De Backer, A., Van Hoey, G., Coates, D., Vanaverbeke, J., Hostens, K., 2014b. Similar diversity-disturbance responses to different physical impacts: three cases of small-scale biodiversity increase in the Belgian part of the North Sea. Mar. Pollut. Bull. 84, 251–262. https://doi.org/10.1016/j.marpolbul.2014.05.006.

De Jong, M.F., Baptist, M.J., Lindeboom, H.J., Hoekstra, P., 2015. Short-term impact of deep sand extraction and ecosystem-based landscaping on macrozoobenthos and sediment characteristics. Mar. Pollut. Bull. 97, 294–308. https://doi.org/10.1016/j.marpolbul.2015.06.002.

Deagle, B.E., Clarke, L.J., Kitchener, J.A., Polanowski, A.M., Davidson, A.T., 2018. Genetic monitoring of open ocean biodiversity: an evaluation of DNA metabarcoding for processing continuous plankton recorder samples. Mol. Ecol. Resour. 18, 391–406. https://doi.org/10.1111/1755-0998.12740.

Dermott, R.M., Paterson, C.G., 1974. Determining dry weight and percentage dry matter of chironomid larvae. Can. J. Zool. 52, 1243–1250. https://doi.org/10.1139/z74-165.

Derycke, S., Maes, S., Van den Bulcke, L., Vanhollebeke, J., Wittoeck, J., Hillewaert, H., Ampe, B., Haegeman, A., Hostens, K., De Backer, A., 2021. Detection of macrobenthos species with metabarcoding is consistent in bulkDNA but dependent on body size and sclerotization in eDNA from the ethanol preservative. Front. Mar. Sci. https://doi.org/10.3389/fmars.2021.637858.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

Duarte, S., Leite, B.R., Feio, M.J., Costa, F.O., Filipe, A.F., 2021. Integration of DNA-based approaches in aquatic ecological assessment using benthic macroinvertebrates. Water 13, 331. https://doi.org/10.3390/w13030331.

Elbrecht, V., Leese, F., 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer Bias and biomass—sequence relationships with an innovative metabarcoding protocol. PLoS One 10, e0130324. https://doi.org/10.1371/journal.pone.0130324.

Elbrecht, V., Leese, F., 2017. Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. Front. Environ. Sci. 5, 11. https://doi.org/10.3389/fenvs.2017.00011.

Ewels, P., Magnusson, M., Lundin, S., Kaller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

Festjens, F., Buyse, J., De Backer, A., Hostens, K., Lefaible, N., Vanaverbeke, J., Van Hoey, G., 2023. Functional trait responses to different anthropogenic pressures. Ecol. Indic. 146, 109854 https://doi.org/10.1016/j.ecolind.2022.109854.

Foden, J., Rogers, S.I., Jones, A.P., 2009. Recovery rates of UK seabed habitats after cessation of aggregate extraction. Mar. Ecol. Prog. Ser. 390, 15–26. https://doi.org/10.3354/meps08169.

Gaston, G.R., Bartlett, J.H.W., McAllister, A.P., Heard, R.W., 1996. Biomass variations of estuarine macrobenthos preserved in ethanol and formalin. Estuaries 19, 674–679. https://doi.org/10.2307/1352527.

Goedefroo, N., Braeckman, U., Hostens, K., Vanaverbeke, J., Moens, T., De Backer, A., 2023. Understanding the impact of sand extraction on benthic ecosystem functioning: a combination of functional indices and biological trait analysis. Front. Mar. Sci. 10 https://doi.org/10.3389/fmars.2023.1268999.

Hollatz, C., Leite, B.R., Lobo, J., Froufe, H., Egas, C., Costa, F.O., 2017. Priming of a DNA metabarcoding approach for species identification and inventory in marine macrobenthic communities. Genome 60, 260–271. https://doi.org/10.1139/gen-2015-0220.

Howmiller, R.P., 1972. Effects of preservatives on weights of some common macrobenthic invertebrates. Trans. Am. Fish. Soc. 101, 743–746. https://doi.org/10.1577/1548-8659(1972)101<743:EOPOWO>2.0.CO;2.

ICES, 2019. Working Group on the Effects of Extraction of Marine Sediments on the Marine Ecosystem (WGEXT). In: ICES Scientific Reports. https://doi.org/10.17895/ices.pub.5733.

ICES, 2024. Working Group on Fisheries Benthic Impact and Trade-offs (WGFBIT; outputs from 2023 meeting). In: ICES Scientific Reports. https://doi.org/10.17895/ices.pub.25603191 (156 pp.).

Jaccard, P., 1912. The distribution of the flora in the alpine zone. 1. New Phytol. 11, 37–50. https://doi.org/10.1111/j.1469-8137.1912.tb05611.x.

Kassambara, A., 2023. 'ggplot2' Based Publication Ready Plots. R package version 0.6.0. URL. https://rpkgs.datanovia.com/ggpubr/.

Klunder, L., van Bleijswijk, J.D.L., Kleine Schaars, L., van der Veer, H.W., Luttikhuizen, P. C., Bijleveld, A.I., 2022. Quantification of marine benthic communities with metabarcoding. Mol. Ecol. Resour. 22, 1043–1054. https://doi.org/10.1111/1755-0998.13536.

Landahl, C.C., Nagell, B., 1978. Influence of the season and of preservation methods on wet- and dry weights of larvae of Chironomus plumosus L. Internationale Revue der gesamten Hydrobiologie und Hydrographie 63, 405–410. https://doi.org/10.1002/iroh.19780630308.

Leray, M., Knowlton, N., 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proc. Natl. Acad. Sci. USA 112, 2076–2081. https://doi.org/10.1073/pnas.1424997112.

Leuven, R.S.E.W., Brock, T.C.M., van Druten, H.A.M., 1985. Effects of preservation on dry- and ash-free dry weight biomass of some common aquatic macro-invertebrates. Hydrobiologia 127, 151–159. https://doi.org/10.1007/BF00004193.

Linlin, Y., 2021. ggvenn: Draw Venn Diagram by 'ggplot2' https://doi.org/10.32614/CRAN.package.ggvenn.

Lobo, J., Shokralla, S., Costa, M.H., Hajibabaei, M., Costa, F.O., 2017. DNA metabarcoding for high-throughput monitoring of estuarine macrobenthic communities. Sci. Rep. 7, 13. https://doi.org/10.1038/s41598-017-15823-6.

Mason, W.T., Lewis, P.A., Weber, C.I., 1983. An evaluation of benthic macroinvertebrate biomass methodology. Environ. Monit. Assess. 3, 29–44. https://doi.org/10.1007/BF00394030.

Newell, R.C., Seiderer, L.J., Simpson, N.M., Robinson, J.E., 2004. Impacts of marine aggregate dredging on benthic macrofauna off the south coast of the United Kingdom. J. Coast. Res. 20, 115–125. URL. http://www.jstor.org/stable/4299272.

Pawlowski, J., Esling, P., Lejzerowicz, F., Cedhagen, T., Wilding, T.A., 2014. Environmental monitoring through protist next-generation sequencing metabarcoding: assessing the impact of fish farming on benthic foraminifera communities. Mol. Ecol. Resour. 14, 1129–1140. https://doi.org/10.1111/1755-0998.12261.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org/.

Searle, S.R., Speed, F.M., Milliken, G.A., 1980. Population marginal means in the linear model: an alternative to least squares means. Am. Stat. 34, 216–221. https://doi.org/10.1080/00031305.1980.10483031.

Stein, E.D., White, B.P., Mazor, R.D., Miller, P.E., Pilgrim, E.M., 2013. Evaluating ethanol-based sample preservation to facilitate use of DNA barcoding in routine freshwater biomonitoring programs using benthic macroinvertebrates. PLoS One 8, e51273. https://doi.org/10.1371/journal.pone.0051273.

Steyaert, M., Priestley, V., Osborne, O., Herraiz, A., Arnold, R., Savolainen, V., 2020. Advances in metabarcoding techniques bring us closer to reliable monitoring of the marine benthos. J. Appl. Ecol. 57, 2234–2245. https://doi.org/10.1111/1365-2664.13729.

Van den Bulcke, L., De Backer, A., Wittoeck, J., Beentjes, K., Maes, S., Christodoulou, M., Martinez Arbizu, P., Sapkota, R., Van der Hoorn, B., Winding, A., Hostens, K., Derycke, S., 2023. DNA metabarcoding on repeat: sequencing data of marine macrobenthos are reproducible and robust across labs and protocols. Ecol. Indic. 150, 110207 https://doi.org/10.1016/j.ecolind.2023.110207.

Van Hoey, G., Degraer, S., Vincx, M., 2004. Macrobenthic community structure of soft-bottom sediments at the Belgian Continental Shelf. Estuar. Coast. Shelf Sci. 59, 599–613. https://doi.org/10.1016/j.ecss.2003.11.005.

Van Hoey, G., Drent, J., Ysebaert, T., Herman, P., 2008a. The Benthic Ecosystem Quality index (BEQI), intercalibration and assessment of Dutch Coastal and Transitional Waters for the Water Framework Directive: Final report. URL. https://api.semanticscholar.org/CorpusID:129407828.

Van Hoey, G., Guilini, K., Rabaut, M., Vincx, M., Degraer, S., 2008b. Ecological implications of the presence of the tube-building polychaete Lanice conchilega on soft-bottom benthic ecosystems. Mar. Biol. 154, 1009–1019. https://doi.org/10.1007/s00227-008-0992-1.

Van Hoey, G., Borja, A., Birchenough, S., Buhl-Mortensen, L., Degraer, S., Fleischer, D., Kerckhof, F., Magni, P., Muxika, I., Reiss, H., Schroder, A., Zettler, M.L., 2010. The use of benthic indicators in Europe: from the Water Framework Directive to the Marine Strategy Framework Directive. Mar. Pollut. Bull. 60, 2187–2196. https://doi.org/10.1016/j.marpolbul.2010.09.015.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/aem.00062-07.

Wetzel, M.A., Leuchs, H., Koop, J.H.E., 2005. Preservation effects on wet weight, dry weight, and ash-free dry weight biomass estimates of four common estuarine macroinvertebrates: no difference between ethanol and formalin. Helgol. Mar. Res. 59, 206–213. https://doi.org/10.1007/s10152-005-0220-z.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Wyns, L., Roche, M., Barette, F., Van Lancker, V., Degrendele, K., Hostens, K., Backer, A., 2021. Near-field changes in the seabed and associated macrobenthic communities due to marine aggregate extraction on tidal sandbanks: a spatially explicit biophysical approach considering geological context and extraction regimes. Cont. Shelf Res. 229, 104546 https://doi.org/10.1016/j.csr.2021.104546.